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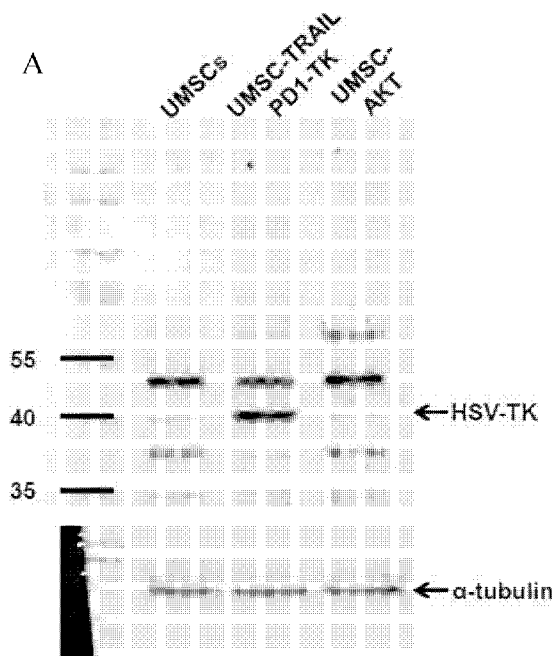


Fig. 3

(57) Abstract: Provided is an engineered stem cell comprising a vector with a polynucleotide comprising a nucleic acid sequence of suicide gene, a nucleic acid sequence of immune checkpoint gene and a natural cytotoxicity triggering receptor or a TNF-related apoptosis-inducing ligand, wherein the stem cell is a tumor-targeting cell. Also provided is a method for treating a cancer or enhancing intratumor immunity or enhancing immunity in tumor microenvironment in a subject, comprising administering an effective amount of the engineered stem cell.



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## ENGINEERING STEM CELLS FOR CANCER THERAPY

### Field of the Invention

5 [0001] The present invention relates to engineered stem cells for the treatment of cancer. Particularly, the engineered stem cells comprise at least a suicide gene and an immune checkpoint gene.

### Background of the Invention

10 [0002] Checkpoint immunotherapy by interacting with PD-1/PD-L1 pathway is at the cutting age of cancer treatment, and giving hope for a cure of cancer. The protein encoded by this gene is a natural cytotoxicity receptor (NCR3) that may aid NK cells in the lysis of tumor cells. However, up to 70% of patients do not respond to the treatment, which even causes severe complications in some clinical cases (The Journal of Clinical Endocrinology & Metabolism 2013, 98(4): 1361-1375). There is urgent need for improvement such that the inhibitors are able to  
15 selectively accumulate within tumors and not cause autoimmune responses in the peripheral normal tissue.

[0003] US20180214544 provides a combination of immune checkpoint blockade and hematopoietic stem cell transplantation and/or hematopoietic stem cell mobilization yielding synergistic effects in disease therapy. However, a need continues to exist for improving the effect  
20 of immune checkpoint inhibitors.

### Summary of the Invention

[0004] The present disclosure provides an engineered stem cell, comprising a vector comprising a polynucleotide comprising a nucleic acid sequence of suicide gene, a nucleic acid

sequence of immune checkpoint gene and a natural cytotoxicity triggering receptor or a TNF-related apoptosis-inducing ligand, wherein the stem cell is a tumor-targeting cell.

[0005] Certain embodiments of the engineered stem cell include embryonic stem cell, marrow stromal cell, hematopoietic stem cell and neural stem cell. A particular embodiment of the engineered stem cell is MSC. A further particular embodiment of the engineered stem cell is umbilical cord mesenchymal stem cell (UMSC).

[0006] Certain embodiments of the suicide gene include cytosine deaminase gene, varicella-zoster virus thymidine kinase gene, nitroreductase gene, Escherichia coli gpt gene, E. coli Deo gene, thymidine kinase gene (TK), caspase 1, caspase 3, caspase 6, caspase 7, caspase 8, caspase 9 and Fas or cytosine deaminase (CD).

[0007] Certain embodiments of the immune checkpoint gene include the E3 ubiquitin ligase Cbl-b, CTLA-4, PD-1, TIM-3, killer inhibitory receptor (KIR), LAG-3, CD73, Fas, the aryl hydrocarbon receptor, Smad2, Smad4, TGF-beta receptor, ILT-3, IDO, KIR, and LAG3.

[0008] Certain embodiments of the natural cytotoxicity triggering receptor include NCR1, NCR2 and NCR3.

[0009] A certain embodiment of the TRAIL gene includes TIC10.

[0010] The present disclosure provides a kit or combination comprising the vector or engineered cell of the present disclosure and optionally an additional active agent.

[0011] The present disclosure also provides a method for treating a cancer or enhancing intratumor immunity in a subject, comprising administering an effective amount of the engineered stem cell of the present disclosure to the subject. In one embodiment, the effective amount ranges from 100,000 ( $1 \times 10^5$ ) ~2,000,000 ( $2 \times 10^6$ ) cells. In one embodiment, the cancer is a metastatic cancer.

### **Brief Description of the Drawing**

[0012] Figures 1A to G show *in vitro* characterization of UMSCs and UMSC-TRAIL-TK-PD-1. **A.** The cell morphology and biological properties of umbilical cord mesenchymal stem cells (UMSCs) from Wharton's jelly (WJ). **B.** The flow cytometry plots show the cells are negative for CD1q, CD3, CD10, CD14, CD31, CD34, CD45, CD49d, CD56, CD117 and HLA-DR, but positive for CD13, CD29, CD44, CD73, CD90, CD105, CD166, CD49b and HLA-ABC. **C.** The results of RFP and PD-1 flowcytometry and transduction with the transgenes (UMSC-PD-1 and UMSC-TRAIL-TK-PD-1). **D-E.** UMSC-TRAIL-TK-PD-1-Luc retains luciferase expression more than 100 days and **E** cell proliferation assay by BrdU incorporation and migration by transwell assay reveal that genetic modification does not affect the UMSC-TRAIL-TK-PD-1 cellular viability (**E-(a)**), cell proliferation (**E-(b)**) or migration (**E-(c)**) *in vitro* compared to unlabeled UMSCs after 14 h of incubation. **F.** The UMSC-TRAIL-TK-PD-1 displays similar behavior to the plain UMSCs without plasmids labeling. **G.** The UMSC-TRAIL-TK-PD-1's neuroglial cell differentiation was identified by immunofluorescence with MAP-2, Tuj-1 and GFAP; the results exhibited refractile cell body morphology with extended neurite-like structures arranged into a network as the plain UMSCs.

[0013] Figures 2A to D show immunological assessment of UMSC-TRAIL-TK-PD-1 *in vitro*. **A.** The binding affinity of HRP-conjugated PD-1 protein is increased significantly in a dose-dependent manner. **B.** The gating strategy is based on the justification of first gate, exclusion of doublets by FSC-A and FSC-H, exclusion of dead cells by selection of 7-AAD<sup>+</sup> (R&D systems)/CD45<sup>+</sup> or FSC-A (**B-(a)** and **B-(b)**). **C.** UMSCs (at a ratio of 1:1) significantly suppress both CD4<sup>+</sup> and CD8<sup>+</sup> T-cell proliferation (**C-(a)** and **C-(b)**). However, at a ratio of either 1:1 or 1:10, UMSC-TRAIL-TK-PD-1 significantly increased both CD4<sup>+</sup> and CD8<sup>+</sup> T-cell proliferation

(**C-(a) and C-(b)**). **D.** UMSC-TRAIL-TK-PD-1 stimulated with CD3-CD28 exhibits significant increase level of CD4<sup>+</sup>INF- $\gamma$ <sup>+</sup> (**D-(a)**) and reduction of CD8<sup>+</sup>CD122<sup>+</sup> (**D-(b)**) compared to that of UMSCs.

[0014] Figures 3A to E show suicide and bystander effect of UMSC-TRAIL-TK-PD-1-GFP *in vitro*. **A.** Increased level of TK is found in the UMSC-TRAIL-TK-PD-1 compared to UMSC-  
5 Akt and UMSCs by Western blot. **B.** GCV itself does not affect cell proliferation of UMSCs. Phosphorylated GCV induces apoptotic-like cell injury in UMSC-TRAIL-TK-PD-1-GFP at 24h and 48h after GCV treatment by immunohistochemistry (white arrow) (**B-(b)**). Cell proliferation of UMSC-TRAIL-TK-PD-1-GFP is inhibited in a dose-dependent manner (**B-(a)**). **C.** UMSC-  
10 TRAIL-TK-PD-1-GFP significantly attenuates the growth of 4T1-Luc cells ((**C-(a)**, **C-(b)**) (Hep55.1C (**C-(c)** and **C-(d)**), Pan18-Luc (**C-(e)** and **C-(f)**), CT26-Luc (**C-(g)** and **C-(h)**) and GL261-Luc (**C-(i)** and **C-(j)**) with the presence of 0, 1, 10, 100  $\mu$ g/mL GCV after co-culture for 24h, 48h and 72h. **D-E.** Cell mortality rate by suicide effect in this co-culture system slowly reaches about one-third of the whole system during the first two days, and then subsequently  
15 accelerates from day 3 to day 6. The same findings show that most 4T1-Luc cells are killed from day 3 to day 5. Furthermore, quantitative assessment of apoptotic cells under this bystander effect by PI/annexin-V staining using flowcytometry shows significant cytotoxicity in a GCV dose-dependent and time-dependent manner (**E-(a) to E-(d)**). Figures 4A to C show UMSC-TRAIL-TK-PD-1 expressing TRAIL display *in vitro* antitumor activity in 4T1-luc and  
20 Hep55.1C-Luc cell. **A.** Genetically modified UMSC-TRAIL-TK-PD-1 allows a relevant TRAIL protein expression (90%) on the cell surface of UMSCs as measured by FACS analysis. **B.** UMSC-TRAIL-TK-PD-1 expressing TRAIL induces cell apoptosis (4T1-Luc, Hep55.1C-Luc), represented by cell shrinkage, reduction of adherent 4T1-Luc cells (**B-(a)**) and Hep55.1C-Luc

(**B-(b)**) with the appearance of cellular debris, which were demonstrated by the propidium iodine staining (PI staining) (**B-(c)**), especially at 72 hours after coculture. **C.** Quantitatively, cell death occurred at 24, 48 and 72 hours (**C-(a)** and **C-(b)**), a large amount of Annexin-V<sup>+</sup>PI<sup>+</sup> dead cells ( $\geq 70\%$ ) were detected in coculture where UMSC-TRAIL-TK-PD-1 is present in a dose-  
5 dependent manner as measured by FACS analysis.

[0015] Figures 5A to G show tumor targeting of UMSC-TRAIL-TK-PD-1-Luc in the some tumors model. **A.** Bioillumiance intensity increased in a UMSC-TRAIL-TK-PD-1-Luc cell dose-dependent manner as measured by IVIS *in vitro*. **B.** UMSC-TRAIL-TK-PD-1-Luc survives and relocates to the subcutaneous 4T1 tumors. The bioluminescent signal of the subcutaneous  
10 tumor area in IVIS image was observed initially at five days after intravenous UMSC-TRAIL-TK-PD-1-Luc injection, gradually increased intensity afterward, and peaked at day 14. **C-E.** Intraarterial UMSC-TRAIL-TK-PD-1-Luc transplantation is directly recruited to the orthotopic 4T1 tumor region (**C**) (also for Hep55.1C (**D**) and pan18 tumor region (**E**) at two hours after intra-femoral artery injection without lung entrapment. Subsequently, UMSC-TRAIL-TK-PD-1-  
15 Luc survives and relocates to the tumors sites. **F.** Metastatic tumor from original 4T1-tumor model significantly recruits the UMSC-TRAIL-TK-PD-1-Luc to increase the bioillumiance intensity as measured by IVIS in the multiple metastatic sites. **G.** By immunohistochemical analysis, numerous GFP<sup>+</sup>Luciferase<sup>+</sup> cells was found in the 4T1 tumor at one day after treatment, which indicated UMSC-TRAIL-TK-PD-1-GFP were recruited into tumor microenvironment.

20 [0016] Figures 6A to G show the therapeutic effect of UMSC-TRAIL-TK-PD-1 in 4T1-Luc model. **A.** The tumoricidal effects in luciferase-expressing 4T1-Luc- and Hep55.1C-Luc-tumor-bearing mice treated with various strategies of gene modified UMSCs are assessed by IVIS, tumor volume and survival time following the *q4dx3* course of treatment protocol. **B.** Prior to

treatment, every group of tested cells is subjected to hypoxia preconditioning culture in 3% O<sub>2</sub>, which induced CXCR4 overexpression by Western blot for enhancing stem cell homing in a time-dependent manner. **C.** UMSC-PD-1 (UP) group and UMSC-TRAIL (UT) group exhibit a therapeutic effect, reducing tumor volumes compared to those in the IgG control group as measured by IVIS. Moreover, the UMSC-TK-PD-1+GCV (UTPG) group, UMSC-TRAIL-TK+GCV (UTTg) group, and UMSC-TRAIL-PD-1 (UTP) group showed stronger antitumoral effects and exhibited inhibition of tumor growth respectively. **D.** Intravenous UTP significantly extends survival time on both 4T1 and Hempa55.1C model compared to the other groups (**D-(a)** to **D-(d)**). **E.** Fewer than 5 nodules of lung metastasis were discovered in UTP-treated mice compared to over 20 metastases in lungs of the control mice. However, UMSC-TK (UT), UP and UTP did not show a significant decrease in metastasis compared to the control group. **F-G.** Next, to verify whether intra-arterial injection of UMSC-TRAIL-TK-PD-1 displayed a significant robust therapeutic effect in 4T1-Luc and Hep55.1C-Luc model following the *q7dx2* course of treatment protocol, four groups (UMSC-TK-PD-1+GCV (UTPG) group, UMSC-TRAIL-TK-PD-1 (UTTP) group, and UMSC-TRAIL-TK-PD-1+GCV (UTTPG) group) were divided to examine the tumor growth and median survival time (**F-(a)**). Before the analysis of intra-arterial injection, intravenous administration of UMSC-TRAIL-TK-PD-1+GCV (UTTPG) group showed stronger antitumoral effects (**F-(b)**) than the other groups of IgG control, UTPG and UTTP, respectively. Importantly, intra-arterial implantation revealed a robustly superior therapeutic effect to the intravenous ones. Moreover, the UTTPG group significantly inhibit tumor growth and enhance the median survival times of the mice than the other groups of IgG control, UTPG and UTTP in 4T1-Luc (**F-(c-d)**) and Hep55.1C-Luc (**G-(a-b)**) model, respectively.

Unfortunately, administration anti-PD-L1 did not show any significantly therapeutic effect in 4T1-Luc model (**F-(c)**).

[0017] Figures 7A to D show UTTPG treatment enhances immunity in the tumor microenvironment (TME). A. The gating strategy is based on the justification of first gate, exclusion of doublets by FSC-A and FSC-H, exclusion of dead cells by selection of 7-AAD<sup>+</sup> (R&D systems)/CD45<sup>+</sup> or FSC-A (**A-(a)**). There was an overall increase in the percentage of tumor-infiltrating CD45<sup>+</sup> leukocytes across the therapeutic groups of UTTPG and the other therapeutic groups (**A-(b)**). The frequencies for both CD3<sup>+</sup>CD8<sup>+</sup> and CD3<sup>+</sup>CD4<sup>+</sup> T cells are significantly enhanced in UTTPG treatment compared to the other groups (**A-(c) and A-(d)**). B-  
10 C. UTTPG induces a significant reduction in Tregs and TAMs (**B-(a) and B-(b)**), and thereby reverses the ratio of CD8<sup>+</sup> (**C-(b)**) and CD4<sup>+</sup> (**C-(a)**) T cells to Tregs within the tumors. Additionally, the number of TAMs dramatically decrease in response to UTTPG treatment, which increases the ratio of CD8<sup>+</sup> (**C-(d)**) and CD4<sup>+</sup> (**C-(c)**) T cells to TAMs in the TME. D. The marked upregulation in intracellular granzyme B (Grb<sup>+</sup>) (**D-(b)**) and Ki67<sup>+</sup> (**D-(a)**) cells indicates  
15 that UTTPG treatment not only increases the antitumor immune population but also effectively achieves activation and proliferation of TILs.

### Detailed Description of the Invention

[0018] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention  
20 pertains. Generally, the nomenclature used herein and the experiment methods, which will be described below, are those well known and commonly employed in the art.

[0019] As used herein, the terms "a," "an," "the" and similar references can be construed to cover both the singular and the plural.

[0020] As used herein, the term "genetically modified cells," "redirected cells," "genetically engineered cells" or "modified cells" refer to cells that express the recombinant polynucleotide of the invention.

[0021] The terms "polynucleotide," "nucleic acid" and "oligonucleotide" are used interchangeably herein to refer to a polymeric form of nucleotides of any length, either  
5 deoxyribonucleotides or ribonucleotides or analogs thereof.

[0022] As used herein, the term "gene" refers to a polynucleotide containing at least one open reading frame (ORF) that is capable of encoding a particular polypeptide or protein after being transcribed and translated.

10 [0023] As used herein, the term "encode" as it is applied to polynucleotides refers to a polynucleotide which is said to "encode" a polypeptide if, in its native state or when manipulated by methods well known to those skilled in the art, it can be transcribed and/or translated to produce the mRNA for the polypeptide and/or a fragment thereof. The antisense strand is the complement of such a nucleic acid, and the encoding sequence can be deduced therefrom.

15 [0024] As used herein, the term "operably linked" refers to functional linkage between a regulatory sequence and a heterologous nucleic acid sequence resulting in expression of the latter. For example, a first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter  
20 affects the transcription or expression of the coding sequence.

[0025] As used herein, the term "expression" refers to the process by which polynucleotides are transcribed into mRNA and/or the process by which the transcribed mRNA is subsequently translated into peptides, polypeptides, or proteins.

[0026] As used herein, the term "expression vector" refers to a vector comprising a recombinant polynucleotide comprising expression control sequences operatively linked to a nucleotide sequence to be expressed. An expression vector comprises sufficient cis-acting elements for expression; other elements for expression can be supplied by the host cell or be in an in vitro expression system.

[0027] As used herein, the term "thymidine kinase" or "TK" means the thymidine kinase suicide gene "TK" that is known in the art to provide biosafety to recombinant vectors. Unless specified otherwise, the term "TK" means wild-type (WT) and/or mutant forms of the gene known in the art.

[0028] As used herein, the term "marrow stromal cell," also referred to as "mesenchymal stem cells," or MSC, is a multipotent stem cell that can differentiate into a variety of cell types.

[0029] As used herein, the term "subject," "individual" or "patient" is used interchangeably, and refers to a vertebrate, preferably a mammal, more preferably a human.

[0030] As used herein, the term "treatment" or "treating" should be understood to include any indicia of success in the treatment, alleviation or amelioration of an injury, pathology or condition. This may include parameters such as abatement, remission, diminishment of symptoms, slowing in the rate of degeneration or decline, making the final point of degeneration less debilitating; improving a patient's physical or mental well-being; or preventing the onset of disease.

[0031] As used herein, the term "therapeutically effective amount" when used in reference to symptoms of a disease/condition refers to the amount and/or concentration of a compound that ameliorates, attenuates, or eliminates one or more symptoms of a disease/condition or prevents or delays the onset of a symptom(s).

[0032] Mesenchymal stem cells (MSCs) are regarded as a cellular vehicle for the expression of therapeutic proteins by gene transfer and show a unique tumor-homing tropism for targeted delivery of anticancer substances to animal models of various tumors, including melanoma, glioblastoma, and breast cancer. There are several advantages, such as easy isolation and expansion, immunotolerant property, and systemic or local delivery. Although current genetic engineering methods by viral transduction of DNA to MSCs can be applied as diagnostic and therapeutic strategies for cancer treatment, they might induce detrimental transformation to increase secondary malignancy risk.

[0033] It is imperative to test whether MSC could represent an efficient vehicle to deliver genetic material for anti-cancer function. Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), which is a promising anticancer death ligand with a sequence homology to TNF and FasL, could mediate the apoptotic effect by binding to its death receptors (DR), as homotrimer, particularly on TRAIL-R1/DR4 and TRAIL-R2/DR5 activation, a protein complex, causes caspase-8 activation, triggering apoptosis (Nat Rev Cancer 2008;8:782–98; Science 1998;281:1305–8; Eur J Cancer 2006;42:2233–40). Furthermore, suicide gene therapy is based on transferring a gene encoding a suicide protein of herpes simplex virus thymidine kinase (HSV-TK), which selectively sensitizes it to the prodrug ganciclovir (GCV) by preferential monophosphorylation of nontoxic GCV into a toxic compound through the viral TK enzyme (Mol Biol Cell 2002;13:4279–95). Chimeric antigen receptor-T cell (CAR-T) immunotherapy combined with suicide gene modification has been demonstrated to not only inhibit tumor outgrowth but improve the safety profile to facilitate clinical development (Journal of Cancer 2011; 2: 378-382).

[0034] It has not been verified whether PD-1- or NCR3-overexpressed MSCs will enhance migration into tumors and immune sensitization, induce tumor death as well as reduce inflammation. The present disclosure develops a natural nanoparticle harboring inherent anti-tumor ability to play an important task in therapeutic gene engineering.

5 [0035] In one aspect, the present disclosure provides an engineered stem cell, comprising a vector comprising a polynucleotide comprising a nucleic acid sequence of suicide gene, a nucleic acid sequence of immune checkpoint gene and a natural cytotoxicity triggering receptor or a TNF-related apoptosis-inducing ligand; wherein the stem cell is a tumor-targeting cell.

[0036] In one embodiment, the tumor-targeting cell is a stem cell selected from the group  
10 consisting of: embryonic stem cell, marrow stromal cell, hematopoietic stem cell and neural stem cell.

[0037] In one embodiment, the stem cell is MSC. In one embodiment the MSC has the phenotype CD34<sup>-</sup>/CD45<sup>-</sup>/CD105<sup>+</sup>/CD90<sup>+</sup>/CD73<sup>+</sup>. MSCs have been shown differentiation *in vitro* or *in vivo*, including osteoblasts, chondrocytes, myocytes, and adipocytes. Mesenchyme is  
15 embryonic connective tissue that is derived from the mesoderm and that differentiates into hematopoietic and connective tissue, whereas MSCs do not differentiate into hematopoietic cells. Stromal cells are connective tissue cells that form the supportive structure in which the functional cells of the tissue reside.

[0038] A suicide gene, in genetics, will cause a cell to kill itself through apoptosis. In some  
20 embodiments, the suicide gene is cytosine deaminase gene, varicella-zoster virus thymidine kinase gene, nitroreductase gene, Escherichia coli gpt gene, E. coli Deo gene, thymidine kinase gene (TK), caspase 1, caspase 3, caspase 6, caspase 7, caspase 8, caspase 9, Fas or cytosine deaminase (CD). In a certain embodiment, the suicide gene is thymidine kinase gene. In one

embodiment, the TK gene is a wild-type TK gene. In another embodiment, the TK gene is a mutated form of the gene. In some embodiments, the thymidine kinase sequence includes, but is not limited to, the following sequences.

HSV1-TK sequence

5 ATGGCCTCGTACCCCGGCCATCAACACGCGTCTGCGTTCGACCAGGCTGCGCGTTCT  
 CGCGGCCATAGCAACCGACGTACGGCGTTGCGCCCTCGCCGGCAGCAAGAAGCCAC  
 GGAAGTCCGCCCCGAGCAGAAAATGCCACGCTACTGCGGGTTTATATAGACGGTC  
 CCCACGGGATGGGGAAAACCACCACCACGCAACTGCTGGTGGCCCTGGGTTCGCGC  
 GACGATATCGTCTACGTACCCGAGCCGATGACTTACTGGCGGGTGCTGGGGGCTTCC  
 10 GAGACAATCGCGAACATCTACACCACACAACACCGCCTCGACCAGGGTGAGATATC  
 GGCCGGGGACGCGGCGGTGGTAATGACAAGCGCCAGATAACAATGGGCATGCCTT  
 ATGCCGTGACCGACGCCGTTCTGGCTCCTCATATCGGGGGGAGGCTGGGAGCTCA  
 CATGCCCCGCCCCCGGCCCTCACCTCATCTTCGACCGCCATCCCATCGCCGCCCTCC  
 TGTGCTACCCGGCCGCGCGGTACCTTATGGGCAGCATGACCCCCAGGCCGTGCTGG  
 15 CGTTCGTGGCCCTCATCCCGCCGACCTTGCCCGGCACCAACATCGTGCTTGGGGCCC  
 TTCCGGAGGACAGACACATCGACCGCCTGGCCAAACGCCAGCGCCCCGGCGAGCGG  
 CTGGACCTGGCTATGCTGGCTGCGATTCGCCGCGTTTACGGGCTACTTGCCAATACG  
 GTGCGGTATCTGCAGTGCGGCGGGTCGTGGCGGGAGGACTGGGGACAGCTTTCGGG  
 GACGGCCGTGCCGCCCCAGGGTGCCGAGCCCCAGAGCAACGCGGGCCCACGACCCC  
 20 ATATCGGGGACACGTTATTTACCCTGTTTCGGGCCCCCGAGTTGCTGGCCCCCAACG  
 GCGACCTGTATAACGTGTTTGCCTGGGCCTTGGACGTCTTGGCCAAACGCCTCCGTT  
 CCATGCACGTCTTTATCCTGGATTACGACCAATCGCCCCGCCGGCTGCCGGGACGCCC  
 TGCTGCAACTTACCTCCGGGATGGTCCAGACCCACGTCACCACCCCCGGCTCCATAC

CGACGATATGCGACCTGGCGCGCACGTTTGCCCCGGGAGATGGGGGAGGCTAACTGA

(SEQ ID NO:1)

CpG free HSV1-TK sequence

ATGGCTTCTTACCCTGGACACCAGCATGCTTCTGCCTTTGACCAGGCTGCCAGATCC  
 5 AGGGGCCACTCCAACAGGAGAACTGCCCTAAGACCCAGAAGACAGCAGGAAGCCA  
 CTGAGGTGAGGCCTGAGCAGAAGATGCCAACCTGCTGAGGGTGTACATTGATGGA  
 CCTCATGGCATGGGCAAGACCACCACCACTCAACTGCTGGTGGCACTGGGCTCCAG  
 GGATGACATTGTGTATGTGCCTGAGCCAATGACCTACTGGAGAGTGCTAGGAGCCTC  
 TGAGACCATTGCCAACATCTACACCACCCAGCACAGGCTGGACCAGGGAGAAATCT  
 10 CTGCTGGAGATGCTGCTGTGGTGTGACCTCTGCCCAGATCACAATGGGAATGCCCT  
 ATGCTGTGACTGATGCTGTTCTGGCTCCTCACATTGGAGGAGAGGCTGGCTCTTCTC  
 ATGCCCTCCACCTGCCCTGACCCTGATCTTTGACAGACACCCATTGCAGCCCTGC  
 TGTGCTACCCAGCAGCAAGGTACCTCATGGGCTCCATGACCCACAGGCTGTGCTGG  
 CTTTTGTGGCCCTGATCCCTCCAACCCTCCCTGGCACCAACATTGTTCTGGGAGCACT  
 15 GCCTGAAGACAGACACATTGACAGGCTGGCAAAGAGGCAGAGACCTGGAGAGAGA  
 CTGGACCTGGCCATGCTGGCTGCAATCAGAAGGGTGTATGGACTGCTGGCAAACAC  
 TGTGAGATACTCCAGTGTGGAGGCTCTTGGAGAGAGGACTGGGGACAGCTCTCTG  
 GAACAGCAGTGCCCCCTCAAGGAGCTGAGCCCCAGTCCAATGCTGGTCCAAGACCC  
 CACATTGGGGACACCCTGTTACCCTGTTTCAGAGCCCCTGAGCTGCTGGCTCCCAAT  
 20 GGAGACCTGTACAATGTGTTTGCCTGGGCTCTGGATGTTCTAGCCAAGAGGCTGAGG  
 TCCATGCATGTGTTTCATCCTGGACTATGACCAGTCCCCTGCTGGATGCAGAGATGCT  
 CTGCTGCAACTAACCTCTGGCATGGTGCAGACCCATGTGACCACCCCTGGCAGCATC

CCCACCATCTGTGACCTAGCCAGAACCTTTGCCAGGGAGATGGGAGAGGCCAACTA  
A (SEQ ID NO:2)

[0039] Immune checkpoints are regulators of the immune system. Immune checkpoint molecules have been considered as targets for cancer immunotherapy due to their potential for use in multiple types of cancers. Examples of the immune checkpoint gene include, but are not limited to, the E3 ubiquitin ligase Cbl-b, CTLA-4, PD-1, TIM-3, killer inhibitory receptor (KIR), LAG-3, CD73, Fas, the aryl hydrocarbon receptor, Smad2, Smad4, TGF-beta receptor, ILT-3, IDO, KIR, and LAG3. In a certain embodiment, the immune checkpoint gene is PD-1. In some embodiments, the PD-1 sequence includes, but is not limited to, the following sequence.

