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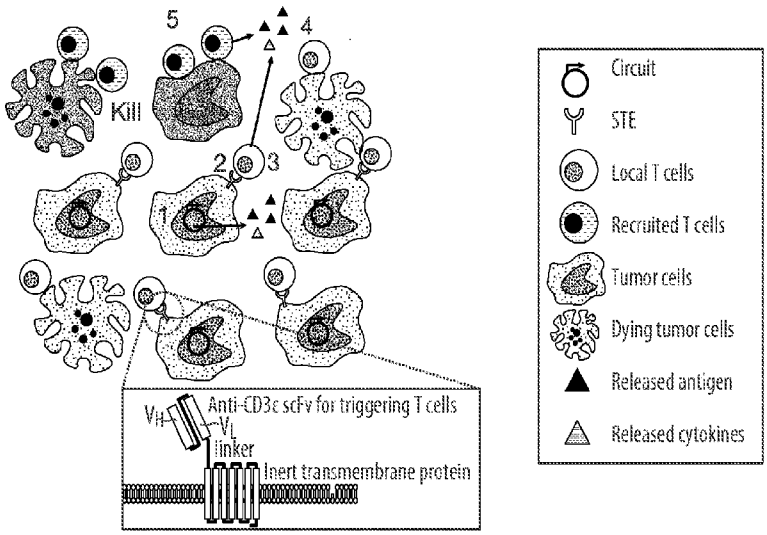

Fig. 2B

(57) Abstract: Aspects of the present disclosure provide a platform that triggers potent and effective immunotherapy against tumors from within tumors themselves, thus overcoming limitations of existing cancer immunotherapies and tumor-detecting gene circuits.

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TUMOR IMMUNOTHERAPY

RELATED APPLICATIONS

This application claims the benefit under 35 U.S.C. § 119(e) of U.S. provisional application number 62/181,906, filed June 19, 2015, and U.S. provisional application number 62/325,314, filed April 20, 2016, each of which is incorporated by reference herein in its entirety.

FIELD OF THE INVENTION

Aspects of the present disclosure relate to the general field of biotechnology and, more particularly, to the fields of synthetic biology and immunology.

BACKGROUND

Existing treatments for many cancers (*e.g.*, ovarian cancer), such as chemotherapies and targeted therapies, are unable to cure metastatic disease and prevent tumor relapse. Further, standard-of-care treatments, such as chemotherapy, can cause significant morbidity and toxicity. New therapeutic strategies are needed to treat primary and metastatic ovarian cancer and to achieve long-term efficacy.

SUMMARY

Provided herein, in some aspects, is a platform that triggers potent and effective immunotherapy against tumors from within tumors themselves, thus overcoming limitations of existing cancer immunotherapies and tumor-detecting gene circuits. Engineered genetic circuits of the present disclosure, in some embodiments, express T-cell-engaging proteins on cancer cell surfaces (referred to as Surface T Cell Engagers (STEs)), which can trigger antigen-independent T cell killing of tumor cells. In some embodiments, engineered genetic circuits are delivered to tumors (*see, e.g.*, Figs. 2A and 2B), and are selectively activated only in cancer cells, resulting in the surface display of STEs and the secretion of other immunomodulatory molecules to recruit T cells to target the tumor. The engineered genetic circuits of the present disclosure, advantageously, can be administered systemically but activated locally only in cancer cells, resulting in enhanced safety and reduced side effects. Thus, the platform of the present disclosure, in some embodiments, combines the advantages

of systemic delivery (*e.g.*, treating metastasis) with the advantages of localized treatment (*e.g.*, safety, minimal side effects).

Existing therapies are hindered by certain limitations that are overcome by the present disclosure. In CAR (chimeric antigen receptor) T cell therapy, for example, the T cells must be custom made for each individual. As another example, bispecific T cell engagers (BiTEs) (Iwahori K. *et al., Molecular Therapy*, 2015, 23(1): 171-178, incorporated herein by reference) are limited by their short half-life, and thus require a continuous intravenous pump infusion for 4-8 weeks. Both therapies target tumor cell surface antigens; however, not all tumor types have ideal surface tumor antigens for detection. Cancer-detecting genetic circuits can harness an intracellular killing mechanism, inducing cell death via a toxin, although delivery of these circuits to all (or most) tumor cells has been virtually impossible.

The present disclosure, in some aspects, provides methods and engineered (recombinant or synthetic) genetic circuits (*e.g.*, engineered mammalian genetic circuits), referred to in some embodiments as "logic gates" that are RNA-based (*e.g.*, the genetic circuits include nucleic acids that comprise primarily RNA, or the genetic include nucleic acids that consist of RNA), thus reducing the likelihood of unwanted immunogenic reactions, as foreign proteins are not introduced into a cell or subject.

In some embodiments, the present disclosure provides methods and engineered genetic circuits for specific detection of cancer cells and production of immunomodulators (*e.g.*, cytokines). In some embodiments, the methods and genetic circuits as provided herein are used for "bystander killing" of cancer cells, whereby memory T cells are triggered to destroy cancer cells that are not directly transformed by engineered genetic circuits of the present disclosure.

In some embodiments, the present disclosure provides methods and engineered genetic circuits for targeted expression of combinatorial immunomodulators released from specific cells (*e.g.*, cancer cells). In some embodiments, the engineered genetic circuits encode molecules that bind to CD3, which when expressed at the surface of targeted cancer cells (anti-CD3 cells), function as synthetic T cell engagers (STEs) to directly recruit T cells to kill the cancers cells targeted/detected by the engineered genetic circuits, resulting in localized and targeted immunotherapy. In other embodiments, the engineered genetic circuits encode bi-directional T cell engagers (BiTEs), which when expressed by a cell and bound to the cell through an antigen-specific region, recruit T cells to kill the cells. BiTEs may be

expressed selectively within specific cell types using engineered genetic circuits (logic gates) that provide for localized production and the same advantages observed with the use of STEs.

In some embodiments, STEs may be used as a general targeted immunotherapy, as BiTEs typically require the recognition of a tumor-specific surface antigen to trigger T cell killing.

The targeted immunotherapies of the present disclosure differ from existing therapies in that they enable systemic delivery with high efficacy and safety. In some embodiments, combination therapies using other cytokines and immunotherapy agents further enhance the efficacy of the target immunotherapy of the present disclosure.

In some embodiments, the present disclosure methods and engineered genetic circuits for the detection of aberrant cell states in diseases (including, but not limited to, autoimmune and neurological diseases) and/or for expression or secretion of immunomodulatory molecules and therapeutic molecules to modulate disease.

In some embodiments, the immunotherapy platform of the present disclosure also includes outputs (*e.g.*, engineered genetic circuits encoding detectable molecules), which may serve as diagnostics.

Some embodiments, provide engineered nucleic acids comprising a cancer-specific promoter operably linked to a nucleic acid encoding a microRNA within an mRNA encoding an immunomodulatory molecule (*e.g.*, a "surface T cell engager," or STE) or a bispecific monoclonal antibody linked to microRNA binding sites.

In some embodiments, the immunomodulatory molecule or bispecific monoclonal antibody is translated only when transcription of the engineered nucleic acid is activated.

Also provided herein are engineered nucleic acid comprising a cancer-specific promoter operably linked to a nucleic acid encoding an mRNA transcript containing microRNA binding sites.

Further provided herein are engineered nucleic acids as depicted in any of Figs. 3A-3D, 4A, 6A, 7A-7H, 9A, 14A, 15A, 16A and 17A.

The present disclosure also provides vectors comprising any of the engineered nucleic acid, as described herein. The present disclosure also provides cells comprising any of the vectors and/or engineered nucleic acid, as described herein.

Some embodiments of the present disclosure provide an engineered genetic circuit, comprising (a) a nucleic acid comprising a promoter operably linked to (i) a nucleotide

sequence encoding an output messenger RNA (mRNA) containing an intronic microRNA (miRNA) and (ii) a nucleotide sequence encoding a miRNA binding site complementary to the miRNA of (a)(i), and (b) a nucleic acid comprising a promoter operably linked to a nucleotide sequence encoding at least one miRNA binding site complementary to the miRNA of (a)(i).

Other embodiments of the present disclosure provide an engineered genetic circuit, comprising (a) a first nucleic acid comprising a promoter operably linked to (i) a nucleotide sequence encoding an output messenger RNA (mRNA) containing an intronic microRNA (miRNA), (ii) a nucleotide sequence encoding an intronic miRNA, and (iii) a nucleotide sequence encoding a miRNA binding site (miRNA-BS); (b) a second nucleic acid comprising a promoter operably linked to (i) a nucleotide sequence encoding an output mRNA containing an intronic miRNA, (ii) a nucleotide sequence encoding an intronic miRNA, and (iii) a nucleotide sequence encoding a miRNA-BS; and (c) a third nucleic acid comprising a promoter operably linked to a nucleotide sequence encoding an output protein linked to a miRNA-BS, wherein the miRNA-BS of (a)(iii) is complementary to the miRNA of (b)(i), the miRNA-BS of (b)(iii) is complementary to the miRNA of (a)(i), and the miRNA-BS of (c) is complementary to the miRNA of (a)(ii) and the miRNA of (b)(ii).

Yet other embodiments of the present disclosure provide an engineered genetic circuit, comprising (a) a first nucleic acid comprising a promoter operably linked to (i) a nucleotide sequence encoding a nascent RNA transcript (*e.g.*, a non-coding RNA transcript) containing an intronic microRNA (miRNA), and (ii) a nucleotide sequence encoding at least one miRNA binding site (miRNA-BS); (b) a second nucleic acid comprising a promoter operably linked to (i) a nucleotide sequence encoding a nascent RNA transcript containing an intronic miRNA, and (ii) a nucleotide sequence encoding at least one miRNA-BS; and (c) a third nucleic acid comprising a promoter operable linked to a nucleotide sequence encoding an output protein linked to (i) a first miRNA-BS and (ii) a second miRNA-BS, wherein the at least one miRNA-BS of (a)(ii) is complementary to the miRNA of (b)(i), the at least one miRNA-BS of (b)(iii) is complementary to the miRNA of (a)(i), the first miRNA-BS of (c)(i) is complementary to the miRNA of (a)(i), and the second miRNA-BS of (c)(ii) is complementary to the miRNA of (b)(i).

Still other embodiments of the present disclosure provide an engineered genetic circuit, comprising (a) a first nucleic acid comprising a promoter operably linked to a

nucleotide sequence encoding a nascent RNA transcript containing an intronic microRNA (miRNA); (b) a second nucleic acid comprising a promoter operably linked to a nucleotide sequence encoding a nascent RNA transcript containing an intronic miRNA; and (c) a third nucleic acid comprising a promoter operable linked to a nucleotide sequence encoding an output protein linked to (i) a first miRNA-BS and (ii) a second miRNA-BS, wherein the first miRNA-BS of (c)(i) is complementary to the miRNA of (a), and the second miRNA-BS of (c)(ii) is complementary to the miRNA of (b).

Further embodiments of the present disclosure provide an engineered genetic circuit, comprising (a) a first nucleic acid comprising a promoter operably linked to a nucleotide sequence encoding a nascent RNA transcript containing an intronic microRNA (miRNA); and (b) a second nucleic acid comprising a promoter operably linked to a nucleotide sequence encoding an output protein linked to a miRNA binding site (miRNA-BS), wherein the miRNA-BS of (b) is complementary to the miRNA of (a).

Other embodiments of the present disclosure provide an engineered genetic circuit, comprising (a) a first nucleic acid comprising a promoter operably linked to (i) a nucleotide sequence encoding an output messenger RNA (mRNA) containing an intronic microRNA (miRNA) and (ii) at least one miRNA binding site (miRNA-BS); and (b) a second nucleic acid comprising a promoter operably linked to (i) a nucleotide sequence encoding an output mRNA containing an intronic miRNA and (ii) at least one miRNA-BS, wherein the at least one miRNA-BS of (a) is complementary to the miRNA of (b), the at least one miRNA-BS of (b) is complementary to the miRNA of (a).

Still other embodiments of the present disclosure provide an engineered genetic circuit, comprising (a) a first nucleic acid comprising a promoter operably linked to a nucleotide sequence encoding a nascent RNA transcript containing an intronic microRNA (miRNA); (b) a second nucleic acid comprising a promoter operably linked to a nucleotide sequence encoding an output protein; and (c) a third nucleic acid comprising a promoter operable linked to a nucleotide sequence encoding an output protein linked to an miRNA binding site, wherein the miRNA-BS of (c) is complementary to the miRNA of (a).

Yet other embodiments of the present disclosure provide an engineered genetic circuit, comprising (a) a first nucleic acid comprising a promoter operably linked to a nucleotide sequence encoding an output protein linked to a microRNA binding site (miRNA-BS); and (b) a second nucleic acid comprising a promoter operably linked to a nucleotide

sequence encoding a nascent RNA transcript containing an intronic miRNA, wherein the miRNA-BS of (a) is complementary to the miRNA of (b).

In some embodiments, the output mRNA encodes a synthetic T cell engager (STE) or a bispecific T cell engager (BiTE).

In some embodiments, the output mRNA encodes an output protein that binds to a T cell surface marker.

In some embodiments, the T cell surface marker is CD3, CD4, CD8 or CD45.

In some embodiments, the output protein is an antibody or antibody fragment that binds specifically to the T cell surface antigen.

In some embodiments, the output mRNA encodes an anti-cancer agent. For example, the output mRNA may encode a chemokine, a cytokine or a checkpoint inhibitor.

In some embodiments, a promoter is an inducible promoter. For example, a promoter may be a tumor-specific promoter (e.g., benign tumor-specific promoter or a malignant tumor-specific promoter) or a cancer-promoter.

In some embodiments, a promoter is SSX1 or H2A1.

In some embodiments, a nucleotide sequence encodes 2-5 or 2-10 micro RNA binding.

In some embodiments, an output protein is a transcription factor.

In some embodiments, an output protein is an anti-cancer agent.

In some embodiments, the output mRNA encodes a transcription factor that can bind to and activate transcription of the promoter of the at least one nucleic acid.

In some embodiments, an engineered genetic circuit comprises nucleic acids that encode a split protein system in which each protein of a functional protein dimer is encoded on a separate nucleic acid and regulated by a separate promoter.

The invention is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments and of being practiced or of being carried out in various ways. Each of the above embodiments and aspects may be linked to any other embodiment or aspect. Also, the phraseology and terminology used herein is for the purpose of description and should not be regarded as limiting. The use of "including," "comprising," or "having," "containing," "involving," and variations thereof herein, is meant to encompass the items listed thereafter and equivalents thereof as well as additional items.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings are not intended to be drawn to scale. For purposes of clarity, not every component may be labeled in every drawing.

Figs. 1A-1C. Examples of prior immunotherapy approaches. (Fig. 1A) Mode of action of chimeric antigen receptor (CAR) T cell therapy. (Figs. 1B and 1C) Mode of action of bispecific T cell engagers.

Figs. 2A-2B. Overview of STRICT therapy. (Fig. 2A) Using STRICT to secrete BiTE. (1) Tumor-identifying circuits are introduced into tumors by local injection or systemic administration. (2) Tumor cells transduced with the circuits secrete BiTEs, which diffuse locally, and other immunomodulatory molecules. (3) BiTEs simultaneously engage HER2 on tumor cells and T-cell receptors on local tumor-infiltrating T cells, thus triggering T cells to directly kill tumor cells. BiTEs can also recruit nearby circulating T cells to traffic to the tumor site. (4) Tumor antigens released by the first wave of killing prime and recruit more tumor-reactive T cells into play. (5) Newly recruited polyclonal T cells can kill more cancer cells, including HER2-negative tumor cells and other heterogeneous tumor cells not killed by the first wave of the anti-tumor immune response. (Fig. 2B) Using STRICT to display surface T cell engager (STE). (1) Tumor-identifying gene circuits are introduced into tumors by local injection or systemic administration. (2) Tumor cells transduced with the circuits express STEs and other immunomodulatory molecules. (3) STEs engage T-cell receptors on local tumor-infiltrating T cells, thus triggering T cells to directly kill tumor cells. (4) Tumor antigens released by the first wave of killing prime and recruit more tumor-reactive T cells into play. (5) Newly recruited polyclonal T cells can kill more cancer cells, including other heterogeneous tumor cells, and metastases, not killed by the first-wave anti-tumor immune response. Immune memory can prevent tumor relapses.

Figs. 3A-3H. The design of RNA-only single-output AND gate. (Figs. 3A-3D) The computation layers of all 4 input states and their and respective output states are shown. The RNA-based logic AND gate integrates the activity of two input promoters, P1 and P2, and generates an output only when both promoters are decidedly active. In this architecture, the output is the Surface T-cells Engager (STE). Promoter P1 is regulating the expression of an STE mRNA which comprises a synthetic miRNA intron (mirFF4). A negative autoregulatory feedback loop was incorporated into the circuit by encoding perfect-match mirFF4 binding sites at the 3' end of the STE/mirFF4 transcript (mirFF4-BS). Consequently, when only

promoter P1 is active the STE mRNA is constantly degraded by the cellular miRNA machinery and no STE protein is produced (State 3). Promoter P2 is regulating the expression of a miRNA sponge that includes a non-coding RNA (Decoy) with multiple bulged mirFF4 binding sites at the 3' end. Therefore, when only promoter P2 is active, no protein output is produced (State 2). When both promoters P1 and P2 are active, the mirFF4 that is produced by the STE/mirFF4 mRNA regulated by promoter P1 is shunted away by mirFF4 sponge regulated by promoter P2, therefore allowing the production of the STE protein (State 1). (Figs. 3E-3H) The 4 input states and their respective outputs states of the AND gate circuit when using a fluorescent protein mKate2 as the output.

Figs. 4A-4B. mKate2 AND gate experiment results. (Fig. 4A) To examine the RNA-based logic AND gate design, it was encoded with mKate2 output. As promoter inputs for this design two human promoters we used, which are over-expressed in many human cancers: SSX1 and H2A1 (Input 1 and Input 2 respectively, whereas Input 1 encodes the mKate2 output and mirFF4). (Fig. 4B) The mKate2 output levels were measured for different designs, with respect to (a) the number of perfect-match FF4-BS encoded in input 1 and (b) two different architectures of sponge design in Input 2. X-axis annotations: M# represents Input 1 with # of FF4-BS encoded downstream to mKate2/mirFF4. For example, M3 represents Input 1 with 3 perfect-match FF4-BS, as shown in the gate illustration. S0, S1 and S2 represent three different sponge designs. S0 is a negative control transcript with no mirFF4-BS. Design S1 is Decoy transcript with 10 bulged FF4-BS encoded on the 3', as shown in the gate illustration. Design S2 is similar to S1, but with an additional circular intron with 10 bulged FF4-BS located upstream to the 10 bulged FF4-BS which are encoded in the transcript 3'. Therefore, the gate illustration represents design M3-S1 (surrounded with green dashed lines in the plot). Results are represented in mean mKate2 expression (P1), which is the average mKate2 for cells gated for SSC/FSC in FACS to remove cell clumps and debris. Error bars represent SEM. We did not test the Input 2 condition since it does not encode the output protein anyway. NT represents non-transfected cells.

Fig. 5. mKate2 AND gate experiment results. To again examine the RNA-based logic AND gate design, it was encoded with mKate2 output. ECFP was encoded in the sponge transcript to measure the degradation of sponge by the miRNA. SSX1 and H2A1 were used promoter inputs for this design: Input 1 and Input 2 respectively, whereas Input 1 encodes the mKate2 output and mirFF4. The mKate2 and ECFP output level for different experimental

settings were measured, with respect to (a) the number of perfect-match FF4-BS encoded in input 1 and (b) two different architectures of sponge design in Input 2. X-axis annotations: M# represents Input 1 with # of FF4-BS encoded downstream to mKate2/mirFF4.

Figs. 6A-6B. The design of multi-output AND-gate circuit. (Fig. 6A) When both promoters P1 and P2 are active, the mirFF4 that is produced by the TF/mirFF4 mRNA regulated by promoter P1 is shunted away by mirFF4 sponge regulated by promoter P2, therefore allowing the production of an artificial transcription factor (TF). The TF will further bind to its promoter and trigger the transcription of multiple user-defined outputs. (Fig. 6B) The output level of multi-output AND-gate is tunable. CXCL10 is CXCL1p regulating a GAL4BD-VP16AD harboring a mirFF4v2B intron and 10 downstream mirFF4-Bs. SSX10 is SSX1p regulating a GAL4BD-VP16AD harboring a mirFF4v2B intron and 10 downstream mirFF4-Bs. SSX*10 is truncated SSX1p in which part of the 5' UTR was removed together with the KOZAK sequence, regulating a GAL4BD-VP16AD harboring a mirFF4v2B intron and 10 downstream mirFF4-Bs. Sponge S0 is a negative control transcript WO mirFF4-BS. Sponge S2 is Decoy transcript with 10 bulged FF4-BS encoded on the 3, with an additional circular intron with 10 bulged mirFF4-BS located upstream to the 10 bulged mirFF4-BS which are encoded in the transcript 3'. In all samples, the mKate2 output is encoded in under a G5p (a promoter containing 5 GAL4 binding sites). The output levels are tunable by using different strength of promoters as P1 and different architecture of sponges.

Figs. 7A-7H. The design of several Boolean logic gates. Schematic illustration of RNA-based designs for AND, NAND, XNOR, NOR, NOT, XOR, IMPLY, NIMPLY gate. OP: Output protein; Nan: nascent RNA transcript.

Fig. 8. Anti-HER2 bispecific T cell engager (BiTE) and surface T cell engager (STE) trigger T cells to mediate robust tumor killing and IFN-γ secretion. HEK-293T (minimally expressing HER2) cells were transfected with various DNA constructs as indicated. 48hrs post transfection, various HEK-293T cells were harvested and co-cultured with human T cells for 5 hrs or 24 hrs. 5hr cytotoxicity by T cells was measured by LDH release assay and 24hr IFN-γ secretion by T cells was measured by IFN-γ ELISA. Data show that T cells mediate robust tumor killing and IFN-γ secretion on BiTE secreting tumor cells (group 1-2). The tumor killing and IFN-γ secretion correlate with HER2 expression level on tumor cells (group 1-2). T cells also mediate robust tumor killing and IFN-γ secretion on STE expressing tumor cells (group 3-6), and the cytotoxicity and IFN-γ secretion are independent of tumor

antigen (HER2) expression (group 3-6). Furthermore, T cells mediate minimal tumor killing and IFN-γ secretion when co-cultured with HEK-293T cells expressing non-BiTE and non-STE control proteins (group 7-9).

Figs. 9A-9C. Single-output AND gate architecture can be harnessed to fine tune T cell killing efficiency of tumor cells. HEK-293T cells were transfected with various DNA constructs as indicated. (Fig. 9A) Design of single-output AND gate driving STE expression. (Fig. 9B) Experiment result of mKate AND gate. (1,0) indicated cells transfected with P1 module only. (1,1) indicated cells transfected with P1 and P2 modules. (0,0) represents non-transfected cells. (Fig. 9C) Experiment result of STE AND gate. (1,0) indicated cells transfected with P1 module only. (1,1) indicated cells transfected with P1 and P2 modules. (0,0) indicated cells transfected with a non-STE protein. Ctrl indicated non-transfected cells. 48hrs post transfection, various HEK-293T cells were harvested and co-cultured with human T cells for 5 hrs. Cytotoxicity by T cells was measured by LDH release assay. Data show that T cells kill 293T transfected with P1 module (column 1) and the killing can be greatly enhanced by the AND gate architecture (column 2). T cells exhibit minimal killing on non-STE expressing cells (column 3 & 4).

Fig. 10. Anti-HER2 bispecific T cell engager (BiTE) and surface T cell engager (STE) trigger T cells to mediate robust tumor killing and IFN-γ secretion. Stable 4T1 cells (HER2-) expressing indicated DNA constructs were co-cultured with human T cells for 5 hrs or 24 hrs. 5 hr cytotoxicity by T cells was measured by LDH release assay and 24hr IFN-γ secretion by T cells was measured by IFN-γ ELISA. Data show that T cells mediate minimal killing and IFN-γ secretion on HER2- or STE- tumor cells. (group 1 & 3). T cells mediate robust tumor killing and IFN-γ secretion on STE-expressing tumor cells. (group 2). T cells also mediate robust tumor killing and IFN-γ secretion when co-cultured with cell mixtures consisting of low numbers of BiTE secreting cells with non-BiTE secreting tumor. This indicates minimal numbers of BiTE secreting cells in the tumor mass can elicit robust tumor mass killing and IFN-γ release (group 4).

Fig. 11. anti-HER2 bispecific T cell engager (BiTE) and surface T cell engager (STE) trigger T cells to mediate robust tumor killing on human breast cancer cell line. Stable MDA-MB453 (HER2+) cell lines were created by lentiviral transduction with various DNA constructs as indicated. Various MDA-MB453 cells were harvested and co-cultured with human T cells for 5 hrs. 5hr cytotoxicity by T cells was measured by LDH release assay.

Data show that T cells mediate robust tumor killing on BiTE secreting tumor cells (group 2). T cells also mediate robust tumor killing on STE expressing tumor cells (group 3-4). Furthermore, T cells mediate minimal tumor killing when co-cultured with parental MDA-MB453 tumor cell line (group 1).

Fig. 12. The design of 2 versions of STE. For version 1 (v1), anti-CD3e scFv is fused with an inert transmembrane protein (DARC). For version 2 (v2), anti-CD3e scFv is fused with human IgG1-Hinge-CH2-CH3 domain, followed by murine B7.1-transmembrane (TM) and cytoplasmic (CYP) domains.

Fig. 13. Surface T cell engager (STE) version 1 (v1) and version 2 (v2) both trigger T cells to mediate robust tumor killing on HEK-293T cells. Various inducible STE expressing HEK-293T cell lines were created by lentiviral transduction. Various HEK-293T cells were harvested and co-cultured with human T cells for 5 hrs. 5hr cytotoxicity by T cells was measured by LDH release assay. Data show that T cells mediate robust tumor killing on transfected STEv1 expressing tumor cells (column 2). T cells also mediate robust tumor killing on inducible STEv1 and STEv2 expressing tumor cells (column 3 and 4). Furthermore, T cells mediate minimal tumor killing when co-cultured with non-STE expressing HEK-293T cell line (column 1).

Fig. 14. AND gate architecture can be harnessed to fine tune T cell killing efficiency of tumor cells. (A) The design of multi-output AND gate for STE expression. (B) HEK-293T cells were transfected with various DNA constructs as indicated. (1,0) indicated cells transfected with STE only. (1,1) indicated cells transfected with STE and sponge. (0,0) indicated cells transfected with a non-STE protein. 48hrs post transfection, various HEK-293T cells were harvested and co-cultured with human T cells for 5 hrs. Cytotoxicity by T cells was measured by LDH release assay. Data show that T cells kill STE expressing (1,0) cells (column 2 and 4) and the killing can be greatly enhanced by the AND gate (1,1) architecture (column 3 and 5). T cells exhibit minimal killing on non-STE expressing cells (column 1).

Fig. 15. AND gate architecture can be harnessed to fine tune T cell killing efficiency of tumor cells. (A) The design of multi-output AND gate for STE expression. (B) HEK-293T cells were transfected with various DNA constructs as indicated. (1,0) indicated cells transfected with STE only. (1,1) indicated cells transfected with STE and sponge. (0,0) indicated cells transfected with a non-STE protein. 48hrs post transfection, various HEK-

293T cells were harvested and co-cultured with human T cells for 5 hrs. Cytotoxicity by T cells was measured by LDH release assay. Data show that T cells kill STE expressing (1,0) cells (column 3 and 5) and the killing can be greatly enhanced by the AND gate (1,1) architecture (column 4 and 6). T cells exhibit minimal killing on non-STE expressing cells (column 1). The killing on (1,0) condition is mainly caused by the leakage of GAL4 promoter output (column 2 v. 3 or 5). Further modification may be made to decrease the leakage of GAL4 promoter output (STE v1). We will decrease the GAL4 promoter leakage by removing the KOZAK sequence of STE v1, making STE v1 output self-degrading by adding miRNA binding sites at 3' end, and the combination of both mechanisms.

Fig. 16. GAL4-gate v2 architecture can be harnessed to fine tune T cell killing efficiency of tumor cells and exhibit less cytotoxicity at (1,0) state. (A) The design of multi-output AND gate for STE expression. (B) HEK-293T cells were transfected with various DNA constructs as indicated. (1,0) indicated cells transfected with STE only. (1,1) indicated cells transfected with STE and sponge. (0,0) indicated cells transfected with a non-STE protein. 48hrs post transfection, various HEK-293T cells were harvested and co-cultured with human T cells for 5 hrs. Cytotoxicity by T cells was measured by LDH release assay. Data show that T cells kill STE expressing (1,0) cells (column 3) and the killing can be enhanced by the AND gate (1,1) architecture (column 4). T cells exhibit minimal killing on not STE expressing cells (column 1). The killing on (1,0) state of this version is improved compared to GAL4 gate v1 architecture (v2 is more closer to basal level (0,0)). Further modification may be made to decrease the killing at (1,0) state. We will decrease the GAL4 promoter output at (1,0) state by adding miR binding sites at 3' end of STE gene.

Fig. 17. GAL4-gate v3 architecture can be harnessed to fine tune T cell killing efficiency of tumor cells and exhibit less cytotoxicity at (1,0) state. (A) The design of multi-output AND gate for STE expression. (B) HEK-293T cells were transfected with various DNA constructs as indicated. (1,0) indicated cells transfected with STE only. (1,1) indicated cells transfected with STE and sponge. (0,0) indicated cells transfected with a non-STE protein. 48hrs post transfection, various HEK-293T cells were harvested and co-cultured with human T cells for 5 hrs. Cytotoxicity by T cells was measured by LDH release assay. Data show that T cells minimally kill STE expressing (1,0) cells (column 3) and only reach efficient killing when the AND gate is active (1,1) (column 4). T cells exhibit minimal killing on not STE expressing cells (column 1). The killing on (1,0) state is as low as (0,0) state.

Further modification, such as increasing GAL4-VP16 output level or increasing GAL4 binding sites, can be done to enhance the killing efficacy of (1,1) state.

Fig. 18. Overview of Synthetic Tumor-Recruited Immuno-Cellular Therapy (STRICT). Panel 1: Tumor-targeting gene circuits, are designed to integrate the activity of two tumor-specific synthetic promoters and generate the expression of synthetic and natural immunomodulators only when both promoters are active, which provides high tumor-selectivity to our circuit; Panel 2: The circuit is delivered in vivo using hydrogel-based delivery; Panel 3: Only transduced cancer cells express synthetic Surface T-cells Engager (STE) and/or native immunomodulators that recruit T-cells to kill tumor cells; Panel 4: tumor cells are eliminated by activated T-cells.

Fig. 19. The design of RNA-only single-output AND gate. The RNA-based logic AND gate integrates the activity of two input promoters, P1 and P2, and generates and output only when both promoters are decidedly active. In this architecture, the output is a fluorescent protein mKate2. Promoter P1 is regulating the expression of an mKate2 mRNA which comprises a synthetic miRNA intron (miR1). We incorporated a negative autoregulatory feedback loop into the circuit by encoding perfect-match miR1 binding sites at the 3′ end of the mKate2/miR1 transcript (miR1-BS). Consequently, only when both promoters P1 and P2 are active, the mirR1 that is produced by the mKate2/miR1 mRNA regulated by promoter P1 is shunted out by the miR1 sponge regulated by promoter P2, therefore allowing the production of the mKate2 protein.

Fig. 20. RNA-only single-output AND gate design. The top panel depicts the design details of RNA-only single-output AND gate. The left table shows that miRNA binding sequences affect the sponging activity. The right panel shows that mKate2 fold-induction by each sponge and the ECFP level reduction by miR1.

Fig. 21. The number of binding sites in the sponge and the abundance of sponge transcripts affect the sponging activity. Left panel shows the design details of module 1 (M1) and various sponges (S67, S73, and S62). Right upper panel shows the raw output level of mKate2 and ECFP of various experimental conditions. Right lower panel shows the mKate2 fold induction by each sponge. SC represents control sponge (no binding sites).

Fig. 22. Sponge architectures affect the sponging activity. Left panel shows the design details of various sponges (S76, S99, S100, and S101). Right upper panel shows the raw output level of mKate2 and ECFP of various experimental conditions. Right lower panel

shows the mKate2 fold induction by each sponge. SC represents control sponge (no binding sites).

Fig. 23. miRNA backbone affects gate performance. Left panel shows the design details of module 1 (M) and various sponges (Sx and S76). Right upper panel shows the raw output level of mKate2 and ECFP of various experimental conditions. Right lower panel shows the mKate2 fold induction of various module 1 constructs (M1, M2A, and M2B are 3 versions of module 1, each consisting of a different miRNA backbone) by various sponges. SC represents control sponge (no binding sites).

Figs. 24A-24B. Doxycycline inducible STE can trigger T cells to efficiently kill OVCAR8 ovarian cancer cells, HEK-293T cells and secrete IFN-g. 3 versions of Dox-inducible STE (STE, STEv2, and STE-snap) all can trigger robust cellular killing and IFN-g secretion by T cell.

Fig. 25. Multiple-output circuit stringently kills tumor cells. (Fig. 25A) GAD outputted by the AND gate can target a third promoter (P3), which can express multiple proteins, such as STE and immunomodulatory molecules. (Fig. 25B) HEK-293T cells transfected with gene circuits encoding: HEK/DARC (0,0) – a non-STE protein; GAD gate (1,0) – the P1+P3 constructs only, where P3 expresses an STE; GAD gate (1,1) – the P1+P2+P3 constructs, where P3 expresses an STE; HEK/const – constitutively expressed STE. 48 h post-transfection, cells were co-cultured with human T cells for 5 hrs. Cytotoxicity was measured by LDH release assay. T cells killed efficiently only when AND gate is ON (1,1). T cells minimally kill STE-negative cells (0,0). Killing in the (1,0) state is as low as on (0,0) state. Increasing GAD expression the number GAD-binding sites may further enhance the efficacy of the (1,1) state.

Fig. 26. Synthetic tumor-specific promoters exhibit higher tumor specificity than native ones. (A) The top panel illustrates the design of synthetic tumor-specific promoters. 16 transcription factor binding sites were cloned in tandem upstream of a minimal promoter (late adenovirus promoter). The lower panel shows that synthetic tumor-specific promoters exhibit higher tumor specificity than native ones. H2A1p is a native tumor-specific promoters. S9 to S19 are selective examples of synthetic promoters and the parentheses denote their transcription factor binding sites. OVCAR8: ovarian cancer cells. IOSE120, IOSE386: immortalized normal ovarian epithelial cells. aHDF: adult human dermal fibroblast. CCD: normal colon fibroblast. MCF10A, MCF12A: immortalized normal breast cells. (B) The top

panel illustrates the design of synthetic tumor-specific promoters. 16 transcription factor binding sites were cloned in tandem upstream of a minimal promoter (late adenovirus promoter). The lower panel shows that synthetic tumor-specific promoters exhibit higher tumor specificity than native ones. SSX1 and H2A1p are native tumor-specific promoters. S9 to S28 are selective examples of synthetic promoters and the parentheses denote their transcription factor binding sites. aHDF: adult human dermal fibroblast. HOV-epi: primary ovarian epithelial cells. OVCAR8: ovarian cancer cells.

Fig. 27. Multi-output AND gate exhibits significantly higher output level in tumor cells than in normal cells. The circuit depicted at the top panel exhibits around 90-fold higher activity in tumor cells (OVCAR8) than in normal cells (ISOE120).

Fig. 28. Multi-output AND gate exhibits significantly higher output level in tumor cells than in normal cells. When both promoters are active, G8-F circuit exhibits around 90-fold higher activity in tumor cells (OVCAR8) than in normal cells (ISOE120). The output level of G8-F gate is also higher than the input promoter activity level.

Fig. 29. The output level of circuit on tumor cells can be tuned by modifying the number of GAD binding sites in the GAD promoter and adjusting the number of miRNA binding sites on the downstream output transcripts. The output of G8-F gate is also higher than the input promoter (S19p) activity.

Fig. 30. Multi-output AND gate exhibits significantly higher output level in tumor cells than in normal cells. When both promoters are active, G8-F circuit exhibits around 90-fold higher activity in tumor cells (OVCAR8) than in normal cells (ISOE120). The output of G8-F gate is also higher than the input promoter activity.

Fig. 31. Multi-output circuit specifically triggers T cells to kill tumors cells and secrete IFN-g. (A) STE triggers robust T- cell killing of circuit-transduced tumor cells (OVCAR8) but not normal cells (aHDF, HOV-epi). Circuit also triggers minimal tumor killing at state (1,0). (B) STE triggers robust T- cell killing of circuit-transduced tumor cells (OVCAR8) but not normal cells (aHDF, HOV-epi). Circuit also triggers minimal tumor killing at state (1,0). (C) T cells mediated strong IFN-g secretion by circuit-transduced tumor cells but not normal cells.

Fig. 32. Different multi-output circuits exhibit different levels of anti-tumor specificity. G8-Fv1 and G14-Fv1 triggers significantly higher tumor cell (OVCAR8) killing

than normal cell (IOSE386) killing. G8 (a promoter containing 8 GAL4 binding sites), G14 (a promoter containing 14 GAL4 binding sites)

Fig. 33. Different multi-output circuits exhibit different levels of anti-tumor specificity. (Fig. 33A) Several gate designs (G5-Fv1, G8-Fv1, G14-Fv1, G5-Fv2, G8-Fv2, G14-Fv2) can trigger significantly higher IFN-g secretion by T cells on tumor cells (OVCAR8) than normal cells (IOSE386). (Fig. 33B) G8-F gate triggers T cells to secrete copious amount of IFN-g on tumor cells (OVCAR8) but not normal cells (aHDF, HOV-epi).

Fig. 34. STEs potently decrease pancreatic tumor burden *in vivo*. NB508 tumor cells displaying doxycycline (Dox)-inducible STEs were injected subcutaneously. 10 days post-inoculation, mice were randomized into Dox-induced or untreated arms. Top panel: Significant growth reduction was observed in Dox-induced tumors (+Dox) vs. untreated controls (nt). Two +Dox mice in were sacrificed prematurely at day 17 due to skin irritation. Lower panel: Whole tumors dissected at day 21 post-treatment are significantly smaller.

Fig. 35. Combination immunotherapies triggered by STRICT reduced tumor burden significantly in an intraperitoneally-disseminated ovarian cancer model. (A) The experimental plan and treatment schedule. (B) Combination therapy triggered by STRICT significantly reduced tumor burden. The left panel represents the tumor burden of control groups. The right panel represents the tumor burden of treated groups. The parentheses denote the combination therapy strategy.

Fig. 36. Combination immunotherapies triggered by STRICT reduced tumor burden significantly in an intraperitoneally-disseminated ovarian cancer model. This is the same data as Fig. 35, but now all the groups are plotted in the same graph.

Fig. 37. Combination immunotherapies triggered by STRICT reduced tumor burden significantly in an intraperitoneally-disseminated ovarian cancer model. This is the same data as Fig. 35, but plotted differently.

Fig. 38. Combination immunotherapies triggered by STRICT reduced tumor burden significantly in an intraperitoneally-disseminated ovarian cancer model. This is the same data as Fig. 35, but plotted differently.

Fig. 39A. Combination immunotherapies triggered by STRICT reduced tumor burden significantly in an intraperitoneally-disseminated ovarian cancer model. This is the same data as Fig. 35, but tumor growth curves of individual mice and the average burden of each group were shown.

Fig. 40. Combination immunotherapies triggered by STRICT reduced tumor burden significantly in an intraperitoneally-disseminated ovarian cancer model. This is the same data as Fig. 35 except group S15p-GAD+G8p-STE-F were not shown, tumor burden of each imaging time point and the average burden of each group were shown. G8p (a promoter containing 8 GAL4 binding sites).

Fig. 41. Combination immunotherapies triggered by STRICT reduced tumor burden significantly in an intraperitoneally-disseminated ovarian cancer model. This is the same data as Fig. 35, but now the bioluminescent images of tumor burden of each individual mouse at day 36 post tumor inoculation were shown.

Fig. 42. Combination immunotherapies triggered by STRICT reduced tumor burden significantly in an intraperitoneally-disseminated ovarian cancer model. This is the same data as Fig. 35, but now the bioluminescent images of tumor burden of each individual mouse at day 43 post tumor inoculation were shown.

Fig. 43. Combination immunotherapies triggered by STRICT reduced tumor burden significantly in an intraperitoneally-disseminated ovarian cancer model. This is the same data as Fig. 35, but now the bioluminescent images of tumor burden of each individual mouse at day 7 and day 43 post tumor inoculation were shown.

Fig. 44. The pipeline of identifying cancer-specific synthetic promoters. A library of synthetic promoters driving mKate2 expression was introduced into normal cells and cancer cells with lentivirus. The mKate2 positive cells were sorted and next generation sequencing was utilized to identify the enriched synthetic promoter sequence for each cell type. The synthetic promoter sequences highly enriched in cancer cell but not in normal cells will be cloned and there tumor-specific activity will be further validated.

Fig. 45. The design of synthetic promoter library. Design 1 constitutive of all permutations of 8mer sequences built in tandem (12 time repeat) without spacer in between each 8mers. Design 2 constitutive of all permutations of 8mer sequences built in tandem (9 time repeat) with a 3mer spacer in between each 8mers. Design 3 constitutive of selective 11mer sequences built in tandem (7 time repeat) without a 3mer spacer in between each 11mers.

Fig. 46. The activity of selected synthetic promoters. The activity of 40 synthetic promoters isolated from FACS sorting was tested on 3 different cancer cell lines. We

observed that these 40 synthetic promoters can provide us a wide range of transcription activity.

Fig. 47. The normalized activity of selected synthetic promoters. The activity of 40 synthetic promoters isolated from FACS sorting were tested on 3 different cancer cell lines. We observed that these 40 synthetic promoters can provide us a wide range of transcription activity. The data is normalized to the constitutive promoter (UbCp) for each cell line.

DETAILED DESCRIPTION

Synthetic Tumor Recruited Immuno-Cellular Therapy (STRICT) of the present disclosure includes cell-specific diagnostic and therapeutic circuits (engineered genetic circuits/logic gates) having, in some embodiments, combinatorial immunomodulatory outputs (*e.g.*, antigens and cytokines). The cell-specific genetic circuits are based primarily on RNA, thus typically do not elicit adverse immunogenic reactions in a subject. The combinatorial immunomodulatory outputs may include, for example, Synthetic T Cell Engagers (STEs), Bi-directional T Cell Engagers (BiTEs), antibodies, antibody fragments, cytokines and other molecules that elicit a cytotoxic T cell response.

In some aspects of the present disclosure, GAL4 gates enable tunable multi-output combinatorial therapy. Additional key immune modulators, as circuit outputs, can be implemented for effective combinatorial therapy. In some embodiments, cytokines may be used to enhance immune cell function; for example, IL-12 may be used to enhance Th1 response and to revert to a suppressive tumor microenvironment. In some embodiments, chemokines may be used to recruit immune cells; for example, CCL21 may be used to recruit CCR7+ T cell populations. In some embodiments, immune checkpoint blockade inhibitors may be used to enhance anti-cancer immunity; for example, anti-PD1 mAb, anti-PDL1 mAb, and anti-CTLA4 mAb).

Further, in some embodiments, anti-HER2 BiTE triggers T cells to mediate robust HER2+ tumor killing and cytokine production. In some embodiments, various STEs can trigger T cell killing of various types of tumor cells. In some embodiments, RNA AND gate architecture can be harnessed to fine tune STE expressing level and T cell tumor killing efficiency. In some embodiments, a low ratio of BiTE secreting cells in whole tumor population is enough to trigger robust tumor killing.

As depicted in Figs. 2A and 2B, the methods provided herein lead to the targeted destruction of cancer cells. For example, tumor-identifying genetic circuits are first introduced into tumors by local injection or systemic administration (Figs. 2A(1) and 2B(1)). Then, tumor cells transduced with the genetic circuits display Surface T-cell Engagers (STEs) and express immunomodulatory molecules (Fig. 2A (2)). STEs engage T-cell receptors on local tumor-infiltrating T cells and trigger the T cells to eradicate tumor cells (Fig. 2A (3)). Tumor antigens released by the first wave of eradication then primes and recruits more tumor-reactive T cells (Fig. 2A (4)). Newly recruited polyclonal T cells eradicate more cancer cells, including other heterogeneous tumor cells and metastases not eradicated by the first-wave anti-tumor immune response (Fig. 2A (5)). Immune memory prevents tumor relapses.

Figs. 3A-3D depict RNA-based logic AND gates. The RNA-based logic AND gate integrates the activity of two input promoters, P1 and P2, and generates and output only when both promoters are decidedly active. In this architecture, the output is the Surface T-cell Engager (STE). Promoter P1 is regulating the expression of an STE mRNA that comprises a synthetic miRNA intron (mirFF4). A negative autoregulatory feedback loop was incorporated into the circuit by encoding perfect-match mirFF4 binding sites at the 3′ end of the STE/mirFF4 transcript (mirFF4-BS). Consequently, when only promoter P1 is active the STE mRNA is constantly degraded by the cellular miRNA machinery and no STE protein is produced (Fig. 3C, State 3). Promoter P2 regulates the expression of a miRNA sponge containing a non-coding RNA (Decoy) with multiple bulged mirFF4 binding sites at the 3′ end. Therefore, when only promoter P2 is active, no protein output is produced (Fig. 3B, State 2). When both promoters P1 and P2 are active, the mirFF4 that is produced by the STE/mirFF4 mRNA regulated by promoter P1 is tittered out by the mirFF4 sponge regulated by promoter P2, therefore allowing the production of the STE protein (Fig. 3A, State 1).

Some embodiments of the present disclosure provide engineered genetic circuits that include (a) a first nucleic acid comprising a first promoter operably linked to (i) a nucleotide sequence encoding an output messenger RNA (mRNA) containing an intronic micro RNA (miRNA) and (ii) a nucleotide sequence encoding at least one miRNA binding site complementary to the miRNA of (a)(i), and (b) a second nucleic acid comprising a second promoter different from the first promoter and operably linked to a nucleotide sequence encoding at least one miRNA binding site complementary to the miRNA of (a)(i).

In some embodiments, the output mRNA encodes an output protein that binds to a T cell surface marker. For example, an output protein may be a protein that elicits a cytotoxic T cell response. Thus, an output protein may be a receptor that binds to an antigen (e.g., a CD3 antigen) on the surface of a T cell. The surface marker may be, for example, CD3, CD4, CD 8 or CD45. Other T cell surface markers are encompassed by the present disclosure. In some embodiments, the output protein is an antibody or antibody fragment that binds specifically to the T cell surface antigen.

Specific non-limiting examples of output proteins are depict in Fig. 12. "STE v1" includes anti-CD3ε scFV V_L and V_H domains for triggering T cells. Thus, in some embodiments, the first nucleic acid of a genetic circuit comprises a first promoter operably linked to a nucleotide sequence encoding an output messenger RNA (mRNA) (containing an intronic micro RNA (miRNA)) that encodes anti-CD3ε scFV V_L and V_H domains of a transmembrane protein. "STE v2" includes anti-CD3e scFv fused with human IgG1-Hinge-CH2-CH3 domain, followed by murine B7.1-transmembrane (TM) and cytoplasmic (CYP) domains. Thus, in some embodiments, the first nucleic acid of a genetic circuit comprises a first promoter operably linked to a nucleotide sequence encoding an output messenger RNA (mRNA) (containing an intronic micro RNA (miRNA)) that encodes anti-CD3e scFv fused with human IgG1-Hinge-CH2-CH3 domain, followed by murine B7.1-transmembrane and cytoplasmic domains.

In some embodiments, the output mRNA encodes a chemokine, a cytokine or a checkpoint inhibitor.

In some embodiments, the first promoter and/or the second promoter is an inducible promoter. Typically, the first promoter is different from the second promoter. For example, the promoters in genetic circuit, in some embodiments, may be regulated by different input signals (*e.g.*, different transcription factors) present in a cell – Input 1 regulates the first promoter, Input 2 regulates the second promoter.

The first and/or second promoter (the first promoter, the second promoter, or both promoters) may be tumor-specific promoters (or disease-specific promoters), meaning that they are regulated by signals that are only expressed by tumor cells or cancer cells (or other disease cell) or by signals that are expressed in tumor/cancer cells at a level that is at least 30% (e.g., at least 40%, 50%, 60%, 70%, 80, 90%) higher than the level expressed in non-tumor/non-cancer cells.

Engineered nucleic acids of the genetic circuits, as provided herein, may include miRNA binding sites. A miRNA binding site is a nucleotide sequence to which a miRNA binds – a miRNA binding site is complementary the miRNA. Thus, a miRNA is said to bind to its cognate miRNA binding site. An engineered nucleic acid may contain 1-50 mirRNA binding sites. In some embodiments, an engineered nucleic acid encoding a decoy molecule (that functions to "soak up" cognate miRNA in a cell) encodes 5-10, 5-20 or 5-30 miRNA binding sites. In some embodiments, an engineered nucleic acid encoding a decoy molecule encodes 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 mRNA binding sites. In some embodiments, an engineered nucleic acid encoding an output mRNA, such as a STE mRNA, encodes 1-5 or 1-10 miRNA binding sites. In some embodiments, an engineered nucleic acid encoding an output mRNA encodes 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 mRNA binding sites. Typically, the number of miRNA binding sites on an mRNA encoding an immunomodulatory molecule is less than the number of miRNA binding sites on a decoy RNA (*e.g.*, a promoter operably linked to a nucleic acid encoding miRNA binding sites and, optionally, non-coding mRNA). The length of an miRNA, and thus a cognate mRNA binding site, may vary. In some embodiments, the length of an miRNA is 15-50, 15-40, 15-30 or 15-20 nucleotides. In some embodiments, the length of an miRNA is 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 nucleotides.

In some embodiments, an output protein is a transcription factor (e.g., a protein that binds to DNA to control the rate of transcription).

Engineered Nucleic Acids and Genetic Circuits

The present disclosure provides engineered genetic circuits that are capable of triggering, from within a tumor/cancer cell, immunotherapy against that tumor/cancer cell and surrounding cancer cells. An "genetic circuit" refers to a collection of molecules (*e.g.*, nucleic acids and proteins, such as transcription factors, co-factors and polymerases) that interact with each other in a cell to control expression of mRNA and proteins. Genetic circuits, as provided herein, typically include at least two nucleic acids, one encoding an output messenger RNA (mRNA) containing and intronic micro RNA (miRNA), and another encoding several miRNA binding sites. An "intronic miRNA" is a miRNA that is positioned within an mRNA transcript between two exons that together encode an output molecule. An intronic miRNA is "spliced out" of the mRNA transcript during transcript maturation. For

example, with reference to Fig. 3A, '*STE-EX1*-mirFF4-*STE-EX2*' (top row) represents a DNA sequence encoding micro RNA mirFF4 positioned between two exons of gene encoding a synthetic T cell engager (STE). The construct in the second row of Fig. 3 represents an mRNA transcript encoding the STE, undergoing maturation, whereby the intronic micro RNA mirFF4 is removed by RNA splicing. The mature mRNA encoding the STE may then be translated to produce the STE protein, depending on whether a decoy molecule (a molecule containing cognate mirFF4 binding sites) is present in the cell.

Thus, an "output messenger RNA" or "output mRNA" refers simply to mRNA encoded by a particular nucleotide sequence of an engineered nucleic acid. Output mRNA, typically including an intronic micro RNA, in some embodiments, encodes a output protein that binds to a T cell surface marker. In some embodiments, an output mRNA encodes an anti-cancer agent. An "anti-cancer" agent is any substance or molecule that, when exposed to a cancer cell, can be used to kill the cancer cell, or reduce the rate of cell division of the cancer cell (*e.g.*, by at least 10%, 20%, 30%, 40% or 50% relative to the cancer cell not exposed to the anti-cancer agent). In some embodiments, an output mRNA encodes a killer gene, a neoantigen, a metabolic enzyme that degrade metabolites on which cancer cells depend for growth and/or survival, a chemokine, a cytokine or a checkpoint inhibitor, as discussed elsewhere herein. Other anti-cancer agents are encompassed by the present disclosure.

Genetic circuits of the present disclosure may also be referred to as, or function as, "logic gates," which typically have two inputs and one output, although more or less inputs and/or outputs are encompassed by the present disclosure. Logic gates (*e.g.*, AND, OR, XOR, NOT, NAND, NOR and XNOR) may be described in terms of an "ON" state, in which an output is produced, and an "OFF" state, in which an output is not produced. With genetic logic gates, each "input" may be regulated by an independent promoter, each promoter responsible for activating transcription of a nucleic acid encoding an output or a molecule that regulates the production of and/or the expression level of an output molecule. For example, Figs. 3A-3D depict an AND logic gate – a genetic circuit that includes two constructs: one regulated by promoter P1, and one regulated by promoter P2. Transcription of the construct on the left, linked to P1, is activated in the presence of Input 1, while transcription of the construct on the right, linked to P2, is activated in the presence of Input 2. With this AND gate, the output molecule, STE protein, is only produced in the presence of

Input 1 and Input 2 (Fig. 3A). In the presence of only Input 2 (Fig. 3B) or in the presence of only Input 1 (Fig. 3C), STE protein is not produced. Likewise, if neither Input 1 nor Input 2 is available, STE protein is not produced (Fig. 3D). In the presence of only Input 1, STE mRNA transcript is produced; however, the presence of the excised intronic miRNA prevents translation of STE mRNA and production of STE protein (Fig. 3C). In the presence of both Input 1 and Input 2, both the STE mRNA transcript and excised intronic miRNA are still produced; however, the excised intronic miRNA is "soaked up" by the decoy miRNA binding sites, the transcription of which is activated by Input 2. Thus, much of the STE mRNA is free from bound miRNA and can be translated to produce STE protein.

Other logic gates are depicted in Figs. 7B-7H.

Fig. 7B depicts a logic gate comprising (a) a first nucleic acid comprising a promoter (P1) operably linked to (i) a nucleotide sequence encoding an output mRNA (OP-EX1---OB-EX2) containing an intronic miRNA (miRNA1), (ii) a nucleotide sequence encoding an intronic miRNA (miRNA3), and (iii) a nucleotide sequence encoding a miRNA binding site (miRNA2-BS (P)); (b) a second nucleic acid comprising a promoter (P2) operably linked to (i) a nucleotide sequence encoding an output mRNA (OP-EX1---OB-EX2) containing an intronic miRNA (miRNA2), (ii) a nucleotide sequence encoding an intronic miRNA (miRNA3) and (iii) a nucleotide sequence encoding a miRNA-BS (mirRNA1-BS (P)); and (c) a third nucleic acid comprising a promoter (Ps) operably linked to a nucleotide sequence encoding an output protein (OP) linked to a miRNA-BS (miRNA3-BS (P)), wherein miRNA1 is complementary to and binds to miRNA1-BS, miRNA3 is complementary to and binds to miRNA3-BS (P), and miRNA2 is complementary to and binds to miRNA2-BS (P).

Fig. 7C depicts a logic gate comprising (a) a first nucleic acid comprising a promoter (P1) operably linked to (i) a nucleotide sequence encoding a nascent RNA transcript (Nan-EX1---Nan-EX2) (*e.g.*, a non-coding RNA transcript or and RNA transcript encoding a protein) containing an intronic miRNA (miRNA1) and (ii) a nucleotide sequence encoding four miRNA binding sites (miRNA2-BS (Bx4)); (b) a second nucleic acid comprising a promoter (P2) operably linked to (i) a nucleotide sequence encoding a nascent RNA transcript (Nan-EX1---Nan-EX2) containing an intronic miRNA (miRNA2), and (ii) a nucleotide sequence encoding four miRNA binding sites (miRNA1-BS (Bx4); and (c) a third nucleic acid comprising a promoter (Ps) operably linked to a nucleic acid encoding an output protein (OP) linked to (i) a first miRNA binding site (miRNA1-BS (P)) and (ii) a second

miRNA binding site (miRNA2-BS (P)), wherein miRNA1 is complementary to and bind to miRNA1-BS (Bx4) and miRNA2 is complementary to and bind to miRNA2-BS (Bx4).

Fig. 7D depicts a logic gate comprising (a) a first nucleic acid comprising a promoter (P1) operably linked to a nucleotide sequence encoding a nascent RNA transcript (Nan-EX1---Nan-EX2) containing an intronic miRNA (miRNA1); (b) a second nucleic acid comprising a promoter (P2) operably linked to a nucleotide sequence encoding a nascent RNA transcript (Nan-EX1---Nan-EX2) containing an intronic miRNA (miRNA2); and (c) a third nucleic acid comprising a promoter (Ps) operably linked to a nucleic acid encoding an output protein (OP) linked to (i) a first miRNA binding site (miRNA1-BS (P)) and (ii) a second miRNA binding site (miRNA2-BS (P)), wherein miRNA1 is complementary to and binds to miRNA1-BS (P) and miRNA2 is complementary to and binds to miRNA2-BS.

Fig. 7E depicts a logic gate comprising (a) a first nucleic acid comprising a promoter (P1) operably linked to a nucleotide sequence encoding a nascent RNA transcript (Nan-EX1---Nan-EX2) containing an intronic microRNA (miRNA); and (b) a second nucleic acid comprising a promoter (Ps) operably linked to a nucleotide sequence encoding an output protein (OP) linked to a miRNA binding site (miRNA1-BS (P)), wherein miRNA1 is complementary to and binds to miRNA-BS (P).

Fig. 7F depicts a logic gate comprising (a) a first nucleic acid comprising a promoter (P1) operably linked to (i) a nucleotide sequence encoding an output mRNA (OP-EX1---OP-EX2) containing an intronic miRNA (miRNA1) and (ii) four miRNA binding sites (miRNA2-BS (Bx4)); and (b) a second nucleic acid comprising a promoter (P2) operably linked to (i) a nucleotide sequence encoding an output mRNA (OP-EX1---OP-EX2) containing an intronic miRNA (miRNA2) and (ii) four miRNA binding sites (miRNA1-BS (Bx4), wherein miRNA1 is complementary to and binds to miRNA1-BS (Bx4) and miRNA2 is complementary to and binds to miRNA2-BS (Bx4).

Fig. 7G depicts a logic gate comprising (a) a first nucleic acid comprising a promoter (P1) operably linked to a nucleotide sequence encoding a nascent RNA transcript (Nan-EX1---Nan-EX2) containing an intronic miRNA (miRNA1); (b) a second nucleic acid comprising a promoter (P2) operably linked to a nucleotide sequence encoding an output protein (OP); and (c) a third nucleic acid comprising a promoter (Ps) encoding an output protein (OP) linked to an miRNA binding site (miRNA1-BS (P), wherein miRNA1 is complementary to and binds to miRNA1-BS (P).

Fig. 7H depicts a logic gate comprising (a) a first nucleic acid comprising a promoter (P1) operably linked to a nucleotide sequence encoding an output protein (OP) linked to a miRNA binding site (miRNA1-BS); and (b) a second nucleic acid comprising a promoter (P2) operably linked to a nucleotide sequence encoding a nascent RNA transcript (Nan-EX1---Nan-EX2) containing an intronic miRNA (miRNA1), wherein miRNA1 is complementary to and binds to miRNA1-BS (P).

A "nucleic acid" is at least two nucleotides covalently linked together, and in some instances, may contain phosphodiester bonds (*e.g.*, a phosphodiester "backbone"). An "engineered nucleic acid" (also referred to as a "construct") is a nucleic acid that does not occur in nature. It should be understood, however, that while an engineered nucleic acid as a whole is not naturally-occurring, it may include nucleotide sequences that occur in nature. In some embodiments, an engineered nucleic acid comprises nucleotide sequences from different organisms (*e.g.*, from different species). For example, in some embodiments, an engineered nucleic acid includes a murine nucleotide sequence, a bacterial nucleotide sequence, a human nucleotide sequence, and/or a viral nucleotide sequence. Engineered nucleic acids include recombinant nucleic acids and synthetic nucleic acids. A "recombinant nucleic acid" is a molecule that is constructed by joining nucleic acids (*e.g.*, isolated nucleic acids, synthetic nucleic acids or a combination thereof) and, in some embodiments, can replicate in a living cell. A "synthetic nucleic acid" is a molecule that is amplified or chemically, or by other means, synthesized. A synthetic nucleic acid includes those that are chemically modified, or otherwise modified, but can base pair with naturally-occurring nucleic acid molecules. Recombinant and synthetic nucleic acids also include those molecules that result from the replication of either of the foregoing.

In some embodiments, a nucleic acid of the present disclosure is considered to be a nucleic acid analog, which may contain, at least in part, other backbones comprising, for example, phosphoramide, phosphorothioate, phosphorodithioate, O-methylphophoroamidite linkages and/or peptide nucleic acids. A nucleic acid may be single-stranded (ss) or double-stranded (ds), as specified, or may contain portions of both single-stranded and double-stranded sequence. In some embodiments, a nucleic acid may contain portions of triple-stranded sequence. A nucleic acid may be DNA, both genomic and/or cDNA, RNA or a hybrid, where the nucleic acid contains any combination of deoxyribonucleotides and ribonucleotides (*e.g.*, artificial or natural), and any combination of bases, including uracil,

adenine, thymine, cytosine, guanine, inosine, xanthine, hypoxanthine, isocytosine and isoguanine.

Nucleic acids of the present disclosure may include one or more genetic elements. A "genetic element" refers to a particular nucleotide sequence that has a role in nucleic acid expression (*e.g.*, promoter, enhancer, terminator) or encodes a discrete product of an engineered nucleic acid (*e.g.*, a nucleotide sequence encoding a guide RNA, a protein and/or an RNA interference molecule, such as siRNA or miRNA).

Nucleic acids of the present disclosure may be produced using standard molecular biology methods (*see, e.g.*, *Green and Sambrook, Molecular Cloning*, A Laboratory Manual, 2012, Cold Spring Harbor Press).

In some embodiments, nucleic acids are produced using GIBSON ASSEMBLY® Cloning (*see, e.g.*, Gibson, D.G. *et al. Nature Methods*, 343–345, 2009; and Gibson, D.G. *et al. Nature Methods*, 901–903, 2010, each of which is incorporated by reference herein). GIBSON ASSEMBLY® typically uses three enzymatic activities in a single-tube reaction: 5′ exonuclease, the 3′ extension activity of a DNA polymerase and DNA ligase activity. The 5′ exonuclease activity chews back the 5′ end sequences and exposes the complementary sequence for annealing. The polymerase activity then fills in the gaps on the annealed regions. A DNA ligase then seals the nick and covalently links the DNA fragments together. The overlapping sequence of adjoining fragments is much longer than those used in Golden Gate Assembly, and therefore results in a higher percentage of correct assemblies.

In some embodiments, an engineered nucleic acid is delivered to a cell on a vector. A "vector" refers to a nucleic acid (*e.g.*, DNA) used as a vehicle to artificially carry genetic material (*e.g.*, an engineered nucleic acid) into a cell where, for example, it can be replicated and/or expressed. In some embodiments, a vector is an episomal vector (*see, e.g.*, Van Craenenbroeck K. *et al. Eur. J. Biochem.* 267, 5665, 2000, incorporated by reference herein). A non-limiting example of a vector is a plasmid (*e.g.*, Fig. 3). Plasmids are double-stranded generally circular DNA sequences that are capable of automatically replicating in a host cell. Plasmid vectors typically contain an origin of replication that allows for semi-independent replication of the plasmid in the host and also the transgene insert. Plasmids may have more features, including, for example, a "multiple cloning site," which includes nucleotide overhangs for insertion of a nucleic acid insert, and multiple restriction enzyme consensus sites to either side of the insert. Another non-limiting example of a vector is a viral vector.

Thus, in some embodiments, engineered genetic circuits are delivered to cells (e.g., cancer cells) using a viral delivery system (e.g., retroviral, adenoviral, adeno-association, helper-dependent adenoviral systems, hybrid adenoviral systems, herpes simplex, pox virus, lentivirus, Epstein–Barr virus) or a non-viral delivery system (e.g., physical: naked DNA, DNA bombardment, electroporation, hydrodynamic, ultrasound or magnetofection; or chemical: cationic lipids, different cationic polymers or lipid polymer) (Nayerossadat N *et al. Adv Biomed Res*. 2012; 1: 27, incorporated herein by reference). In some embodiments, the non-viral based deliver system is a hydrogel-based delivery system (*see, e.g.*, Brandl F, *et al. Journal of Controlled Release*, 2010, 142(2): 221-228, incorporated herein by reference).

A microRNA ("miRNA") is a small non-coding RNA molecule (*e.g.*, containing about 22 nucleotides) found in plants, animals, and some viruses, which typically functions under wild-type conditions in RNA silencing and post-transcriptional regulation of gene expression.

Genetic Elements

Expression of engineered nucleic acids is driven by a promoter operably linked to a nucleic acid containing, for example, a nucleic acid encoding a molecule of interest. A "promoter" refers to a control region of a nucleic acid sequence at which initiation and rate of transcription of the remainder of a nucleic acid sequence are controlled. A promoter drives expression or drives transcription of the nucleic acid sequence that it regulates. A promoter may also contain sub-regions at which regulatory proteins and molecules may bind, such as RNA polymerase and other transcription factors. Promoters may be constitutive, inducible, activatable, repressible, tissue-specific or any combination thereof.

Herein, a promoter is considered to be "operably linked" when it is in a correct functional location and orientation in relation to a nucleic acid sequence it regulates to control ("drive") transcriptional initiation and/or expression of that sequence.

A promoter may be one naturally associated with a gene or sequence, as may be obtained by isolating the 5' non-coding sequences located upstream of the coding segment of a given gene or sequence. Such a promoter can be referred to as "endogenous."

In some embodiments, a coding nucleic acid sequence may be positioned under the control of a recombinant or heterologous promoter, which refers to a promoter that is not normally associated with the encoded sequence in its natural environment. Such promoters

may include promoters of other genes; promoters isolated from any other cell; and synthetic promoters or enhancers that are not "naturally occurring" such as, for example, those that contain different elements of different transcriptional regulatory regions and/or mutations that alter expression through methods of genetic engineering that are known in the art. In addition to producing nucleic acid sequences of promoters and enhancers synthetically, sequences may be produced using recombinant cloning and/or nucleic acid amplification technology, including polymerase chain reaction (PCR) (see U.S. Pat. No. 4,683,202 and U.S. Pat. No. 5,928,906).

In some embodiments, a promoter is an "inducible promoter," which refer to a promoter that is characterized by regulating (*e.g.*, initiating or activating) transcriptional activity when in the presence of, influenced by or contacted by an inducer signal. An inducer signal may be endogenous or a normally exogenous condition (*e.g.*, light), compound (*e.g.*, chemical or non-chemical compound) or protein that contacts an inducible promoter in such a way as to be active in regulating transcriptional activity from the inducible promoter. Thus, a "signal that regulates transcription" of a nucleic acid refers to an inducer signal that acts on an inducible promoter. A signal that regulates transcription may activate or inactivate transcription, depending on the regulatory system used. Activation of transcription may involve directly acting on a promoter to drive transcription or indirectly acting on a promoter by inactivation a repressor that is preventing the promoter from driving transcription. Conversely, deactivation of transcription may involve directly acting on a promoter to prevent transcription or indirectly acting on a promoter by activating a repressor that then acts on the promoter.

The administration or removal of an inducer signal results in a switch between activation and inactivation of the transcription of the operably linked nucleic acid sequence. Thus, the active state of a promoter operably linked to a nucleic acid sequence refers to the state when the promoter is actively regulating transcription of the nucleic acid sequence (i.e., the linked nucleic acid sequence is expressed). Conversely, the inactive state of a promoter operably linked to a nucleic acid sequence refers to the state when the promoter is not actively regulating transcription of the nucleic acid sequence (*i.e.*, the linked nucleic acid sequence is not expressed).

An inducible promoter of the present disclosure may be induced by (or repressed by) one or more physiological condition(s), such as changes in light, pH, temperature, radiation,

osmotic pressure, saline gradients, cell surface binding, and the concentration of one or more extrinsic or intrinsic inducing agent(s). An extrinsic inducer signal or inducing agent may comprise, without limitation, amino acids and amino acid analogs, saccharides and polysaccharides, nucleic acids, protein transcriptional activators and repressors, cytokines, toxins, petroleum-based compounds, metal containing compounds, salts, ions, enzyme substrate analogs, hormones or combinations thereof.

Inducible promoters of the present disclosure include any inducible promoter described herein or known to one of ordinary skill in the art. Examples of inducible promoters include, without limitation, chemically/biochemically-regulated and physically-regulated promoters such as alcohol-regulated promoters, tetracycline-regulated promoters (*e.g.*, anhydrotetracycline (aTc)-responsive promoters and other tetracycline-responsive promoter systems, which include a tetracycline repressor protein (tetR), a tetracycline operator sequence (tetO) and a tetracycline transactivator fusion protein (tTA)), steroid-regulated promoters (*e.g.*, promoters based on the rat glucocorticoid receptor, human estrogen receptor, moth ecdysone receptors, and promoters from the steroid/retinoid/thyroid receptor superfamily), metal-regulated promoters (*e.g.*, promoters derived from metallothionein (proteins that bind and sequester metal ions) genes from yeast, mouse and human), pathogenesis-regulated promoters (*e.g.*, induced by salicylic acid, ethylene or benzothiadiazole (BTH)), temperature/heat-inducible promoters (*e.g.*, heat shock promoters), and light-regulated promoters (*e.g.*, light responsive promoters from plant cells).

In some embodiments, an inducer signal of the present disclosure is isopropyl β-D-1-thiogalactopyranoside (IPTG), which is a molecular mimic of allolactose, a lactose metabolite that triggers transcription of the lac operon, and it is therefore used to induce protein expression where the gene is under the control of the lac operator. IPTG binds to the lac repressor and releases the tetrameric repressor from the lac operator in an allosteric manner, thereby allowing the transcription of genes in the lac operon, such as the gene coding for beta-galactosidase, a hydrolase enzyme that catalyzes the hydrolysis of β-galactosides into monosaccharides. The sulfur (S) atom creates a chemical bond which is non-hydrolyzable by the cell, preventing the cell from metabolizing or degrading the inducer. IPTG is an effective inducer of protein expression, for example, in the concentration range of 100 μM to 1.0 mM. Concentration used depends on the strength of induction required, as well as the genotype of cells or plasmid used. If lacIq, a mutant that over-produces the lac repressor, is

present, then a higher concentration of IPTG may be necessary. In blue-white screen, IPTG is used together with X-gal. Blue-white screen allows colonies that have been transformed with the recombinant plasmid rather than a non-recombinant one to be identified in cloning experiments.

Other inducible promoter systems are known in the art and may be used in accordance with the present disclosure.

Immunomodulatory Agents

An immunomodulatory agent is an agent (*e.g.*, protein) that regulates an immune response. The present disclosure provides, in some embodiments, engineered genetic circuits that include nucleic acids encoding immunomodulatory agents that are expressed at the surface of, or secreted from, a cancerous cell or secreted from a cancerous cell.

In some embodiments, the immunomodulatory agent is a synthetic T cell engager (STE). A "synthetic T cell engager" is a molecule (*e.g.*, protein) that binds to (*e.g.*, through a ligand-receptor binding interaction) a molecule on the surface of a T cell (*e.g.*, a cytotoxic T cell), or otherwise elicits a cytotoxic T cell response. In some embodiments, an STE is a receptor that binds to a ligand on the surface of a T cell. In some embodiments, an STE is an anti-CD3 antibody or antibody fragment. A STE of the present disclosure is typically expressed at the surface of, or secreted from, a cancer cell or other disease cell to which a nucleic acid encoding the STEs is delivered.

Examples of STEs of the present disclosure include antibodies, antibody fragments and receptors that binds to T cell surface antigens. T cell surface antigens include, for example, CD3, CD4, CD 8 and CD45. STEs expressed by the genetic circuits of the present disclosure may also be selected from any of the immunomodulatory agents described below.

In some embodiments, a genetic circuit of the present disclosure modulates expression of a chemokine, a cytokine or a checkpoint inhibitor.

Immunomodulatory agents include immunostimulatory agents and immunoinhibitory agents. As used herein, an immunostimulatory agent is an agent that stimulates an immune response (including enhancing a pre-existing immune response) in a subject to whom it is administered, whether alone or in combination with another agent. Examples include antigens, adjuvants (*e.g.*, TLR ligands such as imiquimod, imidazoquinoline, nucleic acids comprising an unmethylated CpG dinucleotide, monophosphoryl lipid A or other

lipopolysaccharide derivatives, single-stranded or double-stranded RNA, flagellin, muramyl dipeptide), cytokines including interleukins (*e.g.*, IL-2, IL-7, IL-15 (or superagonist/mutant forms of these cytokines), IL-12, IFN-gamma, IFN-alpha, GM-CSF, FLT3-ligand, etc.), immunostimulatory antibodies (*e.g.*, anti-CTLA-4, anti-CD28, anti-CD3, or single chain/antibody fragments of these molecules), and the like.

As used herein, an immunoinhibitory agent is an agent that inhibits an immune response in a subject to whom it is administered, whether alone or in combination with another agent. Examples include steroids, retinoic acid, dexamethasone, cyclophosphamide, anti-CD3 antibody or antibody fragment, and other immunosuppressants.

Antigens may be, without limitation, a cancer antigen, a self-antigen, a microbial antigen, an allergen, or an environmental antigen. An antigen may be peptide, lipid, or carbohydrate in nature, but it is not so limited.

A cancer antigen is an antigen that is expressed preferentially by cancer cells (*e.g.*, it is expressed at higher levels in cancer cells than on non-cancer cells) and in some instances it is expressed solely by cancer cells. The cancer antigen may be expressed within a cancer cell or on the surface of the cancer cell. The cancer antigen may be MART-1/Melan-A, gp100, adenosine deaminase-binding protein (ADAbp), FAP, cyclophilin b, colorectal associated antigen (CRC)--C017-1A/GA733, carcinoembryonic antigen (CEA), CAP-1, CAP-2, etv6, AML1, prostate specific antigen (PSA), PSA-1, PSA-2, PSA-3, prostate-specific membrane antigen (PSMA), T cell receptor/CD3-zeta chain, and CD20. The cancer antigen may be selected from the group consisting of MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A5, MAGE-A6, MAGE-A7, MAGE-A8, MAGE-A9, MAGE-A10, MAGE-A11, MAGE-A12, MAGE-Xp2 (MAGE-B2), MAGE-Xp3 (MAGE-B3), MAGE-Xp4 (MAGE-B4), MAGE-C1, MAGE-C2, MAGE-C3, MAGE-C4, MAGE-C5). The cancer antigen may be selected from the group consisting of GAGE-1, GAGE-2, GAGE-3, GAGE-4, GAGE-5, GAGE-6, GAGE-7, GAGE-8, GAGE-9. The cancer antigen may be selected from the group consisting of BAGE, RAGE, LAGE-1, NAG, GnT-V, MUM-1, CDK4, tyrosinase, p53, MUC family, HER2/neu, p21ras, RCAS1, α-fetoprotein, E-cadherin, α-catenin, β-catenin, γ-catenin, p120ctn, gp100Pmel117, PRAME, NY-ESO-1, cdc27, adenomatous polyposis coli protein (APC), fodrin, Connexin 37, Ig-idiotype, p15, gp75, GM2 ganglioside, GD2 ganglioside, human papilloma virus proteins, Smad family of tumor antigens, lmp-1, P1A, EBV-encoded nuclear antigen (EBNA)-1, brain glycogen phosphorylase, SSX-1, SSX-2

(HOM-MEL-40), SSX-1, SSX-4, SSX-5, SCP-1 and CT-7, CD20, and c-erbB-2.

Cells and Cell Expression

Engineered genetic circuits of the present disclosure are typically delivered systemically and activated (transcription of the circuits are activated) conditionally (based on the presence or absence of input signals) in a particular cell type, such as a cancerous cell, a benign tumor cell or other disease cell. Thus, in some embodiments, genetic circuits (logic gates) are delivered to a subject having tumor cells or cancer cells, and the genetic circuits (logic gates) are expressed in the tumor cells or cancer cells.

A cancerous cell may be any type of cancerous cell, including, but not limited to, premalignant neoplasms, malignant tumors, metastases, or any disease or disorder characterized by uncontrolled cell growth such that it would be considered cancerous or precancerous. The cancer may be a primary or metastatic cancer. Cancers include, but are not limited to, ocular cancer, biliary tract cancer, bladder cancer, pleura cancer, stomach cancer, ovary cancer, meninges cancer, kidney cancer, brain cancer including glioblastomas and medulloblastomas, breast cancer, cervical cancer, choriocarcinoma, colon cancer, endometrial cancer, esophageal cancer, gastric cancer, hematological neoplasms including acute lymphocytic and myelogenous leukemia, multiple myeloma, AIDS-associated leukemias and adult T-cell leukemia lymphoma, intraepithelial neoplasms including Bowen's disease and Paget's disease, liver cancer, lung cancer, lymphomas including Hodgkin's disease and lymphocytic lymphomas, neuroblastomas, oral cancer including squamous cell carcinoma, ovarian cancer including those arising from epithelial cells, stromal cells, germ cells and mesenchymal cells, pancreatic cancer, prostate cancer, rectal cancer, sarcomas including leiomyosarcoma, rhabdomyosarcoma, liposarcoma, fibrosarcoma, and osteosarcoma, skin cancer including melanoma, Kaposi's sarcoma, basocellular cancer, and squamous cell cancer, testicular cancer including germinal tumors such as seminoma, non-seminoma, teratomas, choriocarcinomas, stromal tumors and germ cell tumors, thyroid cancer including thyroid adenocarcinoma and medullar carcinoma, and renal cancer including adenocarcinoma and Wilms' tumor. Commonly encountered cancers include breast, prostate, lung, ovarian, colorectal, and brain cancer. In some embodiments, the tumor is a melanoma, carcinoma, sarcoma, or lymphoma.

Engineered nucleic acids of the present disclosure may be expressed in a broad range of host cell types. In some embodiments, engineered nucleic acids are expressed in mammalian cells (*e.g.*, human cells), bacterial cells (*Escherichia coli* cells), yeast cells, insect cells, or other types of cells. Engineered nucleic acids of the present disclosure may be expressed *in vivo*, *e.g.*, in a subject such as a human subject.

In some embodiments, engineered nucleic acids are expressed in mammalian cells. For example, in some embodiments, engineered nucleic acids are expressed in human cells, primate cells (*e.g.*, vero cells), rat cells (*e.g.*, GH3 cells, OC23 cells) or mouse cells (*e.g.*, MC3T3 cells). There are a variety of human cell lines, including, without limitation, human embryonic kidney (HEK) cells, HeLa cells, cancer cells from the National Cancer Institute's 60 cancer cell lines (NCI60), DU145 (prostate cancer) cells, Lncap (prostate cancer) cells, MCF-7 (breast cancer) cells, MDA-MB-438 (breast cancer) cells, PC3 (prostate cancer) cells, T47D (breast cancer) cells, THP-1 (acute myeloid leukemia) cells, U87 (glioblastoma) cells, SHSY5Y human neuroblastoma cells (cloned from a myeloma) and Saos-2 (bone cancer) cells. In some embodiments, engineered nucleic acids are expressed in human embryonic kidney (HEK) cells (*e.g.*, HEK 293 or HEK 293T cells). In some embodiments, engineered nucleic acids are expressed in stem cells (*e.g.*, human stem cells) such as, for example, pluripotent stem cells (*e.g.*, human pluripotent stem cells including human induced pluripotent stem cells (hiPSCs)). A "stem cell" refers to a cell with the ability to divide for indefinite periods in culture and to give rise to specialized cells. A "pluripotent stem cell" refers to a type of stem cell that is capable of differentiating into all tissues of an organism, but not alone capable of sustaining full organismal development. A "human induced pluripotent stem cell" refers to a somatic (*e.g.*, mature or adult) cell that has been reprogrammed to an embryonic stem cell-like state by being forced to express genes and factors important for maintaining the defining properties of embryonic stem cells (*see, e.g.*, Takahashi and Yamanaka, Cell 126 (4): 663–76, 2006, incorporated by reference herein). Human induced pluripotent stem cell cells express stem cell markers and are capable of generating cells characteristic of all three germ layers (ectoderm, endoderm, mesoderm).

Additional non-limiting examples of cell lines that may be used in accordance with the present disclosure include 293-T, 293-T, 3T3, 4T1, 721, 9L, A-549, A172, A20, A253, A2780, A2780ADR, A2780cis, A431, ALC, B16, B35, BCP-1, BEAS-2B, bEnd.3, BHK-21, BR 293, BxPC3, C2C12, C3H-10T1/2, C6, C6/36, Cal-27, CGR8, CHO, CML T1, CMT,

COR-L23, COR-L23/5010, COR-L23/CPR, COR-L23/R23, COS-7, COV-434, CT26, D17, DH82, DU145, DuCaP, E14Tg2a, EL4, EM2, EM3, EMT6/AR1, EMT6/AR10.0, FM3, H1299, H69, HB54, HB55, HCA2, Hepa1c1c7, High Five cells, HL-60, HMEC, HT-29, HUVEC, J558L cells, Jurkat, JY cells, K562 cells, KCL22, KG1, Ku812, KYO1, LNCap, Ma-Mel 1, 2, 3....48, MC-38, MCF-10A, MCF-7, MDA-MB-231, MDA-MB-435, MDA-MB-468, MDCK II, MG63, MONO-MAC 6, MOR/0.2R, MRC5, MTD-1A, MyEnd, NALM-1, NCI-H69/CPR, NCI-H69/LX10, NCI-H69/LX20, NCI-H69/LX4, NIH-3T3, NW-145, OPCN/OPCT Peer, PNT-1A/PNT 2, PTK2, Raji, RBL cells, RenCa, RIN-5F, RMA/RMAS, S2, Saos-2 cells, Sf21, Sf9, SiHa, SKBR3, SKOV-3, T-47D, T2, T84, THP1, U373, U87, U937, VCaP, WM39, WT-49, X63, YAC-1 and YAR cells.

Cells of the present disclosure, in some embodiments, are modified. A modified cell is a cell that contains an exogenous nucleic acid or a nucleic acid that does not occur in nature. In some embodiments, a modified cell contains a mutation in a genomic nucleic acid. In some embodiments, a modified cell contains an exogenous independently replicating nucleic acid (*e.g.*, an engineered nucleic acid present on an episomal vector). In some embodiments, a modified cell is produced by introducing a foreign or exogenous nucleic acid into a cell. A nucleic acid may be introduced into a cell by conventional methods, such as, for example, electroporation (*see, e.g.*, Heiser W.C. *Transcription Factor Protocols: Methods in Molecular Biology*™ 2000; 130: 117-134), chemical (*e.g.*, calcium phosphate or lipid) transfection (*see, e.g.*, Lewis W.H., *et al.*, *Somatic Cell Genet.* 1980 May; 6(3): 333-47; Chen C., et al., *Mol Cell Biol.* 1987 August; 7(8): 2745–2752), fusion with bacterial protoplasts containing recombinant plasmids (*see, e.g.*, Schaffner W. *Proc Natl Acad Sci USA.* 1980 Apr; 77(4): 2163-7), transduction, conjugation, or microinjection of purified DNA directly into the nucleus of the cell (*see, e.g.*, Capecchi M.R. *Cell.* 1980 Nov; 22(2 Pt 2): 479-88).

In some embodiments, a cell is modified to express a reporter molecule. In some embodiments, a cell is modified to express an inducible promoter operably linked to a reporter molecule (*e.g.*, a fluorescent protein such as green fluorescent protein (GFP) or other reporter molecule).

In some embodiments, a cell is modified to overexpress an endogenous protein of interest (*e.g.*, via introducing or modifying a promoter or other regulatory element near the endogenous gene that encodes the protein of interest to increase its expression level). In some embodiments, a cell is modified by mutagenesis. In some embodiments, a cell is

modified by introducing an engineered nucleic acid into the cell in order to produce a genetic change of interest (*e.g.*, via insertion or homologous recombination).

In some embodiments, an engineered nucleic acid may be codon-optimized, for example, for expression in mammalian cells (*e.g.*, human cells) or other types of cells. Codon optimization is a technique to maximize the protein expression in living organism by increasing the translational efficiency of gene of interest by transforming a DNA sequence of nucleotides of one species into a DNA sequence of nucleotides of another species. Methods of codon optimization are well-known.

Engineered nucleic acids of the present disclosure may be transiently expressed or stably expressed. "Transient cell expression" refers to expression by a cell of a nucleic acid that is not integrated into the nuclear genome of the cell. By comparison, "stable cell expression" refers to expression by a cell of a nucleic acid that remains in the nuclear genome of the cell and its daughter cells. Typically, to achieve stable cell expression, a cell is co-transfected with a marker gene and an exogenous nucleic acid (*e.g.*, engineered nucleic acid) that is intended for stable expression in the cell. The marker gene gives the cell some selectable advantage (*e.g.*, resistance to a toxin, antibiotic, or other factor). Few transfected cells will, by chance, have integrated the exogenous nucleic acid into their genome. If a toxin, for example, is then added to the cell culture, only those few cells with a toxin-resistant marker gene integrated into their genomes will be able to proliferate, while other cells will die. After applying this selective pressure for a period of time, only the cells with a stable transfection remain and can be cultured further. Examples of marker genes and selection agents for use in accordance with the present disclosure include, without limitation, dihydrofolate reductase with methotrexate, glutamine synthetase with methionine sulphoximine, hygromycin phosphotransferase with hygromycin, puromycin N-acetyltransferase with puromycin, and neomycin phosphotransferase with Geneticin, also known as G418. Other marker genes/selection agents are contemplated herein.