10 PD-1 sequence

ATGCAGATCCCACAGGCGCCCTGGCCAGTCGTCTGGGCGGTGCTACAACCTGGGCTG  
GCGGCCAGGATGGTTCTTAGACTCCCCAGACAGGCCCTGGAACCCCCCACCTTCTC  
CCCAGCCCTGCTCGTGGTGACCGAAGGGGACAACGCCACCTTCACCTGCAGCTTCTC  
CAACACATCGGAGAGCTTCGTGCTAAACTGGTACCGCATGAGCCCCAGCAACCAGA  
15 CGGACAAGCTGGCCGCTTTCCCCGAGGACCGCAGCCAGCCCGGCCAGGACTGCCGC  
TTCCGTGTCACAACTGCCAACGGGCGTGACTTCCACATGAGCGTGGTCAGGGCC  
CGGCGCAATGACAGCGGCACCTACCTCTGTGGGGCCATCTCCCTGGCCCCCAAGGC  
GCAGATCAAAGAGAGCCTGCGGGCAGAGCTCAGGGTGACAGAGAGAAGGGCAGAA  
GTGCCACAGCCCACCCAGCCCCTCACCCAGGCCAGCCGGCCAGTTCCAAACCCTG  
20 GTGGTTGGTGTCTGTGGGCGGCCTGCTGGGCAGCCTGGTGCTGCTAGTCTGGGTCCTG  
GCCGTCATCTGCTCCCGGGCCGCACGAGGGACAATAGGAGCCAGGCGCACCGGCCA  
GCCCCTGAAGGAGGACCCCTCAGCCGTGCTGTGTTCTCTGTGGACTATGGGGAGCT  
GGATTTCCAGTGCGGAGAGAAGACCCCGGAGCCCCCGTGCCCTGTGTCCCTGAGC

AGACGGAGTATGCCACCATTGTCTTTCCTAGCGGAATGGGCACCTCATCCCCGCC  
 GCAGGGGCTCAGCCGACGGCCCTCGGAGTGCCCAGCCACTGAGGCCTGAGGATGGA  
 CACTGCTCTTGGCCCCTC (SEQ ID NO:3)

[0040] Natural cytotoxicity triggering receptors also can be used in the vector of the present  
 5 disclosure. Examples of the natural cytotoxicity triggering receptor include, but are not limited to,  
 NCR1, NCR2 and NCR3. In a certain embodiment, the natural cytotoxicity triggering receptor is  
 NCR3. In some embodiments, the NCR3 sequence includes, but is not limited to, the following  
 sequence.

NCR3 sequence

10 ATGGCCTGGATGCTGTTGCTCATCTTGATCATGGTCCATCCAGGATCCTGTGCTCTCT  
 GGGTGTCCCAGCCCCCTGAGATTCGTACCCTGGAAGGATCCTCTGCCTTCCTGCCCT  
 GCTCCTTCAATGCCAGCCAAGGGAGACTGGCCATTGGCTCCGTCACGTGGTTCCGAG  
 ATGAGGTGGTTCCAGGGAAGGAGGTGAGGAATGGAACCCCAGAGTTCAGGGGCCGC  
 CTGGCCCCACTTGCTTCTTCCCGTTTCCTCCATGACCACCAGGCTGAGCTGCACATCC  
 15 GGGACGTGCGAGGCCATGACGCCAGCATCTACGTGTGCAGAGTGGAGGTGCTGGGC  
 CTTGGTGTCTGGGACAGGGAATGGGACTCGGCTGGTGGTGGAGAAAGAACATCCTCA  
 GCTAGGGGCTGGTACAGTCCTCCTCCTTCGGGCTGGATTCTATGCTGTCAGCTTTCTC  
 TCTGTGGCCGTGGGCAGCACCGTCTATTACCAGGGCAAATGTCTGACCTGGAAAGGT  
 CCAAGAAGGCAGCTGCCGGCTGTGGTCCCAGCGCCCCTCCCACCACCATGTGGGAG  
 20 CTCAGCACATCTGCTTCCCCCAGTCCCAGGAGGC (SEQ ID NO:4)

[0041] TNF-related apoptosis-inducing ligand (TRAIL) is a protein functioning as a ligand  
 that induces the process of cell death. Examples of the TRAIL gene include, but are not limited

to, TIC10. In some embodiments, the TIC10 sequence includes, but is not limited to, the following sequence.

TRAIL sequence

ATGGCTATGATGGAGGTCCAGGGGGGACCCAGCCTGGGACAGACCTGCGTGCTGAT  
5 CGTGATCTTCACAGTGCTCCTGCAGTCTCTCTGTGTGGCTGTAACCTACGTGTACTTT  
ACCAACGAGCTGAAGCAGATGCAGGACAAGTACTCCAAAAGTGGCATTGCTTGTTT  
CTTAAAAGAAGATGACAGTTATTGGGACCCCAATGACGAAGAGAGTATGAACAGCC  
CCTGCTGGCAAGTCAAGTGGCAACTCCGTCAGCTCGTTAGAAAGATGATTTTGAGAA  
CCTCTGAGGAAACCATTTCTACAGTTCAAGAAAAGCAACAAAATATTTCTCCCCTAG  
10 TGAGAGAAAGAGGTCCTCAGAGAGTAGCAGCTCACATAACTGGGACCAGAGGAAG  
AAGCAACACATTGTCTTCTCCAAACTCCAAGAATGAAAAGGCTCTGGGCCGCAAAA  
TAAACTCCTGGGAATCATCAAGGAGTGGGCATTCATTCCTGAGCAACTTGCACTTGA  
GGAATGGTGA ACTGGTCATCCATGAAAAAGGGTTTTACTACATCTATTCCCAAACAT  
ACTTTCGATTT CAGGAGGAAATAAAAAGAAAACACAAAGAACGACAAACAAATGGTC  
15 CAATATATTTACAAATACACAAGTTATCCTGACCCTATATTGTTGATGAAAAGTGCT  
AGAAATAGTTGTTGGTCTAAAGATGCAGAATATGGACTCTATTCCATCTATCAAGGG  
GGAATATTTGAGCTTAAGGAAAATGACAGAATTTTTGTTTCTGTAACAAATGAGCAC  
TTGATAGACATGGACCATGAAGCCAGTTTTTTTCGGGGCCTTTTTAGTTGGC (SEQ ID  
NO:5)

20 [0042] The vector of the present disclosures comprises one or more control sequences to regulate the expression of the polynucleotide of the present disclosure. Manipulation of the isolated polynucleotide prior to its insertion into a vector may be desirable or necessary depending on the expression vector utilized. Techniques for modifying polynucleotides and

nucleic acid sequences utilizing recombinant DNA methods are well known in the art. In some embodiments, the control sequences include, among others, promoters, leader sequences, polyadenylation sequences, propeptide sequences, signal peptide sequences, and transcription terminators. In some embodiments, suitable promoters are selected based on the host cells  
5 selection.

[0043] A recombinant expression vector comprising the polynucleotide of the present disclosure is disclosed along with one or more expression regulating regions such as a promoter and a terminator, a replication origin, etc., depending on the type of hosts into which they are to be introduced. Non-limiting examples of constitutive promoters include SFFV, CMV, PKG,  
10 MDNU3, SV40, Efla, UBC, and CAGG.

[0044] In some embodiments, the various nucleic acid and control sequences described herein are joined together to produce recombinant expression vectors which include one or more convenient restriction sites to allow for insertion or substitution of the polynucleotide of the present disclosure at such sites. Alternatively, in some embodiments, the polynucleotide of the  
15 present disclosure is expressed by inserting the polynucleotide or a nucleic acid construct comprising the sequence into an appropriate vector for expression. In some embodiments involving the creation of the expression vector, the coding sequence is located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression. The recombinant expression vector may be any suitable vector (e.g., a plasmid or virus) that can  
20 be conveniently subjected to recombinant DNA procedures and bring about the expression of the polynucleotide of the present disclosures. The choice of the vector typically depends on the compatibility of the vector with the host cell into which the vector is to be introduced. The vector may be a linear or closed circular plasmid. In one embodiment, the vector is a viral vector.

Examples of viral vectors include retroviral vectors, lentiviral vectors, adenovirus vectors, adeno-associated virus vectors, alphavirus vectors and the like. In a certain embodiment, the viral vector is lentiviral vector. Lentiviral vectors are based on or derived from oncoretroviruses (the sub-group of retroviruses containing MLV), and lentiviruses (the sub-group of retroviruses containing HIV). Examples of such include, without limitation, human immunodeficiency virus (HIV), equine infectious anaemia virus (EIAV), simian immunodeficiency virus (SIV) and feline immunodeficiency virus (FIV). Alternatively, it is contemplated that other retroviruses can be used as a basis for a vector backbone such as murine leukemia virus (MLV).

[0045] In some embodiments, the vector used in the present disclosure is pLAS3w, pLAS3w.Ppuro, pLAS3w.Pneo, pLAS3w.Phyg and pLAS3w.Pbsd, pCMV- $\Delta$ R8.91 or pMD.G.

[0046] In another aspect, the present invention provides a kit or combination comprising the vector or engineered cell of the present disclosures and optionally an additional active agent. In one embodiment, the additional active agent is GCV.

[0047] The vector or engineered cell of the present disclosure is typically in combination with another carrier, e.g., compound or composition, inert (for example, a detectable agent or label) or active, such as an adjuvant, diluent, binder, stabilizer, buffers, salts, lipophilic solvents, preservative, adjuvant or the like and include pharmaceutically acceptable carriers. Carriers also include pharmaceutical excipients and additives, proteins, peptides, amino acids, lipids, and carbohydrates (e.g., sugars, including monosaccharides, di-, tri-, tetra-, and oligosaccharides; derivatized sugars such as alditols, aldonic acids, esterified sugars and the like; and polysaccharides or sugar polymers). Exemplary protein excipients include serum albumin such as human serum albumin (HSA), recombinant human albumin (rHA), gelatin, casein, and the like. The carrier further includes a buffer or a pH adjusting agent; typically, the buffer is a salt

prepared from an organic acid or base. Representative buffers include organic acid salts such as salts of citric acid, ascorbic acid, gluconic acid, carbonic acid, tartaric acid, succinic acid, acetic acid, or phthalic acid; Tris, tromethamine hydrochloride, or phosphate buffers.

[0048] Any of the compositions described herein may be comprised in a kit. In a non-limiting example, cells for cell therapy or one or more reagents to produce the cells may be  
5 comprised in a kit. The kits may also comprise a second container means for containing a sterile, pharmaceutically acceptable buffer and/or other diluent. Where there is more than one component in the kit, the kit also will generally contain a second, third or other additional container into which the additional component(s) may be separately placed. However, various  
10 combinations of components may be comprised in a vial. The kit may have a single container means, and/or it may have distinct container means for each compound. The kits of the present invention also will typically include a means for containing any container(s) in close confinement for commercial sale. Such containers may include injection or blow-molded plastic containers in which the desired vials are retained.

[0049] In a further aspect, the present invention provides a method for treating a cancer or  
15 enhancing intratumor immunity in a subject, comprising administering an effective amount of the engineered stem cell of the present disclosure to the subject. In one embodiment, the effective amount ranges from 100,000 ( $1 \times 10^5$ ) ~2,000,000 ( $2 \times 10^6$ ) cells. In some embodiment, the effective amount ranges from  $1 \times 10^5$  to  $1 \times 10^6$  cells.

[0050] In one embodiment, the cancer is a metastatic cancer.  
20

[0051] In one embodiment, the method enhances immunity in the tumor microenvironment through an increase in tumor-specific  $CD8^+IFN-\gamma^+CD44^+$  T cells with central memory potential. In one embodiment, the method induces a significant reduction in Tregs, and thereby reverses the

ratio of CD8<sup>+</sup> and CD4<sup>+</sup> T cells to Tregs within the tumors. The method also decreases the number of TAMs, which increases the ratio of CD8<sup>+</sup> and CD4<sup>+</sup> T cells to TAMs in the TME. In one embodiment, the effective amount ranges from 100,000 ( $1 \times 10^5$ ) ~2,000,000 ( $2 \times 10^6$ ) cells. In some embodiment, the effective amount ranges from  $1 \times 10^5$  to  $1 \times 10^6$  cells.

5 [0052] Exemplary cancers treated using methods and compositions as described herein are breast cancer, colon cancer, rectal cancer, lung cancer, ovarian cancer, prostate cancer, skin cancer, brain cancer, bladder cancer, endometrial cancer, kidney cancer, pancreatic cancer, thyroid cancer, or melanoma or metastatic cancers thereof. Exemplary cancer cells include, but are not limited to, carcinoma, melanoma, leukemia, fibrosarcoma, sarcoma, adenocarcinoma, and  
10 glioma.

[0053] Methods of delivery include but are not limited to intra-arterial, intra-muscular, and intravenous. In a specific embodiment, it may be desirable to administer the pharmaceutical compositions and/or cells of the disclosure locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, by  
15 injection or by means of a catheter. In some embodiments, the compositions or cells are administered by intravenous injection. In a further embodiment, the compositions or cells are administered by intramuscular injection. The compositions may be administered in one injection or in multiple injections. Solutions containing the cells can be prepared in suitable diluents such as water, ethanol, glycerol, liquid polyethylene glycol(s), various oils, and/or mixtures thereof,  
20 and others known to those skilled in the art. In some embodiments, the engineered stem cell of the present disclosure can be intravenously or intra-arterially administered to the subject. The present disclosure unexpectedly found that the above administrations of the engineered stem cell of the present disclosure have advantageous efficacy in treating a cancer, enhancing intratumor

immunity or enhancing immunity in the tumor microenvironment. Particularly, the intra-arterial administration exhibits better efficacy than the intravenous administration.

[0054] In one embodiment, the engineered stem cell of the present disclosure can be administered with an additional active agent. In some embodiments, the engineered stem cell and the additional active agent can be administered concurrently, separately or simultaneously. In one embodiment, the engineered stem cell and the additional active agent can be administered periodically. In a further embodiment, the additional active agent is GCV.

[0055] It is to be understood that if any prior art publication is referred to herein, such reference does not constitute an admission that the publication forms a part of the common general knowledge in the art.

[0056] Although disclosure has been provided in some detail by way of illustration and example for the purposes of clarity of understanding, it will be apparent to those skilled in the art that various changes and modifications can be practiced without departing from the spirit or scope of the disclosure. Accordingly, the foregoing descriptions and examples should not be construed as limiting.

### Examples

[0057] **Methods and Materials:**

[0058] **Preparation, isolation and characterization of UMSCs and Other Stem Cells**

[0059] Collected human umbilical cord tissues approved by the Institutional Review Board (IRB) of the China Medical University Hospital, Taichung were washed three times with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -free PBS (DPBS, Life Technology). They were mechanically cut by scissors in a midline direction and the vessels of the umbilical artery, vein and outlining membrane were dissociated from the Wharton's jelly (WJ). The jelly content was then extensively cut into pieces

smaller than 0.5 cm<sup>3</sup>, treated with collagenase type 1 (Sigma, St Louis, USA) and incubated for 3 h at 37 °C in a 95% air/5% CO<sub>2</sub> humidified atmosphere. The explants then were cultured in DMEM containing 10% fetal calf serum (FCS) and antibiotics at 37 °C in a 95% air/5% CO<sub>2</sub> humidified atmosphere. They were left undisturbed for 5–7 days to allow for migration of the cells from the explants. The cellular morphology of umbilical cord-derived mesenchymal stem cells (UMSCs) became homogenously spindle shaped in cultures after 4–8 passages, and the specific surface molecules of cells from the WJ were characterized by flow cytometric analysis. The cells were detached with 2 mM EDTA in PBS, washed with PBS containing 2% BSA and 0.1% sodium azide (Sigma, USA) and incubated with the respective antibody conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) including CD13, CD29, CD44, CD73, CD90, CD105, CD166, CD49b, CD1q, CD3, CD10, CD14, CD31, CD34, CD45, CD49d, CD56, CD117, HLA-ABC, and HLA-DR (BD, PharMingen). Thereafter, the cells were analyzed using a Becton Dickinson flow cytometer (Becton Dickinson, San Jose, CA).

[0060] Other types of stem cells can be obtained and cultured according to procedures known in the art.

[0061] **Plasmid construction:**

[0062] TK, NCR3, TRAIL, PD-1 and GFP cDNA from plasmids of TK (0.1 µg) (pUNO1-HSV1tk, InvivoGen), NCR3 (0.1 µg) (pLenti-C-mGFP-NCR3, Origene), TRAIL (0.1 µg) (pCMV6-myc-DDK-TRAIL, Origene), or PDCD-1 (0.1 µg) (pLenti-C-Myc-DDK-PDCD1, Origene) were transferred into pIRES (Clontech) or pSF-CMV-CMV-SbfI (Oxford Genetics) by specific restriction enzyme linker (EcoR1 and Nhe1 in TK, BamH1 and Not1 in PD-1) to build as the construct of pIRES-TK-PD-1, pIRES-TK-GFP, pIRES-PD-1-GFP, etc. which was transfected into UMSCs by XtremeGene HP DNA (Roche) per manufacturer's instruction to

engineer as the UMSC-TK-PD-1, UMSC-TK-GFP, and UMSC-PD-1-GFP. The above constructs can be transfected into other types of stem cells.

[0063] **Lenti-viral plasmids:**

[0064] Lentivector (pLAS3w), and packaging (psPAX2)/envelope plasmids (pMD2.G) were  
5 obtained from Academia Sinica, Taiwan. The cDNA encoding full-length human TK, NCR3, TRAIL, PD-1 and control GFP was recombinant from the cDNA (pUNO1-HSV1tk, InvivoGen; pLenti-C-mGFP-NCR3, Origene; pCMV6-myc-DDK-TRAIL, Origene pLenti-C-Myc-DDK-PDCD1, Origene) was transferred into pUltra (Addgene) and pSF-CMV-CMV-Sbf1 (Oxford  
10 Genetics) by specific restriction enzyme linker (EcoR1 and Nhe1 in TK, BamH1 and Not1 in PD-1) to build as the construct of pUltra-TRAIL-TK-PD-1, pUltra-TK-PD-1, pUltra-TK-GFP, pUltra-PD-1-GFP and pUltra-TRAIL-GFP. Subsequently, these templates were amplified by PCR using specific primers, and digested with restriction enzymes sub-cloned to lentiviral vector backbone plasmid pLAS2w and pLAS3w (Academia Sinica, Taiwan) (Lenti-TK-GFP, Lenti-PD-1-GFP, Lenti-TRAIL-GFP, Lenti-TK-PD-1-GFP, Lenti-TRAIL-PD-1-GFP and Lenti-TRAIL-  
15 TK-PD-1-GFP). To produce the recombinant lentivirus carrying TK, PD-1, TRAIL and control GFP, the recombinant plasmid and vector were co-transfected with packaging and enveloping plasmids into 293T cells at a ratio of 3:3:1 by XtremeGene HP DNA (Roche) transfection. The culture supernatant containing the virus particles was collected after 36 hours and again after another 24 hours at half the volume, and was then centrifuged at 15,000 rpm/min for 10 min to  
20 remove debris, and then transferred into a 36-mL ultra-centrifuge tube for ultra-centrifugation at 25,000 rpm/min for 3 h. The pellet containing lentivirus was resuspended. Viruses were thawed immediately before titer and cell transduction. UMSCs were infected with the appropriate lentivirus where gene transfer efficiency reached at least 80%.

**[0065] Lenti-virus Transduction**

[0066] Lenti-viral plasmid transductions were done in six-well plates. Unless otherwise specified, UMSCs were seeded at  $1 \times 10^5$  cells per well in triplicate at a final volume of 1 ml per well with a multiplicity of infection (MOI) of 5. Protamine sulfate (Sigma-Aldrich) from an 8 mg/ml stock solution (in DMEM-LG, sterile filtered) was added to obtain the desired final concentration. Cells were transduced for 24 hours before being replaced with 1.5 ml per well to build as UMSC-TRAIL-TK-PD-1, UMSC-TK-PD-1, UMSC-TRAIL-PD-1, UMSC-TRAIL, UMSC-TK, and UMSC-PD-1. Overgrown cells were inoculated onto a six-well plate for drug screening using 1.0 mg/ml G418 or puromycin solutions (Sigma). The medium was replaced every 2 days. The expression of green fluorescent protein (GFP) was observed using inverted fluorescence microscopy based on the color of the medium and the cell state. Following 7 days of screening, the complete medium without G418 was replaced and cultivation was continued.

**[0067] Construction of the piggyBac transposon system for stable cell lines**

[0068] PiggyBac vector pPB-CMV-MCS-EF1 $\alpha$ -RedPuro, which contains the multiple cloning sites (MCS), piggyBac terminal repeats (PB-TRs), core insulators (CIs) and puromycin selection maker (BSD) fused with RFP driven by the human EF1 $\alpha$ , was used as the base vector (System Bioscience). A DNA fragment containing TRAIL-TK-PD-1, TK-PD-1, TRAIL-PD-1, TRAIL, TK, and PD-1 (from pUltra-TRAIL-TK-PD-1, pUltra-TK-PD-1, pUltra-TRAIL-PD-1, pUltra-TRAIL, pUltra-TK, and pUltra-PD-1) was PCR amplified and subcloned into the pPB-CMV-MCS-EF1 $\alpha$ -RedPuro vector, in front of the coding region of EF1 $\alpha$ . Detailed information regarding vector constructions is available upon request. To generate UMSC stable cells, the above plasmids were co-transfected with a piggyBac transposase expression vector (System Biosciences) into UMSC cells by an electroporation method using Amaxa Nucleofector II

(Lonza). Stably cells (UMSC-TRAIL-TK-PD-1, UMSC-TK-PD-1, UMSC-TRAIL-PD-1, UMSC-TRAIL, UMSC-TK, and UMSC-PD-1) were selected in the presence of puromycin.

[0069] ***In vitro* proliferation, migration and differentiation assays**

[0070] For examining cellular proliferation and migration, bromodeoxyuridine (BrdU) incorporation and transwell migration assays were performed for comparison of UMSC-TRAIL-TK-PD-1 or UMSCs. Proliferation of UMSC-TRAIL-TK-PD-1 or UMSCs was tested by measuring BrdU incorporation (10  $\mu$ M) using a BrdU chemiluminescence immunoassay kit (Roche) and further confirmed by counting Trypan blue cells. After a 4-6 h starvation (incubation in medium lacking serum), UMSCs were incubated in medium for 2 days and pulse loaded with 10  $\mu$ M BrdU for 12 h as previously described (J Clin Invest **2009**;119:1997). UMSCs were then incubated with anti-BrdU-peroxidase for 90 min and staining was developed by incubating with substrate solution for 3 min. Plates were read with an Lmax microplate luminometer (Molecular Devices). Results as shown in Figure S5 were analyzed and presented as percent (%) increase over control.

[0071] Cell migration assay was assessed as described previously with modifications (EMBO Mol Med 2013;5:1227–1246). In brief, UMSC-TK-PD-1 or UMSCs were placed in 100  $\mu$ L in the upper chamber (transwell: 6.5-mm diameter, 5.0-mm pore size) according to manufacturer's instructions (Costar, #3421). We used SDF-1 $\alpha$  (100 ng/mL, R&D System, positive control) in the lower chambers. The assays were conducted over a 4-h incubation period at 37°C in a 5% CO<sub>2</sub> incubator. Because almost all cells stay at the lower side of the membrane after migration, quantification can be performed by simply counting these cells. Adhered cells at the lower side of the membranes were counted under the microscopy as previously described.

[0072] Adipogenic differentiation was induced according to the method described previously (J Orthop Res **2002**;20:1060). In brief, confluent monolayer cultures of UMSC-TK-PD-1 or UMSCs were grown in adipogenic differentiation medium, consisting of DMEM-high glucose (DMEM-HG, Sigma), 100 U/mL penicillin, 100 mg/mL streptomycin, 100 mM insulin (Sigma), 5 500 mM 3-isobutyl-1-methylxanthine (Sigma), 1 mM dexamethasone (Sigma), 100 mM indomethacin (Sigma) and 10% FCS. Cells maintained in ordinary UMSCs medium served as a negative control. The adipogenic differentiation was changed three times per week. To assess adipogenic differentiation, cells were stained with 0.3% oil red O (Sigma) for 10 min at room temperature, (to label intracellular lipid accumulation), and counterstained with haematoxylin.

10 [0073] To induce osteogenic differentiation, confluent monolayer UMSC-TK-PD-1 or UMSCs cultures were grown in DMEM-high glucose (DMEM-HG, Sigma) containing 100 U/mL penicillin (Sigma), 100 mg/mL streptomycin (Sigma), 50 mg/mL L-ascorbic acid 2-phosphate (Sigma), 10 mM b-glycerophosphate (Sigma), 100 nM dexamethasone (Sigma) and 10% FCS. Cells maintained in ordinary UMSC medium served as negative controls. The 15 osteogenic differentiation medium was changed three times per week. Levels of osteogenesis were determined using Alizarin red S staining (1%, Sigma) to detect calcium mineralization (J Biomed Mater Res **1998**,42, 433).

[0074] Chondrogenic differentiation of UMSC-TK-PD-1 or UMSCs was induced using a high-density pellet cell culture system (J Biomed Mater Res **1998**,42, 433). Cells were washed in 20 serum-free chondrogenic differentiation medium consisting of DMEM-HG, 100 U/mL penicillin, 100 mg/mL streptomycin, 50 mg/mL L-ascorbic acid 2-phosphate, 40 mg/mL proline (Sigma), 100 mg/mL sodium pyruvate (Sigma), 100 nM dexamethasone, and ITS-plus (10 mg/ml bovine insulin, 5.5 mg/ml transferrin, 5 mg/ml sodium selenite, 4.7 mg/ml linoleic acid, and 0.5 mg/ml

bovine serum albumin, Sigma). Aliquots of 250,000 cells were resuspended in chondrogenic differentiation medium and centrifuged at 250 ×g, and then 10 ng/mL TGF-β1 (R&D Systems) was added. Pellets maintained in chondrogenic differentiation medium without TGF-β1 served as negative controls. Medium was changed twice per week. Chondrogenic differentiation of pellet  
5 cultures was confirmed histologically using Alcian blue staining (Sigma) of sulfated proteoglycans. In addition, endothelial cells were induced to differentiate to vascular tubes formation by culturing UMSC-TK-PD-1 or UMSCs for 2–3 days in EBM (Cambrex) on 24-well plates precoated with Matrigel (300 μL/well; Becton Dickinson) and vascular endothelial growth factor (VEGF, 10 ng/ml, Sigma) as described previously (Nat Rev Cancer 2002, 2, 826).

10 [0075] To induce neural cell differentiation, UMSC-TK-PD-1 or UMSCs were incubated with DMEM using a three-step method with modification (Stem Cells Transl Med. 2015;4:775-88). Briefly, in the neural induction step, cells were plated at low density on 6-well plates containing fibronectin (Sigma), and then exposed sequentially to (1) DMEM-HG (Sigma), containing 10% FCS, and 10 ng/mL bFGF (R&D System) for 24 h, (2) in the neural commitment  
15 step, DMEM-HG containing, 1 mM β-mercaptoethanol (βME, Sigma), and 10 ng/mL NT-3 (R&D Systems) for 2 days, and (3) in the neural differentiation step, DMEM-HG containing NT-3 (10 ng/mL, R&D Systems), NGF (10 ng/mL, R&D Systems) and BDNF (50 ng/mL, R&D Systems) for 3 to 7 days. Following cell differentiation, the cells were left for immunohistochemical analysis.