Expression of nucleic acids in transiently-transfected and/or stably-transfected cells may be constitutive or inducible. Inducible promoters for use as provided herein are described above.

Some aspects of the present disclosure provide cells that comprises 1 to 10 engineered nucleic acids. In some embodiments, a cell comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more engineered nucleic acids. It should be understood that a cell that "comprises an engineered

nucleic acid" is a cell that comprises copies (more than one) of an engineered nucleic acid. Thus, a cell that "comprises at least two engineered nucleic acids" is a cell that comprises copies of a first engineered nucleic acid and copies of an engineered second nucleic acid, wherein the first engineered nucleic acid is different from the second engineered nucleic acid. Two engineered nucleic acids may differ from each other with respect to, for example, sequence composition (*e.g.*, type, number and arrangement of nucleotides), length, or a combination of sequence composition and length. For example, the SDS sequences of two engineered nucleic acids in the same cells may differ from each other.

Some aspects of the present disclosure provide cells that comprises 1 to 10 episomal vectors, or more, each vector comprising, for example, an engineered nucleic acids. In some embodiments, a cell comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more vectors.

Also provided herein, in some aspects, are methods that comprise introducing into a cell an (*e.g.*, at least one, at least two, at least three, or more) engineered nucleic acid or an episomal vector (*e.g.*, comprising an engineered nucleic acid). As discussed elsewhere herein, an engineered nucleic acid may be introduced into a cell by conventional methods, such as, for example, electroporation, chemical (*e.g.*, calcium phosphate or lipid) transfection, fusion with bacterial protoplasts containing recombinant plasmids, transduction, conjugation, or microinjection of purified DNA directly into the nucleus of the cell.

In Vivo Delivery

Engineered nucleic acids of the present disclosure may be delivered to a subject (*e.g.*, a mammalian subject, such as a human subject) by any *in vivo* delivery method known in the art. For example, engineered nucleic acids may be delivered intravenously. In some embodiments, engineered nucleic acids are delivered in a delivery vehicle (*e.g.*, non-liposomal nanoparticle or liposome). In some embodiments, engineered genetic circuits are delivered systemically to a subject having a cancer or other disease and activated (transcription is activated) specifically in cancer cells or diseased cells of the subject.

Engineered genetic circuits, as discussed above, may be delivered to cells (e.g., cancer cells) of a subject using a viral delivery system (e.g., retroviral, adenoviral, adeno-association, helper-dependent adenoviral systems, hybrid adenoviral systems, herpes simplex, pox virus, lentivirus, Epstein–Barr virus) or a non-viral delivery system (e.g., physical: naked DNA, DNA bombardment, electroporation, hydrodynamic, ultrasound or magnetofection; or

chemical: cationic lipids, different cationic polymers or lipid polymer) (Nayerossadat N *et al. Adv Biomed Res*. 2012; 1: 27, incorporated herein by reference). In some embodiments, the non-viral based deliver system is a hydrogel-based delivery system (*see, e.g.*, Brandl F, *et al. Journal of Controlled Release*, 2010, 142(2): 221-228, incorporated herein by reference).

Synthetic Promoter Libraries

Synthetic promoter libraries are provided that include a plurality of nucleic acids, wherein each nucleic acid in the library comprises a synthetic promoter sequence. Three designs for synthetic promoter libraries are provided. In two of the designs ("Design 1" and "Design 2"), the promoter sequences of the library comprise 8mer nucleotide sequences that are joined in tandem (head-to-tail). In one of these designs ("Design 2"), 3mer nucleotide spacers are placed in between each pair of 8mer nucleotide sequences. In the third design ("Design 3"), the nucleic acid sequences of the library comprise 11mer nucleotide sequences that are joined in tandem (head-to-tail), with 3mer nucleotide spacers placed in between each pair of 11mer nucleotide sequences.

The number of 8mer or 11mer nucleotide sequences in tandem can be at least: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 8mer or 11mer nucleotide sequences. The sequence of each 8mer or 11mer nucleotide sequence in a nucleic acid can be random (i.e., the sequence NNNNNNNN, wherein each N represents any nucleotide) and the 8mer or 11mer nucleotide sequences in any nucleic acid can be randomly selected so that the plurality of nucleic acids in the promoter library represents substantially all possible sequences or all possible sequences of the length of the nucleic acid that is selected for the library. Alternatively, if a particular nucleotide sequences or compositions (e.g., pyrimidine content) are to be favored or required, or disfavored or avoided, then the 8mer or 11mer nucleotide sequences can be designed to have certain nucleotides in certain positions, or certain nucleotide content, as desired. In such cases, the plurality of nucleic acids in the promoter library represents a selected subset of all possible sequences.

In some embodiments, a nucleotide spacer of defined sequence is placed between each 8mer or 11mer nucleotide sequence. The nucleotide spacer preferably is a 3mer nucleotide, but other length spacers can be used, such as 1, 2, 4, or 5 nucleotides. The 3mer nucleotide spacers in some embodiments are selected from AGC, ATC, GAC, ACT, AGT,

GTC, GAT, and GCT. In some embodiments, each nucleotide spacer used in a nucleic acid in the library is different than other nucleotide spacers in the same nucleic acid.

In some embodiments, the nucleic acids in the synthetic promoter library further includes restriction endonuclease sites at the 5' and 3' ends. In some embodiments, the restriction endonuclease site at the 5' end is a SbfI site and the restriction endonuclease site at the 3' end is an AscI site. Other restriction endonuclease sites may be used.

In some embodiments, each of the nucleic acids in the synthetic promoter library further includes a nucleotide sequence encoding an output molecule operably linked to the promoter sequence. The output molecule in some embodiments is a detectable molecule, such as a fluorescent or colored protein (e.g., mKate2), an enzyme, or any other type of detectable nucleic acid or polypeptide known in the art.

The synthetic promoter libraries can be used in method of selecting synthetic promoters. The method includes obtaining a library comprising nucleic acid molecules comprising synthetic promoter sequences operably linked to an output molecule, expressing the library in one or more types of cells, detecting the expression of the output molecule, and isolating the cells in which the output molecule is expressed. Optionally the method also includes determining the sequence of the synthetic promoter sequences in the isolated cells.

In some embodiments, the one or more types of cells are at least two different types of cells, such as cancer cells and matched non-cancer cells, such as ovarian cancer cells and ovarian cells, or breast cancer cells and breast cells, etc..

By comparing the synthetic promoter sequences that drive the expression of the output molecule in each of the at least two different types of cells, synthetic promoter sequences that are more active in one of the at least two different types of cells than in another of the at least two different types of cells can be identified. Thus if the at least two different types of cells are cancer cells and non-cancer cells, then promoters can be identified that are active in cancer cells but not in non-cancer cells, or vice versa.

By "more active in one of the at least two different types of cells than in another of the at least two different types of cells" is meant that the promoter has at least 10%, 50%, 100%, 2-fold, 3-fold, 4-fold, 5-fold, 6 -fold, 7-fold, 8 -fold, 9-fold, 10-fold, 20-fold, 30-fold, 40-fold, 50-fold, 60-fold, 70-fold, 80-fold, 90-fold, 100-fold, 500-fold, or 1000-fold (or even more) greater activity in one of the two types of cells. For example, a synthetic promoter

isolated from a library by these methods can be essentially inactive in one type of cell and active in another type of cell, which provides cell type-specific synthetic promoters.

EXAMPLES

Example 1.

Two human promoters were used as promoter inputs for the engineered genetic circuit used in this Example. These human promoters, SSX1 (Input 1) and H2A1 (Input 2), are over-expressed in many human cancers (Input 1 encodes the mKate2 output containing an intervening mirFF4) (Fig. 4A). The mKate2 output levels were measured for different circuit configurations, with respect to (a) the number of perfect-match mirFF4 binding sites (FF4-BS) encoded in Input 1 (downstream of *mK2*) and (b) two different configurations for the "sponge" construct in Input 2. Fig. 4B, x-axis annotations: M# represents Input 1 with the number of mirFF4 binding sites (FF4-BS) encoded downstream from *mKate2/mirFF4*. For example, M3 represents Input 1 with 3 perfect-match mirFF4 binding sites (FF4-BS) (Fig. 4A). S0, S1 and S2 represent three different sponge/Input 2 configurations. S0 is a negative control transcript with no mirFF4 binding sites. S1 is a Decoy transcript with 10 bulged mirFF4 binding sites encoded at the 3' end of the construct (Fig. 4A). S2 is similar to S1, but with an additional circular intron with 10 bulged FF4-BS located upstream from the 10 bulged mirFF4-BS encoded at the 3' end of the construct. The engineered genetic circuit (logic gate) depicted in Fig. 4A corresponds to M3-S1 in Fig. 4B (highlighted by a dashed box). The results are represented in mean mKate2 expression (P1), which is the average mKate2 for cells gated for SSC/FSC in FACS to remove cell clumps and debris. Error bars represent SEM. NT represents non-transfected cells.

The experiment was repeated with ECFP labeling (Fig. 5).

Example 2.

The engineered genetic circuit (G5) described in this Example is based on the circuit (AND gate) encoding mKate2, described in Example 1, with the exception that the AND gate product is not mKate2, but rather a synthetic transcription factor (annotated "TF" in Fig. 6A). In this example, the TF is the fusion protein GAL4BD-VP16 AD (the yeast GAL4 DNA binding domain fused to the viral VP16 transcription activation domain), although it can be any transcription activator such as rtTA3, TALE-TFs and ZF-TFs. Alternatively, this can

also be a transcriptional repressor such as GAL4BD-KRAB. Because the output is a transcription factor rather than a reporter/effector protein, it can regulate the expression of multiple outputs encoded downstream from the TF target promoter. In this example, the target promoter (annotated P3) is the synthetic G5 promoter that consists of a minimal viral or human promoter with 5 upstream GAL4 DNA binding sites. The I/O curve of this synthetic promoter can be tuned with the number of the GAL4 binding sites. Therefore, the ratio between any multiple outputs, together with the activation threshold for each output can be determined by the number of GAL4 binding sites in the synthetic P3 promoter.

Fig. 6B shows experimental results. CXCL10 is CXCL1p regulating a GAL4BD-VP16AD harboring a mirFF4v2B intron and 10 downstream mirFF4-Bs. SSX10 is SSX1p regulating a GAL4BD-VP16AD harboring a mirFF4v2B intron and 10 downstream mirFF4-Bs. SSX*10 is truncated SSX1p in which part of the 5' UTR was removed together with the KOZAK sequence, regulating a GAL4BD-VP16AD harboring a mirFF4v2B intron and 10 downstream mirFF4-Bs. Sponge S0 is a negative control transcript mirFF4-BS. Sponge S2 is Decoy transcript with 10 bulged FF4-BS encoded on the 3' end, with an additional circular intron with 10 bulged mirFF4-BS located upstream to the 10 bulged mirFF4-BS which are encoded in the transcript3'. In all samples, the mKate2 output is encoded under a G5p.

Example 3. BiTE and STE Trigger Robust Tumor Killing HEK-293T Cells

Anti-HER2 bispecific T cell engager (BiTE) and surface T cell engager (STE) trigger T cells to mediate robust tumor killing and IFN-γ secretion (Fig. 8). HEK-293T (minimally expressing HER2) cells were transfected with various DNA constructs as indicated. 48 hours post transfection, various HEK-293T cells were harvested and co-cultured with human T cells for 5 hrs or 24 hrs. 5 hr cytotoxicity by T cells was measured by lactate dehydrogenase (LDH) release assay (Korzeniewski C and Callewaert DM, *Journal of Immunological Methods*, 1983, 64(3):313-320, incorporated herein by reference) and 24hr IFN-γ secretion by T cells was measured by IFN-γ ELISA. Data show that T cells mediate robust tumor killing and IFN-γ secretion on BiTE secreting tumor cells (group 1-2). The tumor killing and IFN-γ secretion correlate with HER2 expression level on tumor cells (group 1-2). T cells also mediate robust tumor killing and IFN-γ secretion on STE expressing tumor cells (group 3-6), and the cytotoxicity and IFN-γ secretion are independent of tumor antigen (HER2) expression

(group 3-6). Furthermore, T cells mediate minimal tumor killing and IFN-γ secretion when co-cultured with HEK-293T cells expressing non-BiTE and non-STE control proteins (group 7-9).

Stable 4T1 Cells

Stable 4T1 cells (HER2-) expressing indicated DNA constructs (STRICT017 +018) were co-cultured with human T cells for 5 hrs or 24 hrs (Fig. 10). 5 hr cytotoxicity by T cells was measured by LDH release assay and 24hr IFN-γ secretion by T cells was measured by IFN-γ ELISA (Fig. 10A). Data show that T cells mediate minimal killing and IFN-γ secretion on HER2- or STE- tumor cells (groups 1 and 3). T cells mediate robust tumor killing and IFN-γ secretion on STE-expressing tumor cells (group 2). T cells also mediate robust tumor killing and IFN-γ secretion when co-cultured with cell mixtures consisting of low numbers of BiTE secreting cells with non-BiTE secreting tumor. This indicates minimal numbers of BiTE secreting cells in the tumor mass can elicit robust tumor mass killing and IFN-γ release (group 4).

Stable HEK-293T Cells

Stable HEK-293T cells (minimally expressing HER2) expressing indicated DNA constructs were co-cultured with human T cells for 5 hrs or 24 hrs. 5 hr cytotoxicity by T cells was measured by LDH release assay and 24hr IFN-γ secretion by T cells was measured by IFN-γ ELISA (Fig. 10B). Data show that T cells mediate minimal killing and IFN-γ secretion on BiTE- or STE- tumor cells (group 4). T cells mediate robust cytotoxicity and IFN-γ secretion on BiTE secreting tumor cells (group 1). T cells also mediate robust cytotoxicity and IFN-γ secretion on STE-expressing tumor cells (groups 2 and 3). Furthermore, T cells also mediate robust tumor killing and IFN-γ secretion when co-cultured with cell mixtures consisting of low numbers of BiTE secreting cells with non-BiTE secreting tumor cells. This indicates minimal numbers of BiTE secreting cells in the tumor mass can elicit robust tumor mass killing and IFN-γ release (group 5 & 6).

Stable MDA-MB452 (HER2+) Cells (Human Breast Cancer Cell Line)

Anti-HER2 bispecific T cell engager (BiTE) and surface T cell engager (STE) trigger T cells to mediate robust tumor killing on human breast cancer cell line (Fig. 11). Stable

MDA-MB453 (HER2+) cell lines were created by lentiviral transduction with various DNA constructs (STRICT034, 035) as indicated. Donor #2 T cells were used. The E:T ratio was 10:1; 6 x 10^5:6 x 10^4. Various MDA-MB453 cells were harvested and co-cultured with human T cells for 5 hrs. 5hr cytotoxicity by T cells was measured by LDH release assay. Data show that T cells mediate robust tumor killing on BiTE secreting tumor cells (group 2). T cells also mediate robust tumor killing on STE expressing tumor cells (group 3-4). Furthermore, T cells mediate minimal tumor killing when co-cultured with parental MDA-MB453 tumor cell line (group 1).

Example 4. T Cells Kill Doxycycline-induced STE-expressing Cells Efficiently

Surface T cell engager (STE) version 1 (v1) and version 2 (v2) both trigger T cells to mediate robust tumor killing on HEK-293T cells (Fig. 13). Various inducible STE expressing HEK-293T cell lines were created by lentiviral transduction. Various HEK-293T cells were harvested and co-cultured with human T cells for 5 hrs. 5hr cytotoxicity by T cells was measured by LDH release assay. Data show that T cells mediate robust tumor killing on transfected STEv1 expressing tumor cells (column 2). T cells also mediate robust tumor killing on inducible STEv1 and STEv2 expressing tumor cells (columns 3 and 4). Furthermore, T cells mediate minimal tumor killing when co-cultured with non-STE expressing HEK-293T cell line (column 1).

Example 5: Increase in T Cell Killing Efficiency of Tumor Cells

HEK-293T Cells

AND gate architecture was harnessed increase the T cell killing efficiency of tumor cells (Fig. 9). HEK-293T cells were transfected with various DNA constructs (STRICT014) as indicated (Fig. 9A) and Donor #S T cells were used. The E:T ratio was 10:1; 6 x 10^5:6 x 10^4. For the right panel (Fig. 9B), (1,0) indicated cells transfected with STE only). (1,1) indicated cells transfected with STE and sponge. (0,0) indicated cells transfected with a non-STE protein. Ctrl indicated non-transfected cells. 48 hrs post transfection, various HEK-293T cells were harvested and co-cultured with human T cells for 5 hrs. Cytotoxicity by T cells was measured by LDH release assay. Data show that T cells kill 293T/STE expressing cells (column 1) and the killing can be greatly enhanced by the AND gate architecture (column 2). T cells exhibit minimal killing on not STE expressing cells (column 3 & 4). For

the left panel (Fig. 9C), the Input 2 condition was not tested since it does not encode the output protein. (0,0) represents non-transfected cells. An additional experiment is conducted to further decrease the output of the AND gate at state (1,0) by removing the Kozak sequence and the 5' UTR of SSX1 promoter.

HEK-293T Cells (GAL4 Gate v1 for STE)

HEK-293T cells were transfected with various DNA constructs (STRICT037, 039, 040) as indicated and Donor #2's T cells were used (Fig. 14). The E:T ratio was 10:1; 6 x 10^5:6 x 10^4. The left panel showed the circuit used for this T cell cytotoxicity experiment (Fig. 14A). In the right panel, (1,0) indicated cells transfected with STE only. (1,1) indicated cells transfected with STE and sponge. (0,0) indicated cells transfected with a non-STE protein. 48 hrs post transfection, various HEK-293T cells were harvested and co-cultured with human T cells for 5 hrs. Cytotoxicity by T cells was measured by LDH release assay (Fig. 14B). Data show that T cells kill STE expressing (1,0) cells (column 2 and 4) and the killing can be greatly enhanced by the AND gate (1,1) architecture (column 3 and 5). T cells exhibit minimal killing on not STE expressing cells (column 1).

HEK-293T cells were transfected with various DNA constructs (STRICT039, 040) as indicated and Donor #2's T cells were used (Fig. 15). The E:T ratio was 10:1; 6 x 10^5:6 x 10^4. Fig. 15A shows the circuit used for this T cell cytotoxicity experiment. In Fig. 15B, (1,0) indicated cells transfected with STE only. (1,1) indicated cells transfected with STE and sponge. (0,0) indicated cells transfected with a non-STE protein. 48 hrs post transfection, various HEK-293T cells were harvested and co-cultured with human T cells for 5 hrs. Cytotoxicity by T cells was measured by LDH release assay. Data show that T cells kill STE expressing (1,0) cells (column 3 and 5) and the killing can be greatly enhanced by the AND gate (1,1) architecture (column 4 and 6). T cells exhibit minimal killing on not STE expressing cells (column 1). The killing on (1,0) condition is mainly caused by the leakage of GAL4 promoter output (column 2 vs. 3 or 5). An additional experiment is conducted to decrease the GAL4 promoter leakage by removing the Kozak sequence of STE v1, making STE v1 output self-degrading by adding miRNA binding sites at 3' end, and the combination of both mechanisms.

HEK-293T Cells (GAL4 Gate v2 for STE)

GAL4-gate version 2 (v2) architecture can be harnessed to fine tune T cell killing efficiency of tumor cells and exhibit less cytotoxicity at (1,0) state. HEK-293T cells were transfected with various DNA constructs (STRICT039, 040) as indicated and Donor #2's T cells were used (Fig. 16). The E:T ratio was 10:1; 6 x 10^5:6 x 10^4. Fig. 16A shows the circuit used for this T cell cytotoxicity experiment. In Fig. 16B, (1,0) indicated cells transfected with STE only. (1,1) indicated cells transfected with STE and sponge. (0,0) indicated cells transfected with a non-STE protein. 48 hrs post transfection, various HEK-293T cells were harvested and co-cultured with human T cells for 5 hrs. Cytotoxicity by T cells was measured by LDH release assay (Fig. 16B). Data show that T cells kill STE expressing (1,0) cells (column 3) and the killing can be enhanced by the AND gate (1,1) architecture (column 4). T cells exhibit minimal killing on not STE expressing cells (column 1). The killing on (1,0) state of this version is improved compared to GAL4 gate v1 architecture (v2 is more closer to basal level (0,0)). An additional experiment is conducted to decrease the killing at (1,0) state. The GAL4 promoter output at (1,0) state is decreased by adding miR binding sites at 3' end of STE gene.

HEK-293T Cells (GAL4 Gate v3 for STE)

GAL4-gate version 3 (v3) architecture can be harnessed to fine tune T cell killing efficiency of tumor cells and exhibit less cytotoxicity at (1,0) state. HEK-293T cells were transfected with various DNA constructs (STRICT039, 040) as indicated and Donor #2's T cells were used (Fig. 16). The E:T ratio was 10:1; 6 x 10^5:6 x 10^4. Fig. 17A shows the circuit used for this T cell cytotoxicity experiment. In Fig. 17B, (1,0) indicated cells transfected with STE only. (1,1) indicated cells transfected with STE and sponge. (0,0) indicated cells transfected with a non-STE protein. 48 hrs post transfection, various HEK-293T cells were harvested and co-cultured with human T cells for 5 hrs. Cytotoxicity by T cells was measured by LDH release assay (Fig. 17B). Data show that T cells minimally kill STE expressing (1,0) cells (column 3) and only reach efficient killing when the AND gate is active (1,1) (column 4). T cells exhibit minimal killing on not STE expressing cells (column 1). The killing on (1,0) state is as long as (0,0) state. An additional experiment is conducted increase GAL4-VP16 output level or increase GAL4 binding sites to enhance the killing efficacy of (1,1) state.

Example 6

This Example addresses two overarching challenges (Figs. 2A-2B): (1) to create novel breast-cancer therapies that are safe and effective for replacing interventions that have life-threatening toxicities; and (2) to use these new therapies to eliminate the mortality associated with metastatic breast cancer.

Immunotherapy has achieved robust and potentially curative efficacy against cancers in clinical trials. Immunotherapies that harness T cell effector functions, such as chimeric antigen receptor (CAR) T cells or bispecific T-cell engagers (BiTEs), can have potent effects [1, 2]. However, there are major challenges associated with these therapies, especially for solid tumors such as breast cancer. Current CAR-T cell therapy requires custom cell isolation, engineering, and expansion for every patient, which is expensive and challenging to scale. Also, CAR-T cells must traffic to tumor sites to mediate killing and require long-term persistence for robust efficacy, which can pose challenges for solid tumors [3].

BiTEs are fusion proteins that include two single-chain variable fragments (scFvs) fused in tandem to enable engagement of tumor cells by T cells, thus resulting in T-cell-triggered tumor killing. BiTE therapy is potent and can confer tumor killing at a concentration five orders-of-magnitude lower than tumor-targeting antibodies (Abs) [2]. However, even multi-bolus injections cannot maintain high serum BiTE concentrations due to their short half lives *in vivo* (~2 hours) [4]. Successful BiTE clinical trials treating hematological cancers have all required continuous intravenous infusions for 4 to 8 weeks [2]. Since solid tumors are generally less accessible to immune cells than hematological malignancies, successful BiTE therapy for solid tumors will likely require even longer periods of continuous BiTE infusions, which is undesirable due to potential side effects, patient inconvenience, and reduced efficacy. Finally, both CAR T-cells and BiTE therapies target extracellular tumor-specific antigens that are not available in many cancer types, including triple-negative breast cancer. Furthermore, target antigens can be displayed by normal cells and thus immunotherapy can result in off-target immune responses with severe consequences [5].

In addition to harnessing T cell effector function with CARs or BiTEs, an alternative approach is to deliver genetic circuits into tumor cells that express T-cell-engaging proteins on cancer cell surfaces and activate T-cell-based killing. These Surface T cell Engagers (STEs) can trigger antigen-independent T cell killing of tumor cells *in vitro* and *in vivo* [6-

10]. However, previous STE studies were not able to build genetic circuits that were only activated in tumor cells. Thus, to avoid systemic toxicity, these constructs were only limited to intra-tumoral injections, resulting in decreased efficacy and the inability to treat systemic diseases [7, 10]. This is a major limitation, because for many cancers and especially breast cancers, metastatic disease is the main reason for mortality. Thus, a scalable therapy that can harness the immune system to treat systemic and metastatic cancers with high anti-tumor specificity is urgently needed, which is provided herein.

Synthetic biologists have developed gene circuits for highly specific intracellular detection of cancer states based on cancer-specific promoters or miRNA profiles [11, 12]. However, further development is required before these tumor-detecting circuits can be used in the clinic. For example, these synthetic tumor-detecting circuits have only been coupled with intracellular killing mechanisms, which restricts their efficacy against tumors because it is virtually impossible to deliver the circuits to 100% of cancer cells. In addition, high targeting specificity is required to avoid damaging healthy tissues. Finally, past circuits have utilized foreign proteins but minimizing ectopic protein expression is essential to avoid inducing host immune responses in normal cells.

To overcome limitations of existing cancer immunotherapies and tumor-detecting gene circuits, provided herein are Tumor Immunotherapy by Gene-circuit Recruited Immunomodulatory Systems (TIGRIS), also referred to as Synthetic Tumor Recruited Immuno-Cellular Therapy (STRICT), a platform technology to trigger potent and effective immunotherapy against tumors from within tumors themselves. TIGRIS is combination of tumor-detecting gene circuits with anti-cancer immunotherapies. Engineered genetic circuits can be delivered to tumors. These engineered genetic circuits are selectively activated only in cancer cells, resulting in the surface display of STEs and the secretion of other immunomodulatory molecules to recruit T cells to target the tumor. We designed tumor-detecting gene circuits with very high specificity to enable TIGRIS therapy to be administered systemically but only be activated locally in cancer cells, resulting in enhanced safety and reduced side effects. Therefore, TIGRIS combines the advantages of systemic delivery (*e.g.*, treating metastasis) with the advantages of localized treatment (*e.g.*, safety, minimal side effects), and enables the benefits below.

We developed TIGRIS against triple-negative breast cancer (TNBC), a difficult-to-treat subset of breast cancer that exhibits aggressive behavior and is correlated with poorer

prognosis [13-15]. There are no ideal targeted therapies for TNBC since this subset of breast cancers does not express the estrogen receptor, progesterone receptor, or HER2. TIGRIS should overcome key obstacles associated with other therapies, including:

1) *The challenge of breast cancer heterogeneity*. Breast cancers are known to be very inter-tumorally and intra-tumorally heterogeneous [16]. For example, HER2 expression heterogeneity is correlated with poor prognosis [17] and traditional targeted therapies cannot cover entire heterogeneous cancer populations. In contrast, we hypothesize that tumor-specific STE expression will first recruit T cells to kill STE-expressing cancer cells. The initial killing should release immunogenic mutant antigens [18] that should recruit additional waves of T cells with a variety of targeting specificities. This would generate a polyclonal immune response against the tumor antigens, cover the broad mutational landscape of the heterogeneous tumor population, and prevent immunoediting-mediated tumor relapse. In addition, almost all targeted therapy can create target-negative tumor variant outgrowth. Since TIGRIS does not require a known tumor-specific antigen to be expressed by tumor cells, it should not be affected by tumor escape mechanisms that involve downregulation of surface antigens.

2) *Limited targeting spectra*. Unlike CAR-T cell or BiTE therapy, TIGRIS does not depend on the surface expression of tumor-specific antigens that can be hard to identify for many cancers. Rather, TIGRIS is activated by the concerted activity of multiple tumor-specific/tissue-specific promoters via AND gate logic, which results in enhanced specificity versus single promoter systems. These logic circuits can be customized for different promoters and even incorporate tumor-specific/tissue-specific microRNAs for further specificity, thus enabling flexible therapeutic efficacy. Furthermore, these promoters can be identified via tumor cell sequencing and customized for different tumors to overcome immunoedited cancers and heterogeneous cancer cell types.

3) *The deadly consequence of metastasis*. Metastatic tumor cells are difficult to treat and are responsible for 90% of breast cancer deaths [19]. Our gene circuits can be delivered systemically but only have local effects due to their specificity, thus potentially enabling the detection and destruction of metastases. In addition, we expect that anti-cancer T cells activated by TIGRIS will patrol the body to target metastases for destruction.

4) *Evolution of tumor escape variants during targeted therapy*. TIGRIS can initiate epitope spreading, and this phenomenon recruits many T cells bearing different tumor-

targeting specificities. The probability of tumor escape variants will be much smaller than traditional targeted therapy.

5) *The challenge of tumor relapse*. Many advanced breast cancers eventually recur and no predictive or preventive measures for relapse are available. Since T cells can differentiate into memory T cells and reside in the body for a long period of time, TIGRIS can prevent future tumor relapse. Here, we provide, as an example, TNBC, a difficult subset of breast cancer to treat using traditional therapies.

6) *The challenge of therapeutic delivery*. The delivery of discrete therapies, such as nucleic acids or gene circuits using viral or non-viral vectors, is usually unable to target all tumor cells. Since STEs can recruit T cells to initiate tumor killing and initiate epitope-spreading phenomena, this technology can kill surrounding cancer cells as long as the immune response triggered by STE is robust enough, even if our tumor-detecting circuits can only be delivered to a small fraction of tumor cells.

By engineering highly specific cancer-detection circuits to command tumor cells to express STE and other immunomodulators, we can elicit a robust host immune response to eliminate primary tumor cells, target heterogeneous tumors, inhibit local lymph node invasion, and target systemic metastases, while also forming immune memory to protect against future tumor relapse.

Engineer TIGRIS constructs and validate therapeutic efficacy in vitro and in vivo.

We created novel cancer-detection circuits that command tumor cells to display STEs. We test if STEs can trigger robust immune responses and effectively kill breast cancer cells *in vitro* and *in vivo*. The key parameters needed to achieve robust efficacy against solid tumors such as breast cancer with TIGRIS (*e.g.*, the minimal fraction of STE-expressing tumor cells and the minimal STE expression level on tumor cell surfaces) are unknown, so we determine these with *in vitro* and *in vivo* assays. We also test whether the TIGRIS-triggered immune response can enable effective anti-tumor therapy despite intratumoral heterogeneity in breast cancers.

Create and validate cancer-detecting circuits that display STEs on tumor cells. We created human and murine STEs (Fig. 12A (top)) by fusing an scFv derived from an anti-human CD3ε Ab (clone: OKT3) or anti-murine CD3ε Ab (clone: 2C11) with inert membrane anchoring proteins (*e.g.*, cytoplasmic truncated Duffy Antigen/Receptor for Chemokines

(DARC)), respectively. We performed *in vitro* T cell cytotoxicity assays and cytokine release assays to test the functionality of the human STE when expressed by various tumor cell lines representing TNBC, chronic myeloid leukemia, and embryonic kidney tumors (4T1, K562, and HEK-293T, respectively). We observed robust cytotoxicity and IFN-γ production by T cells when T cells were co-cultured with STE-expressing tumor cells (Fig. 24B). Since human and murine STEs should only bind to human and murine T cells, respectively, these constructs enable us to confirm that specific T-cell engagement is necessary for therapeutic efficacy.

In addition, we designed synthetic gene circuits to specifically detect intracellular signatures of cancer. We previously engineered cancer-detecting circuits referred to as Dual Promoter Integrators (DPIs) whose output was only expressed when two cancer-specific promoters were activated beyond a minimal threshold, thus implementing an AND gate [12]. The DPI was implemented using non-human transcription factors, which are not ideal for clinical use since they may introduce foreign proteins that could become immunogenic in normal cells. Here, we create an AND gate using RNA only (Figs. 3A-3D), which have the additional benefit of being more compact than protein-based circuits. This circuit design only expresses an output when two promoters are activated in cancer cells. We constructed and validated the tunability, modularity, and functionality of our RNA-only AND gate architecture using the SSX1 and H2A1 cancer-specific promoters in HEK-293T cells with fluorescent proteins and STEs as outputs (see description below).

We adapt our RNA gates for specifically recognizing breast cancer cells. With our current circuit, there is a ~2-fold enhancement in T-cell-mediated killing between cells that contain both inputs to the AND gate activated in cancer cells (40% lysis, State 1 in Figure 4) over cells that contain just one input active in cancer cells (the one that expresses the STE protein only, State 3 in Figs. 3A-3D). The performance of this circuit (*e.g.*, enhanced ON:OFF ratio) can be further enhanced by increasing the number of miRNA binding sites in the STE transcript, modifying the miRNA backbone for more robust miRNA production, producing multiple miRNA copies per STE transcript, testing libraries of different miRNAs and sponges, modifying sponge sequences and architectures, minimizing leakiness with mRNA degradation tags, implementing trans-cleaving ribozymes for the removal of the miRNA-binding sites in the STE transcript, and including additional miRNA binding sites in

the STE transcript that are bound and repressed by endogenous miRNAs that are highly expressed in normal cells but downregulated in tumor cells [31].

We also test other cancer-specific and tissue-specific promoters (*e.g.*, RPC1 and RRM2 that are highly breast cancer specific and have been validated in TNBC cell lines [32]) and validate that our circuit is activated in 4T1 cancer cells but not in normal cells (*e.g.*, COMMA-1D, EpH4, MCF10A).

We tested circuit functionality by transfecting or stably integrating the circuits into tumor cells. We further encode our circuits in adenoviral, AAV, or HSV vectors in order to enable delivery into 4T1 and normal breast cell lines to verify tumor detection sensitivity, specificity, and tunability. We also leverage oncolytic HSV vectors, such as T-VEC, which have been used for cancer therapy in human patients [33].

If some of the cancer-specific promoters described above, in some instances, do not achieve specific activation in 4T1 cells, additional cancer-specific promoters may be identified with comparative transcriptomics and by screening barcoded promoter libraries for specific activation in target cells using FACS and sequencing. If some RNA-only circuits do not achieve significant ON:OFF ratios, human transcription factors (such as artificial zinc-finger proteins [27]) may be used to minimize the introduction of potentially immunogenic foreign proteins.

Identify the minimal percentage of tumor cells that need to be targeted by TIGRIS for in vivo efficacy. We elucidate the minimal percentage of tumor cells that need to be targeted by our gene circuits to achieve robust therapeutic efficacy *in vivo*. This information is used for designing systemic delivery strategies, since these are unlikely, in some instances, to target 100% of tumor cells. We mix STE-displaying tumor cells (4T1/STE+) with non-STE-displaying counterparts (4T1/STE–) at various ratios and directly implant them into immune-competent BALB/c mice mammary pads to create orthotopic breast cancer models. The 4T1 murine model resembles advanced human TNBC and is highly malignant and metastatic [34, 35]. Tumor growth kinetics will be monitored by measuring tumor volume with calipers every other day. We monitor animal survival over time with experiments that will be kept running for at least two times longer than the mean survival time of control mice. The minimal percentage of STE-expressing tumor cells needed to efficiently inhibit the growth of injected tumor cells will be identified. Tumor cell lines expressing human STEs are used as

controls to validate T-cell-engagement specificity. We utilize 4-6 mice per experimental condition.

When there are sufficient STE-expressing cells, tumor growth should be partially or totally suppressed, resulting in surviving mice that are disease free over long time periods. We use Student's t-test and one-way ANOVA to compare tumor volumes between 2 groups and between >2 groups, respectively. To analyze survival experiments, we use Kaplan-Meier survival analysis. We also adoptively transfer T cells engineered with a dual bioluminescent reporter system to track the dynamics of T-cell tumor infiltration and activation with *in vivo* imaging [36]. We extend this work with C3(1)/SV40 T-antigen transgenic mice [37], a very aggressive spontaneous TNBC model, to verify our findings in a more physiologically relevant tumor model.

We determine the lower limit of tumor cells that need to express STEs to confer robust *in vivo* efficacy. For limits greater than the average gene delivery efficiency, we design new circuits that can simultaneously secrete multiple immunostimulatory effectors. These molecules include chemokines that actively attract T cells (*e.g.*, CCL19 and CCL21) [38], cytokines that are immunostimulatory and can condition tumor microenvironments (*e.g.*, IL-12, IL-15, and IL-21) [39], and immune-checkpoint blockade Abs (*e.g.*, anti-CTLA4 or anti-PD1 Abs) that can unleash brakes in T cell activity [40]. This combinatorial approach should enhance therapeutic efficacy against heterogeneous breast cancers. For example, anti-PD1 Abs have achieved response rates of 20-50% in multiple clinical trials targeting various solid tumor types. However, pre-existing immunity is required for patients to respond to anti-PD1 Abs [41, 42]. By expressing STEs and anti-PD1 Abs together, STEs can help create pre-existing immunity against tumor-associated and mutated antigens while anti-PD1 Abs can enhance T-cell function, proliferation, and infiltration into tumors, especially those that express PD-L1 (PD-1 ligand) to shut down T-cell function [43, 44].

Evaluate TIGRIS against metastatic cancer and relapse.

In advanced breast cancer, tumor cell lymph node infiltration and systemic metastasis is commonly observed and is responsible for 90% of breast cancer mortality. The standard of care after surgery is chemotherapy combined with targeted therapy, but this is not very effective for TNBCs [13-15]. In addition, 20-30% of patients diagnosed with invasive breast cancer will relapse after therapy but there are no preventive measures or diagnostic markers

for early detection of recurrence. We test whether immune cells triggered by TIGRIS can eliminate lymph node and systemic metastasis, and establish long-term immune memory. TIGRIS may obviate the need for systemic chemotherapy and surgical removal of lymph nodes, which is the most common cause of morbidity, and provide protection against tumor relapse.

Determine if TIGRIS can eliminate primary tumors and metastases via systemic delivery. We test if systemic viral delivery of the engineered genetic circuits can eliminate primary and metastatic tumors *in vivo.* We engineer 4T1 cells to express luciferase for *in vivo* imaging. To test for efficacy against metastases, we use the 4T1 orthotopic model from above but only initiate our virally delivered circuit therapy when metastases in lymph nodes and vital organs (expected in lung, liver, bone, and brain) are observed. We monitor the overall tumor burden (primary + metastatic tumors) in the mouse models.

We test different treatment protocols by varying parameters such as viral vector concentration, timing, and types [45]. We track the *in vivo* immune response generated by TIGRIS via live animal imaging. We should see reductions in tumor growth in primary and metastatic tumors after treatment, especially in organs that immune cells can readily enter, such as lung, liver, and bone. Reduction in brain metastases may also be possible since T-cell-based immunotherapy has been shown to infiltrate the cerebral spinal fluid [1]. We compare TIGRIS versus known chemotherapy regimens, such as taxane and anthracycline [46]

If, in some instances, primary tumors are not eliminated with STE expression alone, we augment the therapy with multiple immunostimulatory effectors described above. We also test whether multiple viral injections can enhance therapeutic efficacy. In addition, we surgically remove the primary tumor before and after circuit therapy to mimic common clinical practice and to test how surgical removal of primary tumors may affect the immune response against metastases.

Systemic circuit delivery may, in some instances, pose a challenge for achieving high therapeutic efficacy. We improve viral delivery, in some embodiments, by pseudotyping our vectors (*e.g.*, adenovirus) with small peptides to target other cell surface receptors [47]. In some embodiments, we adapt oncolytic viruses that have been shown to target breast cancers to take advantage of simultaneous tumor lysis and immunotherapy [48]. In some instances, viral particles may only penetrate the tumor periphery in many solid tumors. Thus, we can

express iRGD tumor penetrating peptides as additional circuit outputs [49]. These peptides can significantly enhance the tumor penetration of many therapeutic agents, including Abs, oncolytic viruses, and nanoparticles [49-51].

In addition to testing systemic delivery, we also determine the therapeutic efficacy of localized circuit delivery into primary tumor cells for treating systemic metastases. A localized tumor injection of the immunomodulatory oncolytic virus, T-Vec, can cause shrinkage of uninjected tumors [33]. This finding indicates that localized delivery of TIGRIS circuits, which can be achieved with viral or non-viral vectors, may also confer therapeutic efficacy. By generating a local immune response in injected tumors, TIGRIS may initiate a systemic immune response that could target metastatic tumors.

STE expression should be terminated when all gene-circuit-containing tumor cells are killed. However, to enhance controllability and safety, in some embodiments, we build synthetic safety mechanisms into our gene circuits. In these designs, if the gate is operating properly in normal cells, it should be OFF and should not express any foreign proteins. Thus, only if the gate malfunctions in normal cells or if the gate operates properly in cancer cells would the therapeutic output proteins be expressed along with safety mechanisms that can be externally toggled. First, we engineer inducible circuits to terminate STE expression and/or kill STE-expressing cells. Specifically, the STE output is replaced with a synthetic transcription factor, such as GAL4BD-VP16AD (GAD). In this architecture, genes for the STE, immunostimulatory molecules, and iRGD peptides, together with the conditional killer gene TK1, are regulated by the GAD-responsive promoter, G5p. Thus, foreign proteins are expressed, along with STEs, TK1, and other output genes, only when the logic gate is active. Addition of the TK1 substrate (e.g., ganciclovir or acyclovir) enables the killing of cells in which a circuit is active. Alternatively, we generate inducible transcription factors as outputs of our logic gates (*e.g.*, the doxycycline-responsive transcription factor rtTA3), instead of GAD, to drive therapeutic output expression. In this case, the whole system would not be activated without the administration of exogenous inducers (*e.g.*, doxycycline), thus providing a simple and safe mechanism to control treatment initiation and termination with FDA-approved small molecules. As a final layer of safety, we implement inducible expression of secreted STE antagonists, such as CD3ε on its own, that can titrate out functional STEs.

Test if TIGRIS can elicit immune memory to protect against future tumor relapse. TIGRIS should initiate long-term immune memory against recurrent breast cancer. To show this, we re-challenge long-term survivors (from above) with 4T1 tumor cells via tail vein injection. Tail vein injection of 4T1 tumor cells mainly results in lung metastases, which is a common metastatic site for breast cancers [52]. Live animal imaging is performed to monitor tumor seeding in the lung and other vital organs to determine if there is protective immunity against re-introduced tumor cells.

If, in some instances, initial treatment elicits very robust responses against primary tumors but no significant protection against re-challenges, we design the tumor-detecting circuits to additionally secrete IL-7 and IL-15, since these drive memory T cell formation [53]. Furthermore, the 4T1 tumor model is very immunosuppressive [54]. Thus, incorporating checkpoint-blockade Abs and/or pro-inflammatory cytokines (see above), in some instances, may help to generate a more robust memory response.

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Example 7

Synthetic Tumor Recruited Immuno-Cellular Therapy for Ovarian Cancer. New therapeutic strategies are needed to treat primary and metastatic ovarian cancer and to achieve long-term efficacy. Existing treatments for ovarian cancer, such as chemotherapies and targeted therapies, are unable to cure metastatic disease and prevent tumor relapse. In addition, standard-of-care treatments such as chemotherapy can cause significant morbidity and toxicity.

Provided herein is a transformative new class of immunotherapies for ovarian cancer that is highly specific, effective, and long lasting. This therapeutic strategy, Synthetic Tumor Recruited Immuno-Cellular Therapy (STRICT), leverages tumors themselves to recruit immune cells to destroy the tumors (Figs. 2A-2B), thereby inducing a strong polyclonal anti-tumor response that should be tunable, safe, long lasting, and effective.

Specifically, we provide synthetic gene circuits that are selectively turned on in ovarian cancer cells only when multiple tumor-specific promoters are active (for example, via digital gene circuits that implement AND logic). These synthetic circuits can be delivered systemically via viral vectors or locally into tumors. We utilize recent advances in synthetic biology to design these synthetic gene circuits to be highly compact, RNA-based (to avoid expressing immunogenic foreign proteins in normal cells), and specifically activated only in

ovarian cancer cells (not in any other normal cell type). When activated, these circuits display Surface T-cell Engagers (STEs) and other immunomodulatory molecules, such as checkpoint inhibitors and cytokines, to trigger a robust and targeted anti-tumor immune response. STEs will be designed to engage T-cell receptors on T cells and trigger the T cells to kill the STE-displaying cells. Furthermore, we incorporate safety switches into the gene circuits to enable them to be turned on or off externally.

The first wave of T cells should enact STE-directed killing of tumor cells, followed by secondary waves of polyclonal T cells that target a broader spectrum of cancer antigens released by cell lysis. Thus, the immunotherapy triggered by STRICT may suppress both primary and metastatic tumors, since T cells can provide disseminated immune surveillance throughout the body. Furthermore, these immune responses may enable long-term memory to be established against ovarian cancer. We adapt STRICT to target ovarian adenocarcinoma, the most common and difficult-to treat subset of ovarian cancer that exhibits aggressive behavior and is correlated with poor prognosis (1).

Immunotherapies that harness T-cell effector functions, such as chimeric antigen receptor (CAR) T cells or bispecific T-cell engagers (BiTEs), have achieved potent effects (2, 3). However, the use of these therapies poses significant challenges, especially for solid tumors such as ovarian cancer. Current CAR-T therapy requires custom cell engineering and expansion for every patient, which is expensive and difficult to scale. CAR-T cells need to traffic to tumor sites, target tumor-specific antigens, and persist long-term to mediate robust tumor killing and efficacy (4), which are major challenges for ovarian cancer (5).

BiTEs include of two single-chain variable fragments fused in tandem to enable the engagement and killing of tumor cells by T cells. BiTEs can confer potent and robust tumor killing at concentrations five orders-of-magnitude lower than tumor-targeting antibodies (Abs) (3). However, because BiTEs have short half-lives *in vivo* (~2 hours) (6) and solid tumors are generally less accessible to immune cells than hematological malignancies, successful therapy for solid tumors will likely require long periods of continuous i.v. BiTE infusions, which is challenging due to side effects, patient convenience, and therapeutic efficacy. I n addition, Surface T-cell Engagers (STEs) have been displayed on cancer cells to recruit T-cell-mediated killing (7-11), but such systems have not been specifically targeted to make systemic therapy possible without significant side effects.

Recently, oncolytic viruses, such as T-Vec, have neared FDA approval to treat melanoma. Oncolytic viruses rely on viral replication to kill tumor cells. However, it can be challenging to engineer oncolytic viruses to only replicate in specific tumor cells and oncolytic viruses have not yet demonstrated good efficacy versus ovarian cancers in clinical trials. In addition, synthetic biologists have developed gene circuits for highly specific intracellular detection of cancer cells based on cancer-specific promoters or microRNA profiles (12, 13). However, synthetic tumor-detecting circuits have only been coupled with intracellular killing mechanisms, which limits their efficacy against cancer because it is virtually impossible to deliver the circuits to 100% of cancer cells.

By harnessing synthetic cancer-detection circuits to command tumor cells to display STEs and to secrete other immunomodulators, we can elicit a robust host immune response to eliminate primary tumor cells and trigger secondary polyclonal T-cell responses. We test whether STRICT can inhibit local lymph node invasion, target systemic metastases, and form immune memory to protect against future relapse. Robust immune responses can be effective against cancer and that synthetic gene circuits can be designed to specifically detect cancer cells with intracellular markers.

We provide at least two methods for target primary, metastatic, and recurring ovarian cancer with STRICT:

1) We provide cancer-detection circuits to command tumor cells to display STEs and secrete immunomodulatory effectors. We validate their therapeutic efficacy *in vitro* and *in vivo*. *In vitro*, we measure T-cell induced cytotoxicity and key cytokines secreted by T cells due to STRICT. *In vivo*, we determine the minimal number of STE-displaying tumor cells that need to be targeted in order to achieve efficient tumor clearance by STRICT using the ID8 murine model (14).

2) We evaluate STRICT against primary ovarian tumors, metastases, and relapse in mouse models. We use the ID8 murine model to show that metastatic tumors can be eliminated by STRICT and that STRICT can prevent cancer relapse in mice that have survived after initial treatment. Controls to test the efficacy, specificity, and tunability of STRICT include gene circuits that display inactive STEs, gene circuits that are inactive, testing gene circuits in non-cancerous ovarian cells and other normal tissues, and using human versus murine STEs, as well as human versus murine T cells.

This disclosure provides methods for treating ovarian cancer by turning tumors against themselves. STRICT enables long-term activity against ovarian cancer and disseminated T-cell activity against primary and metastatic tumors. Our therapeutic constructs can be customized against a variety of different ovarian cancers, and are easier to scale and deploy in clinical practice versus engineered cell therapies.

STRICT may achieve strong therapeutic effects against primary and metastatic disease, induce long-lasting immune memory, incorporate safety switches, and reduce the cost, labor, and infrastructure needed for therapeutic application. STRICT may be effective against primary and metastatic tumors and achieve long-term protection against tumor relapse. STRICT should overcome limitations of other treatments by enabling convenient, targeted, and safe induction of polyclonal anti-tumor immune responses and long-lasting immune memory from within tumors. STRICT could ultimately replace standard-of-care treatments for ovarian cancer that have toxicities and side effects, and be broadly extensible to other cancers. STRICT is a transformative new treatment modality that can suppress long-term disease by harnessing the immune system against ovarian cancers.

STRICT may be effective against primary and metastatic tumors and achieve long-term protection against tumor relapse. STRICT may be able to replace standard-of-care treatments for ovarian cancer that have limited efficacy and significant toxicities and side effects. Furthermore, this technology establishes a powerful technology platform that can be broadly applied and reprogrammed against a broad range of cancers.

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Example 8

Synthetic Tumor Recruited Immuno-Cellular Therapy for Lung Cancer. New therapeutic strategies are needed to treat primary and metastatic lung cancer and to achieve long-term efficacy. Existing treatments for lung cancer, such as chemotherapies and targeted therapies, are unable to cure the disease and prevent tumor relapse. In addition, standard-of-care treatments such as chemotherapy can cause significant morbidity and toxicity.

Provided herein, in some embodiments, are immunotherapies for lung cancer that are be highly specific, effective, and long lasting. This therapeutic strategy, Synthetic Tumor Recruited Immuno-Cellular Therapy (STRICT), leverages tumors themselves to recruit immune cells to destroy the tumors (Figs. 2A-2B), thereby inducing a strong polyclonal anti-tumor response that should be tunable, safe, long lasting, and effective.

Specifically, we design synthetic gene circuits that are selectively turned on in lung cancer cells only when multiple tumor-specific promoters are active (for example, via digital gene circuits that implement AND logic). These synthetic circuits can be delivered systemically via viral vectors or locally into tumors. We utilize recent advances in synthetic biology to design these synthetic gene circuits to be highly compact, RNA-based (to avoid expressing immunogenic foreign proteins in normal cells), and specifically activated only in

lung cancer cells (not in any other normal cell type). When activated, these circuits display Surface T-cell Engagers (STEs) and other immunomodulatory molecules, such as checkpoint inhibitors and cytokines, to trigger a robust and targeted anti-tumor immune response. STEs are designed to engage T-cell receptors on T cells and trigger the T cells to kill the STE-displaying cells. Furthermore, we incorporate safety switches into the gene circuits to enable them to be turned on or off externally.

The first wave of T cells should enact STE-directed killing of tumor cells, followed by secondary waves of polyclonal T cells that target a broader spectrum of cancer antigens released by cell lysis. Thus, the immunotherapy triggered by STRICT may be able to suppress both primary and metastatic tumors, since T cells can provide disseminated immune surveillance throughout the body. Furthermore, these immune responses may enable long-term memory to be established against lung cancer.

We adapt STRICT to target non-small-cell lung cancer (NSCLC), the most common and difficult-to treat subset of lung cancer. STRICT should exhibit efficacy against NSCLC since NSCLC is responsive to some immunotherapies, such as with anti-PD-1 immune checkpoint blockade antibodies (Abs), an immunotherapy that activates host T-cell effector functions (*1, 2*).

Although anti-PD-1 Abs are approved by the FDA for treating NSCLC, the enhanced survival benefit of anti-PD-1 Abs is only 3.2 months over docetaxel and needs to be further be improved. Other immunotherapies that harness T-cell effector functions, such as chimeric antigen receptor (CAR) T cells or bispecific T-cell engagers (BiTEs), have achieved potent effects against other cancers (*3, 4*). However, the use of these therapies poses significant challenges for solid tumors such as lung cancer. Current CAR-T therapy requires custom cell engineering and expansion for every patient, which is expensive and difficult to scale. CAR-T cells need to traffic to tumor sites, target tumor-specific antigens, and persist long-term to mediate robust tumor killing and efficacy (*5*), which are major challenges for lung cancer (*6*).

BiTEs include of two single-chain variable fragments fused in tandem to enable the engagement and killing of tumor cells by T cells. BiTEs can confer potent and robust tumor killing at concentrations five orders-of-magnitude lower than tumor-targeting Abs (*4*). However, because BiTEs have short half-lives *in vivo* (~2 hours) (*7*) and solid tumors are generally less accessible to immune cells than hematological malignancies, successful therapy for solid tumors will likely require long periods of continuous i.v. BiTE infusions,

which is challenging due to side effects, patient convenience, and therapeutic efficacy. In addition, Surface T-cell Engagers (STEs) have been displayed on cancer cells to recruit T-cell-mediated killing (*8-12*), but such systems have not been specifically targeted to make systemic therapy possible without significant side effects.

Recently, oncolytic viruses that kill tumor cells based on viral replication, such as T-Vec, have neared FDA approval to treat melanoma. However, it can be challenging to engineer oncolytic viruses to only replicate in specific tumor cells and oncolytic viruses have not yet demonstrated good efficacy versus lung cancers in clinical trials. In addition, synthetic biologists have developed gene circuits for highly specific intracellular detection of cancer cells based on cancer-specific promoters or microRNA profiles (*13, 14*). However, synthetic tumor-detecting circuits have only been coupled with intracellular killing mechanisms, which limits their efficacy against cancer because it is virtually impossible to deliver the circuits to 100% of cancer cells.

By harnessing synthetic cancer-detection circuits to command tumor cells to display STEs and to secrete other immunomodulators, we can elicit a robust host immune response to eliminate primary tumor cells and trigger secondary polyclonal T-cell responses. We show that STRICT can inhibit local lymph node invasion, target systemic metastases, and form immune memory to protect against future relapse. Robust immune responses can be effective against cancer and synthetic gene circuits can be designed to specifically detect cancer cells with intracellular markers.

We provide at least two methods for targeting primary, metastatic, and recurring lung cancer with STRICT:

1) We create cancer-detection circuits to command tumor cells to display STEs and secrete immunomodulatory effectors. We validate their therapeutic efficacy *in vitro* and *in vivo*. *In vitro*, we measure T-cell induced cytotoxicity and key cytokines secreted by T cells due to STRICT. *In vivo*, we determine the minimal number of STE-displaying tumor cells that need to be targeted in order to achieve efficient tumor clearance by STRICT using the A549 xenograft lung cancer model (*15*).

2) We evaluate STRICT against primary lung tumors, metastases, and relapse in mouse models. We use the A549 xenograft model and LSL-KrasG12D spontaneous tumor models (*16*) to show that metastatic tumors can be eliminated by STRICT, and that STRICT can prevent cancer relapse in mice that have survived after initial treatment. Controls to test

the efficacy, specificity, and tunability of STRICT include circuits that display inactive STEs, circuits that are inactive, testing circuits in non-cancerous lung cells and other normal tissues, and using human versus murine STEs, and human versus murine T cells.

This disclosure provides methods for treating lung cancer by turning tumors against themselves. Highly specific cancer-detecting circuits have not yet been integrated with immunotherapy against lung cancer. STRICT should enable long-term activity against lung cancer and disseminated T-cell activity against primary and metastatic tumors. Our therapeutic constructs can be customized against a variety of different lung cancers, and should be easier to scale and deploy in clinical practice versus engineered cell therapies.

STRICT can achieve strong therapeutic effects against primary and metastatic disease, induce long-lasting immune memory, incorporate safety switches, and reduce the cost, labor, and infrastructure needed for therapeutic application. STRICT should be effective against primary and metastatic tumors and achieve long-term protection against tumor relapse. STRICT should overcome limitations of other treatments by enabling convenient, targeted, and safe induction of polyclonal anti-tumor immune responses and long-lasting immune memory from within tumors. STRICT may ultimately replace standard-of-care treatments for lung cancer that have toxicities and side effects, and be broadly extensible to other cancers. Here, we aim to how that STRICT is a transformative new treatment modality that may suppress long-term disease by harnessing the immune system against lung cancers.

This disclosure provides a powerful technology platform that can be broadly applied and reprogrammed against a broad range of cancers, including lung cancer.

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Example 9

Synthetic Promoters Library. Provided herein, in some embodiments, is a simple, fast and cost-efficient method to characterize the post translational regulation of transcription factors. The methods may be used, for example, to identify highly specific and very short synthetic promoters that can be used to target a cell state of interest, which is important both for research and personalized medicine. This may be done, for example, by identifying highly specific binding motifs which are activated in a specific cell state. Current methods such as RNA-Seq and ChIP-Seq can be misleading, since RNA levels are not always correlated with protein activity (p53 is a great example) and binding of TFs to the DNA is not always correlated with transcriptional activation (for example, the TF can function as a repressor). The method of the present disclosure, in some embodiments, provides direct evidence of the binding motifs which are activated in specific cell state and the activation levels of these motifs. The Bioinformatics layer enables characterizing the transcription factors associated with these motifs and therefore deciphering the transcriptional cascades activated in the cell state of interest. For Figs. 46 and 47, synthetic promoters were isolated from NB508-low library.

While several inventive embodiments have been described and illustrated herein, those of ordinary skill in the art will readily envision a variety of other means and/or structures for performing the function and/or obtaining the results and/or one or more of the advantages described herein, and each of such variations and/or modifications is deemed to be within the scope of the inventive embodiments described herein. More generally, those skilled in the art will readily appreciate that all parameters, dimensions, materials, and configurations described herein are meant to be exemplary and that the actual parameters, dimensions, materials, and/or configurations will depend upon the specific application or applications for which the inventive teachings is/are used. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific inventive embodiments described herein. It is, therefore, to be understood that the foregoing embodiments are presented by way of example only and that, within the scope of the appended claims and equivalents thereto, inventive embodiments may be practiced otherwise than as specifically described and claimed. Inventive embodiments of the present disclosure are directed to each individual feature, system, article, material, kit, and/or method described herein. In addition, any combination of two or more such features, systems, articles, materials, kits, and/or methods, if such features, systems, articles, materials, kits, and/or methods are not mutually inconsistent, is included within the inventive scope of the present disclosure.

All definitions, as defined and used herein, should be understood to control over dictionary definitions, definitions in documents incorporated by reference, and/or ordinary meanings of the defined terms.

All references, patents and patent applications disclosed herein are incorporated by reference with respect to the subject matter for which each is cited, which in some cases may encompass the entirety of the document.

The indefinite articles "a" and "an," as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean "at least one."

The phrase "and/or," as used herein in the specification and in the claims, should be understood to mean "either or both" of the elements so conjoined, *i.e.*, elements that are conjunctively present in some cases and disjunctively present in other cases. Multiple elements listed with "and/or" should be construed in the same fashion, *i.e.*, "one or more" of the elements so conjoined. Other elements may optionally be present other than the elements

specifically identified by the "and/or" clause, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, a reference to "A and/or B", when used in conjunction with open-ended language such as "comprising" can refer, in one embodiment, to A only (optionally including elements other than B); in another embodiment, to B only (optionally including elements other than A); in yet another embodiment, to both A and B (optionally including other elements); etc.

As used herein in the specification and in the claims, the phrase "at least one," in reference to a list of one or more elements, should be understood to mean at least one element selected from any one or more of the elements in the list of elements, but not necessarily including at least one of each and every element specifically listed within the list of elements and not excluding any combinations of elements in the list of elements. This definition also allows that elements may optionally be present other than the elements specifically identified within the list of elements to which the phrase "at least one" refers, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, "at least one of A and B" (or, equivalently, "at least one of A or B," or, equivalently "at least one of A and/or B") can refer, in one embodiment, to at least one, optionally including more than one, A, with no B present (and optionally including elements other than B); in another embodiment, to at least one, optionally including more than one, B, with no A present (and optionally including elements other than A); in yet another embodiment, to at least one, optionally including more than one, A, and at least one, optionally including more than one, B (and optionally including other elements); etc.

It should also be understood that, unless clearly indicated to the contrary, in any methods claimed herein that include more than one step or act, the order of the steps or acts of the method is not necessarily limited to the order in which the steps or acts of the method are recited.

In the claims, as well as in the specification above, all transitional phrases such as "comprising," "including," "carrying," "having," "containing," "involving," "holding," "composed of," and the like are to be understood to be open-ended, *i.e.*, to mean including but not limited to. Only the transitional phrases "consisting of" and "consisting essentially of" shall be closed or semi-closed transitional phrases, respectively, as set forth in the United States Patent Office Manual of Patent Examining Procedures, Section 2111.03.

What is claimed is:

CLAIMS

1. An engineered genetic circuit, comprising:

(a) a first nucleic acid comprising a promoter operably linked to (i) a nucleotide sequence encoding an output messenger RNA (mRNA) containing an intronic microRNA (miRNA) and (ii) a nucleotide sequence encoding at least one miRNA binding site complementary to the miRNA of (a)(i); and

(b) a second nucleic acid comprising a promoter operably linked to a nucleotide sequence encoding at least one miRNA binding site complementary to the miRNA of (a)(i).

2. The engineered genetic circuit of claim 1, wherein output mRNA encodes a synthetic T cell engager (STE) or a bispecific T cell engager (BiTE).

3. The engineered genetic circuit of claim 1, wherein the output mRNA encodes an output protein that binds to a T cell surface marker.

4. The engineered genetic circuit of claim 3, wherein the T cell surface marker is CD3, CD4, CD8 or CD45.

5. The engineered genetic circuit of any one of claims 1-4, wherein the output protein is an antibody or antibody fragment that binds specifically to the T cell surface antigen.

6. The engineered genetic circuit of any one of claims 1-5, wherein the output mRNA encodes an anti-cancer agent.

7. The engineered genetic circuit of claim 6, wherein the anti-cancer agent is a chemokine, a cytokine or a checkpoint inhibitor.

8. The engineered genetic circuit of any one of claims 1-7, wherein the promoter of (a) and/or (b) is an inducible promoter.

9. The engineered genetic circuit of claim 8, wherein the promoter of (a) and/or (b) is a tumor-specific promoter or a cancer-promoter.

10. The engineered genetic circuit of claim 9, wherein the promoter of (a) and/or (b) is SSX1 or H2A1.

11. The engineered genetic circuit of any one of claims 1-10, wherein the nucleotide sequence of (a)(ii) encodes 2-5 miRNA binding sites complementary to the miRNA of (a)(i)

12. The engineered genetic circuit of any one of claims 1-11, wherein the nucleotide sequence of (b) encodes 2-10 miRNA binding sites complementary to the miRNA of (a)(i)

13. The engineered genetic circuit of any one of claims 1-12, wherein the output protein is a transcription factor.

14. The engineered genetic circuit of claim 13, further comprising at least one nucleic acid comprising a promoter operably linked to a nucleic acid encoding an output nucleic acid or an output protein.

15. The engineered genetic circuit of claim 14, wherein the output mRNA encodes a transcription factor that can bind to and activate transcription of the promoter of the at least one nucleic acid.

16 The engineered genetic circuit of any one of claims 1-15, further comprising a nucleic acid comprising a promoter operably linked to (i) a nucleotide sequence encoding an additional output messenger RNA (mRNA) containing an intronic microRNA (miRNA) and (ii) a nucleotide sequence encoding at least one miRNA binding site complementary to the miRNA of (a)(i), wherein the additional output mRNA encodes a chemokine, a cytokine, a checkpoint inhibitor or a combination thereof.

17. A cell comprising at least one engineered genetic circuit of any one of claims 1-16.

18. The cell of claim 17, wherein the cell is a tumor cell.

19. A method, comprising administering to a subject having a tumor at least one engineered genetic circuit of any one of claims 1-15.

20. The method of claim 19, wherein the subject has ovarian cancer, breast cancer or lung cancer.

21. The method of claim 19 or 20, wherein the engineered genetic circuit is administered systemically to the subject.

22. The method of any one of claims 19-21, wherein the engineered genetic circuit is delivered using a viral delivery system.

23. The method of claim 22, wherein the viral delivery system is a lentiviral delivery system, an adenoviral delivery system or an adeno-associated viral delivery system.

24. The method of any one of claims 19-21, wherein the engineered genetic circuit is delivered using a non-viral delivery system.

25. The method of claim 19 or 20, wherein the engineered genetic circuit is administered locally to the tumor of the subject.

26. The method of claim 25, wherein the engineered genetic circuit is administered locally to the tumor using a hydrogel-based delivery system.

27. The method of any one of claims 19-26, wherein the output mRNA of at least one of the engineered genetic circuits encodes an output protein that binds to a T cell surface marker, and the output mRNA of at least one other engineered genetic circuit encodes a chemokine, a cytokine or a checkpoint inhibitor.

28. A composition comprising an anti-CD3e scFv antibody fragment fused with an transmembrane protein.

29. The composition of claim 28, wherein the transmembrane protein comprises cytoplasmic truncated Duffy Antigen/Receptor for Chemokines (DARC).

30. A composition comprising an anti-CD3e scFv antibody fragment fused with a human IgG1-Hinge-CH2-CH3 domain, a murine B7.1-transmembrane and a cytoplasmic domain.

31. An engineered genetic circuit, comprising:

(a) a first nucleic acid comprising a first tumor-specific promoter operably linked to (i) a nucleotide sequence encoding an output messenger RNA (mRNA) containing an intronic microRNA (miRNA) and (ii) a nucleotide sequence encoding at least one miRNA binding site complementary to the miRNA of (a)(i), wherein the output mRNA encodes a synthetic T cell engager or a bispecific T cell engager; and

(b) a second nucleic acid comprising a second promoter different from the first promoter and operably linked to a nucleotide sequence encoding at least one miRNA binding site complementary to the miRNA of (a)(i).

32. The engineered genetic circuit of claim 31 further comprising a nucleic acid comprising a tumor-specific promoter operably linked to (i) a nucleotide sequence encoding an additional output messenger RNA (mRNA) containing an intronic microRNA (miRNA) and (ii) a nucleotide sequence encoding at least one miRNA binding site complementary to the miRNA of (a)(i), wherein the additional output mRNA encodes a chemokine, a cytokine, a checkpoint inhibitor or a combination thereof.

33. An engineered genetic circuit, comprising:

(a) a first nucleic acid comprising a promoter operably linked to

(i) a nucleotide sequence encoding an output messenger RNA (mRNA) containing an intronic microRNA (miRNA),

(ii) a nucleotide sequence encoding an intronic miRNA, and

(iii) a nucleotide sequence encoding a miRNA binding site (miRNA-BS);

(b) a second nucleic acid comprising a promoter operably linked to

(i) a nucleotide sequence encoding an output mRNA containing an intronic miRNA,

(ii) a nucleotide sequence encoding an intronic miRNA, and

(iii) a nucleotide sequence encoding a miRNA-BS; and

(c) a third nucleic acid comprising a promoter operably linked to a nucleotide sequence encoding an output protein linked to a miRNA-BS,

wherein the miRNA-BS of (a)(iii) is complementary to the miRNA of (b)(i), the miRNA-BS of (b)(iii) is complementary to the miRNA of (a)(i), and the miRNA-BS of (c) is complementary to the miRNA of (a)(ii) and the miRNA of (b)(ii).

34. An engineered genetic circuit, comprising:

(a) a first nucleic acid comprising a promoter operably linked to

(i) a nucleotide sequence encoding a nascent RNA transcript containing an intronic microRNA (miRNA), and

(ii) a nucleotide sequence encoding at least one miRNA binding site (miRNA-BS);

(b) a second nucleic acid comprising a promoter operably linked to

(i) a nucleotide sequence encoding a nascent RNA transcript containing an intronic miRNA, and

(ii) a nucleotide sequence encoding at least one miRNA-BS; and

(c) a third nucleic acid comprising a promoter operably linked to a nucleic acid encoding an output protein linked to (i) a first miRNA-BS and (ii) a second miRNA-BS,

wherein the at least one miRNA-BS of (a)(ii) is complementary to the miRNA of (b)(i), the at least one miRNA-BS of (b)(ii) is complementary to the miRNA of (a)(i), the first miRNA-BS of (c)(i) is complementary to the miRNA of (a)(i), and the second miRNA-BS of (c)(ii) is complementary to the miRNA of (b)(i).

35. An engineered genetic circuit, comprising:

(a) a first nucleic acid comprising a promoter operably linked to a nucleotide sequence encoding a nascent RNA transcript containing an intronic microRNA (miRNA);

(b) a second nucleic acid comprising a promoter operably linked to a nucleotide sequence encoding a nascent RNA transcript containing an intronic miRNA; and

(c) a third nucleic acid comprising a promoter operable linked to a nucleotide sequence encoding an output protein linked to (i) a first miRNA-BS and (ii) a second miRNA-BS,

wherein the first miRNA-BS of (c)(i) is complementary to the miRNA of (a), and the second miRNA-BS of (c)(ii) is complementary to the miRNA of (b).

36. An engineered genetic circuit, comprising:

(a) a first nucleic acid comprising a promoter operably linked to a nucleotide sequence encoding a nascent RNA transcript containing an intronic microRNA (miRNA); and

(b) a second nucleic acid comprising a promoter operably linked to a nucleotide sequence encoding an output protein linked to a miRNA binding site (miRNA-BS);

wherein the miRNA-BS of (b) is complementary to the miRNA of (a).

37. An engineered genetic circuit, comprising:

(a) a first nucleic acid comprising a promoter operably linked to (i) a nucleotide sequence encoding an output messenger RNA (mRNA) containing an intronic microRNA (miRNA) and (ii) at least one miRNA binding site (miRNA-BS); and

(b) a second nucleic acid comprising a promoter operably linked to (i) a nucleotide sequence encoding an output mRNA containing an intronic miRNA and (ii) at least one miRNA-BS,

wherein the at least one miRNA-BS of (a) is complementary to the miRNA of (b), the at least one miRNA-BS of (b) is complementary to the miRNA of (a).

38. An engineered genetic circuit, comprising:

(a) a first nucleic acid comprising a promoter operably linked to a nucleotide sequence encoding a nascent RNA transcript containing an intronic microRNA (miRNA);

(b) a second nucleic acid comprising a promoter operably linked to a nucleotide sequence encoding an output protein; and

(c) a third nucleic acid comprising a promoter operable linked to a nucleotide sequence encoding an output protein linked to an miRNA binding site,

wherein the miRNA-BS of (c) is complementary to the miRNA of (a).

39. An engineered genetic circuit, comprising:

(a) a first nucleic acid comprising a promoter operably linked to a nucleotide sequence encoding an output protein linked to a microRNA binding site (miRNA-BS); and

(b) a second nucleic acid comprising a promoter operably linked to a nucleotide sequence encoding a nascent RNA transcript containing an intronic miRNA,

wherein the miRNA-BS of (a) is complementary to the miRNA of (b).

40. A synthetic promoter library comprising a plurality of nucleic acids, wherein each nucleic acid comprises a promoter sequence having at least two 8mer nucleotide sequences in tandem without any spacer nucleotides between each 8mer nucleotide sequence.

41. The synthetic promoter library of claim 40, wherein each nucleic acid comprises at least six 8mer nucleotide sequences in tandem without any spacer nucleotides between each 8mer nucleotide sequence.

42. The synthetic promoter library of claim 40 or claim 41, wherein each nucleic acid comprises at least twelve 8mer nucleotide sequences in tandem without any spacer nucleotides between each 8mer nucleotide sequence.

43. The synthetic promoter library of any one of claims 40-42, wherein the 8mer nucleotide sequence is NNNNNNNN, wherein each N represents any nucleotide.

44. The synthetic promoter library of any one of claims 40-43, wherein each of the nucleic acids further comprises a restriction endonuclease site at the 5' and 3' ends.

45. The synthetic promoter library of claim 44, wherein the restriction endonuclease site at the 5' end is a SbfI site and the restriction endonuclease site at the 3' end is an AscI site.

46. The synthetic promoter library of any one of claims 40-45, wherein each of the nucleic acids further comprises a nucleotide sequence encoding an output molecule operably linked to the promoter sequence.

47. The synthetic promoter library of claim 46, wherein the output molecule is a detectable molecule.

48. A synthetic promoter library comprising a plurality of nucleic acids, wherein each nucleic acid comprises a promoter sequence having at least two 8mer nucleotide sequences in tandem with a 3mer nucleotide spacer between each 8mer nucleotide sequence.

49. The synthetic promoter library of claim 48, wherein each nucleic acid comprises at least six 8mer nucleotide sequences in tandem with a 3mer nucleotide spacer between each 8mer nucleotide sequence.

50. The synthetic promoter library of claim 48 or claim 49, wherein each nucleic acid comprises at least nine 8mer nucleotide sequences in tandem with a 3mer nucleotide spacer between each 8mer nucleotide sequence.

51. The synthetic promoter library of any one of claims 48-50, wherein the 8mer nucleotide sequence is NNNNNNNN, wherein each N represents any nucleotide.

52. The synthetic promoter library of any one of claims 48-51, wherein each of the nucleic acids further comprises a restriction endonuclease site at the 5' and 3' ends.

53. The synthetic promoter library of claims 52, wherein the restriction endonuclease site at the 5' end is a SbfI site and the restriction endonuclease site at the 3' end is an AscI site.

54. The synthetic promoter library of any one of claims 48-53, wherein the 3mer nucleotide spacers are selected from AGC, ATC, GAC, ACT, AGT, GTC, GAT, and GCT.

55. The synthetic promoter library of claim 54, wherein each 3mer nucleotide spacer is different.

56. The synthetic promoter library of any one of claims 48-55, wherein each of the nucleic acids further comprises a nucleotide sequence encoding an output molecule operably linked to the promoter sequence.

57. The synthetic promoter library of claim 56, wherein the output molecule is a detectable molecule.

58. A synthetic promoter library comprising a plurality of nucleic acids, wherein each nucleic acid comprises a promoter sequence having at least two 11mer nucleotide sequences in tandem with a 3mer nucleotide spacer between each 11mer nucleotide sequence.

59. The synthetic promoter library of claim 58, wherein each nucleic acid comprises at least four 11mer nucleotide sequences in tandem with a 3mer nucleotide spacer between each 11mer nucleotide sequence.

60. The synthetic promoter library of claim 58 or claim 59, wherein each nucleic acid comprises at least seven 11mer nucleotide sequences in tandem with a 3mer nucleotide spacer between each 11mer nucleotide sequence.

61. The synthetic promoter library of any one of claims 58-60, wherein the 11mer nucleotide sequence is NNNNNNNNNNN, wherein each N represents any nucleotide.

62. The synthetic promoter library of any one of claims 58-61, wherein each of the nucleic acids further comprises a restriction endonuclease site at the 5' and 3' ends.

63. The synthetic promoter library of claims 62, wherein the restriction endonuclease site at the 5' end is a SbfI site and the restriction endonuclease site at the 3' end is an AscI site.

64. The synthetic promoter library of any one of claims 58-63, wherein the 3mer nucleotide spacers are selected from AGC, ATC, GAC, ACT, AGT, GTC, GAT, and GCT.

65. The synthetic promoter library of claim 64, wherein each 3mer nucleotide spacer is different.

66. The synthetic promoter library of any one of claims 58-65, wherein each of the nucleic acids further comprises a nucleotide sequence encoding an output molecule operably linked to the promoter sequence.

67. The synthetic promoter library of claim 66, wherein the output molecule is a detectable molecule.

68. A method of selecting a synthetic promoters comprising

obtaining a library comprising nucleic acid molecules comprising synthetic promoter sequences operably linked to an output molecule,

expressing the library in one or more types of cells,

detecting the expression of the output molecule, and

isolating the cells in which the output molecule is expressed.

69. The method of claim 68, further comprising

determining the sequence of the synthetic promoter sequences in the isolated cells.

70. The method of claim 68 or claim 69, wherein the one or more types of cells are at least two different types of cells.

71. The method of claim 70, further comprising comparing the synthetic promoter sequences that drive the expression of the output molecule in each of the at least two different types of cells to identify synthetic promoter sequences that are more active in one of the at least two different types of cells than in another of the at least two different types of cells.

72. The method of claim 71, wherein the at least two different types of cells are cancer cells and non-cancer cells, and wherein promoters are identified that are more active in cancer cells than in non-cancer cells.

5

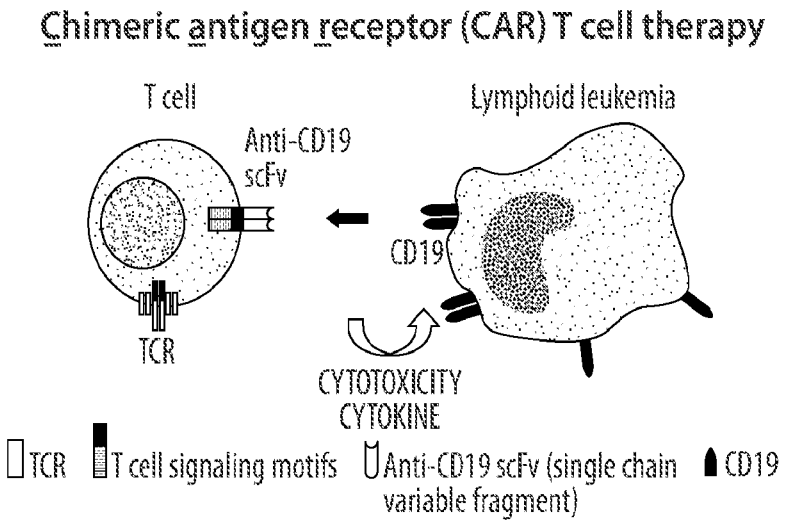


Fig. 1A

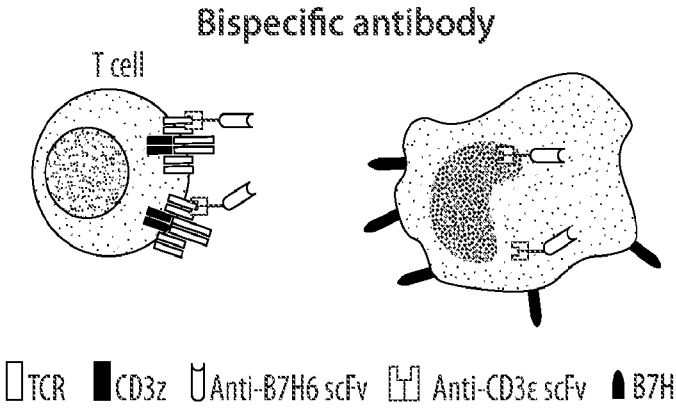


Fig. 1B

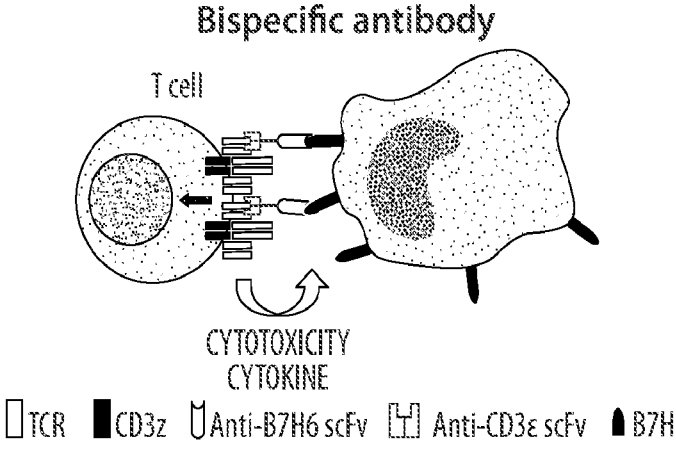


Fig. 1C

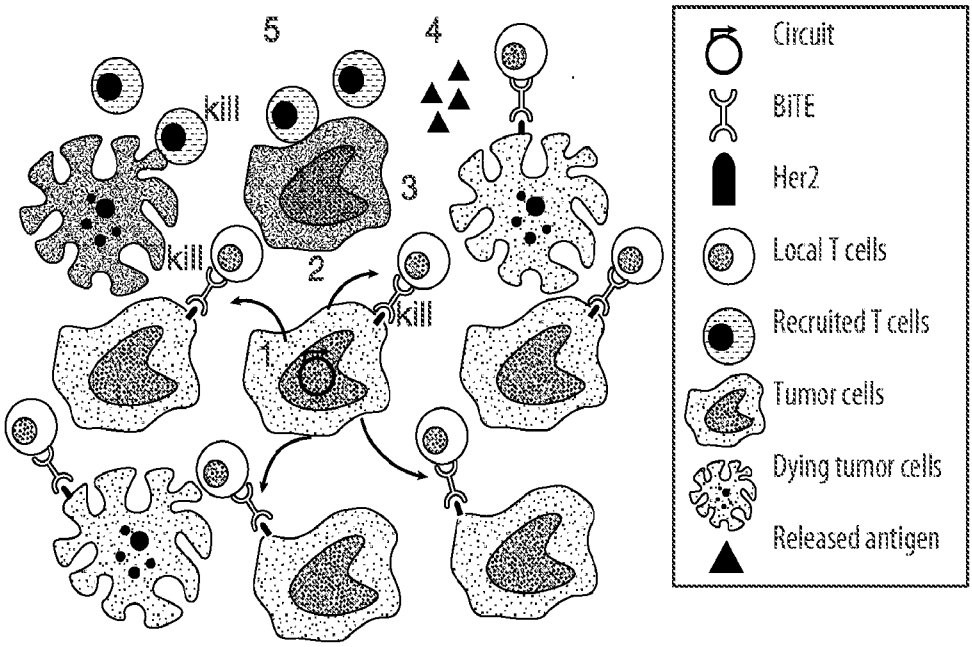

5
4
kill
3
kill
2
kill
1
Circuit
BiTE
Her2
Local T cells
Recruited T cells
Tumor cells
Dying tumor cells
Released antigen

Fig. 2A

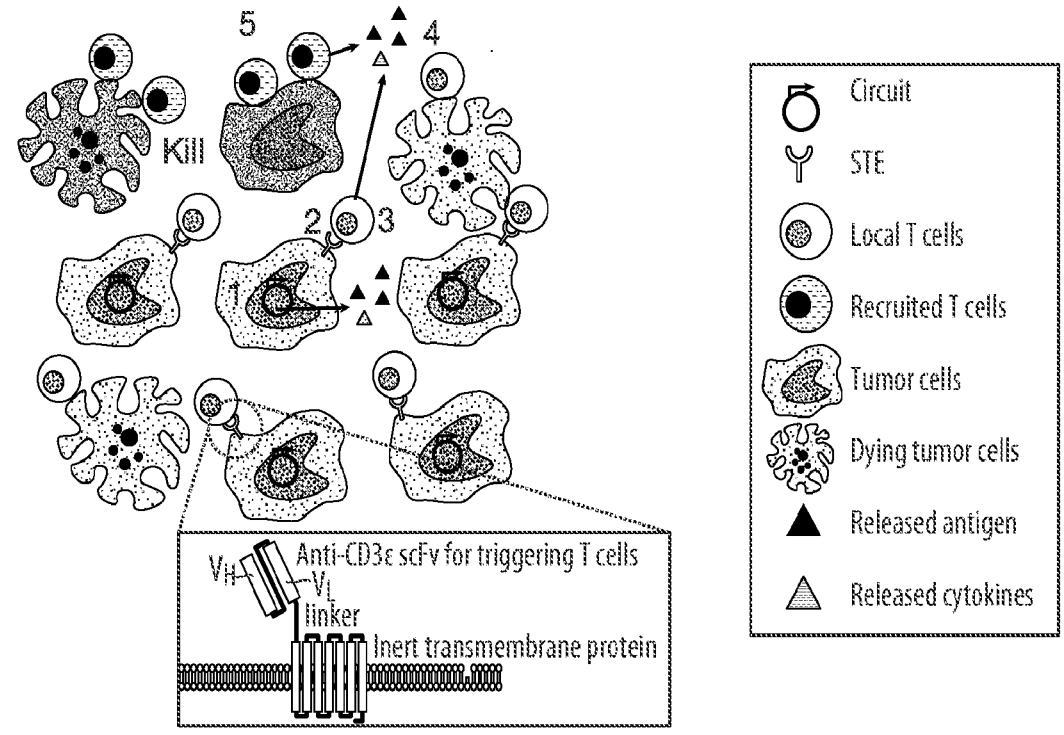

5
4
Kill
2
3
1
Anti-CD3ε scFv for triggering T cells
VH
VL
linker
Inert transmembrane protein
Circuit
STE
Local T cells
Recruited T cells
Tumor cells
Dying tumor cells
Released antigen
Released cytokines

Fig. 2B

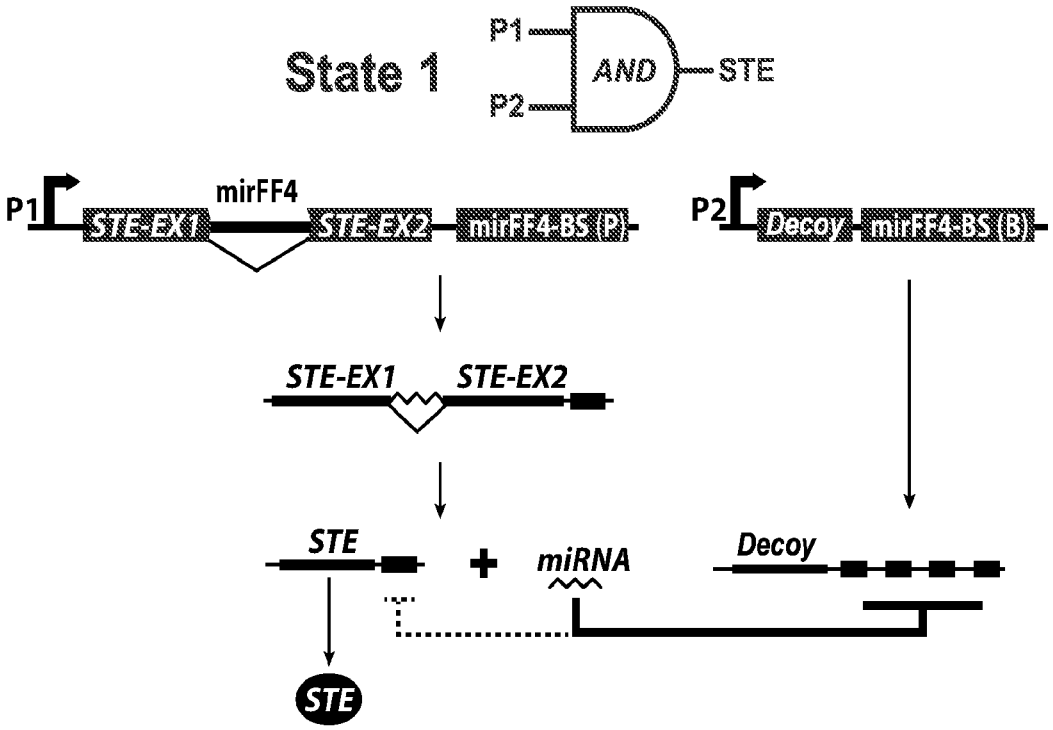

State 1
P1
P2
AND
STE
P1
mirFF4
STE-EX1
STE-EX2
mirFF4-BS (P)
P2
Decoy
mirFF4-BS (B)
STE-EX1
STE-EX2
STE
miRNA
Decoy
STE

Fig. 3A

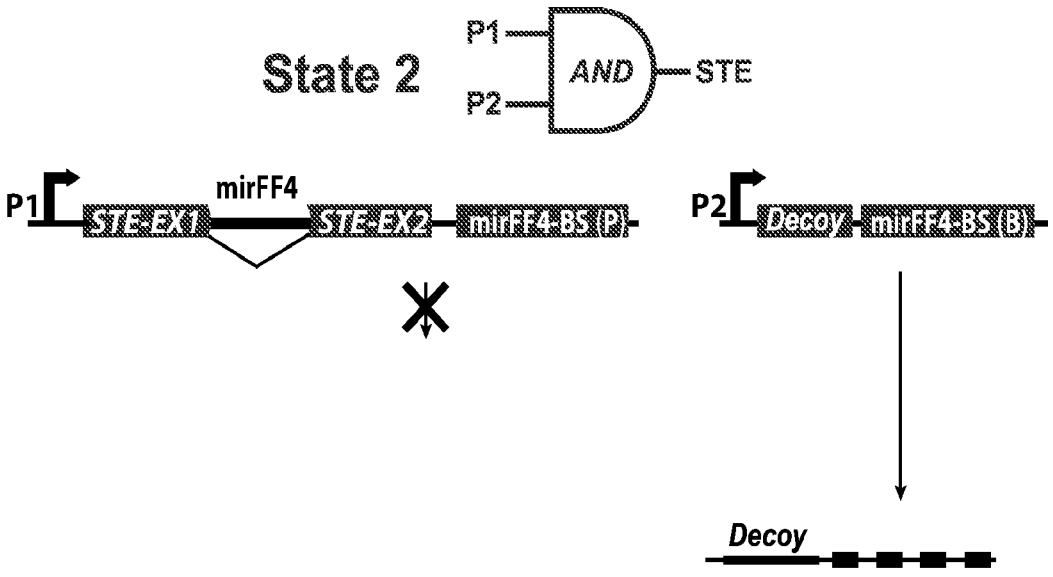

State 2
P1
P2
AND
STE
P1
mirFF4
STE-EX1
STE-EX2
mirFF4-BS (P)
P2
Decoy
mirFF4-BS (B)
Decoy

Fig. 3B

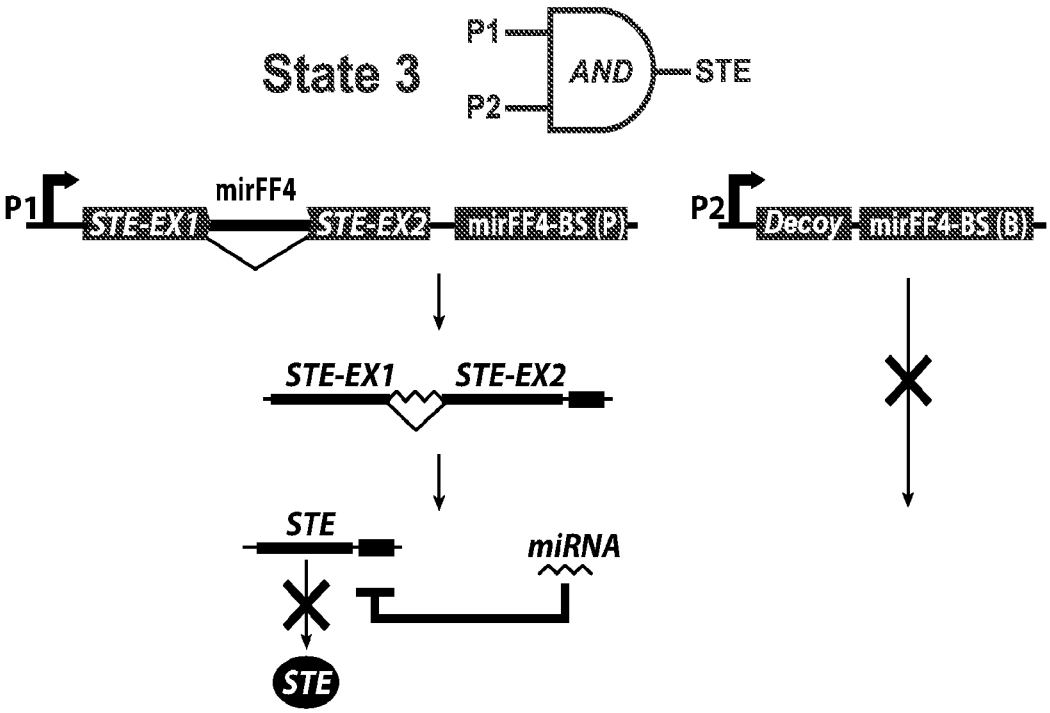

State 3
P1
P2
AND
STE
P1
mirFF4
STE-EX1
STE-EX2
mirFF4-BS (P)
P2
Decoy
mirFF4-BS (B)
STE-EX1
STE-EX2
STE
miRNA
STE

Fig. 3C

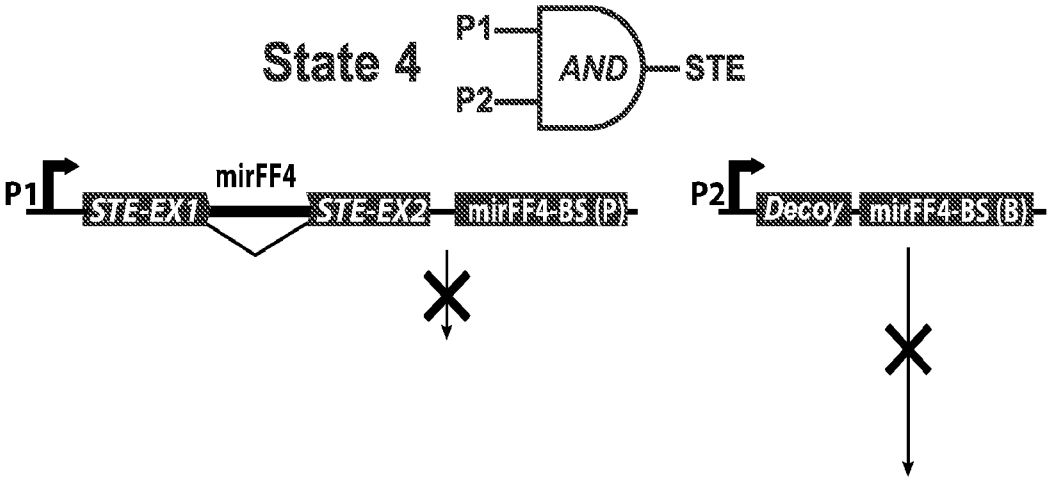

State 4
P1
P2
AND
STE
P1
mirFF4
STE-EX1
STE-EX2
mirFF4-BS (P)
P2
Decoy
mirFF4-BS (B)

Fig. 3D

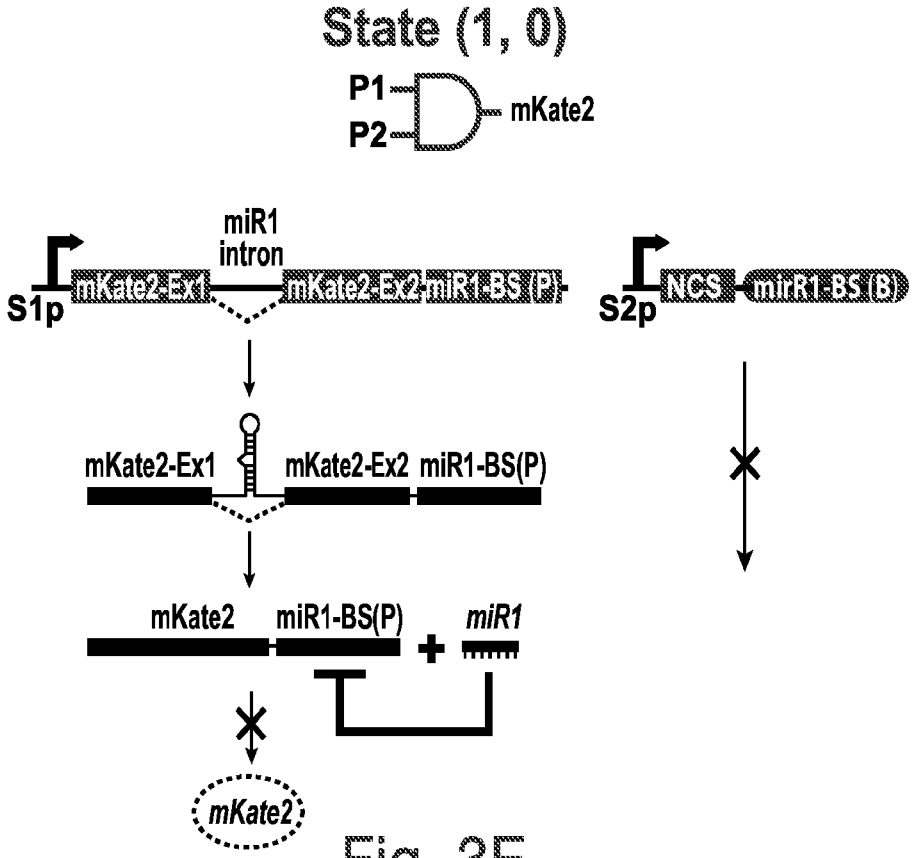

State (1, 0)
P1
P2
mKate2
miR1
intron
S1p
mKate2-Ex1
mKate2-Ex2-miR1-BS(P)
S2p
NCS
miR1-BS(B)
mKate2-Ex1
mKate2-Ex2 miR1-BS(P)
mKate2
miR1-BS(P)
miR1
mKate2

Fig. 3E

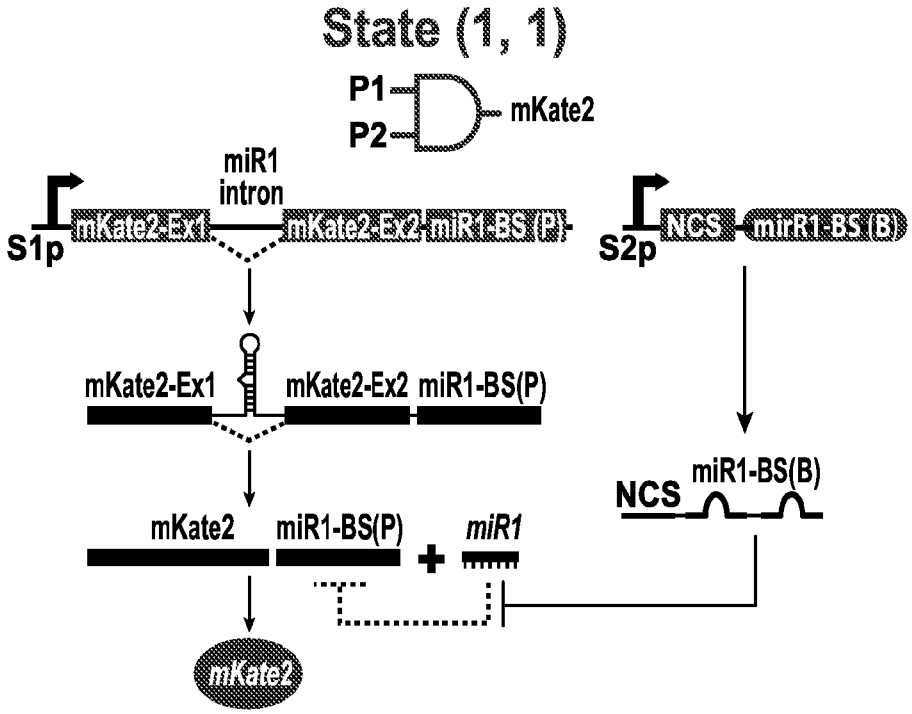

State (1, 1)
P1
P2
mKate2
miR1
intron
S1p
mKate2-Ex1
mKate2-Ex2-miR1-BS(P)
S2p
NCS
miR1-BS(B)
mKate2-Ex1
mKate2-Ex2 miR1-BS(P)
NCS
miR1-BS(B)
mKate2
miR1-BS(P)
miR1
mKate2

Fig. 3F

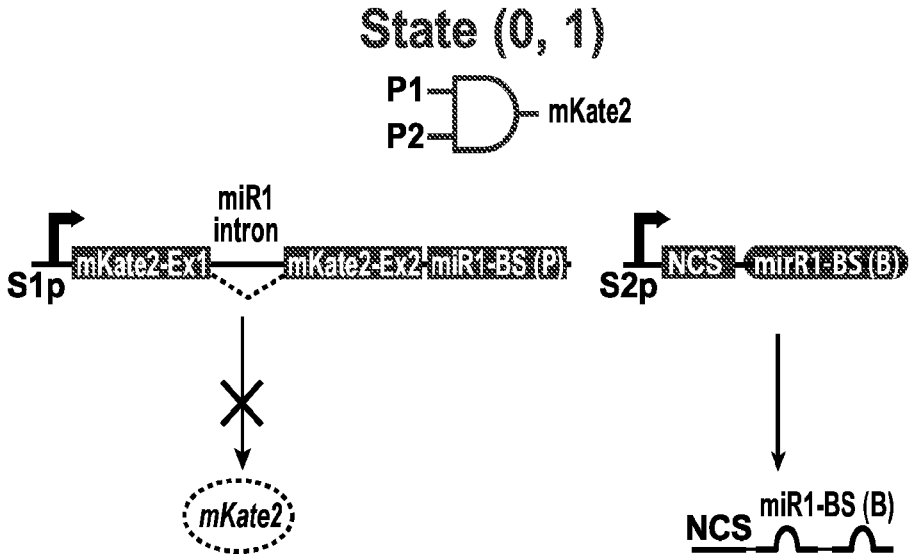

State (0, 1)
P1
P2
mKate2
miR1
intron
S1p
mKate2-Ex1
mKate2-Ex2-miR1-BS (P)
S2p
NCS
miR1-BS (B)
mKate2
NCS
miR1-BS (B)

Fig. 3G

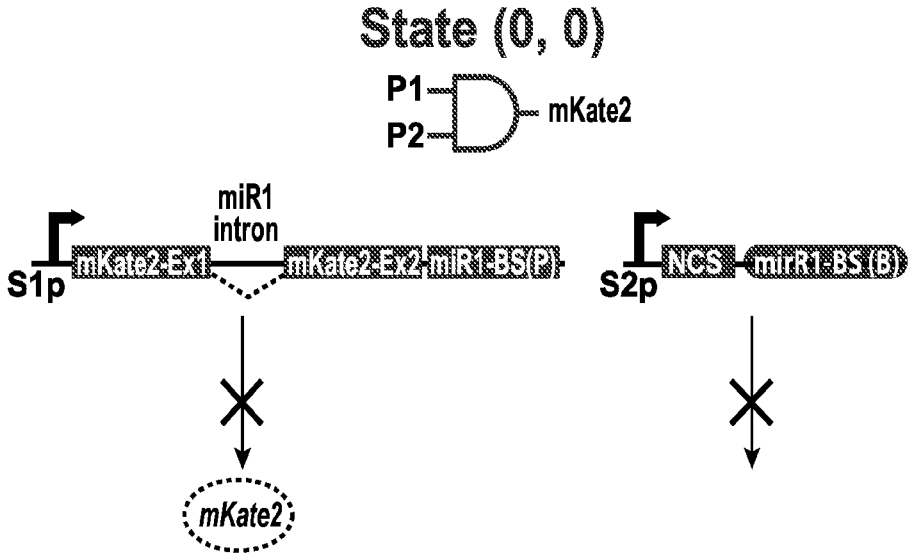

State (0, 0)
P1
P2
mKate2
miR1
intron
S1p
mKate2-Ex1
mKate2-Ex2-miR1-BS(P)
S2p
NCS
miR1-BS (B)
mKate2

Fig. 3H

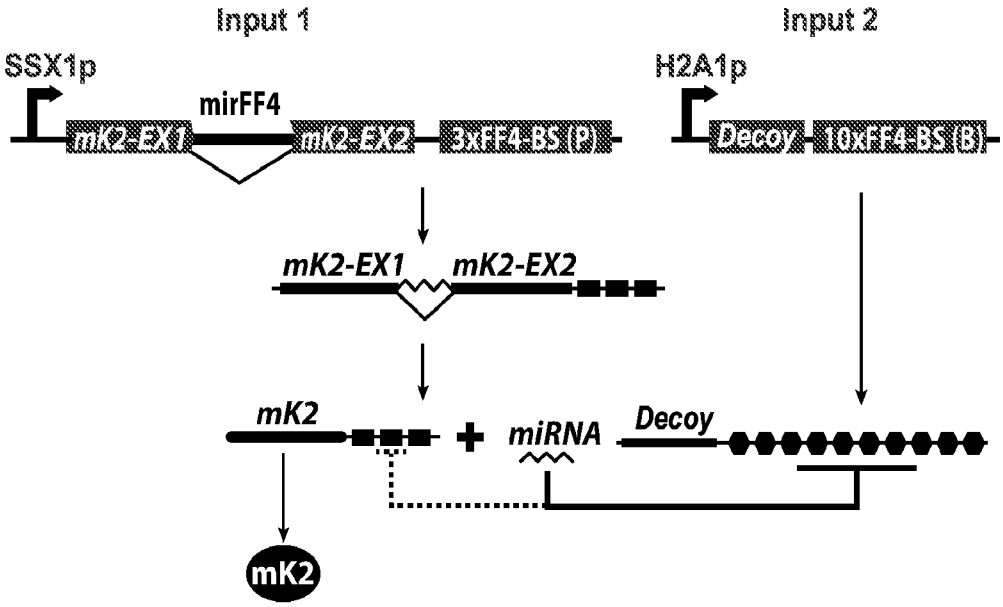

Input 1
Input 2
SSX1p
H2A1p
mirFF4
mK2-EX1
mK2-EX2
3xFF4-BS (P)
Decoy
10xFF4-BS (B)
mK2-EX1
mK2-EX2
mK2
miRNA
Decoy
mK2

Fig. 4A

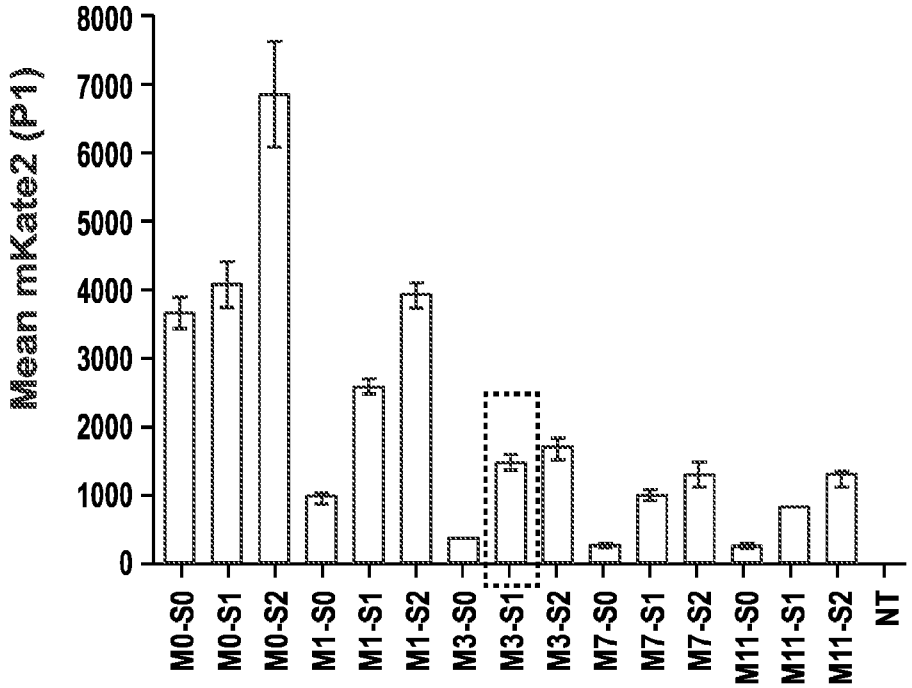

Mean mKate2 (P1)
8000
7000
6000
5000
4000
3000
2000
1000
0
M0-S0
M0-S1
M0-S2
M1-S0
M1-S1
M1-S2
M3-S0
M3-S1
M3-S2
M7-S0
M7-S1
M7-S2
M11-S0
M11-S1
M11-S2
NT

Fig. 4B

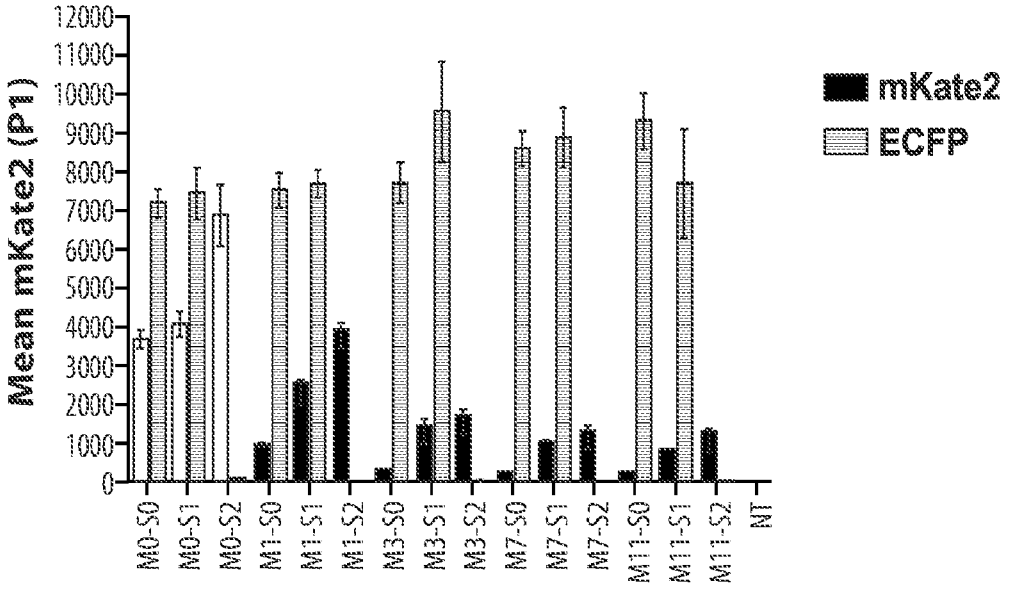

Mean mKate2 (P1)
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
mKate2
ECFP
M0-S0
M0-S1
M0-S2
M1-S0
M1-S1
M1-S2
M3-S0
M3-S1
M3-S2
M7-S0
M7-S1
M7-S2
M11-S0
M11-S1
M11-S2
NT

Fig. 5

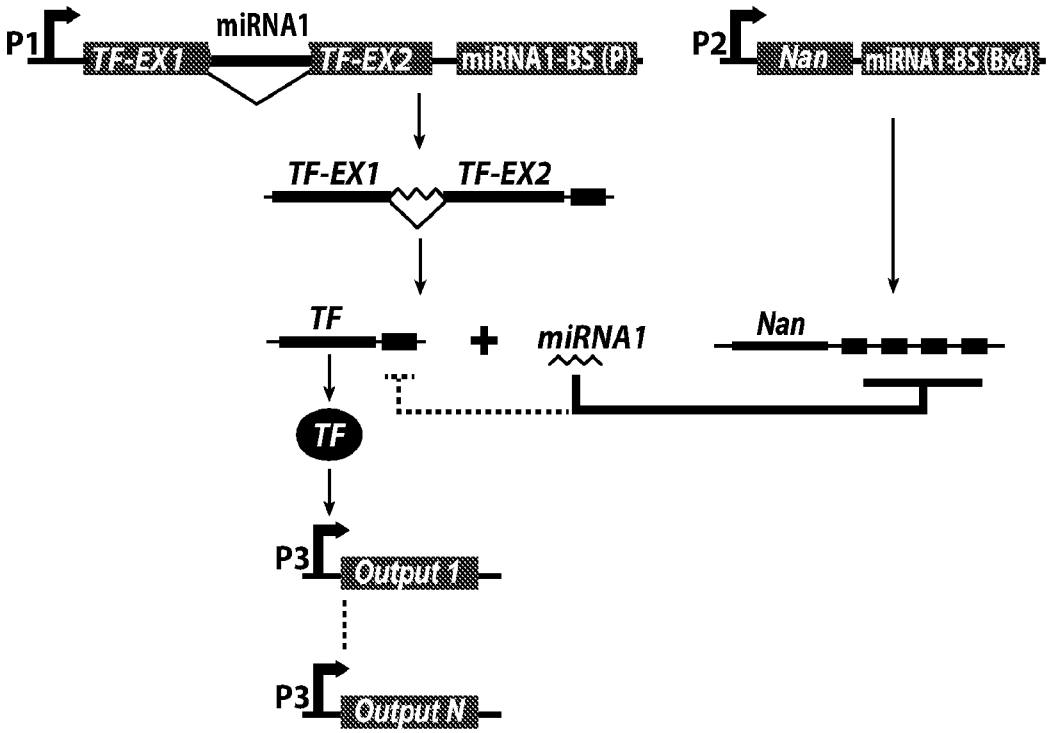

P1
miRNA1
TF-EX1
TF-EX2
miRNA1-BS (P)
P2
Nan
miRNA1 BS (BX4)
TF-EX1
TF-EX2
TF
+
miRNA1
Nan
TF
P3
Output 1
P3
Output N

Fig. 6A

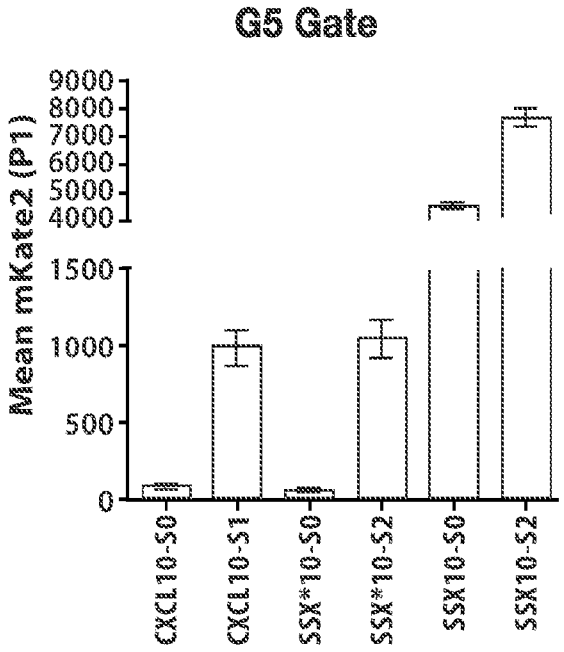

G5 Gate
Mean mKate2 (P1)
9000
8000
7000
6000
5000
4000
1500
1000
500
0
CXCL10-S0
CXCL10-S1
SSX*10-S0
SSX*10-S2
SSX10-S0
SSX10-S2

Fig. 6B

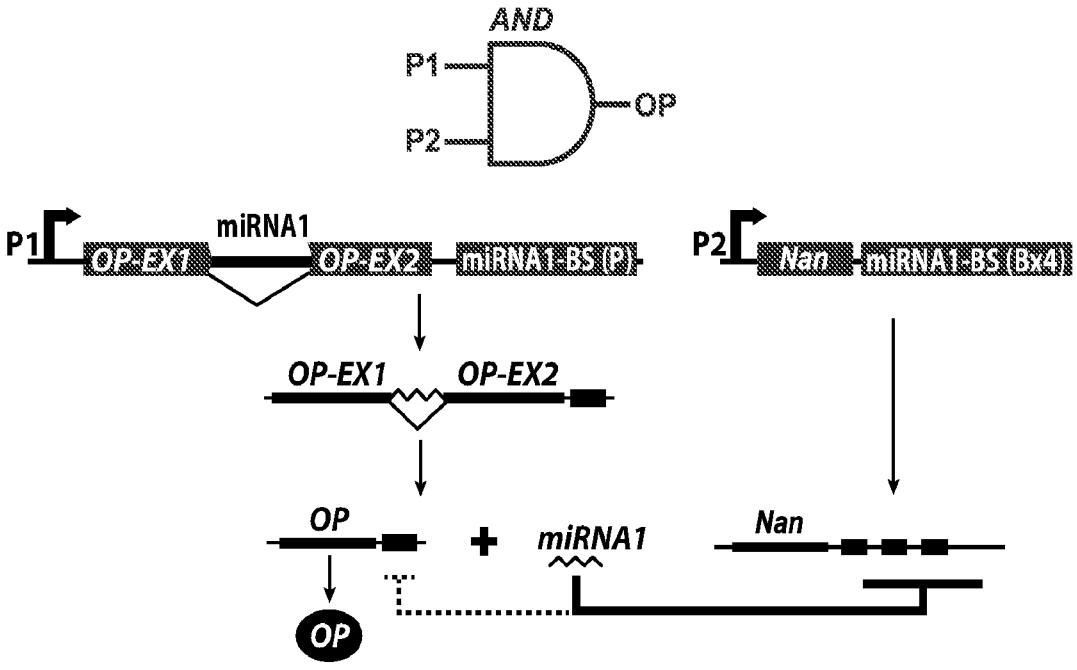

AND
P1
P2
OP
P1
miRNA1
OP-EX1
OP-EX2
miRNA1-BS (P)
P2
Nan
miRNA1-BS (Bx4)
OP-EX1
OP-EX2
OP
+
miRNA1
Nan
OP

Fig. 7A

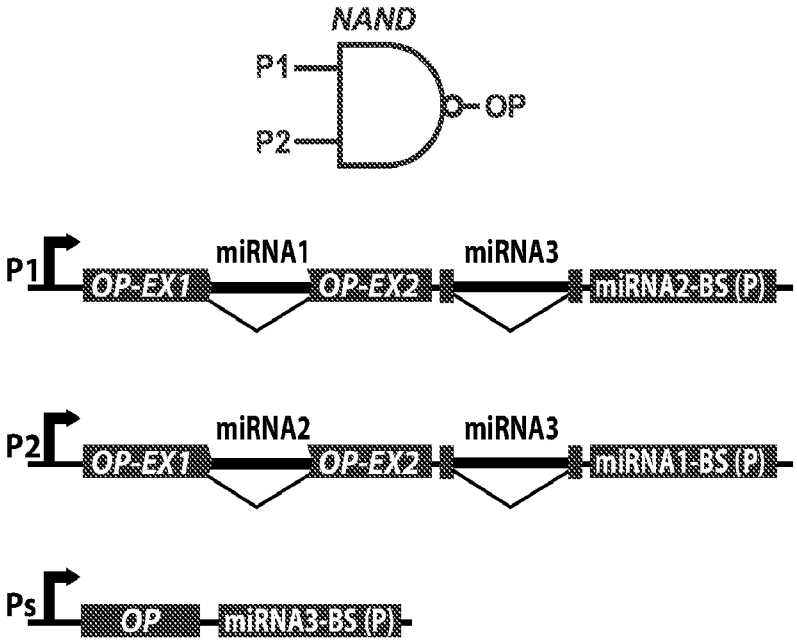

NAND
P1
P2
OP
P1
miRNA1
miRNA3
OP-EX1
OP-EX2
miRNA2-BS (P)
P2
miRNA2
miRNA3
OP-EX1
OP-EX2
miRNA1-BS (P)
Ps
OP
miRNA3-BS (P)

Fig. 7B

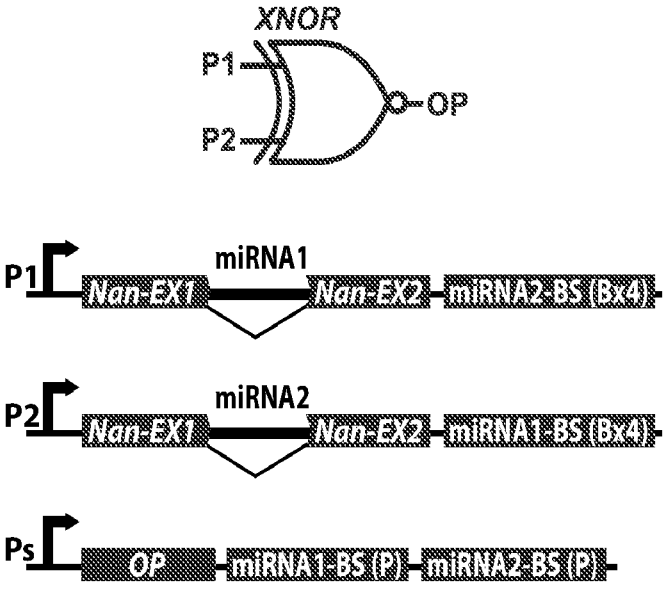

XNOR
P1
P2
OP
P1
miRNA1
Nan-EX1
Nan-EX2
miRNA2-BS (Bx4)
P2
miRNA2
Nan-EX1
Nan-EX2
miRNA1-BS (Bx4)
Ps
OP
miRNA1-BS (P)
miRNA2-BS (P)

Fig. 7C

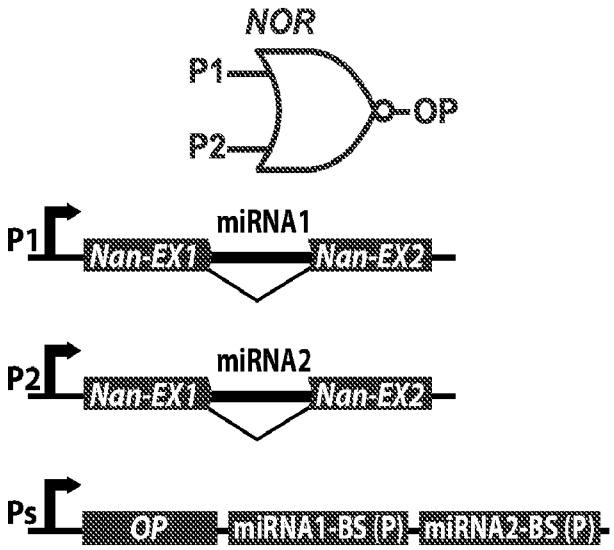

NOR
P1
P2
OP
P1
miRNA1
Nan-EX1
Nan-EX2
P2
miRNA2
Nan-EX1
Nan-EX2
Ps
OP
miRNA1-BS (P)
miRNA2-BS (P)

Fig. 7D

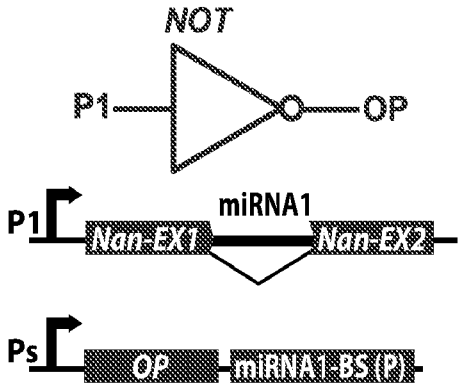

NOT
P1
OP
P1
miRNA1
Nan-EX1
Nan-EX2
Ps
OP
miRNA1-BS (P)

Fig. 7E

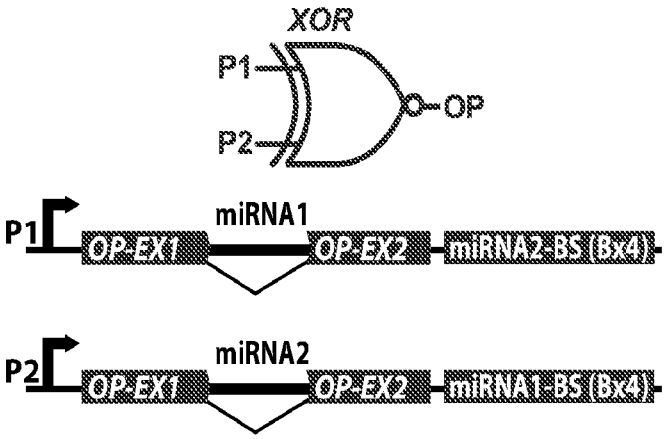

XOR
P1
P2
OP
P1
miRNA1
OP EX1
OP EX2
miRNA2 BS (8x4)
P2
miRNA2
OP EX1
OP EX2
miRNA1 BS (8x4)

Fig. 7F

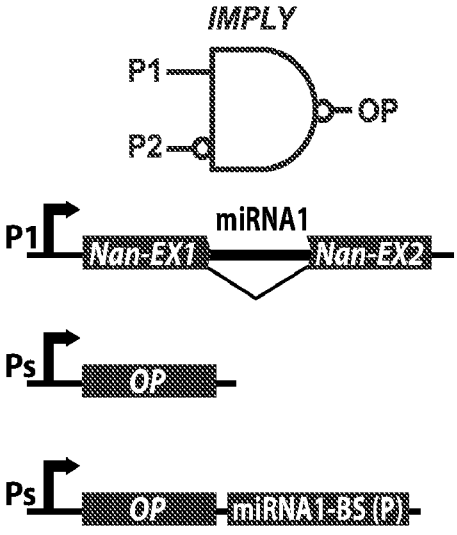

IMPLY
P1
P2
OP
P1
miRNA1
Nan EX1
Nan EX2
Ps
OP
Ps
OP
miRNA1 BS (P)

Fig. 7G

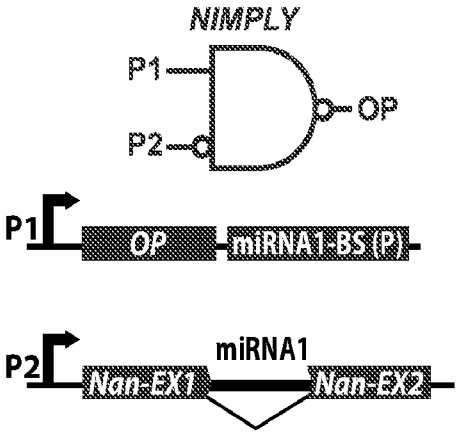

NIMPLY
P1
P2
OP
P1
OP
miRNA1-BS (P)
P2
miRNA1
Nan-EX1
Nan-EX2

Fig. 7H

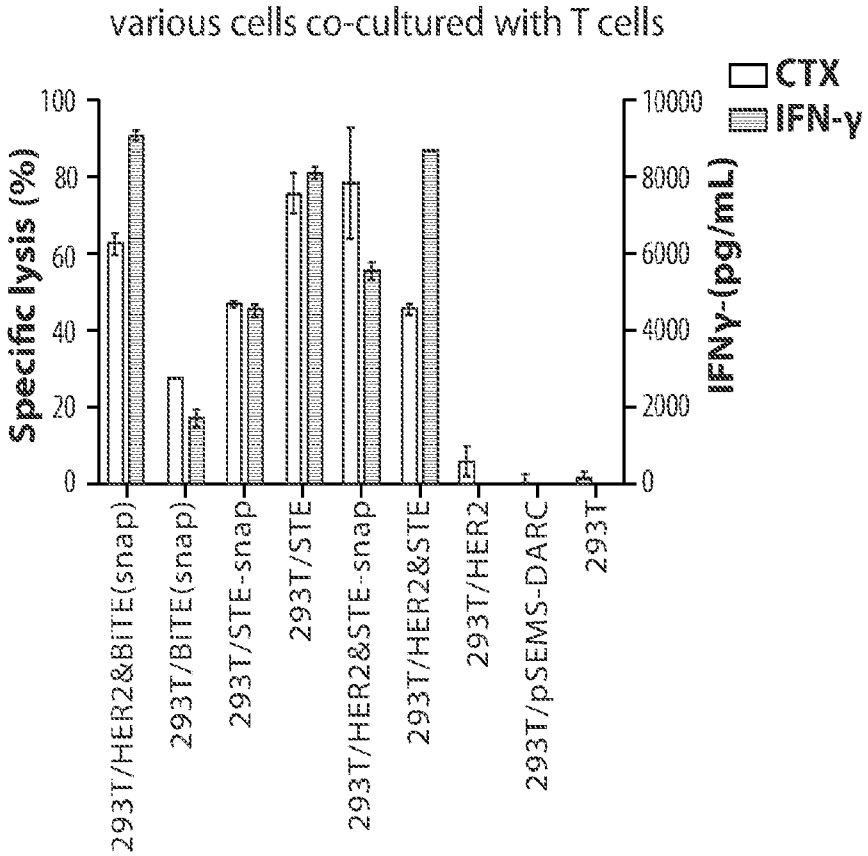

various cells co-cultured with T cells
CTX
IFN-γ
Specific lysis (%)
IFNγ-(pg/mL)
100
80
60
40
20
0
10000
8000
6000
4000
2000
0
293T/HER2&BiTE(snap)
293T/BiTE(snap)
293T/STE-snap
293T/STE
293T/HER2&STE-snap
293T/HER2&STE
293T/HER2
293T/pSEMS-DARC
293T

Fig. 8

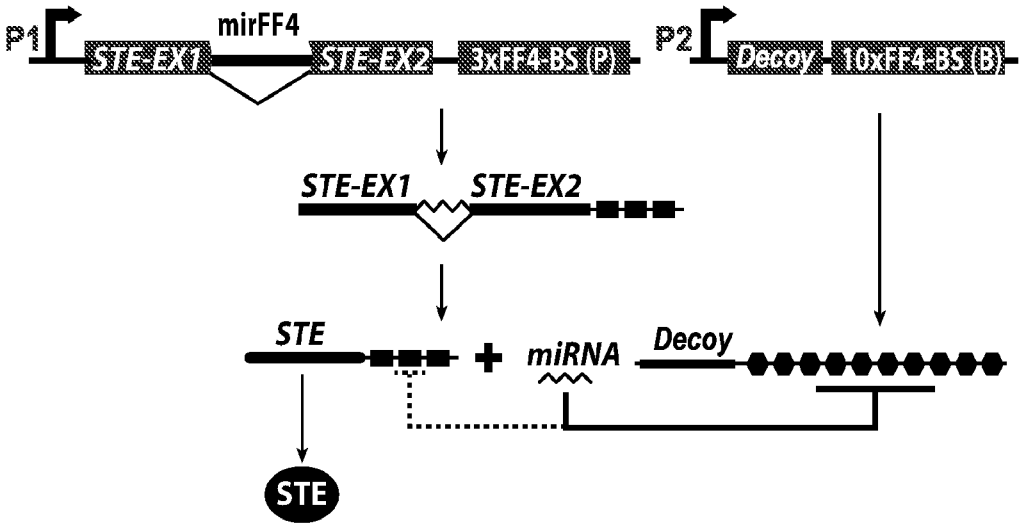


Fig. 9A

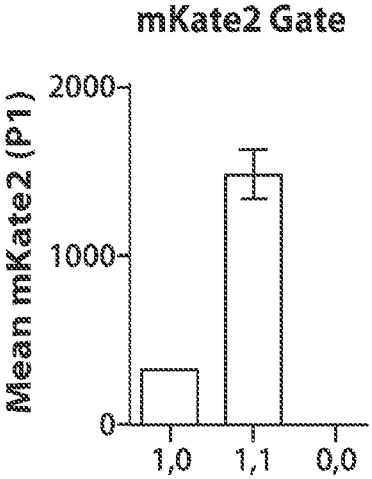


Fig. 9B

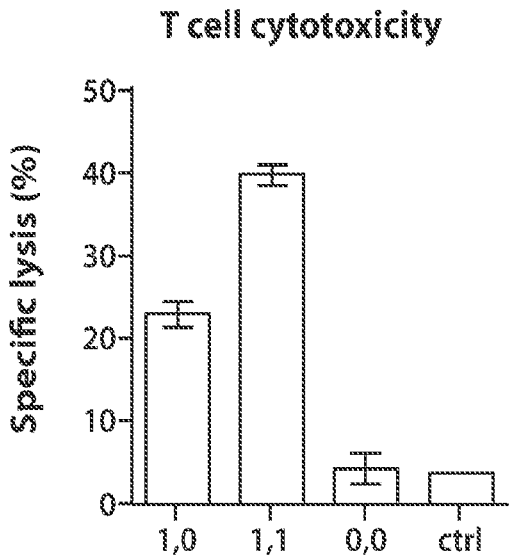


Fig. 9C

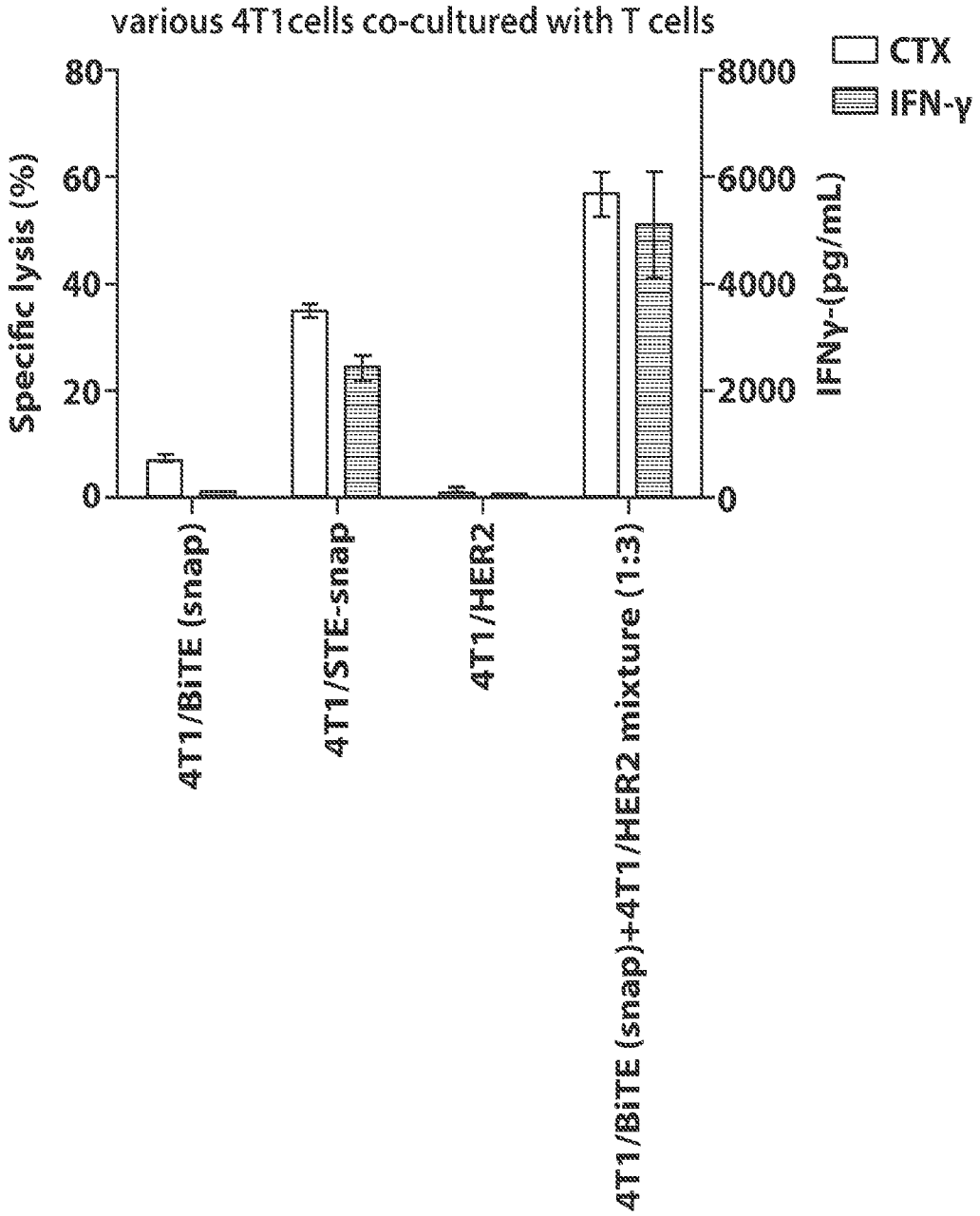

various 4T1cells co-cultured with T cells
CTX
IFN-γ
Specific lysis (%)
IFNγ-(pg/mL)
0
20
40
60
80
0
2000
4000
6000
8000
4T1/BiTE (snap)
4T1/STE-snap
4T1/HER2
4T1/BiTE (snap)+4T1/HER2 mixture (1:3)

Fig. 10

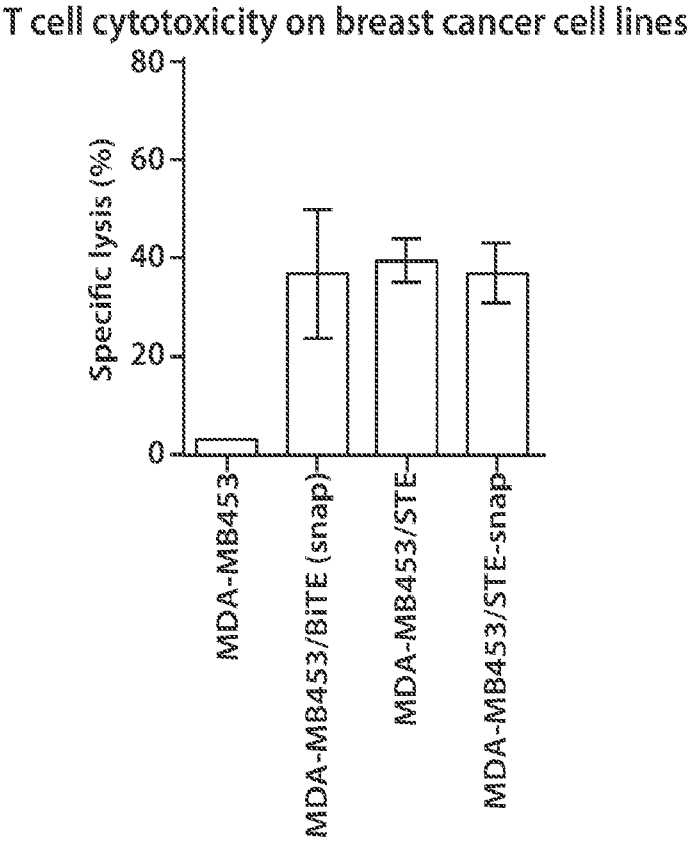

T cell cytotoxicity on breast cancer cell lines
Specific lysis (%)
80
60
40
20
0
MDA-MB453
MDA-MB453/BiTE (snap)
MDA-MB453/STE
MDA-MB453/STE-snap

Fig. 11

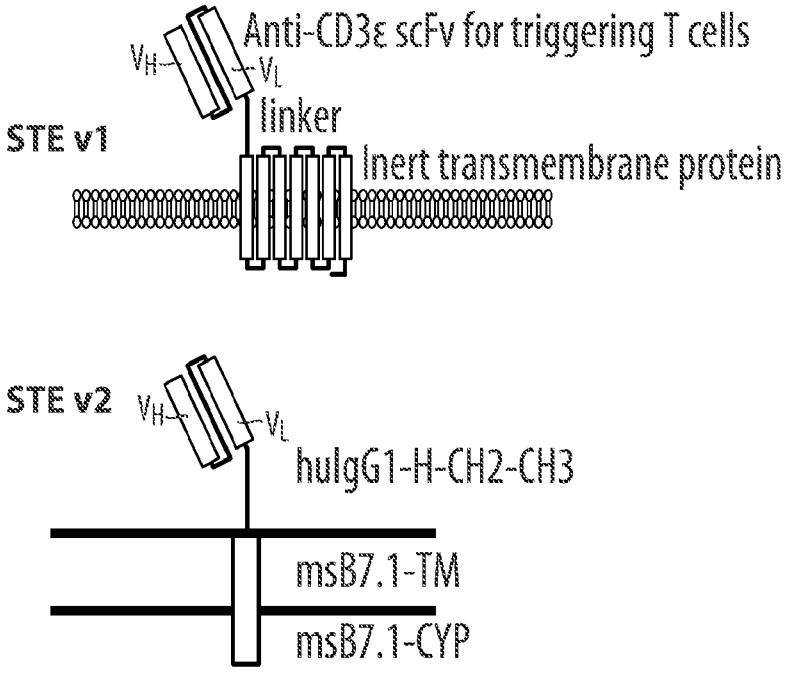

Anti-CD3ε scFv for triggering T cells
VH
VL
linker
STE v1
Inert transmembrane protein
STE v2
VH
VL
huIgG1-H-CH2-CH3
msB7.1-TM
msB7.1-CYP

Fig. 12

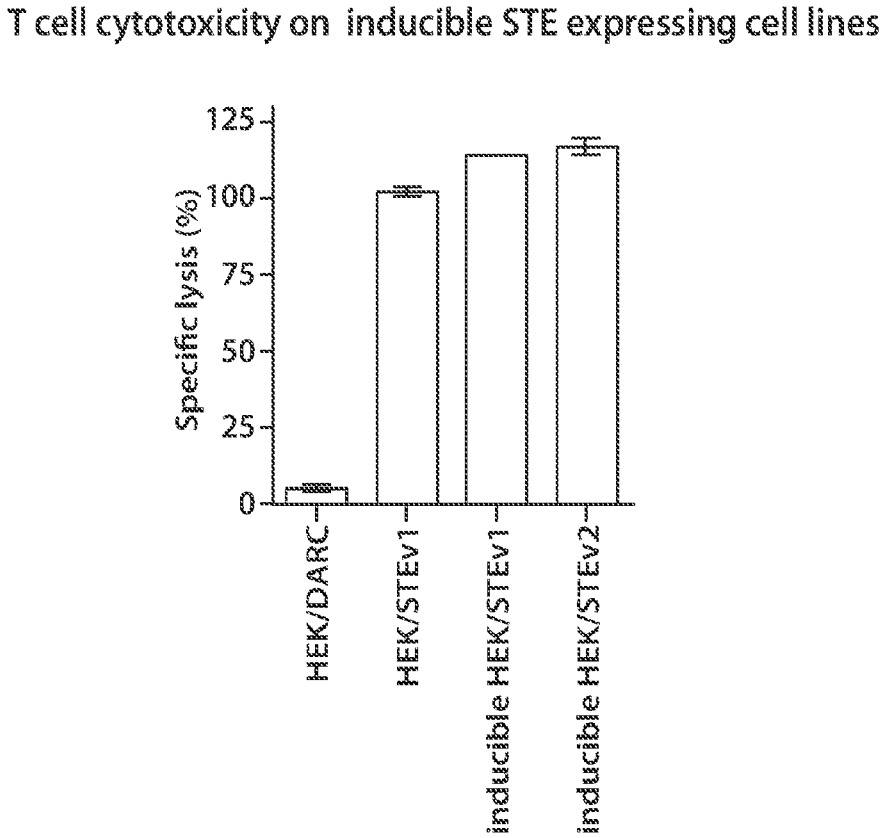

T cell cytotoxicity on inducible STE expressing cell lines
Specific lysis (%)
125
100
75
50
25
0
HEK/DARC
HEK/STEv1
inducible HEK/STEv1
inducible HEK/STEv2

Fig. 13

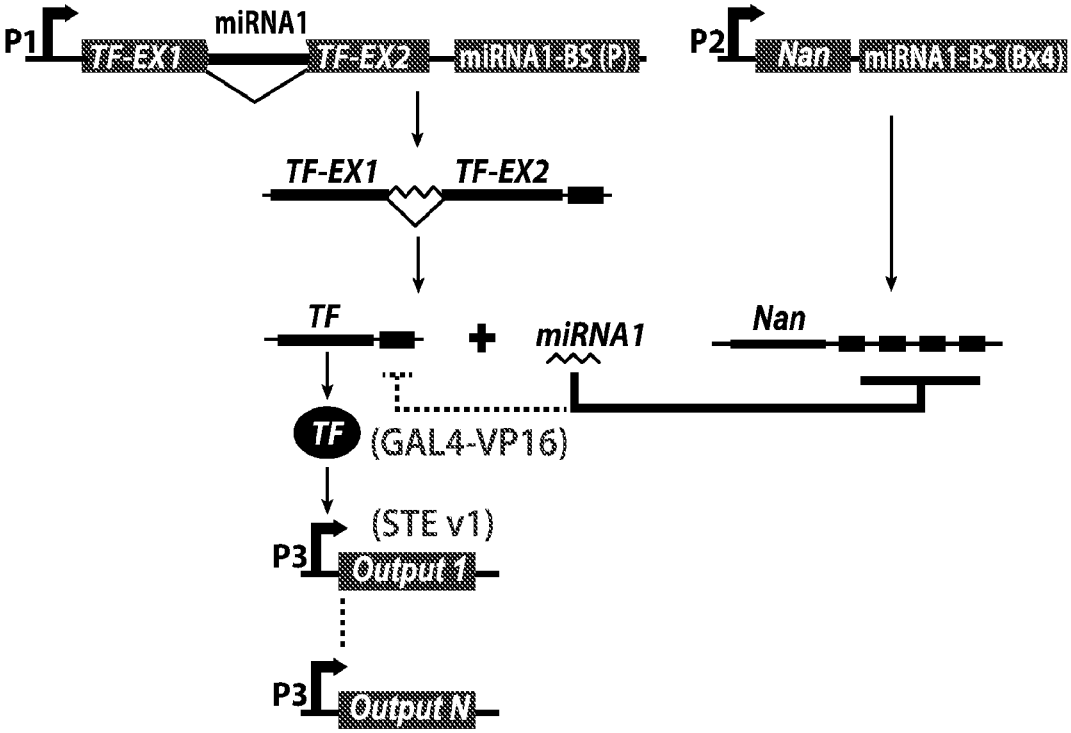


Fig. 14A

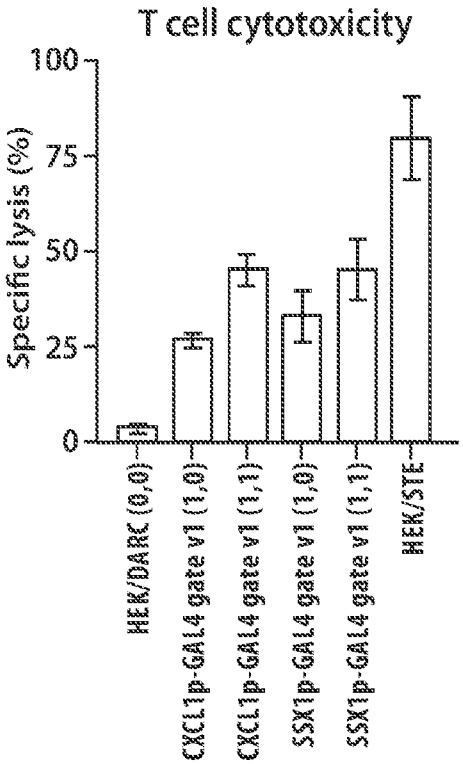


Fig. 14B

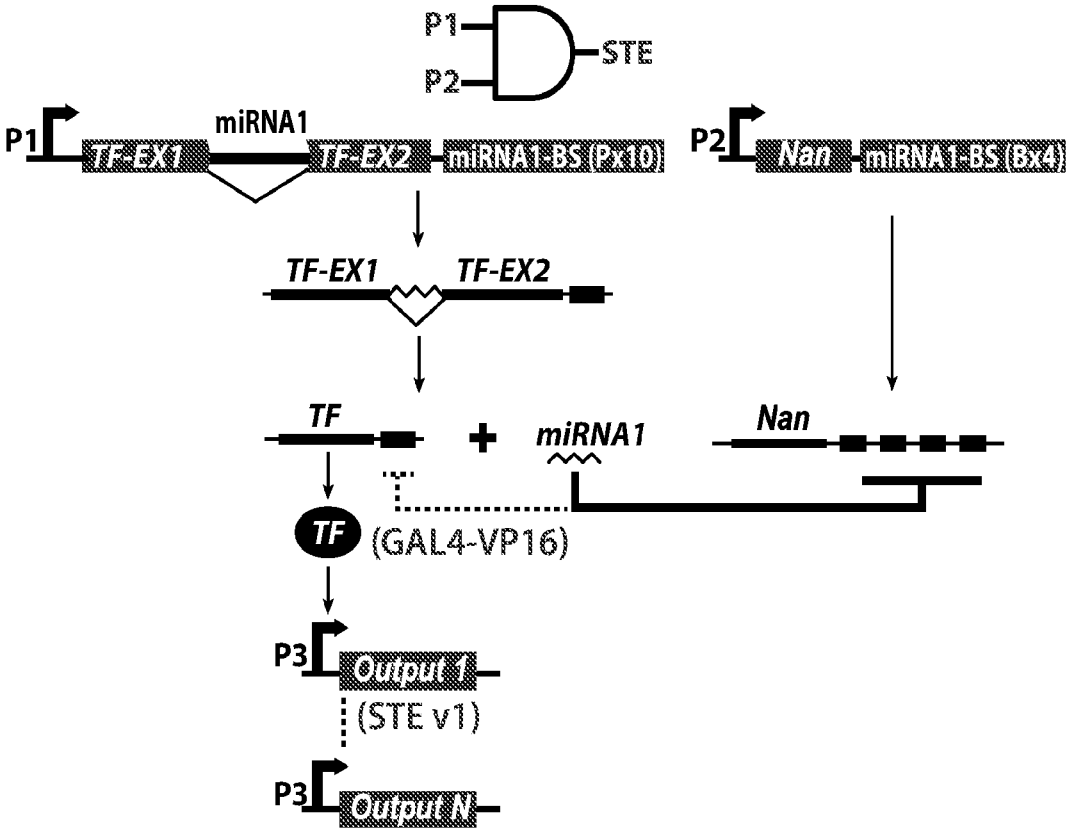


Fig. 15A

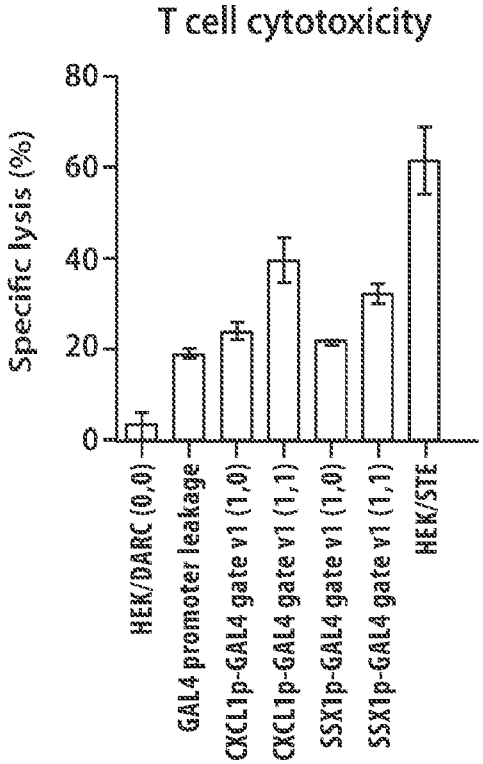


Fig. 15B

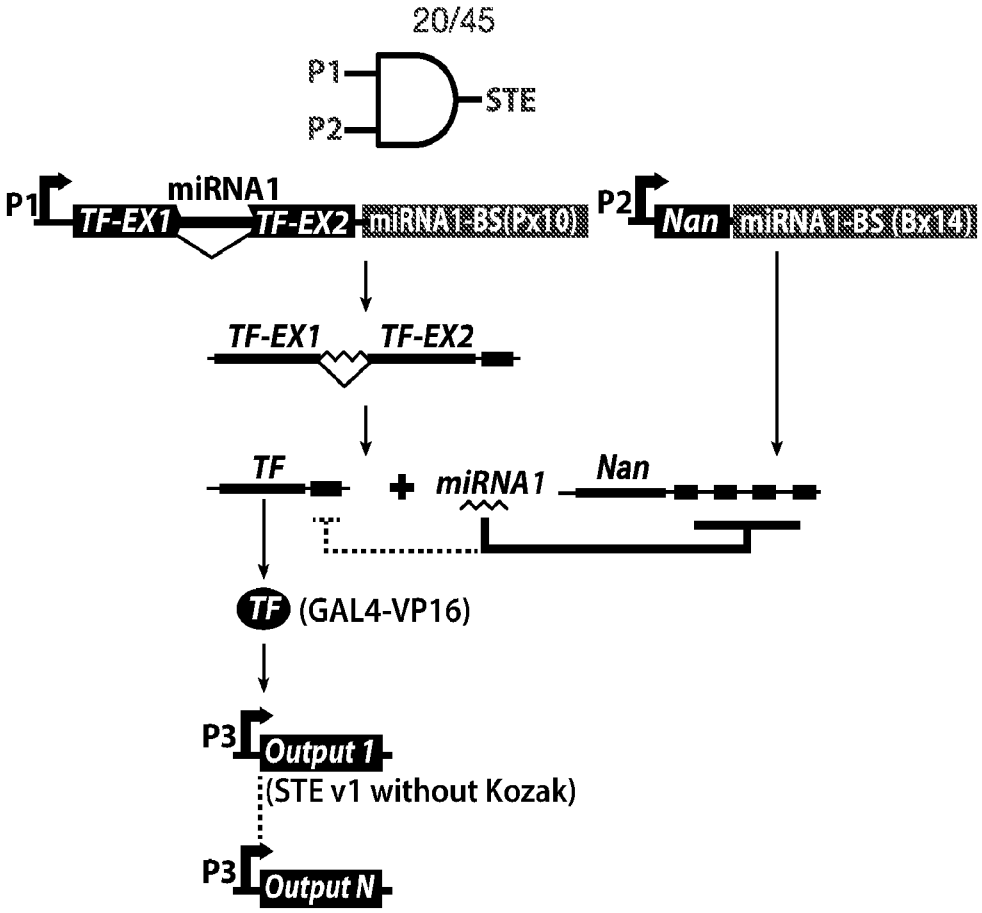


Fig. 16A

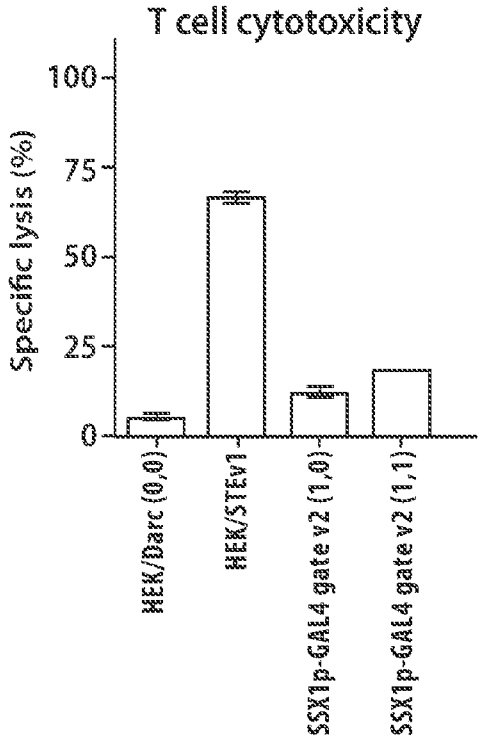


Fig. 16B

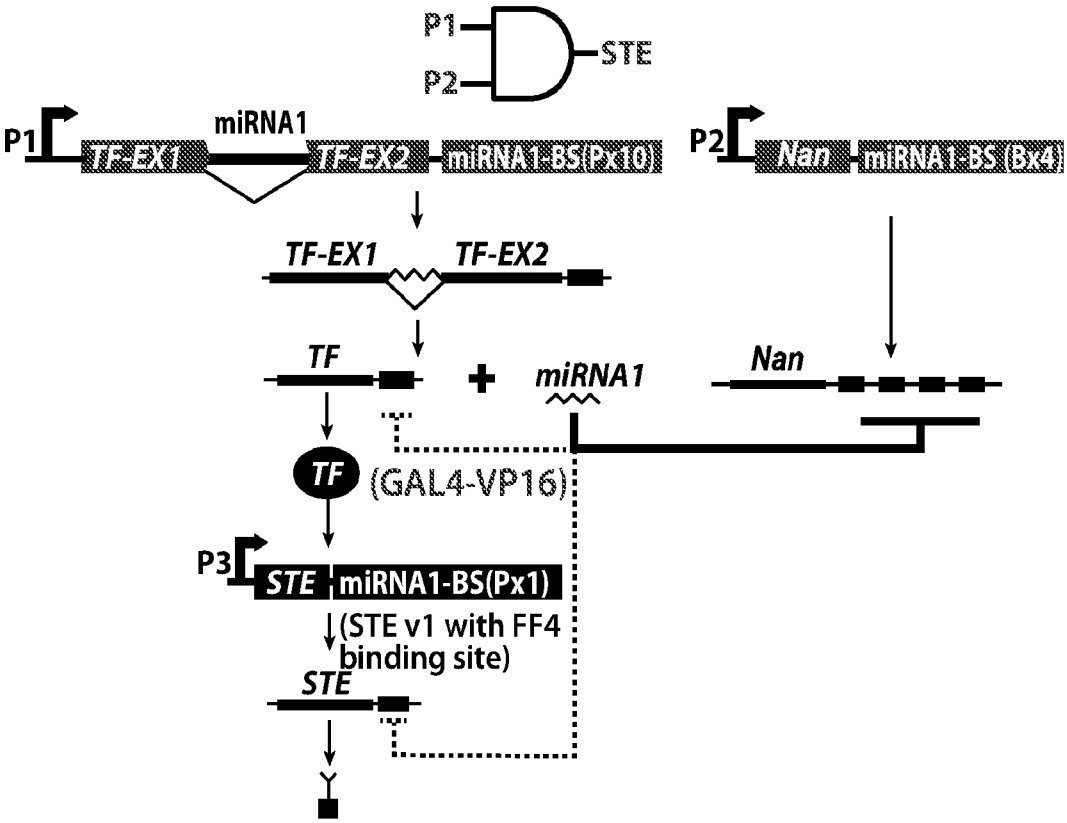


Fig. 17A

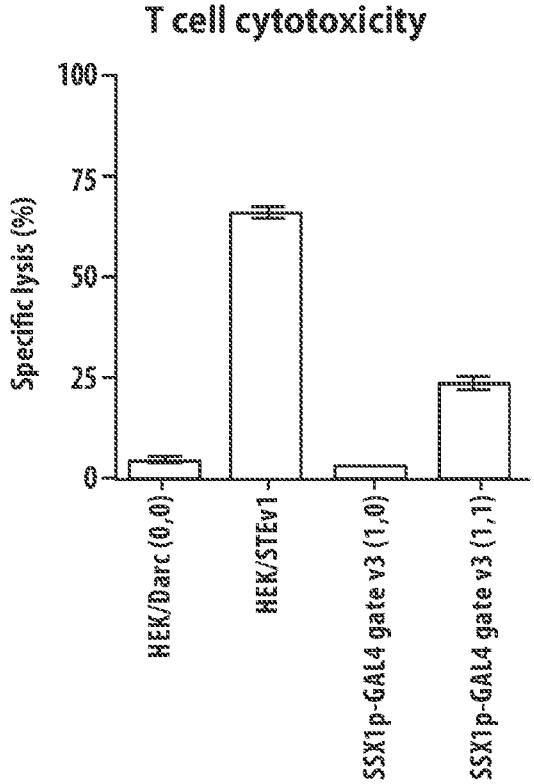


Fig. 17B

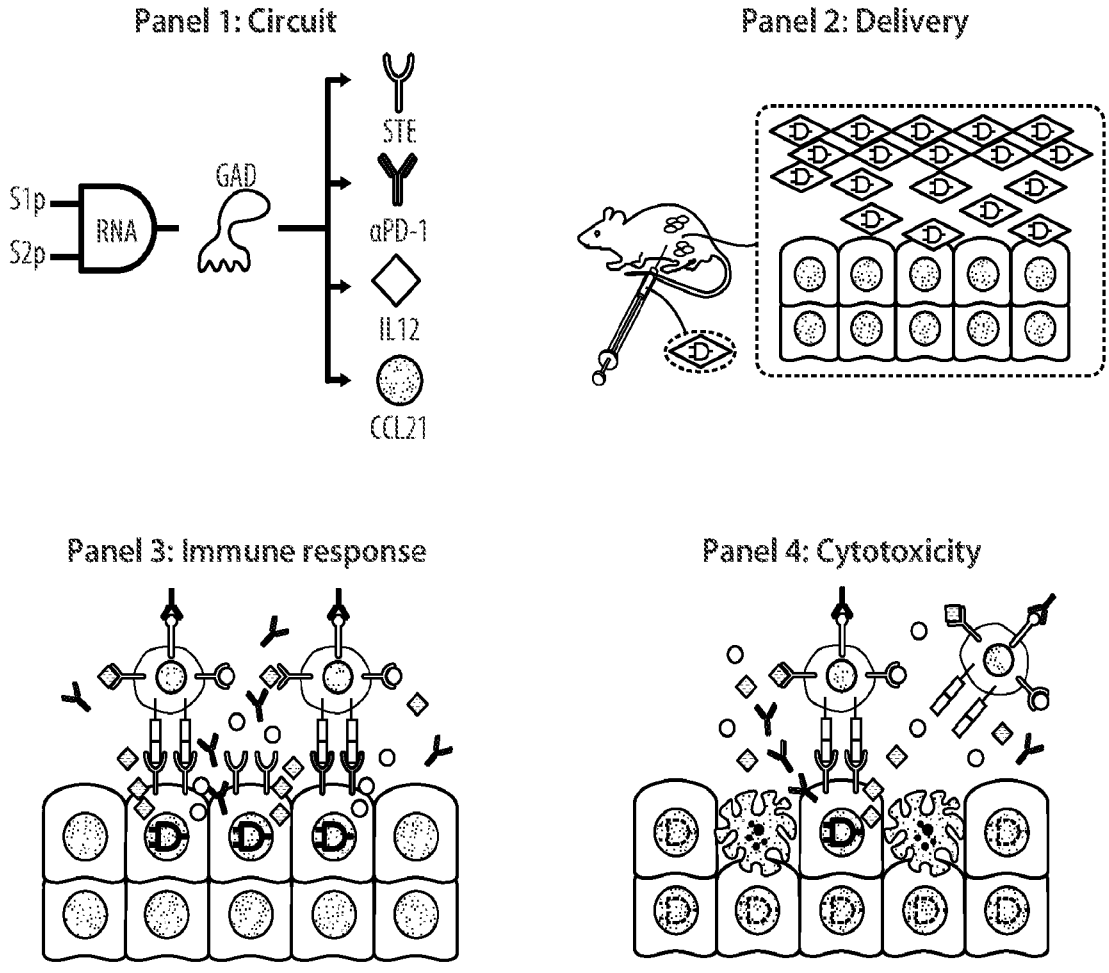

Panel 1: Circuit
S1p
S2p
RNA
GAD
STE
αPD-1
IL12
CCL21
Panel 2: Delivery
Panel 3: Immune response
Panel 4: Cytotoxicity

Fig. 18

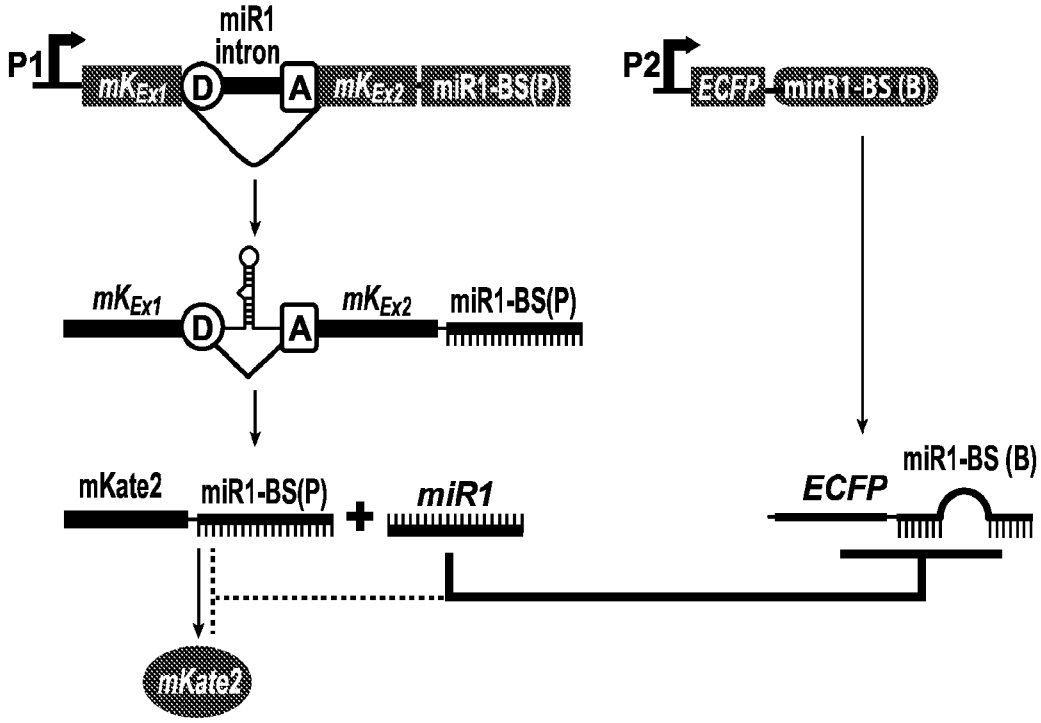
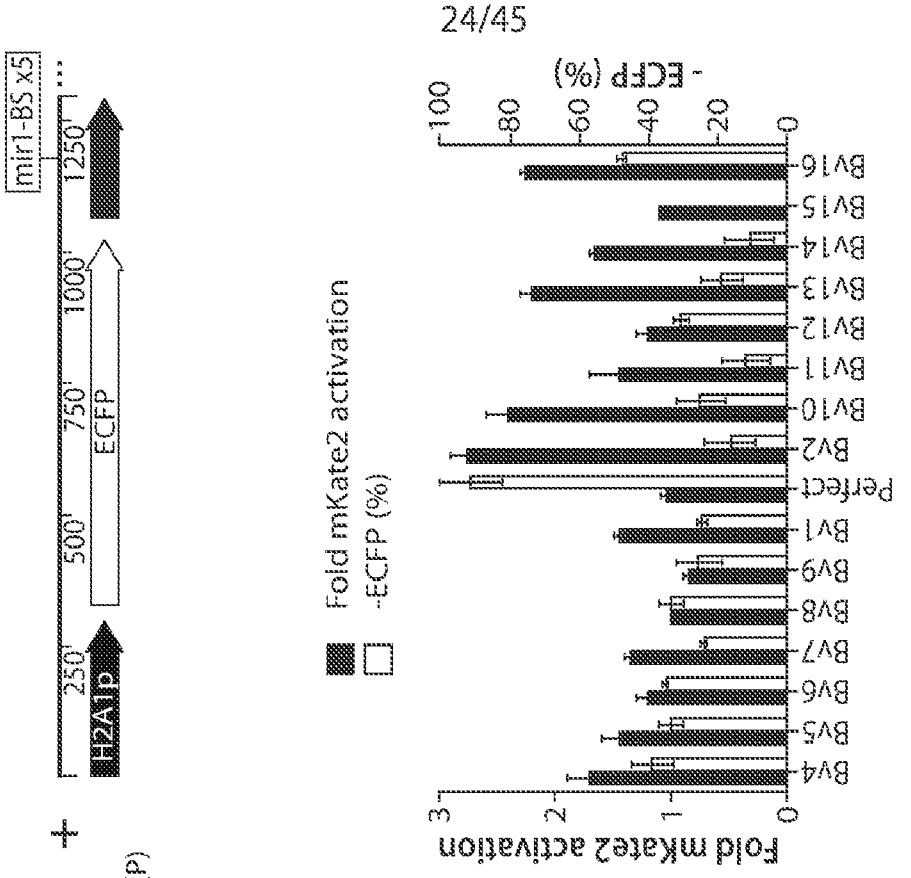

P1
miR1
intron
mKEx1
D
A
mKEx2
miR1-BS(P)
P2
ECFP
miR1-BS (B)
mKEx1
D
A
mKEx2
miR1-BS(P)
mKate2
miR1-BS(P)
miR1
ECFP
miR1-BS (B)
mKate2

Fig. 19



BS	2-2.5 fold mKate2 activation		-ECFP (%)	ddG	Seed
Bv2	5' CCGCTTGAAGACAATTAAA	3'	17	-15	8
Bv10	5' CCGCTTGAAGACAATTAAA	3'	25	-14	7
Bv16	5' CCGCTTGAAGTCTTCTAATTAAA	3'	48	-24	8
Bv13	5' CCGCTTGAAACAATTAAAA	3'	19	-16	8
	~1.5 fold mate2 activation				
Bv4	5' CCGCTTGAAGACAATTAAA	3'	39	-15	8
Bv5	5' CCGCTTGAAGACATTAATTAAA	3'	34	-16	8
Bv14	5' CCGCTTGAACAATTAAA	3'	11	-14	6
Bv1	5' CCGCTTGAAGCTCTTAATTAAA	3'	25	-20	8
Bv7	5' CCGCTTGAACAATTAAA	3'	24	-11	8
	No effect				
Bv12	5' CCGCTTGAAAACAAAAATTAAA	3'	31	-12	7
Bv11	5' CCGCTTGAAAACAATTAAA	3'	12	-13	8
Bv15	5' CCGCTTGAAGGGTCTTAATTAAA	3'	0	-15	8
Bv6	5' CCGCTTGAAGACTTTAATTAAA	3'	35	-15	8
Bv8	5' CCGCTTGAAGACTTTAATTAAA	3'	33	-10	8
Bv9	5' CCGCTTGAACACTTTAATTAAA	3'	26	-11	8
Perf	5' CCGCTTGAAGTCTTTAATTAAA	3'	82	-26	8

Fig. 20

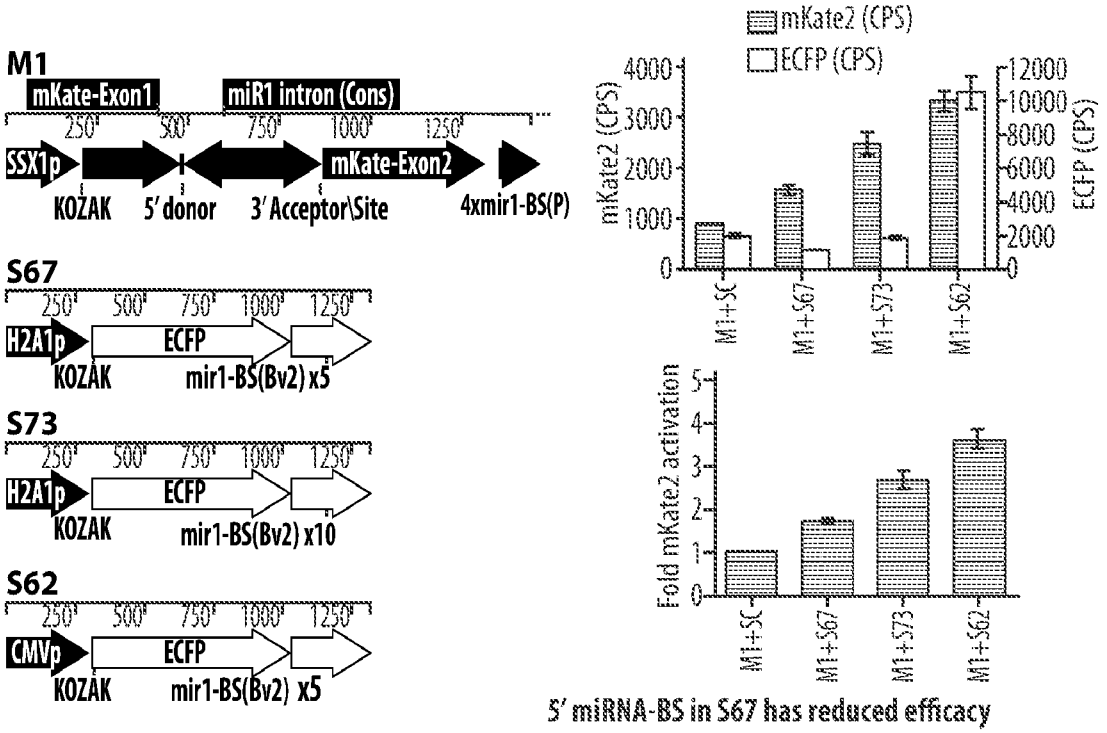

M1
mKate-Exon1
miR1 intron (Cons)
SSX1p
mKate-Exon2
KOZAK
5′donor
3′Acceptor\Site
4xmir1-BS(P)
S67
H2A1p
ECFP
KOZAK
mir1-BS(Bv2) x5
S73
H2A1p
ECFP
KOZAK
mir1-BS(Bv2) x10
S62
CMVp
ECFP
KOZAK
mir1-BS(Bv2) x5
mKate2 (CPS)
ECFP (CPS)
M1+SC
M1+S67
M1+S73
M1+S62
Fold mKate2 activation
5′ miRNA-BS in S67 has reduced efficacy