20 [0076] **Flow-cytometry**

[0077] For the analysis of the cell surface-marker expression, cells were detached with 2 mM EDTA in PBS, washed with PBS containing BSA (2%) and sodium azide (0.1%), and then incubated with the respective antibody conjugated with fluorescein isothiocyanate (FITC) or

phycoerythrin (PE) until analysis. The gating was performed based on the justification of first gate, exclusion of doublets by FSC-A and FSC-H, exclusion of dead cells by selection of 7-AAD<sup>+</sup> (R&D systems)/CD45<sup>+</sup> or FSC-A according to the previous literature (Mucosal Immunol 2013, 6(3): 498-510). As a control, cells were stained with mouse IgG1 isotype-control antibodies. The antibodies to PD-1, PD-L1, CD3, CD8, CD4, CD25, Foxp3, CD44, CD45, CD11b, F4/80, IFN- $\gamma$ , CD206, TRAIL and GFP for flow cytometry were purchased from BD Biosciences. Cells were analyzed using a FACScan (BD) with CellQuest Analysis (BD Biosciences) and FlowJo software v.8.8 (TreeStar Inc.). Results are expressed by the percentage of positively stained cells relative to total cell number. For quantitative comparison of surface protein expression, the fluorescence intensity of each sample is presented as median fluorescence intensity (MFI). For intracellular staining of Ki-67 and granzyme B, TILs were cultured in the presence of 1  $\mu$ g/ml of anti-CD3 for 48 h. Cells were then incubated with anti-CD8 before permeabilization with Triton x100 and then stained with antibody against Ki-67 (Millipore) and Granzyme B. Data were analyzed using a FACScan (BD) with CellQuest Analysis (BD Biosciences) and FlowJo v.8.8 (TreeStar).

[0078] ***In Vitro* analysis of antigen-specific T-cell responses**

[0079] Splenocytes ( $2 \times 10^6$ ) from BALB/c mice were cultured on 24-well plates in RPMI-1640 media (Gibco) supplemented with 10% FBS (Sigma), 1% penicillin/streptomycin (Gibco). Then, splenocytes cocultured with UMSC-TRAIL-TK-PD-1 ( $2 \times 10^5$ ) were either left unstimulated or incubated with CD3-CD28 beads (Dynabeads, Thermo). For proliferation assays, splenocytes were stained with Carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen) as previously described (Nat Protoc, 2007;2:2049-56). We estimated the proliferation/division of cells using the Proliferation Index (PI), which can be calculated by the formula: PI = total

number posterior to proliferation / total number prior to proliferation. After a 6-day-culture cells were harvested and stained to analyze proliferation of Treg, CD4- and CD8- T cell subsets. Alternatively, to analyze proliferation after a 6-day culture in longitudinal samples for which cell numbers were limited, non-CFSE stained splenocytes were cultured as previously described, and  
5 stained with Ki67 or isotype control antibodies. Fold change in proliferation (FC proliferation) was calculated as a ratio of proliferation under UMSC-TRAIL-TK-PD-1 condition divided by proliferation under control condition.

[0080] Moreover, mixed lymphocyte response (MLR) assays were performed by co-culturing  $1 \times 10^5$  cells responder  $CD4^+$  T cells from mice spleen enriched by nylon wood column  
10 (Polysciences) with allogeneic dendritic cells (DC) at a ratio of 10:1 (T:DC) in flat-bottom 96-well microtiter plates.  $CD4^+$  T cells and allogeneic DC were incubated for 6 days in the absence or presence of UMSC-TK-PD-1 ( $10^2$ ,  $10^3$  and  $10^4$ ). The effector T cells were serially stimulated a total of three times. Culture supernatants were harvested on day 5 for ELISA analysis of IFN- $\gamma$  and IL-12 secretion (R&D).

15 [0081] **Suicide effect in UMSC-TK-PD-1 with ganciclovir (GCV) *in vitro***

[0082] To investigate the biological effect *in vitro*, the suicide ability of UMSC-TK-PD-1 combined with GCV was analyzed. After incubation at 37 °C in 5% CO<sub>2</sub> for 24 h, various dosages of GCV (0.1, 1, 10 and 100  $\mu\text{g}/\text{mL}$ ) were added in each well every day for 7 consecutive days. Cell viability was evaluated by MTT assay (Invitrogen) and GFP fluorescent  
20 intensity by luminometer (Promega).

[0083] ***In vitro* bystander effect assay**

[0084] 4T1-Luc (BCRC, Taiwan), CT26-Luc (BCRC, Taiwan) or Hep-55.1C-Luc (BCRC, Taiwan) cells ( $1 \times 10^4$  cells) and various numbers of UMSC-TRAIL-TK-PD-1 cells (UMSC-

TRAIL-TK-PD-1: tumor cells ratios = 1:1, 1:4, 1:16, 1:32, and 1:64) co-incubated at 37 °C in 5% CO<sub>2</sub> in DMEM with 10% FBS were seeded on a 24 well-plate. The medium was replaced every day with fresh medium containing 100 µg/mL GCV for 7 consecutive days. UMSC-TRAIL-TK-PD-1 and 4T1-Luc cells were also seeded in DMEM medium with 10% FBS without GCV as the corresponding control group. After 8 days, luciferase fluorescent intensity by luminometer (Promega) was acquired from 5 random fields to determine cell density. Further investigation on the time course of bystander effect of the above co-culture system was performed in the same number of UMSC-TRAIL-TK-PD-1 and 4T1-Luc cells seeded in the 12-well culture plates with GCV (100 µg/mL) daily. Cell mortality ratio was measured as percentage of the fluorescence intensity of GFP and luciferase through a luminometer (Promega).

[0085] ***In vitro* apoptosis assay**

[0086] To investigate the pro-apoptogen potential toward various tumor cells, we carried out cocultures at 1:2 ratio and evaluated the cytotoxicity at 24 h by Annexin-V-FITC/propidium iodide (PI) staining (eBioscience) using the FACScanto II. The tumor cell population was gated based on forward scatter (FSC) and side scatter (SSC) parameters.

[0087] **Mice model and tumor Inoculation**

[0088] All animal experiments were carried out in accordance with Institutional Guidelines on Animal Research of China Medical University. Six to eight week-old female BALB/c (National Animal Center of Taiwan) mice were utilized for building the mouse cancer model using 4T1, CT26, Hep-55.1C, CT26-Luc, 4T1-Luc or Hep-55.1C-Luc. In brief, 4T1 cells ( $1 \times 10^6$ ) were implanted in the 4<sup>th</sup> mouse mammary fat pad of female BALB/c mice at the right side of the abdomen, and the treatments were started on day 8 after tumor implantation.

[0089] ***In Vivo* UMSCs migration assay**

[0090] To examine the biodistribution of intravenously or intra-arterially injected stem cells, luciferase gene (pHAGE PGK-GFP-IRES-LUC-W, Addgene) was subcloned into pUltra-TRAIL-TK-PD-1 and then pLAS3w to construct Lenti-TRAIL-TK-PD-1-Luc. UMSCs engineered by Lenti-TRAIL-TK-PD-1-Luc (UMSC-TRAIL-TK-PD-1-Luc) ( $2 \times 10^6$  cells) were injected days into the femoral vein or femoral artery of 4T1 tumor-bearing mice at 7 days after tumor inoculation. *Ex vivo* imaging was performed by placing whole animal in the IVIS Lumina Imaging System (Xenogen) at indicated time points after UMSC-TRAIL-TK-PD-1-Luc injection (6h, 1d, 3d, 6d, 9d and 14d) and analyzing fluorescence based on the manufacturer's recommendations. Fluorescence intensity was quantified as photons/sec/cm<sup>2</sup> by Living Image software (Xenogen). Mice were sacrificed at 24 hours after UMSC injection, and then various organs (lung, liver, spleen, heart, kidney, and brain) were isolated. Each organ was minced, treated with collagenase, and prepared for flow-cytometric analysis.

[0091] **Bioluminescent imaging (BLI)**

[0092] Animals were imaged with the IVIS Imaging System 200 Series (Xenogen) to record bioluminescent signal emitted from the 4T1-Luc, CT26-Luc, Hep-55.1C-Luc (luciferase expression). Animals were anesthetized with isoflurane and received intra-peritoneal injection of D-luciferin (Caliper) at a dose of 270 mg/g body weight. Imaging acquisition was performed at 15 min after intraperitoneal injection of luciferin. For BLI analysis, regions of interest encompassing the intracranial area of signal were defined using an IVIS System (Xenogen), and the total photon flux was recorded. To facilitate comparison of cellular engrafted rates, each animal's luminescence scores were normalized against its own luminescence reading at Day14, thereby allowing each mouse to serve as its own control.

[0093] ***In vivo* therapeutic effect of UMSC-TRAIL-TK-PD-1 on tumor-bearing mice**

[0094] In 4T1-Luc and Hep55.1C-Luc mice model, we first examined whether the intravenous injection of UMSC-TRAIL-TK-PD-1 could significantly induce the tumoricidal effect compared to the anti-PD-1 (Roche) or IgG-control. Then, the treatment groups were subdivided into six groups (**Figure 6A**): IgG-control group; UMSC-PD-1 (UP) group; UMSC-TRAIL (UT) group; UMSC-TRAIL-PD-1 (UTP) group; UMSC-TRAIL-TK+GCV (UTTG) group; and UMSC-TK-PD-1+GCV (UTPG) group. Before each treatment, cells were subjected to hypoxia preconditioning protocol with incubation in 3% O<sub>2</sub> level for 24 hours to induce CXCR4 (Millipore) upregulation to enhance the tumor homing effect (Cancer Research 2012;73:2333-2344). Antitumor effects of sequential therapy were evaluated by repeating second and third injections of 5 × 10<sup>5</sup> cells in each group at 10-day interval. GCV (50 mg/kg) was administered intraperitoneally for 7 consecutive days starting on day 2 after each treatment administration.

[0095] Next, to further demonstrate whether the intra-arterial injection of UMSC-TRAIL-TK-PD-1 through femoral artery could significantly induce the tumoricidal effect compared to the UMSC-TK-PD-1, the treatment groups were again subdivided into six groups (**Figure 6F**): IgG-control group; UMSC-TK-PD-1+GCV (UTPG) group; UMSC-TRAIL-TK-PD-1 (UTTP) group; and UMSC-TRAIL-TK-PD-1+GCV (UTTPG) group.

[0096] **Survival study**

[0097] To determine the therapeutic effect of UMSC-TRAIL-TK-PD-1 on the survival of 4T1-Luc and Hep55.1C-Luc tumor-bearing mice in vivo, the mice were treated with six different therapeutic targets via the right femoral vein every 4 days three consecutive times (q4dx3) within 10 days after tumor inoculation (n=8). The tumor volumes were monitored using a digital caliper (Mitutoyo) every 2–3 d using the following equation: Tumor *volume* (mm<sup>3</sup>) =  $\frac{W^2 \times L}{2}$  (Equation

1), where W is the width of the tumor and L is the length of the tumor ( $W < L$ ). Animals were euthanized when the volume was over  $3,000 \text{ mm}^3$  for ethical reasons. Mice were sacrificed when tumor size reached 2 cm at the largest diameter, or when their body weight decreased to less than 80%. The survival rate using Kaplan–Meier survival analysis was reported as the median and mean survival times with a 95% confidence interval. The statistical difference between these different conditions was determined by log-rank analysis ( $n=8$ ).

[0098] Isolation of infiltrating leukocytes (TILs), splenic cells and peripheral blood mononuclear cells (PBMCs)

[0099] Four weeks after the final treatment, leukocytes in the tumors, spleens or peripheral blood were harvested from the freshly euthanized mice. Tumor-infiltrating lymphocytes (TILs) were prepared for single cell suspensions using the method previously described (Blood 2005;06:2339). In brief, TILs were isolated by digesting tumor tissue with collagenase type IV ( $2.5 \text{ mg ml}^{-1}$ , Gibco) for 20 min and concentrated by centrifugation in a discontinuous percoll gradient (GE Healthcare). The  $\text{CD8}^+$  T cells in TIL suspensions were separated by mixing with  $\alpha\text{CD8}$  microbeads (Miltenyi Biotec) on a MACS column or staining with anti-CD8 antibody on a FACS Aria (BD Biosciences) sorter (purity  $>95\%$ ). The total number of infiltrating  $\text{CD8}^+$  T cells/gram of tumor was obtained by multiplying the percentage of  $\text{CD8}^+$  T cells by the total number of lymphocytes obtained from percoll gradient and dividing that number by 100 and by the weight of the tumors. Tumor associated macrophage (TAM,  $\text{CD11b}^+\text{CD206}^+\text{F4/80}^+$  cells) in TIL suspension was examined on a FACS Aria (BD Biosciences) after staining with anti-CD11b, anti-CD206, or anti-F4/80 antibodies (purity  $>95\%$ ). Regulatory T lymphocytes (Treg,  $\text{CD4}^+\text{CD25}^+$ ) were purified using an isolation kit (Miltenyi Biotec) (purity  $> 90\%$ ). Regulatory T lymphocytes ( $\text{CD4}^+\text{CD25}^+\text{Foxp3}^+$  cells) were assayed on a FACS Aria (BD Biosciences) after

staining with anti-CD4, anti-CD25 and anti-Foxp3 antibodies (purity >95%). We teased the spleens apart and filtered them with nylon mesh screen to obtain single cell suspensions. To further produce single cell splenocyte suspensions, we removed red blood cells by using RBC lysis buffer. The splenic CD8<sup>+</sup> T cells were separated by mixing the cells with  $\alpha$ CD8 microbeads (Miltenyi Biotec) on a MACS column or staining with anti-CD8 antibody on a FACS Aria (BD Biosciences) sorter (purity >95%).

[00100] Peripheral blood mononuclear cells (PBMCs) were isolated from each mouse (Blood. 2001; 98: 3520-6). The cells were collected using the Ficoll-Histopaque (Sigma Aldrich) centrifugation method (Science. 1997; 275: 964-7), and washed twice with 1 mM EDTA in PBS for further experimentation.

[00101] **Flow cytometry**

[00102] TILs suspension were washed with PBS containing BSA (2%) and sodium azide (0.1%). Cell were stained with the respective fluorochrome-conjugated monoclonal antibodies to the cell surface markers as follows: anti-PD-L1 (MIH5), anti-CD3 (145-2C11), anti-CD8 (53-6.7), anti-CD11b (M1/70), anti-CD45 (30-F11), anti-IFN- $\gamma$  (XMG1.2), anti-CD44 (IM7.8.1R), anti-CD4 (GK1.5), anti-CD25 (PC61.5), anti-Foxp3 (MF23), anti-F4/80 (BM8) and anti-CD206 (MR5D3). As a control, cells were stained with mouse IgG1 isotype-control or IgG2 isotype-control antibodies. Cells were analyzed using a FACScan (BD) with CellQuest Analysis (BD Biosciences) and FlowJo software v.8.8 (TreeStar Inc.).

[00103] The gating was performed based on the right justification of first gate, exclusion of doublets by FSC-A and FSC-H, exclusion of dead cells and further selected as being 7AAD<sup>+</sup>/CD45<sup>+</sup> (or FSC-A) according to previous literature (*Mucosal Immunol.* 2013;6:498-510). Then, CD8<sup>+</sup> T cells, CD4<sup>+</sup> T cells, Treg and TAM from TIL or splenocyte suspensions were

analyzed using a FACScan (BD) with the CellQuest Analysis (BD Biosciences) and FlowJo software v.8.8 (TreeStar Inc.). Results were expressed by the percentage of positively stained cells relative to total cell number. Differences between groups were evaluated by two-way ANOVA with Newman-Keuls post hoc test. A *P* value < 0.05 was considered significant.

5 [00104] **Isolation of CD8<sup>+</sup>CD44<sup>+</sup>IFN- $\gamma$ <sup>+</sup> T cells from TILs**

[00105] The CD8<sup>+</sup> T cells in TILs suspension were separated by mixing with  $\alpha$ CD8 microbeads (Miltenyi Biotec) on a MACS column. To examine the expression of IFN- $\gamma$  and CD44, the separated CD8<sup>+</sup> T cells were treated using anti-mouse CD28 mAb (0.5  $\mu$ g), Monensin, and brefeldin A for 3 hours. In the meantime, they were co-cultured with  $1 \times 10^6$  irradiated 4T1-Luc cells (at a rate of 84 cGy min<sup>-1</sup> with a 0.5-mm Cu filter, Philips x-ray unit) at 37 °C for 24 h. Flow cytometric analysis of IFN- $\gamma$  and CD44 expression was then performed using BD Cytotfix/Cytoperm Plus Kit following the manufacturer's instructions.

[00106] **Evaluation of Ki-67 and granzyme B expression in CD8<sup>+</sup> T cells**

15 [00107] For intracellular staining of Ki-67 and granzyme B, TILs were cultured in the presence of anti-CD3 (1  $\mu$ g ml<sup>-1</sup>) for 48 h. The cells were then incubated with anti-CD8 before permeabilization with Tritonx100, and stained with antibody against Ki-67 and Granzyme B (Millipore).

[00108] **CFSE test**

20 [00109] Tregs isolated from tumor were co-cultured with carboxy-fluorescein diacetate succinimidyl ester (CFSE)-treated spleen CD8<sup>+</sup> T cells in the presence of CD3 antibodies, and the proliferation of CD8<sup>+</sup> T cells was monitored using the fluorescent intensity of CFSE. The CFSE<sup>low</sup> cells are defined as the cells with lower fluorescent intensity than the original population, which represented the proliferated CD8<sup>+</sup> T cells.

**[00110] Cytokine measurement**

[00111] TILs from mice treated with different protocols were cultured directly at a density of  $2 \times 10^5$  cells/mL in 6-well plates in PRMI-1640 (Invitrogen) medium with 2 mM L-glutamine (Sigma-Aldrich) for 48 h. The level of TNF- $\alpha$ , VEGF, IL-10 and TGF- $\beta$  was measured with Quantikine ELISA kit (R&D Systems). Semi-quantitative analysis of TNF- $\alpha$ , VEGF, IL-10 and TGF- $\beta$  levels in culture supernatants and serum was performed. Optical density was measured using a spectrophotometer (Molecular Devices) and standard curves were generated with the program SOFTmax (Molecular Devices).

**[00112] Evaluation of anti-metastasis ability**

[00113] The mice was tumor-challenged mice by 4T1-Luc, CT26-Luc or Hep-55.1C-Luc were examined for lung metastasis by direct visual counting of the metastatic nodules. Lungs were then excised and washed once in water and fixed further by immersion in 4% PFA, and dehydrated in 30% sucrose at room temperature. Surface metastases subsequently appeared as white nodules and were counted under a microscope.

**[00114] Immunohistochemical assessment**

[00115] Animals were anesthetized with chloral hydrate (0.4 g/kg, ip) and their abdominal skin tissues were fixed by transcardial perfusion with saline followed by immersion in 4% paraformaldehyde. Tissue samples were dehydrated in 30% sucrose, frozen on dry ice, and then cut in a series of adjacent 6- $\mu$ m-thick coronal sections using a cryostat. Sections were stained with H&E and Prussian blue (for identifying the iron) for observation by light microscopy (Nikon, E600). Each section was immunostained with antibodies against CD4 (1:100; BD), CD8 (1:400; BD), using secondary antibodies conjugated with FITC or Cy-3 (1:500; Jackson Immunoresearch) and then analyzed in three-dimensional images using a Carl Zeiss LSM510

laser-scanning confocal microscope. The total number of cells co-stained with a cell type-specific marker was measured as previously described (*J. Cereb. Blood Flow Metab.* 2008;**28**,1804-1810).

**[00116] TUNEL assay**

5 [00117] Cellular apoptosis was assayed by immuno-histochemistry using a commercial TUNEL staining kit (DeadEnd Fluorimetric TUNEL system; Promega) as previously described (*Proceedings of the National Academy of Sciences* 2009;**106**, 9391-9396. The percentage of TUNEL labeling was expressed as the number of TUNEL-positive nuclei divided by the total number of nuclei stained with DAPI (*Nat. Protocols* 2016;**11**:688-713; *PLoS Genet.* 10 2009;**5**:e1000379). The apoptotic index expressed as the percentage of TUNEL-positive apoptotic nuclei divided by the total number of nuclei visualized by counterstaining with DAPI obtained from counts of randomly chosen microscopic fields.

**[00118] Assessment of immune-related adverse events (irAEs)**

[00119] We evaluated irAEs including: (1) weight monitoring, (2) histology, (3) immune cell 15 infiltration, and (4) liver and kidney function after the treatment of each group. The body weight of the mice was monitored during the treatment. In addition, H&E staining of livers, lungs, spleens, kidneys, and colons sections of the mice treated with each group were evaluated at 4 weeks after tumor inoculation (n=6) for histology analysis. The CD8<sup>+</sup> and CD4<sup>+</sup> T cell infiltration (*Cancer Res* 2016;**76**:5288-5301) to liver, colon, kidney, and lung were examined by 20 IHC and scored by counting the number of positive cells in ten high power field per mm<sup>2</sup>. Furthermore, biochemical profiles of the ALT, AST, creatinine and glucose were measured using mouse serum from sequential time points (0, 5 10, 15, 20, 25 and 30 d) of each group (n= 6) by a Beckman Unicell DxC800 analyzer.

[00120] **Statistical analysis**

[00121] All measurements in this study were performed in a blinded design. Results were expressed as mean  $\pm$  SEM. Two-tailed Student's *t* tests was used to evaluate significance of mean differences between the control and the treated group. Differences between groups were  
5 evaluated by two-way ANOVA with the Newman-Keuls post hoc test. A *P* value  $< 0.05$  was considered significant.

**Example 1 In vitro characterization of UMSCs and UMSC-TK-PD-1**

[00122] We prepared the primary cultures of umbilical cord mesenchymal stem cells (UMSCs) from Wharton's jelly (WJ) and analyzed the cell morphology and biological properties (**Figure**  
10 **1A**). The flow cytometry revealed that the cells were negative for CD1q, CD3, CD10, CD14, CD31, CD34, CD45, CD49d, CD56, CD117 and HLA-DR, but positive for CD13, CD29, CD44, CD73, CD90, CD105, CD166, CD49b and HLA-ABC (**Figure 1 B**). These observations indicate that UMSCs have the same surface markers as those of mesenchymal stem cells (MSCs), consistent with observations of bone marrow MSCs (J Cell Sci **2004**, 117, 2971).

15 [00123] To evaluate the UMSCs transfection efficacy, the RFP fluorescence and PD-1 expression level of UMSC-TRAIL-TK-PD-1 were analyzed by flowcytometry study. At 36 h to 48h after transfection, it demonstrated that the uptake efficacy was on average 55-65% via the results of RFP and PD-1 flowcytometry (**Figure 1C**). Subsequently, after 3-5 days puromycin or G418 screening, over 90% of cells were fully transduced with the transgenes (**Figure 1C**).

20 [00124] UMSC-TRAIL-TK-PD-1-Luc retained luciferase expression for over 100 days (**Figure 1D**) and the cell viability by MTT assay, cell proliferation assay by BrdU incorporation and migration by transwell assay (**Figure 1E**) revealed that pLAS3w-TRAIL-TK-PD-1 labeling

did not affect the UMSC-TRAIL-TK-PD-1 cellular viability, cell proliferation or migration *in vitro* compared to unlabeled UMSCs after 14 h of incubation.

[00125] To demonstrate whether UMSC-TRAIL-TK-PD-1 still possessed multipotent differentiation potential, we analyzed the adipogenic, chondrogenic, osteogenic and vascular tube formation, which demonstrated that the UMSC-TRAIL-TK-PD-1 displayed similar behavior to the plain UMSCs without plasmid labeling (**Figure 1F**). The UMSC-TRAIL-TK-PD-1's neuroglial cell differentiation was identified by immunofluorescence with MAP-2, Tuj-1 and GFAP and exhibited refractile cell body morphology with extended neurite-like structures arranged into a network as the plain UMSCs (**Figure 1G**). Therefore, UMSC-TRAIL-TK-PD-1 did not lose cell differentiation potential *in vitro*.

#### **Example 2 Specific protein binding of PD-L1 with UMSC-TRAIL-TK-PD-1 *in vitro***

[00126] Since tumor cells express PD-L1 for the purpose of immune escape (Trends Immunol. 2006;27:195–201), we established a gene-modified UMSCs of UMSC-TRAIL-TK-PD-1 in which presenting PD-1 could catch tumor cells by PD-1/PD-L1 interaction. To illustrate the protein-ligand binding affinity of UMSC-TRAIL-TK-PD-1, we analyzed the RLU of HRP-conjugated PD-1 protein at various concentrations by ELISA. The UMSC-TRAIL-TK-PD-1 was incubated with HRP-conjugated PD-L1 protein at 37°C for 2 hours. The binding affinity of HRP-conjugated PD-1 protein increased significantly in a dose-dependent manner (**Figure 2A**). The result indicates that UMSC-TRAIL-TK-PD-1 have high binding efficiency to PD-L1.

#### **Example 3 *In Vitro* activity of UMSC-TRAIL-TK-PD-1 in human T cells**

[00127] To determine if the stimulatory effect was direct interaction between T cell and MSCs, splenocyte T cells were stimulated with CD3-CD28 beads. The gating strategy was based on the justification of first gate, exclusion of doublets by FSC-A and FSC-H, exclusion of dead cells by

selection of 7-AAD<sup>+</sup> (R&D systems)/CD45<sup>+</sup> or FSC-A depicted in **Figure 2B**. T cells were labeled with CFSE and then co-cultured with either UMSCs or UMSC-TRAIL-TK-PD-1 stimulated with CD3-CD28 beads for 6 days. UMSCs (at a ratio of 1:1) significantly suppressed both CD4<sup>+</sup> and CD8<sup>+</sup> T-cell proliferation (**Figure 2C**), but not at a ratio of 1:10. However, at either ratio of 1:1 or 1:10, UMSC-TRAIL-TK-PD-1 significantly increased both CD4<sup>+</sup> and CD8<sup>+</sup> T-cell proliferation (**Figure 2C**). Moreover, UMSC-TRAIL-TK-PD-1 stimulated with CD3-CD28 beads significantly increased level of CD4<sup>+</sup>INF- $\gamma$ <sup>+</sup> and reduced CD8<sup>+</sup>CD122<sup>+</sup> compared to UMSCs (**Figure 2D**). These results suggest that either ratio of UMSC-TRAIL-TK-PD-1 can support T-cell proliferation, whereas higher ratios are inhibitory.

#### 10 **Example 4 Suicide effect of UMSC-TRAIL-TK-PD-1 *in vitro***

[00128] To investigate the thymidine kinase (TK)-induced cell killing effect in UMSC-TRAIL-TK-PD-1, suicide effect tested by evaluating the cell viability of UMSC-TRAIL-TK-PD-1 in the presence of various concentrations of GCV was performed. First, significantly increased level of TK was found in the UMSC-TRAIL-TK-PD-1 in a time- and dose-dependent manner (**Figure 3A**). GCV itself did not affect cell proliferation of UMSCs (**Figure 3B**). Phosphorylated GCV induced apoptotic-like cell injury in UMSC-TRAIL-TK-PD-1 after GCV treatment (**Figure 3B**). The cell proliferation of UMSC-TRAIL-TK-PD-1 was inhibited in a dose-dependent manner (**Figure 3B**). It indicates that the UMSC-TRAIL-TK-PD-1 could express TK after transfecting plasmid of TRAIL-TK-PD-1 and could activate GCV to its toxic form by inducing cytotoxicity on the UMSCs themselves.

[00129] Sensitivity of tumor cells to the bystander effect of UMSC-TRAIL-TK-PD-1 *in vitro*

[00130] To examine the bystander effect through UMSC-TRAIL-TK-PD-1, both 4T1 (Hep55.1C, Pan18, CT26) and UMSC-TRAIL-TK-PD-1 cell viability were evaluated by direct

co-culture with different ratios of each cell in various concentrations of GCV (**Figure 3C**). UMSC-TRAIL-TK-PD-1 could significantly attenuate the growth of 4T1-Luc cells (Hep55.1C, Pan18-Luc, CT26-Luc and GL261-Luc) ( $n = 3$ ) when the ratio was maximal on 1:32 and minimal on 1:1 with the presence of 100  $\mu\text{g}/\text{mL}$  GCV after co-culturing for 7 days. Moreover, it  
5 demonstrated the best inhibition efficiency was at the ratio of 1:1 (**Figure 3D-E**).

[00131] To further confirm the bystander effect of UMSC-TRAIL-TK-PD-1, the time course (before 7 days) of both the suicide effect and bystander effect of UMSC-TRAIL-TK-PD-1 were investigated. Cell mortality rate by suicide effect in this co-culture system slowly reached about one-third of the whole system during the first two days, and then subsequently accelerated from  
10 day 3 to day 6. In the bystander effect experiments, the same findings showed that most of 4T1-Luc cells were killed from day 3 to day 5 (**Figure 3C-E**). Moreover, flowcytometric study also demonstrated that UMSC-TRAIL-TK-PD-1 cocultured with 4T1 cells significantly increased apoptotic cells ( $\text{PI}^+\text{Annexin-V}^+$  cells) in a GCV dose-dependent manner (**Figure 3C**). Therefore, the suicide effect of UMSC-TRAIL-TK-PD-1 and bystander effect on 4T1-Luc cells (Hep55.1C,  
15 Pan18-Luc, CT26-Luc and GL261-Luc) occurred from day 3 to day 5 in the co-culture system.

[00132] UMSC-TRAIL-TK-PD-1 expressing TRAIL displays an *in vitro* antitumor activity in 4T1-luc cell.

[00133] UMSC-TRAIL-TK-PD-1 can be genetically modified to express high levels of TRAIL. UMSC-TRAIL-TK-PD-1 was transduced by vector encoding for full-length human  
20 TRAIL. FACS analysis shows a relevant TRAIL protein expression (90%) on the cell surface of UMSCs (**Figure 4A**).

[00134] To demonstrate whether the UMSC-TRAIL-TK-PD-1 could exert a tumoricidal effect on cancer cells, coculture experiments between tumor cells and UMSC-TRAIL-TK-PD-1 were

then performed. UMSC-TRAIL-TK-PD-1 expressing TRAIL induces cells apoptosis (4T1-Luc, Hep55.1C-Luc), which was represented by cell shrinkage, reduction of adherent 4T1-Luc cells and Hep55.1C-Luc with the appearance of cellular debris, which were demonstrated by propidium iodine staining (PI staining), especially at 48 hours after coculture (**Figure 4B**). To  
5 quantify cell death at 24, 48 and 72 hours, a large amount of Annexin-V<sup>+</sup>PI<sup>+</sup> dead cells ( $\geq 70\%$ ) was detected in coculture where UMSC-TRAIL-TK-PD-1 are present in a dose-dependent manner as measured by FACS analysis (**Figure 4C**).

#### **Example 5 Tumor targeting of UMSC-TRAIL-TK-PD-1-Luc in the 4T1 Tumor Model**

[00135] To demonstrate the UMSC-TRAIL-TK-PD-1 homing effect, biodistribution of  
10 UMSC-TRAIL-TK-PD-1-Luc after intravenous or intra-arterial implantation was performed using IVIS. First, bioillumiance intensity increased in a cell dose-dependent manner as measured by IVIS *in vitro* (**Figure 5A**). In healthy mice, intravenous UMSC-TRAIL-TK-PD-1-Luc transplantation was initially entrapped in the lung capillary from one day after injection, which showed enhanced bioluminescent image of IVIS in lung (**Figure 5B**). Homing of UMSC-  
15 TRAIL-TK-PD-1-Luc makes the UMSC-TRAIL-TK-PD-1-Luc survive and relocate to the subcutaneous 4T1 tumors. The bioluminescent signal of the subcutaneous tumor area in IVIS image was observed initially at five days after UMSC-TRAIL-TK-PD-1-Luc injection, gradually increased in intensity afterward, and peaked at day 14 (**Figure 5B**).

[00136] UMSC-TRAIL-TK-PD-1-Luc transplantation, was directly recruited to the orthotopic  
20 4T1 tumor region (also for Hep55.1C and pan18 tumor region) two hours after intra-femoral artery injection without lung entrapment, which showed enhanced bioluminescent image of IVIS (**Figure 5C-E**). Subsequently, homing of UMSC-TRAIL-TK-PD-1-Luc makes UMSC-TRAIL-TK-PD-1-Luc survive and relocate to the tumors sites.

[00137] To further demonstrate whether UMSC-TRAIL-TK-PD-1-Luc could track the metastatic loci originating from 4T1-tumor model, intra-arterial implantation of UMSC-TRAIL-TK-PD-1-Luc was performed 21 days after induction of the 4T1-tumor model. Consistently, metastatic lung tumor from the original 4T1-tumor model significantly recruited the UMSC-TRAIL-TK-PD-1-Luc to increase the bioillumination intensity as measured by IVIS in the multiple metastatic sites (**Figure 5F**). Through immunohistochemical analysis, numerous GFP<sup>+</sup>Luciferase<sup>+</sup> cells were found in the 4T1 tumor at one day after treatment, which indicated UMSC-TRAIL-TK-PD-1-GFP was recruited into the tumor microenvironment (**Figure 5G**).

#### **Example 6 Therapeutic effect of UMSC-TRAIL-TK-PD-1 on 4T1-Luc model**

[00138] The tumoricidal effects in luciferase-expressing 4T1-Luc- and Hep55.1C-Luc-tumor-bearing mice treated with various strategies of gene modified UMSCs were assessed by IVIS, tumor volumes and survival time following the *q4dx3* course of treatment protocol (**Figure 6A**). Prior to treatment, every group of tested cells was subjected to hypoxia preconditioning culture in 3% O<sub>2</sub>, which induced CXCR4 overexpression for enhancing stem cell homing in a time-dependent manner (**Figure 6B**). Clearly, the UMSC-PD-1 (UP) group and UMSC-TRAIL (UT) group exhibited a therapeutic effect, reducing tumor volumes compared to those in the IgG control group as measured by IVIS (**Figure 6C**). Moreover, the UMSC-TK-PD-1+GCV (UTPG) group, UMSC-TRAIL-TK+GCV (UTTg) group, and UMSC-TRAIL-PD-1 (UTP) group showed stronger antitumoral effects and each of them exhibited inhibition of tumor growth respectively (**Figure 6C**). The median survival times of the mice treated with IgG, UP, UT, UTPG, UTTg, and UTP were 24, 32, 34, 34, 43, and 44 days, respectively (**Figure 6D**). UTP significantly extended survival time to 63 days compared to the other groups (**Figure 6D**). Furthermore, UTP significantly prevented tumor metastasis in lungs compared with other treatments (**Figure 6E**).

On average, fewer than 5 nodules of lung metastasis were discovered in UTP-treated mice compared to over 20 metastases in lungs of the control mice. However, UMSC-TK (UT), UP and UTP did not show a significant decrease in metastasis compared to the control group. As a result, we hypothesized that the metastasis was not only inhibited by UTP-induced bystander effect, but largely affected by the immune-enhancing effect from UTP in the TME, which led us to systemically analyze the intratumoral immunity.

[00139] Next, to verify whether intra-arterial injection of UMSC-TRAIL-TK-PD-1 displayed a significant therapeutic effect in 4T1-Luc and Hep55.1C-Luc model following the *q7dx2* course of treatment protocol (**Figure 6F**), four groups (UMSC-TK-PD-1+GCV (UTPG) group, UMSC-TRAIL-TK-PD-1 (UTTP) group, and UMSC-TRAIL-TK-PD-1+GCV (UTTPG) group) were examined the tumor growth and median survival time. Before the analysis of intra-arterial injection, intravenous administration of UMSC-TK-PD-1+GCV (UTPG) group, UMSC-TRAIL-TK-PD-1 (UTTP) group, and UMSC-TRAIL-TK-PD-1+GCV (UTTPG) group showed stronger antitumoral effects and exhibited inhibition of tumor growth respectively (**Figure 6F-G**).

Importantly, intra-arterial implantation revealed a robustly superior therapeutic effect to the intravenous ones. Moreover, the UTTPG group significantly inhibits tumor growth and enhances the median survival times of the mice than the other groups of IgG control, UTPG and UTTP in 4T1-Luc and Hep55.1C-Luc model, respectively (**Figure 6F-G**). Unfortunately, administration of anti-PD-L1 did not show any significant therapeutic effect in 4T1-Luc and Hepa55.1C model (**Figure 6F-G**).

#### **Example 7 UTPG treatment enhances immunity in tumor microenvironment (TME)**

[00140] Encouraged by the therapeutic outcomes, we evaluated the immunological properties of TME in 4T1 tumor model. Importantly, UTTPG could reverse the immune decline

in the TME. There was an overall increase in the percentage of tumor-infiltrating CD45<sup>+</sup> leukocytes across the therapeutic groups of UTTPG and the other therapeutic groups (**Figure 7A**). The results revealed that the frequencies for both CD3<sup>+</sup>CD8<sup>+</sup> and CD3<sup>+</sup>CD4<sup>+</sup> T cells were significantly enhanced in UTTPG treatment compared to the other groups (**Figure 7A**). UTTPG  
5 also induced a significant reduction in Tregs (**Figure 7B**), and thereby reversed the ratio of CD8<sup>+</sup> and CD4<sup>+</sup> T cells to Tregs within the tumors (**Figure 7C**). Additionally, the number of TAMs dramatically decreased in response to UTTPG treatment (**Figure 7B**), which increased the ratio of CD8<sup>+</sup> and CD4<sup>+</sup> T cells to TAMs in the TME (**Figure 7C**). Of note, the marked upregulation in intracellular granzyme B (Grb<sup>+</sup>) and Ki67<sup>+</sup> cells indicates that UTTPG treatment not only  
10 increased the antitumor immune population but also effectively achieved activation and proliferation of TILs (**Figure 7D**).

## Claims

### What is claimed is:

1. An engineered stem cell, comprising a vector comprising a polynucleotide comprising a nucleic acid sequence of suicide gene, a nucleic acid sequence of immune checkpoint gene and  
5 a natural cytotoxicity triggering receptor or a TNF-related apoptosis-inducing ligand (TRAIL);  
wherein the stem cell is a tumor-targeting cell.
2. The engineered stem cell of Claim 1, wherein the suicide gene is cytosine deaminase gene, varicella-zoster virus thymidine kinase gene, nitroreductase gene, Escherichia coli gpt gene, E. coli Deo gene, thymidine kinase gene (TK), caspase 1, caspase 3, caspase 6, caspase 7,  
10 caspase 8, caspase 9, Fas or cytosine deaminase (CD).
3. The engineered stem cell of Claim 1, wherein the suicide gene is TK.
4. The engineered stem cell of Claim 3, wherein the TK gene comprises a sequence as set forth in SEQ ID NO:1 or 2.
5. The engineered stem cell of Claim 1, wherein the immune checkpoint gene is the E3  
15 ubiquitin ligase Cbl-b, CTLA-4, PD-1, TIM-3, killer inhibitory receptor (KIR), LAG-3, CD73, Fas, the aryl hydrocarbon receptor, Smad2, Smad4, TGF-beta receptor, ILT-3, IDO, KIR, or LAG3.
6. The engineered stem cell of Claim 1, wherein the immune checkpoint gene is PD-1.
7. The engineered stem cell of Claim 6, wherein the PD-1 gene has a sequence as set  
20 forth in SEQ ID NO:3.
8. The engineered stem cell of Claim 1, wherein the natural cytotoxicity triggering receptor is NCR1, NCR2 or NCR3.

9. The engineered stem cell of Claim 1, wherein the natural cytotoxicity triggering receptor is NCR3.

10. The engineered stem cell of Claim 9, wherein the NCR3 gene comprises a sequence as set forth in SEQ ID NO:4.

5 11. The engineered stem cell of Claim 1, wherein the TRAIL is TIC10.

12. The engineered stem cell of Claim 1, wherein the TRAIL comprises a sequence as set forth in SEQ ID NO:5.

13. The engineered cell of Claim 1, wherein the stem cell is selected from the group consisting of: embryonic stem cell, marrow stromal cell, hematopoietic stem cell and neural stem  
10 cell.

14. The engineered cell of Claim 13, wherein the stem cell is MSC.

15. A combination comprising an engineered cell of any one of Claims 1 to 14 and optionally an additional active agent.

16. Use of an engineered cell of any one of Claims 1 to 14 or a combination of Claim 15  
15 in the manufacture of a medicament for treating a cancer or enhancing intratumor immunity in a subject.

17. The use of Claim 16, wherein the cancer is breast cancer, colon cancer, rectal cancer, lung cancer, ovarian cancer, prostate cancer, skin cancer, brain cancer, bladder cancer, endometrial cancer, kidney cancer, pancreatic cancer, thyroid cancer, melanoma, leukemia,  
20 fibrosarcoma, sarcoma, adenocarcinoma, or glioma.

18. The use of Claim 17, wherein the cancer is a metastatic cancer.

19. The use of Claim 16, wherein the effective amount ranges from 100,000 ( $1 \times 10^5$ )  
~2,000,000 ( $2 \times 10^6$ ) cells.

20. The use of Claim 16, wherein the method enhances immunity in tumor microenvironment through an increase in tumor-specific CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup>CD44<sup>+</sup> T cells with central memory potential.

21. The use of Claim 16, wherein the method induces a significant reduction in Tregs, and thereby reverses the ratio of CD8<sup>+</sup> and CD4<sup>+</sup> T cells to Tregs within the tumors. The method also decreases the number of TAMs, which increases the ratio of CD8<sup>+</sup> and CD4<sup>+</sup> T cells to TAMs in the TME.

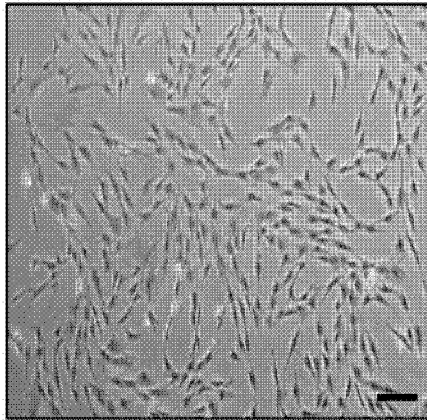
22. The use of Claim 16, wherein the engineered stem cell of any one of Claims 1 to 14 to the subject or a combination of Claim 15 can be intravenously or intra-arterially administered to the subject.

23. The use of Claim 16, wherein the engineered stem cell can be administrated in combination with an additional active agent.

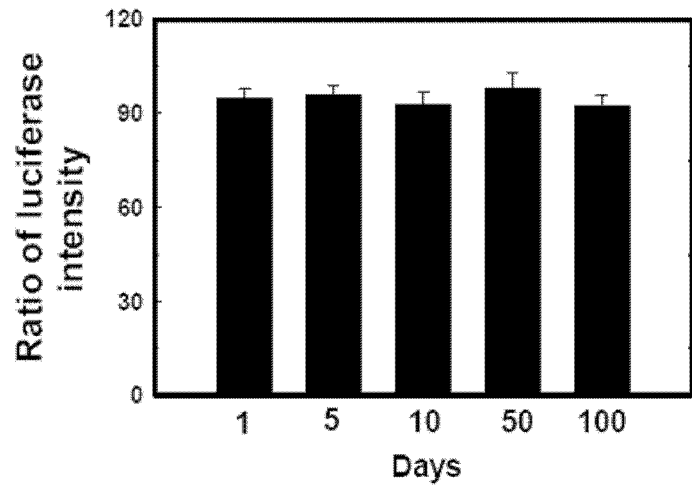
24. The use of Claim 16, wherein the additional active agent is GCV.

25. The use of Claim 16, wherein the engineered stem cell and the additional active agent are administered separately, simultaneously or concurrently.

A



D



B

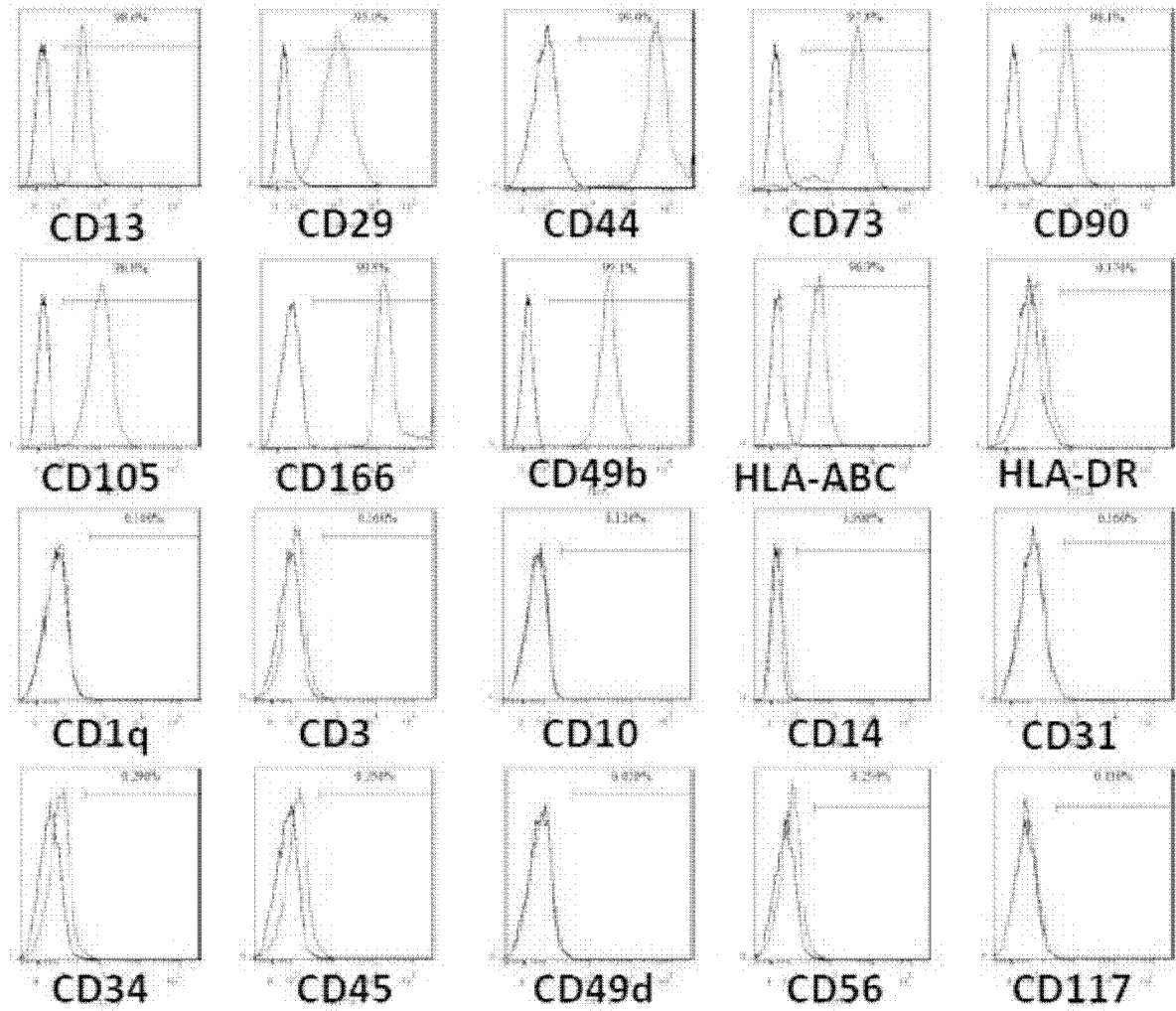
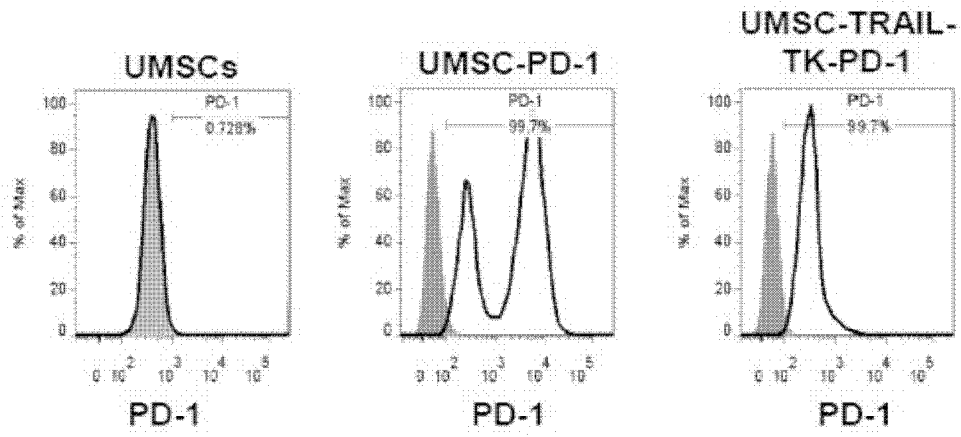
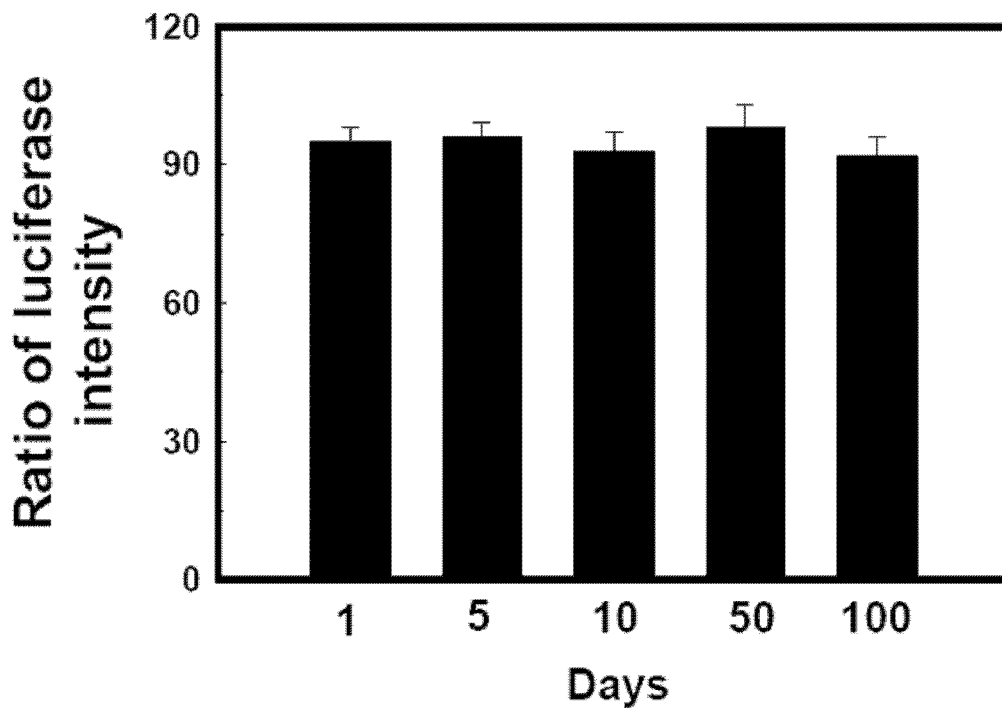


Fig. 1

C



D



E-(a)

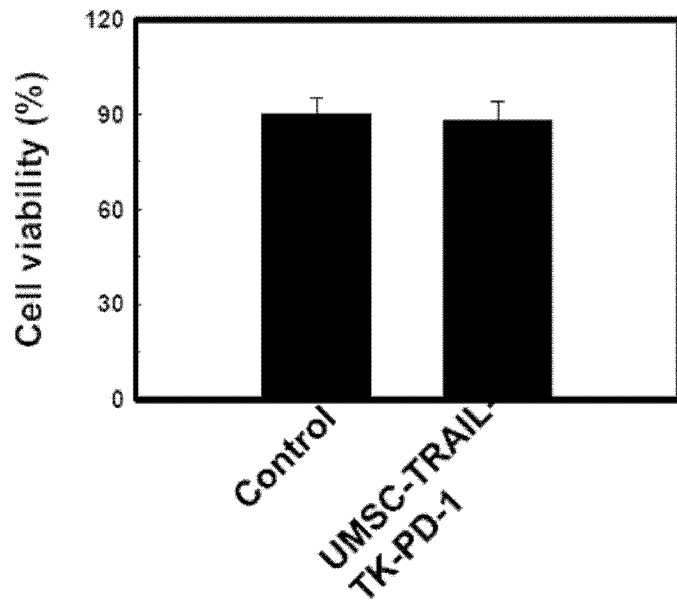
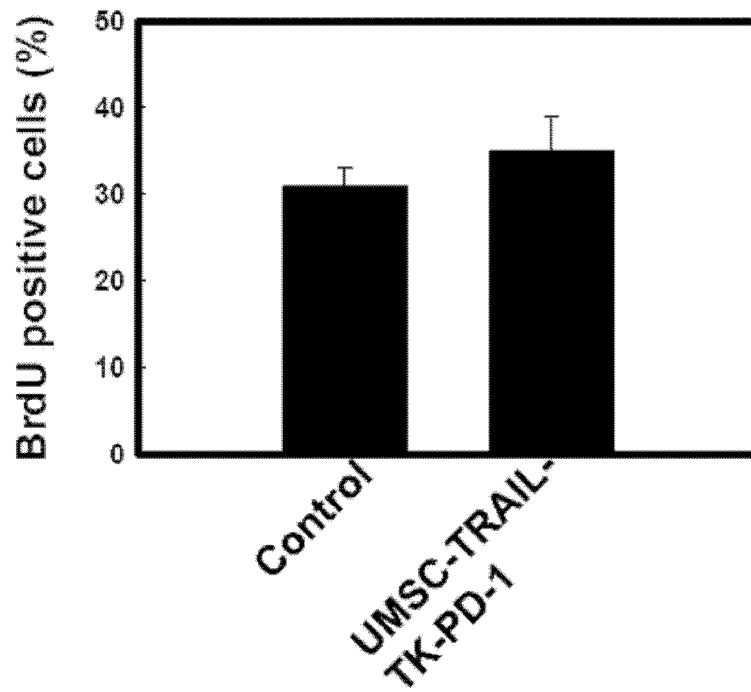


Fig. 1

E-(b)



E-(c)

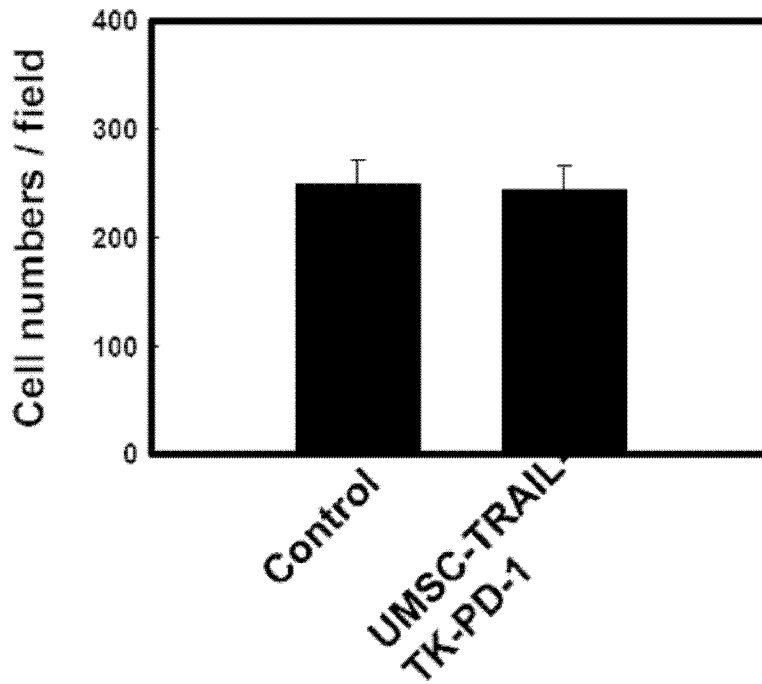
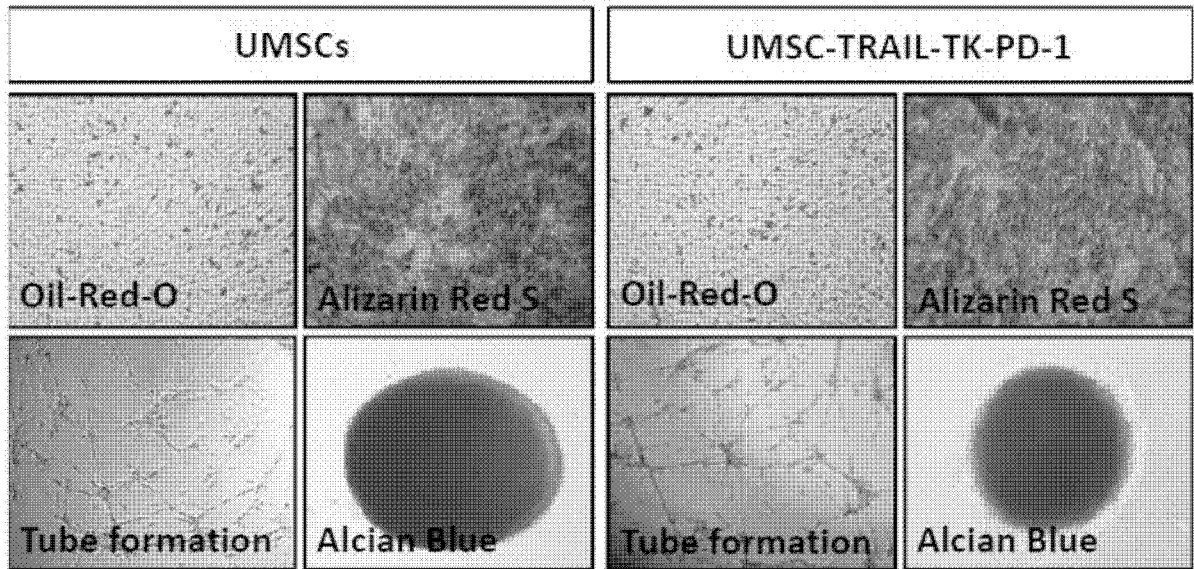