Fig. 21

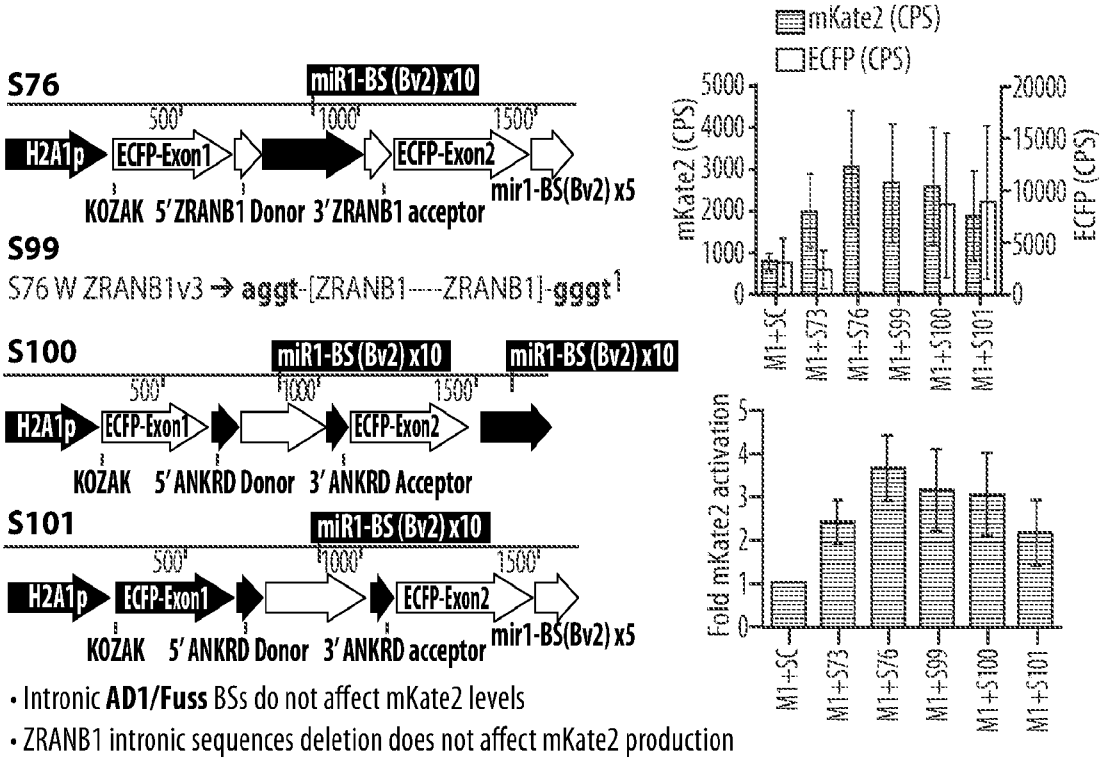

S76
miR1-BS (Bv2) x10
H2A1p
ECFP-Exon1
ECFP-Exon2
mir1-BS(Bv2) x5
KOZAK 5′ZRANB1 Donor 3′ZRANB1 acceptor
S99
S76 W ZRANB1v3 → aggt-[ZRANB1----ZRANB1]-gggt[1]
S100
miR1-BS (Bv2) x10
miR1-BS (Bv2) x10
KOZAK 5′ANKRD Donor 3′ANKRD Acceptor
S101
KOZAK 5′ANKRD Donor 3′ANKRD acceptor
mKate2 (CPS)
ECFP (CPS)
M1+SC
M1+S73
M1+S76
M1+S99
M1+S100
M1+S101
Fold mKate2 activation
• Intronic AD1/Fuss BSs do not affect mKate2 levels
• ZRANB1 intronic sequences deletion does not affect mKate2 production

Fig. 22

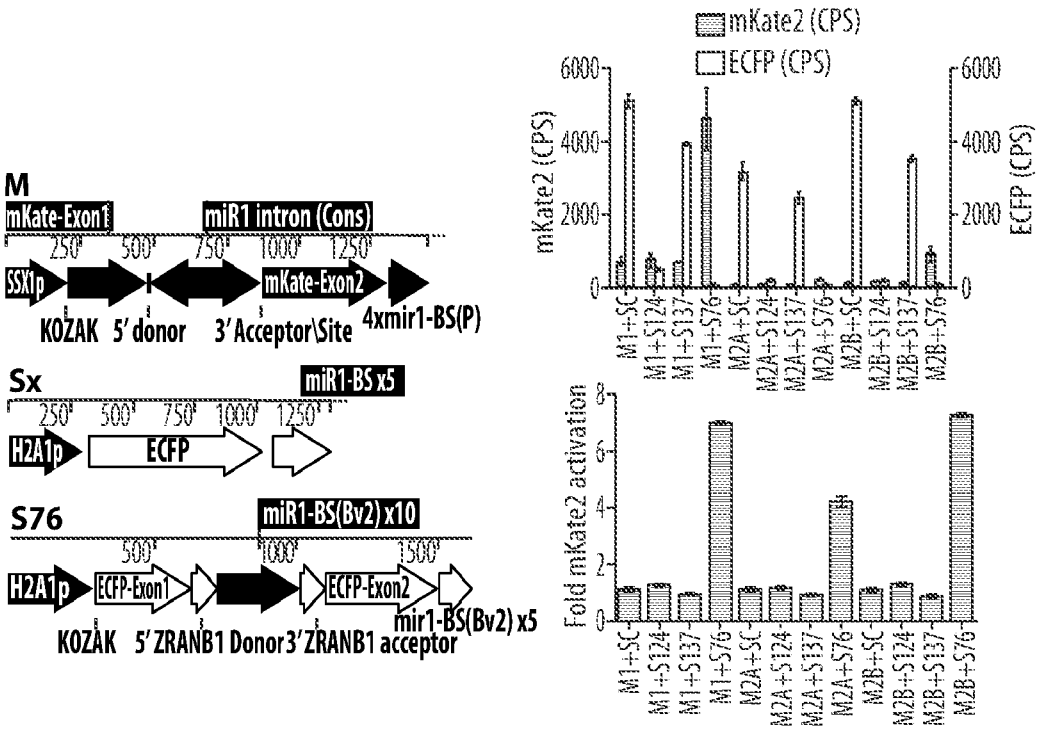


Fig. 23

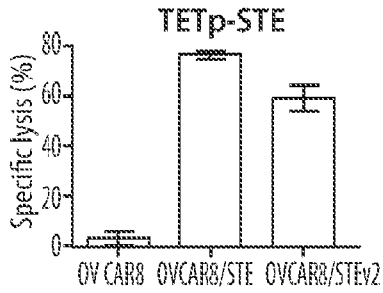


Fig. 24A

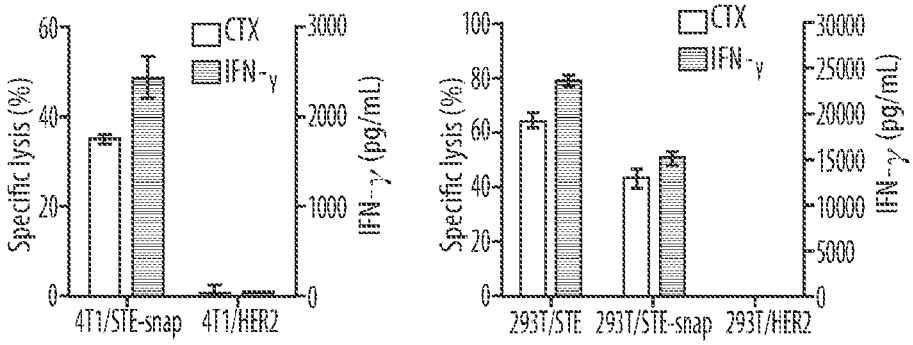


Fig. 24B

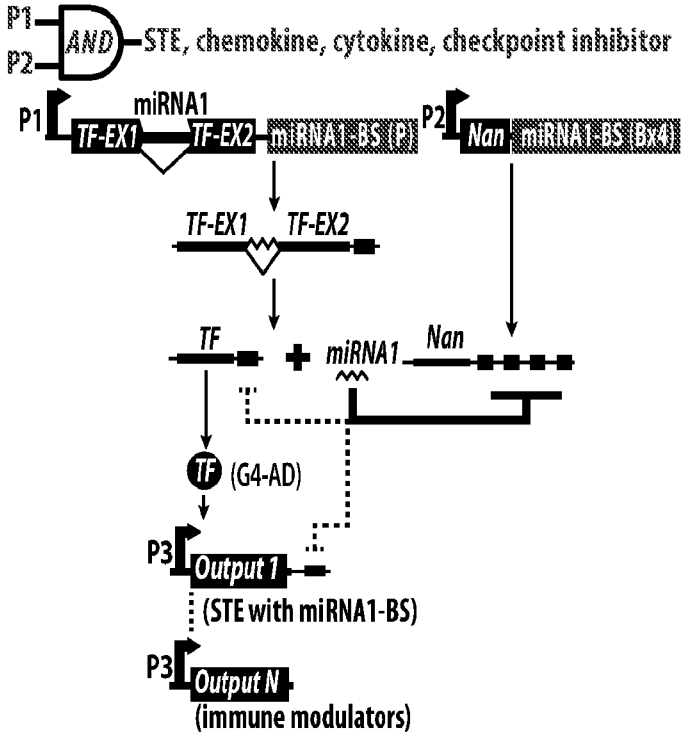


Fig. 25A

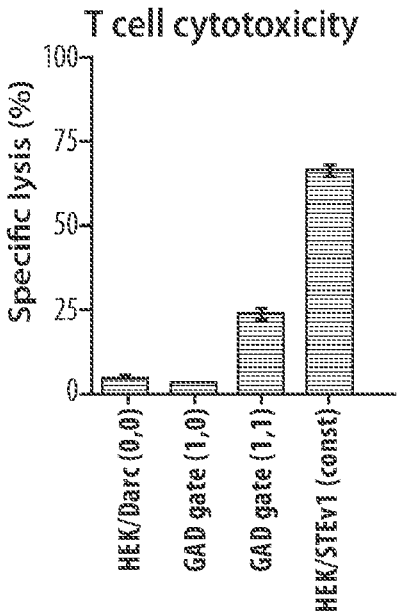


Fig. 25B

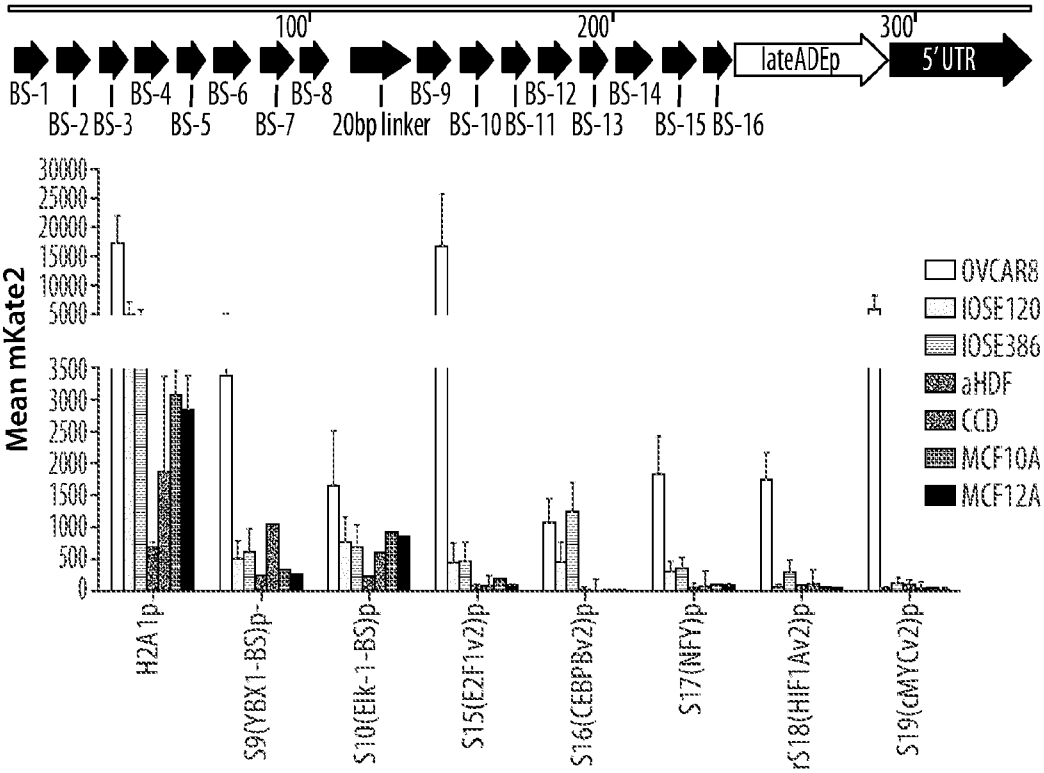


Fig. 26A

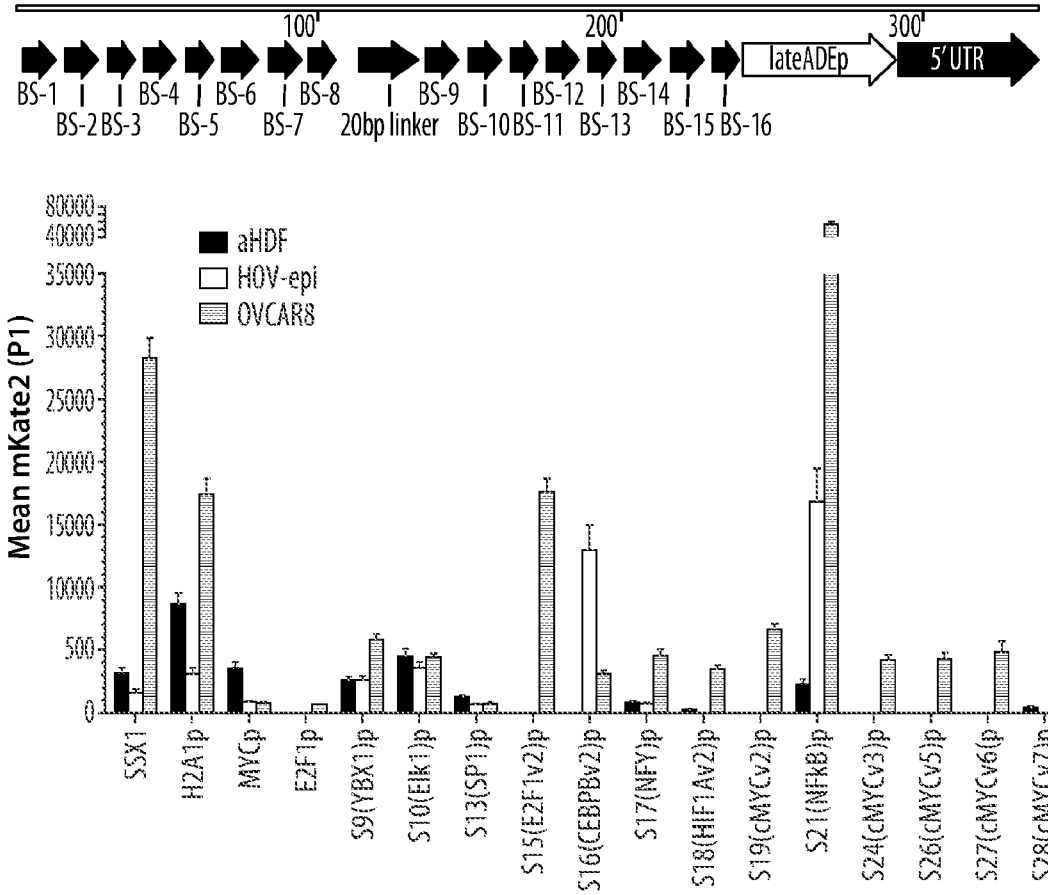


Fig. 26B

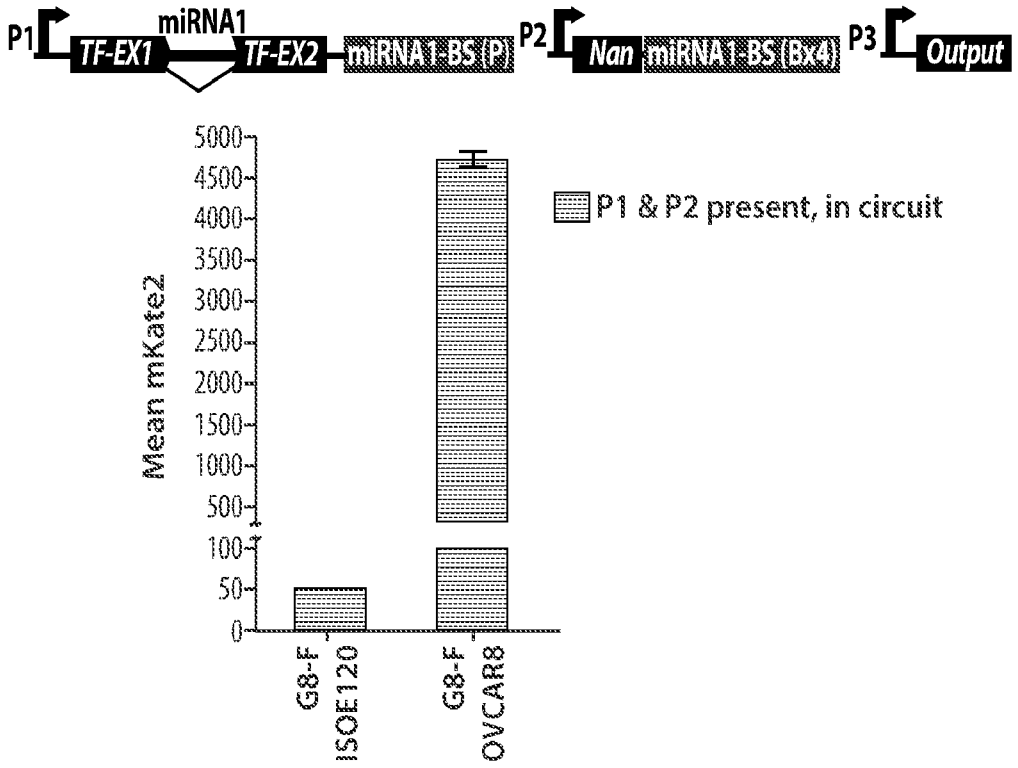


Fig. 27

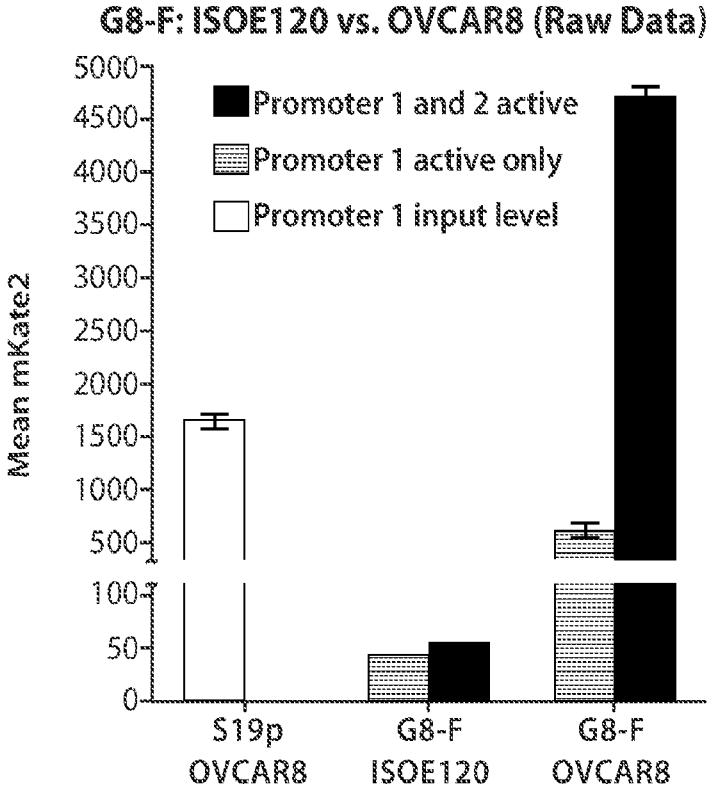

G8-F: ISOE120 vs. OVCAR8 (Raw Data)
Promoter 1 and 2 active
Promoter 1 active only
Promoter 1 input level
Mean mKate2
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
100
50
0
S19p OVCAR8
G8-F ISOE120
G8-F OVCAR8

Fig. 28

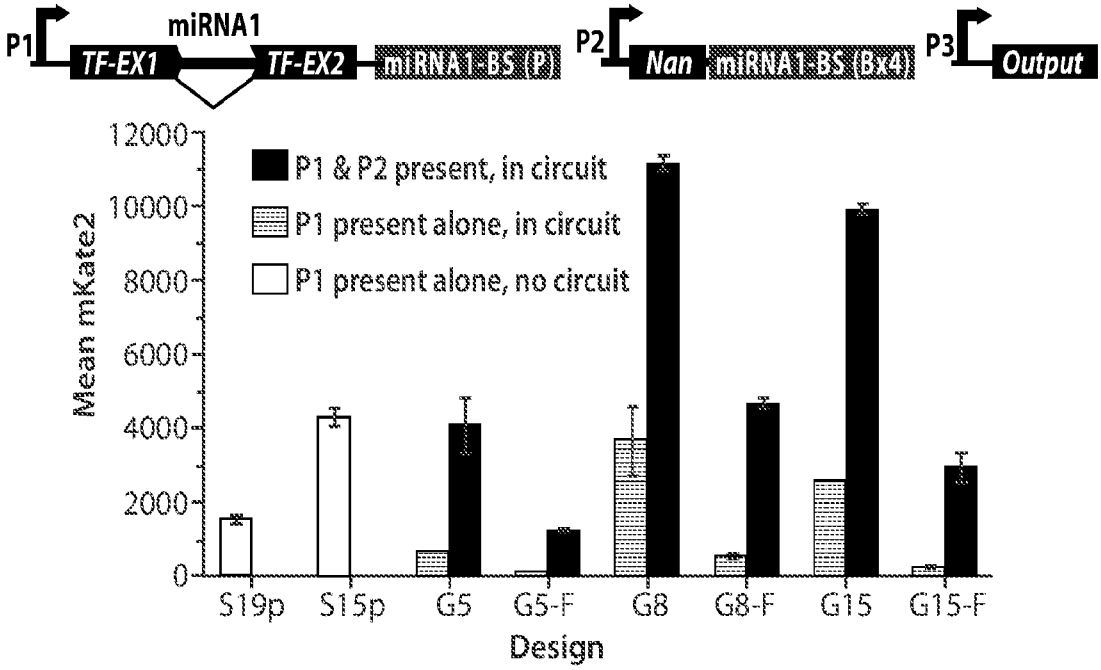

P1
miRNA1
TF-EX1
TF-EX2
miRNA 1 BS (P)
P2
Nan
miRNA 1 BS (Bx4)
P3
Output
P1 & P2 present, in circuit
P1 present alone, in circuit
P1 present alone, no circuit
Mean mKate2
12000
10000
8000
6000
4000
2000
0
S19p
S15p
G5
G5-F
G8
G8-F
G15
G15-F
Design

Fig. 29

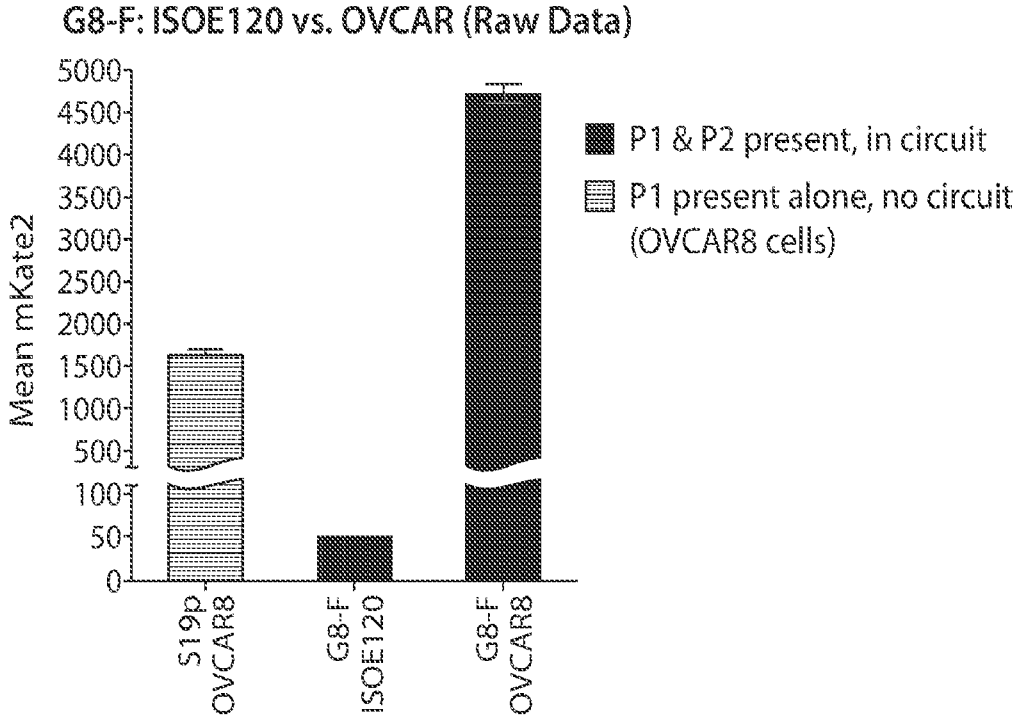


Fig. 30

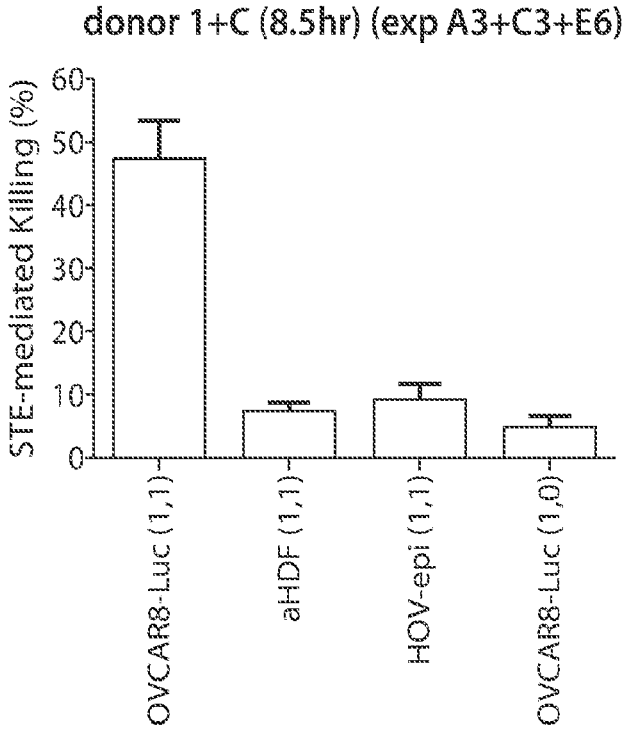


Fig. 31A

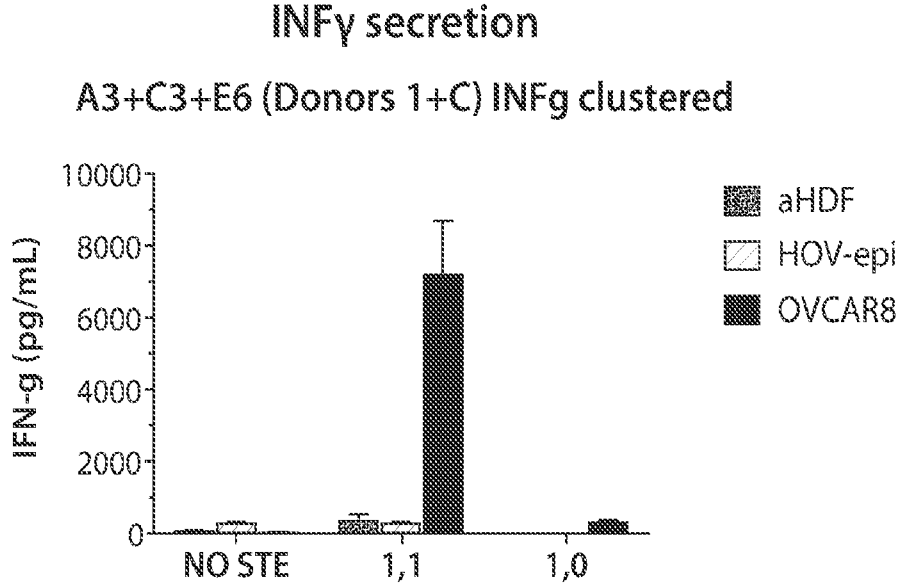


Fig. 31B

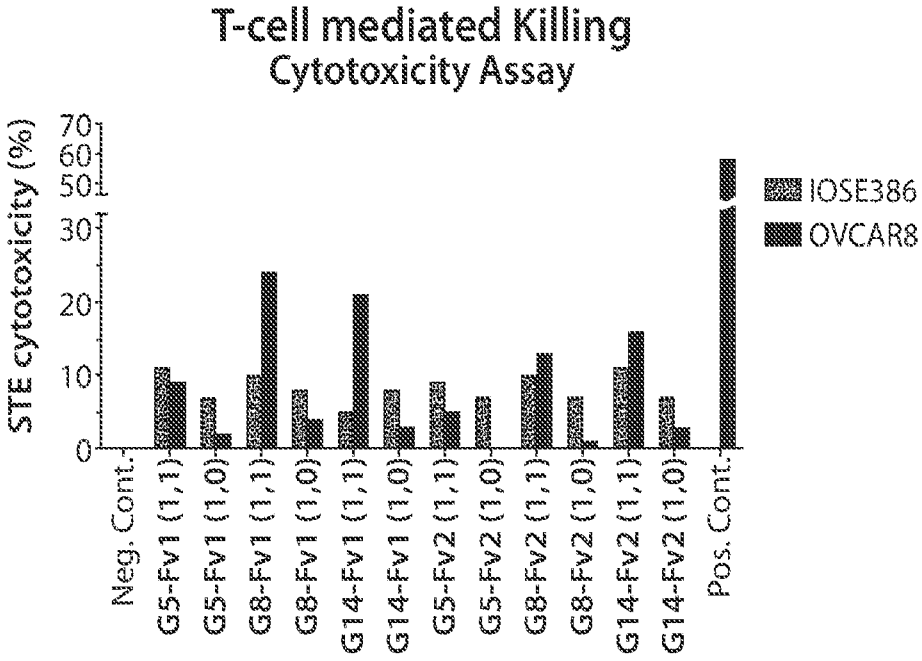


Fig. 32

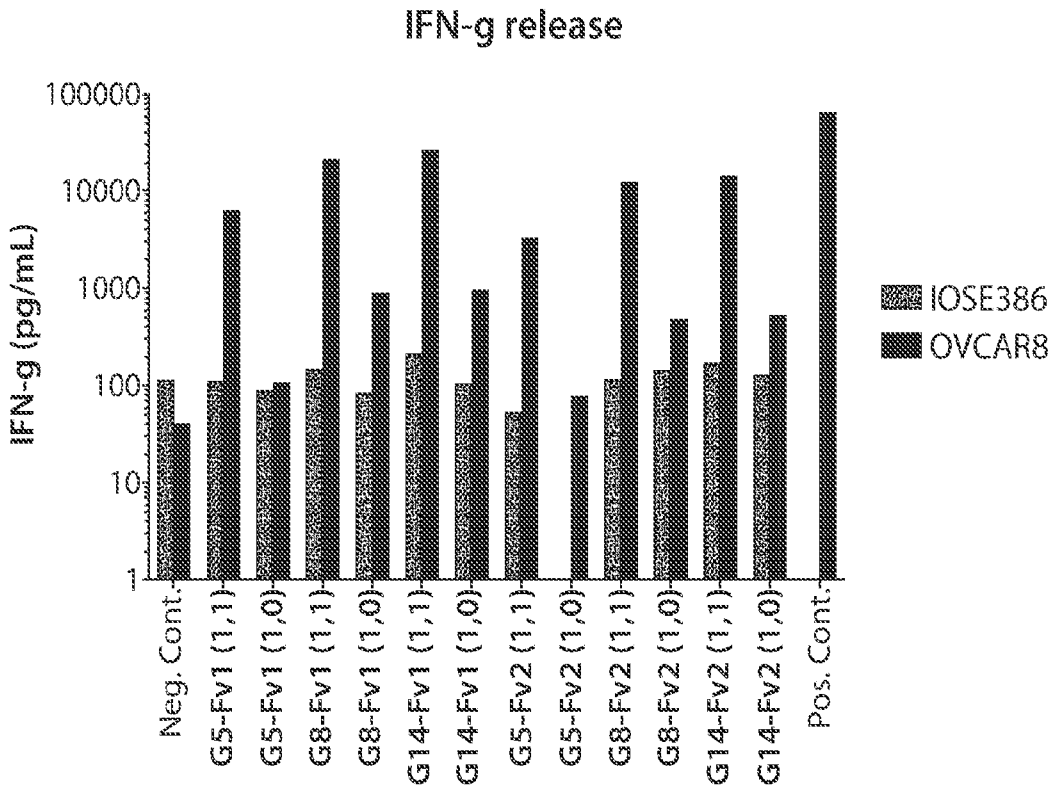

IFN-g release
IFN-g (pg/mL)
100000
10000
1000
100
10
1
IOSE386
OVCAR8
Neg. Cont.
G5-Fv1 (1,1)
G5-Fv1 (1,0)
G8-Fv1 (1,1)
G8-Fv1 (1,0)
G14-Fv1 (1,1)
G14-Fv1 (1,0)
G5-Fv2 (1,1)
G5-Fv2 (1,0)
G8-Fv2 (1,1)
G8-Fv2 (1,0)
G14-Fv2 (1,1)
G14-Fv2 (1,0)
Pos. Cont.

Fig. 33A

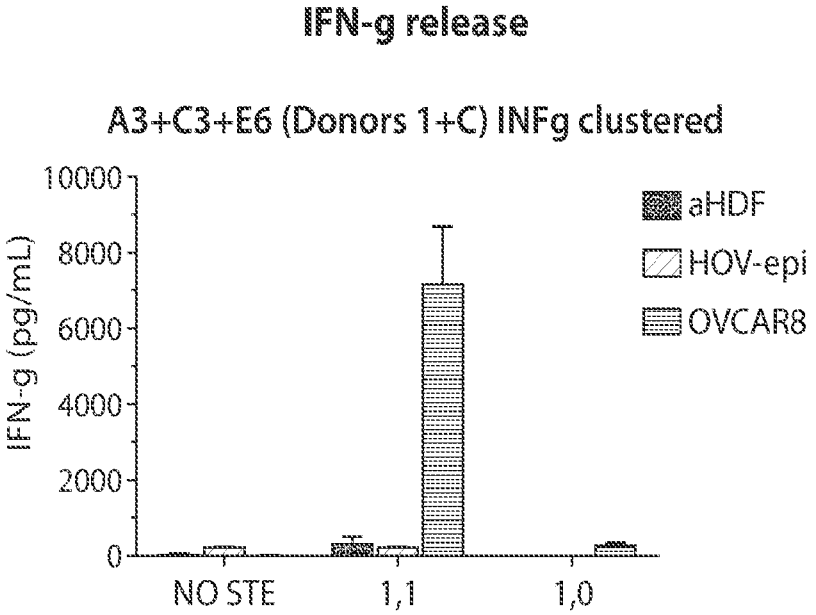


Fig. 33B

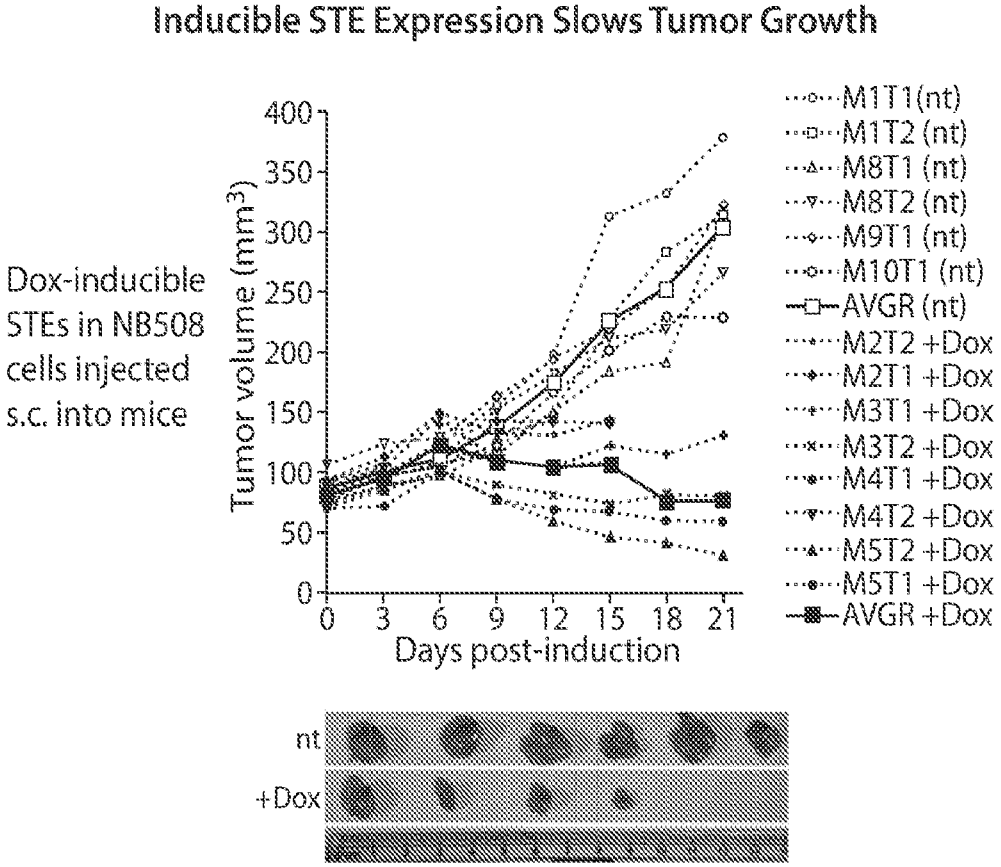


Fig. 34

#	Annotation	DNA	T Cells	Reag	Comments
1	NT No T cells	-	-	-	Maximal tumor growth
2	NT	-	+	-	MHC-mediated killing
3	Reagent only	-	-	+	Reagent cytotoxicity
4	S15p-GAD	S15p-GAD	+	+	T-cells + reagent cytotoxicity
5	S15p-GAD+G8p-STE-F	S15p-GAD+ G8-STE-F	+	+	High STE therapeutic efficacy
6	GATE (STE)	G8-F gate, STE	+	+	G8-F gate + STE
7	GATE (STE, L12)	G8F gate, STE + IL12	+	+	G8-F gate + STE + IL12
8	GATE (STE, CCL21)	G8F gate, STE + CCL21	+	+	G8-F gate + STE + CCL21
9	GATE (STE, αPD1)	G8F gate, STE + αPD1	+	+	G8F gate + STE + αPD1
10	GATE (STE, IL12,CCL21, αPD1)	G8F gate, STE+IL12+CCL21 +αPD1	+	+	TG8-F gate + STE+IL12+CCL21 +αPD1

Treatment:

- **Day 0:** IP injection of $0.5x10^6$ **OVCAR8-Luc cells**
- **Day 10:** IP injection of **DNA/material**[1]
- **Day 12, 19, 26:** IP injection of $10x10^6$ human activated **T cells**

Readout:

Tumor burden estimation in EVOS, **total flux [p/s]**

Fig. 35A

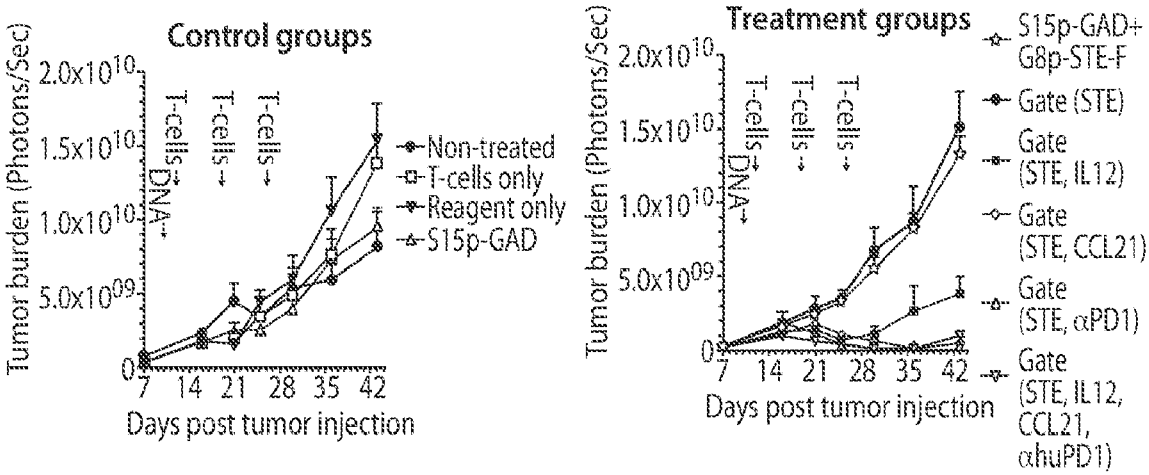


Fig. 35B

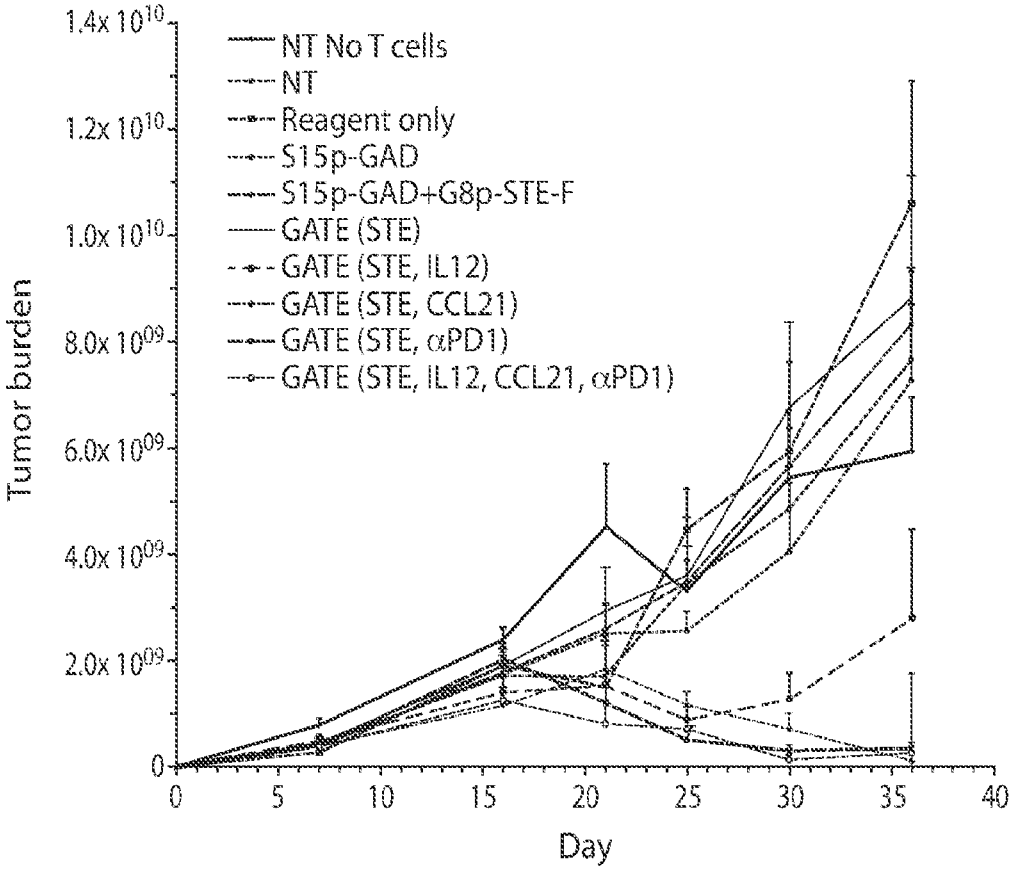

1.4x 10^10
1.2x 10^10
1.0x 10^10
8.0x 10^09
6.0x 10^09
4.0x 10^09
2.0x 10^09
0
Tumor burden
0
5
10
15
20
25
30
35
40
Day
NT No T cells
NT
Reagent only
S15p-GAD
S15p-GAD+G8p-STE-F
GATE (STE)
GATE (STE, IL12)
GATE (STE, CCL21)
GATE (STE, αPD1)
GATE (STE, IL12, CCL21, αPD1)

Fig. 36

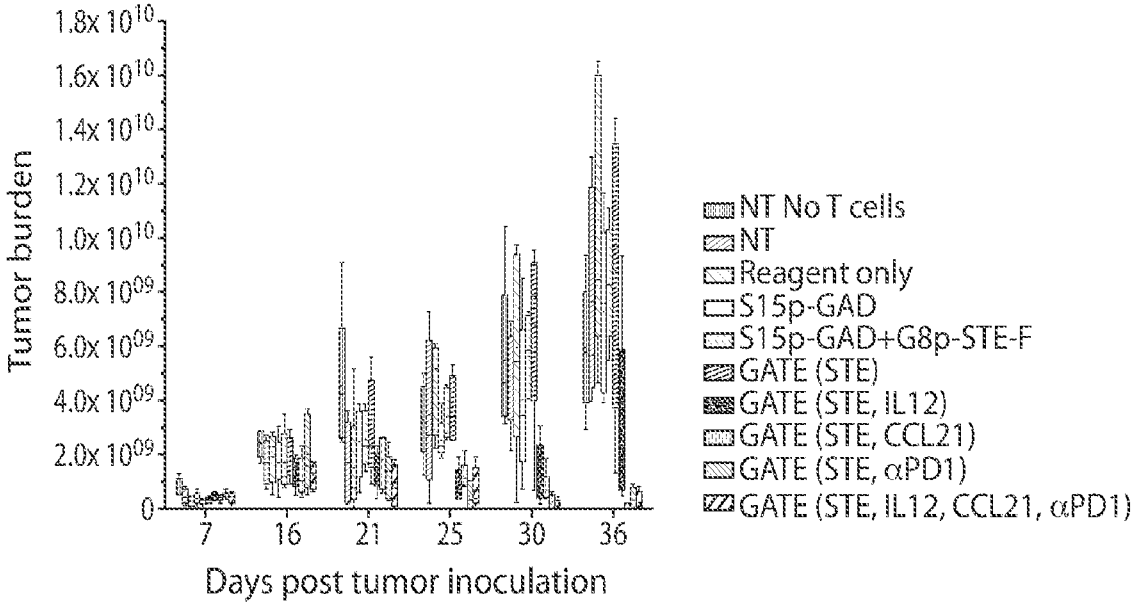

1.8x 10^10
1.6x 10^10
1.4x 10^10
1.2x 10^10
1.0x 10^10
8.0x 10^09
6.0x 10^09
4.0x 10^09
2.0x 10^09
0
Tumor burden
7
16
21
25
30
36
Days post tumor inoculation
NT No T cells
NT
Reagent only
S15p-GAD
S15p-GAD+G8p-STE-F
GATE (STE)
GATE (STE, IL12)
GATE (STE, CCL21)
GATE (STE, αPD1)
GATE (STE, IL12, CCL21, αPD1)

Fig. 37

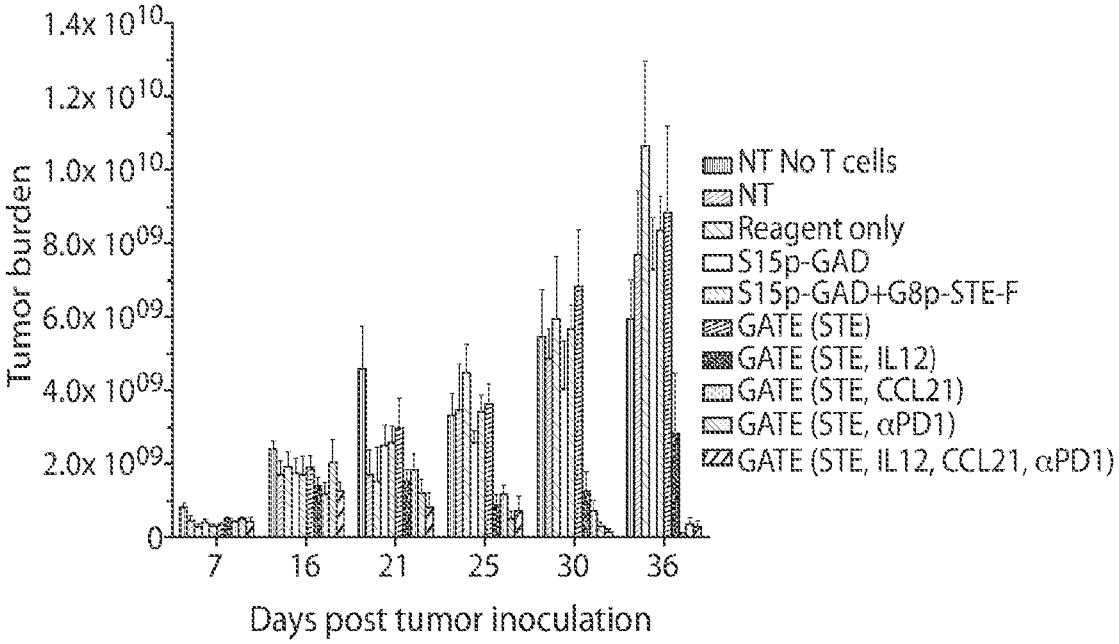

1.4x 10^10
1.2x 10^10
1.0x 10^10
8.0x 10^09
6.0x 10^09
4.0x 10^09
2.0x 10^09
0
Tumor burden
7
16
21
25
30
36
Days post tumor inoculation
NT No T cells
NT
Reagent only
S15p-GAD
S15p-GAD+G8p-STE-F
GATE (STE)
GATE (STE, IL12)
GATE (STE, CCL21)
GATE (STE, αPD1)
GATE (STE, IL12, CCL21, αPD1)

FIG. 38

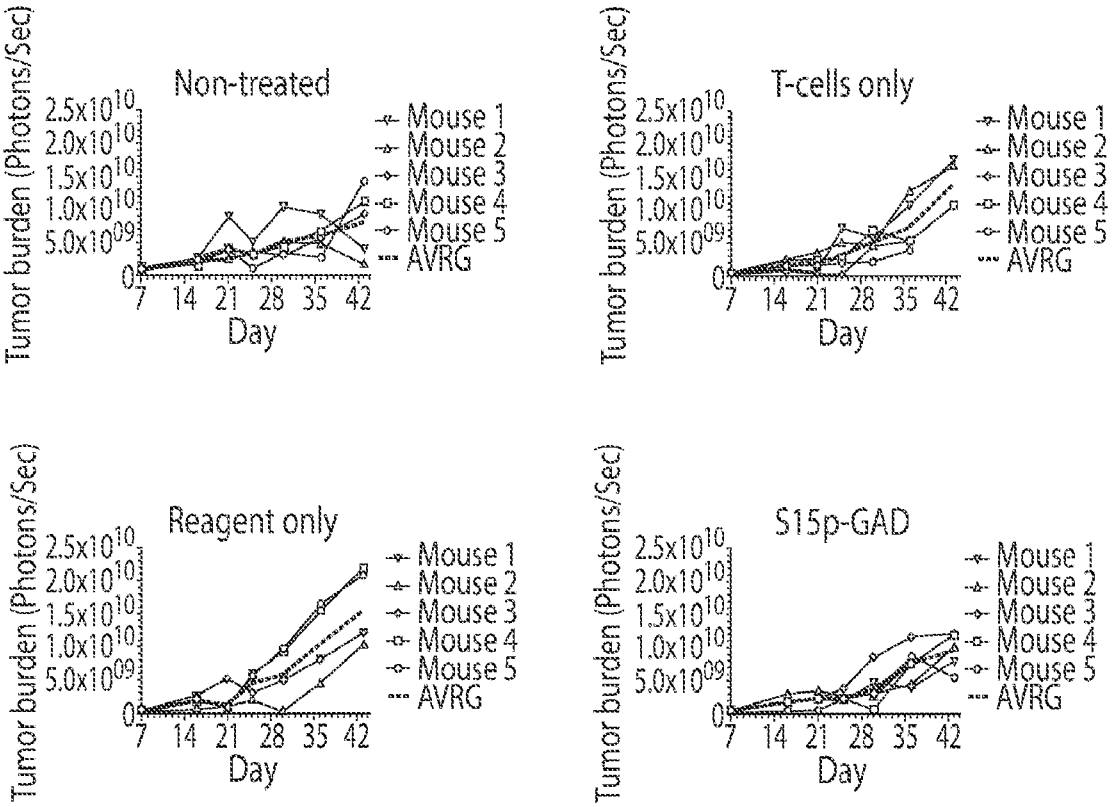

Non-treated
T-cells only
Reagent only
S15p-GAD
Tumor burden (Photons/Sec)
2.5x10^10
2.0x10^10
1.5x10^10
1.0x10^10
5.0x10^09
0
7 14 21 28 35 42
Day
Mouse 1
Mouse 2
Mouse 3
Mouse 4
Mouse 5
AVRG

Fig. 39

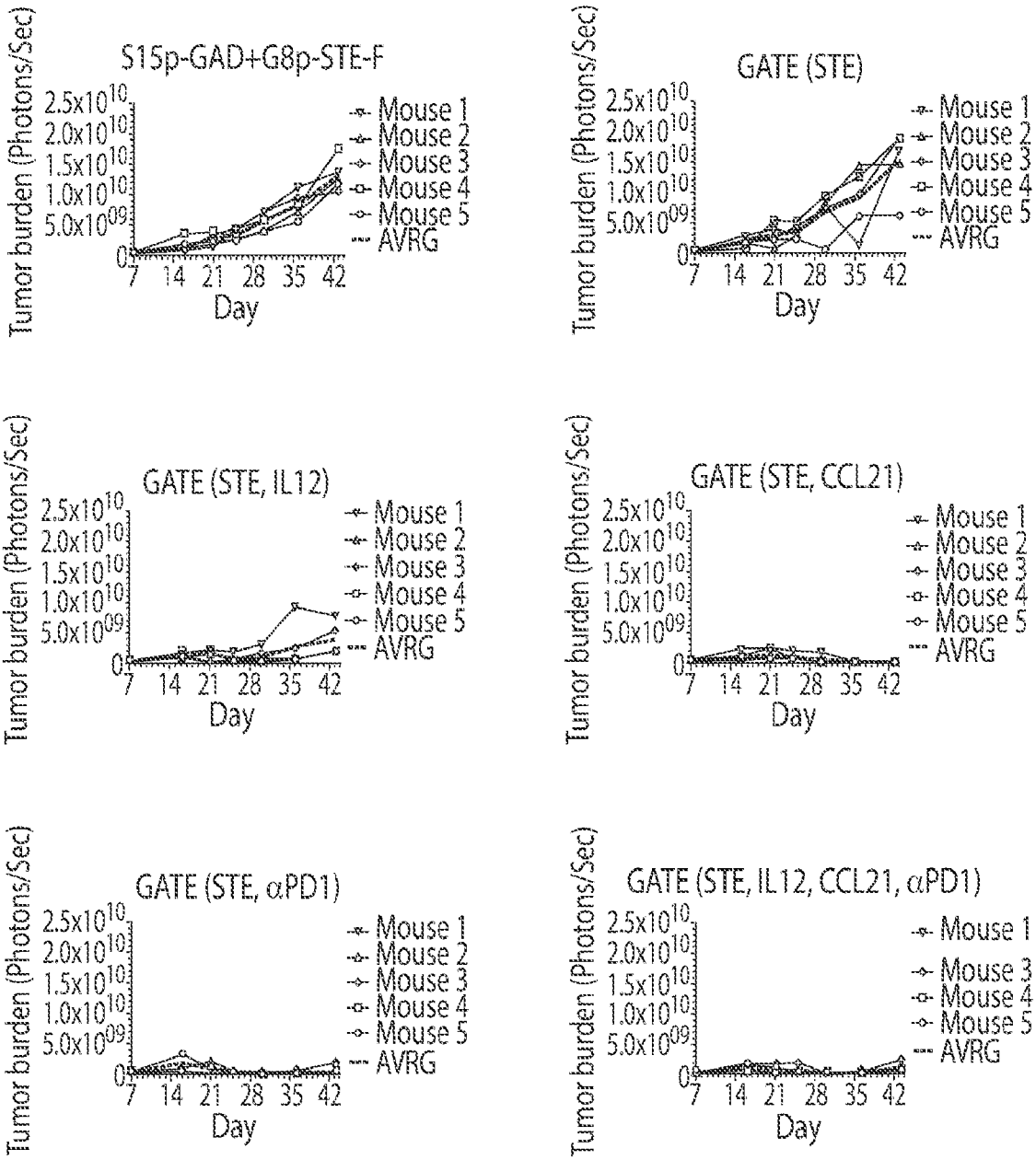


Fig. 39
(continued)

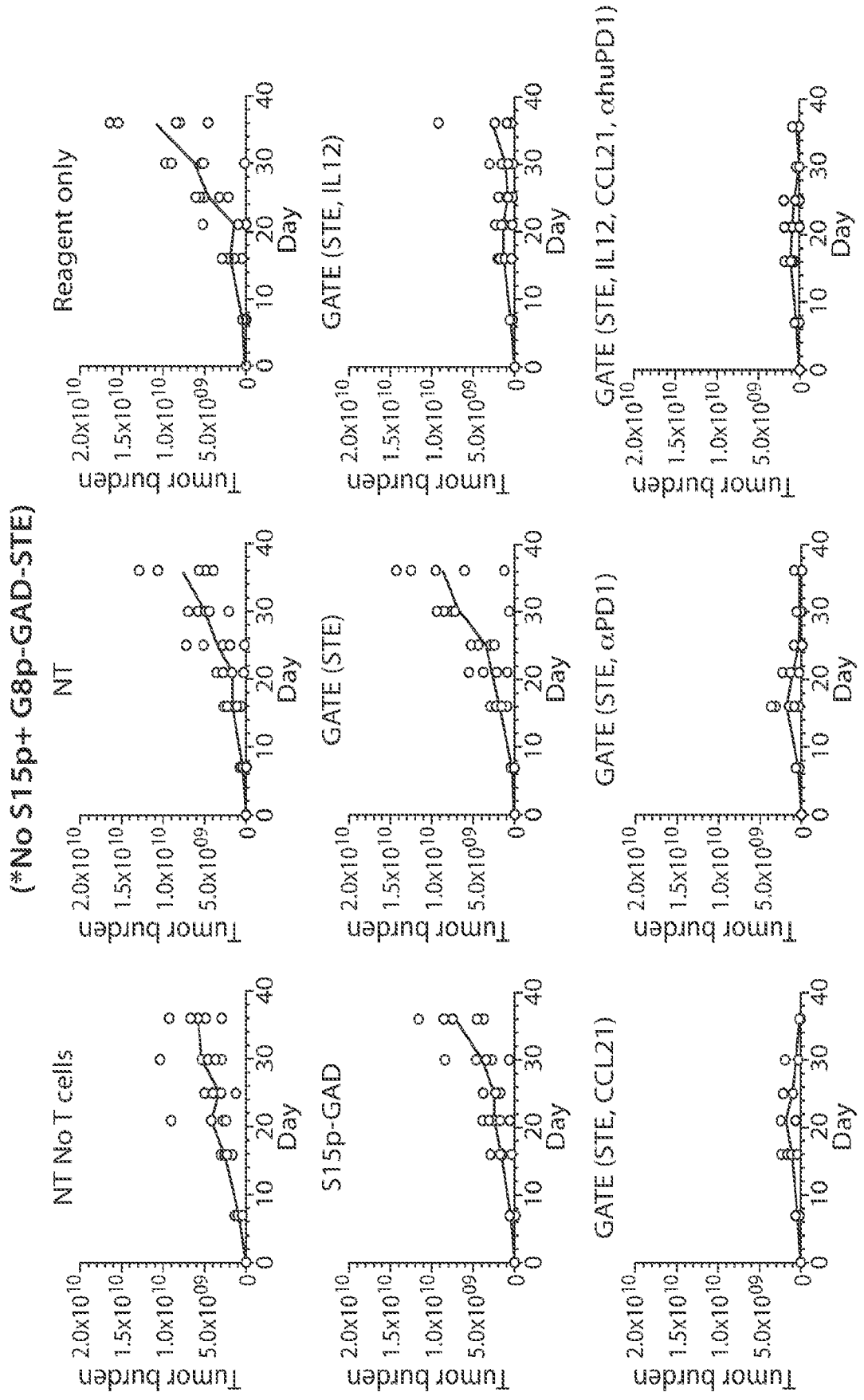

(*No S15p+ G8p-GAD-STE)
Reagent only
NT
GATE (STE, IL12)
GATE (STE)
GATE (STE, IL12, CCL21, αhuPD1)
GATE (STE, αPD1)
NT No T cells
S15p-GAD
GATE (STE, CCL21)
Tumor burden
Day

Fig. 40

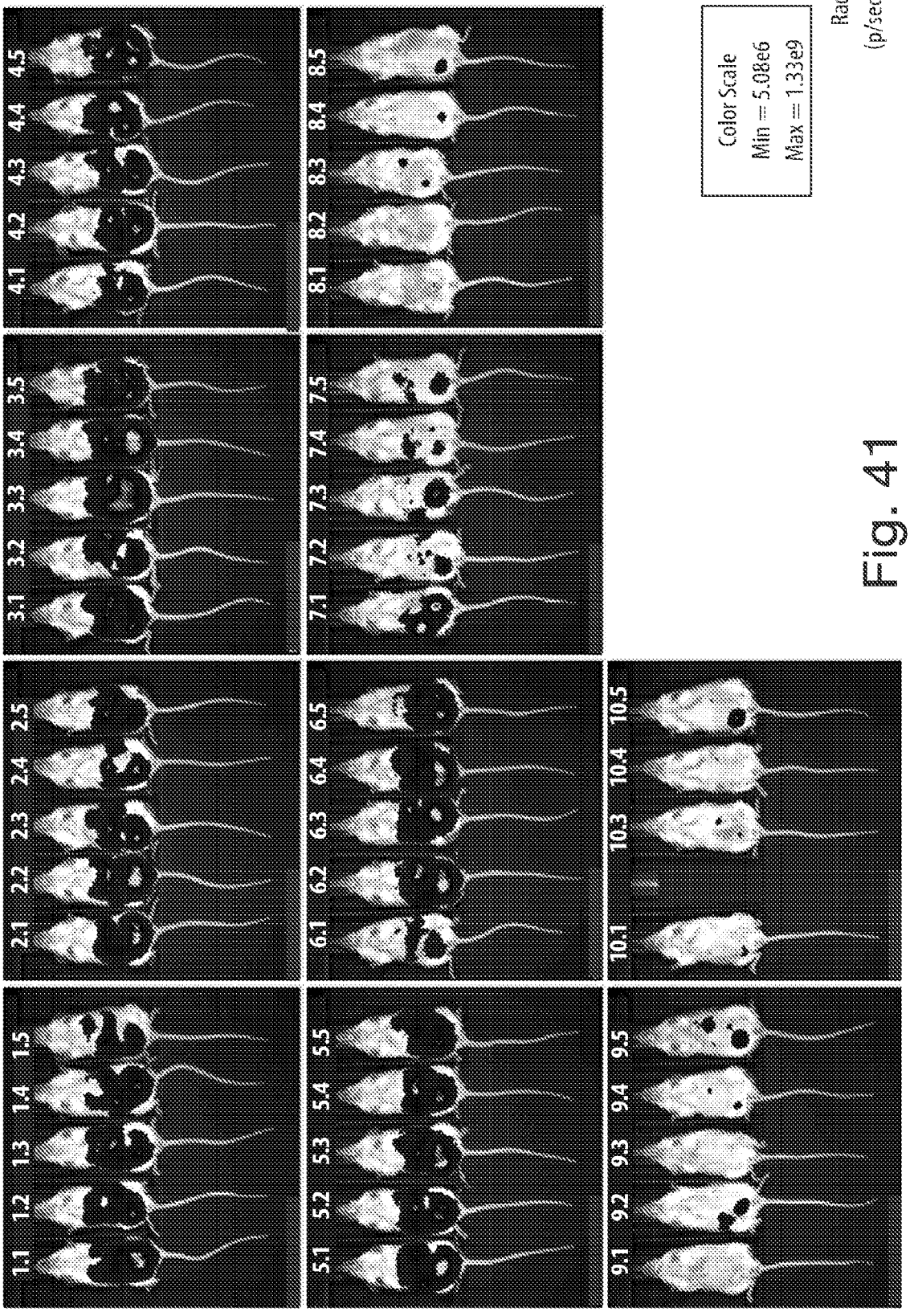

1.2
1.0
0.8
x10^9
0.6
0.4
0.2
Radiance
(p/sec/cm²/sr)
Color Scale
Min = 5.08e6
Max = 1.33e9
1.1 1.2 1.3 1.4 1.5
2.1 2.2 2.3 2.4 2.5
3.1 3.2 3.3 3.4 3.5
4.1 4.2 4.3 4.4 4.5
5.1 5.2 5.3 5.4 5.5
6.1 6.2 6.3 6.4 6.5
7.1 7.2 7.3 7.4 7.5
8.1 8.2 8.3 8.4 8.5
9.1 9.2 9.3 9.4 9.5
10.1 10.3 10.4 10.5

Fig. 41

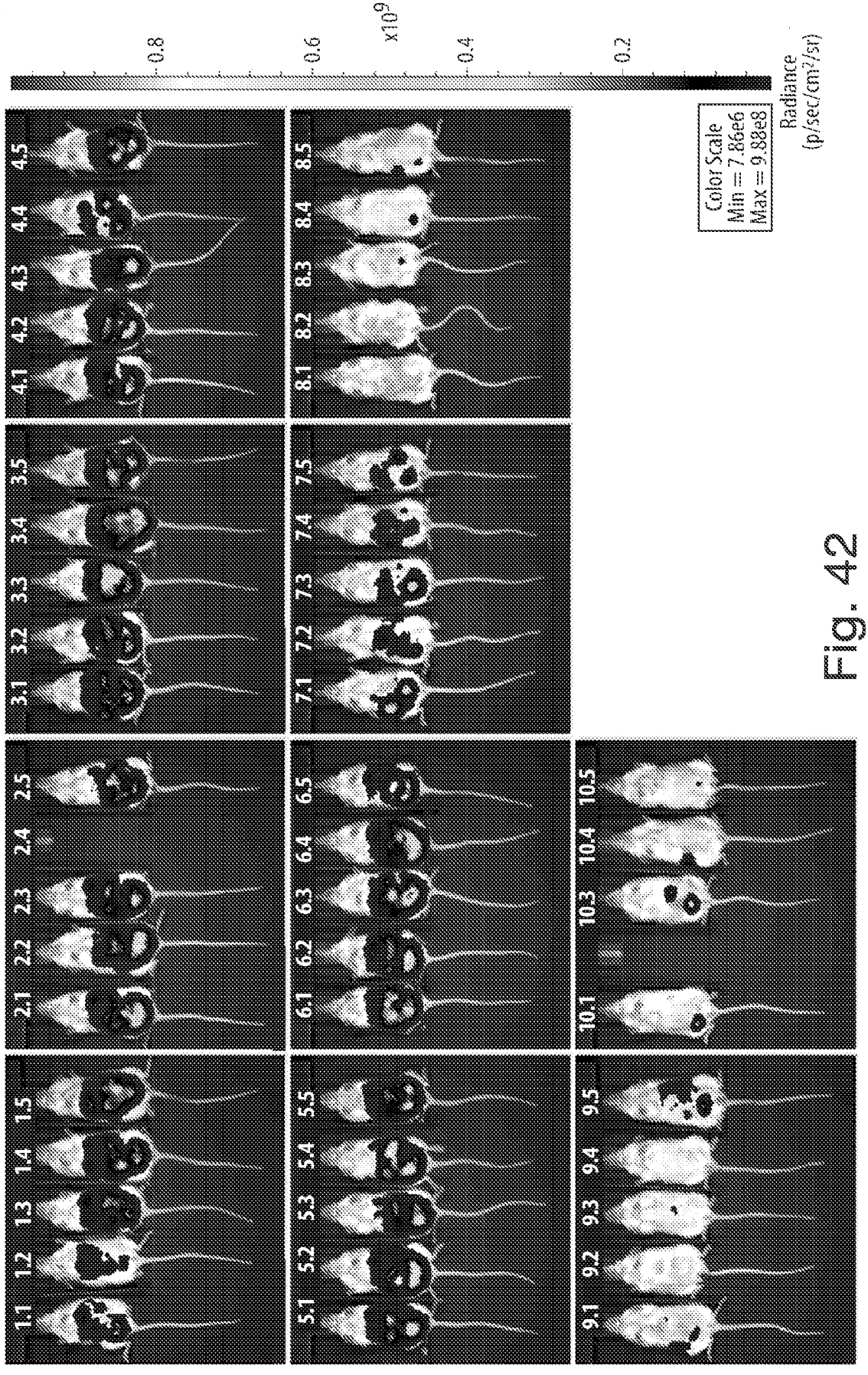

0.8
0.6
x10^9
0.4
0.2
Radiance (p/sec/cm²/sr)
Color Scale
Min = 7.86e6
Max = 9.88e8
1.1 1.2 1.3 1.4 1.5
2.1 2.2 2.3 2.4 2.5
3.1 3.2 3.3 3.4 3.5
4.1 4.2 4.3 4.4 4.5
5.1 5.2 5.3 5.4 5.5
6.1 6.2 6.3 6.4 6.5
7.1 7.2 7.3 7.4 7.5
8.1 8.2 8.3 8.4 8.5
9.1 9.2 9.3 9.4 9.5
10.1 10.3 10.4 10.5

Fig. 42

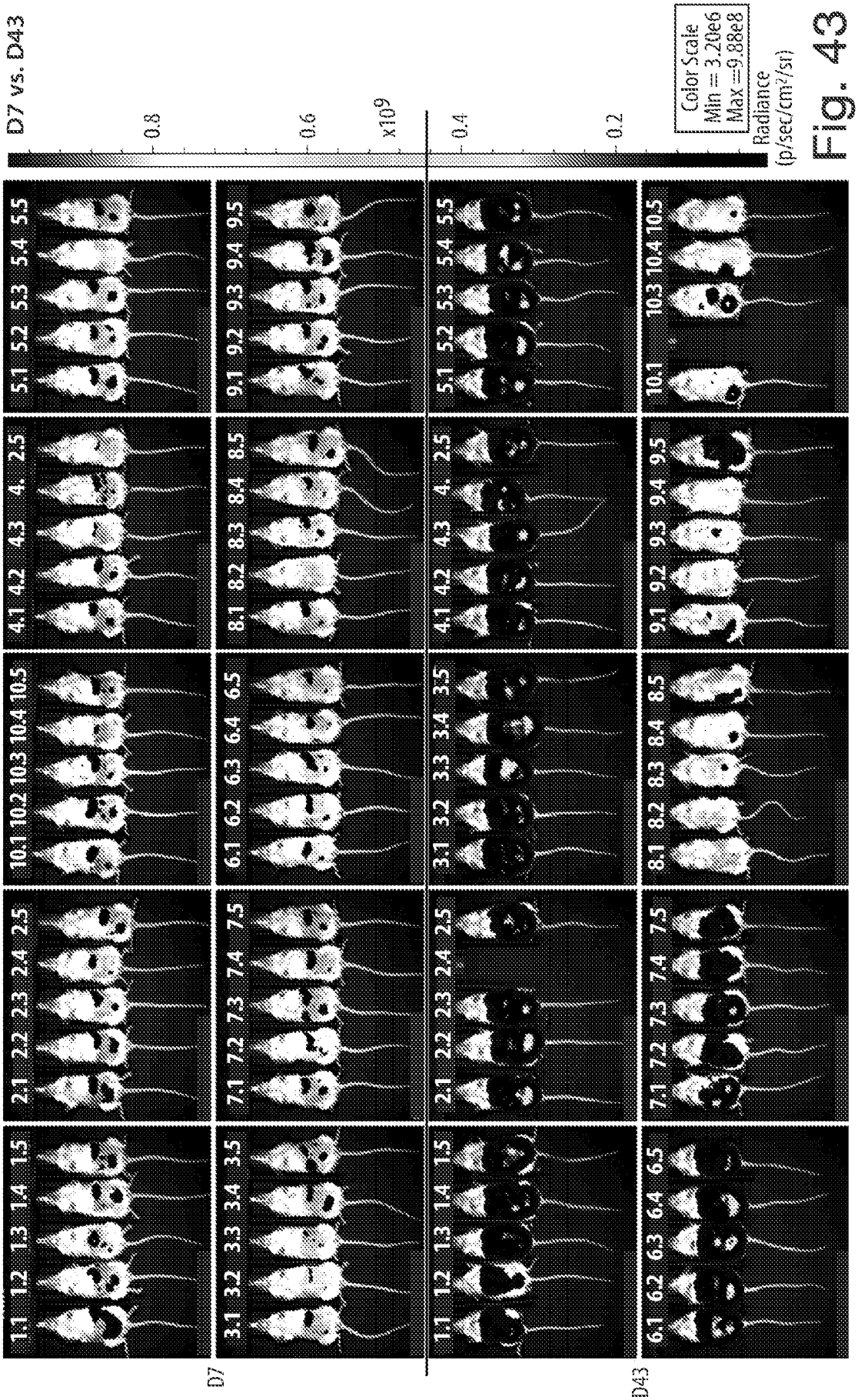

D7 vs. D43
0.8
0.6
x10^9
0.4
0.2
Color Scale
Min = 3.20e6
Max = 9.88e8
Radiance
(p/sec/cm²/sr)
D7
D43

Fig. 43

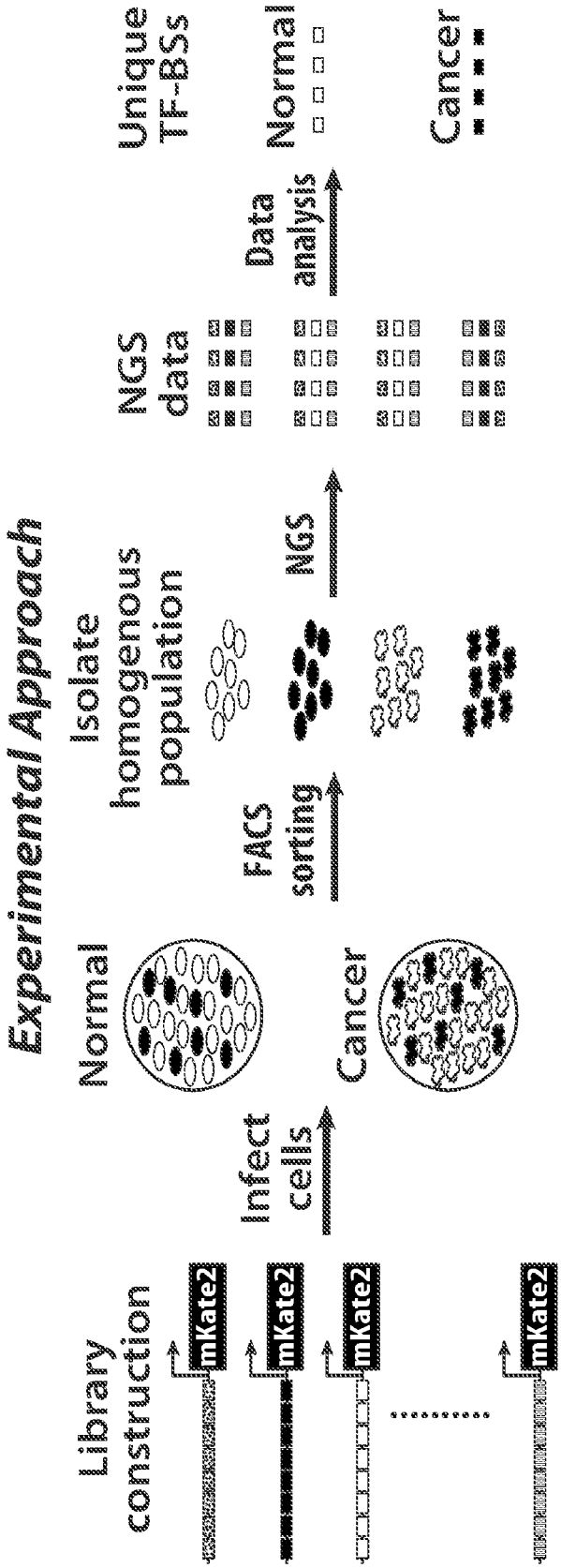

Experimental Approach
Library construction
mKate2
Infect cells
Normal
Cancer
FACS sorting
Isolate homogenous population
NGS
NGS data
Data analysis
Unique TF-BSs
Normal
Cancer

Fig. 44

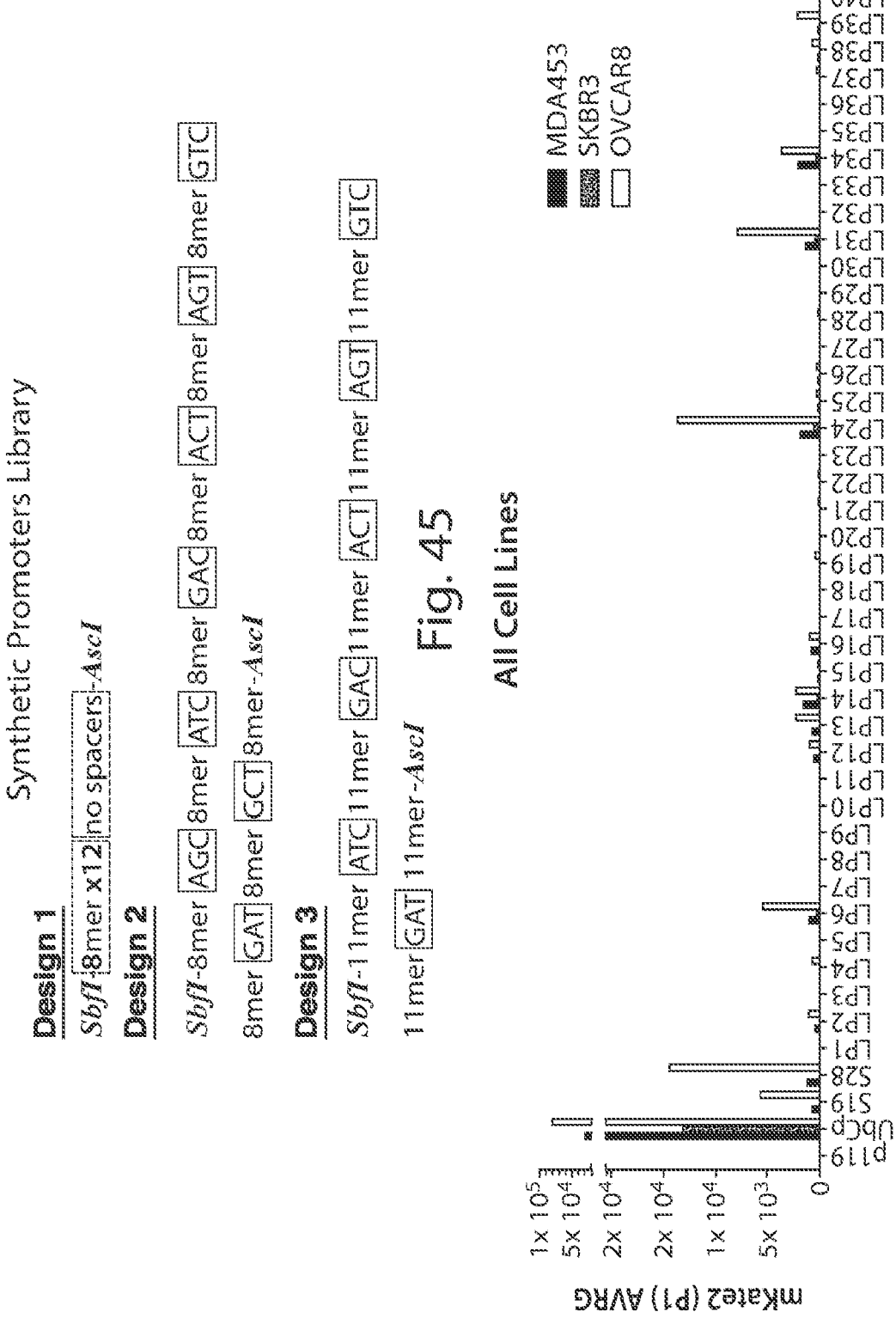
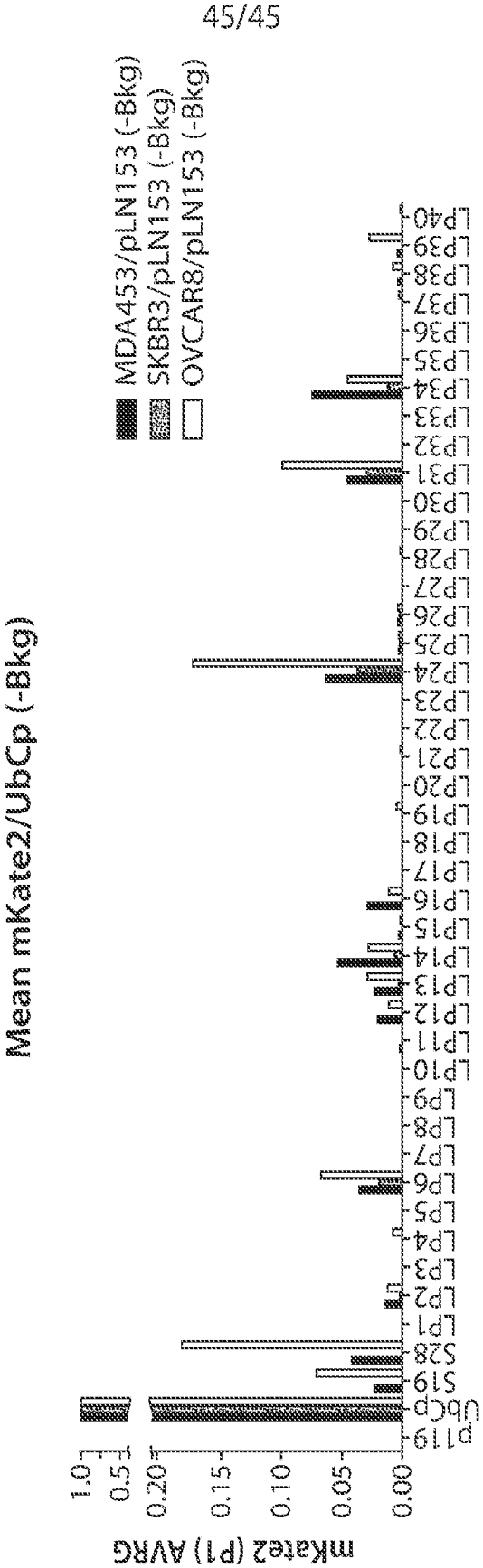

Synthetic Promoters Library
Design 1
SbfI-8mer x12-no spacers-AscI
Design 2
SbfI-8mer-AGC-8mer-ATC-8mer-GAC-8mer-ACT-8mer-AGT-8mer-GTC
8mer-GAT-8mer-GCT-8mer-AscI
Design 3
SbfI-11mer-ATC-11mer-GAC-11mer-ACT-11mer-AGT-11mer-GTC
11mer-GAT-11mer-AscI

Fig. 45

All Cell Lines
MDA453
SKBR3
OVCAR8
mKate2 (P1) AVRG
Promoter

Fig. 46


MDA453/pLN153 (-Bkg)
SKBR3/pLN153 (-Bkg)
OVCAR8/pLN153 (-Bkg)
Mean mKate2/UbCp (-Bkg)
mKate2 (P1) AVRG
1.0
0.5
0.20
0.15
0.10
0.05
0.00
p119
UbCp
S19
S28
LP1
LP2
LP3
LP4
LP5
LP6
LP7
LP8
LP9
LP10
LP11
LP12
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LP39
LP40
Promoter

Fig. 47

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

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[Continued on next page]

(54) Title: TUMOR IMMUNOTHERAPY

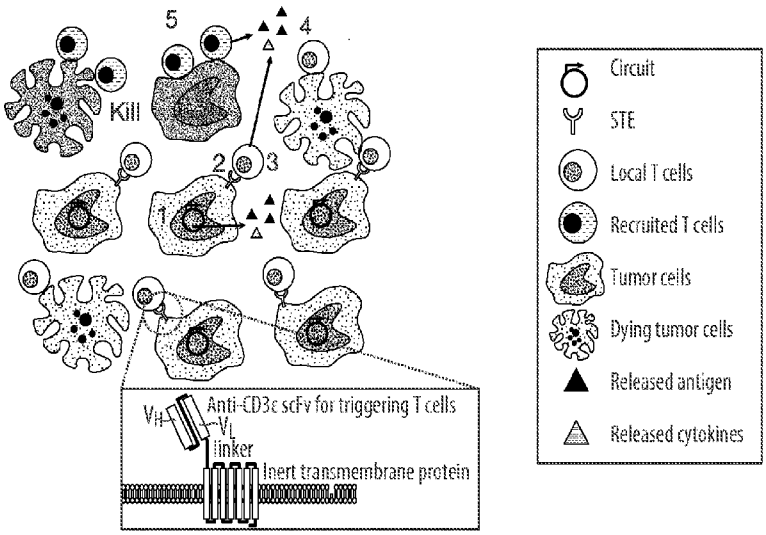

Fig. 2B

(57) Abstract: Aspects of the present disclosure provide a platform that triggers potent and effective immunotherapy against tumors from within tumors themselves, thus overcoming limitations of existing cancer immunotherapies and tumor-detecting gene circuits.