Fig. 1

F



G

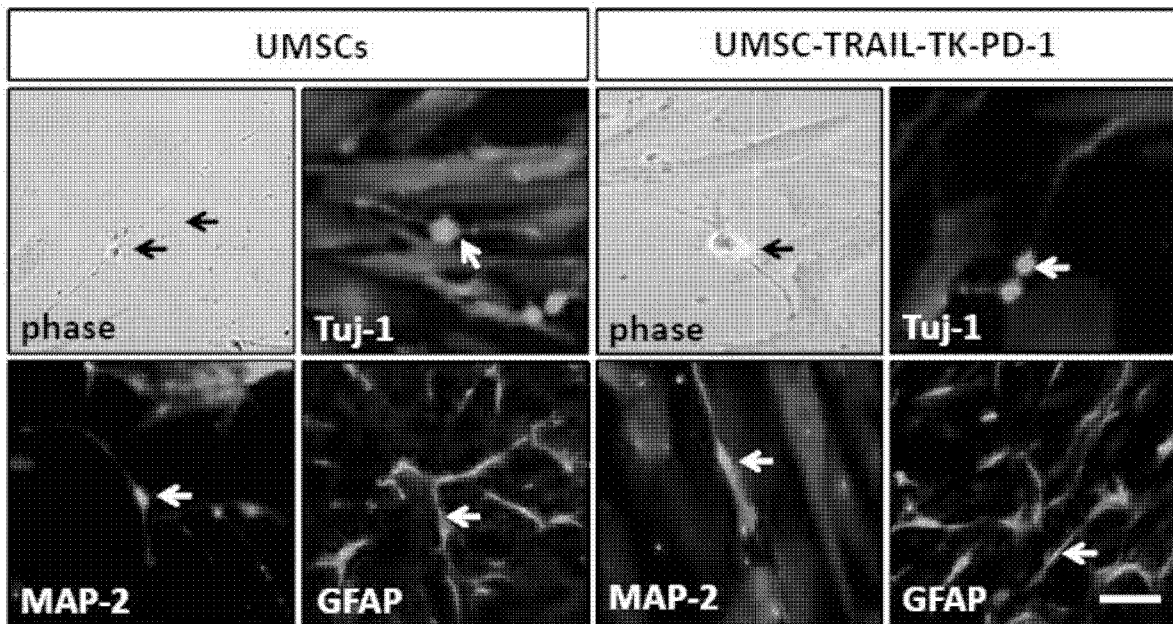
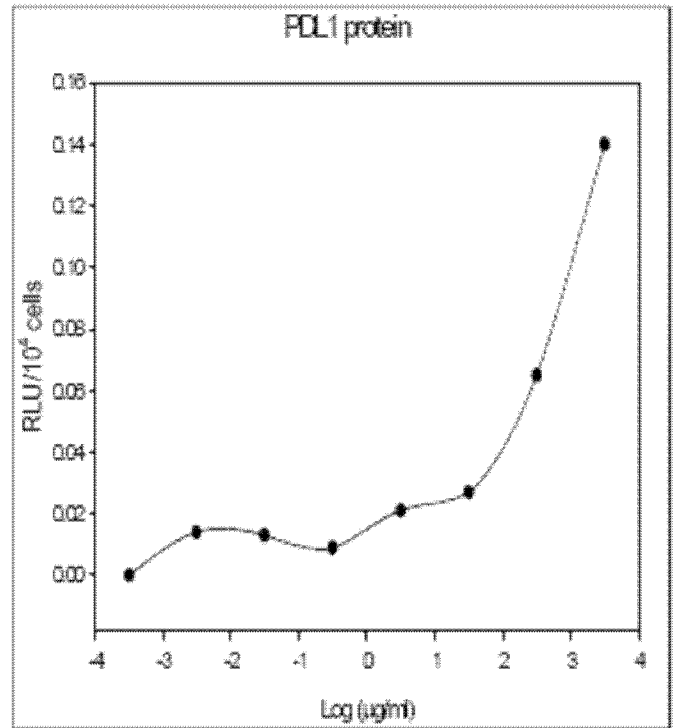


Fig. 1

A

		UMSCs	UMSC-TRAIL-TK-PD-1
PD-L1 (ng/ul)	0.0005	0	0
	0.005	-0.001	0.014
	0.05	0.015	0.013
	0.5	0.006	0.009
	5	0.012	0.021
	50	0.011	0.027
	500	0.05	0.065
	5000	0.124	0.14

UMSC-TRAIL-TK-PD-1



B-(a)

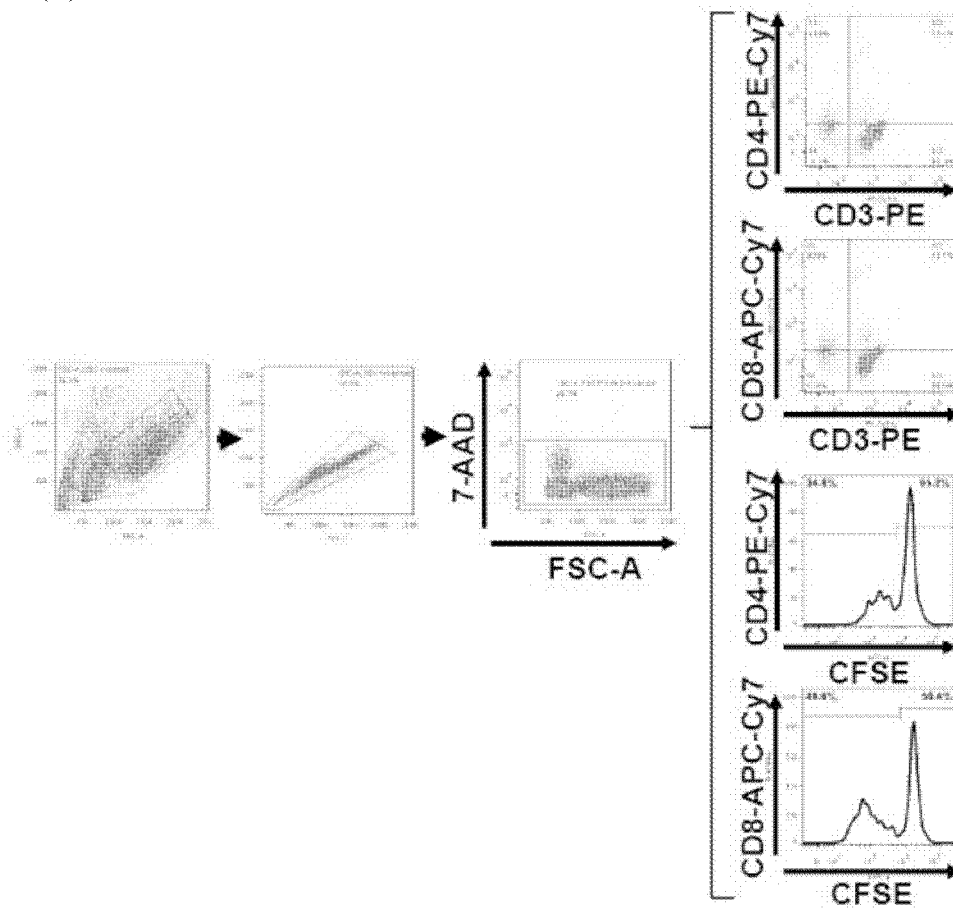
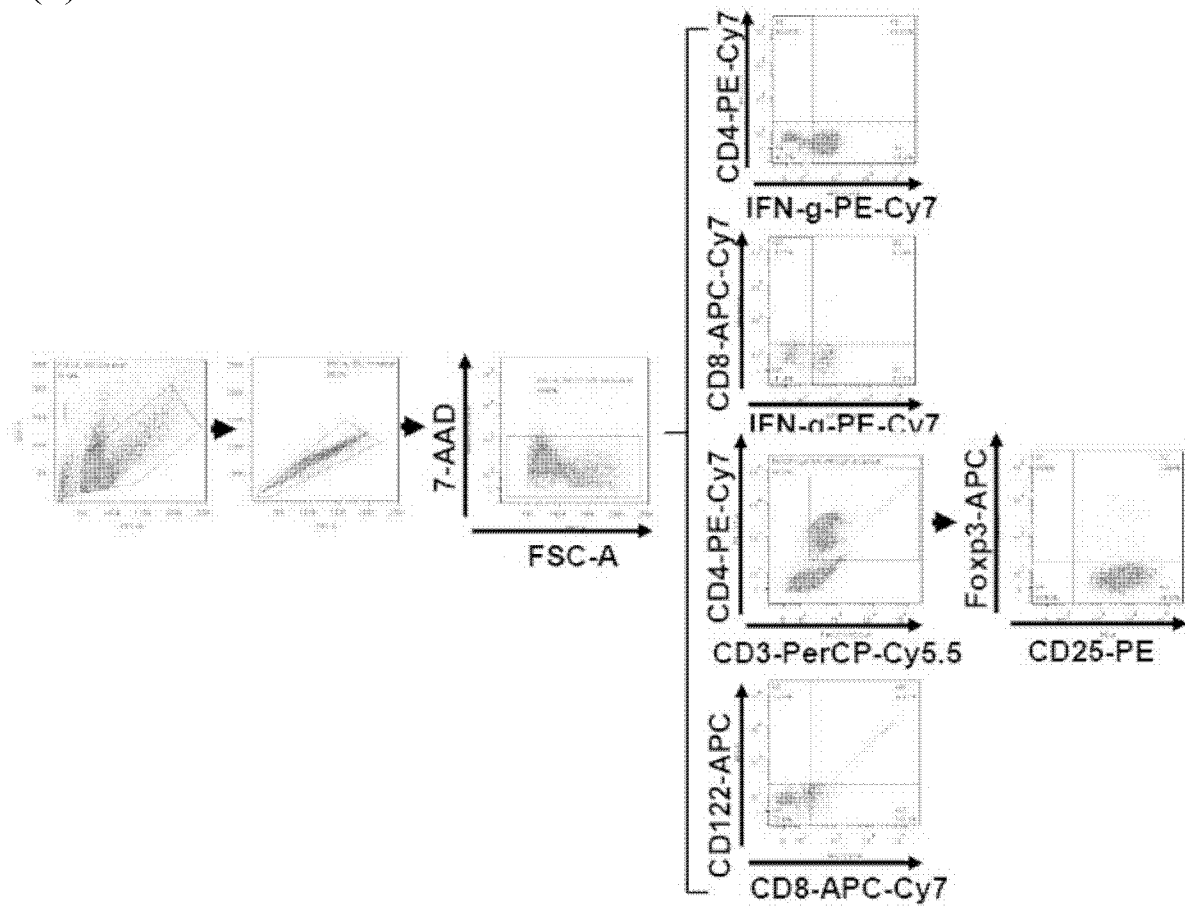
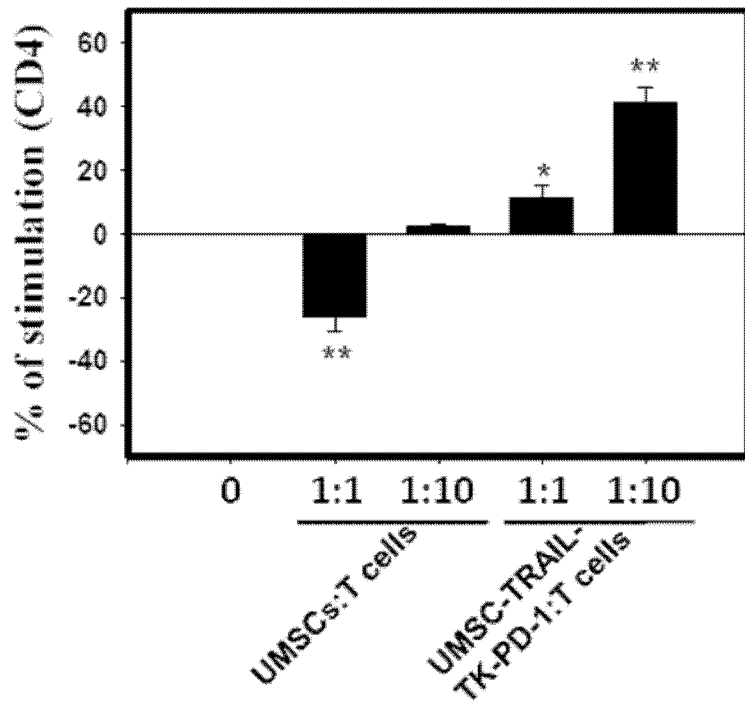


Fig. 2

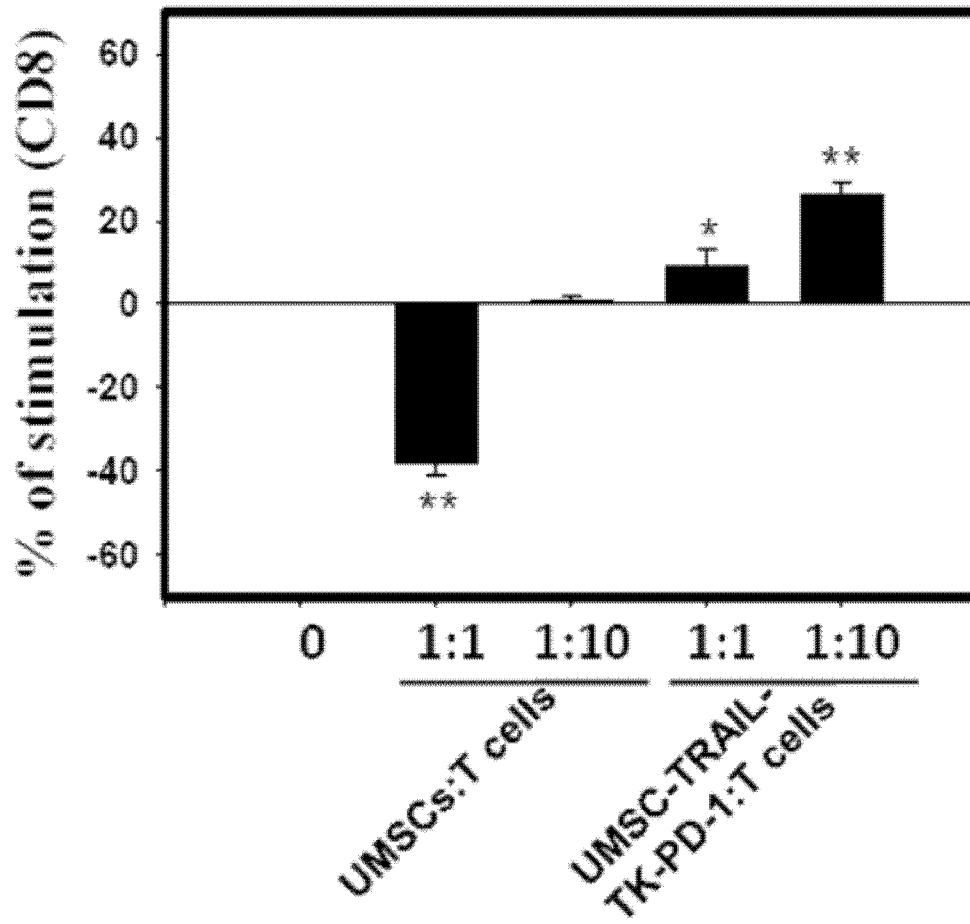
B-(b)



C-(a)



C-(b)



D-(a)

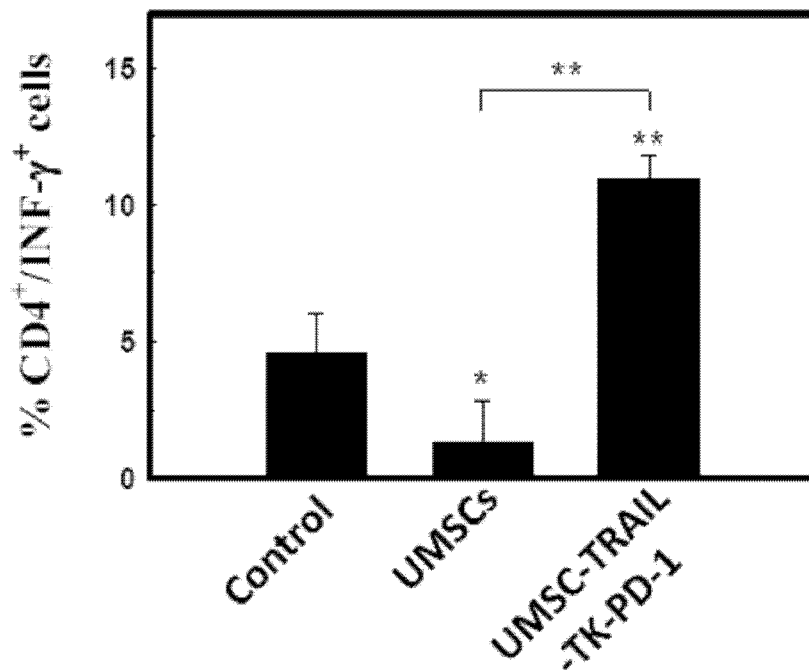


Fig. 2

D-(b)

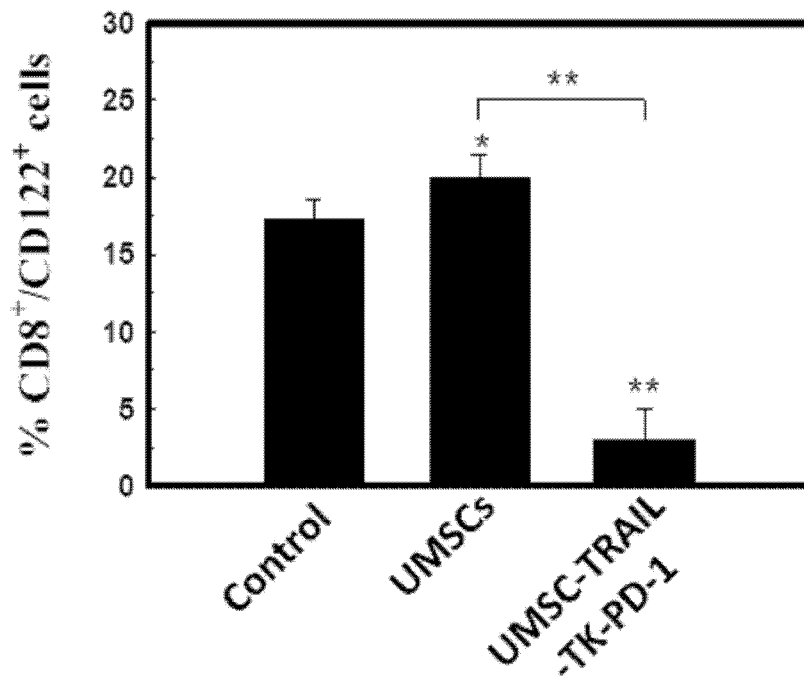


Fig. 2

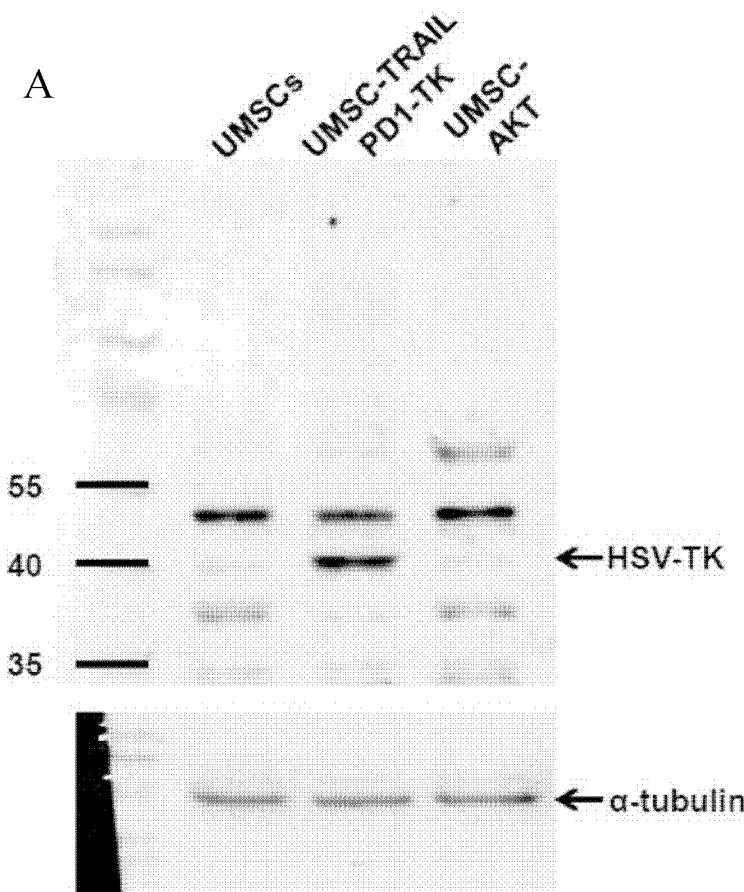
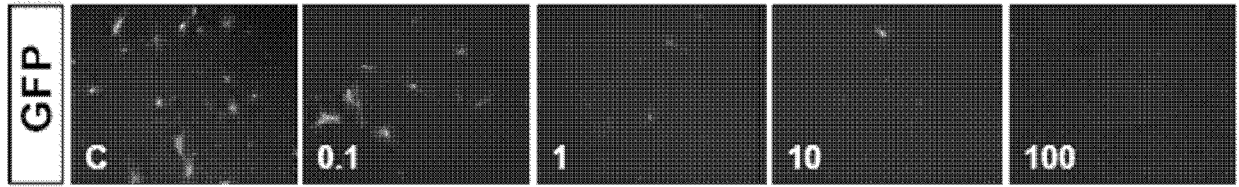


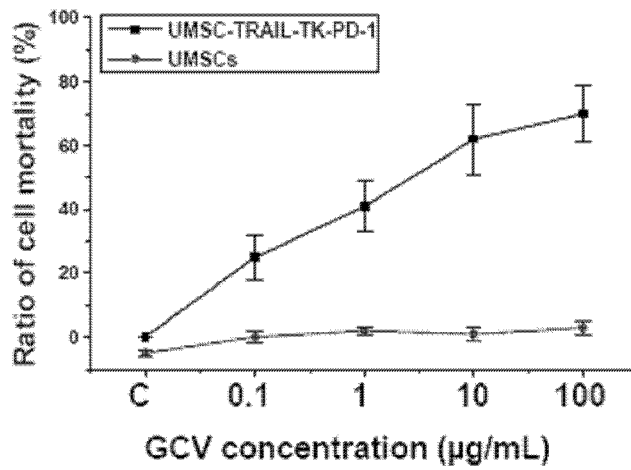
Fig. 3

B-(a)

### Suicide effect



GCV concentration ( $\mu\text{g/mL}$ )



B-(b)

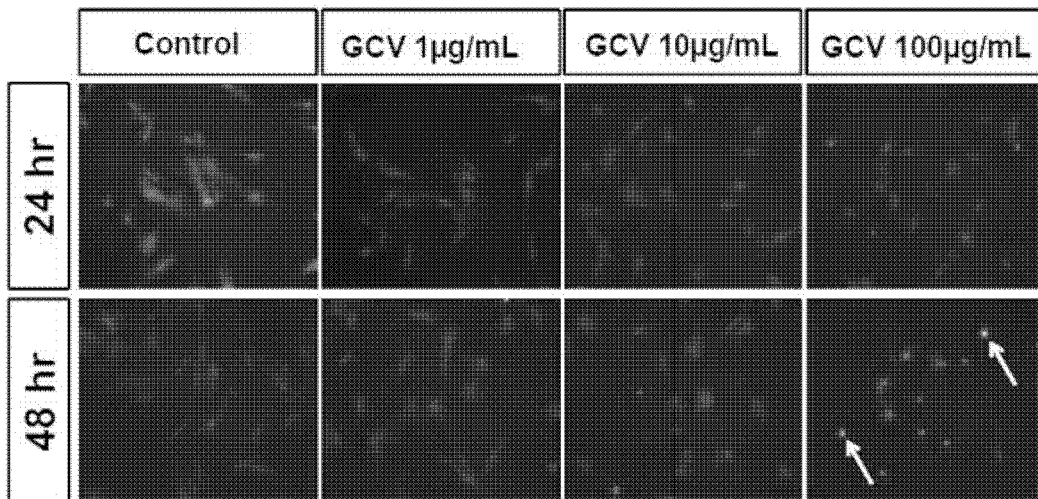
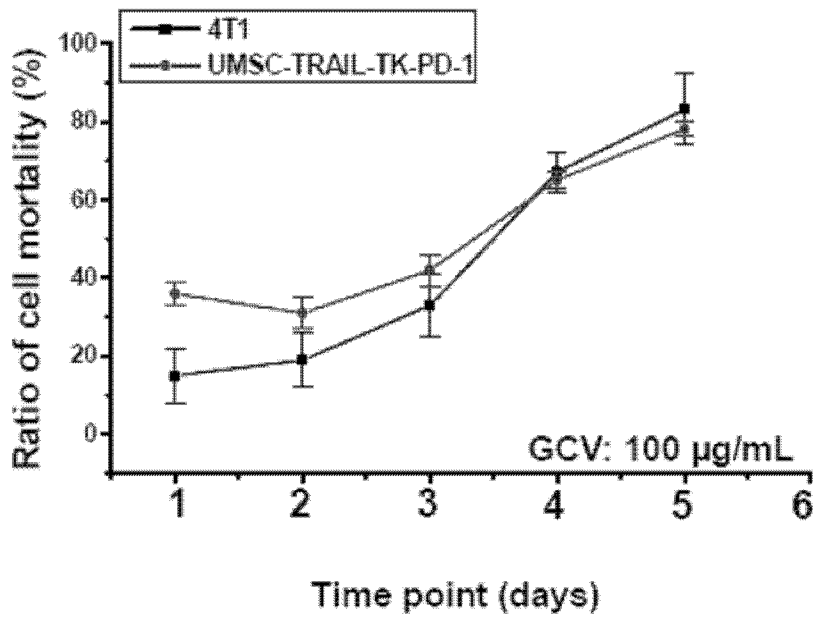


Fig. 3

C-(a)

# Bystander effect



C-(b)

## 4T1/UMSC-TRAIL-TK-PD-1-GFP co-culture

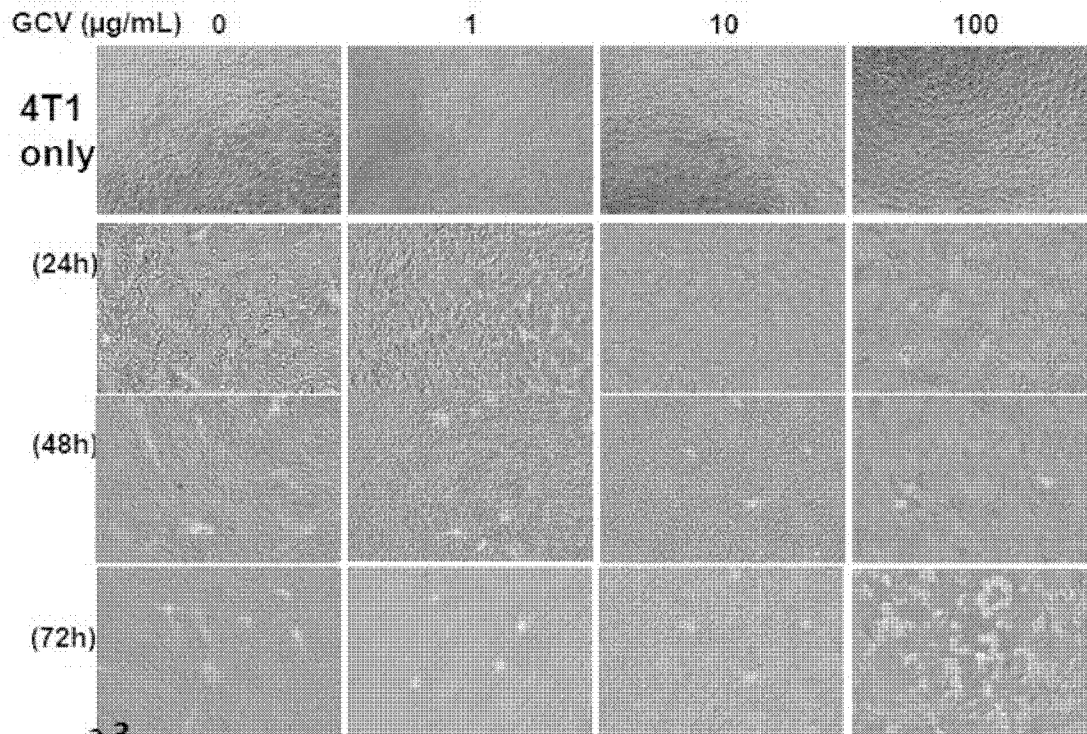
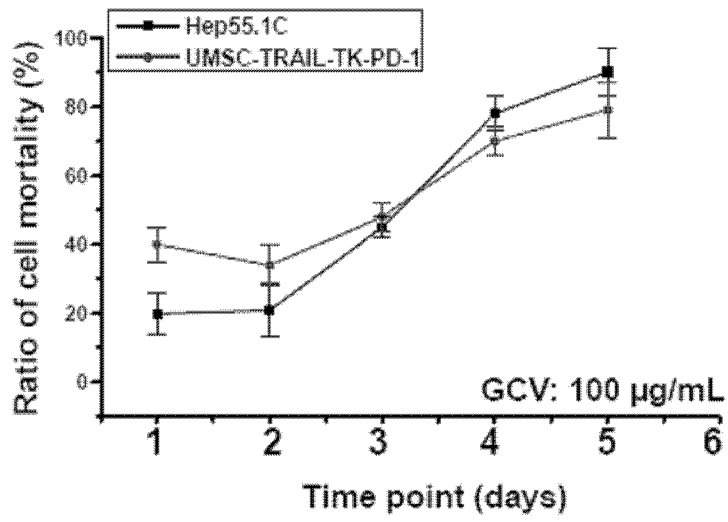


Fig. 3

C-(c)



C-(d)

Hep55.1C/UMSC-TRAIL-TK-PD-1-GFP co-culture

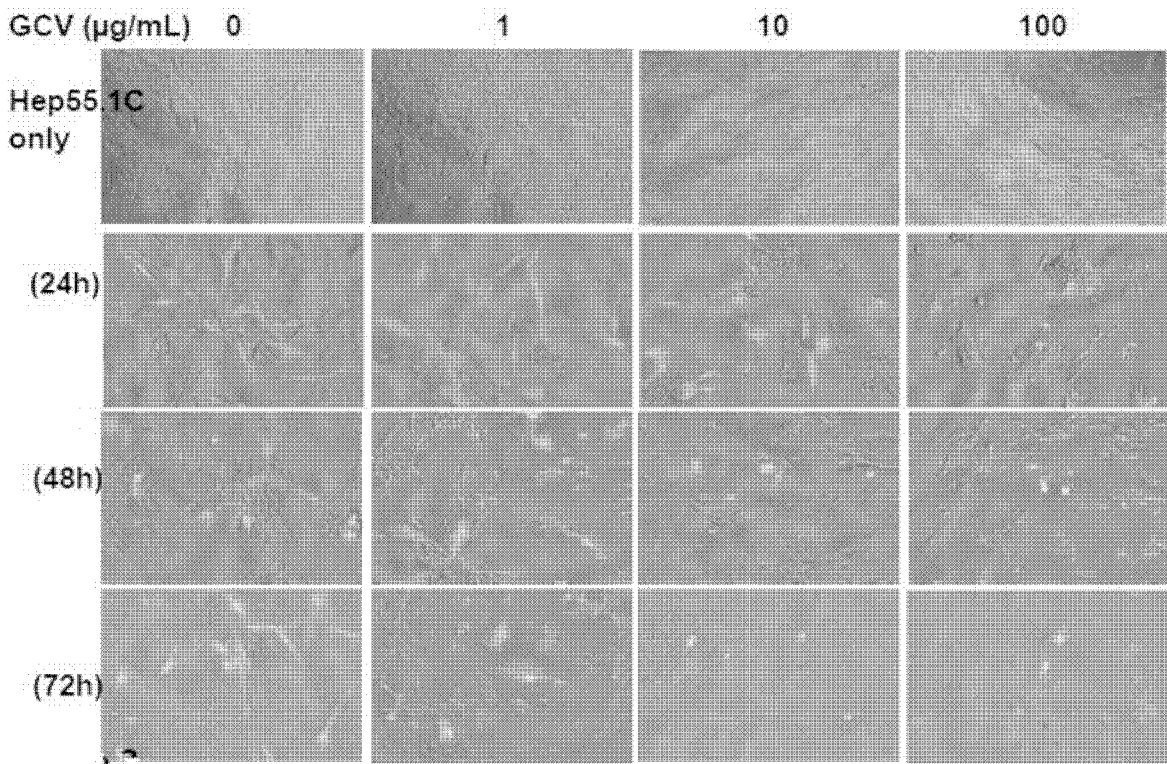
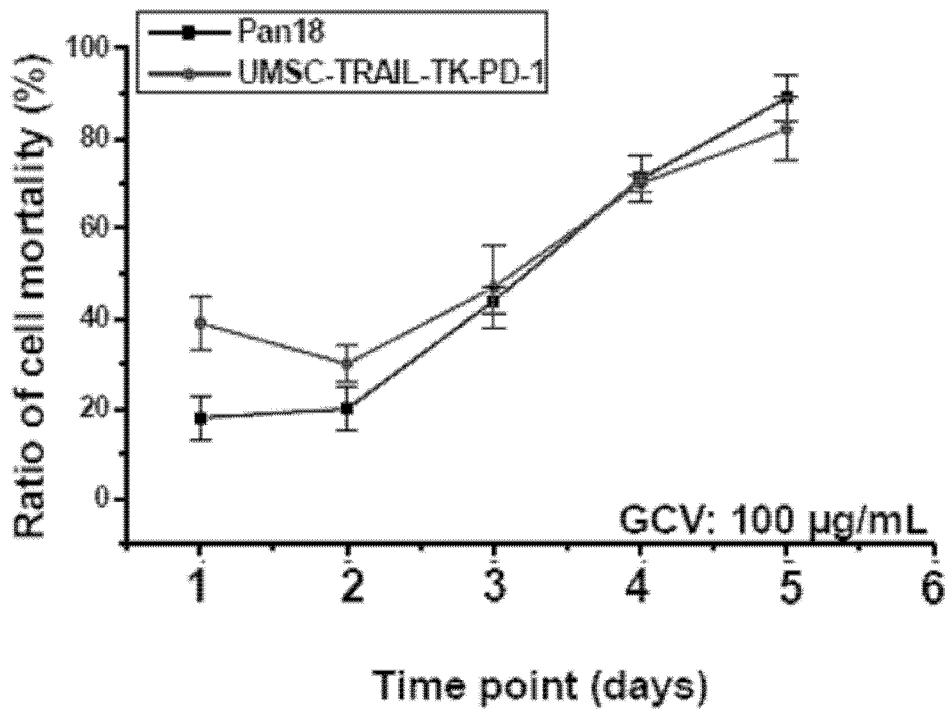


Fig. 3

C-(e)



C-(f)

Pan18/UMSC-TRAIL-TK-PD-1-GFP co-culture

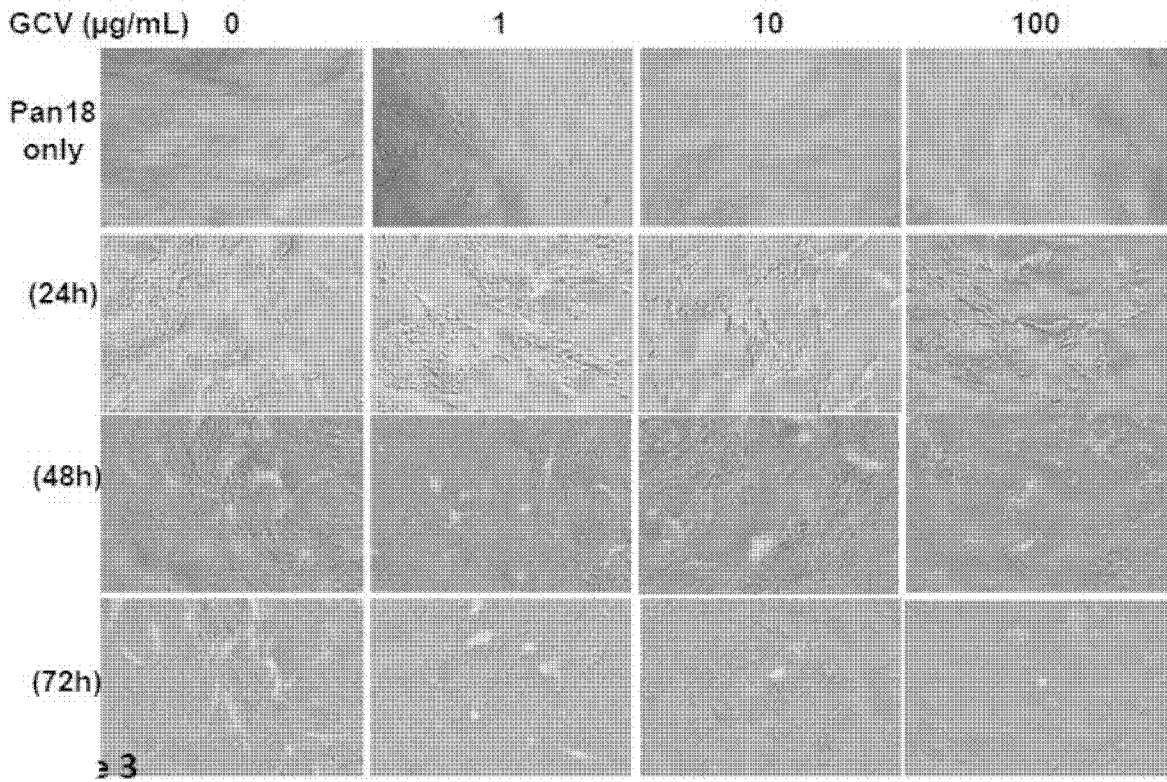
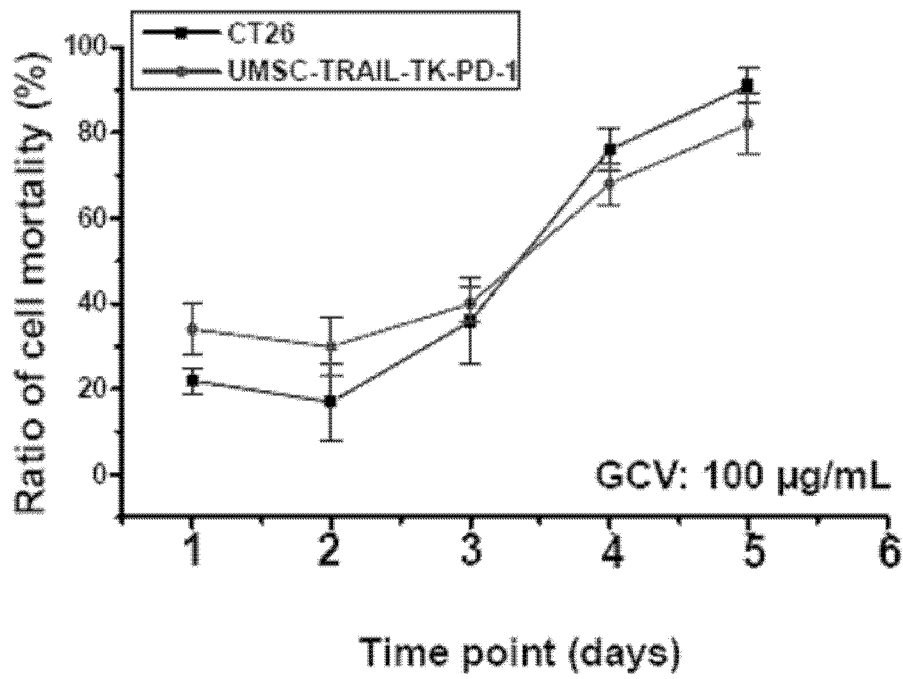


Fig. 3

C-(g)



C-(h)

CT26/UMSC-TRAIL-TK-PD-1-GFP co-culture

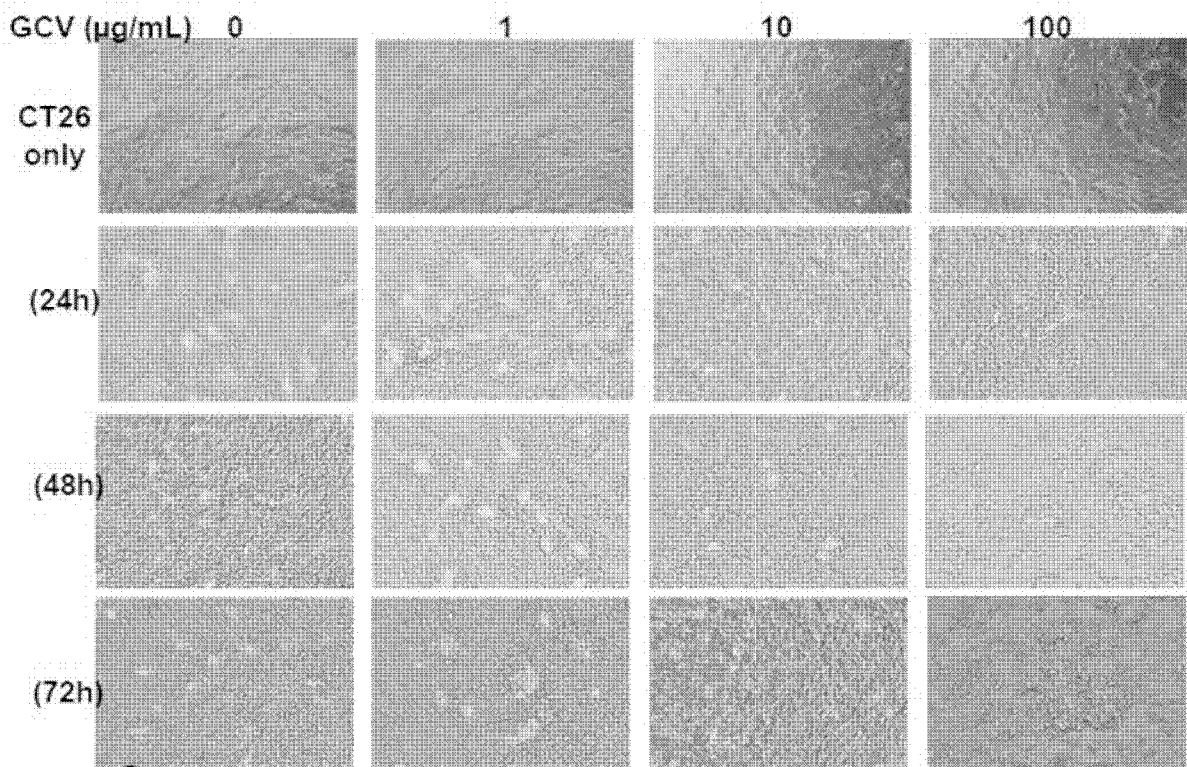
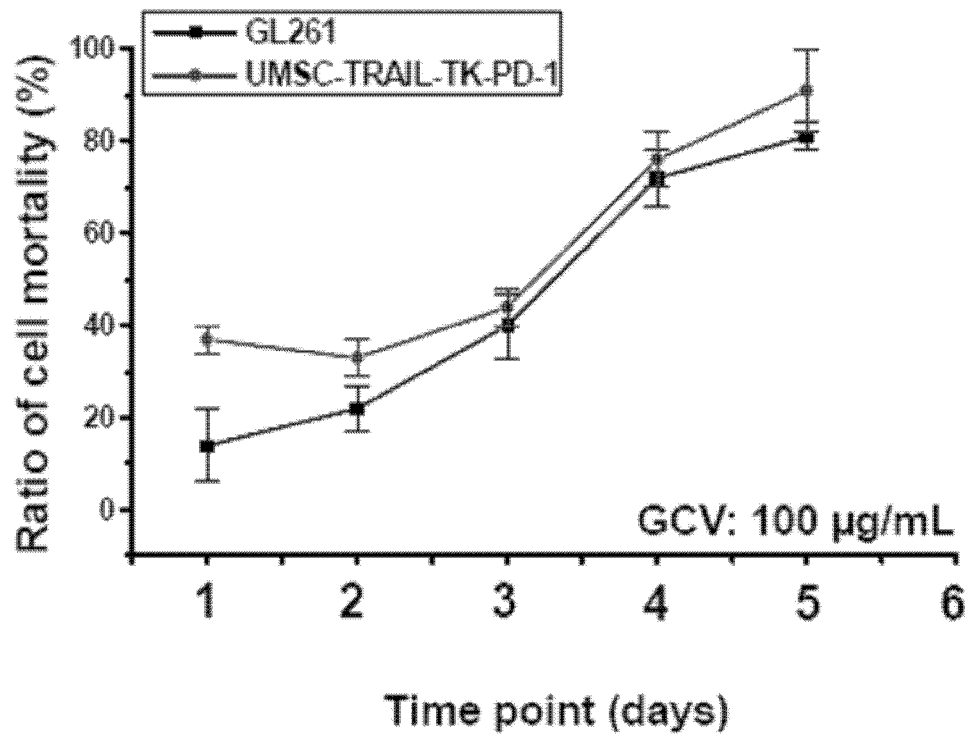


Fig. 3

C-(i)



C-(j)

GL261/UMSC-TRAIL-TK-PD-1-GFP co-culture

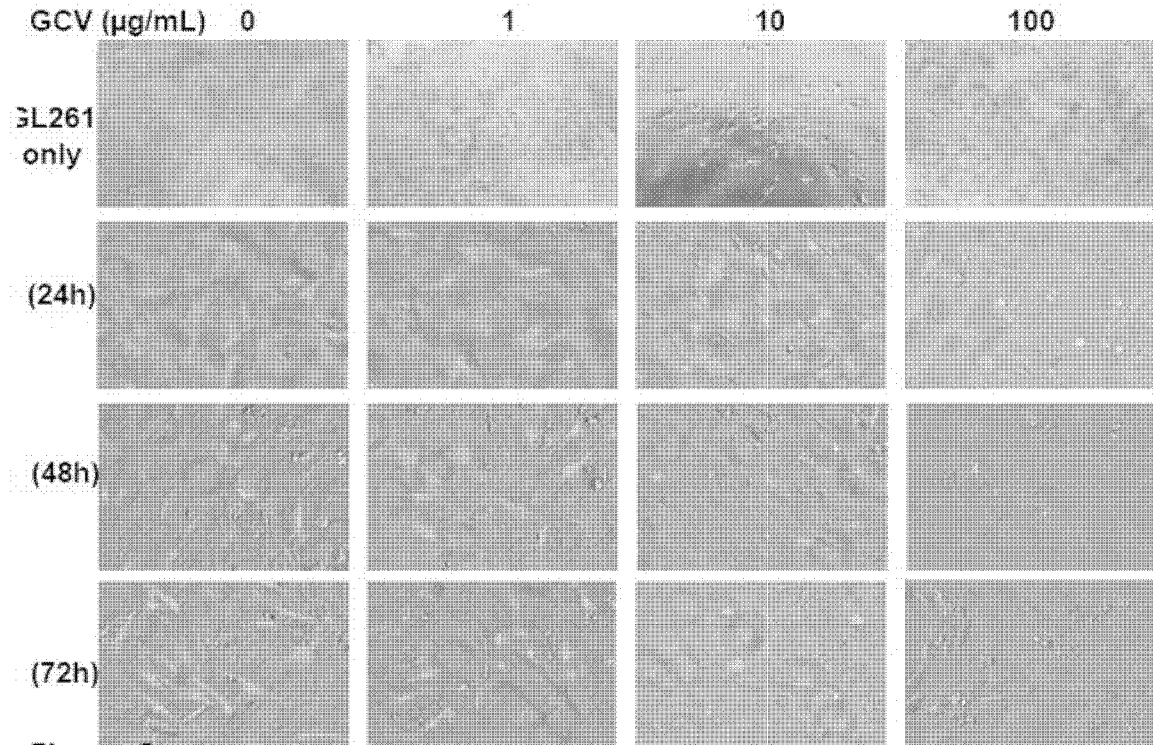
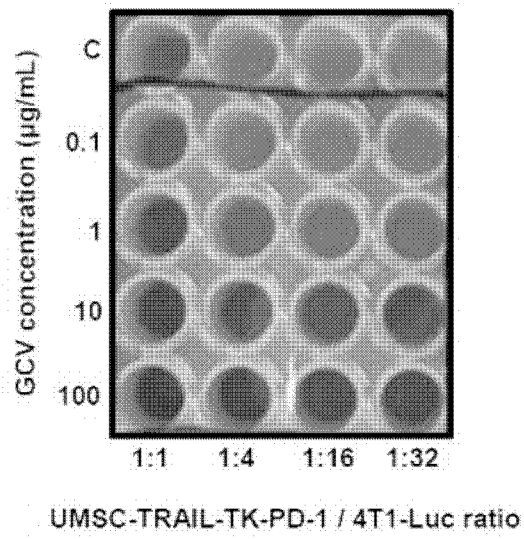


Fig. 3

D



E-(a)

### Bystander effect

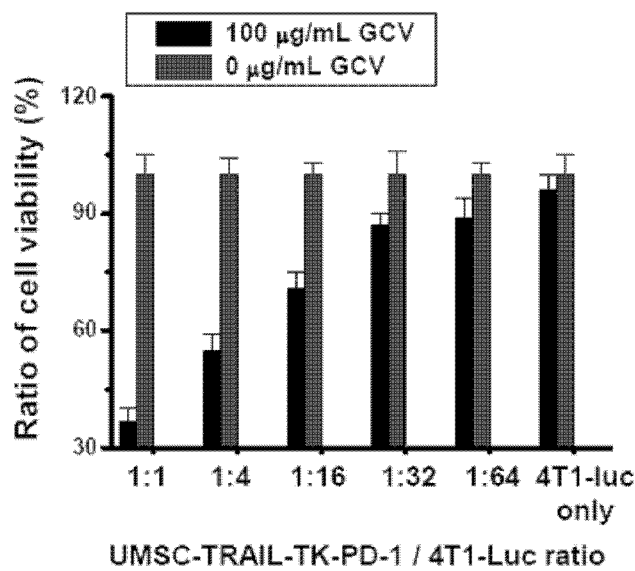
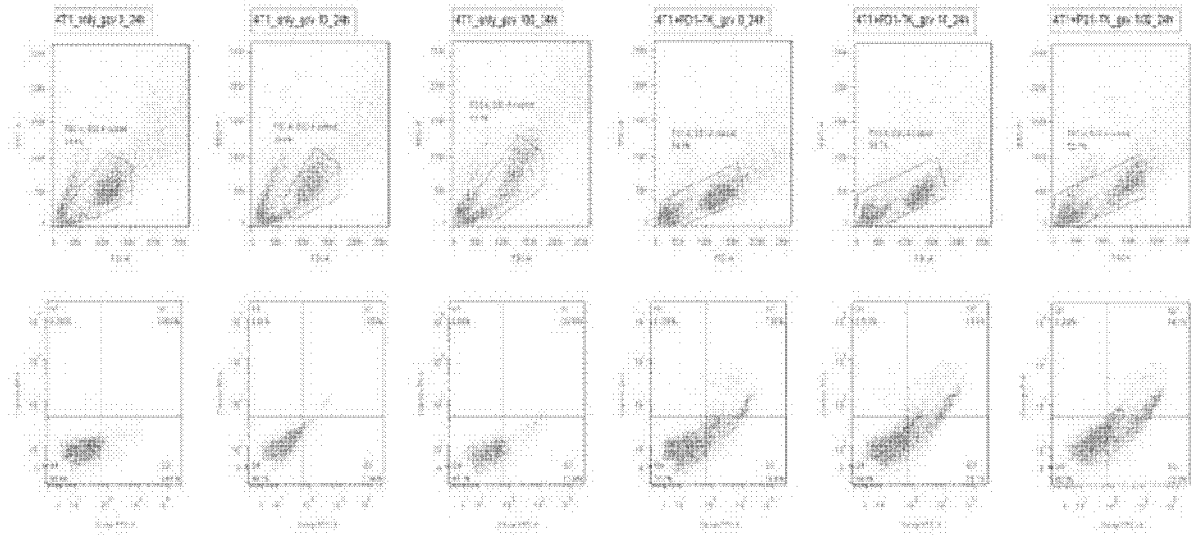


Fig. 3

E-(b)

4T1/UMSC-TRAIL-TK-PD-1, co-culture for 24h  
(PI/Annexin-V)



E-(c)

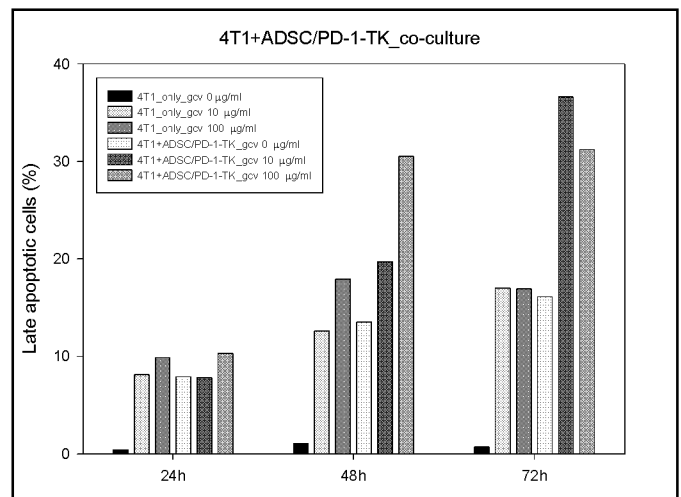
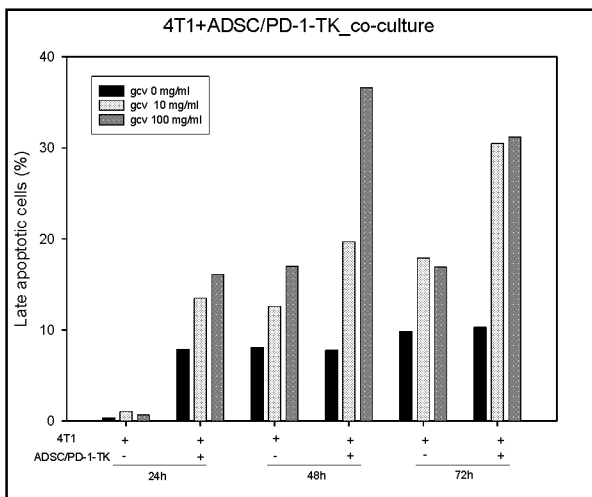


Fig. 3

E-(d)

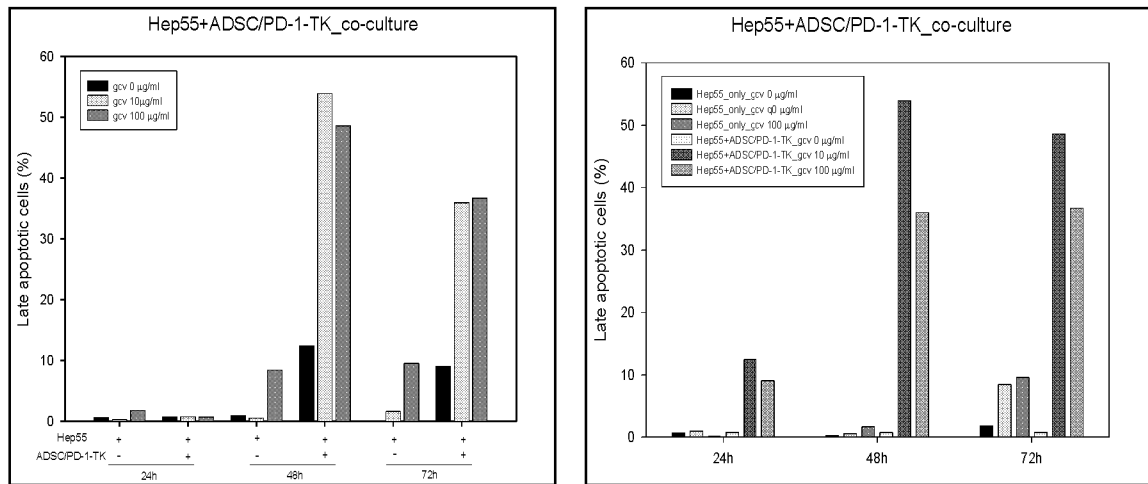
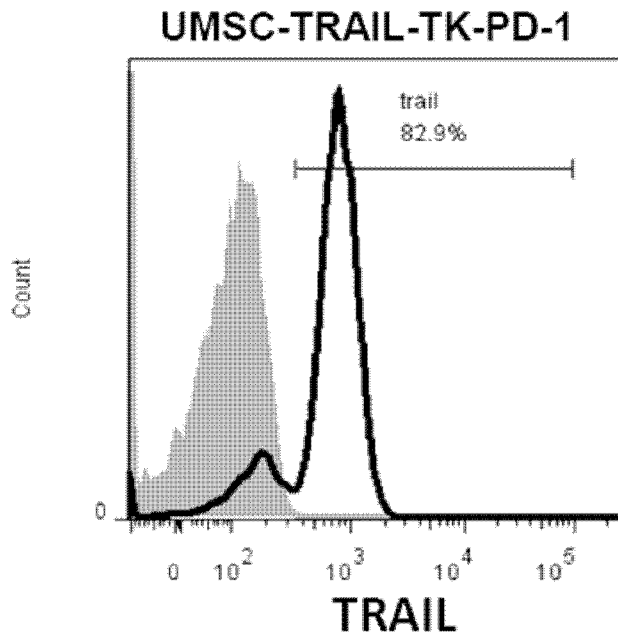