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WO 2016/205737 A3

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

(88) Date of publication of the international search report:
2 February 2017

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/38222

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☒ furnished subsequent to the international filing date for the purposes of international search only:

      ☒ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/38222

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 6-27, 43-47, 51-57, 61-67
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
----go to Extra Sheet for continuation-------

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Claims 1-5, 31, 32, 35, 36, 38

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/38222

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 15/00, 15/10, 15/11, 15/63, 15/113; C12Q 1/68; A61K 39/395 (2016.01)
CPC - C12N 15/00, 15/11, 15/63, 15/113, 2810/00; C07K 16/00, 2317/622, 2316/95
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): C12N 15/00, 15/10, 15/11, 15/63, 15/113; C12Q 1/68; A61K 39/395 (2016.01)
CPC: C12N 15/00, 15/11, 15/63, 15/113, 2810/00; C07K 16/00, 2317/622, 2316/95

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC: C12N 15/00, 15/11, 15/63, 15/113, 2810/00; C07K 16/00, 2317/622, 2316/95 (text search)
USPC: 435/320.1, 6.14; 514/44A; 536/23.1, 24.1; 424/130.1, 135.1

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Electronic data bases: PatBase; Google Patents; Google Scholar
Search terms: Exongenous molecular circuit, intronic miRNA, miRNA binding site, cancer cell promoter (e.g. H2A1, SSX1), synthetic T cell engager (STE) (e.g. anti-CD3 scFv)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	US 2010/0197006 A1 (BENENSON et al.) 5 August 2010 (05.08.2010). Especially para [0005], [0008], [0130], [0138], sheet 28 fig 14D.	1, 35, 36, 38 ---------- 2-5, 31, 32
Y	US 2004/0058445 A1 (LEDBETTER et al.) 25 March 2004 (25.03.2004). Especially para [0017]	2-5, 31, 32
Y	ZHU et al. Transcriptional targeting of tumors with a novel tumor-specific survivin promoter. Cancer Gene Ther April 2004 Vol 11 No 4 Pages 256-262. Especially abstract.	31, 32

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 November 2016	01 DEC 2016
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/38222

------continuation of Box III (Lack of Unity of Invention)--------

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-5, 31, 32, 35, 36, 38, drawn to an engineered genetic circuit design for single output AND gate comprising:
(a) a first nucleic acid comprising promoter-mRNA-intronic miRNA1-mRNA-miRNA1 binding site (miRNA BS);
(b) a second nucleic acid comprising promoter-miRNA1 BS.
[see instant Specific, pg 7, ln 20 to pg 8, ln 9, and Figs. 3A-3H, especially sheet 5 Fig 3E]

Group II: Claims 28-30, drawn to a composition comprising an anti-CD3e scFv protein.

Group III: Claims 33, 34, 37, 39, drawn to an engineered genetic circuit design for Boolean logic gates, comprising
(a) a first nucleic acid comprising promoter-mRNA1-intronic miRNA1-mRNA1-miRNA2 BS;
(b) a second nucleic acid comprising promoter-mRNA2-intronic miRNA2-mRNA2-miRNA1 BS;
(c) a third nucleic acid comprising promoter--miRNA1 BS and promoter-miRNA2 BS
[see instant Specific, pg 9, ln 20-22, and Figs. 7A-7H, especially sheet 12 Fig 7F]

Group IV: Claims 40-42, 48-50, 58-60, 68-72 drawn to a synthetic promoter library composition or a method of using one.

The inventions listed as Groups I-IV do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features:
Group I has the special technical feature of an engineered genetic circuit design for single output AND gate comprising: a second nucleic acid comprising: promoter-miRNA BS and function as decoy/sink for miRNA. [e.g see instant Specification sheet 5 Fig 3E], not required by Groups II-IV.

Group II has the special technical feature of a composition comprising a scFv antibody fragment protein, not required in Groups I, III or IV.

Group III has the special technical feature of an engineered genetic circuit design for Boolean logic gates, comprising a first nucleic acid and a second nucleic that crosstalk to each other via the interaction of miRNA-miRNA BS located on separate nucleic acid molecules, [e.g. see instant Specification sheet 12 Fig 7F], not required by Groups I, II or IV.

Group IV has the special technical feature of a synthetic promoter library, not required by Groups I-III.

Common Technical Feature:
1. Groups I and III share the common technical feature of an engineered genetic circuit.
2. Groups I and III share the common technical feature of comprising a promoter operably linked to a nucleotide sequence encoding an RNA transcript (e.g., mRNA) containing an intronic microRNA (miRNA) and also includes a miRNA-BS.
3. Groups I and III share the common technical feature of an additional nucleic acid molecule that encodes only a miRNA-BS and where its "target" is the miRNA in another nucleic acid molecule.
4. Groups I, III and IV share the common technical feature of a promoter.

However, said common technical features do not represent a contribution over the prior art, and is anticipated by US 2010/0197006 A1 to Benenson. (hereinafter "Benenson").

As to common technical features #1, #2, and #4, Benenson teaches #1, an engineered genetic circuit (para [0130]; "FIG. 14D depicts a molecular implementation of the motif in FIG. 14A, wherein each transcript is spliced into a functional mRNA element and an auxiliary miRNA processed from an intron. The miRNA is designed to target the spliced mRNA after both are exported to the cytoplasm, the export being required for proper miRNA processing. This auto-repression is weak enough to maintain enough mRNA to send the ON output signal (a fluorescent protein), but the repression by circuit-related miRNAs (grey color) is strong enough to convert the output to OFF" sheet 28 fig 14D), and further teaches #2, a promoter (sheet 28 fig 14D; constitutive pol II promoter) operably linked to a nucleotide sequence encoding an RNA transcript (e.g., mRNA) (sheet 28 fig 14D; gene of interest exon 1 and exon 2) containing an intronic microRNA (miRNA) sheet 28 fig 14D; synthetic intron containing pre-miRNA sequence) and also includes a miRNA-BS (sheet 28 fig 14D; para [0130]; "The miRNA is designed to target the spliced mRNA after both are exported to the cytoplasm") and further teaches #4, a promoter (sheet 28 fig 14D; constitutive pol II promoter).

As to common technical feature #3, Benenson teaches an additional nucleic acid construct that encodes only a miRNA-BS and where its "target" is the miRNA in another construct (para [0138]; "In some embodiments, individual components (e.g., an output, a mediator, or a regulatory protein) can be separately encoded in different vectors and introduced into one or more cells separately. For example, a molecular circuit can be introduced into a cell encoded on multiple vectors, each vector encoding one or more components of an operational molecular circuit").

As the common technical feature was known in the art at the time of the invention, this cannot be considered a common special technical feature that would otherwise unify the groups. The inventions lack unity with one another.

Therefore, Groups I-IV lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.

Note concerning item 4: Claims 6-27, 43-47, 51-57, 61-67 are multiple dependent claims and are not drafted according to the second and third sentences of PCT Rule 6.4(a).

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C12Q 1/6886(2018.01)
A61K 39/395(2006.01)

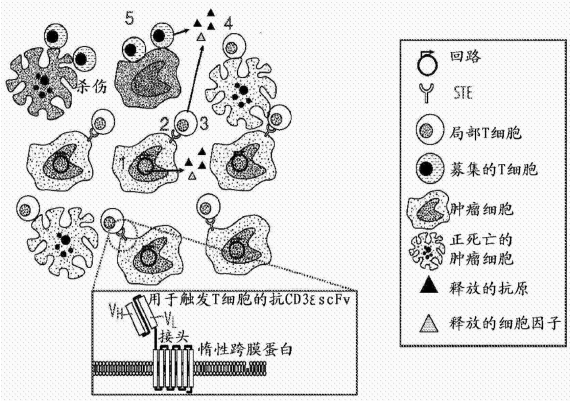
权利要求书6页 说明书43页 附图44页

(54)发明名称

肿瘤免疫治疗

(57)摘要

本公开内容的一些方面提供了这样的平台，其从肿瘤自身内触发针对肿瘤的有力且有效的免疫治疗，因此克服了现有癌症免疫治疗和肿瘤检测基因回路的局限性。



CN 108350445 A

1.工程改造的遗传回路,其包含:

(a)第一核酸,其包含与以下可操作地连接的启动子:(i)编码含有内含子微小RNA(miRNA)的输出信使RNA(mRNA)的核苷酸序列和(ii)编码与(a)(i)的miRNA互补的至少一个mRNA结合位点的核苷酸序列;和

(b)第二核酸,其包含与以下可操作地连接的启动子:编码与(a)(i)的miRNA互补的至少一个miRNA结合位点的核苷酸序列。

2.权利要求1所述的工程改造的遗传回路,其中输出mRNA编码合成的T细胞衔接器(STE)或双特异性T细胞衔接器(BiTE)。

3.权利要求1所述的工程改造的遗传回路,其中所述输出mRNA编码与T细胞表面标志物结合的输出蛋白。

4.权利要求3所述的工程改造的遗传回路,其中所述T细胞表面标志物是CD3、CD4、CD8或CD45。

5.权利要求1至4中任一项所述的工程改造的遗传回路,其中所述输出蛋白是与T细胞表面抗原特异性结合的抗体或抗体片段。

6.权利要求1至5中任一项所述的工程改造的遗传回路,其中所述输出mRNA编码抗癌剂。

7.权利要求6所述的工程改造的遗传回路,其中所述抗癌剂是趋化因子、细胞因子或检查点抑制剂。

8.权利要求1至7中任一项所述的工程改造的遗传回路,其中(a)和/或(b)的所述启动子是诱导型启动子。

9.权利要求8所述的工程改造的遗传回路,其中(a)和/或(b)的启动子是肿瘤特异性启动子或癌症启动子。

10.权利要求9所述的工程改造的遗传回路,其中(a)和/或(b)的启动子是SSX1或H2A1。

11.权利要求1至10中任一项所述的工程改造的遗传回路,其中(a)(ii)的核苷酸序列编码与(a)(i)的miRNA互补的2至5个miRNA结合位点。

12.权利要求1至11中任一项所述的工程改造的遗传回路,其中(b)的核苷酸序列编码与(a)(i)的miRNA互补的2至10个miRNA结合位点。

13.权利要求1至12中任一项所述的工程改造的遗传回路,其中所述输出蛋白是转录因子。

14.权利要求13所述的工程改造的遗传回路,其还包含至少一种核酸,所述核酸包含与编码输出核酸或输出蛋白的核酸可操作地连接的启动子。

15.权利要求14所述的工程改造的遗传回路,其中所述输出mRNA编码能够与所述至少一种核酸的启动子结合并活化其转录的转录因子。

16.权利要求1至15中任一项所述的工程改造的遗传回路,其还包含核酸,所述核酸包含与以下可操作地连接的启动子:(i)编码含有内含子微小RNA(miRNA)的另外的输出信使RNA(mRNA)的核苷酸序列和(ii)编码与(a)(i)的miRNA互补的至少一个miRNA结合位点的核苷酸序列,其中所述另外的输出mRNA编码趋化因子、细胞因子、检查点抑制剂或其组合。

17.细胞,其包含权利要求1至16中任一项的至少一种工程改造的遗传回路。

18.权利要求17所述的细胞,其中所述细胞是肿瘤细胞。

19.方法,其包括向患有肿瘤的对象施用权利要求1至15中任一项的至少一种工程改造的遗传回路。

20.权利要求19所述的方法,其中所述对象患有卵巢癌、乳腺癌或肺癌。

21.权利要求19或20所述的方法,其中向所述对象全身地施用所述工程改造的遗传回路。

22.权利要求19至21中任一项所述的方法,其中使用病毒递送系统递送所述工程改造的遗传回路。

23.权利要求22所述的方法,其中所述病毒递送系统是慢病毒递送系统、腺病毒递送系统或腺相关病毒递送系统。

24.权利要求19至21中任一项所述的方法,其中使用非病毒递送系统递送所述工程改造的遗传回路。

25.权利要求19或20所述的方法,其中向所述对象的肿瘤局部地施用所述工程改造的遗传回路。

26.权利要求25所述的方法,其中使用基于水凝胶的递送系统向所述肿瘤局部地施用所述工程改造的遗传回路。

27.权利要求19至26中任一项所述的方法,其中至少一种所述工程改造的遗传回路的输出mRNA编码与T细胞表面标志物结合的输出蛋白,并且至少一种其他的工程改造的遗传回路的输出mRNA编码趋化因子、细胞因子或检查点抑制剂。

28.组合物,其包含与跨膜蛋白融合的抗CD3e scFv抗体片段。

29.权利要求28所述的组合物,其中所述跨膜蛋白包含细胞质截短的达菲抗原/趋化因子受体(DARC)。

30.组合物,其包含与人IgG1-铰链-CH2-CH3结构域、鼠B7.1-跨膜和胞质结构域融合的抗CD3e scFv抗体片段。

31.工程改造的遗传回路,其包括:

(a)第一核酸,其包含与以下可操作地连接的第一肿瘤特异性启动子:(i)编码含有内含子微小RNA(miRNA)的输出信使RNA(mRNA)的核苷酸序列和(ii)编码与(a)(i)的miRNA互补的至少一个miRNA结合位点的核苷酸序列,其中所述输出mRNA编码合成的T细胞衔接器或双特异性T细胞衔接器;和

(b)第二核酸,其包含与第一启动子不同并且与以下可操作地连接的第二启动子:编码与(a)(i)的miRNA互补的至少一个miRNA结合位点的核苷酸序列。

32.权利要求31所述的工程改造的遗传回路,其还包含核酸,所述核酸包含与以下可操作地连接的肿瘤特异性启动子:(i)编码含有内含子微小RNA(miRNA)的另外的输出信使RNA(mRNA)的核苷酸序列和(ii)编码与(a)(i)的miRNA互补的至少一个miRNA结合位点的核苷酸序列,其中所述另外的输出mRNA编码趋化因子、细胞因子、检查点抑制剂或其组合。

33.工程改造的遗传回路,其包含:

(a)第一核酸,其包含与以下可操作地连接的启动子:

(i)编码含有内含子微小RNA(miRNA)的输出信使RNA(mRNA)的核苷酸序列,

(ii)编码内含子miRNA的核苷酸序列,和

(iii)编码miRNA结合位点(miRNA-BS)的核苷酸序列;

(b)第二核酸,其包含与以下可操作地连接的启动子:

(i)编码含有内含子miRNA的输出mRNA的核苷酸序列,

(ii)编码内含子miRNA的核苷酸序列,和

(iii)编码miRNA-BS的核苷酸序列;以及

(c)第三核酸,其包含与编码输出蛋白的核苷酸序列可操作地连接的启动子,所述输出蛋白与miRNA-BS连接,

其中(a)(iii)的miRNA-BS与(b)(i)的miRNA互补,(b)(iii)的miRNA-BS与(a)(i)的miRNA互补,并且(c)的miRNA-BS与(a)(ii)的miRNA和(b)(ii)的miRNA互补。

34.工程改造的遗传回路,其包含:

(a)第一核酸,其包含与以下可操作地连接的启动子:

(i)编码含有内含子微小RNA(miRNA)的新生RNA转录物的核苷酸序列,和

(ii)编码至少一个miRNA结合位点(miRNA-BS)的核苷酸序列;

(b)第二核酸,其包含与以下可操作地连接的启动子:

(i)编码含有内含子miRNA的新生RNA转录物的核苷酸序列,和

(ii)编码至少一个miRNA-BS的核苷酸序列;以及

(c)第三核酸,其包含与编码输出蛋白的核酸可操作地连接的启动子,所述输出蛋白与(i)第一miRNA-BS和(ii)第二miRNA-BS连接,

其中(a)(ii)的所述至少一个miRNA-BS与(b)(i)的miRNA互补,(b)(ii)的所述至少一个miRNA-BS与(a)(i)的miRNA互补,(c)(i)的第一miRNA-BS与(a)(i)的miRNA互补,并且(c)(ii)的第二miRNA-BS与(b)(i)的miRNA互补。

35.工程改造的遗传回路,其包含:

(a)第一核酸,其包含与编码含有内含子微小RNA(miRNA)的新生RNA转录物的核苷酸序列可操作地连接的启动子;

(b)第二核酸,其包含与编码含有内含子miRNA的新生RNA转录物的核苷酸序列可操作地连接的启动子;以及

(c)第三核酸,其包含与编码输出蛋白的核苷酸序列可操作地连接的启动子,所述输出蛋白与(i)第一miRNA-BS和(ii)第二miRNA-BS连接,

其中(c)(i)的第一miRNA-BS与(a)的miRNA互补,并且(c)(ii)的第二miRNA-BS与(b)的miRNA互补。

36.工程改造的遗传回路,其包含:

(a)第一核酸,其包含与编码含有内含子微小RNA(miRNA)的新生RNA转录物的核苷酸序列可操作地连接的启动子;和

(b)第二核酸,其包含与编码输出蛋白的核苷酸序列可操作地连接的启动子,所述输出蛋白与miRNA结合位点(miRNA-BS)连接;

其中(b)的miRNA-BS与(a)的miRNA互补。

37.工程改造的遗传回路,其包含:

(a)第一核酸,其包含与以下可操作地连接的启动子:(i)编码含有内含子微小RNA(miRNA)的输出信使RNA(mRNA)的核苷酸序列和(ii)至少一个miRNA结合位点(miRNA-BS);以及

(b)第二核酸,其包含与以下可操作地连接的启动子:(i)编码含有内含子miRNA的输出mRNA的核苷酸序列和(ii)至少一个miRNA-BS,

其中(a)的所述至少一个miRNA-BS与(b)的miRNA互补,(b)的所述至少一个miRNA-BS与(a)的miRNA互补。

38.工程改造的遗传回路,其包含:

(a)第一核酸,其包含与编码含有内含子微小RNA(miRNA)的新生RNA转录物的核苷酸序列可操作地连接的启动子;

(b)第二核酸,其包含与编码输出蛋白的核苷酸序列可操作地连接的启动子;以及

(c)第三核酸,其包含与编码输出蛋白的核苷酸序列可操作地连接的启动子,所述输出蛋白与miRNA结合位点连接,

其中(c)的miRNA-BS与(a)的miRNA互补。

39.工程改造的遗传回路,其包含:

(a)第一核酸,其包含与编码输出蛋白的核苷酸序列可操作地连接的启动子,所述输出蛋白与微小RNA结合位点(miRNA-BS)连接;以及

(b)第二核酸,其包含与编码含有内含子miRNA的新生RNA转录物的核苷酸序列可操作地连接的启动子,

其中(a)的miRNA-BS与(b)的miRNA互补。

40.合成的启动子文库,其包含多个核酸,其中每个核酸包含具有串联的至少两个8聚体核苷酸序列的启动子序列,在每个8聚体核苷酸序列之间没有任何间隔物核苷酸。

41.权利要求40所述的合成的启动子文库,其中每个核酸包含串联的至少六个8聚体核苷酸序列,在每个8聚体核苷酸序列之间没有任何间隔物核苷酸。

42.权利要求40或权利要求41所述的合成的启动子文库,其中每个核酸包含串联的至少十二个8聚体核苷酸序列,在每个8聚体核苷酸序列之间没有任何间隔物核苷酸。

43.权利要求40至42中任一项所述的合成的启动子文库,其中所述8聚体核苷酸序列是NNNNNNNN,其中每个N表示任意核苷酸。

44.权利要求40至43中任一项所述的合成的启动子文库,其中每个所述核酸还包含在5'和3'端的限制性内切核酸酶位点。

45.权利要求44所述的合成的启动子文库,其中在5'端的限制性内切核酸酶位点是SbfI位点,在3'端的限制性内切核酸酶位点是AscI位点。

46.权利要求40至45中任一项所述的合成的启动子文库,其中每个所述核酸还包含编码输出分子的核苷酸序列,所述核苷酸序列与所述启动子序列可操作地连接。

47.权利要求46所述的合成的启动子文库,其中所述输出分子是可检测分子。

48.合成的启动子文库,其包含多个核酸,其中每个核酸包含具有串联的至少两个8聚体核苷酸序列的启动子序列,在每个8聚体核苷酸序列之间具有3聚体核苷酸间隔物。

49.权利要求48所述的合成的启动子文库,其中每个核酸包含串联的至少六个8聚体核苷酸序列,在每个8聚体核苷酸序列之间具有3聚体核苷酸间隔物。

50.权利要求48或权利要求49所述的合成的启动子文库,其中每个核酸包含串联的至少九个8聚体核苷酸序列,在每个8聚体核苷酸序列之间具有3聚体核苷酸间隔物。

51.权利要求48至50中任一项所述的合成的启动子文库,其中所述8聚体核苷酸序列是

NNNNNNNN,其中每个N表示任意核苷酸。

52.权利要求48至51中任一项所述的合成的启动子文库,其中每个所述核酸还包含在5'和3'端的限制性内切核酸酶位点。

53.权利要求52所述的合成的启动子文库,其中在5'端的限制性内切核酸酶位点是SbfI位点,在3'端的限制性内切核酸酶位点是AscI位点。

54.权利要求48至53中任一项所述的合成的启动子文库,其中所述3聚体核苷酸间隔物选自AGC、ATC、GAC、ACT、AGT、GTC、GAT和GCT。

55.权利要求54所述的合成的启动子文库,其中每个3聚体核苷酸间隔物是不同的。

56.权利要求48至55中任一项所述的合成的启动子文库,其中每个所述核酸还包含编码输出分子的核苷酸序列,所述核苷酸序列与所述启动子序列可操作地连接。

57.权利要求56所述的合成的启动子文库,其中所述输出分子是可检测分子。

58.合成的启动子文库,其包含多个核酸,其中每个核酸包含具有串联的至少两个11聚体核苷酸序列的启动子序列,在每个11聚体核苷酸序列之间具有3聚体核苷酸间隔物。

59.权利要求58所述的合成的启动子文库,其中每个核酸包含串联的至少四个11聚体核苷酸序列,在每个11聚体核苷酸序列之间具有3聚体核苷酸间隔物。

60.权利要求58或权利要求59所述的合成的启动子文库,其中每个核酸包含串联的至少七个11聚体核苷酸序列,在每个11聚体核苷酸序列之间具有3聚体核苷酸间隔物。

61.权利要求58至60中任一项所述的合成的启动子文库,其中所述11聚体核苷酸序列是NNNNNNNNNNN,其中每个N表示任意核苷酸。

62.权利要求58至61中任一项所述的合成的启动子文库,其中每个所述核酸还包含在5'和3'端的限制性内切核酸酶位点。

63.权利要求62所述的合成的启动子文库,其中在5'端的限制性内切核酸酶位点是SbfI位点,在3'端的限制性内切核酸酶位点是AscI位点。

64.权利要求58至63中任一项所述的合成的启动子文库,其中所述3聚体核苷酸间隔物选自AGC、ATC、GAC、ACT、AGT、GTC、GAT和GCT。

65.权利要求64所述的合成的启动子文库,其中每个3聚体核苷酸间隔物是不同的。

66.权利要求58至65中任一项所述的合成的启动子文库,其中每个所述核酸还包含编码输出分子的核苷酸序列,所述核苷酸序列与所述启动子序列可操作地连接。

67.权利要求66所述的合成的启动子文库,其中所述输出分子是可检测分子。

68.选择合成的启动子的方法,其包括:

获得包含核酸分子的文库,所述核酸分子包含与输出分子可操作地连接的合成的启动子序列,

在一种或更多种类型的细胞中表达所述文库,

检测所述输出分子的表达,以及

分离其中表达所述输出分子的细胞。

69.权利要求68所述的方法,其还包括:

确定所分离的细胞中的所述合成的启动子序列的序列。

70.权利要求68或权利要求69所述的方法,其中所述一种或更多种类型的细胞是至少两种不同类型的细胞。

71.权利要求70所述的方法，其还包括将在所述至少两种不同类型的细胞的每种中驱动输出分子之表达的合成的启动子序列进行比较，以鉴定在至少两种不同类型的细胞的一种中比在至少两种不同类型的细胞的另一种中更具活性的合成的启动子序列。

72.权利要求71所述的方法，其中所述至少两种不同类型的细胞是癌细胞和非癌细胞，并且其中鉴定出在癌细胞中比在非癌细胞中更具活性的启动子。

肿瘤免疫治疗

[0001] 相关申请

[0002] 本申请根据35 U.S.C.§119(e)要求2015年6月19日提交的美国临时申请号62/181,906和2016年4月20日提交的美国临时申请号62/325,314的权益,其各自通过引用整体并入本文。

技术领域

[0003] 本公开内容的一些方面涉及生物技术的一般领域,并且更具体地涉及合成生物学和免疫学的领域。

背景技术

[0004] 许多癌症(例如,卵巢癌)的现有治疗(例如化学疗法和靶向疗法)无法治愈转移性疾病以及防止肿瘤复发。此外,标准护理治疗(例如化学疗法)可引起显著的发病率和毒性。需要新的治疗策略来治疗原发性和转移性卵巢癌并实现长期的效力。

发明内容

[0005] 在一些方面,本文中提供了这样的平台,其从肿瘤自身内触发针对肿瘤的有力且有效的免疫治疗,因此克服了现有癌症免疫治疗和肿瘤检测基因回路(gene circuit)的局限性。在一些实施方案中,本公开内容的工程改造的遗传回路(genetic circuit)在癌细胞表面上表达T细胞接合蛋白(T-cell-engaging protein)(称为表面T细胞衔接器(Surface T Cell Engager,STE)),其可以触发肿瘤细胞的抗原非依赖性T细胞杀伤。在一些实施方案中,工程改造的遗传回路被递送至肿瘤(参见,例如,图2A和2B),并且仅在癌细胞中选择性地活化,这导致表面展示STE和分泌其他免疫调节分子以募集T细胞靶向肿瘤。有利地,本公开内容的工程改造的遗传回路可以全身性施用,但仅在癌细胞中被局部活化,这导致安全性增强以及副作用降低。因此,在一些实施方案中,本公开内容的平台将全身性递送(例如,治疗转移)的优点与局部治疗的优点(例如安全性、最小的副作用)相结合。

[0006] 现有疗法受到某些局限性的阻碍,所述局限性被本公开内容所克服。例如,在CAR(chimeric antigen receptor,嵌合抗原受体)T细胞治疗中,T细胞必须对每个个体定制。作为另一个实例,双特异性T细胞衔接器(bispecific T cell engager,BiTE)(Iwahori K.等,Molecular Therapy,2015,23(1):171-178,其通过引用并入本文)受其短半衰期的限制,因此需要连续静脉内泵输注4至8周。两种疗法均靶向肿瘤细胞表面抗原;然而,并不是所有的肿瘤类型都具有理想的用于检测的表面肿瘤抗原。癌症检测遗传回路可利用细胞内杀伤机制,通过毒素诱导细胞死亡,但是将这些回路递送至全部(或大部分)肿瘤细胞几乎是不可能的。

[0007] 在一些方面,本公开内容提供了方法和工程改造的(重组或合成的)遗传回路(例如,工程改造的哺乳动物遗传回路),在一些实施方案中其被称为基于RNA的“逻辑门(logic gate)”(例如,遗传回路包括主要包含RNA的核酸,或遗传回路包括由RNA组成的核酸),因此

降低了不期望的免疫原性反应的可能性，因为没有外来蛋白被引入细胞或对象。

[0008] 在一些实施方案中，本公开内容提供了用于特异性检测癌细胞和生产免疫调节剂(例如，细胞因子)的方法和工程改造的遗传回路。在一些实施方案中，如本文中提供的方法和遗传回路用于癌细胞的“旁观者杀伤(bystander killing)”，由此触发记忆T细胞以破坏不被本公开内容的工程改造的遗传回路直接转化的癌细胞。

[0009] 在一些实施方案中，本公开内容提供了用于靶向性表达从特定细胞(例如，癌细胞)释放的组合免疫调节剂的方法和工程改造的遗传回路。在一些实施方案中，工程改造的遗传回路编码与CD3结合的分子，当其在靶向癌细胞(抗CD3细胞)的表面上表达时，作为合成的T细胞衔接器(STE)发挥作用以直接募集T细胞从而杀伤由工程改造的遗传回路靶向/检测到的癌细胞，产生局部和靶向的免疫治疗。在另一些实施方案中，工程改造的遗传回路编码双向T细胞衔接器(bi-directional T cell engager，BiTE)，当其由细胞表达并通过抗原特异性区域与所述细胞结合时，其募集T细胞以杀伤细胞。可以使用工程改造的遗传回路(逻辑门)在特定细胞类型内选择性表达BiTE，其使得具有局部性的生产以及使用STE所观察到的同样的优点。

[0010] 在一些实施方案中，STE可用作通用的靶向性免疫治疗，而BiTE通常需要识别肿瘤特异性表面抗原以触发T细胞杀伤。

[0011] 本公开内容的靶向性免疫治疗与现有疗法的不同之处在于其能够以高效力和安全性进行全身性递送。在一些实施方案中，使用其他细胞因子和免疫治疗剂的组合疗法进一步增强本公开内容的靶向性免疫治疗的效力。

[0012] 在一些实施方案中，本公开内容提供了用于检测疾病(包括但不限于自身免疫疾病和神经疾病)中的异常细胞状态和/或用于表达或分泌免疫调节分子和治疗性分子以调节疾病的方法和工程改造的遗传回路。

[0013] 在一些实施方案中，本公开内容的免疫治疗平台还包括可用作诊断的输出(例如，编码可检测分子的工程改造的遗传回路)。

[0014] 一些实施方案提供了包含癌症特异性启动子的工程改造的核酸，所述癌症特异性启动子与以下的核酸可操作地连接：编码mRNA内的微小RNA的核酸，所述mRNA编码免疫调节分子(例如“表面T细胞衔接器”或STE)或与微小RNA结合位点连接的双特异性单克隆抗体。

[0015] 在一些实施方案中，免疫调节分子或双特异性单克隆抗体仅在工程改造的核酸的转录活化时才被翻译。

[0016] 本文中还提供了包含癌症特异性启动子的工程改造的核酸，所述癌症特异性启动子与编码含有微小RNA结合位点的mRNA转录物的核酸可操作地连接。

[0017] 本文中还提供了如图3A-3D、4A、6A、7A-7H、9A、14A、15A、16A和17A中任一幅图中所示的工程改造的核酸。

[0018] 本公开内容还提供了包含如本文中所述的任何工程改造的核酸的载体。本公开内容还提供了包含如本文中所述的任何载体和/或工程改造的核酸的细胞。

[0019] 本公开内容的一些实施方案提供了工程改造的遗传回路，其包含(a)核酸，其包含与以下可操作地连接的启动子：(i)编码含有内含子微小RNA(miRNA)的输出信使RNA(mRNA)的核苷酸序列和(ii)编码与(a)(i)的miRNA互补的miRNA结合位点的核苷酸序列，以及(b)核酸，所述核酸包含与以下可操作地连接的启动子：编码与(a)(i)的miRNA互补的至少一个

miRNA结合位点的核苷酸序列。

[0020] 本公开内容的另一些实施方案提供了工程改造的遗传回路，所述工程改造的遗传回路包含(a)第一核酸，其包含与以下可操作地连接的启动子：(i)编码含有内含子微小RNA(miRNA)的输出信使RNA(mRNA)的核苷酸序列，(ii)编码内含子miRNA的核苷酸序列，和(iii)编码miRNA结合位点(miRNA-BS)的核苷酸序列；(b)第二核酸，其包含与以下可操作地连接的启动子：(i)编码含有内含子miRNA的输出mRNA的核苷酸序列，(ii)编码内含子miRNA的核苷酸序列，和(iii)编码miRNA-BS的核苷酸序列；以及(c)第三核酸，其包含与编码输出蛋白的核苷酸序列可操作地连接的启动子，所述输出蛋白与miRNA-BS连接，其中(a)(iii)的miRNA-BS与(b)(i)的miRNA互补，(b)(iii)的miRNA-BS与(a)(i)的miRNA互补，以及(c)的miRNA-BS与(a)(ii)的miRNA和(b)(ii)的miRNA互补。

[0021] 本公开内容的另一些实施方案提供了工程改造的遗传回路，所述工程改造的遗传回路包含(a)第一核酸，其包含与以下可操作地连接的启动子：(i)编码含有内含子微小RNA(miRNA)的新生RNA转录物(例如，非编码RNA转录物)的核苷酸序列，和(ii)编码至少一个miRNA结合位点(miRNA-BS)的核苷酸序列；(b)第二核酸，其包含与以下可操作地连接的启动子：(i)编码含有内含子miRNA的新生RNA转录物的核苷酸序列和(ii)编码至少一个miRNA-BS的核苷酸序列；以及(c)第三核酸，其包含与编码输出蛋白的核苷酸序列可操作地连接的启动子，所述输出蛋白与(i)第一miRNA-BS和(ii)第二miRNA-BS连接，其中(a)(ii)的至少一个miRNA-BS与(b)(i)的miRNA互补，(b)(iii)的至少一个miRNA-BS与(a)(i)的miRNA互补，(c)(i)的第一miRNA-BS与(a)(i)的miRNA互补，并且(c)(ii)的第二miRNA-BS与(b)(i)的miRNA互补。

[0022] 本公开内容的另一些实施方案提供了工程改造的遗传回路，所述工程改造的遗传回路包含(a)第一核酸，其包含与编码含有内含子微小RNA(miRNA)的新生RNA转录物的核苷酸序列可操作地连接的启动子；(b)第二核酸，其包含与编码含有内含子miRNA的新生RNA转录物的核苷酸序列可操作地连接的启动子；以及(c)第三核酸，其包含与编码输出蛋白的核苷酸序列可操作地连接的启动子，所述输出蛋白与(i)第一miRNA-BS和(ii)第二miRNA-BS连接，其中(c)(i)的第一miRNA-BS与(a)的miRNA互补，并且(c)(ii)的第二miRNA-BS与(b)的miRNA互补。

[0023] 本公开内容的另一些实施方案提供了工程改造的遗传回路，所述工程改造的遗传回路包含(a)第一核酸，其包含与编码含有内含子微小RNA(miRNA)的新生RNA转录物的核苷酸序列可操作地连接的启动子；和(b)第二核酸，其包含与编码输出蛋白的核苷酸序列可操作地连接的启动子，所述输出蛋白与miRNA结合位点(miRNA-BS)连接，其中(b)的miRNA-BS与(a)的miRNA互补。

[0024] 本公开内容的另一些实施方案提供了工程改造的遗传回路，所述工程改造的遗传回路包含(a)第一核酸，其包含与以下可操作地连接的启动子：(i)编码含有内含子微小RNA(miRNA)的输出信使RNA(mRNA)的核苷酸序列和(ii)至少一个miRNA结合位点(miRNA-BS)；以及(b)第二核酸，其包含与以下可操作地连接的启动子：(i)编码含有内含子miRNA的输出mRNA的核苷酸序列和(ii)至少一个miRNA-BS，其中(a)的至少一个miRNA-BS与(b)的miRNA互补，(b)的至少一个miRNA-BS与(a)的miRNA互补。

[0025] 本公开内容的另一些实施方案提供了工程改造的遗传回路，所述工程改造的遗传

回路包含(a)第一核酸,其包含与编码含有内含子微小RNA(miRNA)的新生RNA转录物的核苷酸序列可操作地连接的启动子;(b)第二核酸,其包含与编码输出蛋白的核苷酸序列可操作地连接的启动子;以及(c)第三核酸,其包含与编码输出蛋白的核苷酸序列可操作地连接的启动子,所述输出蛋白与miRNA结合位点连接,其中(c)的miRNA-BS与(a)的miRNA互补。

[0026] 本公开内容的另一些实施方案提供了工程改造的遗传回路,所述工程改造的遗传回路包含(a)第一核酸,其包含与编码输出蛋白的核苷酸序列可操作地连接的启动子,所述输出蛋白与微小RNA结合位点(miRNA-BS)连接;以及(b)第二核酸,其包含与编码含有内含子miRNA的新生RNA转录物的核苷酸序列可操作地连接的启动子,其中(a)的miRNA-BS与(b)的miRNA互补。

[0027] 在一些实施方案中,输出mRNA编码合成的T细胞衔接器(STE)或双特异性T细胞衔接器(BiTE)。

[0028] 在一些实施方案中,输出mRNA编码与T细胞表面标志物结合的输出蛋白。

[0029] 在一些实施方案中,T细胞表面标志物是CD3、CD4、CD8或CD45。

[0030] 在一些实施方案中,输出蛋白是与T细胞表面抗原特异性结合的抗体或抗体片段。

[0031] 在一些实施方案中,输出mRNA编码抗癌剂。例如,输出mRNA可编码趋化因子、细胞因子或检查点抑制剂(checkpoint inhibitor)。

[0032] 在一些实施方案中,启动子是诱导型启动子。例如,启动子可以是肿瘤特异性启动子(例如,良性肿瘤特异性启动子或恶性肿瘤特异性启动子)或癌症启动子。

[0033] 在一些实施方案中,启动子是SSX1或H2A1。

[0034] 在一些实施方案中,核苷酸序列编码2至5或2至10个微小RNA结合。

[0035] 在一些实施方案中,输出蛋白是转录因子。

[0036] 在一些实施方案中,输出蛋白是抗癌剂。

[0037] 在一些实施方案中,输出mRNA编码转录因子,所述转录因子可结合至少一种核酸的启动子并活化其转录。

[0038] 在一些实施方案中,工程改造的遗传回路包含编码脱落蛋白质系统(split protein system)的核酸,其中功能性蛋白质二聚体的每个蛋白在单独的核酸上编码并通过单独的启动子调节。

[0039] 本发明并不将其应用局限于以下说明书中阐述的或者附图中示出的结构和组件布置的细节。本发明能够具有其他实施方案并且能够以多种方式实践或实施。以上实施方案和方面中的每一个可以与任何其他实施方案或方面联合。此外,本文中使用的措辞和术语是为了描述的目的,而不应被认为是限制性的。“包括(including)”,“包含(comprising)”或“具有(having)”,“含有(containing)”,“涉及(involving)”及其变化形式的使用意在涵盖其后列出的项目及其等同物以及附加项目。

[0040] 附图简述

[0041] 附图并不旨在按比例绘制。为了清楚起见,并非每个元件都在每幅图中标出。

[0042] 图1A-1C.现有免疫治疗方法的实例。(图1A)嵌合抗原受体(CAR)T细胞治疗的作用模式。(图1B和1C)双特异性T细胞衔接器的作用模式。

[0043] 图2A-2B.STRICT治疗的概述。(图2A)使用STRICT来分泌BiTE。(1)通过局部注射或全身性施用将肿瘤鉴定回路引入肿瘤。(2)经回路转导的肿瘤细胞分泌局部扩散的BiTE和

其他免疫调节分子。(3)BiTE同时接合肿瘤细胞上的HER2和局部肿瘤浸润性T细胞上的T细胞受体,从而触发T细胞以直接杀伤肿瘤细胞。BiTE还可以募集附近的循环T细胞以运输到肿瘤部位。(4)通过第一波杀伤释放的肿瘤抗原致敏(prime)并募集更多的肿瘤反应性T细胞发挥作用。(5)新募集的多克隆T细胞可以杀伤更多的癌细胞,包括HER2阴性肿瘤细胞和不被第一波抗肿瘤免疫应答所杀伤的其他异质肿瘤细胞。(图2B)使用STRICT来展示表面T细胞衔接器(STE)。(1)通过局部注射或全身性施用将肿瘤鉴定基因回路引入肿瘤。(2)经回路转导的肿瘤细胞表达STE和其他免疫调节分子。(3)STE接合局部肿瘤浸润性T细胞上的T细胞受体,从而触发T细胞以直接杀伤肿瘤细胞。(4)通过第一波杀伤释放的肿瘤抗原致敏并募集更多的肿瘤反应性T细胞发挥作用。(5)新募集的多克隆T细胞可以杀伤更多的癌细胞,包括不被第一波抗肿瘤免疫应答所杀伤的其他异质肿瘤细胞和转移瘤。免疫记忆可以防止肿瘤复发。

[0044] 图3A-3H.仅RNA的单输出AND门的设计。(图3A-3D)示出了所有4个输入状态及其各自的输出状态的计算层。基于RNA的逻辑AND门整合了两个输入启动子P1和P2的活性,并且只有当两个启动子都明确有活性时才产生输出。在这种结构中,输出是表面T细胞衔接器(STE)。启动子P1调节包含合成的miRNA内含子的STE mRNA(mirFF4)的表达。通过在STE/mirFF4转录物的3'端编码完全匹配的mirFF4结合位点(mirFF4-BS)将负向自动调节反馈环并入回路中。因此,当仅启动子P1有活性时,STE mRNA通过细胞miRNA机制被不断降解,并且不产生STE蛋白(状态3)。启动子P2调节miRNA海绵(miRNA sponge)的表达,所述miRNA海绵在3'端含有具有多个凸起的mirFF4结合位点的非编码RNA(诱饵)。因此,当仅启动子P2有活性时,不产生蛋白质输出(状态2)。当两个启动子P1和P2都有活性时,由启动子P1调节的STE/mirFF4mRNA所产生的mirFF4被由启动子P2调节的mirFF4海绵分离开,因此允许产生STE蛋白(状态1)。(图3E-3H)当使用荧光蛋白mKate2作为输出时,AND门回路的4种输入状态及其各自的输出状态。

[0045] 图4A-4B.mKate2AND门实验结果。(图4A)为了检查基于RNA的逻辑AND门设计,用mKate2输出进行编码。作为该设计的启动子输入,我们使用了在许多人癌症中过表达的两种人启动子:SSX1和H2A1(分别为输入1和输入2,而输入1编码mKate2输出和mirFF4)。(图4B)相对于(a)输入1中编码的完全匹配FF4-BS的数目和(b)输入2中海绵设计的两种不同的结构,测量不同的设计的mKate2输出水平。X轴注释:M#表示输入1具有#个在mKate2/mirFF4下游编码的FF4-BS。例如,M3表示输入1具有3个完全匹配FF4-BS,如门示图中所示。S0、S1和S2表示三种不同的海绵设计。S0是无mirFF4-BS的阴性对照转录物。设计S1是在3'上编码的具有10个凸起的FF4-BS的诱饵转录物,如门示图中所示。设计S2与S1类似,但具有另外的环状内含子,在位于转录物3'中编码的10个凸起的FF4-BS的上游具有10个凸起的FF4-BS。因此,门示图表示设计M3-S1(在图中用绿色虚线环绕)。结果以平均mKate2表达(P1)表示,其是在FACS中对SSC/FSC设门以除去细胞团块和碎片的细胞的平均mKate2。误差条表示SEM。我们没有测试输入2条件,因为它无论如何不编码输出蛋白。NT表示未转染的细胞。

[0046] 图5.mKate2 AND门实验结果。为了再次检查基于RNA的逻辑AND门设计,将其用mKate2输出编码。在海绵转录物中编码ECFP以测量通过miRNA的海绵的降解。SSX1和H2A1用作本设计的启动子输入:分别为输入1和输入2,而输入1编码mKate2输出和mirFF4。相对于(a)在输入1中编码的完全匹配FF4-BS的数目和(b)在输入2中海绵设计的两种不同的结构,

测量不同实验设置的mKate2和ECFP输出水平。X轴注释:M#表示输入1具有#个在mKate2/mirFF4的下游编码的FF4-BS。

[0047] 图6A-6B.多输出AND门回路的设计。(图6A)当启动子P1和P2都具有活性时,由启动子P1调节的TF/mirFF4mRNA所产生的mirFF4被由启动子P2调节的mirFF4海绵分离开,因此允许产生人工转录因子(transcription factor,TF)。TF将进一步与其启动子结合并触发多个用户定义输出的转录。(图6B)多输出AND门的输出水平是可调的。CXCL10是CXCL1p,其调节含有mirFF4v2B内含子和10个下游mirFF4-Bs的GAL4BD-VP16AD。SSX10是SSX1p,其调节含有mirFF4v2B内含子和10个下游mirFF4-Bs的GAL4BD-VP16AD。SSX＊10是截短的SSX1p,其中部分5'UTR与KOZAK序列一起被去除,其调节含有mirFF4v2B内含子和10个下游mirFF4-Bs的GAL4BD-VP16AD。海绵S0是阴性对照转录物WO mirFF4-BS。海绵S2是具有在3上编码的10个凸起的FF4-BS的诱饵转录物,具有另外的环状内含子,其在位于转录物3'中编码的10个凸起的mirFF4-BS上游有10个凸起的mirFF4-BS。在所有样品中,mKate2输出在G5p(含有5个GAL4结合位点的启动子)下编码。通过使用与P1不同强度的启动子和不同的海绵结构可以调节输出水平。

[0048] 图7A-7H.几个布尔(Boolean)逻辑门的设计。AND、NAND、XNOR、NOR、NOT、XOR、IMPLY、NIMPLY门的基于RNA的设计的示意图。OP:输出蛋白;Nan:新生RNA转录物。

[0049] 图8.抗HER2双特异性T细胞衔接器(BiTE)和表面T细胞衔接器(STE)触发T细胞以介导稳健的肿瘤杀伤和IFN-γ分泌。如所示用多种DNA构建体转染HEK-293T(最低程度表达HER2)细胞。转染后48小时,收集多种HEK-293T细胞并与人T细胞共培养5小时或24小时。通过LDH释放测定法测量通过T细胞的5小时细胞毒性,并且通过IFN-γELISA测量通过T细胞的24小时IFN-γ分泌。数据显示T细胞介导对BiTE分泌性肿瘤细胞的稳健的肿瘤杀伤和IFN-γ分泌(第1组至第2组)。肿瘤杀伤和IFN-γ分泌与肿瘤细胞上的HER2表达水平相关(第1组至第2组)。T细胞还介导对STE表达性肿瘤细胞的稳健肿瘤杀伤和IFN-γ分泌(第3组至第6组),并且细胞毒性和IFN-γ分泌独立于肿瘤抗原(HER2)表达(第3组至第6组)。此外,当与表达非BiTE和非STE对照蛋白的HEK-293T细胞共培养时,T细胞介导最小的肿瘤杀伤和IFN-γ分泌(第7组至第9组)。

[0050] 图9A-9C.单输出AND门结构可用于微调肿瘤细胞的T细胞杀伤效率。如所示用多种DNA构建体转染HEK-293T细胞。(图9A)驱动STE表达的单输出AND门的设计。(图9B)mKate AND门的实验结果。(1,0)表示仅用P1组件(module)转染的细胞。(1,1)表示用P1和P2组件转染的细胞。(0,0)表示未转染的细胞。(图9C)STE AND门的实验结果。(1,0)表示仅用P1组件转染的细胞。(1,1)表示用P1和P2组件转染的细胞。(0,0)表示用非STE蛋白转染的细胞。Ctrl表示未转染的细胞。转染后48小时,收集多种HEK-293T细胞并与人T细胞共培养5小时。通过LDH释放测定法测量通过T细胞的细胞毒性。数据显示T细胞杀伤经P1组件转染的293T(第1列),并且通过AND门结构可以大大增强杀伤作用(第2列)。T细胞在非STE表达细胞上表现出最小的杀伤作用(第3列和第4列)。

[0051] 图10.抗HER2双特异性T细胞衔接器(BiTE)和表面T细胞衔接器(STE)触发T细胞以介导稳健的肿瘤杀伤和IFN-γ分泌。将表达所示DNA构建体的稳定的4T1细胞(HER2-)与人T细胞共培养5小时或24小时。通过LDH释放测定法测量通过T细胞的5小时细胞毒性以及通过IFN-γ ELISA测量通过T细胞的24小时IFN-γ分泌。数据显示T细胞介导对HER2-或STE-肿

瘤细胞的最小杀伤和IFN-γ分泌。(第1组和第3组)。T细胞介导对STE-表达性肿瘤细胞的稳健的肿瘤杀伤和IFN-γ分泌。(第2组)。当与由少量BiTE分泌细胞和非BiTE分泌性肿瘤组成的细胞混合物共培养时,T细胞也介导稳健的肿瘤杀伤和IFN-γ分泌。这表明在肿瘤块(tumor mass)中最小数量的BiTE分泌细胞可以引起稳健的肿瘤块杀伤和IFN-γ释放(第4组)。

[0052] 图11.抗HER2双特异性T细胞衔接器(BiTE)和表面T细胞衔接器(STE)触发T细胞以介导对人乳腺癌细胞系的稳健的肿瘤杀伤。如所示通过用多种DNA构建体进行慢病毒转导来产生稳定的MDA-MB453(HER2+)细胞系。收集多种MDA-MB453细胞并与人T细胞共培养5小时。通过LDH释放测定法测量通过T细胞5小时的细胞毒性。数据显示T细胞介导对BiTE分泌性肿瘤细胞的稳健的肿瘤杀伤(第2组)。T细胞还介导对STE表达性肿瘤细胞的稳健的肿瘤杀伤作用(第3组至第4组)。此外,当与亲本MDA-MB453肿瘤细胞系共培养时,T细胞介导最小的肿瘤杀伤(第1组)。

[0053] 图12.两种形式的STE的设计。对于形式1(v1),抗CD3e scFv与惰性跨膜蛋白(DARC)融合。对于形式2(v2),抗CD3e scFv与人IgG1-铰链-CH2-CH3结构域,之后是鼠B7.1-跨膜(TM)和细胞质(CYP)结构域融合。

[0054] 图13.表面T细胞衔接器(STE)形式1(v1)和形式2(v2)二者都触发T细胞以介导对HEK-293T细胞的稳健的肿瘤杀伤。通过慢病毒转导产生多种诱导型STE表达性HEK-293T细胞系。收集多种HEK-293T细胞并与人T细胞共培养5小时。通过LDH释放测定法测量通过T细胞5小时的细胞毒性。数据显示T细胞介导对转染的STEv1表达性肿瘤细胞的稳健的肿瘤杀伤(第2列)。T细胞还介导对诱导型STEv1和STEv2表达性肿瘤细胞的稳健的肿瘤杀伤(第3列和第4列)。此外,当与非STE表达性HEK-293T细胞系共培养时,T细胞介导最小的肿瘤杀伤(第1列)。

[0055] 图14.AND门结构可以用来微调对肿瘤细胞的T细胞杀伤效率。(A)用于STE表达的多输出AND门的设计。(B)如所示用多种DNA构建体转染HEK-293T细胞。(1,0)表示仅用STE转染的细胞。(1,1)表示用STE和海绵转染的细胞。(0,0)表示用非STE蛋白转染的细胞。转染后48小时,收集多种HEK-293T细胞并与人T细胞共培养5小时。通过LDH释放测定法测量经T细胞的细胞毒性。数据显示T细胞杀伤STE表达性(1,0)细胞(第2列和第4列),并且通过AND门(1,1)结构可以大大增强杀伤(第3列和第5列)。T细胞对非STE表达性细胞表现出最小的杀伤(第1列)。

[0056] 图15.AND门结构可以用来微调对肿瘤细胞的T细胞杀伤效率。(A)用于STE表达的多输出AND门的设计。(B)如所示用多种DNA构建体转染HEK-293T细胞。(1,0)表示仅用STE转染的细胞。(1,1)表示用STE和海绵转染的细胞。(0,0)表示用非STE蛋白转染的细胞。转染后48小时,收集多种HEK-293T细胞并与人T细胞共培养5小时。通过LDH释放测定法测量经T细胞的细胞毒性。数据显示T细胞杀伤STE表达性(1,0)细胞(第3和5列),并且通过AND门(1,1)结构可以大大增强杀伤(第4列和第6列)。T细胞在非STE表达性细胞上表现出最小的杀伤(第1列)。在(1,0)条件下的杀伤主要是由于GAL4启动子输出的泄漏(leakage)引起的(第2v.3或5列)。可进行进一步修饰以降低GAL4启动子输出的泄漏(STE v1)。我们将通过去除STE v1的KOZAK序列、通过在3'端添加miRNA结合位点使得STE v1输出自我降解、以及两种机制的组合来降低GAL4启动子的泄漏。

[0057] 图16.GAL4门v2结构可用于微调肿瘤细胞的T细胞杀伤效率并在(1,0)状态下表现出较小的细胞毒性。(A)用于STE表达的多输出AND门的设计。(B)如所示用多种DNA构建体转染HEK-293T细胞。(1,0)表示仅用STE转染的细胞。(1,1)表示用STE和海绵转染的细胞。(0,0)表示用非STE蛋白转染的细胞。转染后48小时,收集多种HEK-293T细胞并与人T细胞共培养5小时。通过LDH释放测定法测量经T细胞的细胞毒性。数据显示T细胞杀伤STE表达性(1,0)细胞(第3列),并且通过AND门(1,1)结构可以增强杀伤(第4列)。T细胞对非STE表达性细胞表现出最小的杀伤(第1列)。与GAL4门v1结构(v2更接近于基础水平(0,0))相比,在此形式的(1,0)状态下的杀伤得到改善。可以进行进一步修饰以降低在(1,0)状态下的杀伤。我们将通过在STE基因的3'端添加miR结合位点来降低(1,0)状态下的GAL4启动子输出。

[0058] 图17.GAL4门v3结构可用于微调肿瘤细胞的T细胞杀伤效率并且在(1,0)状态下表现出较小的细胞毒性。(A)用于STE表达的多输出AND门的设计。(B)如所示用多种DNA构建体转染HEK-293T细胞。(1,0)表示仅用STE转染的细胞。(1,1)表示用STE和海绵转染的细胞。(0,0)表示用非STE蛋白转染的细胞。转染后48小时,收集多种HEK-293T细胞并与人T细胞共培养5小时。通过LDH释放测定法测量经T细胞的细胞毒性。数据显示T细胞最低程度地杀伤STE表达性(1,0)细胞(第3列),并且仅在当AND门有活性时(1,1)才达到有效杀伤(第4列)。T细胞对非STE表达性细胞表现出最小的杀伤(第1列)。在(1,0)状态下的杀伤与(0,0)状态一样低。可以进行进一步修饰(例如提高GAL4-VP16输出水平或增加GAL4结合位点)以增强(1,1)状态的杀伤效力。

[0059] 图18.合成的肿瘤募集免疫细胞治疗(Synthetic Tumor-Recruited Immuno-Cellular Therapy,STRICT)的概述。栏1:设计肿瘤靶向基因回路以整合两种肿瘤特异性的合成启动子的活性,并且仅当两种启动子都有活性时才产生合成的和天然的免疫调节剂的表达,这为我们的回路提供了高的肿瘤选择性;栏2:使用基于水凝胶的递送在体内递送回路;栏3:仅转导的癌细胞表达合成的表面T细胞衔接器(STE)和/或天然免疫调节剂,其募集T细胞以杀伤肿瘤细胞;栏4:肿瘤细胞被活化的T细胞消除。

[0060] 图19.仅RNA单输出AND门的设计。基于RNA的逻辑AND门整合了两个输入启动子P1和P2的活性,并且只有当两个启动子都明确有活性时才产生并输出。在这个结构中,输出是荧光蛋白mKate2。启动子P1调节包含合成的miRNA内含子(miR1)的mKate2 mRNA的表达。我们通过在mKate2/miR1转录物的3'端编码完全匹配的miR1结合位点(miR1-BS),将负向自动调节反馈环并入回路中。因此,只有当启动子P1和P2二者都具有活性时,由启动子P1调节的mKate2/miR1 mRNA产生的mirR1被由启动子P2调节的miR1海绵分离开,因此允许产生mKate2蛋白。

[0061] 图20.仅RNA单输出AND门设计。上面的图描绘了仅RNA单输出AND门的设计细节。左表示出了miRNA结合序列影响海绵活性。右图示出由每种海绵得到的mKate2诱导倍数和由miR1得到的ECFP水平降低。

[0062] 图21.海绵中结合位点的数目和海绵转录物的丰度影响海绵活性。左栏示出了组件1(M1)和多种海绵(S67、S73和S62)的设计细节。右上栏示出了多种实验条件的mKate2和ECFP的原始输出水平。右下栏示出了由每种海绵得到的mKate2诱导倍数。SC表示对照海绵(无结合位点)。

[0063] 图22.海绵结构影响海绵活性。左栏示出了多种海绵(S76、S99、S100和S101)的设

计细节。右上栏示出了多种实验条件的mKate2和ECFP的原始输出水平。右下栏示出了由每种海绵得到的mKate2诱导倍数。SC表示对照海绵(无结合位点)。

[0064] 图23.miRNA骨架影响门性能。左栏示出组件1(M)和多种海绵(Sx和S76)的设计细节。右上栏示出了多种实验条件的mKate2和ECFP的原始输出水平。右下栏示出了多种组件1构建体(M1、M2A和M2B是组件1的3种形式,其各自由不同的miRNA骨架组成)由多种海绵得到的mKate2诱导倍数。SC表示对照海绵(无结合位点)。

[0065] 图24A-24B.多西环素诱导型STE可触发T细胞以高效杀伤OVCAR8卵巢癌细胞、HEK-293T细胞并分泌IFN-g。Dox诱导型STE(STE、STEv2和STE-snap)的3种形式均可触发通过T细胞的稳健的细胞杀伤和IFN-g分泌。

[0066] 图25.多输出回路严格杀伤肿瘤细胞。(图25A)通过AND门输出的GAD可以靶向能够表达多种蛋白质(例如STE和免疫调节分子)的第三启动子(P3)。(图25B)用编码以下的基因回路转染的HEK-293T细胞:HEK/DARC(0,0)-非STE蛋白;GAD门(1,0)-仅P1+P3构建体,其中P3表达STE;GAD门(1,1)-P1+P2+P3构建体,其中P3表达STE;HEK/const-组成型表达的STE。转染后48小时,将细胞与人T细胞共培养5小时。通过LDH释放测定法测量细胞毒性。只有当AND门打开(ON)(1,1)时,T细胞才能有效地杀伤。T细胞最低程度地杀伤STE阴性细胞(0,0)。在(1,0)状态中的杀伤与在(0,0)状态中一样低。增加GAD表达、GAD结合位点的数目可以进一步增强(1,1)状态的效力。

[0067] 图26.合成的肿瘤特异性启动子比天然启动子显示出更高的肿瘤特异性。(A)上栏举例说明了合成的肿瘤特异性启动子的设计。在最小启动子(晚期腺病毒启动子)的上游串联克隆了16个转录因子结合位点。下栏示出了合成的肿瘤特异性启动子比天然启动子显示出更高的肿瘤特异性。H2A1p是一种天然的肿瘤特异性启动子。S9至S19是合成的启动子的选择性实例,括号表示它们的转录因子结合位点。OVCAR8:卵巢癌细胞。IOSE120,IOSE386:永生化的正常卵巢上皮细胞。aHDF:成人真皮成纤维细胞。CCD:正常结肠成纤维细胞。MCF10A,MCF12A:永生化的正常乳腺细胞。(B)上栏示出了合成的肿瘤特异性启动子的设计。在最小启动子(晚期腺病毒启动子)的上游串联克隆了16个转录因子结合位点。下栏示出了合成的肿瘤特异性启动子比天然特异性启动子显示出更高的肿瘤特异性。SSX1和H2A1p是天然的肿瘤特异性启动子。S9至S28是合成的启动子的选择性实例,括号表示它们的转录因子结合位点。aHDF:成人真皮成纤维细胞。HOV-epi:原发性卵巢上皮细胞。OVCAR8:卵巢癌细胞。

[0068] 图27.多输出AND门在肿瘤细胞中比在正常细胞中显示出显著更高的输出水平。上图描绘的回路在肿瘤细胞(OVCAR8)中比在正常细胞(ISOE120)中显示出约90倍更高的活性。

[0069] 图28.多输出AND门在肿瘤细胞中比在正常细胞中显示出显著更高的输出水平。当两种启动子均有活性时,G8-F回路在肿瘤细胞(OVCAR8)中比在正常细胞(ISOE120)中显示出约90倍更高的活性。G8-F门的输出水平也高于输入启动子活性水平。

[0070] 图29.可以通过修改GAD启动子中GAD结合位点的数目并调整下游输出转录物上的miRNA结合位点的数目来调节肿瘤细胞上回路的输出水平。G8-F门的输出也高于输入启动子(S19p)活性。

[0071] 图30.多输出AND门在肿瘤细胞中比在正常细胞中显示出显著更高的输出水平。当

两种启动子均有活性时，G8-F回路在肿瘤细胞(OVCAR8)中比在正常细胞(ISOE120)中显示约90倍更高的活性。G8-F门的输出也高于输入启动子活性。

[0072] 图31.多输出回路特异性地触发T细胞以杀伤肿瘤细胞并分泌IFN-g。(A)STE触发对回路转导的肿瘤细胞(OVCAR8)而非正常细胞(aHDF，HOV-epi)的稳健的T细胞杀伤。回路还在状态(1，0)下触发最小的肿瘤杀伤。(B)STE触发对回路转导的肿瘤细胞(OVCAR8)而非正常细胞(aHDF，HOV-epi)的稳健的T细胞杀伤。回路还在状态(1，0)下触发最小的肿瘤杀伤。(C)T细胞介导由经回路转导的肿瘤细胞(而非正常细胞)产生的强力的IFN-g分泌。

[0073] 图32.不同的多输出回路显示出不同的抗肿瘤特异性水平。G8-Fv1和G14-Fv1触发相比于对正常细胞(IOSE386)杀伤显著更高的对肿瘤细胞(OVCAR8)杀伤。G8(含有8个GAL4结合位点的启动子)，G14(含有14个GAL4结合位点的启动子)。

[0074] 图33.不同的多输出回路显示出不同的抗肿瘤特异性水平。(图33A)几种门设计(G5-Fv1、G8-Fv1、G14-Fv1、G5-Fv2、G8-Fv2、G14-Fv2)对肿瘤细胞(OVCAR8)相比于正常细胞(IOSE386)可以触发T细胞的显著更高的IFN-g分泌。(图33B)G8-F门触发T细胞针对肿瘤细胞(OVCAR8)而非正常细胞(aHDF，HOV-epi)分泌大量的IFN-g。

[0075] 图34.STE在体内有力地降低胰腺肿瘤负荷。经皮下注射展示多西环素(Dox)诱导型STE的NB508肿瘤细胞。接种10天后，将小鼠随机分到Dox诱导的或未处理的组中。上栏：相对于未处理的对照(nt)，在Dox诱导的肿瘤(+Dox)中观察到显著的生长降低。由于皮肤刺激性，在第17天过早地处死两只+Dox小鼠。下图：在治疗后第21天解剖的所有肿瘤明显较小。

[0076] 图35.由STRICT触发的组合免疫治疗显著降低了腹膜内播散性卵巢癌模型中的肿瘤负荷。(A)实验计划和治疗时间表。(B)由STRICT触发的组合治疗显著降低肿瘤负荷。左栏表示对照组的肿瘤负荷。右栏表示处理组的肿瘤负荷。括号表示组合治疗策略。

[0077] 图36.由STRICT触发的组合免疫治疗显著降低了腹膜内播散性卵巢癌模型中的肿瘤负荷。这与图35中的数据相同，但是现在所有的组都绘制在相同的图中。

[0078] 图37.由STRICT触发的组合免疫治疗显著降低了腹膜内播散性卵巢癌模型中的肿瘤负荷。这与图35中的数据相同，但绘制方式不同。

[0079] 图38.由STRICT触发的组合免疫治疗显著降低了腹膜内播散性卵巢癌模型中的肿瘤负荷。这与图35中的数据相同，但绘制方式不同。

[0080] 图39A.由STRICT触发的组合免疫治疗显著降低了腹膜内播散性卵巢癌模型中的肿瘤负荷。这与图35的数据相同，但是示出了单个小鼠的肿瘤生长曲线，以及每组的平均负荷。

[0081] 图40.由STRICT触发的组合免疫治疗显著降低了腹膜内播散性卵巢癌模型中的肿瘤负荷。这与图35的数据相同，除了S15p-GAD+G8p-STE-F组未示出之外；示出了各成像时间点的肿瘤负荷和各组的平均负荷。G8p(含有8个GAL4结合位点的启动子)。

[0082] 图41.由STRICT触发的组合免疫治疗显著降低了腹膜内播散性卵巢癌模型中的肿瘤负荷。这与图35的数据相同，但是现在示出了在肿瘤接种后第36天每只小鼠的肿瘤负荷的生物发光图像。

[0083] 图42.由STRICT触发的组合免疫治疗显著降低了腹膜内播散性卵巢癌模型中的肿瘤负荷。这与图35的数据相同，但是现在示出了在肿瘤接种后第43天每只小鼠的肿瘤负荷的生物发光图像。

[0084] 图43.由STRICT触发的组合免疫治疗显著降低了腹膜内播散性卵巢癌模型中的肿瘤负荷。这与图35的数据相同，但是现在示出了在肿瘤接种后第7天和第43天每只小鼠的肿瘤负荷的生物发光图像。

[0085] 图44.鉴定癌症特异性的合成启动子的流水线。将驱动mKate2表达的合成启动子文库引入正常细胞和具有慢病毒的癌细胞中。分选mKate2阳性细胞，并利用下一代测序来鉴定每种细胞类型的富集的合成启动子序列。克隆在癌细胞中而非正常细胞中的高度富集的合成启动子序列，并且将进一步验证肿瘤特异性活性。

[0086] 图45.合成的启动子文库的设计。设计1由串联(12次重复)构建的8聚体序列的所有排列构成，在每个8聚体(mer)之间没有间隔物。设计2由串联(9次重复)构建的8聚体序列的所有排列构成，在每个8聚体之间具有3聚体间隔物。设计3由串联(7次重复)构建的选择性11聚体序列构成，每个11聚体之间没有3聚体间隔物。

[0087] 图46.所选择的合成的启动子的活性。在3种不同的癌细胞系上测试了从FACS分选分离的40种合成的启动子的活性。我们观察到，这40种合成的启动子可以为我们提供广泛的转录活性。

[0088] 图47.所选择的合成的启动子的归一化活性。在3种不同的癌细胞系上测试从FACS分选分离的40种合成的启动子的活性。我们观察到，这40种合成的启动子可以为我们提供广泛的转录活性。对于每个细胞系，将数据相对于组成型启动子(UbCp)进行归一化。

[0089] 发明详述

[0090] 本公开内容的合成的肿瘤募集免疫细胞治疗(STRICT)包括在一些实施方案中具有组合免疫调节输出(例如，抗原和细胞因子)的细胞特异性诊断和治疗回路(工程改造的遗传回路/逻辑门)。细胞特异性遗传回路主要基于RNA，因此通常不会在对象中引起不利的免疫原性反应。组合免疫调节输出可包括例如，合成的T细胞衔接器(STE)、双向T细胞衔接器(BiTE)、抗体、抗体片段、细胞因子和引发细胞毒性T细胞应答的其他分子。

[0091] 在本公开内容的一些方面中，GAL4门能够调节多输出组合治疗。对于有效的组合治疗，可以实施另外的关键免疫调节剂(作为回路输出)。在一些实施方案中，细胞因子可用于增强免疫细胞功能；例如，IL-12可用于增强Th1应答并使得回归至抑制性肿瘤微环境。在一些实施方案中，趋化因子可用于募集免疫细胞；例如，CCL21可用于募集CCR7+T细胞群。在一些实施方案中，免疫检查点阻断抑制剂可用于增强抗癌免疫性(例如抗PD1 mAb、抗PDL1 mAb和抗CTLA4mAb)。

[0092] 此外，在一些实施方案中，抗HER2 BiTE触发T细胞以介导稳健的HER2+肿瘤杀伤和细胞因子产生。在一些实施方案中，多种STE可触发对多种类型的肿瘤细胞的T细胞杀伤。在一些实施方案中，可利用RNA AND门构造来微调STE表达水平和T细胞肿瘤杀伤效率。在一些实施方案中，在整个肿瘤群体中低比例的BiTE分泌细胞足以触发稳健的肿瘤杀伤。

[0093] 如图2A和2B所示，本文中提供的方法导致癌细胞的靶向破坏。例如，首先通过局部注射或全身性施用将肿瘤鉴定遗传回路引入肿瘤中(图2A(1)和2B(1))。然后，以遗传回路转导的肿瘤细胞展示表面T细胞衔接器(STE)并表达免疫调节分子(图2A(2))。STE接合局部肿瘤浸润性T细胞上的T细胞受体并触发T细胞以根除肿瘤细胞(图2A(3))。然后通过第一波根除释放的肿瘤抗原致敏并募集更多的肿瘤反应性T细胞(图2A(4))。新募集的多克隆T细胞根除更多的癌细胞，包括没有被第一波抗肿瘤免疫应答根除的其他异质肿瘤细胞和转移

瘤(图2A(5))。免疫记忆防止肿瘤复发。

[0094] 图3A-3D描绘了基于RNA的逻辑AND门。基于RNA的逻辑AND门整合了两个输入启动子P1和P2的活性,并且只有当两个启动子都明确有活性时才产生并输出。在这个构造中,输出是表面T细胞衔接器(STE)。启动子P1调节包含合成miRNA内含子的STE mRNA(mirFF4)的表达。通过在STE/mirFF4转录物(mirFF4-BS)的3'端编码完全匹配的mirFF4结合位点,将负向自动调节反馈环并入回路中。因此,当仅启动子P1有活性时,STE mRNA被细胞miRNA机制不断地降解,并且不产生STE蛋白(图3C,状态3)。启动子P2调节miRNA海绵的表达,所述miRNA海绵在3'端含有具有多个凸起的mirFF4结合位点的非编码RNA(诱饵)。因此,当仅启动子P2有活性时,不产生蛋白质输出(图3B,状态2)。当启动子P1和P2都具有活性时,由启动子P1调节的STE/mirFF4mRNA产生的mirFF4被由启动子P2调节的mirFF4海绵所拉出(tittered out),因此允许产生STE蛋白(图3A,状态1)。

[0095] 本公开内容的一些实施方案提供了工程改造的遗传回路,所述工程改造的遗传回路包含(a)第一核酸,其包含与以下可操作地连接的第一启动子:(i)编码含有内含子微小RNA(miRNA)的输出信使RNA(mRNA)的核苷酸序列和ii)编码与(a)(i)的miRNA互补的至少一个miRNA结合位点的核苷酸序列,以及(b)第二核酸,其包含与第一启动子不同并且与以下可操作地连接的第二启动子:编码与(a)(i)的miRNA互补的至少一个miRNA结合位点的核苷酸序列。

[0096] 在一些实施方案中,输出mRNA编码与T细胞表面标志物结合的输出蛋白。例如,输出蛋白可以是引发细胞毒性T细胞应答的蛋白。因此,输出蛋白可以是与T细胞表面上的抗原(例如,CD3抗原)结合的受体。表面标志物可以是例如CD3、CD4、CD8或CD45。其他T细胞表面标志物包括在本公开内容中。在一些实施方案中,输出蛋白是与T细胞表面抗原特异性结合的抗体或抗体片段。

[0097] 输出蛋白的具体非限制性实例描绘于图12中。"STE v1"包括用于触发T细胞的抗CD3ε scFV V_L和V_H结构域。因此,在一些实施方案中,遗传回路的第一核酸包含与编码输出信使RNA(mRNA)(含有内含子微小RNA(miRNA))的核苷酸序列可操作地连接的第一启动子,所述输出信使RNA(mRNA)编码跨膜蛋白的抗CD3ε scFV V_L和V_H结构域。"STE v2"包括与人IgG1-铰链-CH2-CH3结构域,之后是鼠B7.1-跨膜(TM)和细胞质(CYP)结构域融合的抗CD3e scFv。因此,在一些实施方案中,遗传回路的第一核酸包含与编码输出信使RNA(mRNA)(含有内含子微小RNA(miRNA))的核苷酸序列可操作地连接的第一启动子,所述输出信使RNA编码与人IgG1-铰链-CH2-CH3结构域、之后是鼠B7.1-跨膜和细胞质结构域融合的抗CD3e scFv。

[0098] 在一些实施方案中,输出mRNA编码趋化因子、细胞因子或检查点抑制剂。

[0099] 在一些实施方案中,第一启动子和/或第二启动子是诱导型启动子。通常,第一启动子不同于第二启动子。例如,在一些实施方案中,遗传回路中的启动子可通过存在于细胞中的不同输入信号(例如,不同的转录因子)来调节(输入1调节第一启动子,输入2调节第二启动子)。

[0100] 第一和/或第二启动子(第一启动子、第二启动子或两种启动子共同)可以是肿瘤特异性启动子(或疾病特异性启动子),这意味着它们受仅由肿瘤细胞或癌症细胞(或其他疾病细胞)表达的信号所调节或者由在肿瘤/癌细胞中表达的水平比在非肿瘤/非癌细胞中表达的水平高至少30%(例如至少40%、50%、60%、70%、80%、90%)的信号所调节。

[0101] 如本文中所提供的遗传回路的工程改造的核酸可包括miRNA结合位点。miRNA结合位点是miRNA结合的核苷酸序列(miRNA结合位点与miRNA互补)。因此,miRNA被认为与其同源的miRNA结合位点结合。工程改造的核酸可含有1至50个mirRNA结合位点。在一些实施方案中,编码诱饵分子(其功能是"吸收(soaked up)"细胞中的同源miRNA)的工程改造的核酸编码5至10个、5至20个或5至30个miRNA结合位点。在一些实施方案中,编码诱饵分子的工程改造的核酸编码5、6、7、8、9、10、11、12、13、14、15、16、17、18、19或20个mRNA结合位点。在一些实施方案中,编码输出mRNA(例如,STE mRNA)的工程改造的核酸编码1至5或1至10个miRNA结合位点。在一些实施方案中,编码输出mRNA的工程改造的核酸编码1、2、3、4、5、6、7、8、9或10个mRNA结合位点。通常,编码免疫调节分子的mRNA上的miRNA结合位点的数目少于诱饵RNA(例如,与编码miRNA结合位点的核酸可操作地连接的启动子,以及任选的非编码mRNA)上的miRNA结合位点的数目。miRNA的长度可能不同,并且因此同源mRNA结合位点可能不同。在一些实施方案中,miRNA的长度是15至50、15至40、15至30或15至20个核苷酸。在一些实施方案中,miRNA的长度是15、16、17、18、19、20、21、22、23、24、25、26、27、28、29或30个核苷酸。

[0102] 在一些实施方案中,输出蛋白是转录因子(例如,与DNA结合以控制转录速率的蛋白质)。

[0103] 工程改造的核酸和遗传回路

[0104] 本公开内容提供了能够从肿瘤/癌细胞内触发针对所述肿瘤/癌细胞和周围癌细胞的免疫治疗的工程改造的遗传回路。"遗传回路"是指在细胞中彼此相互作用以控制mRNA和蛋白质的表达的分子(例如,核酸和蛋白质,例如转录因子、辅因子和聚合酶)的集合。如本文中提供的遗传回路通常包括至少两种核酸,一种编码含有内含子微小RNA(miRNA)的输出信使RNA(mRNA),另一种编码数个miRNA结合位点。"内含子miRNA"是位于共同编码输出分子的两个外显子之间的mRNA转录物内的miRNA。内含子miRNA在转录物成熟过程中被"剪接出"mRNA转录物。例如,参考图3A,"STE-EX1-mirFF4-STE-EX2"(顶行)表示编码位于编码合成的T细胞衔接器(STE)的基因的两个外显子之间的微小RNA mirFF4的DNA序列。图3第二行中的构建体表示编码STE的mRNA转录物,其经历成熟,从而通过RNA剪接去除内含子微小RNA mirFF4。然后编码STE的成熟mRNA可被翻译以产生STE蛋白,这取决于诱饵分子(含有同源mirFF4结合位点的分子)是否存在于细胞中。

[0105] 因此,"输出信使RNA"或"输出mRNA"仅指由工程改造的核酸的特定核苷酸序列编码的mRNA。在一些实施方案中,输出mRNA(通常包括内含子微小RNA)编码与T细胞表面标志物结合的输出蛋白。在一些实施方案中,输出mRNA编码抗癌剂。"抗癌"剂是当暴露于癌细胞时可用于杀伤癌细胞或降低癌细胞的细胞分裂速率(例如至少10%、20%、30%、40%或50%)的任何物质或分子。在一些实施方案中,输出mRNA编码杀伤基因、新抗原(neoantigen)、降解代谢物(癌细胞依赖其生长和/或存活)的代谢酶、趋化因子、细胞因子或检查点抑制剂,如本文中其他地方所讨论的。其他抗癌剂包括在本公开内容中。

[0106] 本公开内容的遗传回路还可被称为"逻辑门"或起到"逻辑门"的作用,其通常具有两个输入和一个输出,尽管本公开内容也包括更多或更少的输入和/或输出。逻辑门(例如AND、OR、XOR、NOT、NAND、NOR和XNOR)可以根据"打开(ON)"状态(其中产生输出)和"关闭(OFF)"状态(其中不产生输出)来描述。对于遗传逻辑门,每个"输入"可以由独立的启动子

调节，每个启动子负责活化编码输出或调节输出分子的产生和/或表达水平的分子的核酸的转录。例如，图3A-3D描绘了AND逻辑门，包括两个构建体的遗传回路：一个由启动子P1调节，另一个由启动子P2调节。在左侧与P1连接的构建体的转录在输入1的存在下被活化，而在右侧与P2连接的构建体的转录在输入2的存在下被活化。对于该AND门，输出分子STE蛋白仅在输入1和输入2的存在下产生(图3A)。在仅输入2存在(图3B)或仅输入1存在(图3C)的情况下，不产生STE蛋白。同样，如果输入1和输入2都不可用，则不产生STE蛋白(图3D)。在仅输入1存在的情况下，产生STE mRNA转录物；然而，切除的内含子miRNA的存在阻止了STE mRNA的翻译和STE蛋白的产生(图3C)。在输入1和输入2两者都存在的情况下，仍然产生STE mRNA转录物和切除的内含子miRNA二者；然而，经切除的内含子miRNA被诱饵miRNA结合位点“吸收”，其转录被输入2活化。因此，大部分STE mRNA不含结合的miRNA并可被翻译以产生STE蛋白。

[0107] 其他逻辑门描绘于图7B-7H中。

[0108] 图7B描绘了包含以下的逻辑门：(a)第一核酸，其包含与以下可操作地连接的启动子(P1)：(i)编码含有内含子miRNA(miRNA1)的输出mRNA(OP-EX1---OB-EX2)的核苷酸序列：(ii)编码内含子miRNA(miRNA3)的核苷酸序列，和(iii)编码miRNA结合位点(miRNA2-BS(P))的核苷酸序列；(b)第二核酸，其包含与以下可操作地连接的启动子(P2)：(i)编码含有内含子miRNA(miRNA2)的输出mRNA(OP-EX1---OB-EX2)的核苷酸序列，(ii)编码内含子miRNA(miRNA3)的核苷酸序列，和(iii)编码miRNA-BS(mirRNA1-BS(P))的核苷酸序列；以及(c)第三核酸，其包含与编码输出蛋白(OP)的核苷酸序列可操作地连接的启动子(Ps)，所述输出蛋白(OP)与miRNA-BS(miRNA3-BS(P))连接，其中miRNA1与miRNA1-BS互补并结合，miRNA3与miRNA3-BS(P)互补并结合，并且miRNA2与miRNA2-BS(P)互补并结合。

[0109] 图7C描绘了包含以下的逻辑门：(a)第一核酸，其包含与以下可操作地连接的启动子(P1)：(i)编码含有内含子微小RNA(miRNA1)的新生RNA转录物(Nan-EX1---Nan-EX2)(例如，非编码RNA转录物或/和编码蛋白质的RNA转录物)的核苷酸序列和(ii)编码四个miRNA结合位点(miRNA2-BS(Bx4))的核苷酸序列；(b)第二核酸，其包含与以下可操作地连接的启动子(P2)：(i)编码含有内含子miRNA(miRNA2)的新生RNA转录物(Nan-EX1---Nan-EX2)的核苷酸序列，和(ii)编码四个miRNA结合位点(miRNA1-BS(Bx4)的核苷酸序列；以及(c)第三核酸，其包含与编码输出蛋白(OP)的核酸可操作地连接的启动子(Ps)：所述输出蛋白(OP)与(i)第一miRNA结合位点(miRNA1-BS(P))和(ii)第二miRNA结合位点(miRNA2-BS(P))连接，其中miRNA1与miRNA1-BS(Bx4)互补并结合，并且miRNA2与miRNA2-BS(Bx4)互补并结合。

[0110] 图7D描绘了包含以下的逻辑门：(a)第一核酸，其包含与编码含有内含子miRNA(miRNA1)的新生RNA转录物(Nan-EX1---Nan-EX2)的核苷酸序列可操作地连接的启动子(P1)；(b)第二核酸，其包含与编码含有内含子miRNA(miRNA2)的新生RNA转录物(Nan-EX1-Nan-EX2)的核苷酸序列可操作地连接的启动子(P2)；以及(c)第三核酸，其包含与编码输出蛋白(OP)的核酸可操作地连接的启动子(P)，所述输出蛋白与(i)第一miRNA结合位点(miRNA1-BS(P))、(ii)第二miRNA结合位点(miRNA2-BS(P))连接，其中miRNA1与miRNA1-BS(P)互补并结合，并且miRNA2与miRNA2-BS互补并结合。

[0111] 图7E描绘了包含以下的逻辑门：(a)第一核酸，其包含与编码含有内含子微小RNA(miRNA)的新生RNA转录物(Nan-EX1---Nan-EX2)的核苷酸序列可操作地连接的启动子

(P1)；和(b)第二核酸，其包含与编码输出蛋白(OP)的核苷酸序列可操作地连接的启动子(Ps)，所述输出蛋白与miRNA结合位点(miRNA1-BS(P))连接，其中miRNAI与miRNA-BS(P)互补并结合。

[0112] 图7F描绘了包含以下的逻辑门：(a)第一核酸，其包含与以下可操作地连接的启动子(P1)：(i)编码含有内含子miRNA(miRNA1)的输出mRNA(OP-EX1---OP-EX2)的核苷酸序列和(ii)四个miRNA结合位点(miRNA2-BS(Bx4))；和(b)第二核酸，其包含与以下可操作地连接的启动子(P2)：(i)编码含有内含子miRNA(miRNA2)的输出mRNA(OP-EX1---OP-EX2)的核苷酸序列和(ii)四个miRNA结合位点(miRNA1-BS(Bx4))，其中miRNA1与miRNA1-BS(Bx4)互补并结合，并且miRNA2与miRNA2-BS(Bx4)互补并结合。

[0113] 图7G描绘了包含以下的逻辑门：(a)第一核酸，其包含与编码含有内含子miRNA(miRNA1)的新生RNA转录物(Nan-EX1---Nan-EX2)的核苷酸序列可操作地连接的启动子(P1)；(b)第二核酸，其包含与编码输出蛋白(OP)的核苷酸序列可操作地连接的启动子(P2)；以及(c)第三核酸，其包含编码输出蛋白(OP)的启动子(Ps)，所述输出蛋白与miRNA结合位点(miRNA1-BS(P))连接，其中miRNA1与miRNA1-BS(P)互补并结合。

[0114] 图7H描绘了包含以下的逻辑门：(a)第一核酸，其包含与编码输出蛋白(OP)的核苷酸序列可操作地连接的启动子(P1)，所述输出蛋白与miRNA结合位点(miRNA1-BS)连接；和(b)第二核酸，其包含与编码含有内含子miRNA(miRNA1)的新生RNA转录物(Nan-EX1---Nan-EX2)的核苷酸序列可操作地连接的启动子(P2)，其中miRNA1与miRNA1-BS(P)互补并结合。

[0115] “核酸”是共价连接在一起的至少两个核苷酸，并且在一些情况下，可含有磷酸二酯键(例如，磷酸二酯“骨架”)。“工程改造的核酸”(也称为“构建体”)是不天然存在的核酸。然而，应理解，尽管工程改造的核酸作为整体是不天然存在的，但其可包括天然存在的核苷酸序列。在一些实施方案中，工程改造的核酸包含来自不同生物体(例如，来自不同物种)的核苷酸序列。例如，在一些实施方案中，工程改造的核酸包括鼠核苷酸序列、细菌核苷酸序列、人核苷酸序列和/或病毒核苷酸序列。工程改造的核酸包括重组核酸和合成核酸。“重组核酸”是通过连接核酸(例如，分离的核酸、合成的核酸或其组合)而构建的分子，并且在一些实施方案中，其可以在活细胞中复制。“合成核酸”是通过扩增的或经化学或通过其他手段合成的分子。合成的核酸包括经化学修饰或以其他方式修饰，但可以与天然存在的核酸分子碱基配对的的那些。重组核酸和合成核酸还包括由前述任一种的复制产生的那些分子。

[0116] 在一些实施方案中，认为本公开内容的核酸是核酸类似物，其可以至少部分包含其他骨架，所述其他骨架包括例如磷酰胺、硫代磷酸酯、二硫代磷酸酯、O-甲基亚磷酰胺键和/或肽核酸。根据指定，核酸可以是单链(single-stranded，ss)或双链(double-stranded，ds)，或者可含有单链和双链序列二者的一部分。在一些实施方案中，核酸可含有三链序列的一部分。核酸可以是DNA(基因组DNA和/或cDNA、RNA或杂合体二者)，其中核酸含有脱氧核糖核苷酸和核糖核苷酸(例如，人造或天然的)的任何组合，以及碱基的任何组合，包括尿嘧啶、腺嘌呤、胸腺嘧啶、胞嘧啶、鸟嘌呤、肌苷、黄嘌呤、次黄嘌呤、异胞嘧啶和异鸟嘌呤。