Fig. 3

A



B-(a)

Cell line: 4T1, 500 cells

UMSC-TRAIL-TK-PD-1, 500/1000/2500 cells

PI staining

4T1 & UMSC-TRAIL-TK-PD-1

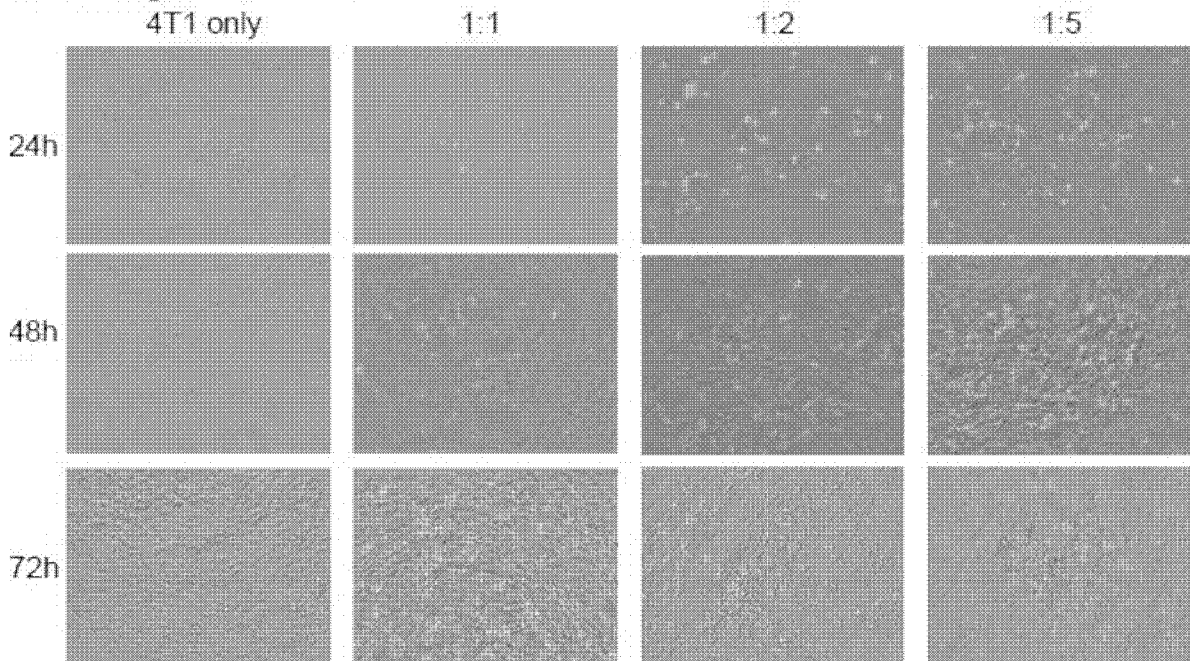
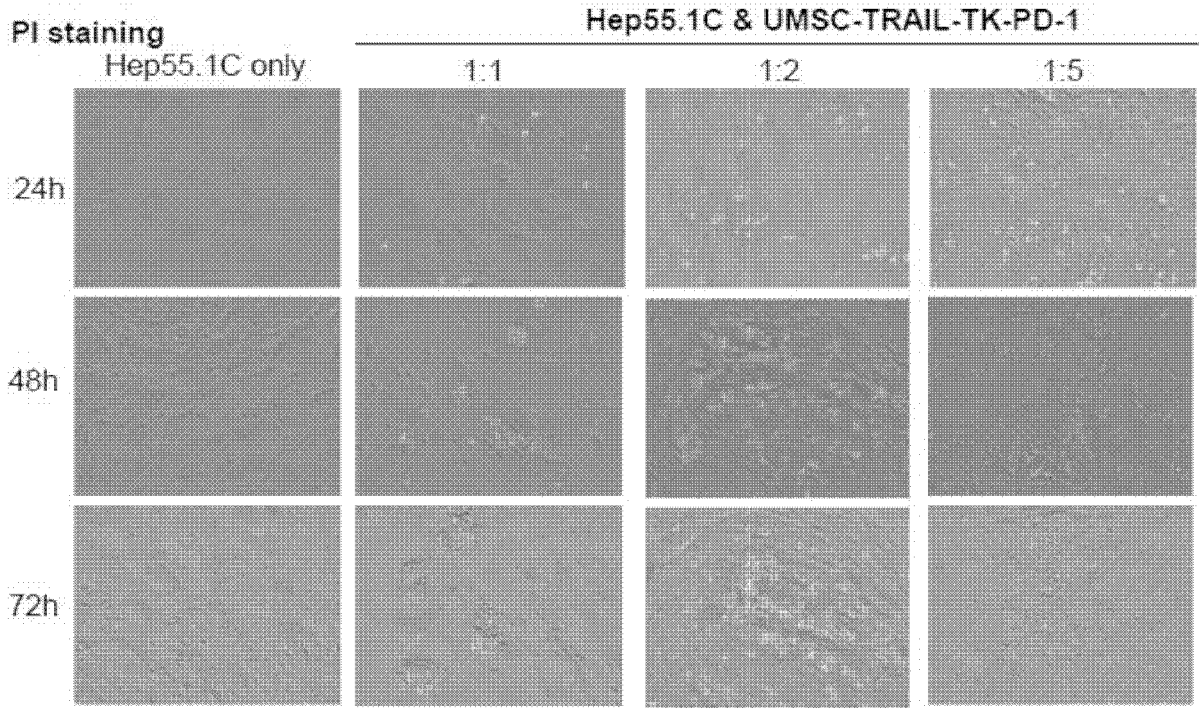


Fig. 4

B-(b)

Cell line: Hep55.1C, 500 cells  
 UMSC-TRAIL-TK-PD-1, 500/1000/2500 cells



B-(c)

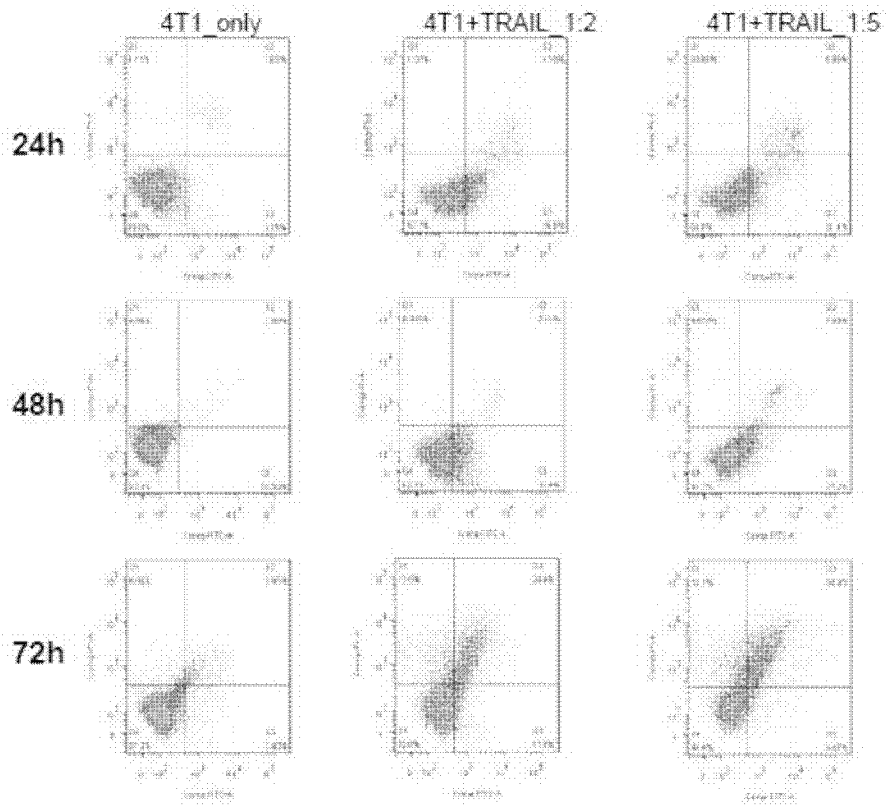
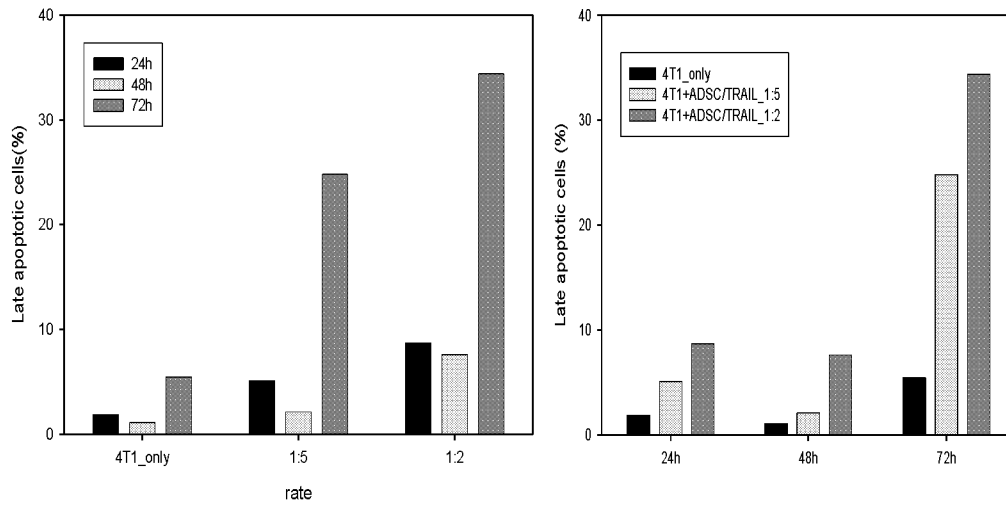


Fig. 4

C-(a)



C-(b)

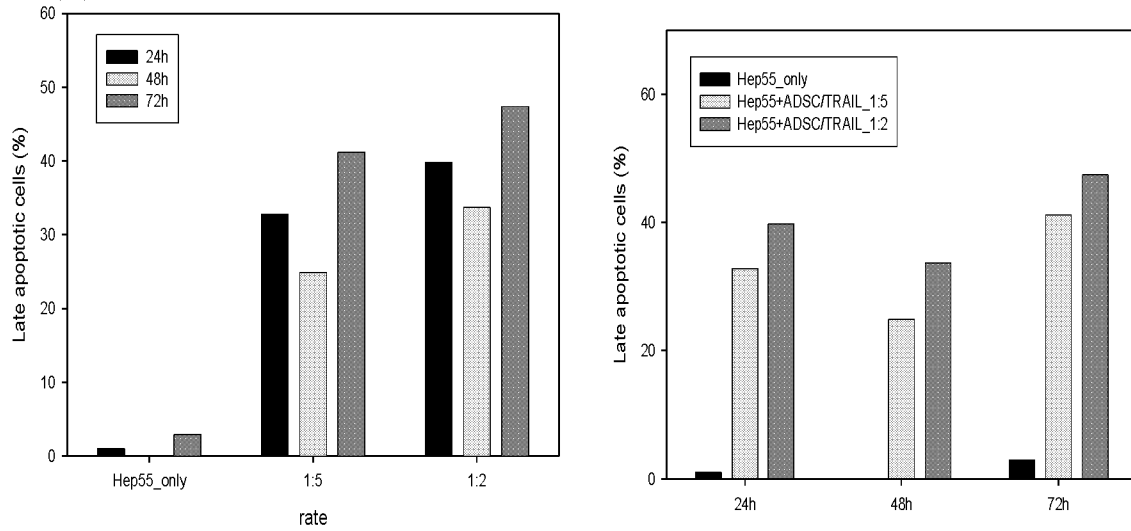
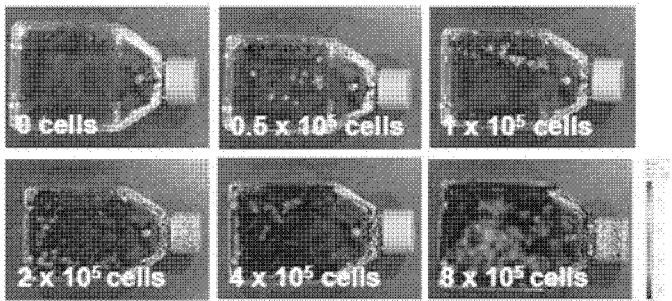


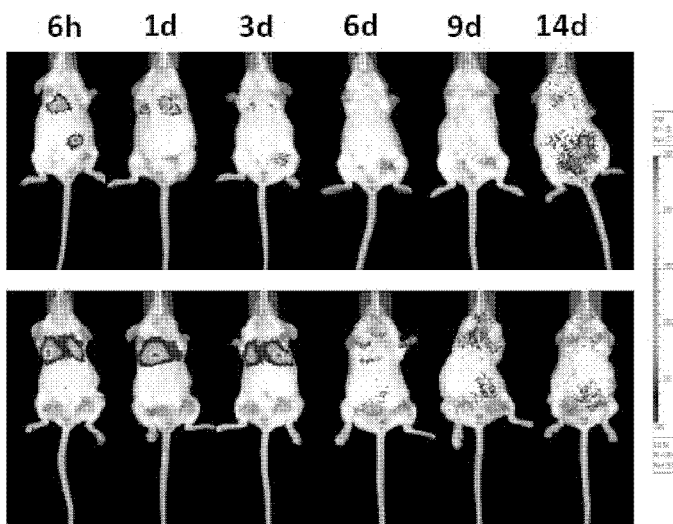
Fig. 4

A



B

**Intravenous injection (4T1)**



C

**Intra-arterial injection (4T1)**

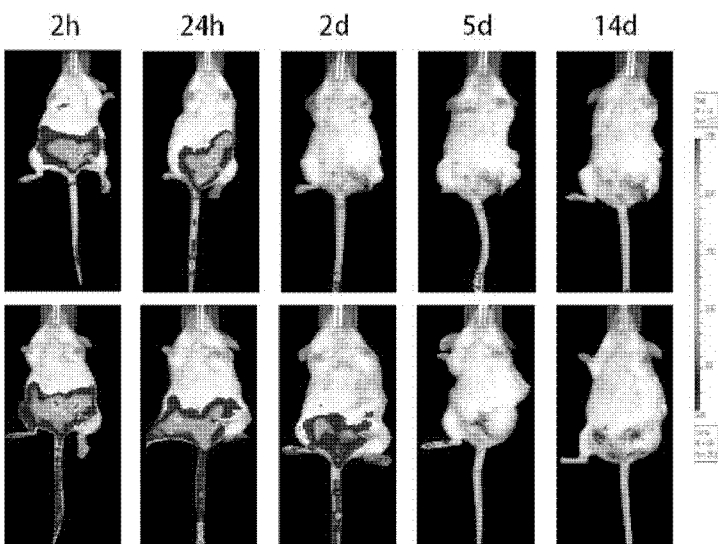
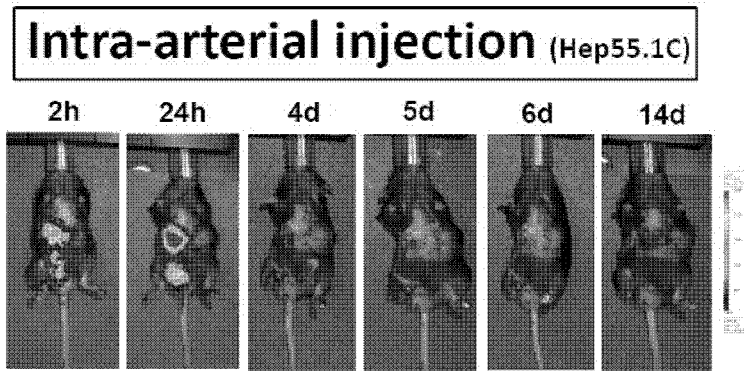
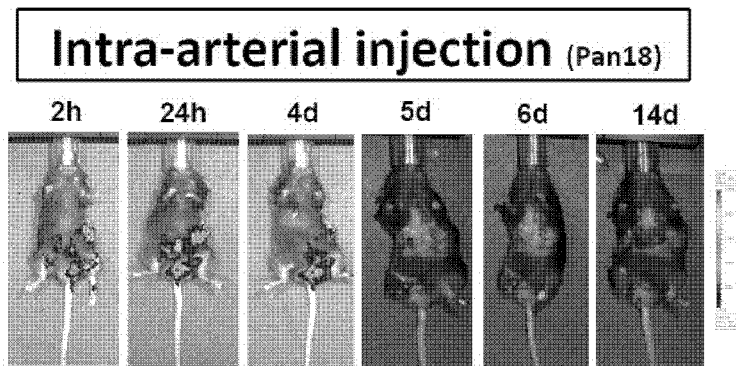


Fig. 5

D



E



F

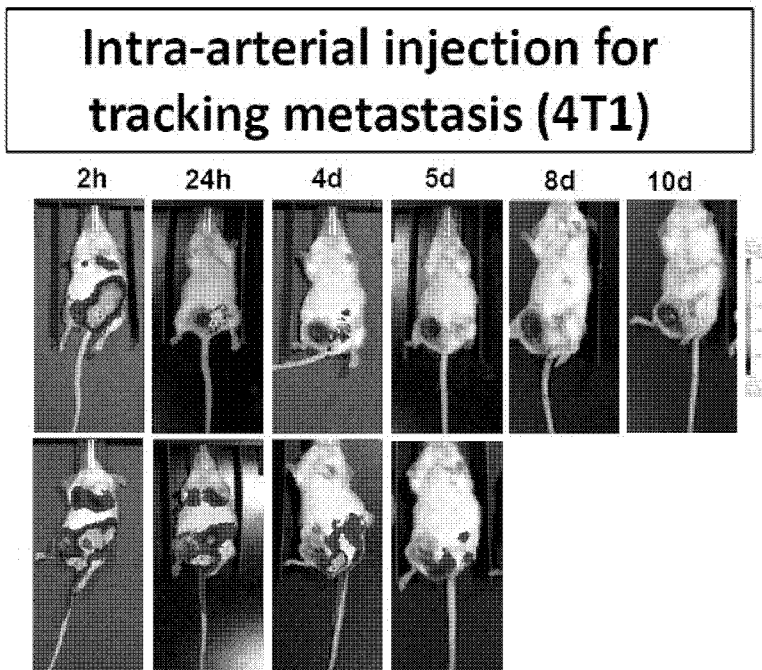


Fig. 5

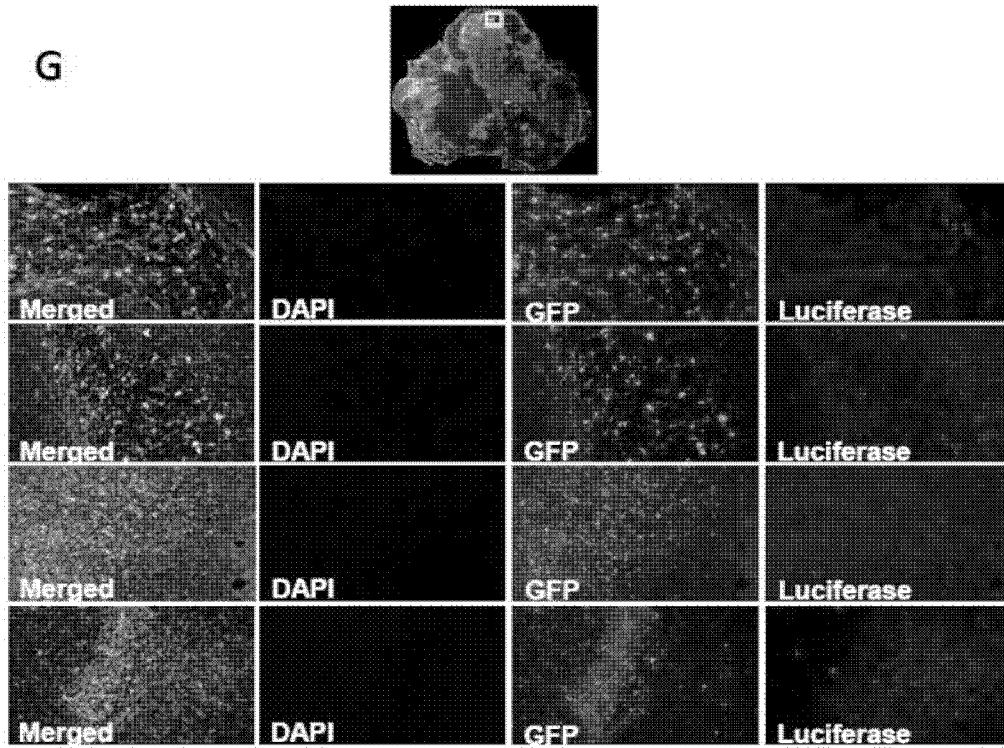


Fig. 5

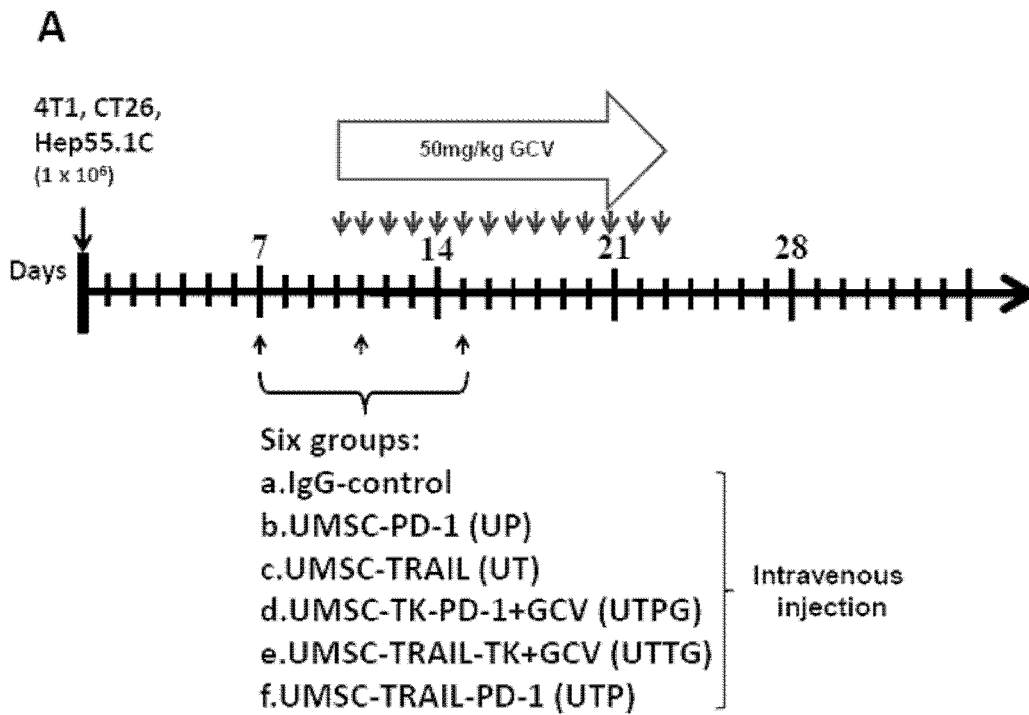
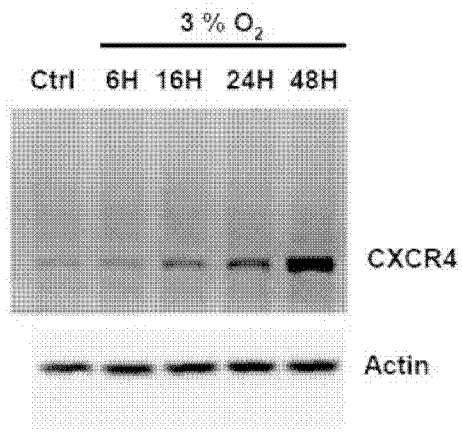
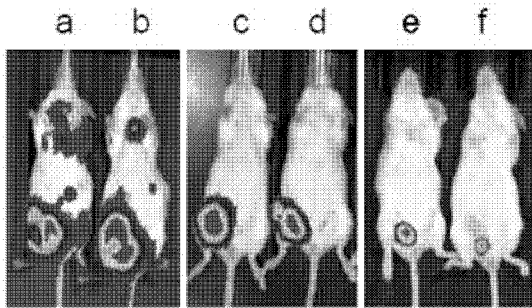


Fig. 6

B



C



D-(a)

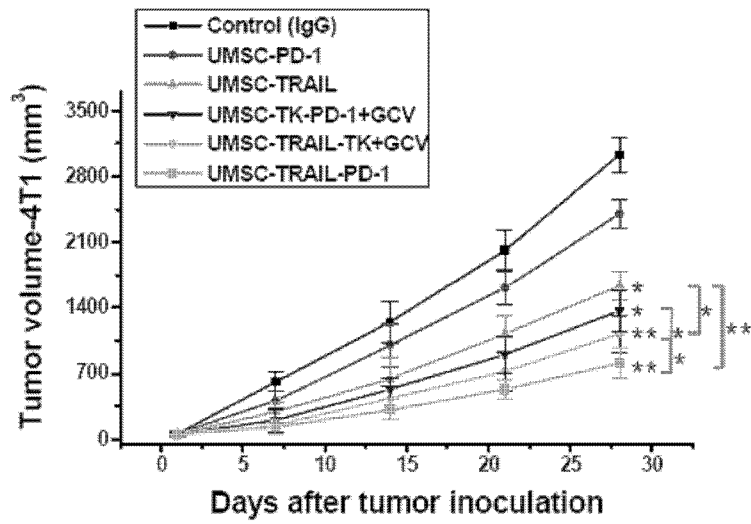
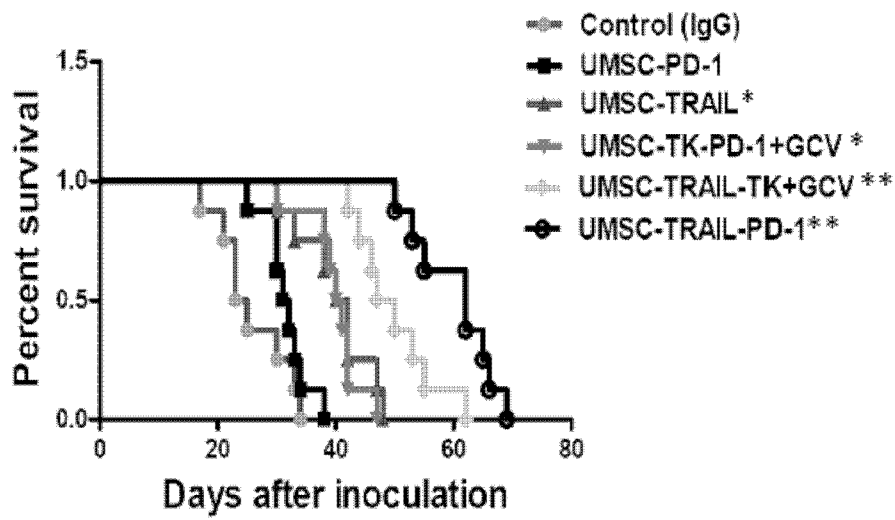
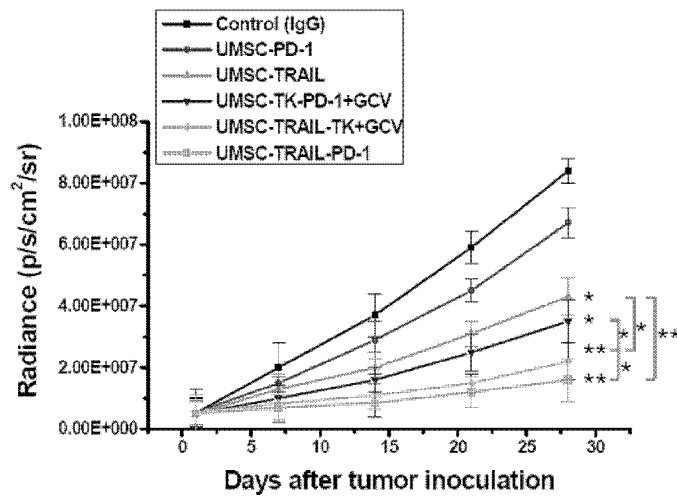