[0117] 本公开内容的核酸可包括一个或更多个遗传元件。“遗传元件”是指在核酸表达中具有作用的特定核苷酸序列(例如启动子、增强子、终止子)或编码工程改造的核酸的离散

产物的特定核苷酸序列(例如,编码引导RNA、蛋白质和/或RNA干扰分子(如siRNA或miRNA)的核苷酸序列)。

[0118] 可使用标准分子生物学方法(参见,例如Green and Sambrook,Molecular Cloning,A Laboratory Manual,2012,Cold Spring Harbor Press)生产本公开内容的核酸。

[0119] 在一些实施方案中,使用GIBSON ASSEMBLY®克隆(参见,例如Gibson,D.G.等.Nature Methods,343-345,2009;和Gibson,D.G.等.Nature Methods,901-903,2010,其各自通过引入并入本文)生产核酸。GIBSON ASSEMBLY®通常在单管反应中使用三种酶活性:5'外切核酸酶、DNA聚合酶的3'延伸活性和DNA连接酶活性。5'外切核酸酶活性消化(chews back)5,末端序列并暴露出用于退火的互补序列。然后聚合酶活性填补退火区域的缺口。然后DNA连接酶封闭切口并将DNA片段共价连接在一起。邻接片段的重叠序列比在Golden Gate组装中使用的序列长得多,因此导致更高百分比的正确组装。在一些实施方案中,在载体上将工程改造的核酸递送至细胞。"载体"是指用作人工携带遗传物质(例如,工程改造的核酸)到细胞中(在那里其可以例如复制和/或表达)的载体的核酸(例如DNA)。在一些实施方案中,载体是附加体型载体(episomal vector)(参见,例如,Van Craenenbroeck K.等.Eur.J.Biochem.267,5665,2000,其通过引用并入本文)。载体的非限制性实例是质粒(例如,图3)。质粒是能够在宿主细胞中自主复制的双链的通常为环状的DNA序列。质粒载体通常含有允许质粒在宿主中进行半独立复制的复制起点,并且还包含转基因插入物。质粒可具有更多特征,包括例如"多克隆位点",其包括用于插入核酸插入物的核苷酸突出端和插入物两侧的多个限制性酶共有位点。载体的另一个非限制性实例是病毒载体。

[0120] 因此,在一些实施方案中,使用病毒递送系统(例如,逆转录病毒、腺病毒、腺相关、辅助依赖性腺病毒系统、混合型腺病毒系统、单纯疱疹、痘病毒、慢病毒、Epstein-Barr病毒)或非病毒递送系统(例如,物理的:裸DNA、DNA轰击、电穿孔、流体动力学、超声波或磁转染;或化学的:阳离子脂质、不同阳离子聚合物或脂质聚合物)(Nayerossadat N等.Adv Biomed Res.2012;1:27,其通过引用并入本文)将工程改造的遗传回路递送至细胞(例如,癌细胞)。在一些实施方案中,基于非病毒的递送系统是基于水凝胶的递送系统(参见,例如,Brandl F等,Brandl F,等.Journal of Controlled Release,2010,142(2):221-228,其通过引用并入本文)。

[0121] 微小RNA("miRNA")是在植物、动物和一些病毒中发现的小的非编码RNA分子(例如,含有约22个核苷酸),其通常在野生型条件下在RNA沉默和基因表达的转录后调节中发挥作用。

[0122] 遗传元件

[0123] 工程改造的核酸的表达由与核酸(其含有例如编码目的分子的核酸)可操作地连接的启动子驱动。"启动子"是指核酸序列的控制区域,在该处控制核酸序列的其余部分之转录的起始和速率。启动子驱动所调节的核酸序列的表达或驱动其转录。启动子还可含有调节蛋白和分子可结合的亚区,例如RNA聚合酶和其他转录因子。启动子可以是组成型的、诱导型的、可活化的、可阻抑的、组织特异性的或其任何组合。

[0124] 本文中,当启动子相对于其调节以控制("驱动")该序列的转录起始和/或表达的

核酸序列处于正确的功能位置和方向时，则认为启动子是“可操作地连接的”。

[0125] 启动子可以是与基因或序列天然缔合的启动子，如可通过分离位于给定基因或序列的编码区段上游的5’非编码序列来获得的。这样的启动子可被称为“内源的”。

[0126] 在一些实施方案中，编码核酸序列可位于重组或异源启动子的控制下，所述启动子是指在其天然环境中通常不与编码序列缔合的启动子。这样的启动子可包括其他基因的启动子；从任何其他细胞分离的启动子；以及不是“天然存在”的合成的启动子或增强子，例如，含有不同转录调节区的不同元件和/或通过本领域已知的遗传工程方法改变表达的突变的那些。除了经合成产生启动子和增强子的核酸序列之外，可以使用重组克隆和/或核酸扩增技术(包括聚合酶链式反应(PCR)(参见，美国专利No.4,683,202和美国专利No.5,928,906)来产生序列。

[0127] 在一些实施方案中，启动子是“诱导型启动子”，其是指特征在于当诱导物信号存在、受其影响或与其接触时调节(例如启动或活化)转录活性的启动子。诱导物信号可以是内源的或正常的外源条件(例如，光)、化合物(例如，化学或非化学化合物)或蛋白质，其以这样的方式与诱导型启动子接触，从而具有活性以从诱导型启动子调节转录活性。因此，核酸的“调节转录的信号”是指作用于诱导型启动子的诱导物信号。调节转录的信号可以使转录活化或失活，这取决于所使用的调节系统。转录的活化可涉及直接作用于启动子以驱动转录，或通过使阻遏物(其阻止启动子驱动转录)失活而间接作用于启动子。相反地，转录的失活可能涉及直接作用于启动子以阻止转录或通过活化阻遏物(阻遏物然后作用于启动子)而间接作用于启动子。

[0128] 诱导物信号的施加或去除导致对可操作地连接的核酸序列的转录的活化和失活之间的转换。因此，与核酸序列可操作地连接的启动子的活性状态是指启动子主动调节核酸序列转录时(即，表达连接的核酸序列)的状态。相反，与核酸序列可操作地连接的启动子的失活状态是指启动子不主动调节核酸序列的转录时(即，不表达连接的核酸序列)的状态。

[0129] 本公开内容的诱导型启动子可通过一种或更多种生理条件，例如光、pH、温度、辐射、渗透压、生理盐水梯度、细胞表面结合以及一种或更多种外在或内在诱导剂的浓度的变化来诱导(或抑制)。外来诱导物信号或诱导剂可包括但不限于：氨基酸和氨基酸类似物、糖和多糖、核酸、蛋白质转录活化剂和阻遏物、细胞因子、毒素、基于石油的化合物、含金属化合物、盐、离子、酶底物类似物、激素或其组合。

[0130] 本公开内容的诱导型启动子包括本文中描述的或本领域普通技术人员已知的任何诱导型启动子。诱导型启动子的实例包括但不限于：化学/生物化学调节的和物理调节的启动子，例如醇调节启动子、四环素调节启动子(例如，脱水四环素(aTc)响应启动子和其他四环素响应启动子系统，其包括四环素阻遏物蛋白(tetR)、四环素操纵子基因序列(tetO)和四环素反式活化因子融合蛋白(tTA))、甾类化合物调节的启动子(例如，基于大鼠糖皮质激素受体的启动子、人雌激素受体、蜕皮激素受体以及来自甾类化合物/类维生素A/甲状腺受体超家族的启动子)、金属调节的启动子(例如衍生自酵母、小鼠和人的金属硫蛋白(结合并螯合金属离子的蛋白)基因的启动子)，发病原调节的启动子(通过水杨酸、乙烯或苯并噻二唑(BTH)诱导的)、温度/热诱导型启动子(例如，热激启动子)和光调节的启动子(例如，来自植物细胞的光响应启动子)。

[0131] 在一些实施方案中，本公开内容的诱导物信号是异丙基β-D-1-硫代吡喃半乳糖苷(IPTG)，其是异乳糖的分子模拟物、一种触发lac操纵子的转录的乳糖代谢物，因此其用于诱导蛋白质(其中基因在lac操纵子的控制下)的表达。IPTG与lac阻遏物结合并以变构方式从lac操纵子释放四聚体阻遏物，由此允许转录lac操纵子中的基因，例如编码β-半乳糖苷酶(一种催化β半乳糖苷转化成单糖的水解酶)的基因。硫(S)原子产生不能被细胞水解的化学键，防止细胞代谢或降解诱导物。IPTG是蛋白质表达的有效诱导剂，例如，在100μM至1.0mM的浓度范围。使用的浓度取决于所需诱导的强度，以及使用的细胞或质粒的基因型。如果存在lacIq(一种过度产生lac阻遏物的突变体)，则可能需要更高浓度的IPTG。在蓝白筛选中，IPTG与X-gal一起使用。蓝白筛选允许在克隆实验中鉴定已用重组质粒而不是以非重组质粒转化的克隆。

[0132] 其他诱导型启动子系统是本领域已知的并且可以根据本公开内容使用。

[0133] 免疫调节剂

[0134] 免疫调节剂是调节免疫应答的物质(例如，蛋白质)。在一些实施方案中，本公开内容提供了工程改造的遗传回路，其包括编码在癌细胞表面表达或由癌细胞分泌或从癌细胞分泌的免疫调节剂的核酸。

[0135] 在一些实施方案中，免疫调节剂是合成的T细胞衔接器(STE)。“合成的T细胞接合器”是与T细胞(例如，细胞毒性T细胞)表面上的分子结合(例如，通过配体-受体结合相互作用)或以其他方式引发细胞毒性T细胞应答的分子(例如，蛋白质)。在一些实施方案中，STE是与T细胞表面上的配体结合的受体。在一些实施方案中，STE是抗CD3抗体或抗体片段。本公开内容的STE通常在癌细胞或其他疾病细胞的表面上表达或由癌细胞或其他疾病细胞分泌(编码STE的核酸被递送至所述癌细胞或其他疾病细胞)。

[0136] 本公开内容的STE的实例包括与T细胞表面抗原结合的抗体、抗体片段和受体。T细胞表面抗原包括例如CD3、CD4、CD8和CD45。由本公开内容的遗传回路表达的STE也可选自下文所述的任何免疫调节剂。

[0137] 在一些实施方案中，本公开内容的遗传回路调节趋化因子、细胞因子或检查点抑制剂的表达。

[0138] 免疫调节剂包括免疫刺激剂和免疫抑制剂。如本文中使用的免疫刺激剂是在其施用的对象中刺激免疫应答(包括增强预先存在的免疫应答)的试剂，无论其是单独还是与另一种试剂组合。实例包括抗原、佐剂(例如，TLR配体例如咪喹莫特、咪唑并喹啉，包含未甲基化CpG二核苷酸的核酸、单磷酰脂质A或其他脂多糖衍生物、单链或双链RNA、鞭毛蛋白、胞壁酰二肽)、细胞因子包括白细胞介素(例如，IL-2、IL-7、IL-15/或这些细胞因子的超激动剂/突变体形式)、IL-12、IFN-γ、IFN-α、GM-CSF、FLT3-配体等)、免疫刺激抗体(例如，这些分子的抗CTLA-4、抗CD28、抗CD3或单链/抗体片段)等。

[0139] 如本文中使用的免疫抑制剂是在其施用的对象中抑制免疫应答的试剂，无论是单独施用还是与其他试剂组合施用。实例包括甾类化合物、视黄酸、地塞米松、环磷酰胺、抗CD3抗体或抗体片段以及其他免疫抑制剂。

[0140] 抗原可以是但不限于：癌抗原、自身抗原、微生物抗原、过敏原或环境抗原。抗原在性质上可以是肽、脂质或碳水化合物，但不限于此。

[0141] 癌抗原是优先由癌细胞表达的抗原(例如，在癌细胞中比在非癌细胞中以更高的

水平表达)，并且在某些情况下，其仅由癌细胞表达。癌抗原可以在癌细胞内或在癌细胞的表面上表达。癌抗原可以是MART-1/Melan-A、gp100、腺苷脱氨酶结合蛋白(ADAbp)、FAP、亲环蛋白b、结直肠相关抗原(CRC)--C017-1A/GA733、癌胚抗原(carcinoembryonic antigen，CEA)、CAP-1、CAP-2、etv6、AML1、前列腺特异性抗原(prostate specific antigen,PSA)、PSA-1、PSA-2、PSA-3、前列腺特异性膜抗原(prostate-specific membrane antigen，PSMA)、T细胞受体/CD3-ζ链和CD20。癌抗原可以选自:MAGE-A1、MAGE-A2、MAGE-A3、MAGE-A4、MAGE-A5、MAGE-A6、MAGE-A7、MAGE-A8、MAGE-A9、MAGE-A10、MAGE-A11、MAGE-A12、MAGE-Xp2(MAGE-B2)、MAGE-Xp3(MAGE-B3)、MAGE-Xp4(MAGE-B4)、MAGE-C1、MAGE-C2、MAGE-C3、MAGE-C4、MAGE-C5)。癌抗原可以选自:GAGE-1、GAGE-2、GAGE-3、GAGE-4、GAGE-5、GAGE-6、GAGE-7、GAGE-8、GAGE-9。癌抗原可以选自:BAGE、RAGE、LAGE-1、NAG、GnT-V、MUM-1、CDK4、酪氨酸酶、p53、MUC家族、HER2/neu、p21raS、RCAS1、α-胎蛋白、E-钙粘蛋白、α-连环蛋白、β-连环蛋白、γ-连环蛋白、p120ctn、gp100Pmel117、PRAME、NY-ESO-1、cdc27、腺瘤性结肠息肉蛋白(adenomatous polyposis coli protein,APC)、胞衬蛋白、连接蛋白37、Ig-独特型(idiotype)、p15、gp75、GM2神经节苷脂、GD2神经节苷脂、人乳头瘤病毒蛋白、肿瘤抗原的Smad家族、lmp-1、P1A、EBV编码的核抗原(EBNA)-1、脑糖原磷酸化酶、SSX-1、SSX-2(HOM-MEL-40)、SSX-1、SSX-4、SSX-5、SCP-1和CT-7、CD20以及c-erbB-2。

[0142] 细胞和细胞表达

[0143] 本公开内容的工程改造的遗传回路通常在特定细胞类型(例如癌细胞、良性肿瘤细胞或者其他疾病细胞)中被有条件地(基于输入信号的存在或不存在)全身性递送并活化(活化回路的转录)。因此，在一些实施方案中，遗传回路(逻辑门)被递送至具有肿瘤细胞或癌细胞的对象，并且遗传回路(逻辑门)在肿瘤细胞或癌细胞中表达。

[0144] 癌性细胞可以是任何类型的癌性细胞，包括但不限于:恶化前的赘生物(neoplasm)、恶性肿瘤、转移瘤或以不受控制的细胞生长为特征以使得其被视为癌性或癌前病变性的任何疾病或病症。癌症可以是原发性癌或转移性癌。癌症包括但不限于:眼癌、胆道癌、膀胱癌、胸膜癌、胃癌、卵巢癌、脑膜癌、肾癌、脑癌(包括成胶质细胞瘤和成神经管细胞瘤)、乳腺癌、宫颈癌、绒毛膜癌、结肠癌、子宫内膜癌、食管癌、胃癌、血液肿瘤(包括急性淋巴细胞和髓性白血病)、多发性骨髓瘤、AIDS相关白血病和成人T细胞白血病淋巴瘤、上皮内瘤(包括博文氏病和佩吉特氏病)、肝癌、肺癌、淋巴瘤(包括霍奇金病和淋巴细胞性淋巴瘤)、成神经细胞瘤、口腔癌(包括鳞状细胞癌)、卵巢癌(包括来自上皮细胞、基质细胞、生殖细胞和间充质细胞的那些)、胰腺癌、前列腺癌、直肠癌、肉瘤(包括平滑肌肉瘤、横纹肌肉瘤、脂肪肉瘤、纤维肉瘤和骨肉瘤)、皮肤癌(包括黑色素瘤、卡波西肉瘤、基底细胞癌和鳞状细胞癌)、睾丸癌(包括生殖性肿瘤例如精原细胞瘤、非精原细胞瘤)、畸胎瘤、绒毛膜癌、基质瘤和生殖细胞瘤、甲状腺癌(包括甲状腺腺癌和髓质癌)以及肾癌(包括腺癌和韦尔姆斯氏瘤)。常见的癌症包括乳腺癌、前列腺癌、肺癌、卵巢癌、结直肠癌和脑癌。在一些实施方案中、肿瘤是黑素瘤、癌、肉瘤或淋巴瘤。

[0145] 本公开内容的工程改造的核酸可以在宽范围的宿主细胞类型中表达。在一些实施方案中，工程改造的核酸在哺乳动物细胞(例如，人细胞)、细菌细胞(大肠杆菌(Escherichia coli)细胞)、酵母细胞、昆虫细胞或其他类型的细胞中表达。本公开内容的工程改造的核酸可以在对象(例如人对象)中在体内表达。

[0146] 在一些实施方案中，工程改造的核酸在哺乳动物细胞中表达。例如，在一些实施方案中，工程改造的核酸在人细胞、灵长类细胞(例如，vero细胞)、大鼠细胞(例如GH3细胞、OC23细胞)或小鼠细胞(例如，MC3T3细胞)中表达。有多种人细胞系，包括但不限于：人胚胎肾(human embryonic kidney，HEK)细胞、HeLa细胞、来自国家癌症研究所的60个癌细胞系(NCI60)的癌细胞、DU145(前列腺癌)细胞、Lncap(前列腺癌)细胞、MCF-7(乳腺癌)细胞、MDA-MB-438(乳腺癌)细胞、PC3(前列腺癌)细胞、T47D(乳腺癌)细胞、THP-1(急性骨髓性白血病)细胞、U87(胶质母细胞瘤)细胞、SHSY5Y人成神经细胞瘤细胞(从骨髓瘤克隆)和Saos-2(骨癌)细胞。在一些实施方案中，工程改造的核酸在人胚胎肾(HEK)细胞(例如，HEK 293或HEK 293T细胞)中表达。在一些实施方案中，工程改造的核酸在干细胞(例如，人干细胞)例如多能干细胞(例如，人多能干细胞，包括人诱导多能干细胞(human induced pluripotent stem cell，hiPSC))中表达。“干细胞”是指具有在培养物中无限期分裂并产生特化细胞的能力的细胞。“多能干细胞”是指能够分化成生物体的所有组织但不能够单独维持全部的生物体发育的一类干细胞。“人诱导多能干细胞”是指通过强制表达维持胚胎干细胞定义性质的重要基因和因子，从而已经重编程为胚胎干细胞样状态的体(例如，成熟或成人的)细胞(参见，例如Takahashi和Yamanaka，Cell 126(4)：663-76，2006，其通过引用并入本文)。人诱导多能干细胞表达干细胞标志物并且能够产生所有三个胚层(外胚层、内胚层、中胚层)的细胞特征。

[0147] 可以根据本公开内容使用的细胞系的另外的非限制性实例包括293-T、293-T、3T3、4T1、721、9L、A-549、A172、A20、A253、A2780、A2780ADR、A2780cis、A431、ALC、B16、B35、BCP-1、BEAS-2B、bEnd.3、BHK-21、BR 293、BxPC3、C2C12、C3H-10T1/2、C6、C6/36、Cal-27、CGR8、CHO、CML T1、CMT、COR-L23、COR-L23/5010、COR-L23/CPR、COR-L23/R23、COS-7、COV-434、CT26、D17、DH82、DU145、DuCaP、E14Tg2a、EL4、EM2、EM3、EMT6/AR1、EMT6/AR10.0、FM3、H1299、H69、HB54、HB55、HCA2、Hepa1c1c7、High Five细胞、HL-60、HMEC、HT-29、HUVEC、J558L细胞、Jurkat、JY细胞、K562细胞、KCL22、KG1、Ku812、KYO1、LNCap、Ma-Mel 1、2、3....48、MC-38、MCF-10A、MCF-7、MDA-MB-231、MDA-MB-435、MDA-MB-468、MDCK II、MG63、MONO-MAC 6、MOR/0.2R、MRC5、MTD-1A、MyEnd、NALM-1、NCI-H69/CPR、NCI-H69/LX10、NCI-H69/LX20、NCI-H69/LX4、NIH-3T3、NW-145、OPCN/OPCT Peer、PNT-1A/PNT 2、PTK2、Raji、RBL细胞、RenCa、RIN-5F、RMA/RMAS、S2、Saos-2细胞、Sf21、Sf9、SiHa、SKBR3、SKOV-3、T-47D、T2、T84、THP1、U373、U87、U937、VCaP、WM39、WT-49、X63、YAC-1和YAR细胞。

[0148] 在一些实施方案中，本公开内容的细胞是经修饰的。经修饰细胞是含有外源核酸或天然不存在的核酸的细胞。在一些实施方案中，经修饰的细胞含有在基因组核酸中的突变。在一些实施方案中，经修饰的细胞含有外源独立复制核酸(例如，存在于附加体载体上的工程改造的核酸)。在一些实施方案中，通过将外来或外源核酸引入细胞来产生经修饰的细胞。可通过常规方法将核酸引入细胞，例如电穿孔(参见，例如，Heiser W.C.Transcription Factor Protocols:Methods in Molecular Biology™2000;130:117-134，化学方法(例如，磷酸钙或脂质)转染(参见，例如，Lewis W.H.，等，Somatic Cell Genet.1980年5月;6(3)：333-47;Chen C.，等，Mol Cell Biol.1987年8月;7(8)：2745-2752)，与含有重组质粒的细菌原生质体融合(参见，例如，Schaffner W.Proc Natl Acad Sci USA.1980年4月;77(4)：2163-7)、转导、缀合或者将DNA直接显微注射到细胞的细胞核

中(参见,例如,Capecchi M.R.Cell.1980年11月;22(2Pt 2):479-88)。

[0149] 在一些实施方案中,细胞被修饰成表达报道分子。在一些实施方案中,细胞被修饰成表达与报道分子(例如,荧光蛋白,例如绿色荧光蛋白(GFP)或其他报道分子)可操作地连接的诱导型启动子。

[0150] 在一些实施方案中,细胞被修饰成过表达目的内源蛋白(例如,通过在编码目的蛋白的内源基因附近引入或修饰启动子或其他调节元件以提高其表达水平)。在一些实施方案中,通过诱变来修饰细胞。在一些实施方案中,通过将工程改造的核酸引入细胞中来修饰细胞以产生目的遗传变化(例如,通过插入或同源重组)。

[0151] 在一些实施方案中,工程改造的核酸可以是密码子优化的,例如用于在哺乳动物细胞(例如,人细胞)或其他类型的细胞中表达。密码子优化是通过将一个物种的核苷酸的DNA序列转化成另一个物种的核苷酸的DNA序列来提高目的基因的翻译效率以使得活生物体中的蛋白质表达最大化的技术。密码子优化的方法是公知的。

[0152] 本公开内容的工程改造的核酸可瞬时表达或稳定地表达。“瞬时细胞表达”是指核酸未整合到细胞的核基因组中的细胞的表达。相比之下,“稳定的细胞表达”是指核酸在细胞核基因组及其子细胞中保留的细胞的表达。通常,为了实现稳定的细胞表达,将细胞与标志物基因和旨在在细胞中稳定表达的外源核酸(例如,工程改造的核酸)共转染。标志物基因给予细胞一些可选的优点(例如,对毒素、抗生素或其他因子的抗性)。很少的经转染细胞会偶然地将外源核酸整合到其基因组中。例如,如果之后将毒素添加到细胞培养物中,那么只有少数具有毒素抗性的标志物基因整合到其基因组中的细胞才能够增殖,而其他细胞则会死亡。在施加该选择压力一段时间之后,只有具有稳定转染的细胞保留下来,并且可以进一步培养。根据本公开内容使用的标志物基因和选择剂的实例包括但不限于:二氢叶酸还原酶与甲氨蝶呤、谷氨酰胺合成酶与甲硫氨酸砜亚胺(methionine sulphoximine)、潮霉素磷酸转移酶与潮霉素、嘌呤霉素N-乙酰转移酶与嘌呤霉素,以及新霉素磷酸转移酶与遗传霉素,也被称为G418。在本文中还构想其他标志物基因/选择剂。

[0153] 在瞬时转染和/或稳定转染的细胞中核酸的表达可以是组成型或诱导型的。以上描述了如本文所提供的诱导型启动子。

[0154] 本公开内容的一些方面提供了包含1至10种工程改造的核酸的细胞。在一些实施方案中,细胞包含1、2、3、4、5、6、7、8、9、10种或更多种工程改造的核酸。应理解,“包含工程改造的核酸”的细胞是包含工程改造的核酸的拷贝(多于一个)的细胞。因此,“包含至少两种工程改造的核酸”的细胞是包含第一工程改造的核酸的拷贝和第二工程改造的核酸的拷贝的细胞,其中第一工程改造的核酸不同于第二工程改造的核酸。就例如序列组成(例如,核苷酸的类型、数目和排列)、长度或序列组成和长度的组合而言,两种工程改造的核酸可以彼此不同。例如,相同细胞中两种工程改造的核酸的SDS序列可能彼此不同。

[0155] 本公开内容的一些方面提供了包含1至10个附加体载体或更多附加体载体的细胞,每个载体包含例如工程改造的核酸。在一些实施方案中,细胞包含1、2、3、4、5、6、7、8、9、10个或更多个载体。

[0156] 在一些方面,本文中还提供了包括将(例如,至少一种、至少两种、至少三种或更多种)工程改造的核酸或附加型载体(例如,包含工程改造的核酸)引入细胞中的方法。如本文其他地方所讨论的,可以通过常规方法将工程改造的核酸引入细胞中,例如电穿孔、化学

(例如,磷酸钙或脂质)转染、与含有重组质粒的细菌原生质体融合、转导、缀合或者将纯化的DNA直接显微注射到细胞的细胞核中。

[0157] 体内递送

[0158] 可通过本领域已知的任何体内递送方法将本公开内容的工程改造的核酸递送至对象(例如,哺乳动物对象,例如人对象)。例如,工程改造的核酸可以经静脉内递送。在一些实施方案中,工程改造的核酸在递送载体(例如,非脂质体纳米颗粒或脂质体)中递送。在一些实施方案中,将工程改造的的遗传回路全身地递送至患有癌症或其他疾病的对象,并且其在对象的癌细胞或患病细胞中被特异性地活化(活化转录)。

[0159] 如上文所讨论的,工程改造的遗传回路可以使用病毒递送系统(例如,逆转录病毒、腺病毒、腺相关、辅助依赖性腺病毒系统、混合型腺病毒系统、单纯疱疹、痘病毒、慢病毒、Epstein-Barr病毒)或非病毒递送系统(例如,物理的:裸DNA、DNA轰击、电穿孔、流体动力学、超声波或磁转染;或化学的:阳离子脂质、不同阳离子聚合物或脂质聚合物)(Nayerossadat N等.Adv BiomedRes.2012;1:27,其通过引用并入本文)将工程改造的遗传回路递送至对象的细胞(例如,癌细胞)。在一些实施方案中,基于非病毒的递送系统是基于水凝胶的递送系统(参见,例如,Brandl F等.Journal of Controlled Release,2010,142(2):221-228,其通过引用并入本文)。

[0160] 合成的启动子库

[0161] 提供了包含多个核酸的合成的启动子文库,其中文库中的每个核酸包含合成的启动子序列。提供了用于合成的启动子文库的三种设计。在两种设计(“设计1”和“设计2”)中,文库的启动子序列包含串联(头对尾)连接的8聚体核苷酸序列。在这些设计之一(“设计2”)中,将3聚体核苷酸间隔物置于每对8聚体核苷酸序列之间。在第三种设计(“设计3”)中,文库的核酸序列包含串联(头对尾)连接的11聚体核苷酸序列,在每对11聚体核苷酸序列之间设置有3聚体核苷酸间隔物。

[0162] 串联的8聚体或11聚体核苷酸序列的数目可以是至少:2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19或20个8聚体或11聚体核苷酸序列。核酸中每个8聚体或11聚体核苷酸序列的序列可以是随机的(即,序列NNNNNNNN,其中每个N表示任意核苷酸),并且任何核酸中的8聚体或11聚体核苷酸序列可以是随机选择的,使得启动子文库中的多个核酸代表文库所选择的核酸的基本上所有可能的序列或所有可能的序列长度。或者,如果特定的核苷酸序列或组成(例如,嘧啶含量)是有利的或需要的,或不利的或要避免的,则可将8聚体或11聚体核苷酸序列设计成在某些位置具有如所期望的某些核苷酸或某些核苷酸含量。在这样的情况下,启动子文库中的多个核酸代表所有可能序列的选定子集。

[0163] 在一些实施方案中,将限定序列的核苷酸间隔物置于每个8聚体或11聚体核苷酸序列之间。核苷酸间隔物优选是3聚体核苷酸,但也可以使用其他长度的间隔物,例如1、2、4或5个核苷酸。在一些实施方案中,3聚体核苷酸间隔物选自:AGC、ATC、GAC、ACT、AGT、GTC、GAT和GCT。在一些实施方案中,文库中的核酸中使用的每个核苷酸间隔物不同于相同核酸中的其他核苷酸间隔物。

[0164] 在一些实施方案中,合成的启动子文库中的核酸在5’和3’端还包含限制性内切核酸酶位点。在一些实施方案中,5’末端的限制性内切核酸酶位点是SbfI位点,3’末端的限制性内切核酸酶位点是AscI位点。也可以使用其他限制性内切核酸酶位点。

[0165] 在一些实施方案中，合成的启动子文库中的每个核酸还包含编码输出分子的核苷酸序列，其与启动子序列可操作地连接。在一些实施方案中，输出分子是可检测的分子，例如荧光或有色蛋白质(例如，mKate2)、酶或本领域已知的任何其他类型的可检测的核酸或多肽。

[0166] 在选择合成的启动子的方法中可使用合成的启动子文库。该方法包括获得包含核酸分子的文库，所述核酸分子含有与输出分子可操作地连接的合成的启动子序列；在一种或更多种类型的细胞中表达文库；检测输出分子的表达；以及分离其中表达输出分子的细胞。任选地，该方法还包括确定分离的细胞中合成的启动子序列的序列。

[0167] 在一些实施方案中，一种或更多种类型的细胞是至少两种不同类型的细胞，例如癌细胞和匹配的非癌细胞，例如卵巢癌细胞和卵巢细胞，或乳腺癌细胞和乳腺细胞等。

[0168] 通过比较驱动至少两种不同类型的细胞的每种中的输出分子的表达的合成的启动子序列，鉴定在至少两种不同类型的细胞的一种中比在至少两种不同类型的细胞的另一种中更具活性的合成的启动子序列。因此，如果至少两种不同类型的细胞是癌细胞和非癌细胞，则可以鉴定在癌细胞中有活性而在非癌细胞中无活性的启动子，反之亦然。

[0169] “在至少两种不同类型的细胞的一种中比在至少两种不同类型的细胞的另一种中更具活性”是指在两种类型的细胞的一种中启动子具有至少10％、50％、100％、2倍、3倍、4倍、5倍、6倍、7倍、8倍、9倍、10倍、20倍、30倍、40倍、50倍、60倍、70倍、80倍、90倍、100倍、500倍或1000倍(或甚至更高)更高的活性。例如，通过这些方法从文库中分离的合成的启动子可以在一种类型的细胞中基本无活性并且在另一类型的细胞中有活性，这提供了细胞类型特异性的合成的启动子。

实施例

[0170] 实施例1

[0171] 使用两种人启动子作为用于在该实施例中使用的工程改造的遗传回路的启动子输入。这些人启动子SSX1(输入1)和H2A1(输入2)在许多人癌症中是过表达的(输入1编码包含间插mirFF4的mKate2输出)(图4A)。相对于(a)输入1(mK2下游)中编码的完全匹配mirFF4结合位点(FF4-BS)的数目和(b)输入2中的“海绵”构建体的两种不同配置，测量不同回路配置的mKate2输出水平。图4B，x轴注释：M#代表输入1具有的在mKate2/mirFF4下游编码的mirFF4结合位点(FF4-BS)的数目。例如，M3代表输入1具有3个完全匹配mirFF4结合位点(FF4-BS)(图4A)。S0、S1和S2代表三种不同海绵/输入2配置。S0为不具有mirFF4结合位点的阴性对照转录物。S1为具有在构建体3’端编码的10个凸起mirFF4结合位点的诱饵转录物(Decoy transcript)(图4A)。S2与S1相似，但是具有额外的环状内含子，具有位于在构建体3’端编码的10个凸起mirFF4-BS的上游的10个凸起FF4-BS。图4A中描绘的工程改造的遗传回路(逻辑门)对应于图4B中的M3-S1(通过虚线框突出显示)。结果以平均mKate2表达(P1)表示，其是在FACS中对SSC/FSC设门以除去细胞块和碎片的细胞的平均mKate2。误差条代表SEM。NT代表未经转染的细胞。

[0172] 用ECFP标记重复实验(图5)。

[0173] 实施例2

[0174] 该实施例中描述的工程改造的遗传回路(G5)是基于实施例1中描述的编码mKate2

的回路(AND门),不同之处在于AND门产物不是mKate2,而是合成转录因子(在图6A中注释为“TF”)。尽管TF可以为任何转录活化剂,例如rtTA3、TALE-TF和ZF-TF,但是在该实施例中,TF为融合蛋白GAL4BD-VP16AD(与病毒VP16转录活化结构域融合的酵母GAL4 DNA结合结构域)。或者,这也可以是转录阻遏物,例如GAL4BD-KRAB。因为输出是转录因子而不是报道子/效应蛋白,故它可以调节在TF靶启动子的下游编码的多个输出的表达。在该实施例中,靶启动子(被注释为P3)为由具有5个上游GAL4DNA结合位点的最小病毒或人启动子组成的合成G5启动子。该合成的启动子的I/O曲线可以用GAL4结合位点的数量调整。因此,任何多个输出之间的比例以及每个输出的活化阈值可以通过合成P3启动子中的GAL4结合位点的数目确定。

[0175] 图6B示出了实验结果。CXCL10为调节含有mirFF4v2B内含子和10个下游mirFF4-Bs的GAL4BD-VP16AD的CXCL1p。SSX10为调节含有mirFF4v2B内含子和10个下游mirFF4-Bs的GAL4BD-VP16AD的SSX1p。SSX＊10为调节含有mirFF4v2B内含子和10个下游mirFF4-Bs的GAL4BD-VP16AD的截短SSX1p,其中5’UTR的一部分与KOZAK序列一起被除去。海绵SO为阴性对照转录物mirFF4-BS。海绵S2为具有额外的环状内含子的具有在3’端上编码的10个凸起FF4-BS的诱饵转录物,该环状内含子具有位于在转录物3’中编码的10个凸起mirFF4-BS的上游的10个凸起mirFF4-BS。在所有样品中,在G5p下编码mKate2输出。

[0176] 实施例3.BiTE和STE触发稳健的肿瘤杀伤

[0177] HEK-293T细胞

[0178] 抗HER2双特异性T细胞衔接器(BiTE)和表面T细胞衔接器(STE)触发T细胞以介导稳健的肿瘤杀伤和IFN-γ分泌(图8)。用所示的多种DNA构建体转染HEK-293T(最低限度表达HER2)细胞。转染后48小时,收集多种HEK-293T细胞并与人T细胞共培养5小时或24小时。通过乳酸脱氢酶(lactate dehydrogenase,LDH)释放测定法测量T细胞的5小时细胞毒性(Korzeniewski C和Callewaert DM,Journal of Immunological Methods,1983,64(3):313-320,通过引用并入本文),并且通过IFN-γELISA测量T细胞的24小时IFN-γ分泌。数据表明T细胞介导了对BiTE分泌性肿瘤细胞的稳健的肿瘤杀伤和IFN-γ分泌(第1组至第2组)。肿瘤杀伤和IFN-γ分泌与肿瘤细胞上的HER2表达水平相关(第1组至第2组)。T细胞还介导了对STE表达性肿瘤细胞的稳健的肿瘤杀伤和IFN-γ分泌(第3组至第6组),并且细胞毒性和IFN-γ分泌不依赖于肿瘤抗原(HER2)表达(第3组至第6组)。此外,当与表达非BiTE和非STE对照蛋白的HEK-293T细胞共培养时,T细胞介导最低的肿瘤杀伤和IFN-γ分泌(第7组至第9组)。

[0179] 稳定的4T1细胞

[0180] 将表达指示DNA构建体(STRICT017 +018)的稳定4T1细胞(HER2-)与人T细胞共培养5小时或24小时(图10)。LDH释放测定法测量T细胞的5小时细胞毒性,并且通过IFN-γELISA测量T细胞的24小时IFN-γ分泌(图10A)。数据表明T细胞介导了对HER2-或STE-肿瘤细胞的最低的杀伤和IFN-γ分泌(第1组和第3组)。T细胞介导了对STE表达性肿瘤细胞的稳健的肿瘤杀伤和IFN-γ分泌(第2组)。当与由少量BiTE分泌细胞和非BiTE分泌肿瘤组成的细胞混合物共培养时,T细胞也介导了稳健的肿瘤杀伤和IFN-γ分泌。这表明肿瘤块中最低量的BiTE分泌细胞可以引起稳健的肿瘤块杀伤和IFN-γ释放(第4组)。

[0181] 稳定的HEK-293T细胞

[0182] 将表达指示DNA构建体的稳定HEK-293T细胞(最低限度表达HER2)与人T细胞共培养5小时或24小时。通过LDH释放测定测量T细胞的5小时细胞毒性,并且通过IFN-γ ELISA测量T细胞的24小时IFN-γ分泌(图10B)。数据表明T细胞介导了对BiTE-或STE-肿瘤细胞的最低的杀伤和IFN-γ分泌(第4组)。T细胞介导了对BiTE分泌性肿瘤细胞的稳健的细胞毒性和IFN-γ分泌(第1组)。T细胞也介导了对STE表达性肿瘤细胞的稳健的细胞毒性和IFN-γ分泌(第2组和第3组)。此外,当与由少量BiTE分泌细胞和非BiTE分泌性肿瘤细胞组成的细胞混合物共培养时,T细胞也介导了稳健的肿瘤杀伤和IFN-γ分泌。这表明肿瘤块中最低量的BiTE分泌细胞可引起稳健的肿瘤块杀伤和IFN-γ释放(第5组和第6组)。

[0183] 稳定的MDA-MB452(HER2+)细胞(人乳腺癌细胞系)

[0184] 抗HER2双特异性T细胞衔接器(BiTE)和表面T细胞衔接器(STE)触发T细胞介导对人乳腺癌细胞系的稳健的肿瘤杀伤(图11)。通过用所示的多种DNA构建体(STRICT034、035)的慢病毒转导产生了稳定的MDA-MB453(HER2+)细胞系。使用供体#2T细胞。E:T比为10:1;6x 10^5:6x 10^4。收集多种MDA-MB453细胞并与人T细胞共培养5小时。通过LDH释放测定测量T细胞的5小时细胞毒性。数据表明T细胞介导了对BiTE分泌性肿瘤细胞的稳健的肿瘤杀伤(第2组)。T细胞还介导了对STE表达性肿瘤细胞的稳健的肿瘤杀伤(第3组至第4组)。此外,当与亲本MDA-MB453肿瘤细胞系共培养时,T细胞介导了最低的肿瘤杀伤(组1)。

[0185] 实施例4.T细胞有效杀伤多西环素诱导的STE表达细胞

[0186] 表面T细胞衔接器(STE)形式1(v1)和形式2(v2)二者都触发T细胞介导对HEK-293T细胞的稳健的肿瘤杀伤(图13)。通过慢病毒转导产生了多种诱导型STE表达性HEK-293T细胞系。收集多种HEK-293T细胞并与人T细胞共培养5小时。通过LDH释放测定测量T细胞的5小时细胞毒性。数据表明T细胞介导了对经转染的STEv1表达性肿瘤细胞的稳健的肿瘤杀伤(第2列)。T细胞也介导了对诱导型STEv1和STEv2表达性肿瘤细胞的稳健的肿瘤杀伤(第3列和第4列)。此外,当与非STE表达性HEK-293T细胞系共培养时,T细胞介导最低的肿瘤杀伤(第1列)。

[0187] 实施例5:T细胞对肿瘤细胞的杀伤效率的提高

[0188] HEK-293T细胞

[0189] 利用AND门构造提高T细胞对肿瘤细胞的杀伤效率(图9)。用所示的多种DNA构建体(STRICT014)(图9A)转染HEK-293T细胞,并且使用供体#S T细胞。E:T比为10:1;6x 10^5:6x 10^4。关于右栏(图9B),(1,0)表示仅用STE转染的细胞。(1,1)表示用STE和海绵转染的细胞。(0,0)表示用非STE蛋白转染的细胞。Ctr1表示未经转染的细胞。转染后48小时,收集多种HEK-293T细胞并与人T细胞共培养5小时。通过LDH释放测定测量T细胞的细胞毒性。数据表明T细胞杀伤293T/STE表达细胞(第1列),并且杀伤可以通过AND门结构大大地增强(第2列)。T细胞对非STE表达细胞表现出最低杀伤(第3列和第4列)。对于左栏(图9C),没有测试输入2条件,因为它不编码输出蛋白。(0,0)表示未经转染的细胞。进行另外的实验以通过除去Kozak序列和SSX1启动子的5'UTR进一步降低AND门在状态(1,0)下的输出。

[0190] HEK-293T细胞(STE的GAL4门v1)

[0191] 用所示的多种DNA构建体(STRICT037、039、040)转染HEK-293T细胞,并且使用供体#2的T细胞(图14)。E:T比为10:1;6x 10^5:6x 10^4。左栏示出了用于该T细胞细胞毒性实验的回路(图14A)。在右栏中,(1,0)表示仅用STE转染的细胞。(1,1)表示用STE和海绵转染的

细胞。(0,0)表示用非STE蛋白转染的细胞。转染后48小时,收集多种HEK-293T细胞并与人T细胞共培养5小时。通过LDH释放测定测量T细胞的细胞毒性(图14B)。数据表明T细胞杀伤STE表达(1,0)细胞(第2列和第4列),并且杀伤可以通过AND门(1,1)结构大大地增强(第3列和第5列)。T细胞对非STE表达细胞表现出最低杀伤(第1列)。

[0192] 用所示的多种DNA构建体(STRICT039、040)转染HEK-293T细胞,并且使用供体#2的T细胞(图15)。E:T比为10:1;6x 10^5:6x 10^4。图15A示出了用于该T细胞细胞毒性实验的回路。在图15B中,(1,0)表示仅用STE转染的细胞。(1,1)表示用STE和海绵转染的细胞。(0,0)表示用非STE蛋白转染的细胞。转染后48小时,收集多种HEK-293T细胞并与人T细胞共培养5小时。通过LDH释放测定测量T细胞的细胞毒性。数据表明T细胞杀伤STE表达(1,0)细胞(第3列和第5列),并且杀伤可以通过AND门(1,1)结构大大地增强(第4列和第6列)。T细胞对非STE表达细胞(第1列)表现出最低杀伤。在(1,0)条件下的杀伤主要是由GAL4启动子输出的泄漏引起的(第2列相对于第3列或第5列)。进行另外的实验以通过除去STE v1的Kozak序列、通过在3'端添加miRNA结合位点使STE v1输出自降解以及两种机制的组合降低GAL4启动子泄漏。

[0193] HEK-293T细胞(STE的GAL4门v2)

[0194] 可以利用GAL4门形式2(v2)结构以微调肿瘤细胞的T细胞杀伤效率并在(1,0)状态下表现出更小的细胞毒性。用所示的多种DNA构建体(STRICT039、040)转染HEK-293T细胞,并且使用供体#2的T细胞(图16)。E:T比为10:1;6x 10^5:6x 10^4。图16A示出了用于该T细胞细胞毒性实验的回路。在图16B中,(1,0)表示仅用STE转染的细胞。(1,1)表示用STE和海绵转染的细胞。(0,0)表示用非STE蛋白转染的细胞。转染后48小时,收集多种HEK-293T细胞并与人T细胞共培养5小时。通过LDH释放测定测量T细胞的细胞毒性(图16B)。数据表明T细胞杀伤STE表达(1,0)细胞(第3列),并且杀伤可通过AND门(1,1)结构增强(第4列)。T细胞对非STE表达细胞表现出最低杀伤(第1列)。与GAL4门v1结构相比,该形式在(1,0)状态下的杀伤提高(v2更接近基础水平(0,0))。进行另外的实验以降低(1,0)状态下的杀伤。在(1,0)状态下的GAL4启动子输出通过在STE基因的3'端添加miR结合位点而降低。

[0195] HEK-293T细胞(STE的GAL4门v3)

[0196] 可以利用GAL4门形式3(v3)结构以微调肿瘤细胞的T细胞杀伤效率并在(1,0)状态下表现出更小的细胞毒性。用所示的多种DNA构建体(STRICT039、040)转染HEK-293T细胞,并且使用供体#2的T细胞(图16)。E:T比为10:1;6x 10^5:6x 10^4。图17A示出了用于该T细胞细胞毒性实验的回路。在图17B中,(1,0)表示仅用STE转染的细胞。(1,1)表示用STE和海绵转染的细胞。(0,0)表示用非STE蛋白转染的细胞。转染后48小时,收集多种HEK-293T细胞并与人T细胞共培养5小时。通过LDH释放测定测量T细胞的细胞毒性(图17B)。数据表明T细胞最低限度地杀伤STE表达(1,0)细胞(第3列),并且仅在AND门有活性(1,1)时达到高效杀伤(第4列)。T细胞对非STE表达细胞表现出最低杀伤(第1列)。在(1,0)状态下的杀伤与(0,0)状态的一样长。进行另外的实验提高GAL4-VP16输出水平或增加GAL4结合位点来增强(1,1)状态的杀伤效率。

[0197] 实施例6

[0198] 该实施例解决了两个首要挑战(图2A至图2B):(1)建立新的乳腺癌疗法,其安全且有效地替代具有危及生命的毒性的介入;以及(2)使用这些新的疗法来消除与转移性乳腺

癌相关的死亡率。

[0199] 免疫治疗已经在临床试验中实现了针对癌症的稳健且潜在治愈的效力。利用T细胞效应物功能(例如嵌合抗原受体(CAR)T细胞或双特异性T细胞衔接器(BiTE))的免疫治疗可具有有力的效力[1,2]。然而,存在与这些疗法相关的重要挑战,特别是针对实体瘤,例如乳腺癌。目前的CAR-T细胞治疗需要对每位患者定制的细胞分离、工程改造的和扩增,这是昂贵且难以扩展的。此外,CAR-T细胞必须运输到肿瘤部位以介导杀伤,并且需要长期持续存在以获得稳健的效力,这可对实体瘤提出了挑战[3]。

[0200] BiTE是包含两个串联融合的单链可变片段(scFv)以能够通过T细胞接合肿瘤细胞从而导致T细胞触发的肿瘤杀伤的融合蛋白。BiTE疗法是有力的并且可以在比肿瘤靶向抗体(Ab)低5个数量级的浓度下赋予肿瘤杀伤[2]。然而,即使多次推注也不能维持高的血清BiTE浓度,因为其在体内的半衰期短(约2小时)[4]。成功的治疗血液性癌症的BiTE临床试验都需要连续静脉输注4至8周[2]。由于实体瘤通常比血液性恶性肿瘤更难以接近免疫细胞,故成功的针对实体瘤的BiTE疗法可能需要甚至更长时间的连续BiTE输注,由于潜在的副作用、患者不便和降低的效力,这是不期望的。最后,CAR T细胞和BiTE疗法二者都靶向细胞外肿瘤特异性抗原,这在许多癌症类型(包括三阴性乳腺癌)中是不可行的。此外,靶抗原可能通过正常细胞展示,因此免疫治疗可能导致脱靶免疫应答,产生严重后果[5]。

[0201] 除了利用采用CAR或BiTE的T细胞效应物功能之外,替代的方法是将表达癌细胞表面上的T细胞接合蛋白并且活化基于T细胞的杀伤的遗传回路递送到肿瘤细胞中。这些表面T细胞衔接器(STE)可以在体外和体内触发肿瘤细胞的抗原非依赖性T细胞杀伤[6-10]。然而,之前的STE研究无法构建仅在肿瘤细胞中被活化的遗传回路。因此,为了避免全身毒性,这些构建体仅限于肿瘤内注射,导致效力降低和无法治疗全身性疾病[7,10]。这是一个主要限制,因为对于许多癌症,特别是乳腺癌,转移性疾病是致死的主要原因。因此,迫切需要可以利用免疫系统来治疗全身性癌症和转移性癌症的具有高抗肿瘤特异性的可扩展疗法,这在本文中被提供。

[0202] 合成生物学家已经开发了基于癌症特异性启动子或miRNA谱的高度特异性细胞内检测癌症状态的遗传回路[11,12]。然而,在这些肿瘤检测回路可以用于临床之前,还需要进一步的开发。例如,这些合成的肿瘤检测回路仅与细胞内杀伤机制偶联,这限制了其对肿瘤的效力,因为其实际上不可能将回路递送至100%的癌细胞。此外,需要高靶向特异性以避免损伤健康组织。最后,过去的回路利用了外来蛋白,但是使异位蛋白表达最小化是对于避免在正常细胞中诱导宿主免疫应答所必不可少的。

[0203] 为了克服现有癌症免疫治疗和肿瘤检测遗传回路的限制,本文中提供了通过遗传回路募集免疫调节系统的肿瘤免疫治疗(Tumor Immunotherapy by Gene-circuit Recruited Immunomodulatory System,TIGRIS),也被称为合成的肿瘤募集免疫细胞治疗(STRICT),一种针对来自肿瘤自身内部的肿瘤触发有力且有效的免疫治疗的平台技术。TIGRIS是肿瘤检测遗传回路与抗癌免疫治疗的组合。可以将工程改造的遗传回路递送到肿瘤。这些工程改造的遗传回路仅在癌细胞中被选择性活化,导致STE的表面展示和其他免疫调节分子的分泌来募集靶向肿瘤的T细胞。我们设计了具有非常高的特异性的肿瘤检测基因回路以使TIGRIS疗法能够全身施用但只在癌细胞中局部活化,产生提高的安全性和降低的副作用。因此,TIGRIS将全身递送的优点(例如,治疗转移)与局部治疗的优点(例如安全

性、最小副作用)结合,并且实现了以下益处。

[0204] 我们开发了针对三阴性乳腺癌(triple-negative breast cancer,TNBC)的TIGRIS,三阴性乳腺癌是一种难以治疗的乳腺癌亚组,其表现出侵袭性行为并且与较差的预后相关[13-15]。对于TNBC没有理想的靶向疗法,因为该乳腺癌亚组不表达雌激素受体、孕激素受体或HER2。TIGRIS克服了其他疗法相关的关键障碍,其包括:

[0205] 1)乳腺癌异质性的挑战。已知乳腺癌具有非常的肿瘤间和肿瘤内异质性[16]。例如,HER2表达异质性与差的预后相关[17],而传统的靶向疗法不能覆盖整个异质癌症群。相反,我们假设肿瘤特异性STE表达首先募集T细胞来杀伤STE表达癌细胞。最初的杀伤释放免疫原性突变抗原[18],其募集另外的波的具有各种靶向特异性的T细胞。这会产生针对肿瘤抗原的多克隆免疫应答、覆盖广泛的异质肿瘤群的突变情形和防止免疫编辑介导的肿瘤复发。此外,几乎所有的靶向疗法都可产生靶阴性肿瘤变体过度增生(target-negative tumor variant outgrowth)。由于TIGRIS不需要已知的由肿瘤细胞表达的肿瘤特异性抗原,故其不受涉及表面抗原下调的肿瘤逃逸机制的影响。

[0206] 2)有限的靶向谱。与CAR-T细胞或BiTE疗法不同,TIGRIS不依赖于对于许多癌症可能难以鉴定的肿瘤特异性抗原的表面表达。相反,TIGRIS是通过凭借AND门逻辑的多个肿瘤特异性/组织特异性启动子的协同活化而被活化的,这导致相对于单个启动子系统的增强的特异性。这些逻辑回路可针对不同启动子定制,并且甚至导入肿瘤特异性/组织特异性微小RNA以获得进一步的特异性,从而实现了灵活的治疗效力。此外,可以通过肿瘤细胞测序鉴定这些启动子并针对不同肿瘤定制以克服经免疫编辑的癌症和异质癌细胞类型。

[0207] 3)转移的致死后果。转移性肿瘤细胞难以治疗,并且导致90%的乳腺癌死亡[19]。我们的基因回路可以全身递送但是由于其特异性仅具有局部作用,因此潜在地能够检测并破坏转移。此外,我们期望由TIGRIS活化的抗癌T细胞巡查机体以靶向转移以进行破坏。

[0208] 4)在靶向治疗期间肿瘤逃逸变体的演化。TIGRIS可以启动表位扩散,并且该现象募集许多具有不同肿瘤靶向特异性的T细胞。肿瘤逃逸变异的可能性比传统的靶向治疗小得多。

[0209] 5)肿瘤复发的挑战。许多晚期乳腺癌最终复发,对于复发没有可行的预测或预防措施。由于T细胞可以分化为记忆T细胞并长期存在于机体中,故TIGRIS可以预防未来的肿瘤复发。在此,作为一个实例,我们提供了TNBC,使用传统疗法难以治疗的乳腺癌亚组。

[0210] 6)治疗递送的挑战。非连续治疗递送(例如使用病毒或非病毒载体的核酸或遗传回路)通常不能靶向所有肿瘤细胞。由于STE可以募集T细胞来启动肿瘤杀伤并启动表位扩散现象,故即使我们的肿瘤检测回路只可以递送至一小部分的肿瘤细胞,只要由STE触发的免疫应答足够稳健,该技术就可以杀伤周围的癌细胞。

[0211] 通过工程改造的高特异性癌症检测回路来命令肿瘤细胞表达STE和其他免疫调节剂,我们可以引起稳健的宿主免疫应答以消除原发性肿瘤细胞、靶向异质肿瘤、抑制局部淋巴结侵袭(invasion)以及靶向全身性转移,同时还形成免疫记忆防止未来的肿瘤复发。

[0212] 工程改造的TIGRIS构建体并在体外和体内验证治疗效力

[0213] 我们创建了新的癌症检测回路,其命令肿瘤细胞展示STE。我们测试了STE是否可触发稳健的免疫应答并在体外和体内有效地杀伤乳腺癌细胞。采用TIGRIS取得针对实体瘤(例如乳腺癌)的稳健的效力所需的关键参数(例如,STE表达性肿瘤细胞的最小部分和肿瘤

细胞表面上的最小STE表达水平)是未知的,因此我们采用在体外和体内测定确定这些参数。我们还测试了无论乳腺癌中的肿瘤内异质性如何,经TIGRIS触发的免疫应答是否可以实现有效的抗肿瘤治疗。

[0214] 创建并验证在肿瘤细胞上展示STE的癌症检测回路。我们通过分别使衍生自抗人CD3εAb(克隆:OKT3)或抗鼠CD3εAb(克隆:2C11)的scFv与惰性膜锚定蛋白(例如,细胞质截短的Duffy抗原/趋化因子受体(Duffy Antigen/Receptor for Chemokine,DARC))融合创建了人和鼠STE(图12A(顶部))。我们进行了体外T细胞细胞毒性测定和细胞因子释放测定以测试在由代表TNBC、慢性粒细胞白血病和胚胎肾肿瘤的多种肿瘤细胞系表达时(分别地4T1、K562和HEK-293T)人STE的功能性。当T细胞与STE表达性肿瘤细胞共培养时,我们观察到T细胞的稳健的细胞毒性和IFN-γ产生(图24B)。由于人STE和鼠STE分别只与人和鼠T细胞结合,故这些构建体使我们能够证实特异性T细胞接合是治疗效力所必需的。

[0215] 此外,我们设计了特异性检测癌症的细胞内标记的合成基因回路。我们之前工程改造了被称为双启动子积分器(Dual Promoter Integrator,DPI)的癌症检测回路,其输出仅在两个癌症特异性启动子被活化超过最小阈值时被表达,因此执行AND门[12]。DPI是使用非人转录因子执行的,其对于临床应用不理想,因为他们可能引入可在正常细胞中变成免疫原性的外来蛋白。在此,我们创建了仅使用RNA的AND门(图3A至图3D),其具有比基于蛋白质的回路更紧凑的额外益处。该回路设计仅当两个启动子在癌细胞中被活化时表达输出。我们在HEK-293T细胞中使用SSX1和H2A1癌症特异性启动子用荧光蛋白和STE作为输出,构建并验证了我们的仅RNA AND门结构的可调性、模块性和功能性(参见以下描述)。

[0216] 我们调整我们的RNA门以特异性识别乳腺癌细胞。在我们目前的回路下,含有在癌细胞中活化的AND门的两个输入的细胞(40%溶解,图4中的情况1)相较于含有在癌细胞中有活性的仅一个输入的细胞(仅表达STE蛋白的细胞,图3A至3D中的情况3),T细胞介导的杀伤增强了约2倍。该回路的性能(例如增强的ON:OFF比)通过以下可以进一步提高:增加STE转录物中的miRNA结合位点的数量、修饰miRNA骨架用于更稳健的miRNA产生、每STE转录物产生多个miRNA拷贝、测试不同miRNA和海绵的文库、修饰海绵序列和结构、用mRNA降解标签使泄漏最小化、实施反式切割核酶以用于除去STE转录物中的miRNA结合位点以及在STE转录物中包含另外的miRNA结合位点,其通过在正常细胞中高度表达但在肿瘤细胞中下调的内源miRNA被结合并抑制[31]。

[0217] 我们还测试了其他癌症特异性和组织特异性启动子(例如,高乳腺癌特异性的并且已经在TNBC细胞系中验证的RPC1和RRM2[32]),并且验证了我们的回路在4T1癌细胞中被活化但在正常细胞(例如COMMA-1D、EpH4、MCF10A)中不被活化。

[0218] 我们通过将回路转染或稳定地整合到肿瘤细胞中测试回路功能性。我们还在腺病毒、AAV或HSV载体中编码我们的回路以能够递送到4T1和正常乳腺细胞系中来验证肿瘤检测灵敏性、特异性和可调性。我们还利用溶瘤HSV载体,例如T-VEC,其已经用于人患者中的癌症治疗[33]。

[0219] 如果在一些情况下上述癌症特异性启动子中的一些在4T1细胞中没有取得特异性活化,则可用比较转录组学和通过使用FACS和测序筛选在靶细胞中特异性活化的条码化的启动子文库来鉴定另外的癌症特异性启动子。如果一些仅RNA回路不能达到显著的ON:OFF比,则可使用人转录因子(例如人工锌指蛋白[27])使潜在的免疫原性外来蛋白的引入最小

化。

[0220] 鉴定需要通过TIGRIS靶向以获得体内效力的肿瘤细胞的最小百分比。我们阐明了需要通过我们的遗传回路靶向以在体内取得稳健的治疗效力的肿瘤细胞的最小百分比。该信息用于设计全身递送策略，因为在一些情况下，他们不可能靶向100％的肿瘤细胞。我们以不同比例将STE展示性肿瘤细胞(4T1/STE+)与非STE展示性对应物(4T1/STE-)混合，并将其直接移植到有免疫能力的BALB/c小鼠乳腺垫中以产生常位乳腺癌模型。4T1鼠模型类似于晚期人TNBC并且是高度恶性和转移性的[34,35]。通过每隔一天用卡尺测量肿瘤体积监测肿瘤生长动力学。我们用以下实验随着时间推移监测动物存活，该实验保持运行了是对照小鼠的平均存活时间的至少两倍长。鉴定了有效抑制经注射的肿瘤细胞的生长所需的STE表达性肿瘤细胞的最小百分比。使用表达人STE的肿瘤细胞系作为对照来验证T细胞接合特异性。每个实验条件我们利用了4至6只小鼠。

[0221] 当有足够的STE表达性细胞时，肿瘤生长部分或全部被抑制，导致得到长期无病的存活小鼠。我们分别使用Student’s t-检验和单向ANOVA来比较两组之间和多于两组之间的肿瘤体积。为了分析存活实验，我们使用Kaplan-Meier存活分析。我们还过继性转移经双生物发光报道系统工程改造的T细胞以用体内成像追踪T细胞肿瘤浸润和活化的动力学[36]。我们用C3(1)/SV40T抗原转基因小鼠(一种非常具侵袭性自发TNBC模型)扩展了该工作[37]，以在更加生理相关的肿瘤模型中验证我们的发现。

[0222] 我们确定了需要表达STE以赋予稳健的体内效力的肿瘤细胞的下限。对于大于平均基因递送效率的限制，我们设计了新的可以同时分泌多种免疫刺激效应物的回路。这些分子包括主动吸引T细胞的趋化因子(例如，CCL19和CCL21)[38]、免疫刺激的并且可以调节肿瘤微环境的细胞因子(例如IL-12、IL-15和IL-21)[39]和可释放T细胞活性的闸的免疫检查点阻断Ab(例如，抗CTLA4或抗PD1 Ab)[40]。该组合方法增强了针对异质乳腺癌的治疗效力。例如，在靶向多种实体瘤类型的多次临床试验中，抗PD1 Ab已经取得了20％至50％的应答率。然而，预先存在的免疫力是患者响应抗PD1 Ab所需的[41,42]。通过共同表达STE和抗PD1 Ab，STE可帮助产生针对肿瘤相关和突变的抗原的预先存在免疫性，同时抗PD1 Ab可以增强T细胞功能、增殖和浸润到肿瘤，特别是表达PD-L1(PD-1配体)来关闭T细胞功能的那些[43,44]。

[0223] 评价针对转移性癌症和复发的TIGRIS

[0224] 在晚期乳腺癌中，通常观察到肿瘤细胞淋巴结侵袭和全身性转移，并且导致90％的乳腺癌死亡。手术后的护理标准是化学疗法联合靶向疗法，但是这对于TNBC不是很有效[13-15]。此外，20％至30％的诊断患有浸润性乳腺癌的患者在治疗后复发，但没有用于早期检测复发的预防措施或诊断标志物。我们测试了通过TIGRIS触发的免疫细胞是否可以消除淋巴结和全身性转移并建立长期的免疫记忆。TIGRIS可避免需要全身化学治疗和手术切除淋巴结，这是发病的最常见原因，并且提供防止肿瘤复发的保护。

[0225] 确定TIGRIS是否可通过全身递送消除原发性肿瘤和转移。我们测试了工程改造的遗传回路的全身病毒递送是否可以在体内消除原发性和转移性肿瘤。我们工程改造了4T1细胞以表达用于体内成像的萤光素酶。为了测试针对转移的效力，我们使用上述4T1常位模型，但是只当在淋巴结和重要器官(期望在肺、肝、骨和脑中)中观察到转移时开始我们的通过病毒递送的回路治疗。我们监测了小鼠模型中的总肿瘤负荷(原发性+转移性肿瘤)。

[0226] 我们通过改变参数(例如,病毒载体浓度、时间和类型)测试不同的治疗方案[45]。我们通过活体动物成像追踪在体内通过TIGRIS产生的免疫应答。我们看到治疗后原发性和转移性肿瘤的肿瘤生长降低,特别是在免疫细胞可以容易进入的器官,例如肺、肝和骨中。脑转移的降低也是可能的,因为基于T细胞的免疫治疗已经显示出浸润脑脊液[1]。我们比较TIGRIS相对于已知的化学治疗方案,例如紫杉烷和蒽环类抗生素[46]。

[0227] 如果在一些情况下只用STE表达没有消除原发性肿瘤,则我们增加了用上述多种免疫刺激效应物的治疗。我们还测试了多次病毒注射是否可以提高治疗效力。此外,我们在回路治疗之前和之后通过外科手术切除原发肿瘤以模拟常见的临床实践并测试原发性肿瘤的外科手术切除可如何影响针对转移的免疫应答。

[0228] 在一些情况下,全身回路递送可对取得高治疗效力造成挑战。在一些实施方案中,我们通过用小的肽使我们的载体(例如,腺病毒)假型化以靶向其他细胞表面受体来改善病毒递送[47]。在一些实施方案中,我们调整已经显示出靶向乳腺癌的溶瘤病毒以同时利用肿瘤溶解和免疫治疗[48]。在一些情况下,病毒颗粒可仅穿透许多实体瘤的肿瘤周边。因此,我们可以表达iRGD肿瘤穿膜肽作为另外的回路输出[49]。这些肽可以显著增强许多治疗剂(包括Ab、溶瘤病毒和纳米颗粒)的肿瘤渗透[49-51]。

[0229] 除了测试全身递送之外,我们还确定了局部回路递送至原发性肿瘤细胞以治疗全身性转移的治疗效力。免疫调节性溶瘤病毒T-Vec的局部肿瘤注射可引起未经注射的肿瘤缩小[33]。该发现表明可以用病毒或非病毒载体实现的TIGRIS回路的局部递送也可赋予治疗效力。通过在经注射的肿瘤中产生局部免疫应答,TIGRIS可启动靶向转移性肿瘤的全身免疫应答。

[0230] 当所有含有基因回路的肿瘤细胞被杀伤时,STE表达应被终止。然而,为了增强可控性和安全性,在一些实施方案中,我们向我们的基因回路中建立了合成的安全机制。在这些设计中,如果门在正常细胞中正常工作,则应是关闭的,不应表达任何外来蛋白。因此,只有门在正常细胞中失灵或门在癌细胞中正常工作,伴随着可以在外部切换的安全机制,治疗性输出蛋白才被表达。首先,我们工程改造了诱导型回路以终止STE表达和/或杀伤STE表达细胞。具体地,STE输出被合成转录因子(例如GAL4BD-VP16AD(GAD))替换。在这种结构中,STE、免疫刺激分子和iRGD肽的基因以及条件杀伤基因TK1共同通过GAD响应性启动子G5p调节。因此,仅当逻辑门有活性时,外来蛋白才与STE、TK1和其他输出基因共同表达。TK1底物(例如,更昔洛韦或阿昔洛韦)的添加能够杀伤其中回路有活性的细胞。或者,我们产生诱导型转录因子(例如多西环素响应性转录因子rtTA3)作为我们逻辑门的输出而不是GAD以驱动治疗性输出表达。在这种情况下,在没有施用外源诱导剂(例如,多西环素)下整个系统不会被活化,从而提供了一种简单且安全的用FDA批准的小分子控制治疗开始和终止的机制。作为安全性的最后一层,我们实施了分泌的STE拮抗剂(例如CD3ε自身,其可以滴定出功能性STE)的诱导型表达。

[0231] 测试TIGRIS是否可引起免疫记忆以防止未来的肿瘤复发。TIGRIS启动针对复发性乳腺癌的长期免疫记忆。为了表明这一点,我们通过尾静脉注射用4T1肿瘤细胞再次攻击了(来自上述的)长期存活者。4T1肿瘤细胞的尾静脉注射主要导致肺转移,其是乳腺癌的常见转移部位[52]。进行活体动物成像来监测在肺和其他重要器官中的肿瘤种植以确定是否存在针对再引入的肿瘤细胞的保护性免疫。

[0232] 如果在一些情况下,初始治疗引起非常稳健的针对原发性肿瘤的应答但没有针对再次攻击的显著保护,则我们设计另外分泌IL-7和IL-15的肿瘤检测回路,因为这些驱动记忆T细胞形成[53]。此外,4T1肿瘤模型是非常具有免疫抑制性的[54]。因此,在一些情况下,导入检查点阻断Ab和/或促炎细胞因子(参见上文)可帮助产生更稳健的记忆应答。

[0233] 实施例6的参考文献

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[0288] 实施例7

[0289] 卵巢癌的合成的肿瘤寡集免疫细胞治疗。需要新的治疗策略来治疗原发性和转移性卵巢癌并取得长期效力。现有卵巢癌治疗(例如化学疗法和靶向疗法)不能治愈转移性疾病和防止肿瘤复发。此外,护理标准治疗(例如化学疗法)可引起显著的发病率和毒性。

[0290] 本文中提供了变革性的新类型的高度特异性、有效且持久的卵巢癌免疫治疗。该治疗策略,合成的肿瘤募集免疫细胞治疗(STRICT)利用肿瘤自身来募集免疫细胞以破坏肿瘤(图2A至图2B),从而诱导可调节的、安全、持久且有效的强的多克隆抗肿瘤应答。

[0291] 具体地,我们提供了仅当多个肿瘤特异性启动子有活性时才在卵巢癌细胞中选择性打开的合成基因回路(例如,通过执行AND逻辑的数字基因回路)。这些合成回路可通过病毒载体全身递送或局部递送到肿瘤中。我们利用合成生物学的最新进展将这些合成基因回路设计成高度紧凑的、基于RNA的(以避免在正常细胞中表达免疫原性外来蛋白)并且仅在卵巢癌细胞中被特异性活化(不在任何其他正常细胞类型中)。当被活化时,这些回路展示表面T细胞衔接器(STE)和其他免疫调节分子(例如检查点抑制剂和细胞因子)以触发稳健

且靶向性的抗肿瘤免疫应答。STE会被设计成接合T细胞上的T细胞受体并触发T细胞以杀伤STE展示性细胞。此外，我们向基因回路中导入安全开关以使其能够从外部打开或关闭。

[0292] 第一波T细胞应进行STE指导的肿瘤细胞杀伤，然后是靶向那些通过细胞裂解释放的更广谱的癌症抗原的第二波多克隆T细胞。因此，通过STRICT触发的免疫治疗可抑制原发性和转移性肿瘤二者，因为T细胞可提供在整个机体内散布的免疫监视。此外，这些免疫应答可使得能够针对卵巢癌建立长期记忆。我们调整了STRICT以靶向卵巢腺癌，这是表现出侵袭性行为并且与差的预后相关的最常见且难以治疗的卵巢癌亚组(1)。

[0293] 利用T细胞效应物功能(例如嵌合抗原受体(CAR)T细胞或双特异性T细胞衔接器(BiTE))的免疫治疗已经取得了有力效果(2,3)。然而，这些疗法的使用造成了重大挑战，特别是针对实体瘤(例如卵巢癌)。目前的CAR-T疗法需要针对每个患者的定制的细胞工程改造和扩增，这是昂贵且难以扩展的。CAR-T细胞需要运输到肿瘤部位、靶向肿瘤特异性抗原并且长期持续介导稳健的肿瘤杀伤和效力[4]，这是对于卵巢癌的主要挑战[5]。

[0294] BiTE包含两个串联融合的单链可变片段，从而能够接合并通过T细胞杀伤肿瘤细胞。BiTE可在比肿瘤靶向性抗体(Ab)低5个数量级的浓度下赋予有效且稳健的肿瘤杀伤(3)。然而，由于BiTE在体内的半衰期短(约2小时)(6)并且实体瘤通常比血液性恶性肿瘤更难以接近免疫细胞，故成功的针对实体瘤的疗法可能需要长期连续静脉BiTE输注，由于副作用、患者方便性和治疗效力，这是具有挑战性的。此外，已经在癌细胞上展示表面T细胞衔接器(STE)来募集T细胞介导的杀伤(7-11)，但是这样的系统还没有被特异性靶向以使得可以全身治疗而没有显著的副作用。

[0295] 最近，溶瘤病毒(例如T-Vec)已经接近获得FDA批准以治疗黑素瘤。溶瘤病毒依靠病毒复制来杀伤肿瘤细胞。然而，将溶瘤病毒改造成仅在特定的肿瘤细胞中复制可能是具有挑战性的，并且在临床试验中溶瘤病毒还没有证明具有良好的抗卵巢癌效力。此外，合成生物学家已经基于癌症特异性启动子或微小RNA谱开发了用于高度特异性细胞内检测癌细胞的基因回路(12,13)。然而，合成的肿瘤检测回路仅与细胞内杀伤机制偶联，这限制了其针对癌症的效力，因为实际上不可能将回路递送至100％的癌细胞。

[0296] 通过利用合成的癌症检测回路来命令肿瘤细胞展示STE并分泌其他免疫调节剂，我们可引起稳健的宿主免疫应答以消除原发性肿瘤细胞，并触发继发性多克隆T细胞应答。我们测试了STRICT是否可抑制局部淋巴结侵袭、靶向全身性转移以及形成免疫记忆以防止未来复发。稳健的免疫应答可有效针对癌症，并且该合成基因回路可设计成用细胞内标志物特异性检测癌细胞。

[0297] 我们提供了至少两种用STRICT靶向原发性、转移性和复发性卵巢癌的方法：

[0298] 1)我们提供了命令肿瘤细胞展示STE并分泌免疫调节效应物的癌症检测回路。我们验证了其在体外和体内的治疗效力。在体外，我们测量了由于STRICT得到的T细胞诱导的细胞毒性和由T细胞分泌的关键细胞因子。在体内，我们使用ID8鼠模型确定了为了通过STRICT实现有效肿瘤清除而需要被靶向的STE展示性肿瘤细胞的最低数量(14)。

[0299] 2)我们在小鼠模型中评价了针对原发性卵巢肿瘤、转移和复发的STRICT。我们使用ID8鼠模型以表明，转移性肿瘤可通过STRICT消除并且STRICT可在初始治疗后存活的小鼠中防止癌症复发。测试STRICT的效力、特异性和可调性的对照包括展示无活性STE的基因回路、无活性的基因回路、在非癌性卵巢细胞和其他正常组织中的测试遗传回路，以及使用

人相对于鼠STE和人相对于鼠T细胞。

[0300] 本公开内容提供了通过使肿瘤转而针对其自身来治疗卵巢癌的方法。STRICT实现了针对卵巢癌的长期活性和针对原发性和转移性肿瘤的散布的T细胞活性。我们的治疗性构建体可以针对多种不同的卵巢癌定制,并且相对于工程改造的细胞治疗在临床实践中更容易扩展和部署。

[0301] STRICT可取得针对原发性和转移性疾病的强力的治疗效果,诱导持久的免疫记忆、导入安全开关并且降低治疗应用所需的成本、人力和基础设施。STRICT可有效针对原发性和转移性肿瘤,并且取得了针对肿瘤复发的长期保护。STRICT通过从肿瘤内实现多克隆抗肿瘤免疫应答和持久免疫记忆的方便、靶向性且安全的诱导,克服了其他治疗的限制。STRICT最终可替代具有毒性和副作用的用于卵巢癌的护理标准治疗,并且广泛地扩展到其他癌症。STRICT是一种变革性的新的治疗方式,其通过利用针对卵巢癌的免疫系统可抑制长期疾病。

[0302] STRICT可有效针对原发性和转移性肿瘤并且取得针对肿瘤复发的长期保护。STRICT可使得能够替代具有有限效力及显著毒性和副作用的用于卵巢癌的护理标准治疗。此外,该技术建立了强大的可以针对大范围癌症得到广泛使用和重新设计的技术平台。

[0303] 实施例7中引用的参考文献

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[0318] 实施例8

[0319] 肺癌的合成肿瘤募集免疫细胞治疗。需要新的治疗策略来治疗原发性和转移性肺癌并取得长期效力。现有肺癌疗法(例如化学疗法和靶向疗法)不能治愈疾病和防止肿瘤复发。此外,护理标准治疗(例如化学疗法)可引起显著的发病率和毒性。

[0320] 在一些实施方案中,本文中提供了高特异性、有效且持久的用于肺癌的免疫治疗。该治疗策略,合成的肿瘤募集免疫细胞治疗(STRICT)利用肿瘤自身募集免疫细胞来破坏肿瘤(图2A至图2B),从而诱导可调节的、安全、持久且有效的强力的多克隆抗肿瘤应答。

[0321] 具体地,我们设计了仅当多个肿瘤特异性启动子有活性时才在肺癌细胞中选择性打开的合成基因回路(例如,通过执行AND逻辑的数字基因回路)。这些合成回路可以通过病毒载体全身递送或局部递送到肿瘤中。我们利用合成生物学的最新进展将这些合成基因回路设计成高度紧凑的、基于RNA的(以避免在正常细胞中表达免疫原性外来蛋白)并且仅在

肺癌细胞中(不在任何其他正常细胞类型中)被特异性活化。当被活化时,这些回路展示表面T细胞衔接器(STE)和其他免疫调节分子(例如检查点抑制剂和细胞因子)以触发稳健且靶向性的抗肿瘤免疫应答。STE被设计成接合T细胞上的T细胞受体并触发T细胞以杀伤STE展示细胞。此外,我们向基因回路中导入安全开关以使其能够从外部打开或关闭。

[0322] 第一波T细胞应进行STE指导的肿瘤细胞杀伤,然后是靶向那些通过细胞裂解释放的更广谱的癌症抗原的第二波多克隆T细胞。因此,通过STRICT触发的免疫治疗能够抑制原发性和转移性肿瘤二者,因为T细胞可提供在整个机体内散布的免疫监视。此外,这些免疫应答可使得能够针对肺癌建立长期记忆。

[0323] 我们调整了STRICT以靶向非小细胞肺癌(non-small-cell lungcancer,NSCLC),这是最常见且难以治疗的肺癌亚组。由于NSCLC响应一些免疫治疗(例如用抗PD-1免疫检查点阻断抗体(Ab),一种活化宿主T细胞效应物功能的免疫治疗(1,2)),故STRICT表现出针对NSCLC的效力。

[0324] 尽管抗PD-1Ab被FDA批准用于治疗NSCLC,但是抗PD-1Ab的增强的存活益处仅比多西他赛长3.2个月,需要进一步提高。利用T细胞效应物功能(例如嵌合抗原受体(CAR)T细胞或双特异性T细胞衔接器(BiTE))的其他免疫治疗已经取得了针对其他癌症的有利效果(3,4)。然而,这些疗法的使用对于实体瘤(例如肺癌)造成了重大挑战。目前的CAR-T治疗需要针对每个患者进行定制的细胞工程改造和扩增,这是昂贵且难以扩展的。CAR-T细胞需要运输到肿瘤部位、靶向肿瘤特异性抗原并且长期持续介导稳健的肿瘤杀伤和效力(5),这是对于肺癌的主要挑战(6)。

[0325] BiTE包含两个串联融合的单链可变片段,以使得能够接合并通过T细胞杀伤肿瘤细胞。BiTE可以在比肿瘤靶向性Ab低5个数量级的浓度下赋予有效且稳健的肿瘤杀伤(4)。然而,由于BiTE在体内的半衰期短(约2小时)(7)并且实体瘤通常比血液性恶性肿瘤更难以接近免疫细胞,故成功的针对实体瘤的疗法可需要长期连续静脉BiTE输注,由于副作用、患者方便和治疗效力,这是具有挑战性的。此外,已经在癌细胞上展示表面T细胞衔接器(STE)来募集T细胞介导的杀伤(8-12),但是这样的系统还没有被特异性靶向以使得可以全身治疗而没有显著的副作用。

[0326] 最近,基于病毒复制杀伤肿瘤细胞的溶瘤病毒(例如T-Vec)已经接近获得FDA批准以治疗黑素瘤。然而,将溶瘤病毒改造成仅在特定的肿瘤细胞中复制可能是具有挑战性的,并且在临床试验中溶瘤病毒还没有证明具有良好的抗肺癌效力。另外,合成生物学家已经基于癌症特异性启动子或微小RNA谱开发了用于高特异性细胞内检测癌细胞的基因回路(13,14)。然而,合成的肿瘤检测回路仅与细胞内杀伤机制偶联,这限制了其针对癌症的效力,因为实际上不可能将回路递送至100%的癌细胞。

[0327] 通过利用合成的癌症检测回路来命令肿瘤细胞展示STE并分泌其他免疫调节剂,我们可引起稳健的宿主免疫应答以消除原发性肿瘤细胞并触发继发性多克隆T细胞应答。我们表明了STRICT可抑制局部淋巴结侵袭、靶向全身性转移并且形成免疫记忆以防止未来复发。稳健的免疫应答可以有效针对癌症,并且合成的基因回路可设计成用细胞内标志物特异性检测癌细胞。

[0328] 我们提供至少两种用STRICT靶向原发性、转移性和复发性肺癌的方法:

[0329] 1)我们创建了命令肿瘤细胞展示STE并分泌免疫调节效应物的癌症检测回路。我

们验证了其在体外和体内的治疗效力。在体外，我们测量了由STRICT造成的T细胞诱导的细胞毒性和由T细胞分泌的关键细胞因子。在体内，我们使用A549异种移植肺癌模型确定了为了通过STRICT实现有效肿瘤清除而需要被靶向的STE展示性肿瘤细胞的最低数目(15)。

[0330] 2)我们在小鼠模型中评价了针对原发性肺肿瘤、转移和复发的STRICT。我们使用A549异种移植模型和LSL-KrasG12D自发肿瘤模型(16)，表明转移性肿瘤可以通STRICT消除并且STRICT可在初始治疗后存活的小鼠中防止癌症复发。测试STRICT的效力、特异性和可调性的对照包括展示无活性STE的回路、无活性的回路、在非癌性肺细胞和其他正常组织中测试回路，以及使用人相对于鼠STE和人相对于鼠T细胞。

[0331] 本公开内容提供了通过使肿瘤转而针对其自身来治疗肺癌的方法。高特异性癌症检测回路尚未与针对肺癌的免疫治疗整合。STRICT应实现了针对肺癌的长期活性以及针对原发性和转移性肿瘤的散布的T细胞活性。我们的治疗性构建体可针对多种不同的肺癌定制，并且相对于工程改造的细胞治疗在临床实践中更容易扩展和部署。

[0332] STRICT可取得针对原发性和转移性疾病的强力的治疗效果、诱导持久的免疫记忆、导入安全开关以及降低治疗应用所需的成本、人力和基础设施。STRICT有效针对原发性和转移性肿瘤，并且取得了针对肿瘤复发的长期保护。STRICT通过从肿瘤内实现多克隆抗肿瘤免疫应答和持久免疫记忆的方便、靶向性且安全的诱导，克服了其他治疗的限制。STRICT最终可替代具有毒性和副作用的用于肺癌的护理标准治疗，并且广泛地扩展到其他癌症。在此，我们的目标是表明STRICT如何是一种变革性的新的治疗方式，其通过利用针对肺癌的免疫系统可抑制长期疾病。

[0333] 本公开内容提供了强大的可针对大范围癌症(包括肺癌)被广泛使用和重新设计的技术平台。

[0334] 实施例8中引用的参考文献

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[0351] 实施例9

[0352] 合成的启动子文库。在一些实施方案中,本文中提供了简单、快速且成本有效的表征转录因子的翻译后调节的方法。所述方法可用于例如鉴定可以用于靶向目标细胞状态的高特异性且很短的合成启动子,这对于研究和个体化医学二者都是重要的。这可例如通过鉴定在特定细胞状态下被活化的高特异性结合基序来完成。目前的方法(例如RNA-Seq和ChIP-Seq)可产生误导,因为RNA水平不总是与蛋白质活性相关(p53是一个很好的实例),并且TF与DNA的结合不总是与转录活化相关(例如,TF可以起阻遏物的作用)。在一些实施方案中,本公开内容的方法提供了在特定细胞状态下被活化的结合基序的直接证据和这些基序的活化水平。生物信息学层面使得能够表征与这些基序有关的转录因子,并且从而能够破译在目标细胞状态下被活化的转录级联。关于图46和图47,从NB508-low文库中分离合成的

启动子。

[0353] 尽管本文中已经描述和说明了多个发明实施方案，但是本领域普通技术人员将容易预见到用于执行本文中所述的功能和/或获得本文中所述的结果和/或一个或更多个优点的多种其他手段和/或结构，并且每个这样的变化和/或修改都视为在本文中所述的发明实施方案的范围内。更一般地，本领域技术人员将容易理解，本文中所述的所有参数、尺寸、材料和配置意指为示例性的，并且实际的参数、尺寸、材料和/或配置将取决于使用发明教导的具体应用。本领域技术人员使用常规实验就将认识到或者能够确定本文中所述的具体发明实施方案的多个等同方案。因此应理解，前述实施方案仅作为实例呈现，并且在所附权利要求及其等同方案的范围内，发明实施方案可以以除具体描述和要求保护的方式之外的方式实施。本公开内容的发明实施方案涉及本文中所述的各个单独的特征、系统、制品、材料、试剂盒/药盒和/或方法。此外，如果这样的特征、系统、制品、材料、试剂盒/药盒和/或方法不互相矛盾，则两种或更多种这样的特征、系统、制品、材料、试剂盒/药盒和/或方法的任意组合包括在本公开内容的发明范围内。

[0354] 本文中限定和使用的所有定义应理解为涵盖按照字典定义、通过引用并入的文献中的定义和/或所限定的术语的普通含义。

[0355] 本文中公开的所有参考文献、专利和专利申请相对于每一个所引用的主题通过引用并入，在一些情况下可涵盖整个文献。

[0356] 除非清楚相反地指出，否则本文中在说明书和权利要求书中未使用数量词修饰的描述应理解为意指“至少一个/种”。

[0357] 如本文中在说明书和权利要求书中使用的短语“和/或”应理解为意指如此连接的要素中的“任一者或两者”，即，在一些情况下共同存在而在另一些情况下分开存在的要素。用“和/或”列举的多个要素应以相同的方式理解，即如此连接的要素中“一个或更多个”。除了由“和/或”子句具体确认的要素之外，可以任选地存在其他要素，无论与那些具体确认的要素有关还是无关。因此，作为非限制性实例，当与开放式语言如“包括”一起使用时，提到的“A和/或B”在一个实施方案中可指仅A(任选地包括除B之外的要素)；在另一个实施方案中可指仅B(任选地包括除A之外的要素)；在又一个实施方案中可指A和B两者(任选地包括其他要素)；等。

[0358] 如本文中在说明书和权利要求书中所使用的，提及一个或更多个要素的列表的短语“至少一个”应理解为意指从要素列表中的任一个或更多个要素中选择的至少一个要素，但不一定包括要素列表中具体列举的每个要素中的至少一个，也不排除要素列表中要素的任意组合。该定义还允许任选地存在除了在短语“至少一个”所提及的要素列表中具体确认的要素之外的要素，无论其与具体确认的那些要素有关还是无关。因此，作为非限制性实例，“A和B中的至少一个”(或者等效地“A或B中的至少一个”，或者等效地“A和/或B中的至少一个”)在一个实施方案中可指至少一个A，任选地包括多于一个A，而不存在B(并且任选地包括除B之外的要素)；在另一个实施方案中可指至少一个B，任选地包括多于一个B，而不存在A(并且任选地包括除A之外的要素)；在又一个实施方案中可指至少一个A，任选地包括多于一个A，以及至少一个B，任选地包括多于一个B(并且任选地包括其他要素)；等。

[0359] 还应理解，除非清楚相反地指出，否则在本文中所要求保护的包括多于一个步骤或动作的任何方法中，该方法的步骤或动作的顺序不一定限于记载该方法的步骤或动作的

顺序。

[0360] 在权利要求书中以及以上的说明书中，所有的过渡短语例如“包含”、“包括”、“携带”、“具有”、“含有”“涉及”、“容纳”、“由...构成”等都理解为开放式的，即，意指包括但不限于。只有过渡短语“由...组成”和“基本上由...组成”分别是封闭式或半封闭式的过渡短语，如在美国专利局专利审查程序指南第2111.03节中所阐明的。

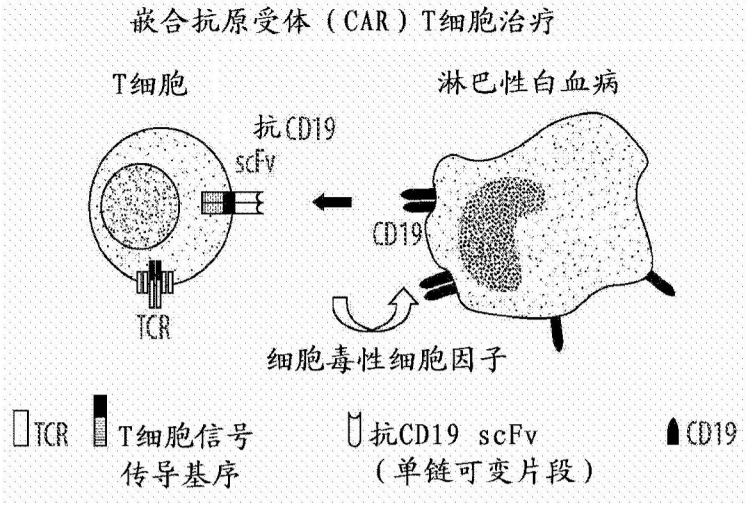
嵌合抗原受体（CAR）T细胞治疗
T细胞
淋巴性白血病
抗CD19
scFv
CD19
TCR
细胞毒性细胞因子
TCR
T细胞信号
传导基序
抗CD19 scFv
（单链可变片段）
CD19

图1A

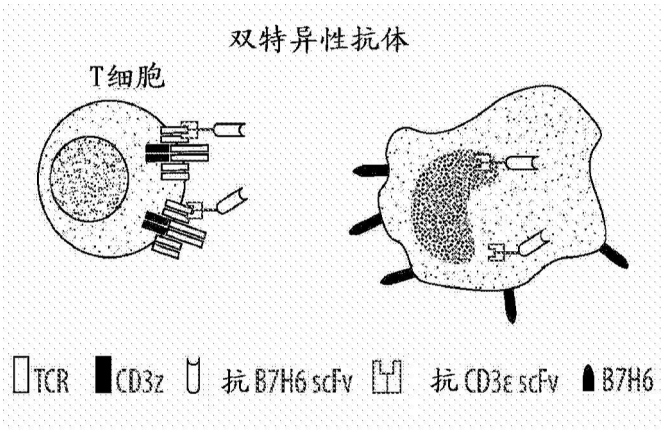
双特异性抗体
T细胞
TCR
CD3z
抗B7H6 scFv
抗CD3ε scFv
B7H6

图1B

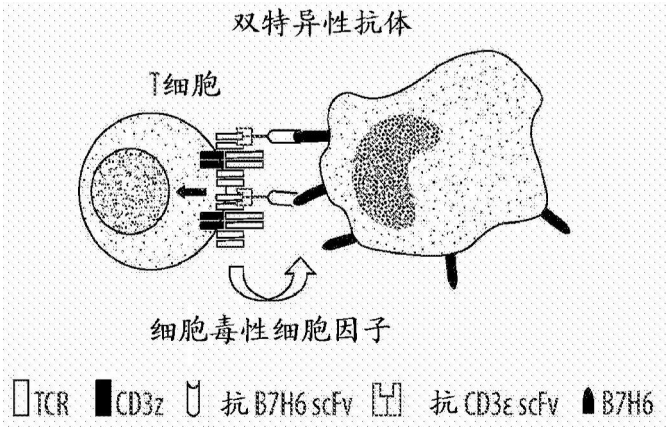
双特异性抗体
T细胞
细胞毒性细胞因子
TCR
CD3z
抗B7H6 scFv
抗CD3ε scFv
B7H6

图1C

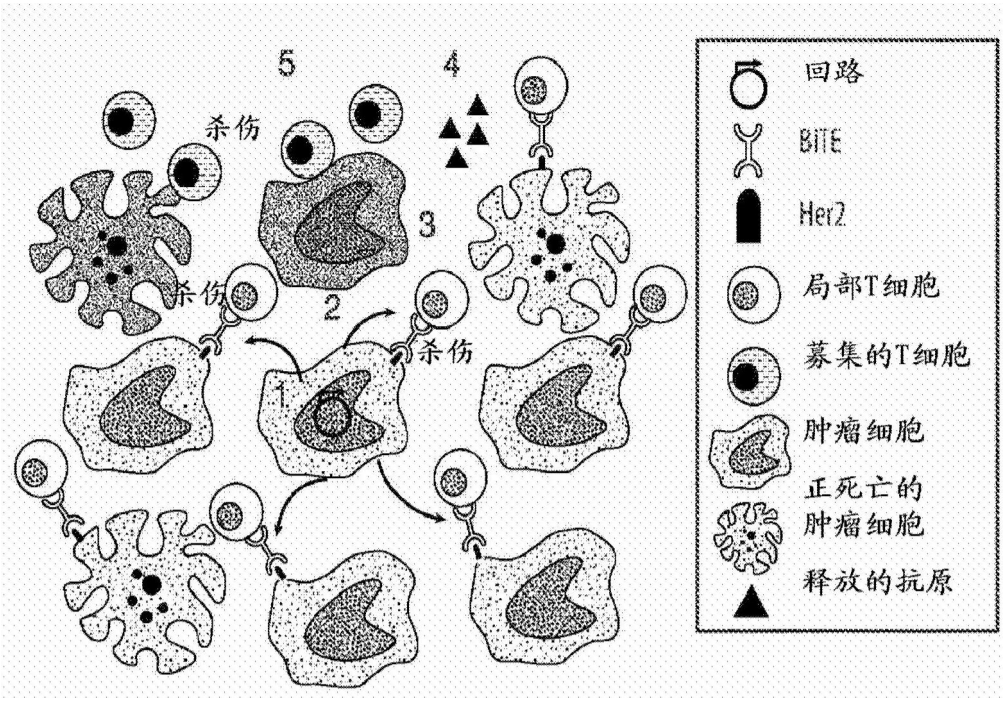
5
4
杀伤
3
杀伤
2
杀伤
1
回路
BiTE
Her2
局部T细胞
募集的T细胞
肿瘤细胞
正死亡的
肿瘤细胞
释放的抗原

图2A

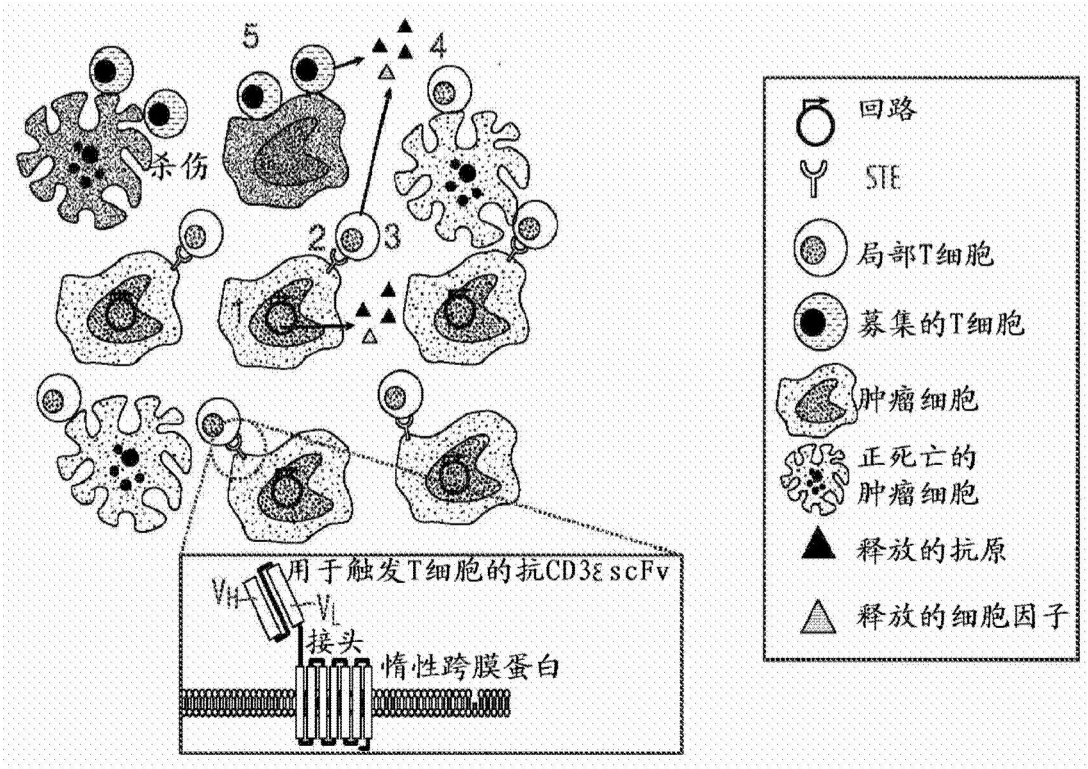
5
4
杀伤
2
3
1
用于触发T细胞的抗CD3εscFv
VH
VL
接头
情性跨膜蛋白
回路
STE
局部T细胞
募集的T细胞
肿瘤细胞
正死亡的
肿瘤细胞
释放的抗原
释放的细胞因子

图2B

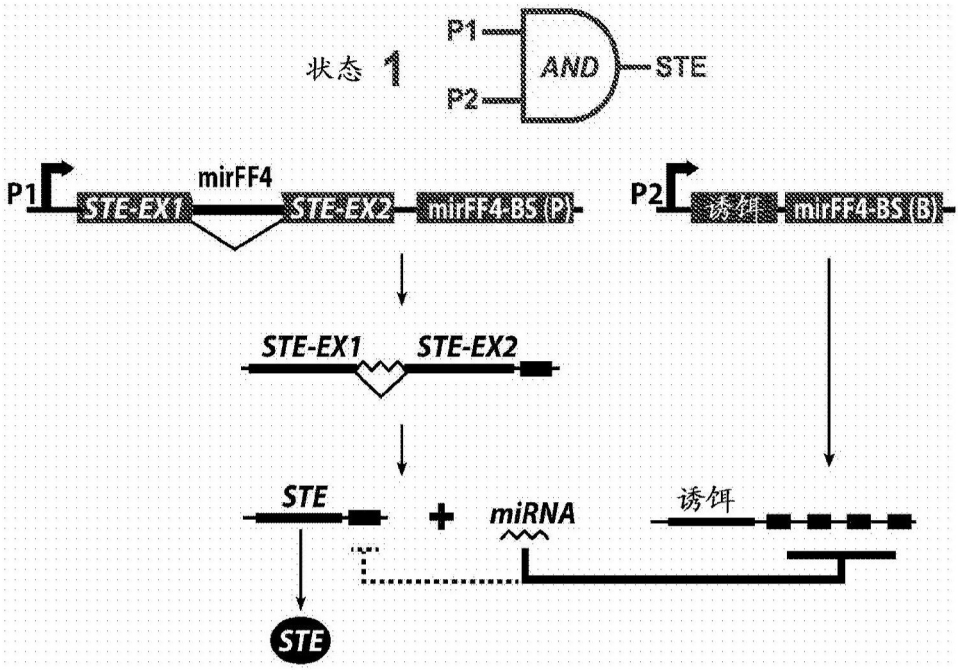
状态 1
P1
P2
AND
STE
P1
STE-EX1
mirFF4
STE-EX2
mirFF4-BS (P)
P2
诱饵
mirFF4-BS (B)
STE-EX1
STE-EX2
STE
miRNA
诱饵
STE

图3A

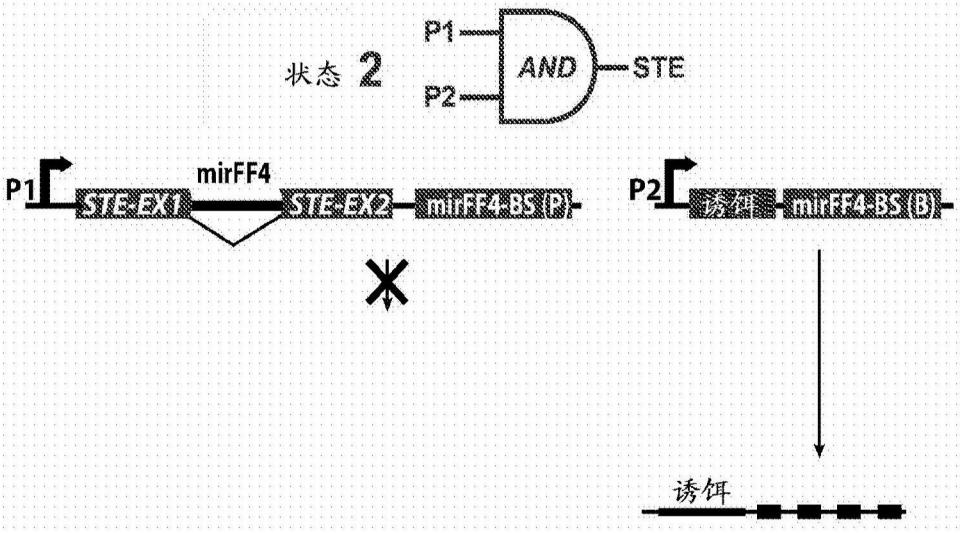
状态 2
P1
P2
AND
STE
P1
STE-EX1
mirFF4
STE-EX2
mirFF4-BS (P)
P2
诱饵
mirFF4-BS (B)
诱饵

图3B

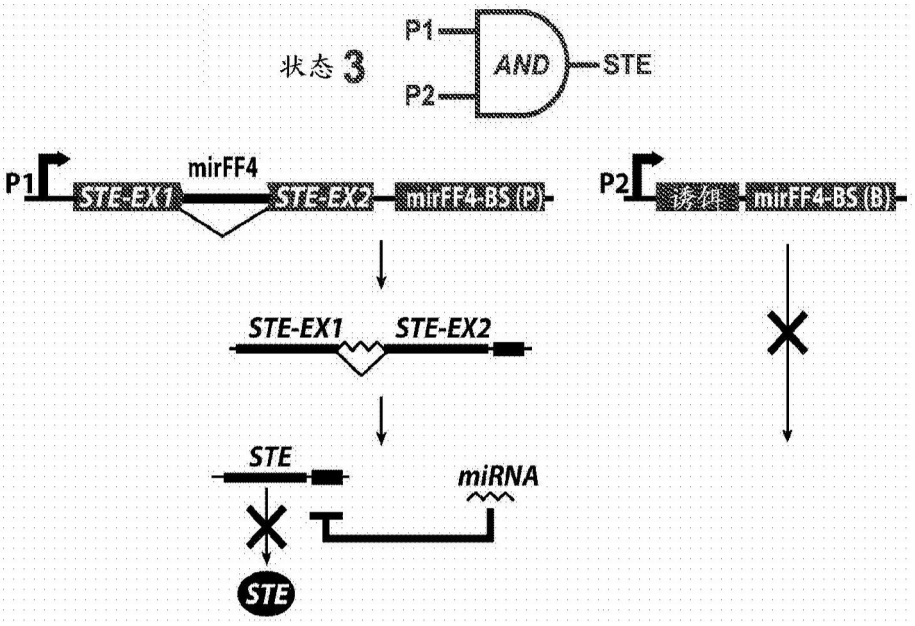
状态 3
P1
P2
AND
STE
P1
mirFF4
STE-EX1
STE-EX2
mirFF4-BS(P)
P2
诱饵
mirFF4-BS(B)
STE-EX1
STE-EX2
STE
miRNA
STE

图3C

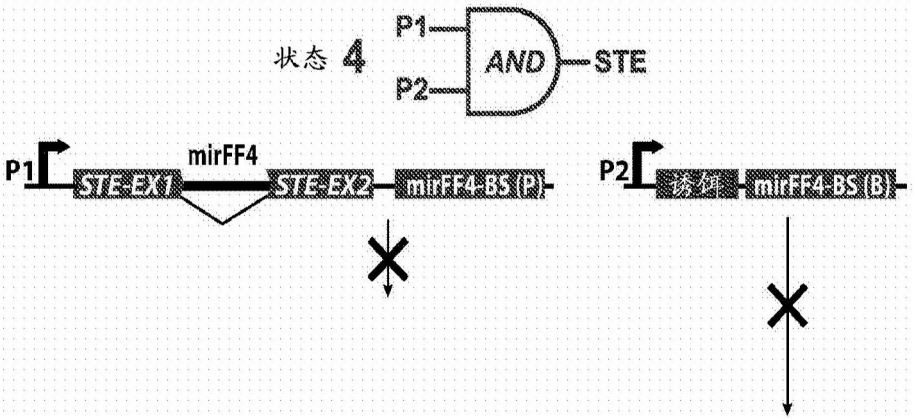
状态 4
P1
P2
AND
STE
P1
mirFF4
STE-EX1
STE-EX2
mirFF4-BS(P)
P2
诱饵
mirFF4-BS(B)

图3D

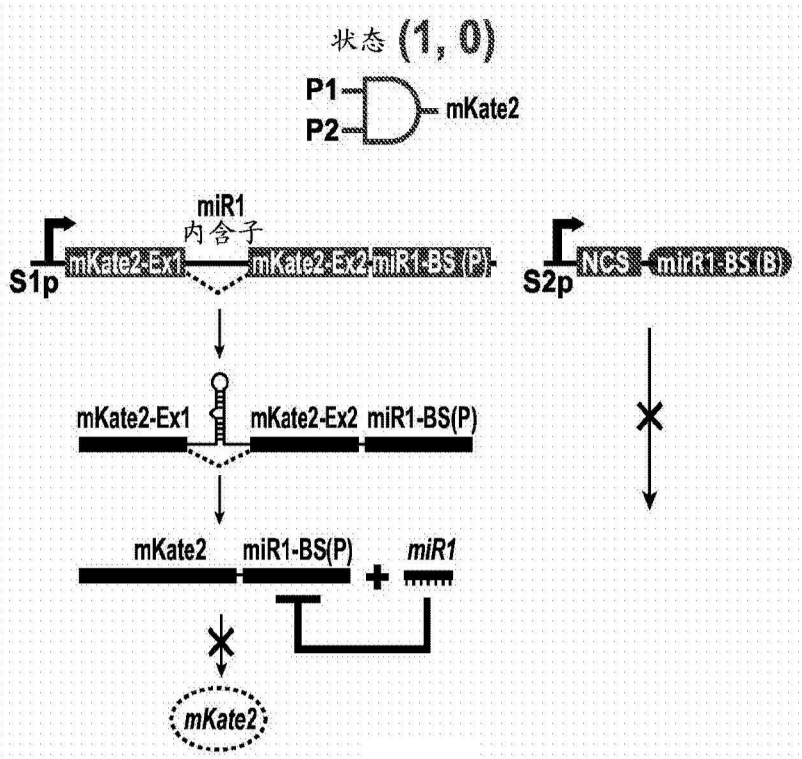
状态 (1, 0)
P1
P2
mKate2
miR1
内含子
S1p
mKate2-Ex1
mKate2-Ex2
miR1-BS (P)
S2p
NCS
mirR1-BS (B)
mKate2-Ex1
mKate2-Ex2
miR1-BS(P)
mKate2
miR1-BS(P)
miR1
mKate2

图3E

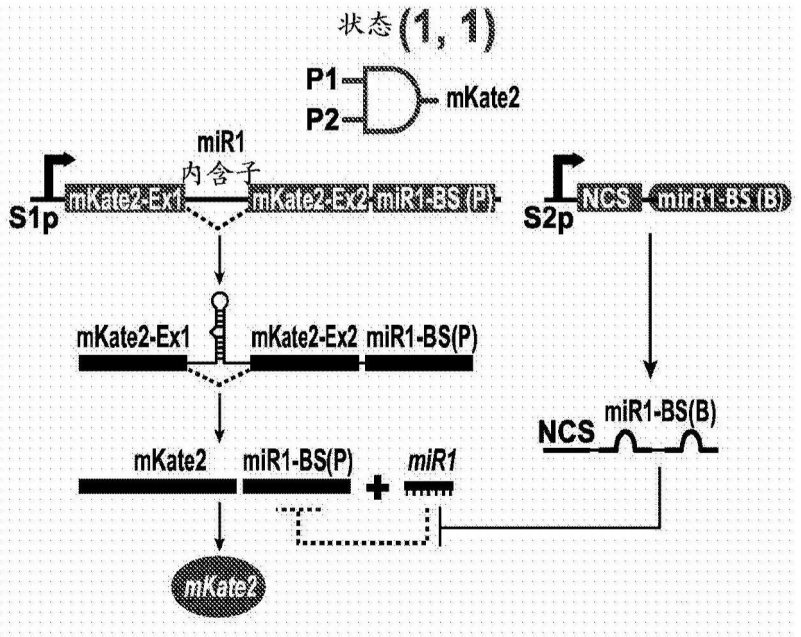
状态 (1, 1)
P1
P2
mKate2
miR1
内含子
S1p
mKate2-Ex1
mKate2-Ex2
miR1-BS (P)
S2p
NCS
mirR1-BS (B)
mKate2-Ex1
mKate2-Ex2
miR1-BS(P)
NCS
miR1-BS(B)
mKate2
miR1-BS(P)
miR1
mKate2

图3F

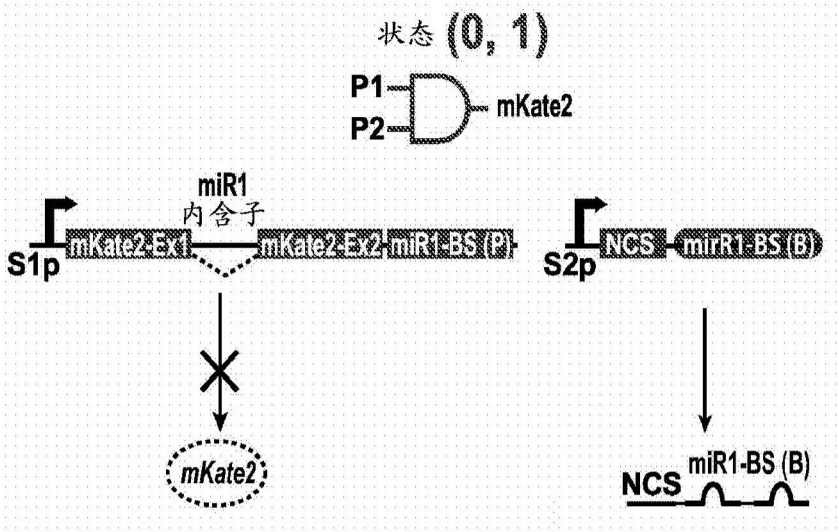
状态 (0, 1)
P1
P2
mKate2
miR1
内含子
S1p
mKate2-Ex1
mKate2-Ex2
miR1-BS (P)
S2p
NCS
miR1-BS (B)
mKate2
NCS
miR1-BS (B)

图3G

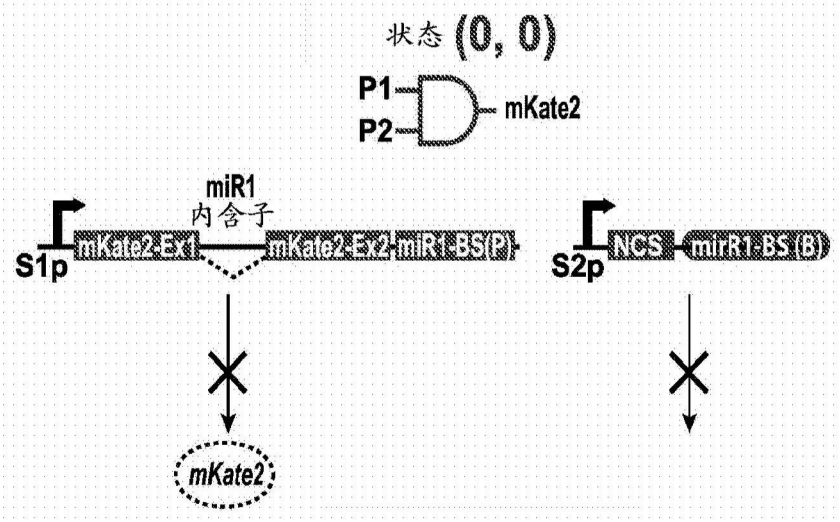
状态 (0, 0)
P1
P2
mKate2
miR1
内含子
S1p
mKate2-Ex1
mKate2-Ex2
miR1-BS(P)
S2p
NCS
miR1-BS (B)
mKate2

图3H

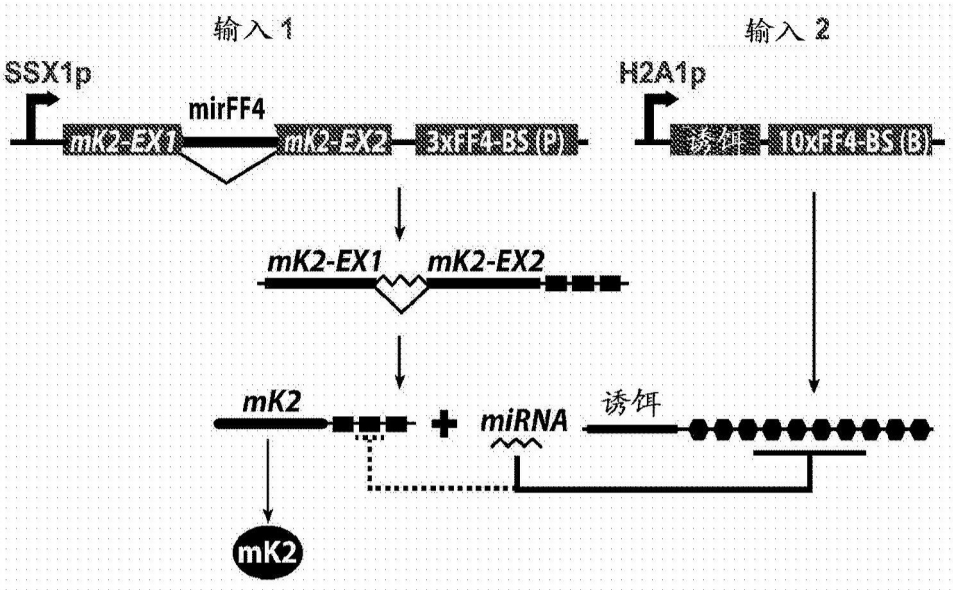
输入 1
输入 2
SSX1p
mirFF4
mK2-EX1
mK2-EX2
3xFF4 BS (R)
H2A1p
诱饵
10xFF4 BS (R)
mK2-EX1
mK2-EX2
mK2
miRNA
诱饵
mK2

图4A

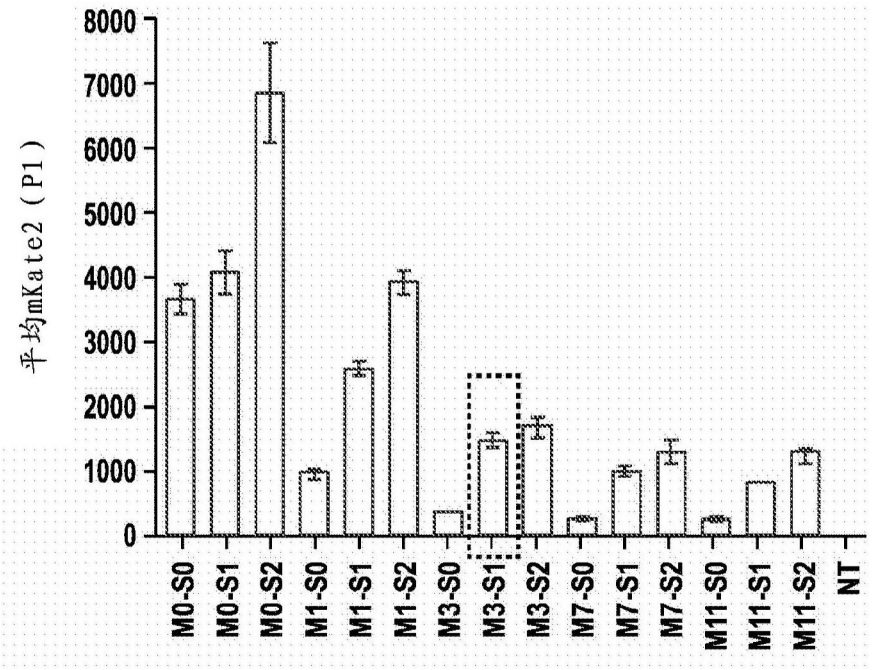
平均mKate2 (P1)
8000
7000
6000
5000
4000
3000
2000
1000
0
M0-S0
M0-S1
M0-S2
M1-S0
M1-S1
M1-S2
M3-S0
M3-S1
M3-S2
M7-S0
M7-S1
M7-S2
M11-S0
M11-S1
M11-S2
NT

图4B

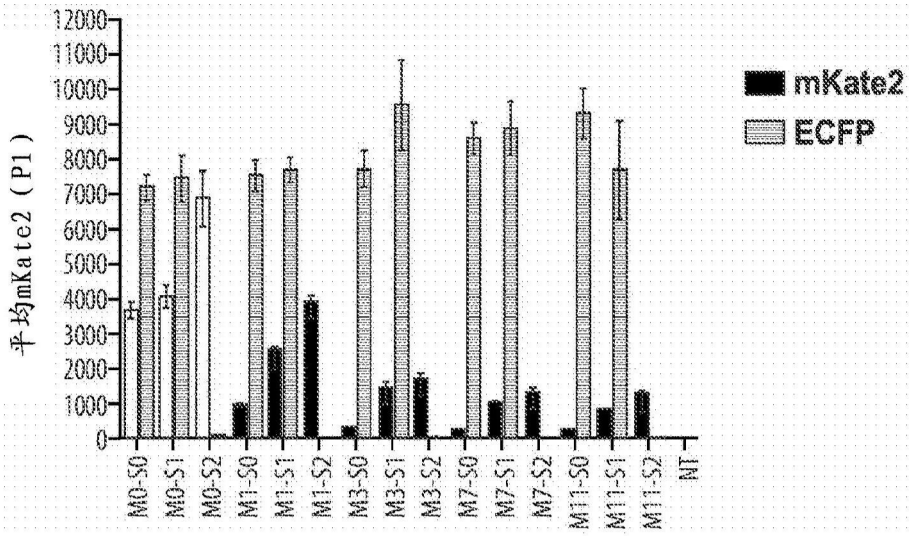
平均mKate2（P1）
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
mKate2
ECFP
M0-S0
M0-S1
M0-S2
M1-S0
M1-S1
M1-S2
M3-S0
M3-S1
M3-S2
M7-S0
M7-S1
M7-S2
M11-S0
M11-S1
M11-S2
NT

图5

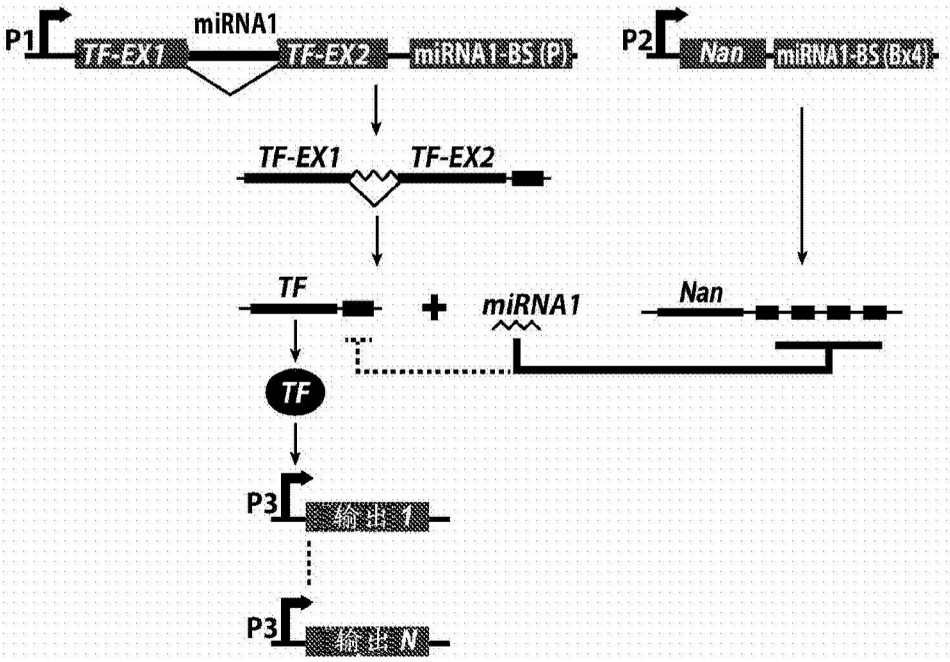
P1
miRNA1
TF-EX1
TF-EX2
miRNA1-BS(P)
P2
Nan
miRNA1-BS(Bx4)
TF-EX1
TF-EX2
TF
miRNA1
Nan
TF
P3
输出 1
P3
输出 N

图6A

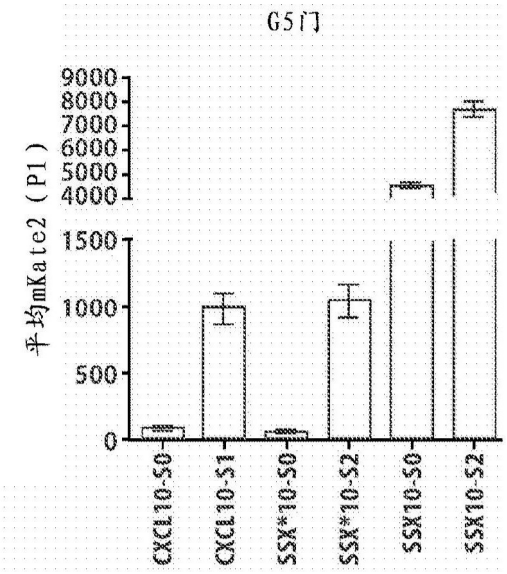
G5门
平均mKate2（P1）
9000
8000
7000
6000
5000
4000
1500
1000
500
0
CXCL10-S0
CXCL10-S1
SSX*10-S0
SSX*10-S2
SSX10-S0
SSX10-S2

图6B

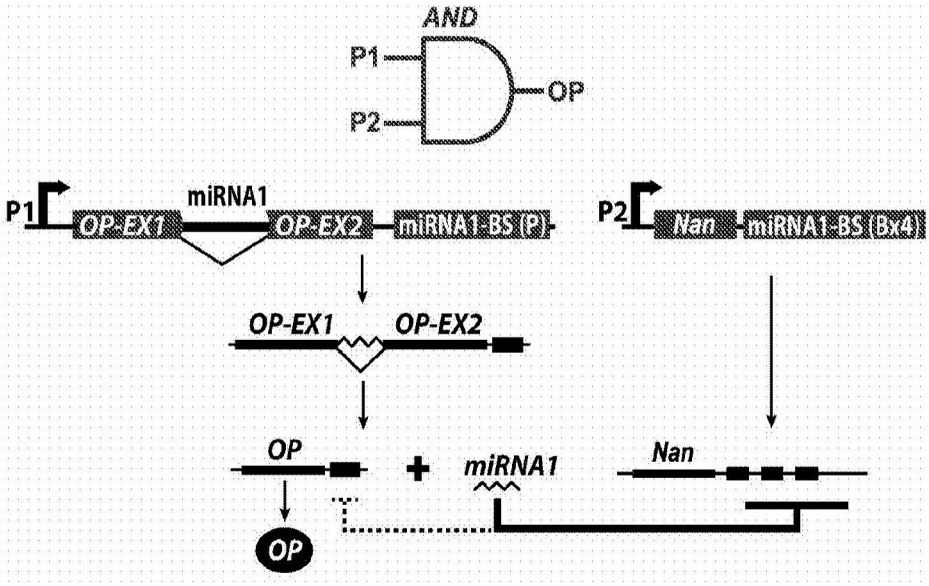
AND
P1
P2
OP
P1
miRNA1
OP-EX1
OP-EX2
miRNA1-BS (P)
P2
Nan
miRNA1-BS (Bx4)
OP-EX1
OP-EX2
OP
miRNA1
Nan
OP

图7A

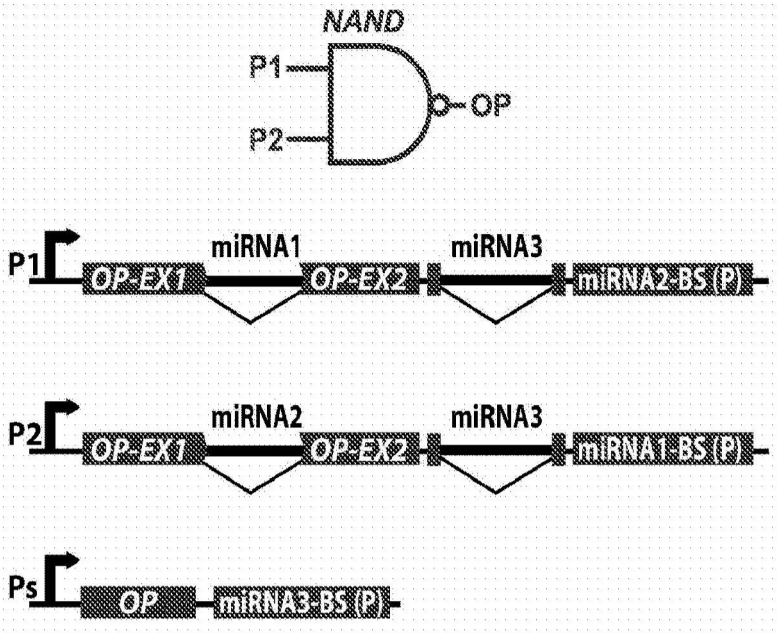
NAND
P1
P2
OP
P1
miRNA1
miRNA3
OP-EX1
OP-EX2
miRNA2-BS (P)
P2
miRNA2
miRNA3
OP-EX1
OP-EX2
miRNA1-BS (P)
Ps
OP
miRNA3-BS (P)

图7B

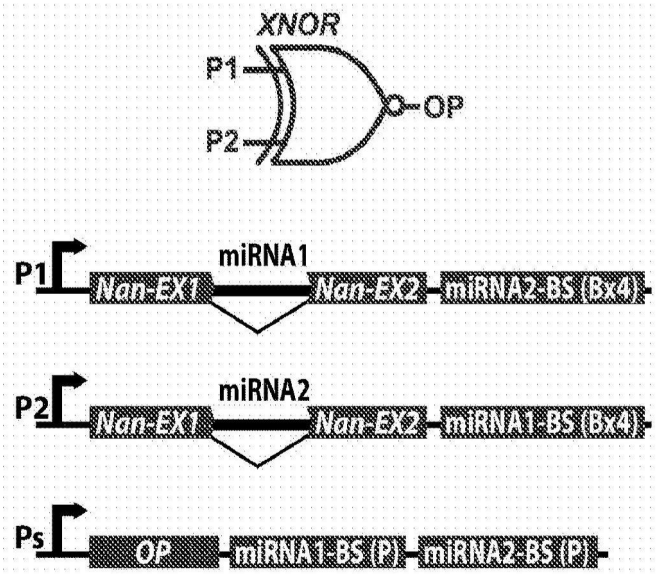
XNOR
P1
P2
OP
P1
miRNA1
Nan-EX1
Nan-EX2
miRNA2-BS (Bx4)
P2
miRNA2
Nan-EX1
Nan-EX2
miRNA1-BS (Bx4)
Ps
OP
miRNA1-BS (P)
miRNA2-BS (P)

图7C

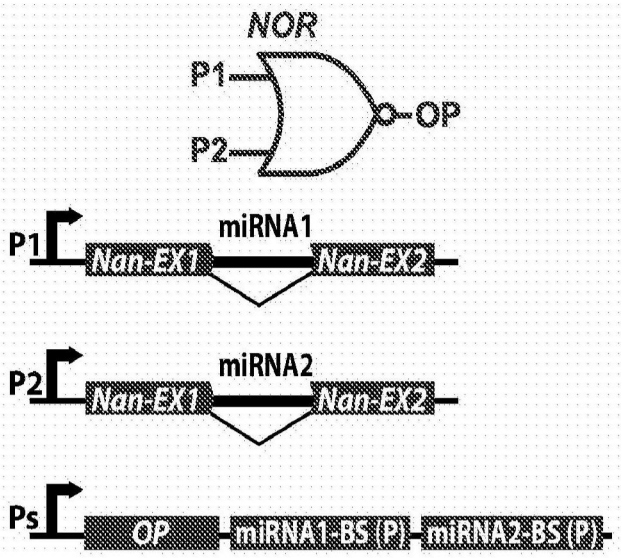
NOR
P1
P2
OP
P1
miRNA1
Nan-EX1
Nan-EX2
P2
miRNA2
Nan-EX1
Nan-EX2
Ps
OP
miRNA1-BS (P)
miRNA2-BS (P)

图7D

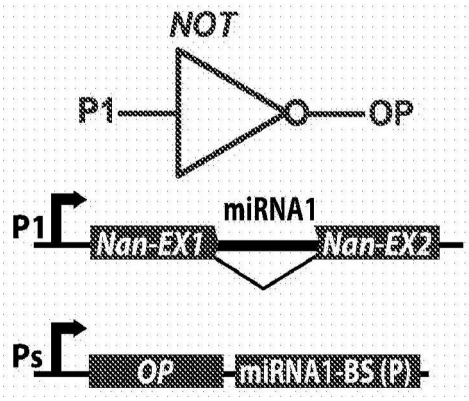
NOT
P1
OP
P1
miRNA1
Nan-EX1
Nan-EX2
Ps
OP
miRNA1-BS (P)

图7E

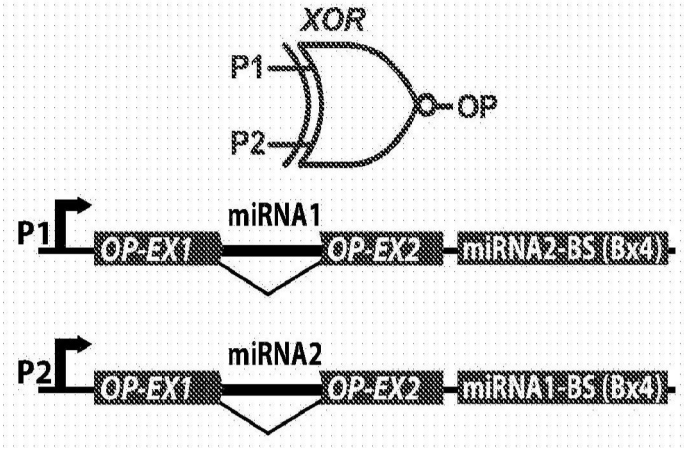
XOR
P1
P2
OP
P1
miRNA1
OP-EX1
OP-EX2
miRNA2-BS (Bx4)
P2
miRNA2
OP-EX1
OP-EX2
miRNA1-BS (Bx4)

图7F

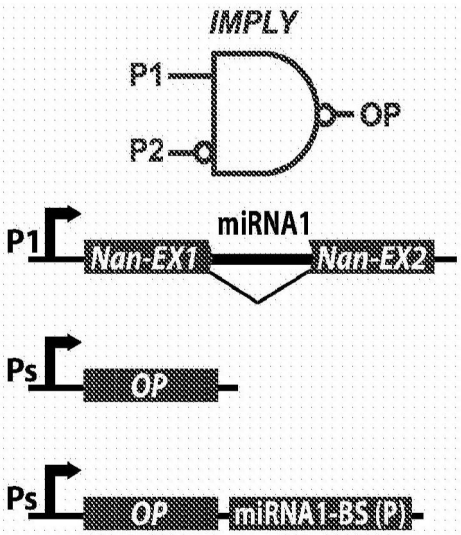
IMPLY
P1
P2
OP
P1
miRNA1
Nan-EX1
Nan-EX2
Ps
OP
Ps
OP
miRNA1-BS(P)

图7G

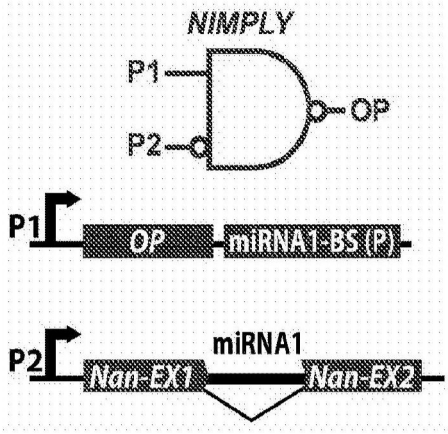
NIMPLY
P1
P2
OP
P1
OP
miRNA1-BS(P)
P2
miRNA1
Nan-EX1
Nan-EX2

图7H

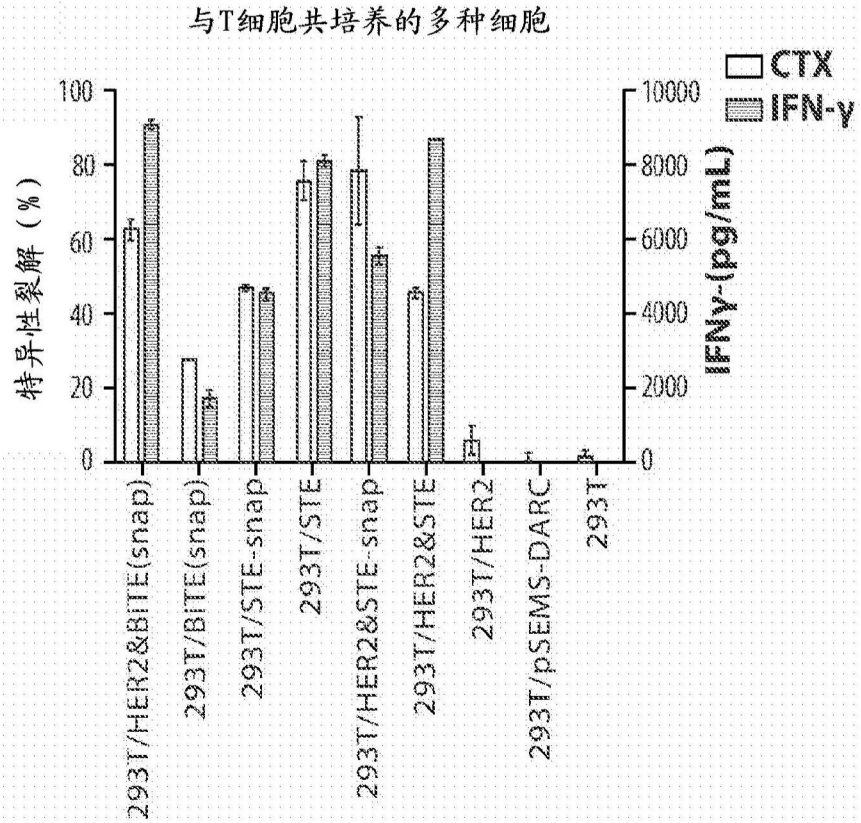
与T细胞共培养的多种细胞
CTX
IFN-γ
特异性裂解（%）
IFNγ-(pg/mL)
100
80
60
40
20
0
10000
8000
6000
4000
2000
0
293T/HER2&BITE(snap)
293T/BITE(snap)
293T/STE-snap
293T/STE
293T/HER2&STE-snap
293T/HER2&STE
293T/HER2
293T/pSEMS-DARC
293T

图8

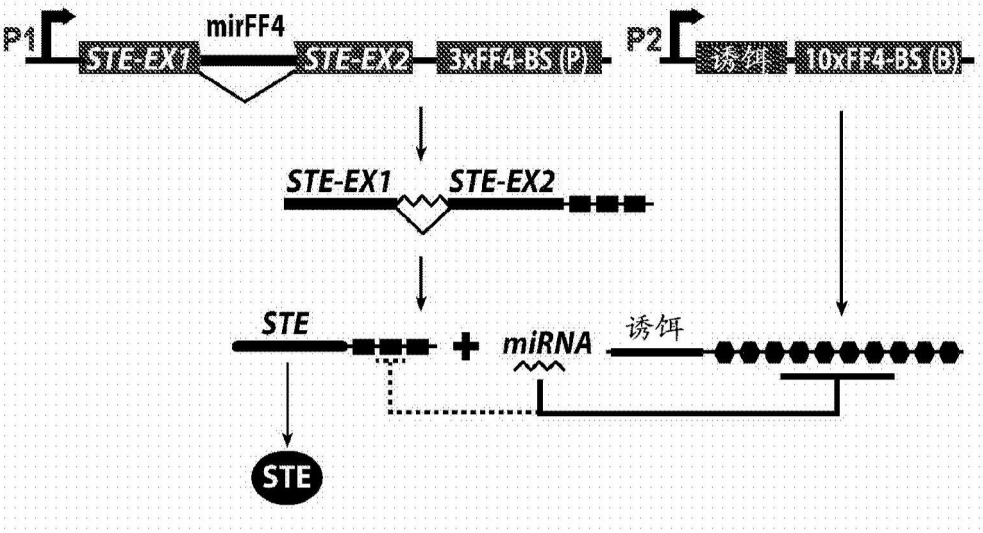
P1
mirFF4
STE-EX1
STE-EX2
3xFF4-BS (P)
P2
10xFF4-BS (B)
STE-EX1
STE-EX2
STE
miRNA
诱饵
STE

图9A

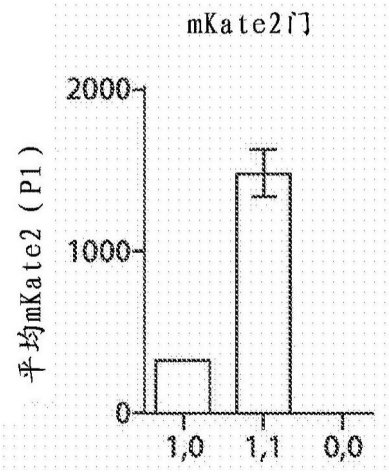
mKate2门
平均mKate2（P1）
2000
1000
0
1,0
1,1
0,0

图9B

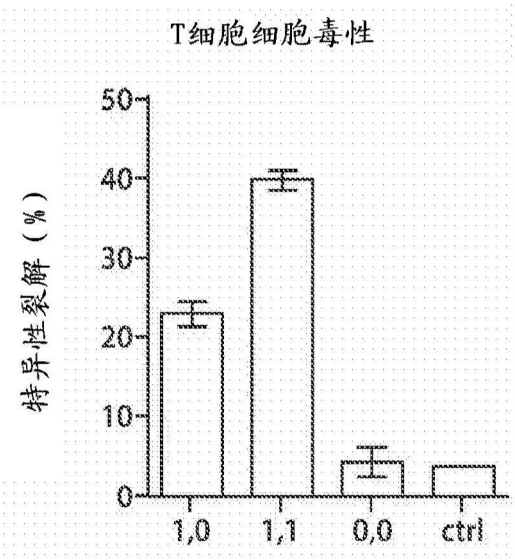
T细胞细胞毒性
特异性裂解（%）
50
40
30
20
10
0
1,0
1,1
0,0
ctrl

图9C

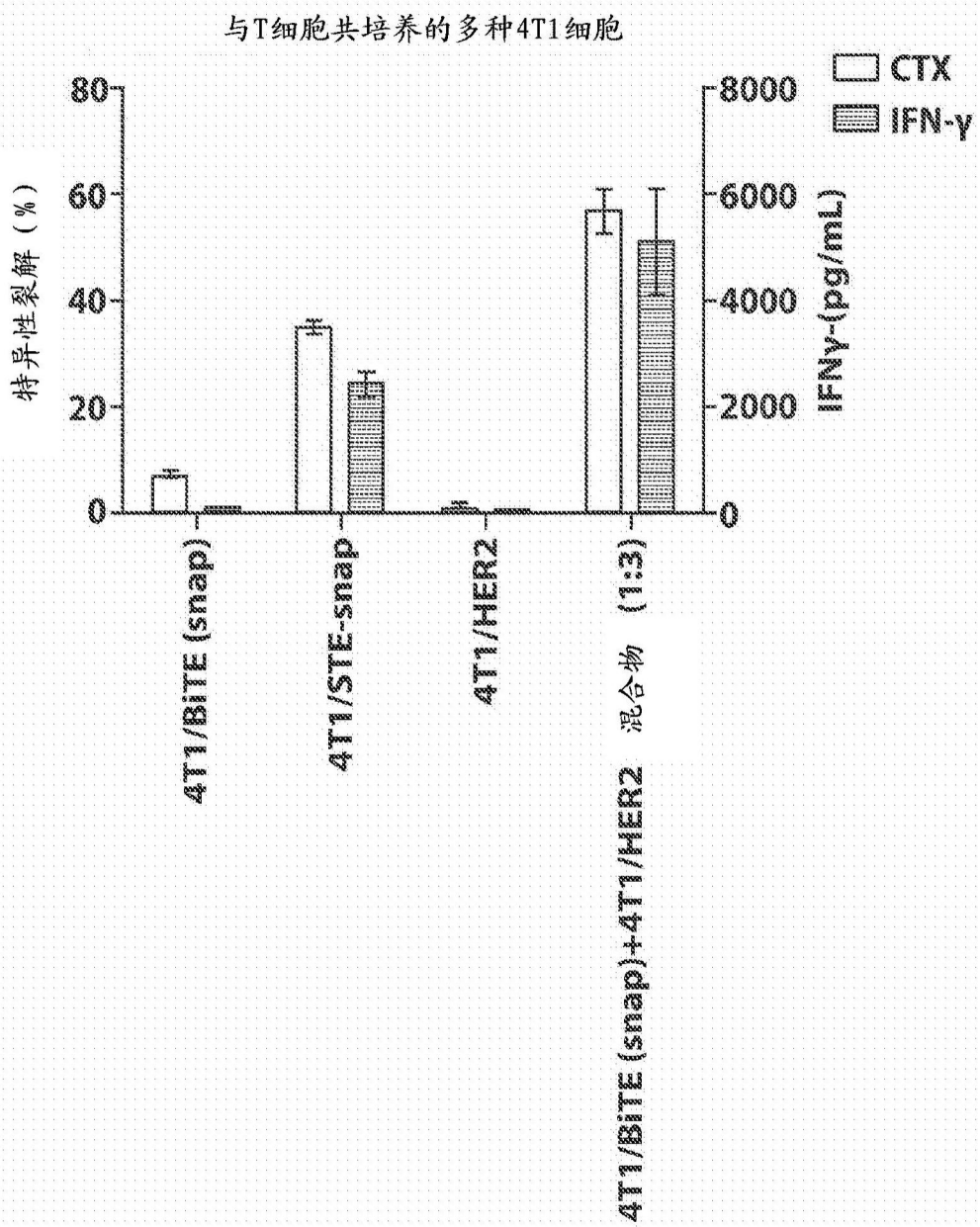
与T细胞共培养的多种4T1细胞
特异性裂解（%）
IFNγ-(pg/mL)
CTX
IFN-γ
80
60
40
20
0
8000
6000
4000
2000
0
4T1/BiTE(snap)
4T1/STE-snap
4T1/HER2
4T1/BiTE(snap)+4T1/HER2 混合物 (1:3)

图10

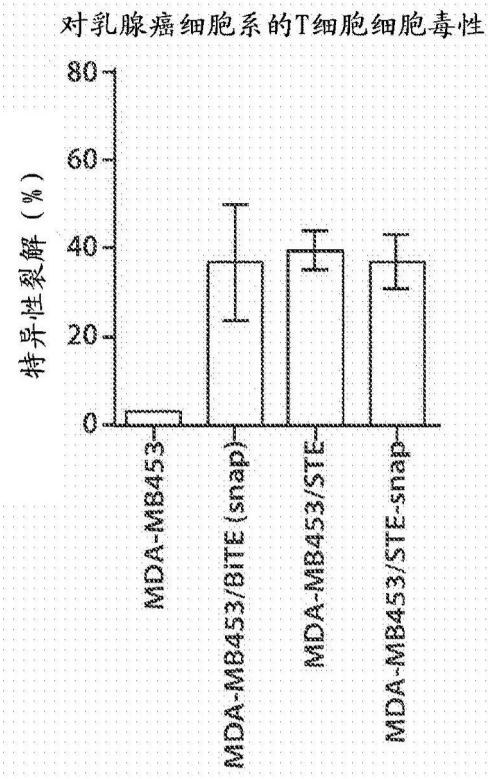
对乳腺癌细胞系的T细胞细胞毒性
特异性裂解（%）
80
60
40
20
0
MDA-MB453
MDA-MB453/BiTE (snap)
MDA-MB453/STE
MDA-MB453/STE-snap

图11

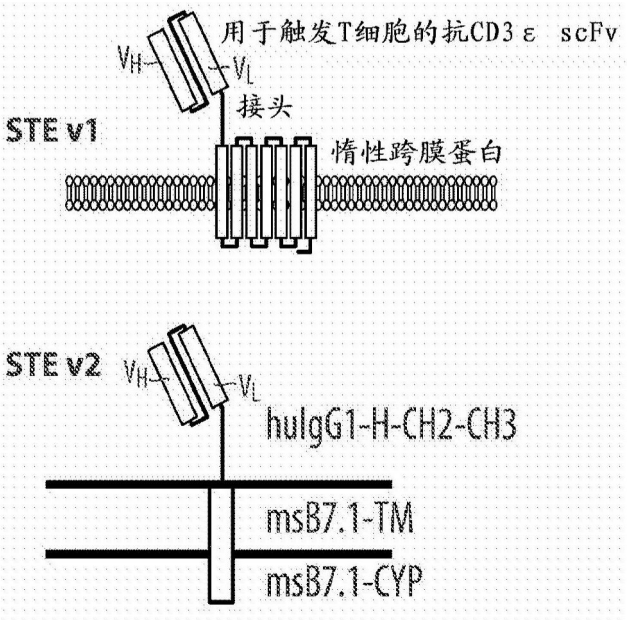
用于触发T细胞的抗CD3 ε scFv
VH
VL
接头
STE v1
惰性跨膜蛋白
STE v2
VH
VL
huIgG1-H-CH2-CH3
msB7.1-TM
msB7.1-CYP

图12

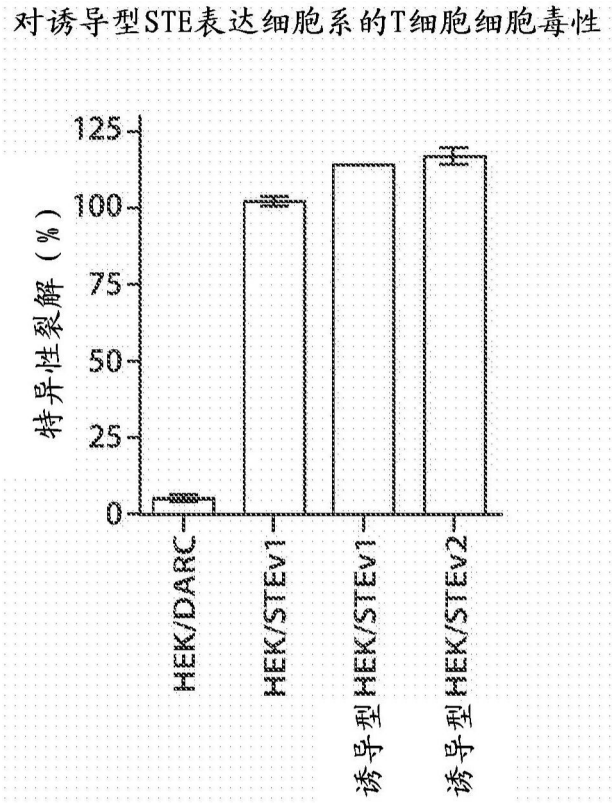
对诱导型STE表达细胞系的T细胞细胞毒性
特异性裂解（%）
125
100
75
50
25
0
HEK/DARC
HEK/STEv1
诱导型HEK/STEv1
诱导型HEK/STEv2

图13

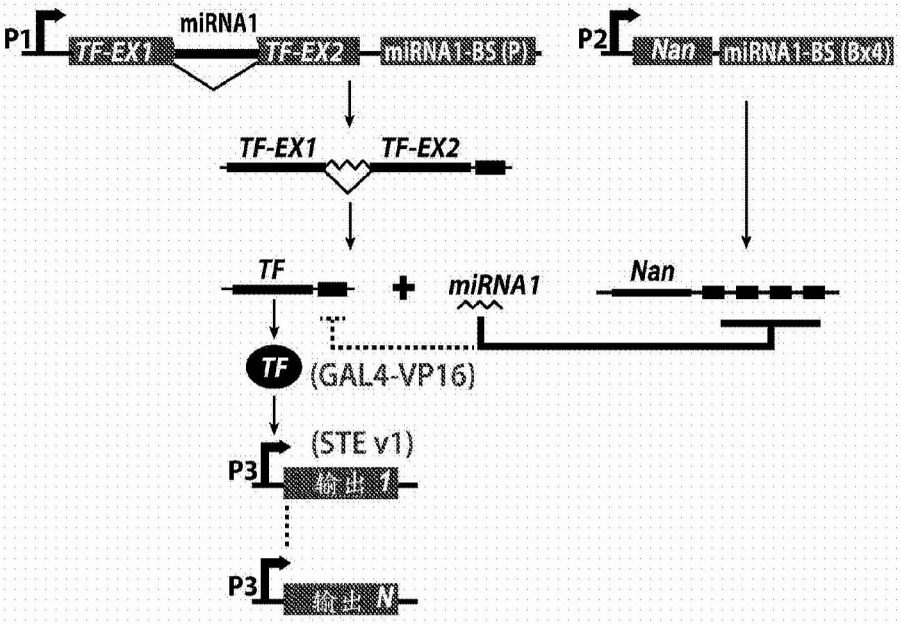
P1
TF-EX1
miRNA1
TF-EX2
miRNA1-BS(P)
P2
Nan
miRNA1-BS(Bx4)
TF-EX1
TF-EX2
TF
miRNA1
Nan
TF
(GAL4-VP16)
(STE v1)
P3
输出 1
P3
输出 N

图14A

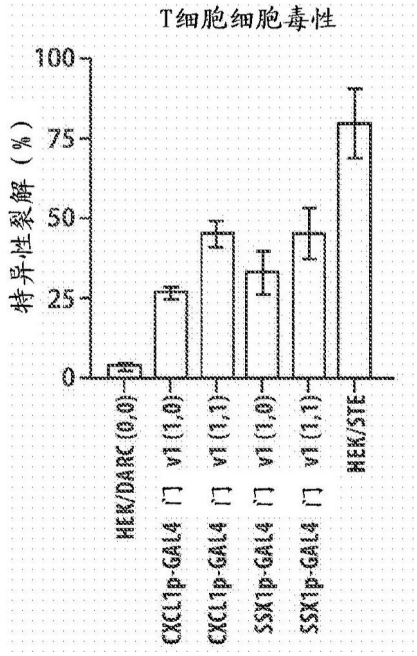
T细胞细胞毒性
特异性裂解（%）
100
75
50
25
0
HEK/DARC (0,0)
v1 (1,0) [] CXCL1p-GAL4
v1 (1,1) [] CXCL1p-GAL4
v1 (1,0) [] SSX1p-GAL4
v1 (1,1) [] SSX1p-GAL4
HEK/STE

图14B

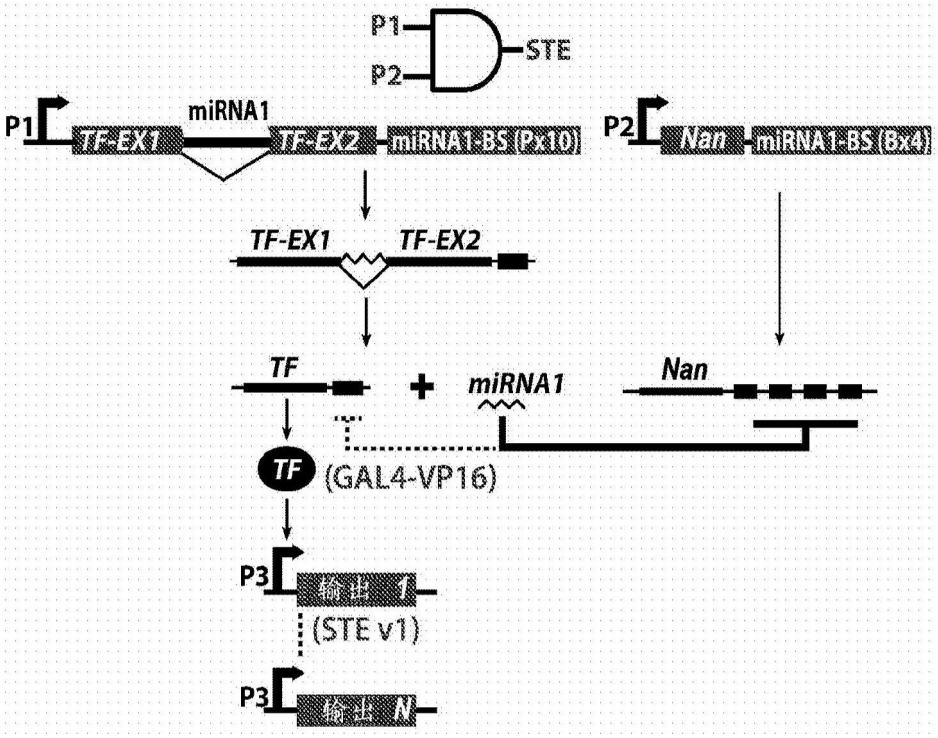
P1
P2
STE
P1
miRNA1
TF-EX1
TF-EX2
miRNA1-BS (Px10)
P2
Nan
miRNA1-BS (Bx4)
TF-EX1
TF-EX2
TF
miRNA1
Nan
TF
(GAL4-VP16)
P3
输出 1
(STE v1)
P3
输出 N

图15A

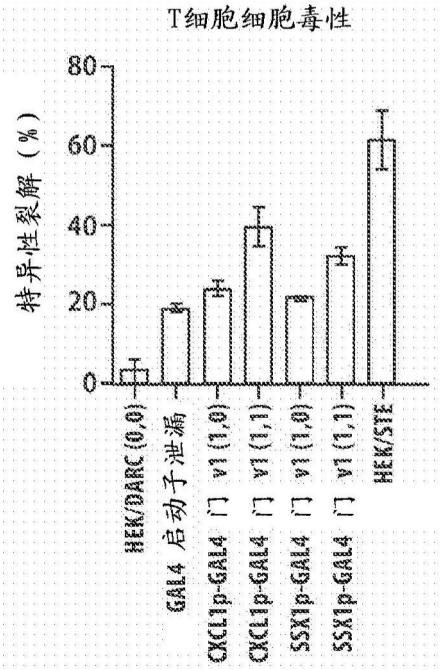
T细胞细胞毒性
特异性裂解（%）
80
60
40
20
0
HEK/DARC(0,0)
GAL4 启动子泄漏
CXCL1p-GAL4 门 v1(1,0)
CXCL1p-GAL4 门 v1(1,1)
SSX1p-GAL4 门 v1(1,0)
SSX1p-GAL4 门 v1(1,1)
HEK/STE

图15B

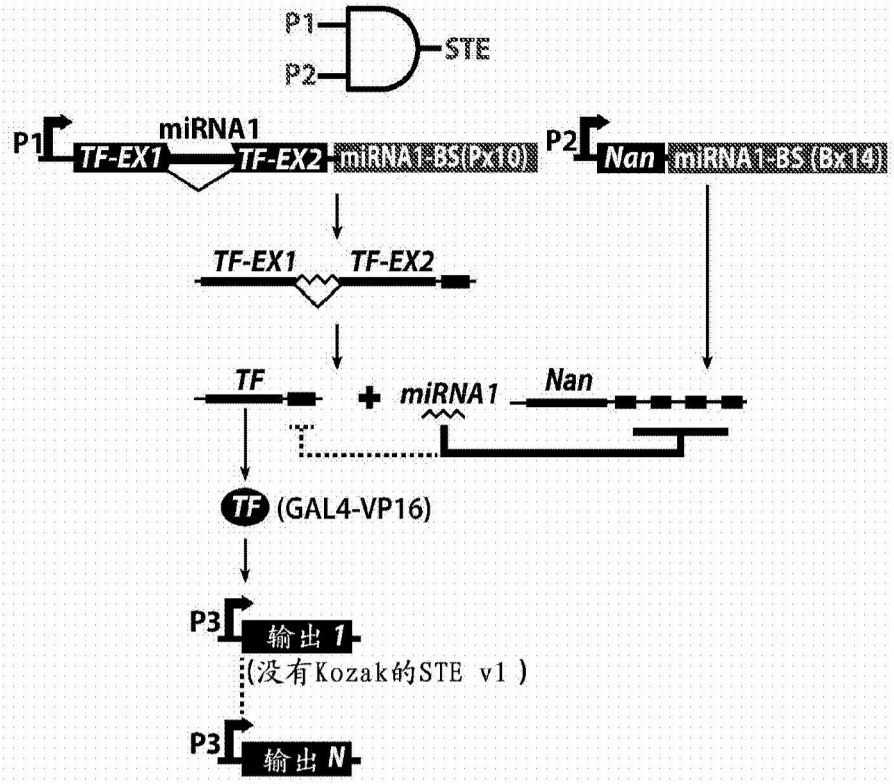
P1
P2
STE
P1
miRNA1
TF-EX1
TF-EX2
miRNA1-BS(Px10)
P2
Nan
miRNA1-BS(Bx14)
TF-EX1
TF-EX2
TF
miRNA1
Nan
TF
(GAL4-VP16)
P3
输出 1
(没有Kozak的STE v1)
P3
输出 N

图16A

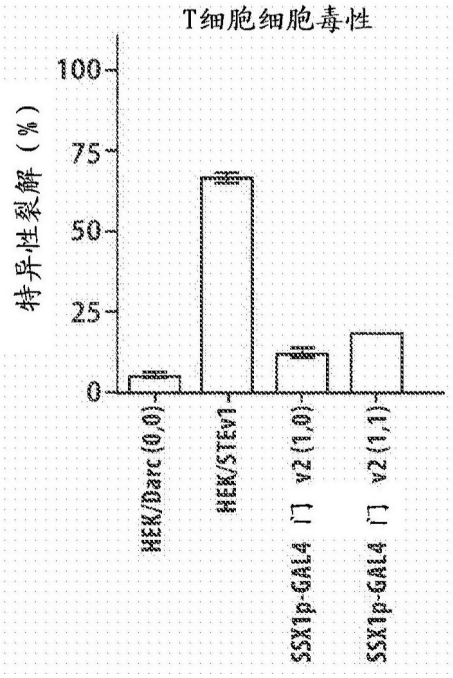
T细胞细胞毒性
特异性裂解（%）
100
75
50
25
0
HEK/Darc(0,0)
HEK/STEv1
SSX1p-GAL4 门 v2(1,0)
SSX1p-GAL4 门 v2(1,1)

图16B

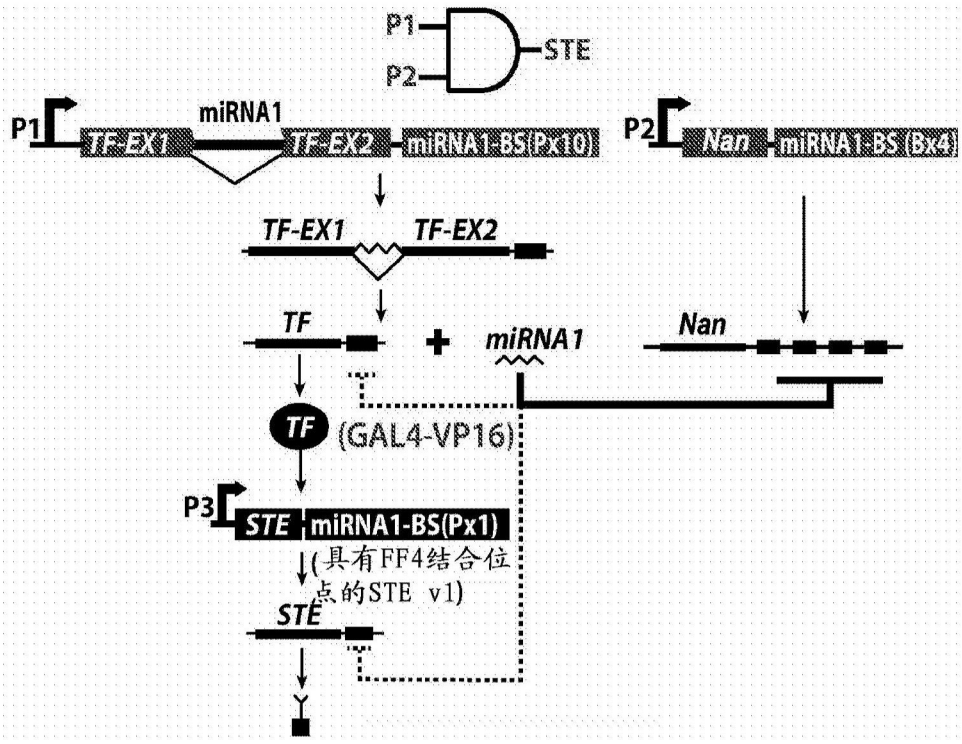
P1
P2
STE
P1
miRNA1
TF-EX1
TF-EX2
miRNA1-BS(Px10)
P2
Nan
miRNA1-BS(Bx4)
TF-EX1
TF-EX2
TF
miRNA1
Nan
TF
(GAL4-VP16)
P3
STE
miRNA1-BS(Px1)
(具有FF4结合位点的STE v1)
STE

图17A

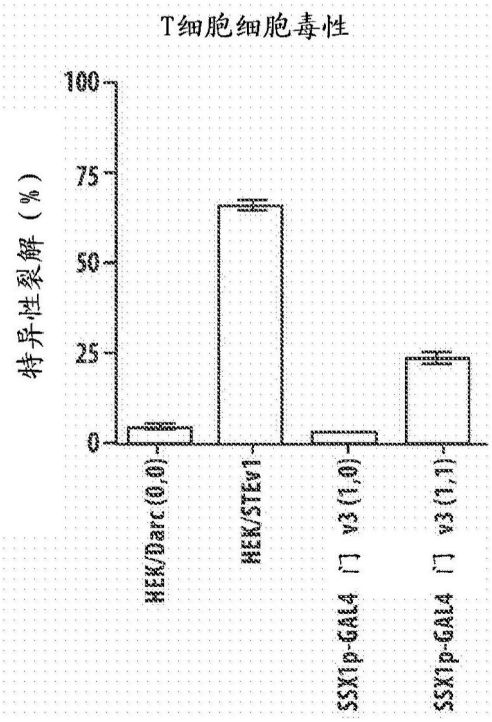
T细胞细胞毒性
特异性裂解（%）
100
75
50
25
0
HEK/Darc(0,0)
HEK/STEv1
SSX1p-GAL4 门 v3(1,0)
SSX1p-GAL4 门 v3(1,1)

图17B

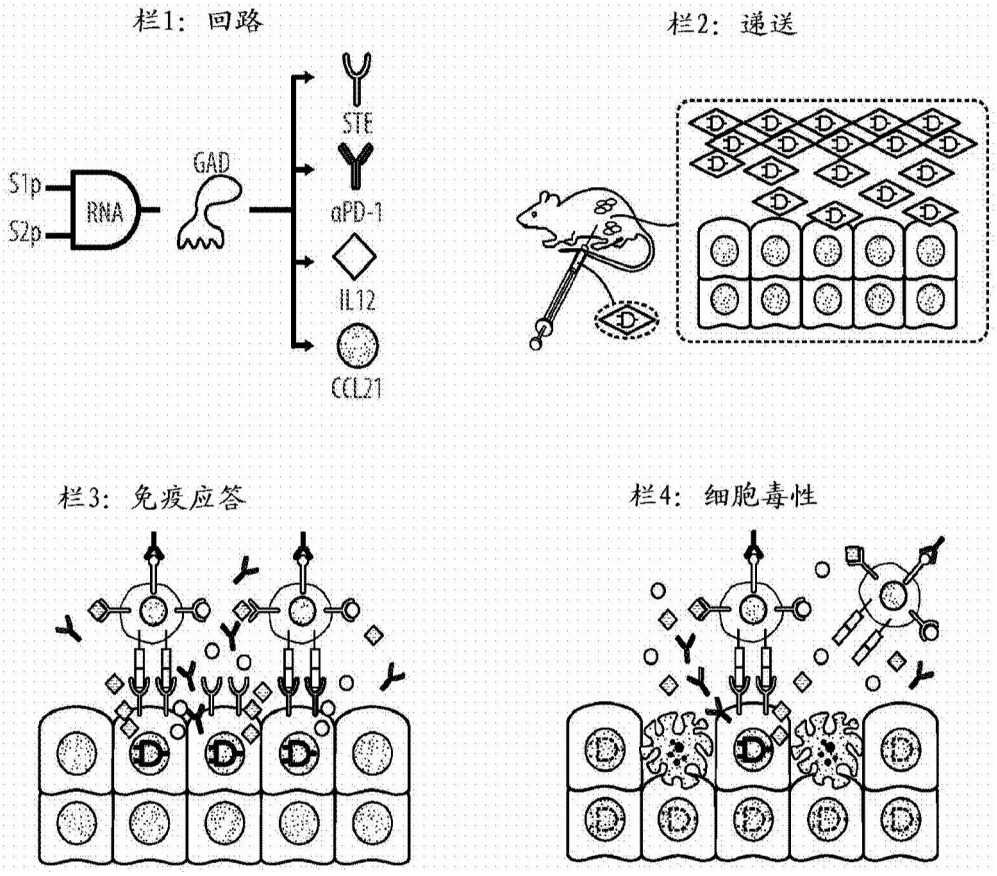
栏1：回路
S1p
S2p
RNA
GAD
STE
αPD-1
IL12
CCL21
栏2：递送
栏3：免疫应答
栏4：细胞毒性

图18

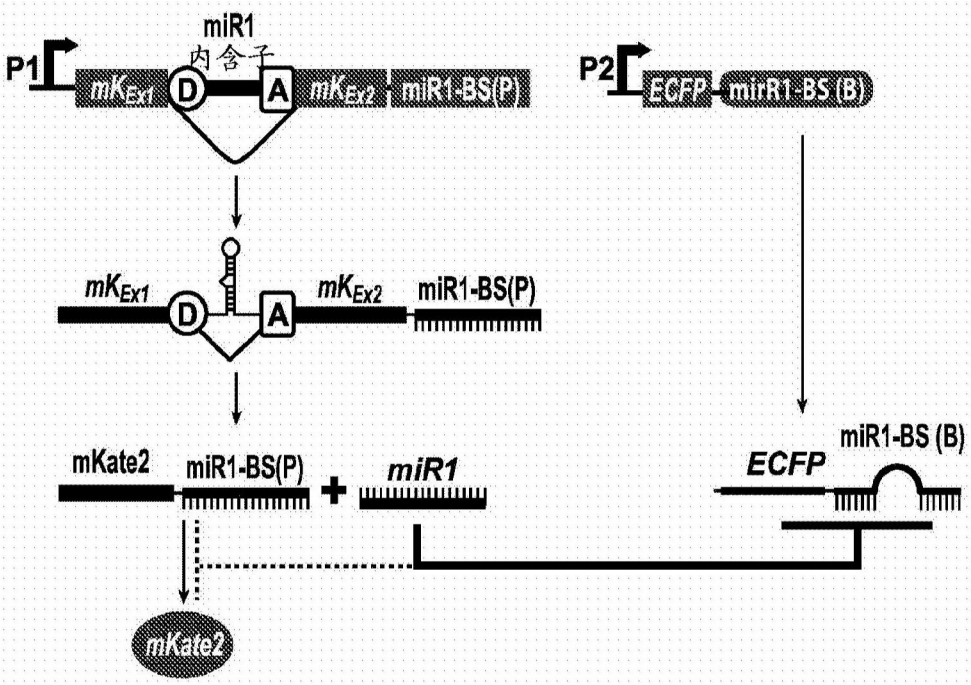
miR1
内含子
P1
mKEx1
D
A
mKEx2
miR1-BS(P)
P2
ECFP
miR1-BS(B)
mKEx1
D
A
mKEx2
miR1-BS(P)
mKate2
miR1-BS(P)
miR1
ECFP
miR1-BS (B)
mKate2

图19

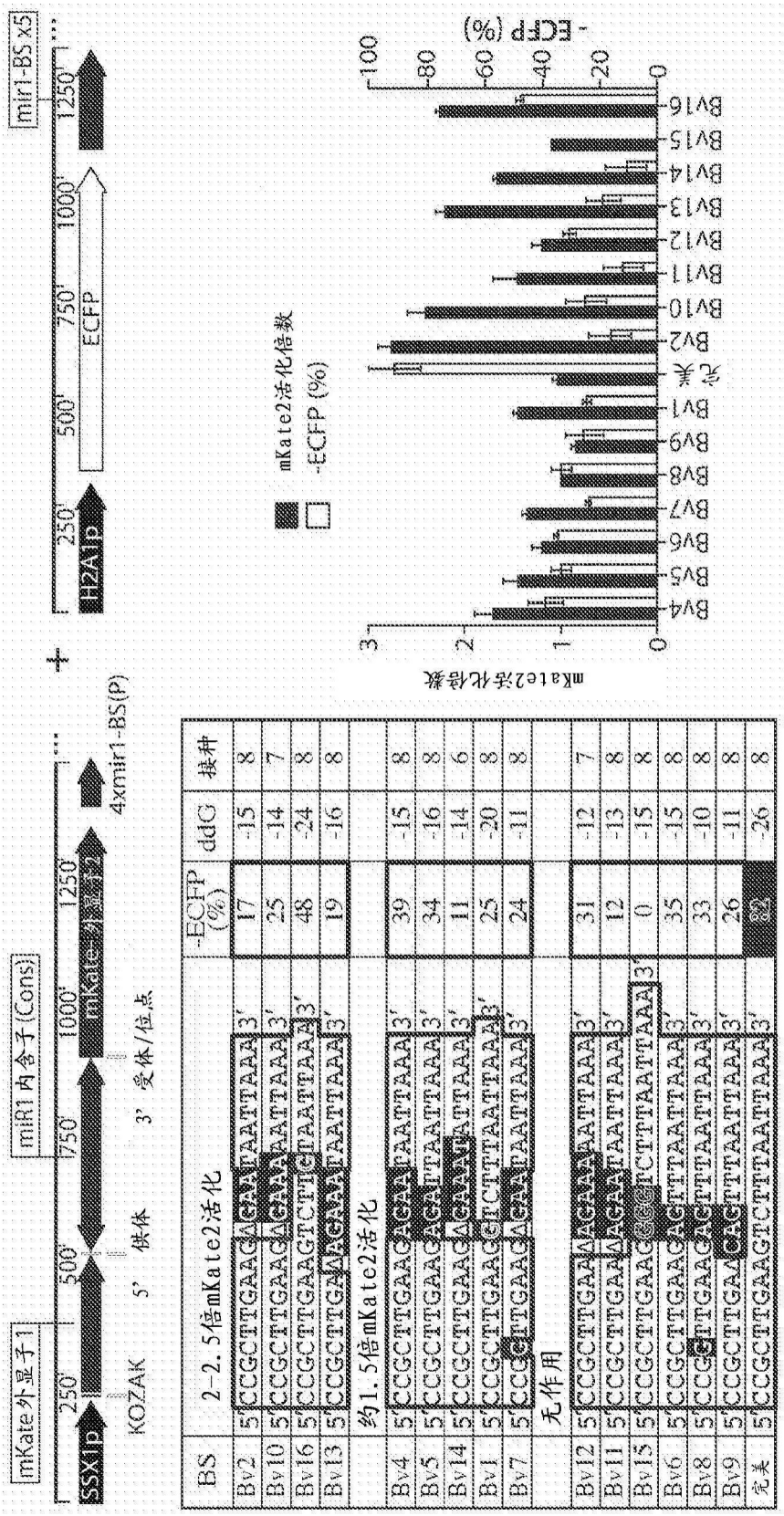
mKate外显子1
miR1 内含子(Cons)
mKate 外显子2
4xmir1-BS(P)
SSX1p
KOZAK
5’ 供体
3’ 受体/位点
H2A1p
ECFP
mir1-BS x5
250’
500’
750’
1000’
1250’
mKate2活化倍数
-ECFP (%)
Bv4
Bv5
Bv14
Bv1
Bv7
Bv12
Bv11
Bv15
Bv6
Bv8
Bv9
Bv2
Bv10
Bv16
Bv13
BS
2-2.5倍mKate2活化
约1.5倍mKate2活化
无作用
-ECFP (%)
ddG
株种
5’CCGCTTGAAGACAATTAAA3’
5’CCGCTTGAAGACAATTAAA3’
5’CCGCTTGAAGACAATTAAA3’
5’CCGCTTGAAGACAATTAAA3’
5’CCGCTTGAAGACAATTAAA3’
5’CCGCTTGAAGACAATTAAA3’
5’CCGCTTGAAGACAATTAAA3’
5’CCGCTTGAAGACAATTAAA3’
5’CCGCTTGAAGACAATTAAA3’
5’CCGCTTGAAGACAATTAAA3’
5’CCGCTTGAAGACAATTAAA3’
5’CCGCTTGAAGACAATTAAA3’
5’CCGCTTGAAGACAATTAAA3’
5’CCGCTTGAAGACAATTAAA3’
5’CCGCTTGAAGACAATTAAA3’
17
25
48
19
39
34
11
25
24
31
12
0
35
33
26
82
-15
-14
-24
-16
-15
-16
-14
-20
-11
-12
-13
-15
-15
-10
-11
-26
8
7
8
8
8
8
6
8
8
7
8
8
8
8
8
8