Fig. 6

D-(b)

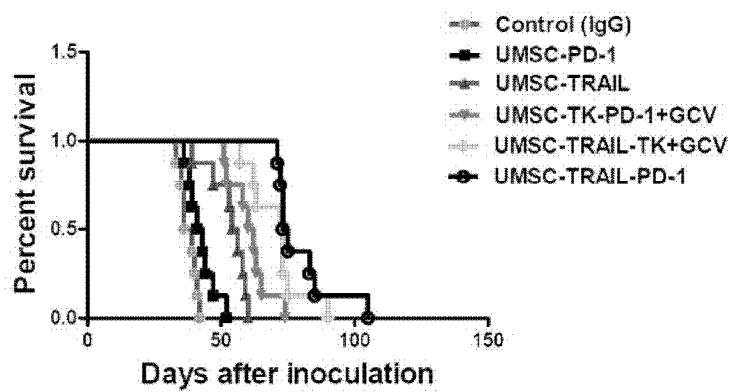


D-(c)



Hep55.1C

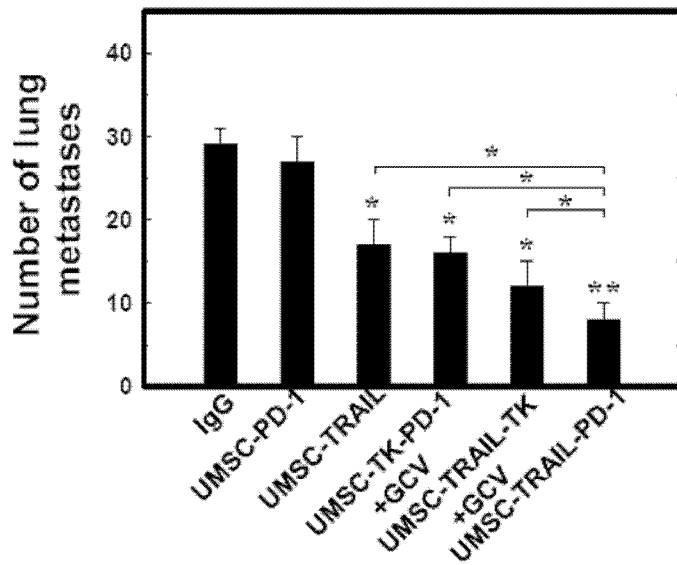
D-(d)



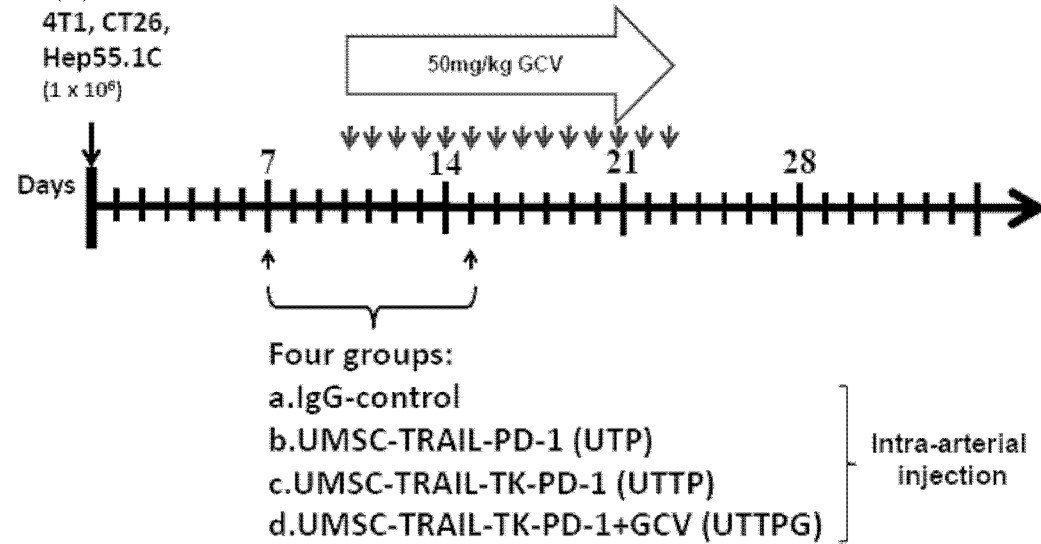
Hep55.1C

Fig. 6

E



F-(a)



F-(b)

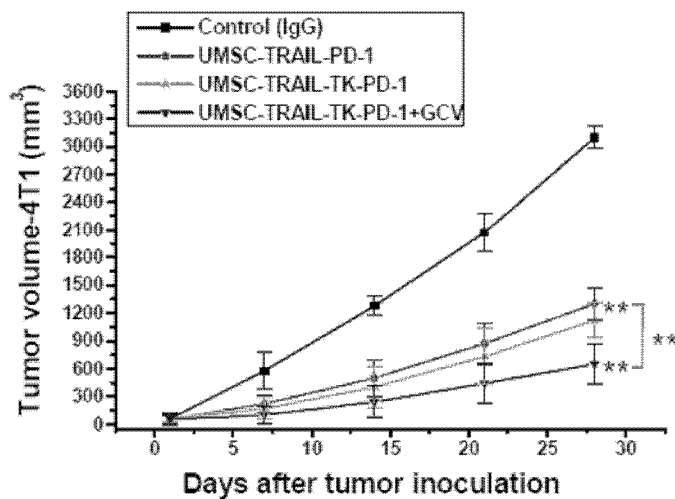
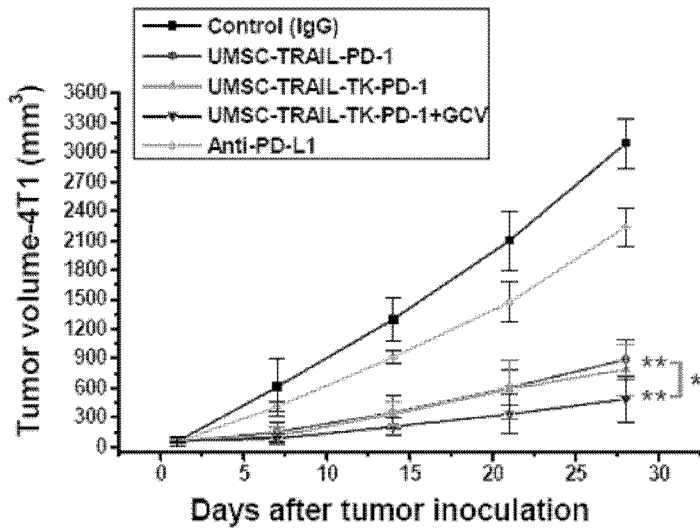
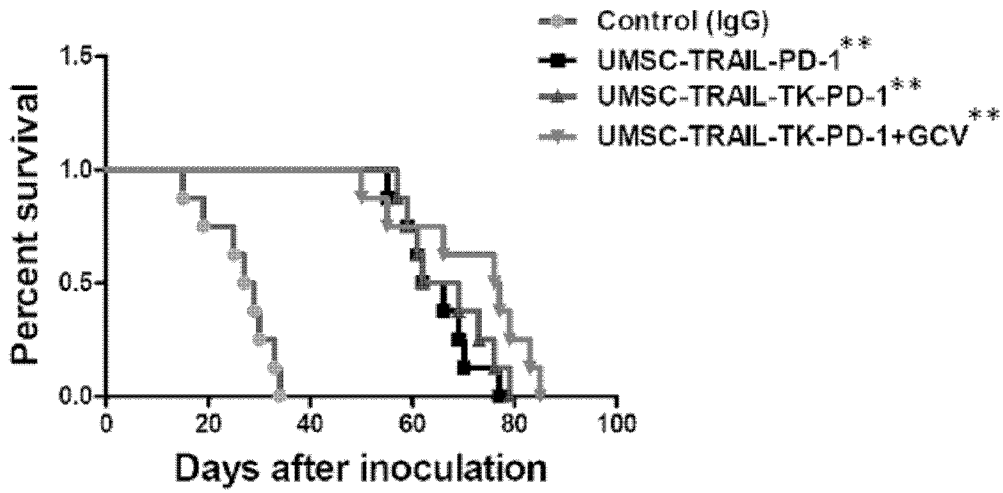


Fig. 6

F-(c)



F-(d)



G-(a)

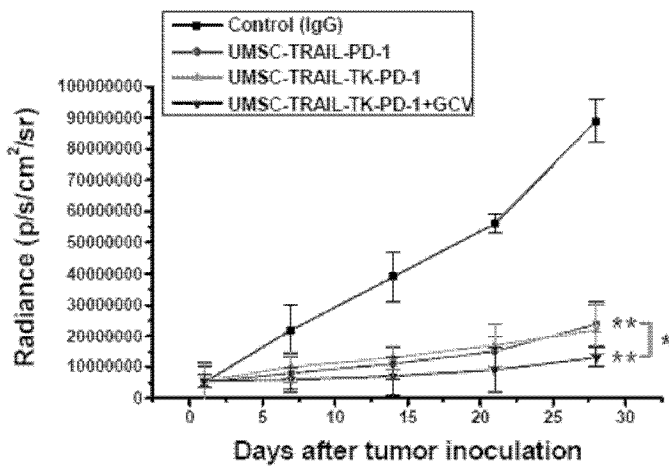


Fig. 6

G-(b)

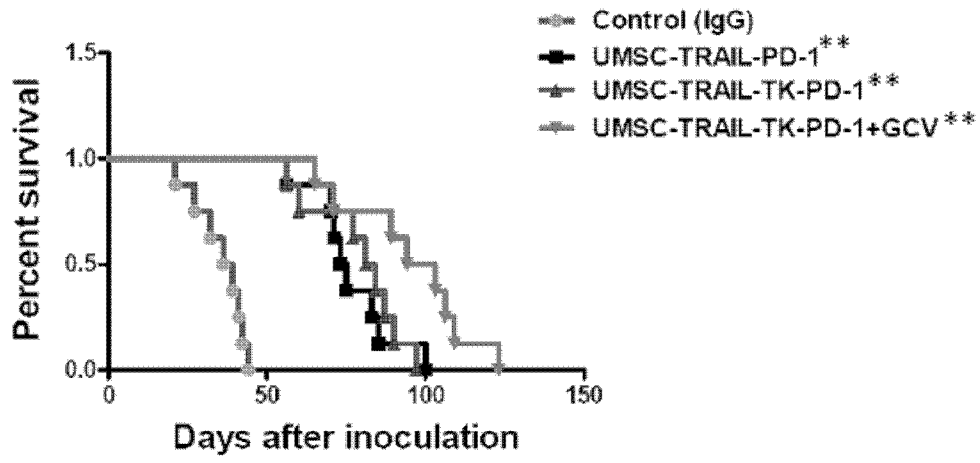
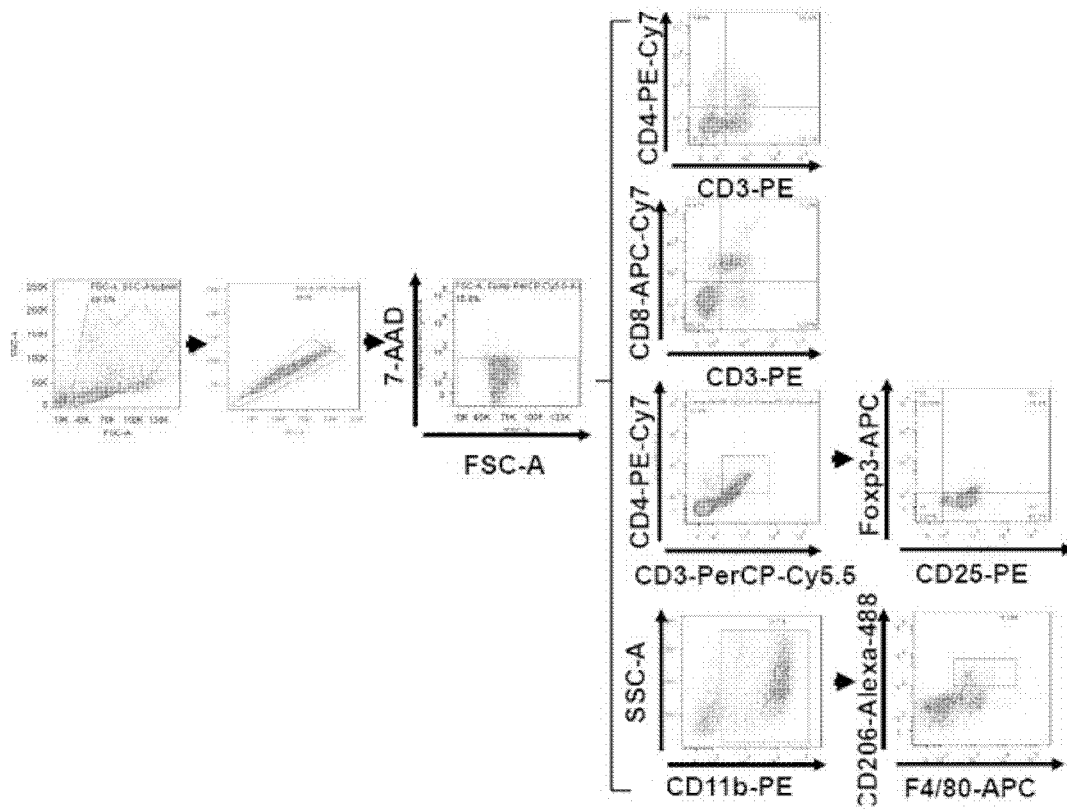


Fig. 6

A-(a)



A-(b)

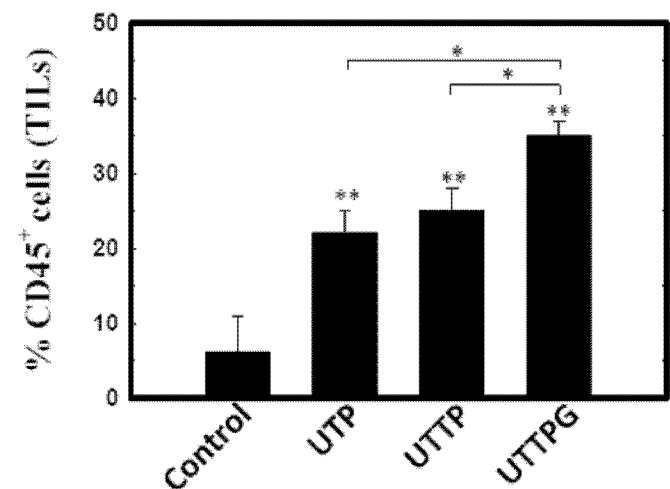
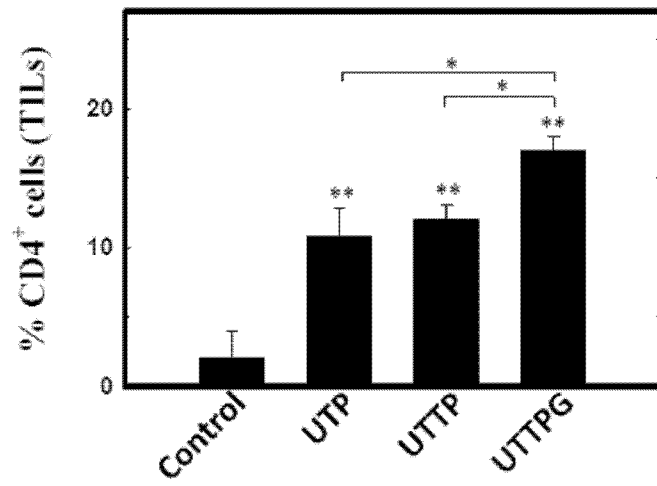
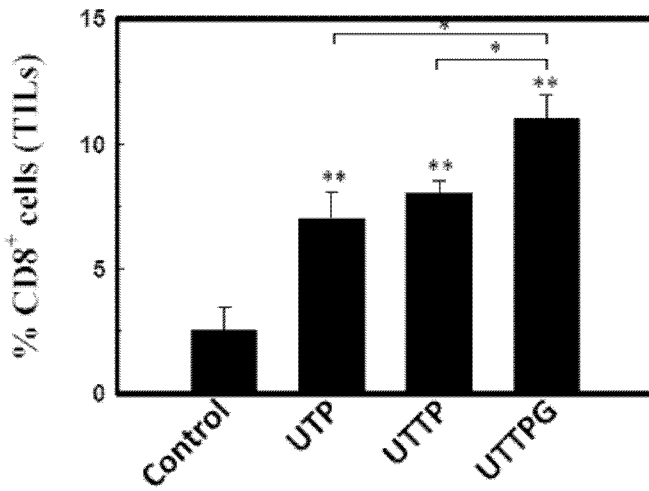


Fig. 7

A-(c)



A-(d)



B-(a)

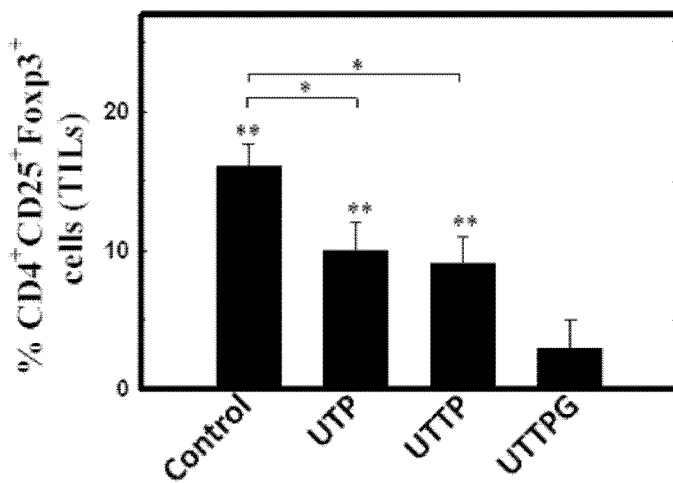
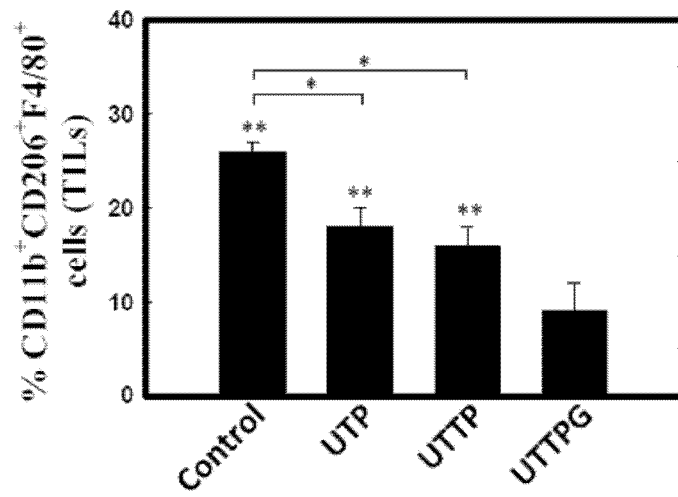
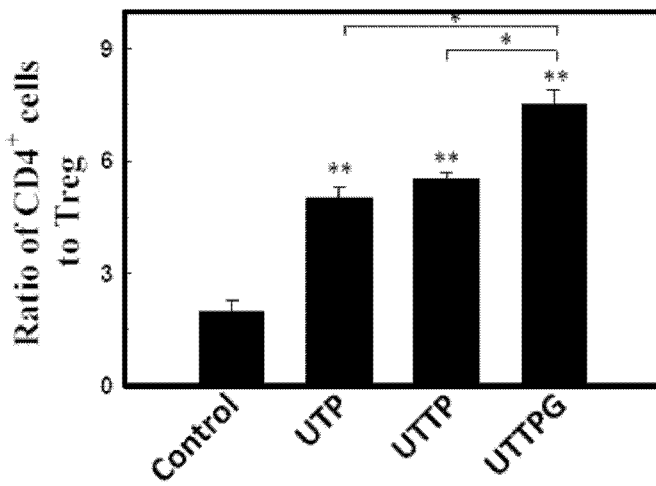


Fig. 7

B-(b)



C-(a)



C-(b)

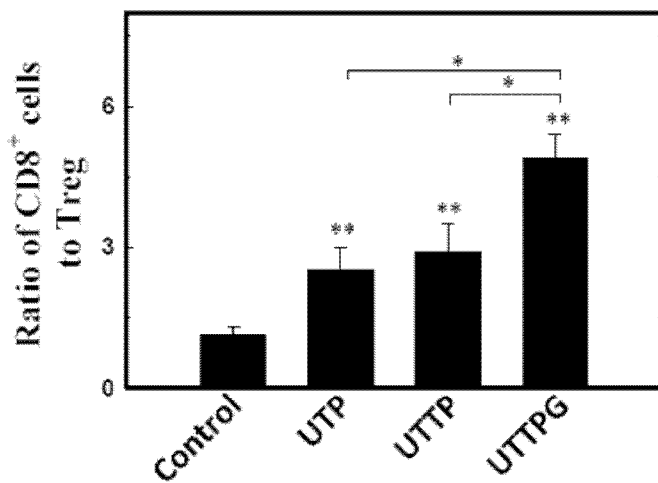
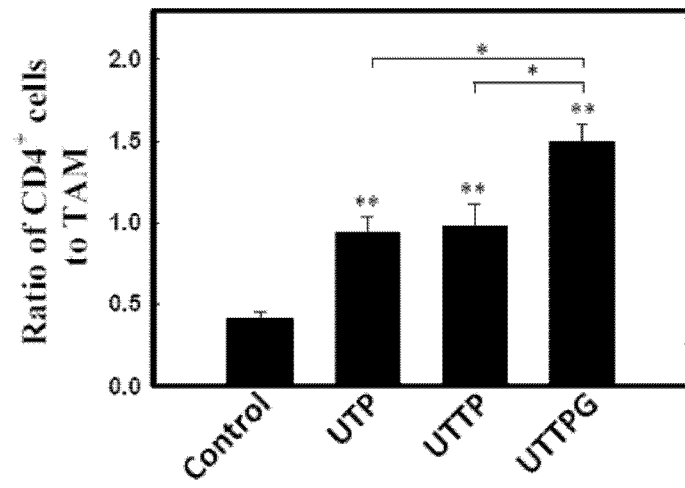
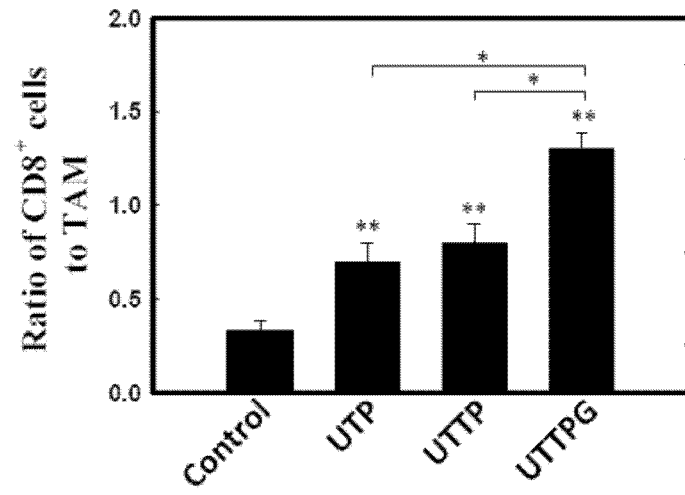


Fig. 7

C-(c)



C-(d)



D-(a)

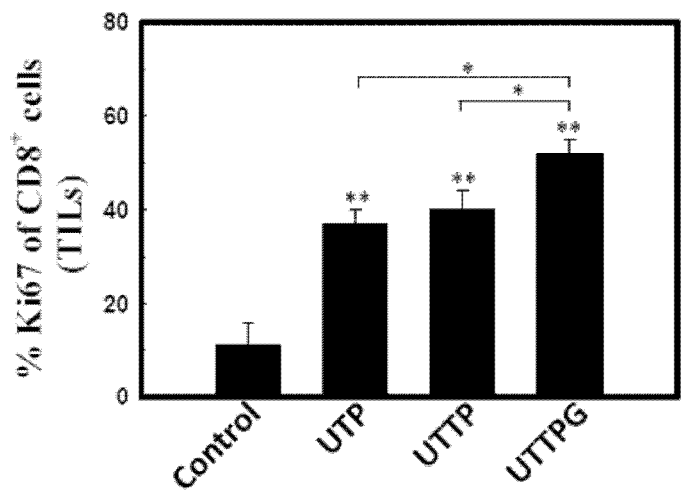


Fig. 7

D-(b)

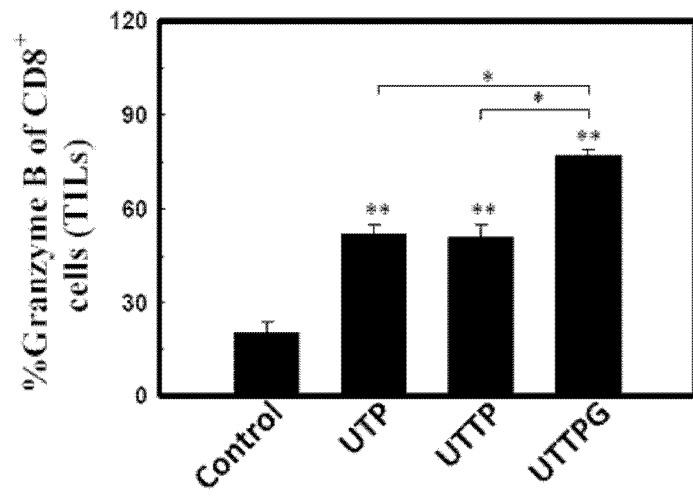


Fig. 7

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2019/078555

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>		
C12N 15/12(2006.01)i; A61K 35/12(2015.01)i; C12N 7/01(2006.01)i; C12N 15/861(2006.01)i		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b>		
Minimum documentation searched (classification system followed by classification symbols) C12N; A61K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) DWPI, SIPOABS, CNABS, CNKI,CNTXT,WOTXT,EPTXT,USTXT,PubMed,ISI web of Knowledge, GenBank,EBI, STN,mesenchymal stem cell,MSC,TRAIL,checkpoint, Cbl-b,CTLA-4,PD-1,PD-L1m,TIM-3,KIR, LAG-3,CD73,Fas,the aryl hydrocarbon receptor,Smad2,Smad4,TGF-beta receptor,ILT-3,IDOTK,thymidine kinase,ganciclovir,GCV,natural cytotoxicity triggering receptor,NCR1,NCR2,NCR3,suicide gene,caspase 1,caspase 3,caspase 6, caspase 7,caspase 8,caspase 9,Fas,cancer, tumor,SEQ ID NOs:1-5		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KIM, S.W.et al. "Complete Regression of Metastatic Renal Cell Carcinoma by Multiple Injections of Engineered Mesenchymal Stem Cells Expressing Dodecameric TRAIL and HSV-TK." <i>Clinical Cancer Research.</i> , Vol. 19, No. 2, 30 November 2012 (2012-11-30), pages 415-427	1-25
Y	HENDRIKS, D.et al. "Programmed Death Ligand 1 (PD-L1)-targeted TRAIL combines PD-L1-mediated checkpoint inhibition with TRAIL-mediated apoptosis induction." <i>Oncoimmunology.</i> , Vol. 5, No. 8, 06 July 2016 (2016-07-06), pages e1202390-1-e1202390-13	1-25
Y	US 2014369979 A1 (POSTECH ACADEMY-INDUSTRY FOUNDATION ET AL.) 18 December 2014 (2014-12-18) description, examples 1-7	1-25
A	WO 2016026854 A2 (APCETH GMBH & CO. KG) 25 February 2016 (2016-02-25) the whole document	1-25
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.		
* 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 <b>31 May 2019</b>		Date of mailing of the international search report <b>17 June 2019</b>
Name and mailing address of the ISA/CN <b>National Intellectual Property Administration, PRC 6, Xitucheng Rd., Jimen Bridge, Haidian District, Beijing 100088 China</b>		Authorized officer <b>WU,Xuemei</b>
Facsimile No. <b>(86-10)62019451</b>		Telephone No. <b>86-(10)-53961961</b>

**INTERNATIONAL SEARCH REPORT**

International application No.

**PCT/CN2019/078555****C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CN 106636000 A (GUANGDONG SHENGSAI BIOTECHNOLOGY CO., LTD) 10 May 2017 (2017-05-10) the whole document	1-25
A	CN 103182089 A (THE CHINESE UNIVERSITY OF HONG KONG) 03 July 2013 (2013-07-03) the whole document	1-25

**INTERNATIONAL SEARCH REPORT**  
**Information on patent family members**

International application No.

**PCT/CN2019/078555**

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				JP	2017093426	A	01 June 2017
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