图20

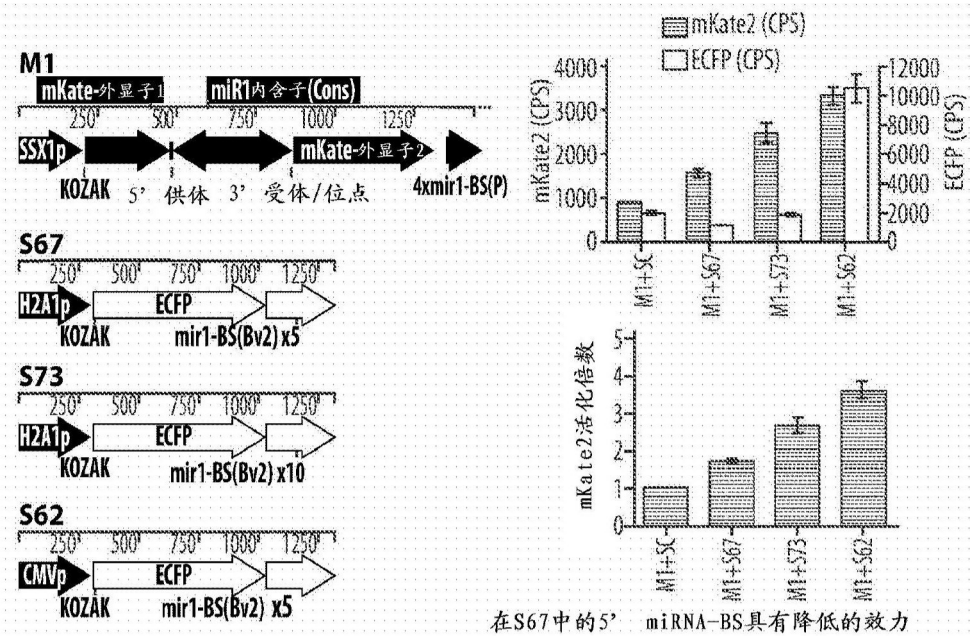
M1
mKate-外显子1
miR1内含子(Cons)
SSX1p
mKate-外显子2
KOZAK
5' 供体
3' 受体/位点
4xmir1-BS(P)
S67
H2A1p
ECFP
KOZAK
mir1-BS(Bv2)x5
S73
mir1-BS(Bv2)x10
S62
CMVp
mir1-BS(Bv2) x5
mKate2 (CPS)
ECFP (CPS)
M1+SC
M1+S67
M1+S73
M1+S62
mKate2活化倍数
在S67中的5' miRNA-BS具有降低的效力

图21

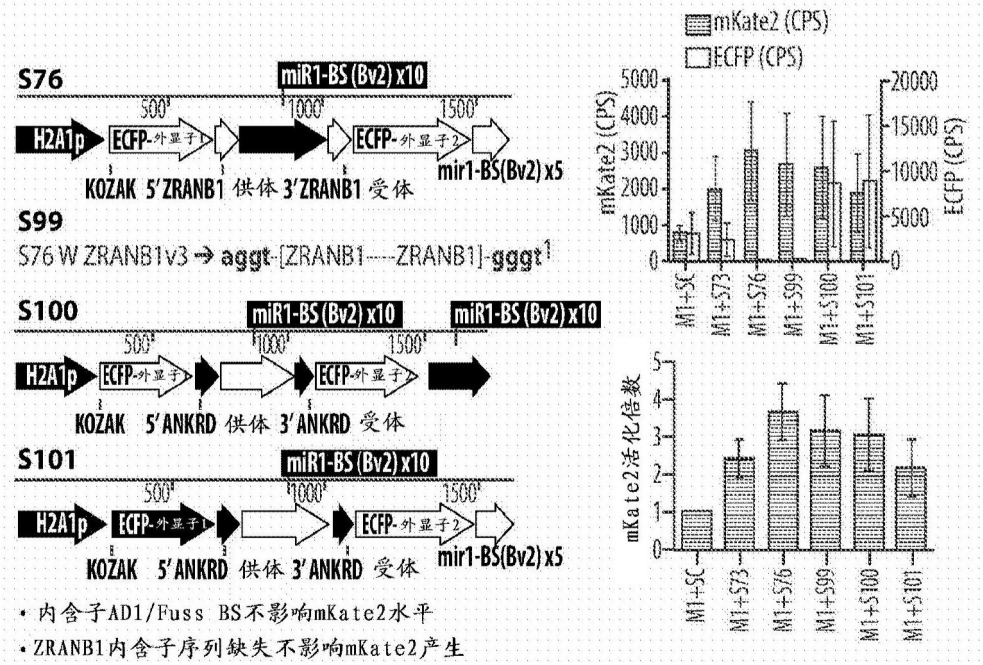
S76
miR1-BS(Bv2) x10
H2A1p
ECFP-外显子1
ECFP-外显子2
mir1-BS(Bv2)x5
KOZAK 5'ZRANB1 供体 3'ZRANB1 受体
S99
S76 W ZRANB1v3 → aggt-[ZRANB1----ZRANB1]-gggt
S100
KOZAK 5'ANKRD 供体 3'ANKRD 受体
S101
• 内含子AD1/Fuss BS不影响mKate2水平
• ZRANB1内含子序列缺失不影响mKate2产生
mKate2 (CPS)
ECFP (CPS)
M1+SC
M1+S73
M1+S76
M1+S99
M1+S100
M1+S101
mKate2活化倍数

图22

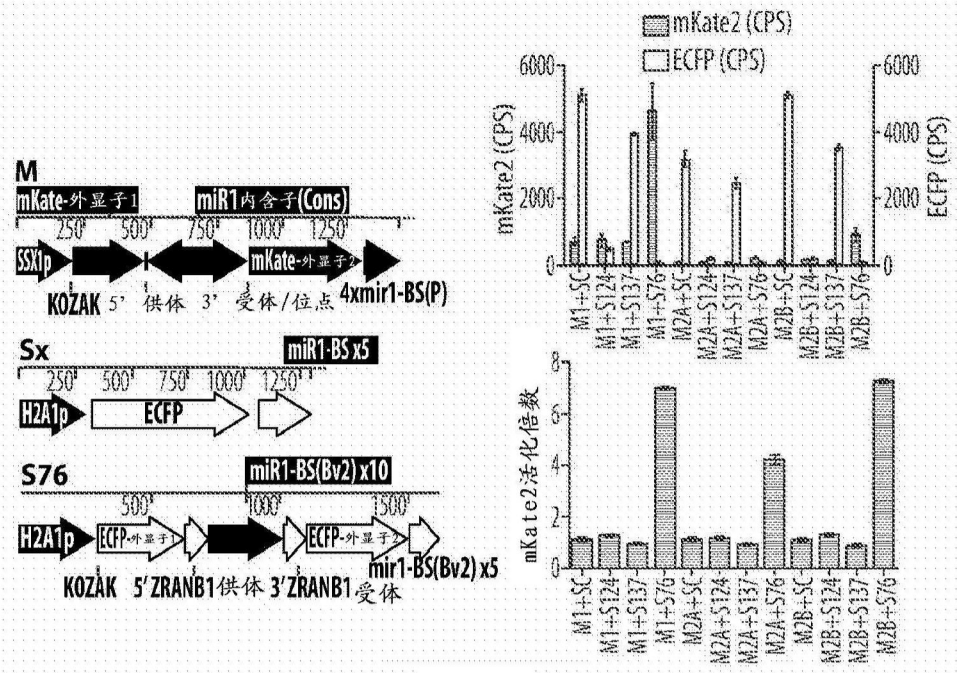

图23

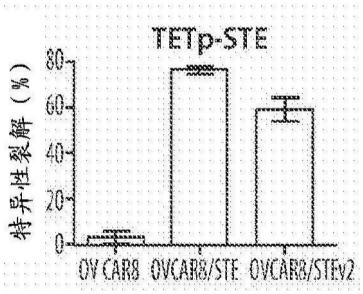

图24A

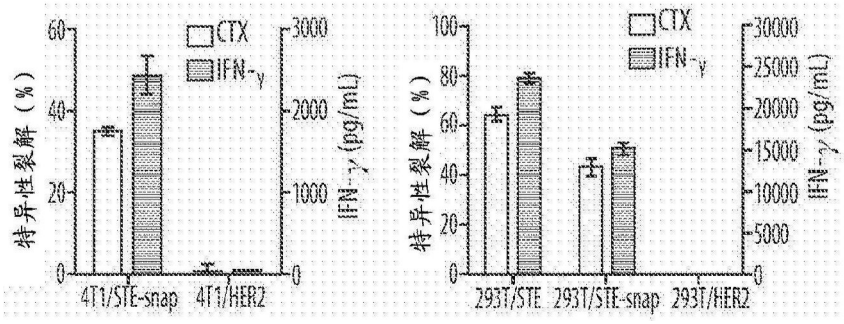

图24B

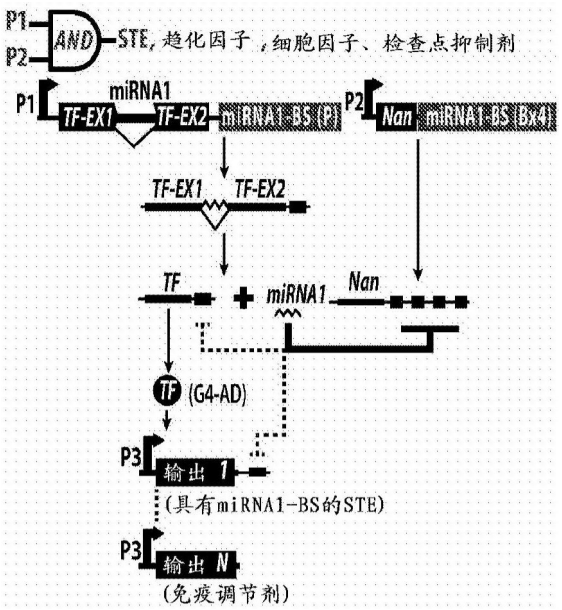
P1
P2
AND
STE,趋化因子,细胞因子、检查点抑制剂
miRNA1
P1
TF-EX1
TF-EX2
miRNA1 BS (F)
P2
Nan
miRNA1 BS (Rx4)
TF-EX1
TF-EX2
TF
miRNA1
Nan
TF
(G4-AD)
P3
输出 1
(具有miRNA1-BS的STE)
P3
输出 N
(免疫调节剂)

图25A

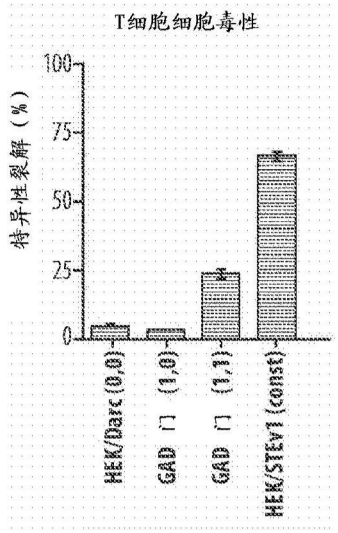
T细胞细胞毒性
特异性裂解（%）
100
75
50
25
0
HEK/Darc (0,0)
GAD (1,0)
GAD (1,1)
HEK/STEv1 (const)

图25B

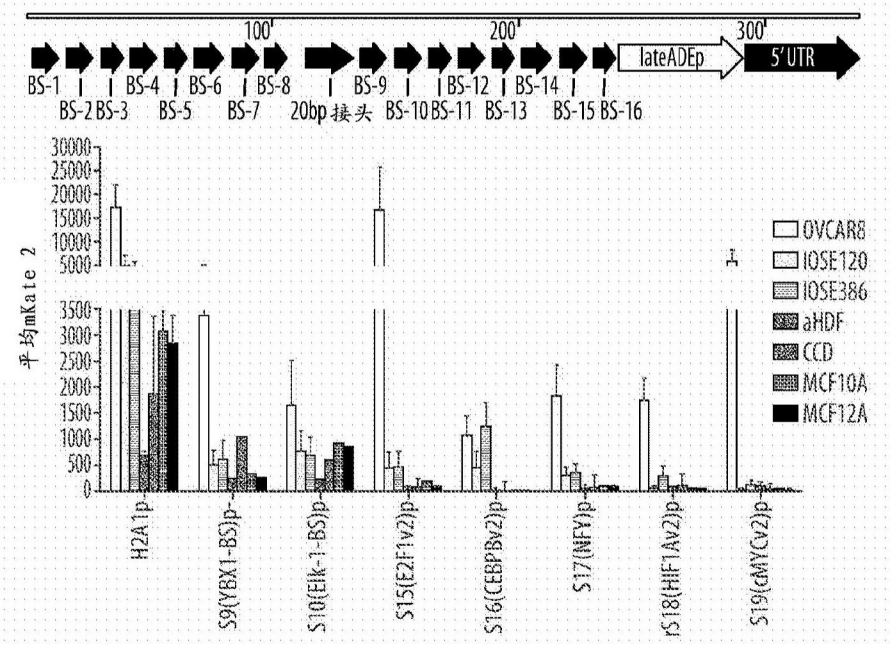
100'
200'
300'
lateADEp
5'UTR
BS-1
BS-2
BS-3
BS-4
BS-5
BS-6
BS-7
BS-8
20bp接头
BS-9
BS-10
BS-11
BS-12
BS-13
BS-14
BS-15
BS-16
平均mKate 2
OVCAR8
IOSE120
IOSE386
aHDF
CCD
MCF10A
MCF12A
H2A1p
S9(YBX1-BS)p
S10(Elk-1-BS)p
S15(E2F1v2)p
S16(CEBPBv2)p
S17(NFY)p
rS18(HIF1Av2)p
S19(cMYCv2)p

图26A

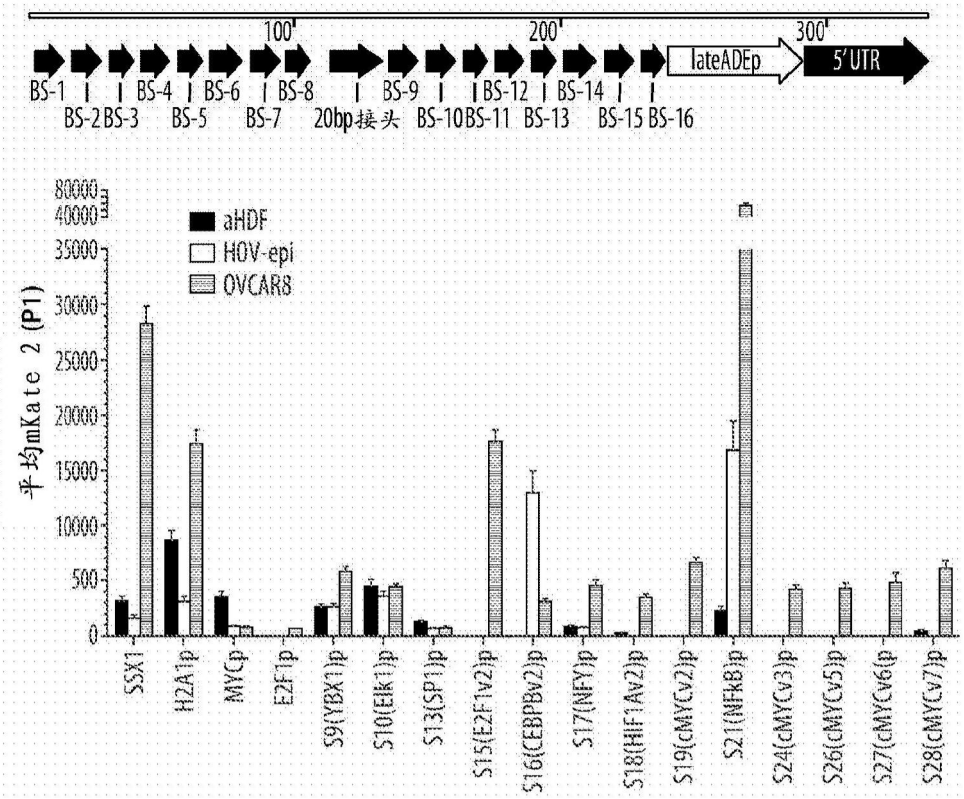
100'
200'
300'
lateADEp
5'UTR
BS-1
BS-2
BS-3
BS-4
BS-5
BS-6
BS-7
BS-8
20bp接头
BS-9
BS-10
BS-11
BS-12
BS-13
BS-14
BS-15
BS-16
平均mKate 2 (P1)
aHDF
HOV-epi
OVCAR8
SSX1
H2A1p
MYCp
E2F1p
S9(YBX1)p
S10(Elk1)p
S13(SP1)p
S15(E2F1v2)p
S16(CEBPBv2)p
S17(NFY)p
S18(HIF1Av2)p
S19(cMYCv2)p
S21(NFkB)p
S24(cMYCv3)p
S26(cMYCv5)p
S27(cMYCv6)p
S28(cMYCv7)p

图26B

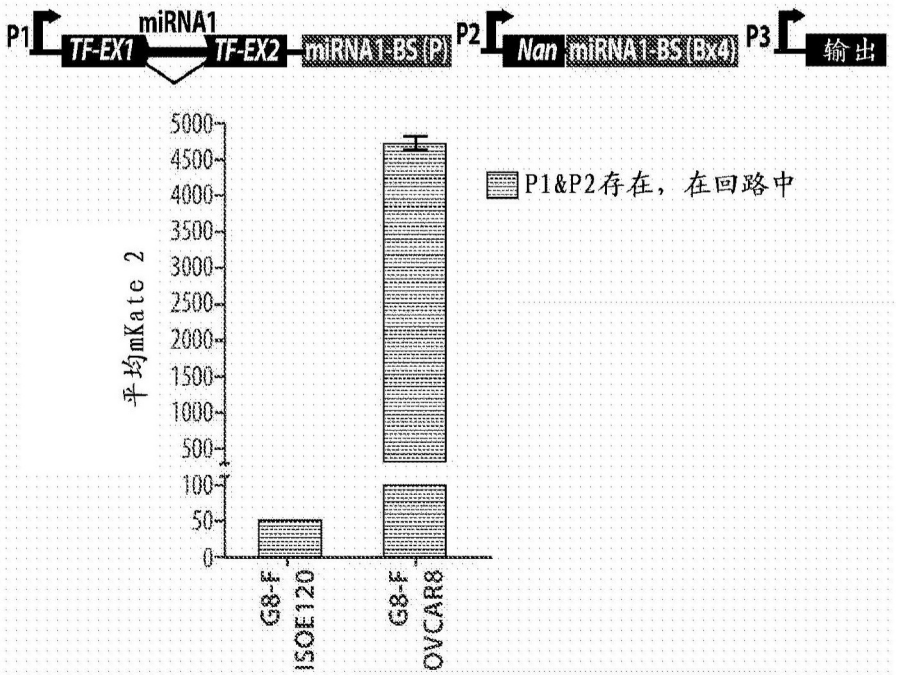
P1
miRNA1
TF-EX1
TF-EX2
miRNA1-BS(P)
P2
Nan
miRNA1-BS(Bx4)
P3
输出
平均mKate 2
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
100
50
0
P1&P2存在，在回路中
G8-F ISOE120
G8-F OVCAR8

图27

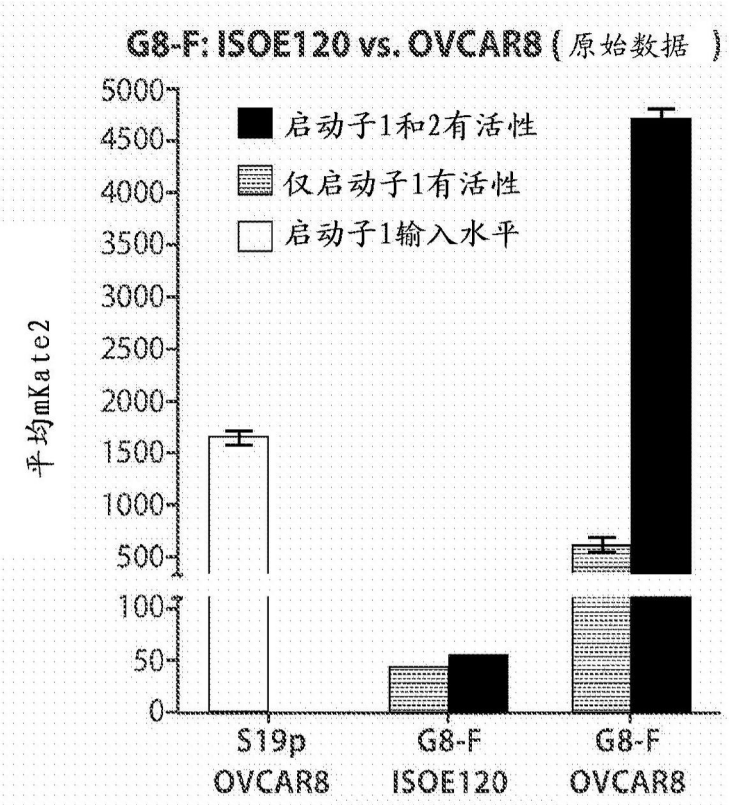
G8-F: ISOE120 vs. OVCAR8 (原始数据)
平均mKate2
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
100
50
0
启动子1和2有活性
仅启动子1有活性
启动子1输入水平
S19p OVCAR8
G8-F ISOE120
G8-F OVCAR8

图28

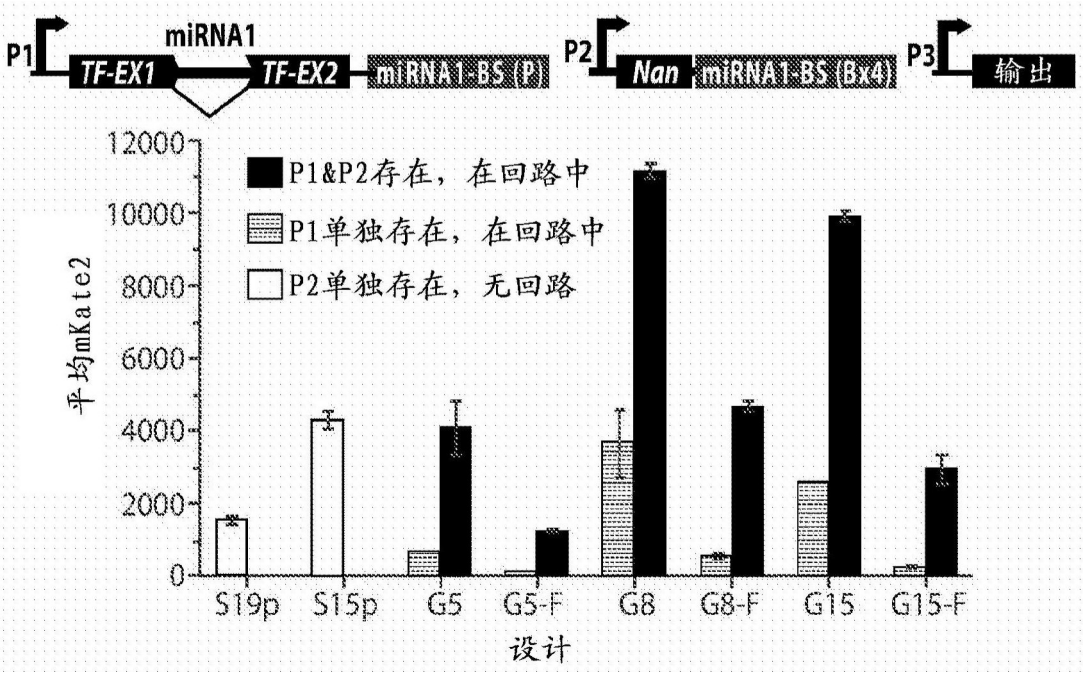
P1
miRNA1
TF-EX1
TF-EX2
miRNA1-BS (P)
P2
Nan
miRNA1-BS (Bx4)
P3
输出
12000
10000
8000
6000
4000
2000
0
平均mKate2
P1&P2存在，在回路中
P1单独存在，在回路中
P2单独存在，无回路
S19p
S15p
G5
G5-F
G8
G8-F
G15
G15-F
设计

图29

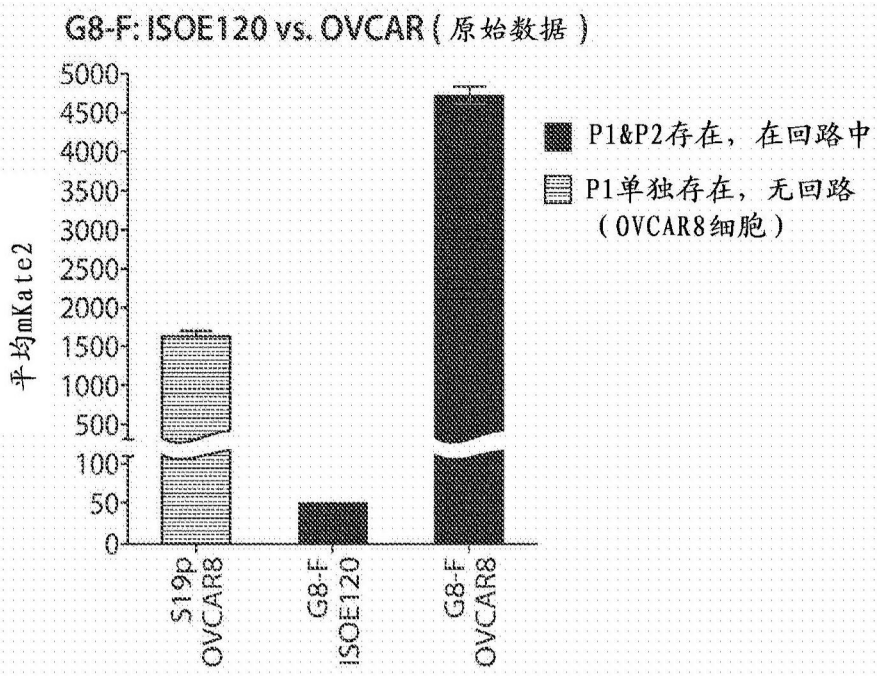
G8-F: ISOE120 vs. OVCAR (原始数据)
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
100
50
0
平均mKate2
P1&P2存在，在回路中
P1单独存在，无回路
(OVCAR8细胞)
S19p
OVCAR8
G8-F
ISOE120
G8-F
OVCAR8

图30

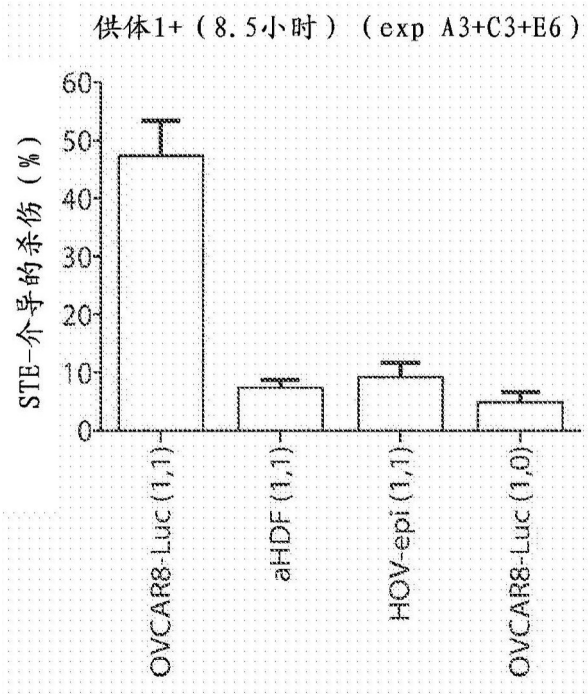
供体1+（8.5小时）（exp A3+C3+E6）
STE-介导的杀伤（%）
60
50
40
30
20
10
0
OVCAR8-Luc (1,1)
aHDF (1,1)
HOV-epi (1,1)
OVCAR8-Luc (1,0)

图31A

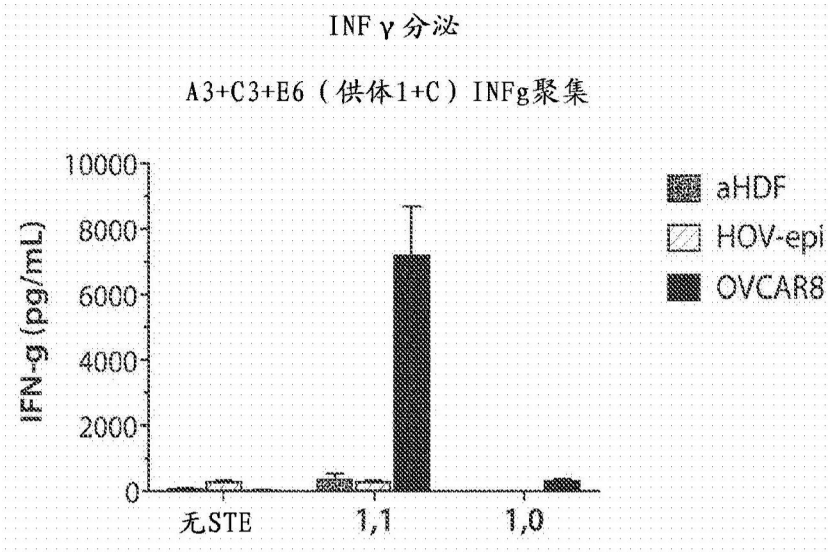
INFγ分泌
A3+C3+E6（供体1+C）INFg聚集
IFN-g (pg/mL)
10000
8000
6000
4000
2000
0
aHDF
HOV-epi
OVCAR8
无STE
1,1
1,0

图31B

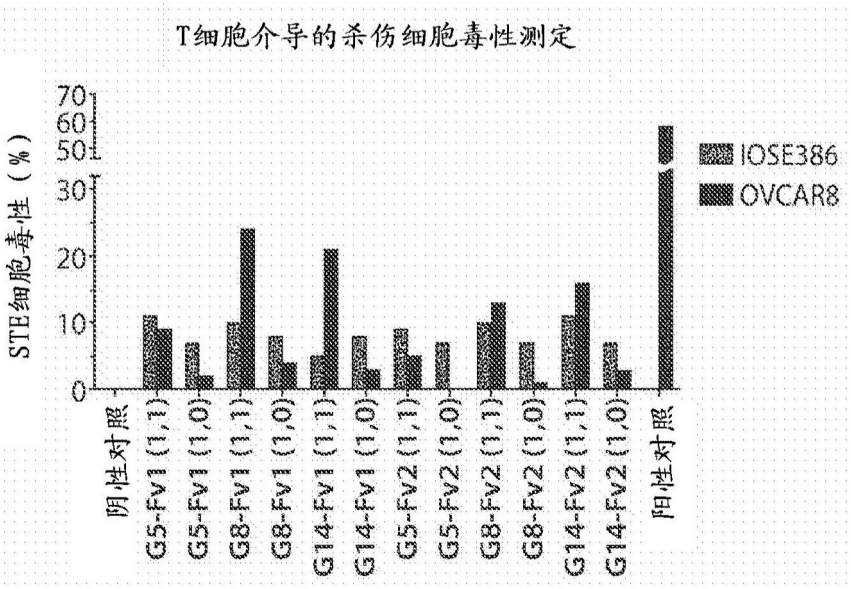
T细胞介导的杀伤细胞毒性测定
STE细胞毒性（%）
70
60
50
30
20
10
0
IOSE386
OVCAR8
阴性对照
G5-Fv1 (1,1)
G5-Fv1 (1,0)
G8-Fv1 (1,1)
G8-Fv1 (1,0)
G14-Fv1 (1,1)
G14-Fv1 (1,0)
G5-Fv2 (1,1)
G5-Fv2 (1,0)
G8-Fv2 (1,1)
G8-Fv2 (1,0)
G14-Fv2 (1,1)
G14-Fv2 (1,0)
阳性对照

图32

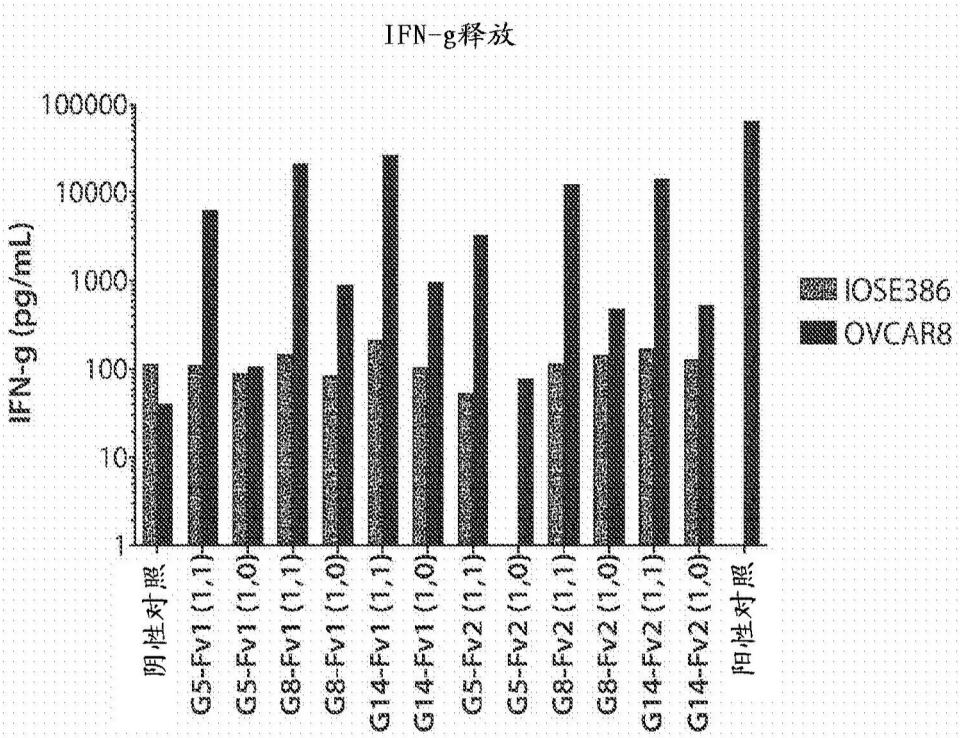
IFN-g释放
IFN-g (pg/mL)
100000
10000
1000
100
10
1
IOSE386
OVCAR8
阴性对照
G5-Fv1 (1,1)
G5-Fv1 (1,0)
G8-Fv1 (1,1)
G8-Fv1 (1,0)
G14-Fv1 (1,1)
G14-Fv1 (1,0)
G5-Fv2 (1,1)
G5-Fv2 (1,0)
G8-Fv2 (1,1)
G8-Fv2 (1,0)
G14-Fv2 (1,1)
G14-Fv2 (1,0)
阳性对照

图33A

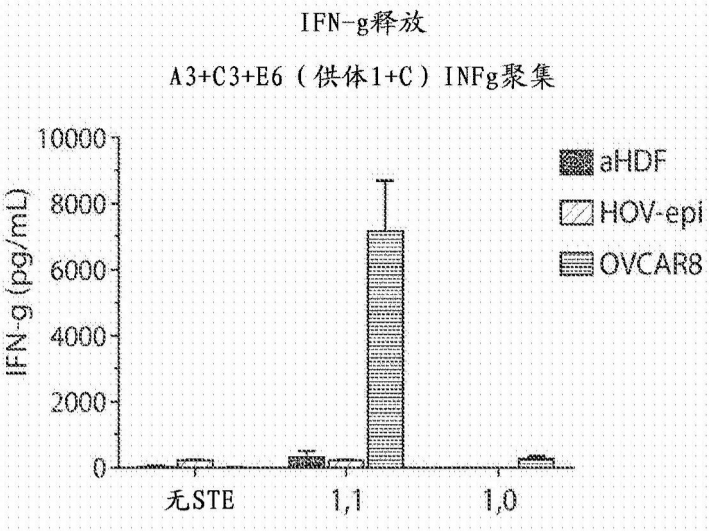
IFN-g释放
A3+C3+E6（供体1+C）INFg聚集
IFN-g (pg/mL)
10000
8000
6000
4000
2000
0
aHDF
HOV-epi
OVCAR8
无STE
1,1
1,0

图33B

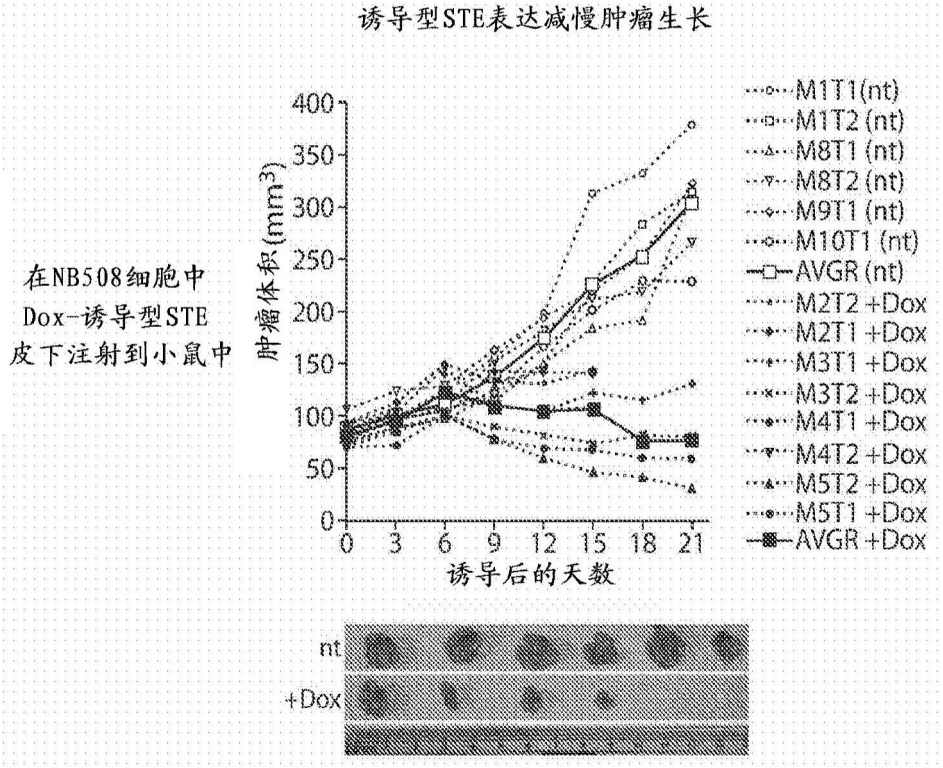
诱导型STE表达减慢肿瘤生长
在NB508细胞中
Dox-诱导型STE
皮下注射到小鼠中
肿瘤体积(mm3)
400
350
300
250
200
150
100
50
0
0 3 6 9 12 15 18 21
诱导后的天数
M1T1(nt)
M1T2 (nt)
M8T1 (nt)
M8T2 (nt)
M9T1 (nt)
M10T1 (nt)
AVGR (nt)
M2T2 +Dox
M2T1 +Dox
M3T1 +Dox
M3T2 +Dox
M4T1 +Dox
M4T2 +Dox
M5T2 +Dox
M5T1 +Dox
AVGR +Dox
nt
+Dox

图34

#	注释	DNA	T细胞	试剂	注解
1	未转染 无T细胞	-	-	-	最大肿瘤生长
2	未转染	-	+	-	MHC-介导的杀伤
3	仅试剂	-	-	+	试剂细胞毒性
4	S15p-GAD	S15p-GAD	+	+	T细胞+试剂细胞毒性
5	S15p-GAD+G8p-STE-F	S15p-GAD+ G8-STE-F	+	+	高STE治疗效力
6	门 (STE)	G8-F 门 , STE	+	+	G8-F 门 + STE
7	门 (STE, L12)	G8F 门 , STE + IL12	+	+	G8-F 门 + STE + IL12
8	门 (STE, CCL21)	G8F 门 , STE + CCL21	+	+	G8-F 门 + STE + CCL21
9	门 (STE, αPD1)	G8F 门 , STE + αPD1	+	+	G8F 门 + STE + αPD1
10	门 (STE, IL12, CCL21, αPD1)	G8F 门 , STE + IL12 + CCL21 + αPD1	+	+	TG8-F 门 + STE + IL12 + CCL21 + αPD1

处理:

- 第0天: IP注射0.5×10^{6} OVCAR-Luc细胞
- 第10天: IP注射DNA/材料[1]
- 第12天: IP注射10×10^{6}人活化的T细胞

读出:

VOS中的肿瘤负荷估计, 总流量[p/s]

图35A

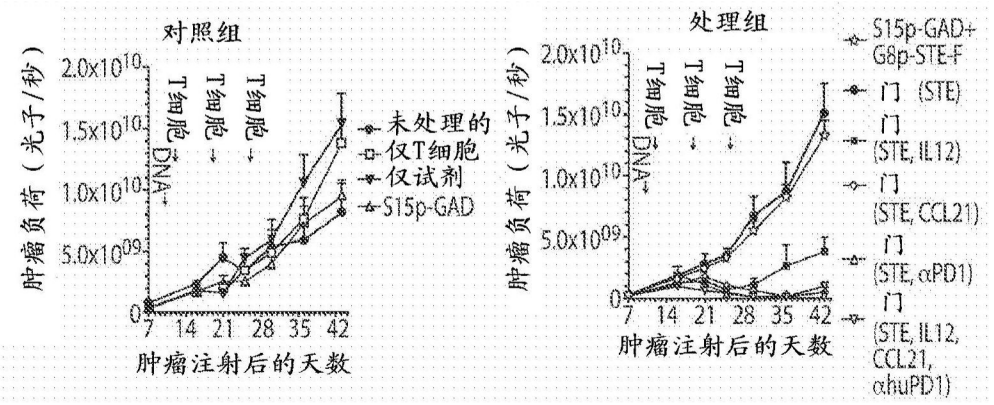

图35B

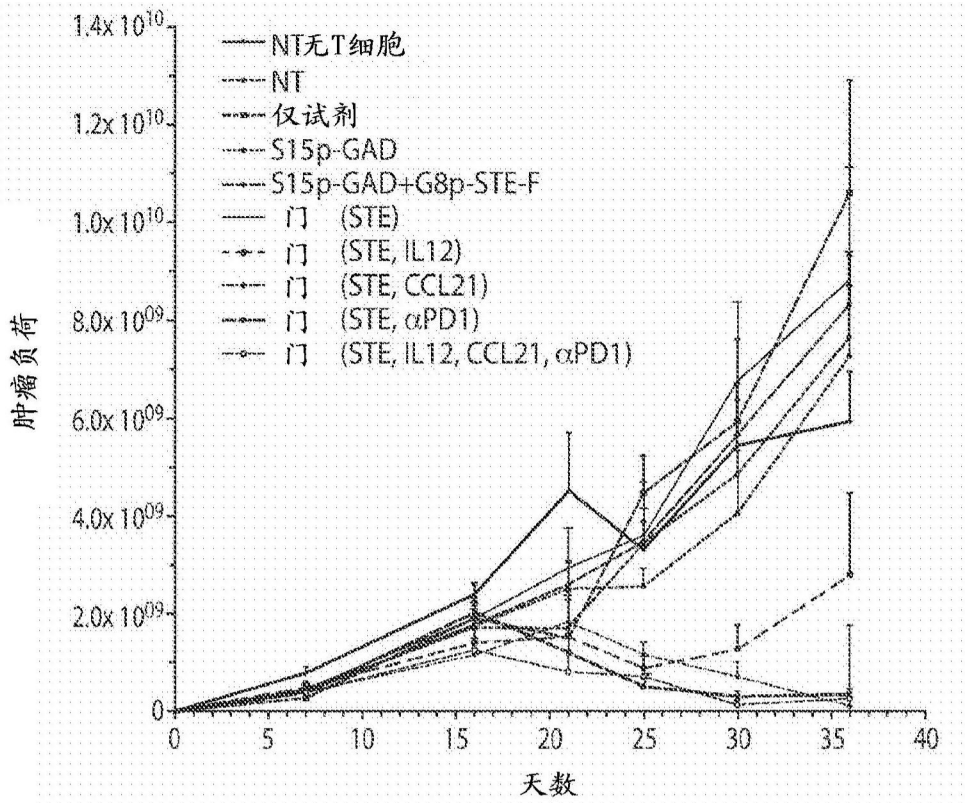
1.4x10^10
1.2x10^10
1.0x10^10
8.0x10^09
6.0x10^09
4.0x10^09
2.0x10^09
0
肿瘤负荷
0
5
10
15
20
25
30
35
40
天数
NT无T细胞
NT
仅试剂
S15p-GAD
S15p-GAD+G8p-STE-F
门 (STE)
门 (STE, IL12)
门 (STE, CCL21)
门 (STE, αPD1)
门 (STE, IL12, CCL21, αPD1)

图36

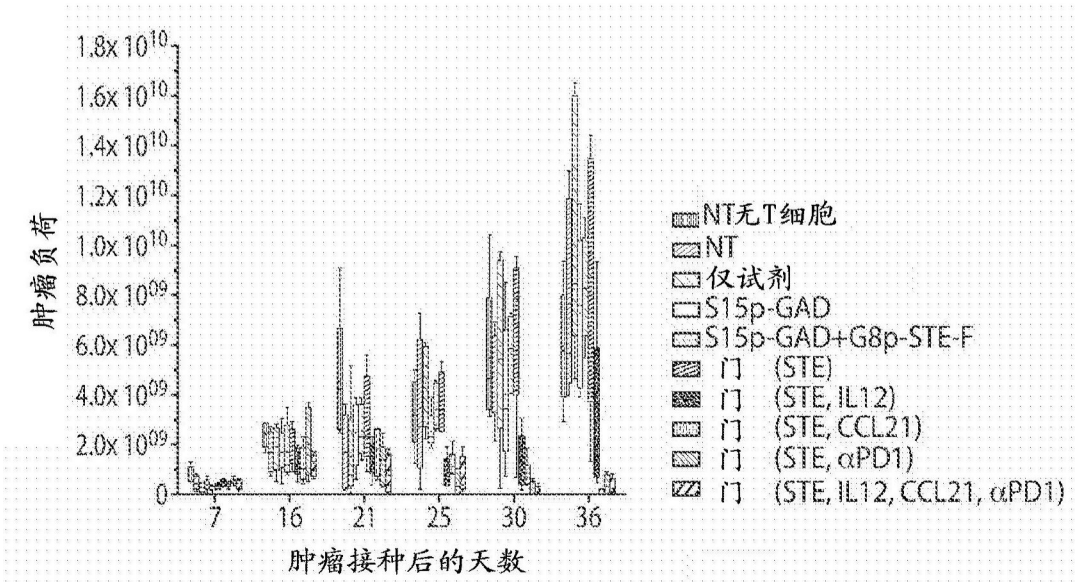
1.8x10^10
1.6x10^10
1.4x10^10
1.2x10^10
1.0x10^10
8.0x10^09
6.0x10^09
4.0x10^09
2.0x10^09
0
肿瘤负荷
7
16
21
25
30
36
肿瘤接种后的天数
NT无T细胞
NT
仅试剂
S15p-GAD
S15p-GAD+G8p-STE-F
门 (STE)
门 (STE, IL12)
门 (STE, CCL21)
门 (STE, αPD1)
门 (STE, IL12, CCL21, αPD1)

图37

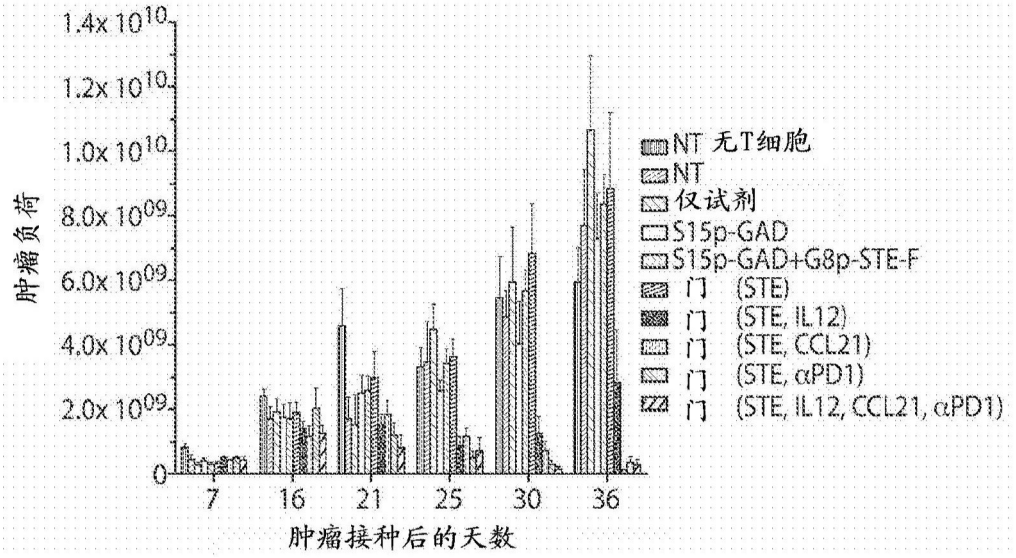
肿瘤负荷
1.4x10^10
1.2x10^10
1.0x10^10
8.0x10^09
6.0x10^09
4.0x10^09
2.0x10^09
0
7
16
21
25
30
36
肿瘤接种后的天数
NT 无T细胞
NT
仅试剂
S15p-GAD
S15p-GAD+G8p-STE-F
门 (STE)
门 (STE, IL12)
门 (STE, CCL21)
门 (STE, αPD1)
门 (STE, IL12, CCL21, αPD1)

图38

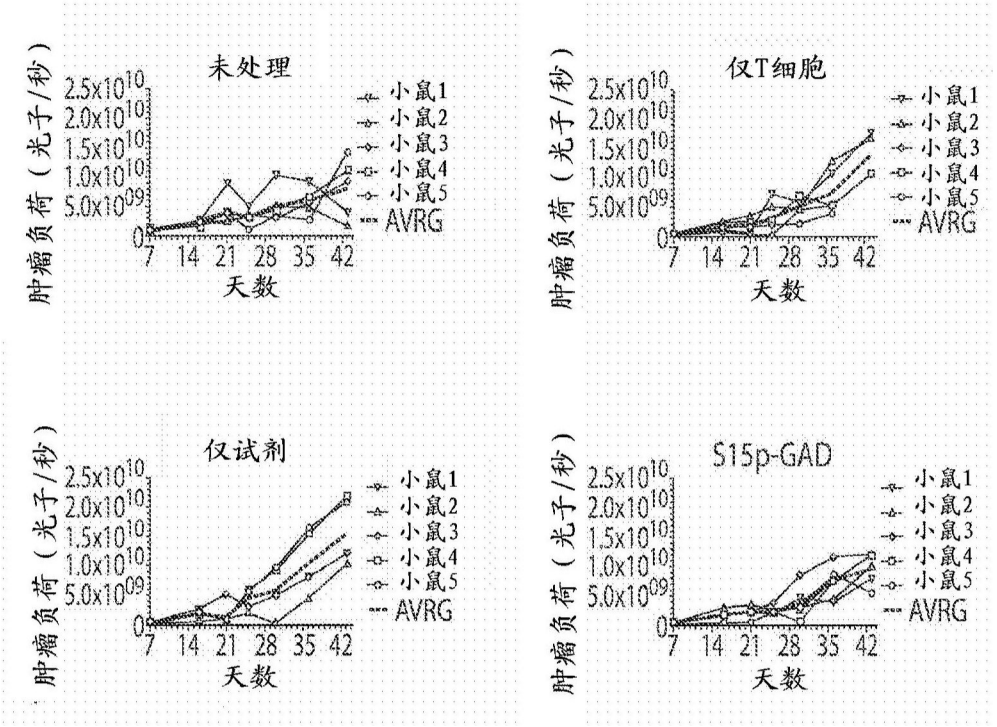
未处理
仅T细胞
仅试剂
S15p-GAD
肿瘤负荷（光子/秒）
2.5x10^10
2.0x10^10
1.5x10^10
1.0x10^10
5.0x10^09
0
7 14 21 28 35 42
天数
小鼠1
小鼠2
小鼠3
小鼠4
小鼠5
AVRG

图39

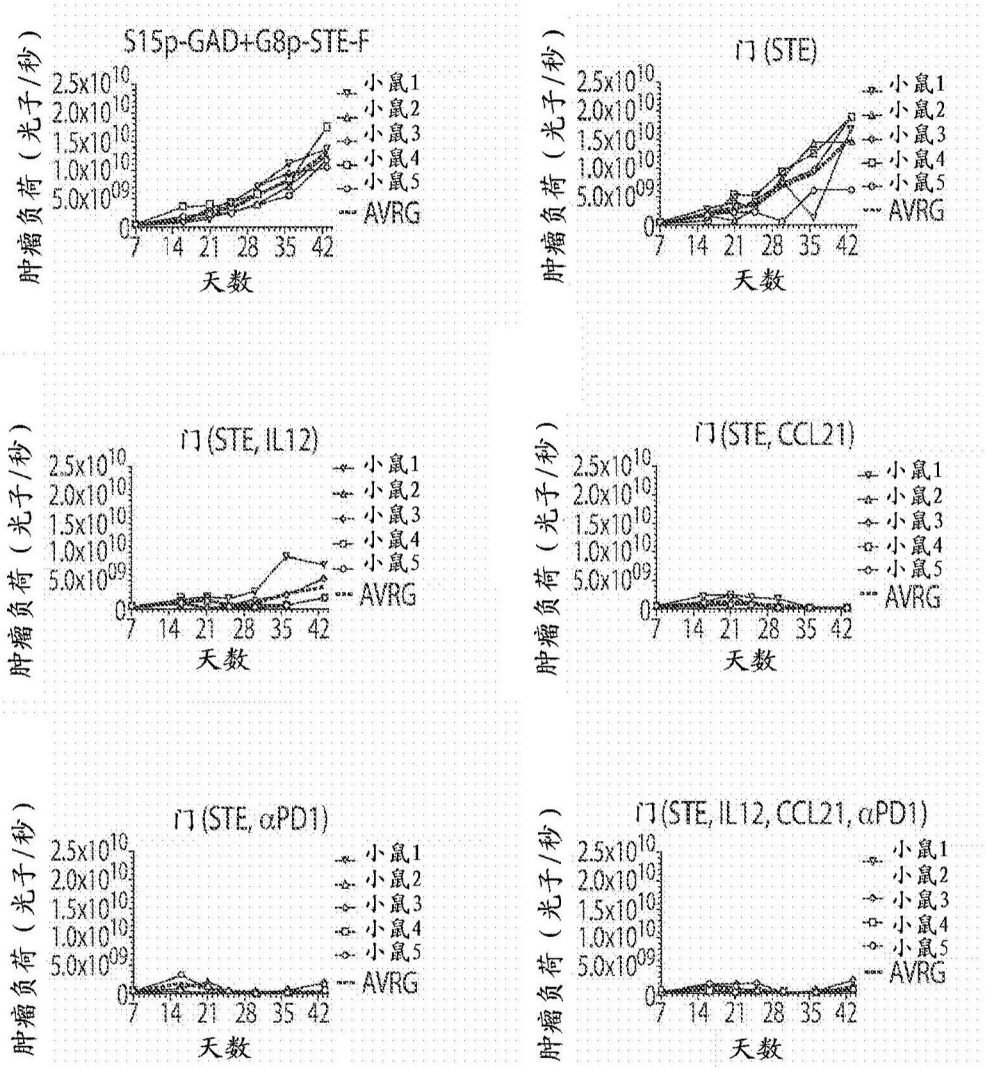
S15p-GAD+G8p-STE-F
门(STE)
门(STE, IL12)
门(STE, CCL21)
门(STE, αPD1)
门(STE, IL12, CCL21, αPD1)
肿瘤负荷（光子/秒）
天数
小鼠1
小鼠2
小鼠3
小鼠4
小鼠5
AVRG

图39(续)

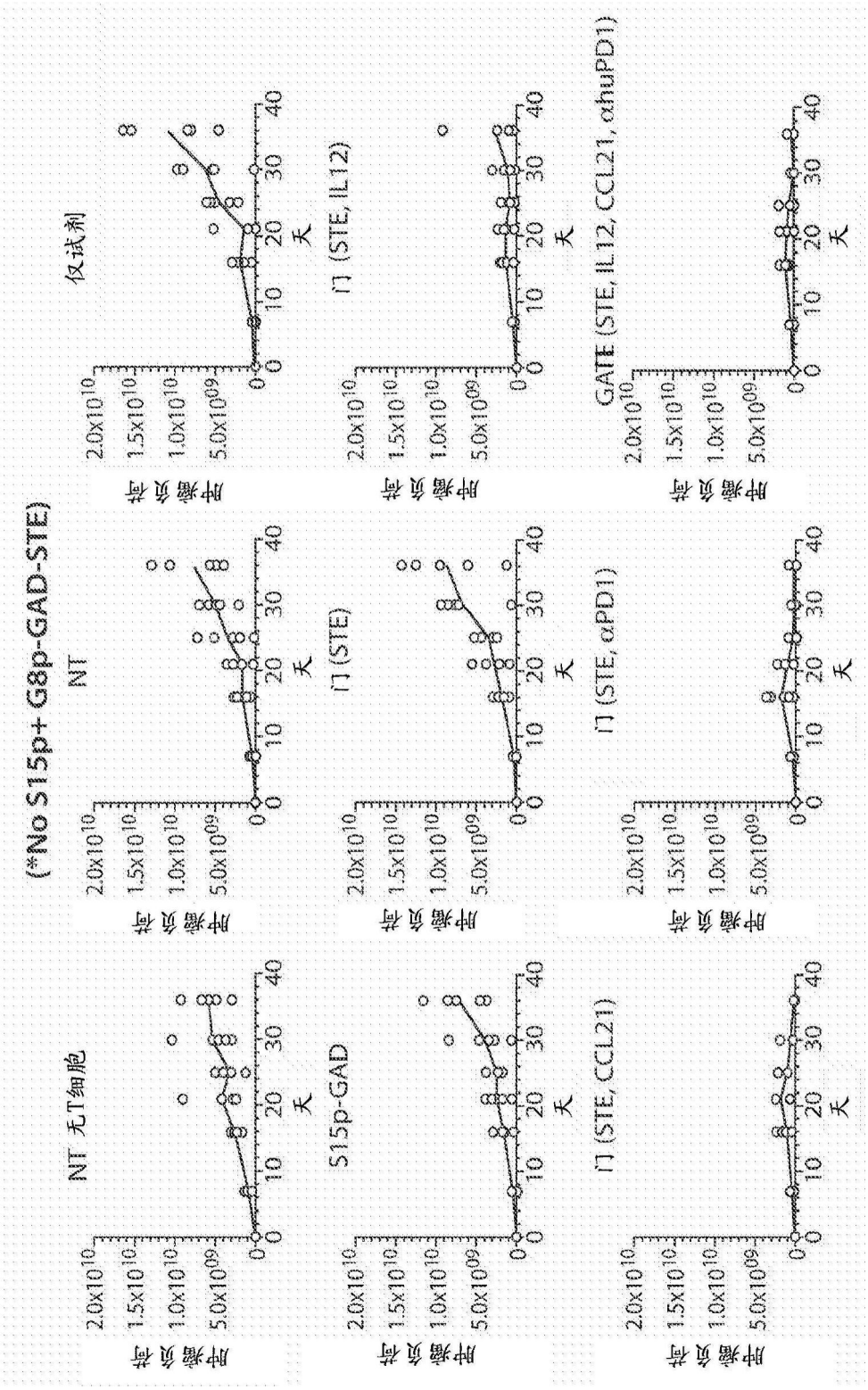
仅试剂
门 (STE, IL12)
GATE (STE, IL12, CCL21, αhuPD1)
(*No S15p+ G8p-GAD-STE)
NT
门 (STE)
门 (STE, αPD1)
NT 无T细胞
S15p-GAD
门 (STE, CCL21)
肿瘤负荷
天
0
10
20
30
40
2.0x10^10
1.5x10^10
1.0x10^10
5.0x10^09

图40

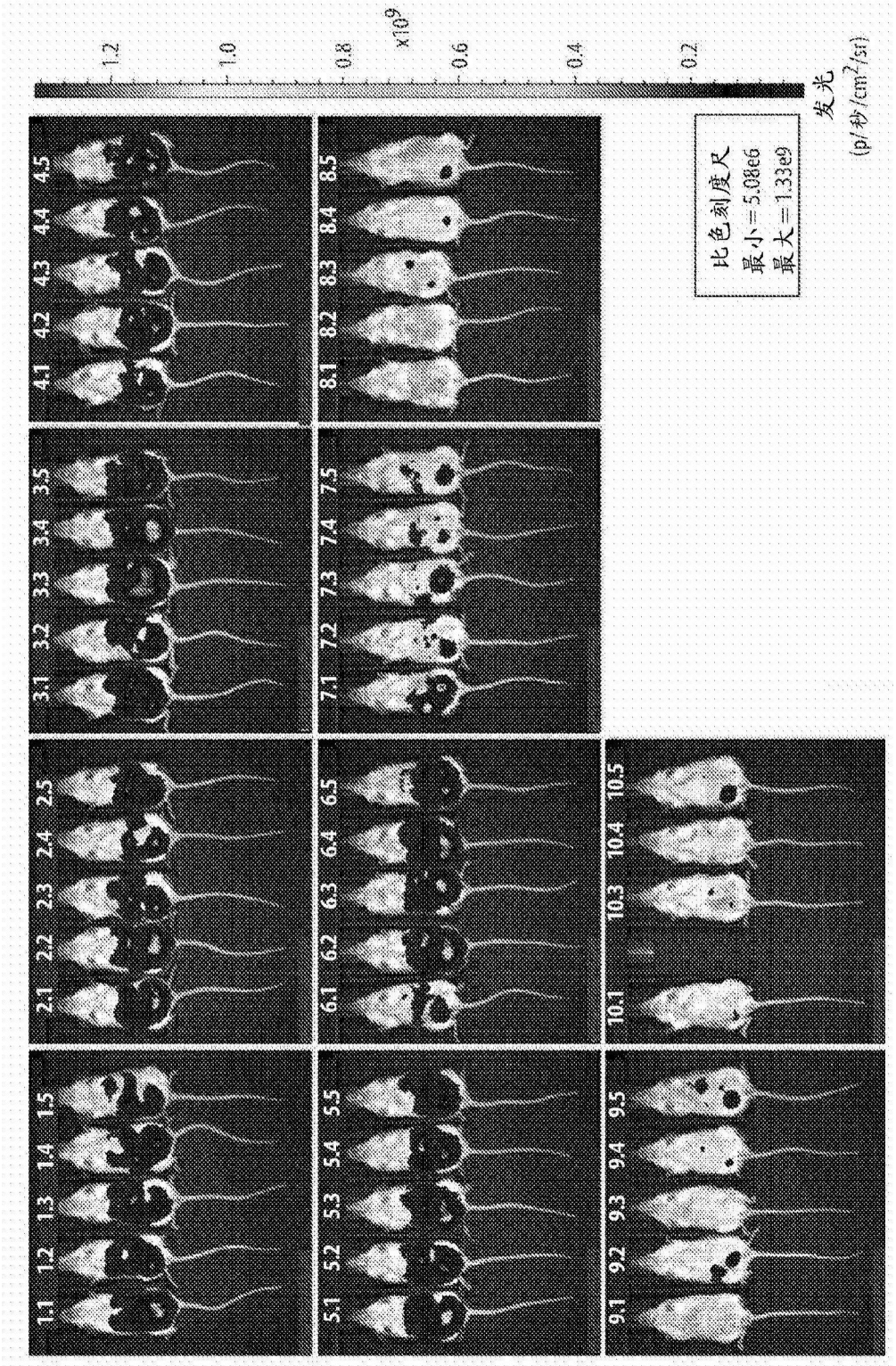
1.2
1.0
0.8
x10^9
0.6
0.4
0.2
发光
(p/秒/cm²/sr)
比色刻度尺
最小 = 5.08e6
最大 = 1.33e9
1.1 1.2 1.3 1.4 1.5
2.1 2.2 2.3 2.4 2.5
3.1 3.2 3.3 3.4 3.5
4.1 4.2 4.3 4.4 4.5
5.1 5.2 5.3 5.4 5.5
6.1 6.2 6.3 6.4 6.5
7.1 7.2 7.3 7.4 7.5
8.1 8.2 8.3 8.4 8.5
9.1 9.2 9.3 9.4 9.5
10.1 10.3 10.4 10.5

图41

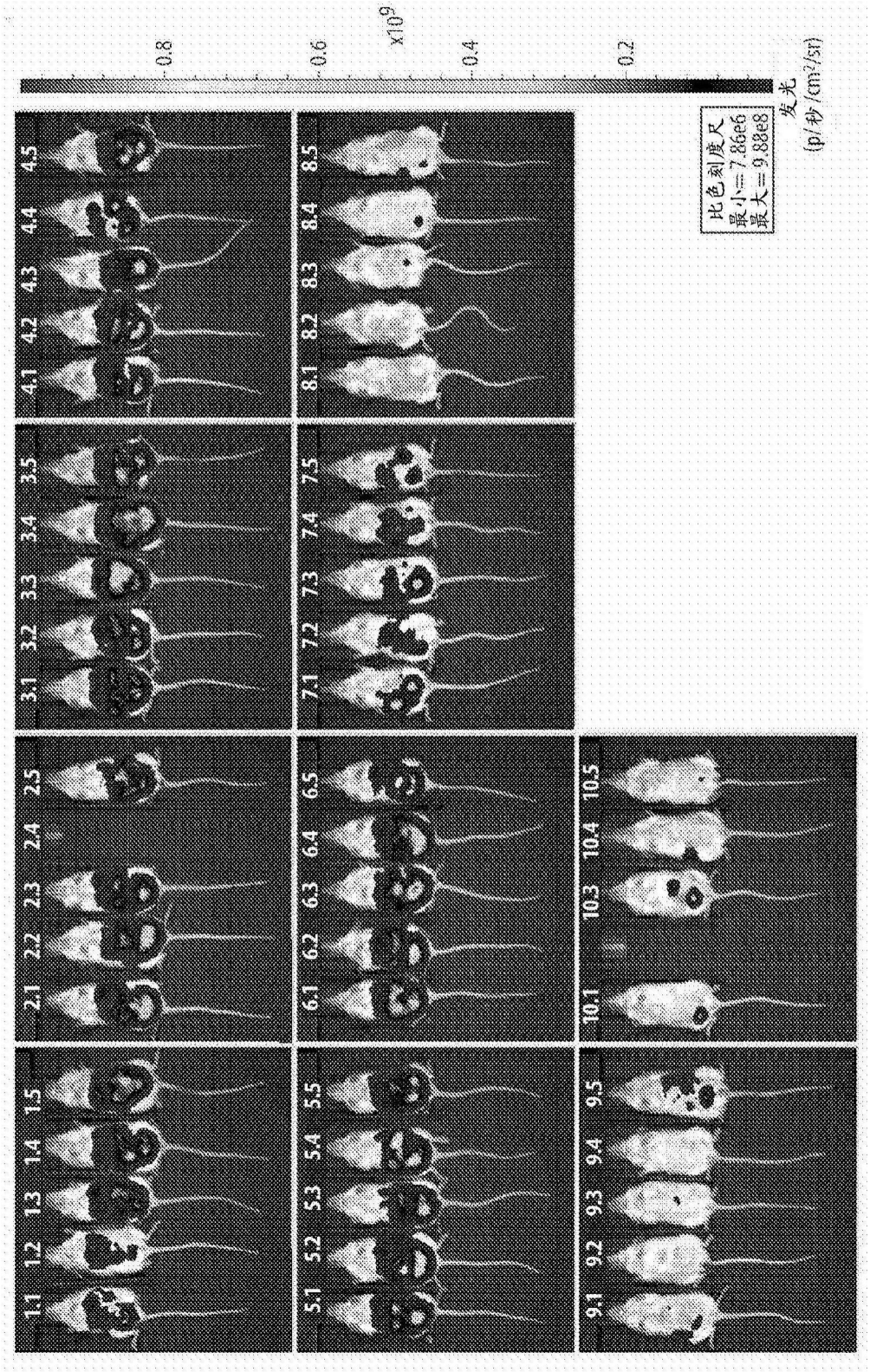
x10^9
0.8
0.6
0.4
0.2
比色刻度尺
最小= 7.86e6
最大= 9.88e8
发光
(p/秒/cm²/sr)
1.1 1.2 1.3 1.4 1.5
2.1 2.2 2.3 2.4 2.5
3.1 3.2 3.3 3.4 3.5
4.1 4.2 4.3 4.4 4.5
5.1 5.2 5.3 5.4 5.5
6.1 6.2 6.3 6.4 6.5
7.1 7.2 7.3 7.4 7.5
8.1 8.2 8.3 8.4 8.5
9.1 9.2 9.3 9.4 9.5
10.1 10.3 10.4 10.5

图42

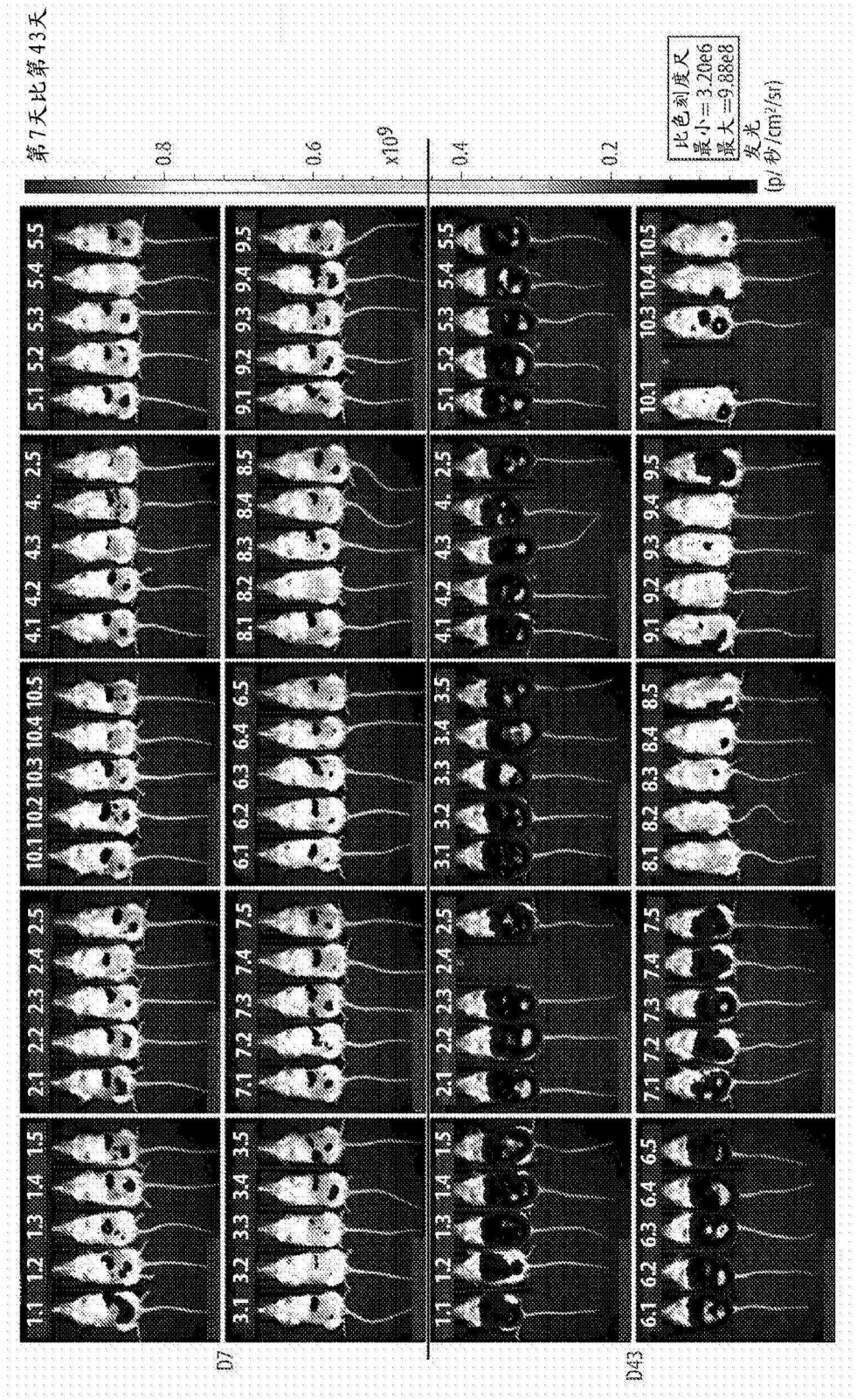
第7天比第43天
0.8
0.6
x10⁹
0.4
0.2
比色刻度尺
最小=3.20e6
最大=9.88e8
发光
(p/秒/cm²/sr)
D7
D43

图43

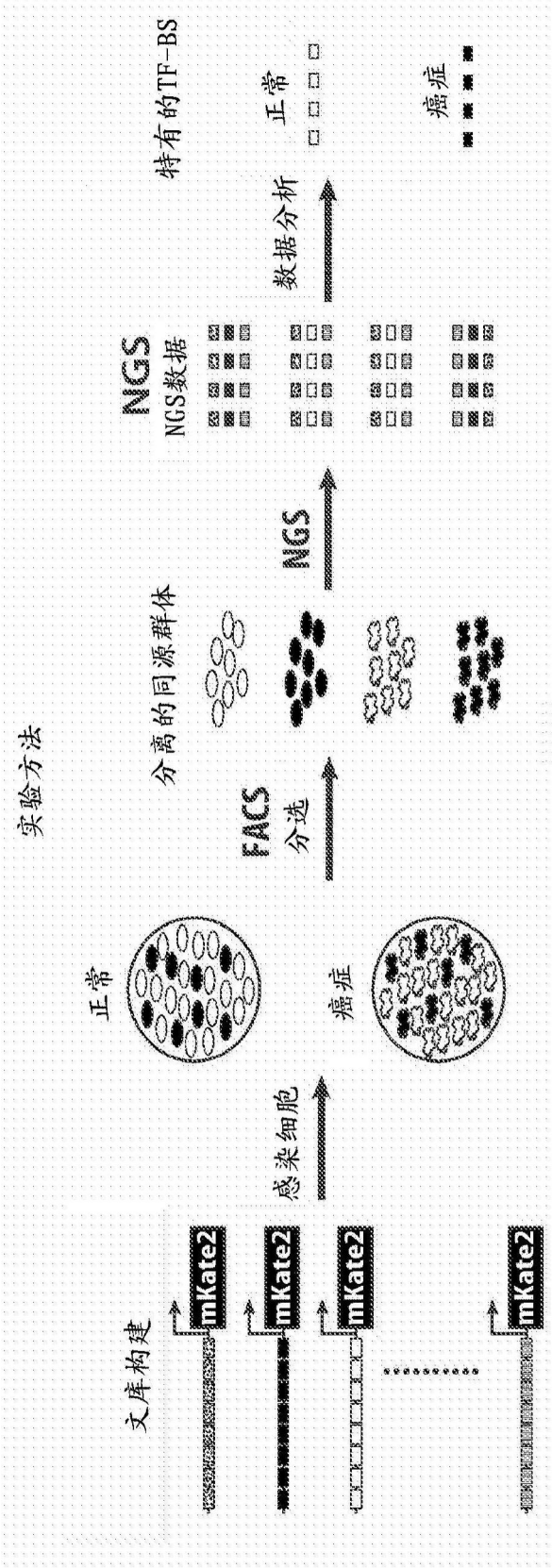
实验方法
文库构建
mKate2
感染细胞
正常
癌症
FACS
分选
分离的同源群体
NGS
NGS
NGS数据
数据分析
特有的TF-BS
正常
癌症

图44

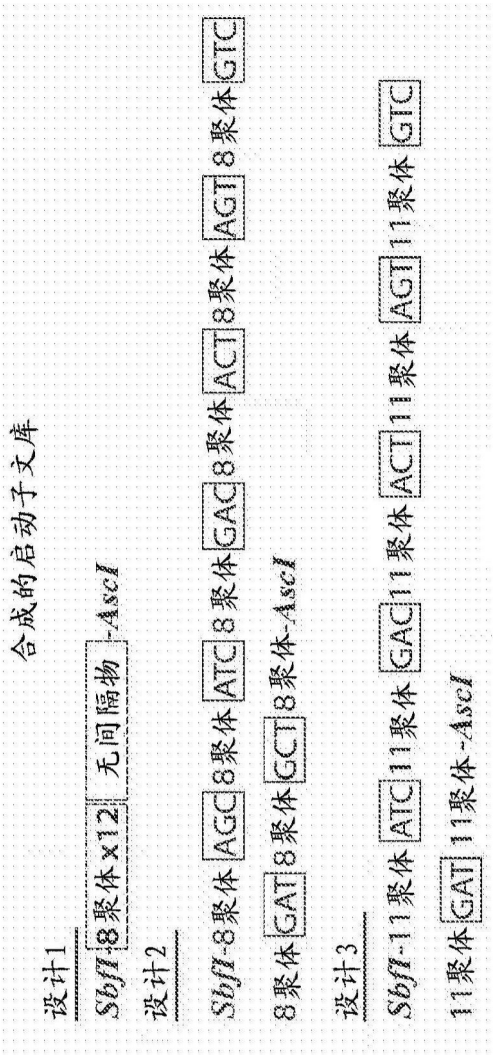
合成的启动子文库
设计1
Sbfl-8聚体x12 无间隔物 -AscI
设计2
Sbfl-8聚体 AGC 8聚体 ATC 8聚体 GAC 8聚体 ACT 8聚体 AGT 8聚体 GTC
8聚体 GAT 8聚体 GCT 8聚体-AscI
设计3
Sbfl-11聚体 ATC 11聚体 GAC 11聚体 ACT 11聚体 AGT 11聚体 GTC
11聚体 GAT 11聚体-AscI

图45

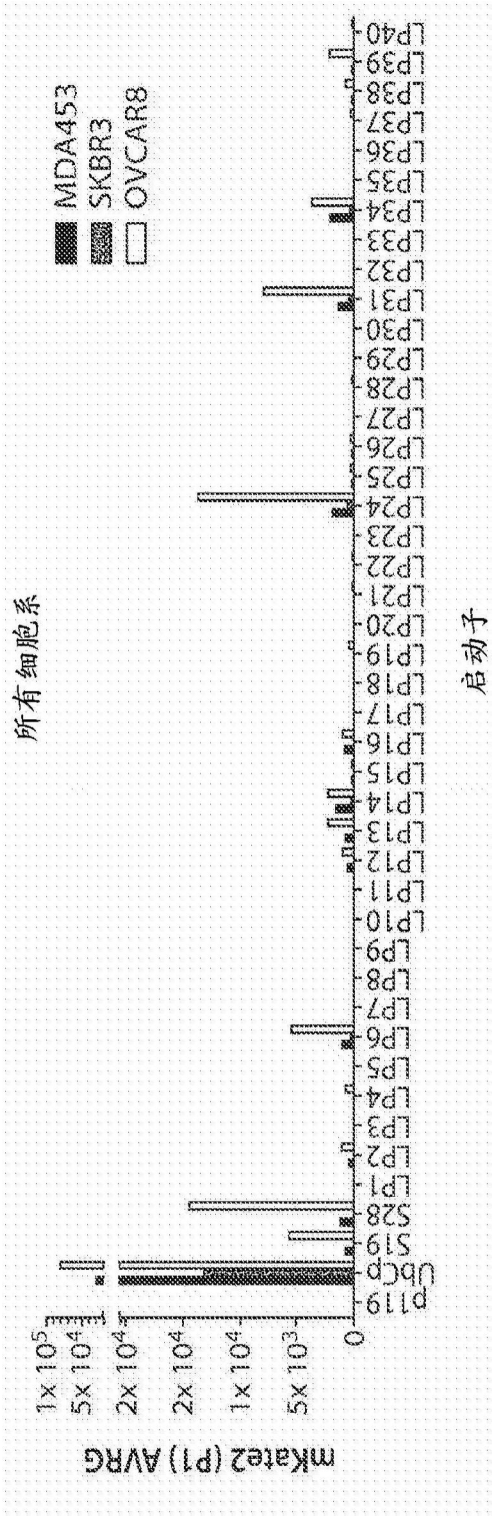
MDA453
SKBR3
OVCAR8
所有细胞系
启动子
mKate2 (P1) AVRG
1x 10^5
5x 10^4
2x 10^4
2x 10^4
1x 10^4
5x 10^3
0
p119
UbCp
S19
S28
LP1
LP2
LP3
LP4
LP5
LP6
LP7
LP8
LP9
LP10
LP11
LP12
LP13
LP14
LP15
LP16
LP17
LP18
LP19
LP20
LP21
LP22
LP23
LP24
LP25
LP26
LP27
LP28
LP29
LP30
LP31
LP32
LP33
LP34
LP35
LP36
LP37
LP38
LP39
LP40

图46

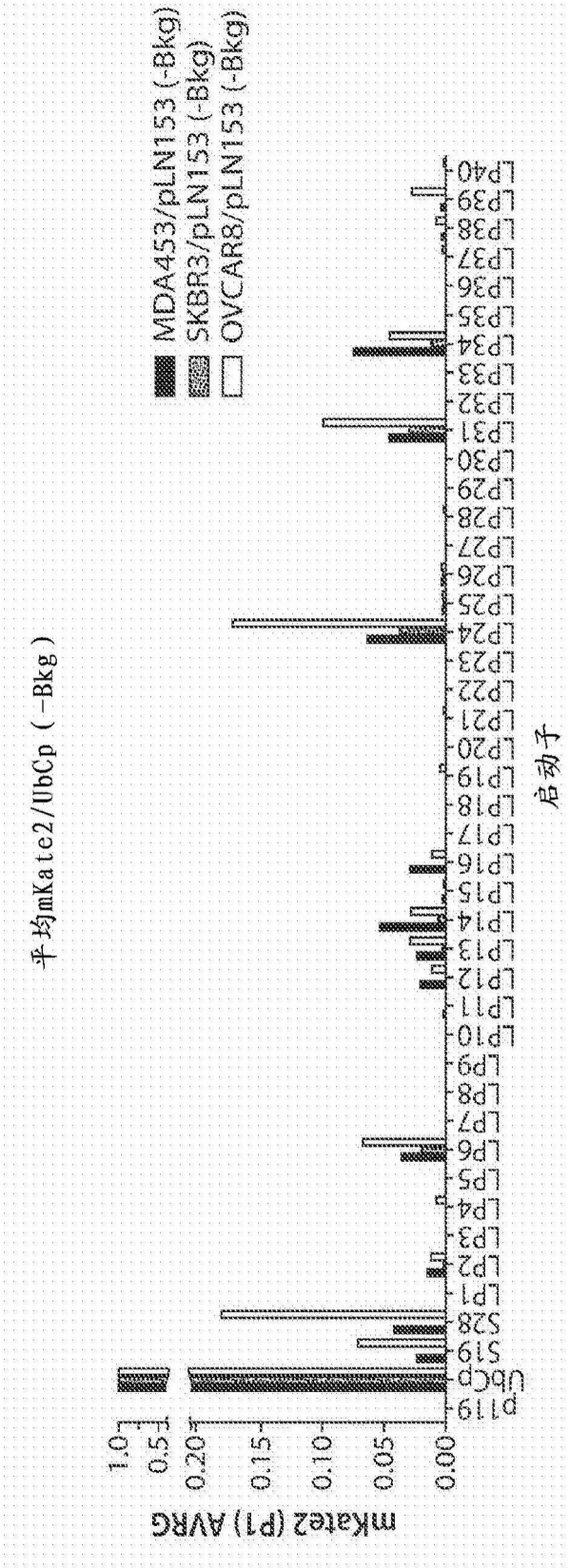
MDA453/pLN153 (-Bkg)
SKBR3/pLN153 (-Bkg)
OVCAR8/pLN153 (-Bkg)
平均mKate2/UbCp (-Bkg)
mKate2 (P1) AVRG
1.0
0.5
0.20
0.15
0.10
0.05
0.00
p119
UbCp
S19
S28
LP1
LP2
LP3
LP4
LP5
LP6
LP7
LP8
LP9
LP10
LP11
LP12
LP13
LP14
LP15
LP16
LP17
LP18
LP19
LP20
LP21
LP22
LP23
LP24
LP25
LP26
LP27
LP28
LP29
LP30
LP31
LP32
LP33
LP34
LP35
LP36
LP37
LP38
LP39
LP40
启动子

图47