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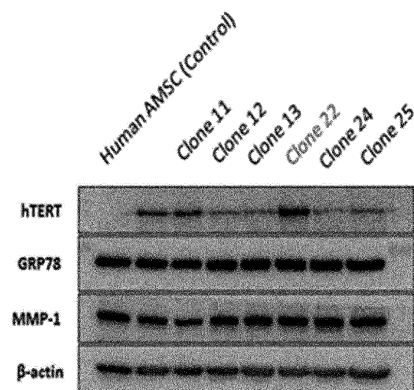
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(54) **MESENCHYMAL STEM CELLS THAT ENABLE TUMOR TARGETING IMPROVEMENT AND VIRUS MASS-PRODUCTION**

(57) The present invention relates to the preparation of mesenchymal stem cells, which are for the proliferation of viral vectors and viruses, and enable virus production and release timing control such that tumor targeting can be improved and viruses can be mass-produced. In addition, the tumorigenesis of mesenchymal stem cells of the present invention can be fundamentally blocked, safety is secured by enabling virus production and release to be controlled at desired time points, and antitumor effects can be maximized.

[FIG. 1]



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Description

[Technical Field]

5 **[0001]** The present invention relates to the preparation of mesenchymal stem cells, which are for the proliferation of viral vectors and viruses, and enable virus production and release timing control such that tumor targeting can be improved and viruses can be mass-produced.

[Background Art]

10 **[0002]** As it was reported that in the case of vesicular stomatitis virus, the rate of reaching a tumor was 0.01% within 24 hours compared to an intravenously administered virus (input virus) [Non-Patent Document 1], and the biggest constraint was the very low efficiency of delivery to a target during intravenous injection of the virus.

15 **[0003]** Since the majority of mortality in cancer patients is caused by metastatic tumors, it is essential that there is tropism to tumors and a sufficient amount of tumor-killing (oncolytic) virus be delivered to tumors relative to the administered virus.

20 **[0004]** However, upon intravascular injection, most viruses are absorbed by Kupffer cells in the liver, rapidly cleared by mechanical deposition, easily destroyed by intravascular virus-neutralizing antibodies and complements, and further, there is a problem in that the proportion of intravascular viruses released at tumor sites is considerably low. Due to these problems, in particular, the introduction of an adenovirus alone by intravenous injection could not be expected to be effective.

25 **[0005]** Solutions to the above problems include genetic modification of viruses, the introduction of nanotechnology, the use of mesenchymal stem cells with tropism to tumor sites, and the like. In particular, around 2010, research had been conducted on tumor-tropic cells such as mesenchymal stem cells [Non-Patent Document 2]. However, although mesenchymal stem cells (MSCs) have many advantages in ethical and technical convenience, low immunogenicity, and genetic stability, problems of decreases in adenovirus infection rate and replication rate for MSCs have emerged.

30 **[0006]** In order to become a carrier and amplifier of an oncolytic adenovirus, infection and replication with respect to MSCs need to be maintained at a certain level or higher, but these problems, the present inventors prepared a genetically modified MSC to get a patent registration in Korea [Patent Document 2]. However, it was confirmed that passage extension was not easy for these MSCs, and the state of a cell line deteriorated as the passage number increased.

[0007] In addition, Non-Patent Document 3 confirmed that the intravascular delivery of MSCs for the delivery of oncolytic viruses is accessible for treating multiple brain tumors, but there are problems such as the tumor growth potential of MSCs.

35 **[0008]** Meanwhile, a technique of constructing a platform that temporarily releases a virus whose production has increased at desired time points after the virus has migrated to tumors has been reported [Non-Patent Document 4]. When an MSC is used as a viral vector, carrier cells (MSCs) loaded with genes that induce cell death are quickly lysed before reaching a tumor site to release the virus, so the technique raised various problems which may be caused by the release of the virus, above all, safety problems, and the like.

40 **[0009]** Therefore, in reality, most deaths from cancer are caused by recurrence and metastasis, and there is a need for a technique capable of overcoming the major obstacles which have not yet been solved in the treatment of tumors with oncolytic viruses.

[Related Art Documents]

[Patent Documents]

45 **[0010]** (Patent Document 1) Korean Patent No. 10-2169798 (October 26, 2020)

[Non-Patent Documents]

50 **[0011]**

(Non-Patent Document 1) Silva, N et al., Double trouble for tumors: Exploiting the tumor microenvironment to enhance anticancer effect of oncolytic viruses, Cytokine & Growth Factor Reviews 21: 135, 2010

55 (Non-Patent Document 2) Xia, X et al, Mesenchymal stem cells as carriers and amplifiers in CRAd delivery to tumors, Molecular Cancer 10: 134, 2011

(Non-Patent Document 3) Kerrigan BCP, Shimizu Y, Andreeff M, Lang FF. Mesenchymal stromal cells for the delivery of oncolytic viruses in gliomas. Cytotherapy 2017; 19:445-457

(Non-Patent Document 4) Nakashima H, Kaur B, Chiocca EA. Directing systemic oncolytic viral delivery to tumors

via carrier cells. Cytokine Growth Factor Rev 2010; 21:119-126

[Disclosure]

5 [Technical Problem]

[0012] Thus, the present inventors have conducted studies and made efforts to solve the above-described problems, and as a result, developed mesenchymal stem cells capable of controlling the timing of virus production and release to improve the tumor targeting of mesenchymal stem cells for the proliferation of viral vectors and viruses and to enable
10 the mass production of viruses, thereby completing the present invention.

[0013] Accordingly, an object of the present invention is to provide mesenchymal stem cells for delivering an oncolytic virus, having improved tumor-targeting ability, into which a glucose regulated protein 78 (GRP78) gene is introduced.

[0014] Further, another object of the present invention is to provide mesenchymal stem cells for delivering an oncolytic virus into which an E1B55K gene is introduced such that the expression thereof is induced by an expression inducer.

15 **[0015]** In addition, still another object of the present invention is to provide mesenchymal stem cells for delivering an oncolytic virus, including a glucose regulated protein 78 (GRP78) gene and an E1B55K gene, in which the E1B55K gene is expressed by an expression inducer.

[0016] Furthermore, yet another object of the present invention is to provide a composition for delivering an anticancer gene, including the mesenchymal stem cells and the oncolytic virus.

20 **[0017]** Further, yet another object of the present invention is to provide an anticancer pharmaceutical composition including the mesenchymal stem cells and the oncolytic virus.

[0018] In addition, yet another object of the present invention is to provide a composition for diagnosing cancer, including the mesenchymal stem cells and the oncolytic virus.

25 **[0019]** Furthermore, yet another object of the present invention is to provide a method for treating cancer, the method including: administering a therapeutically effective amount of a pharmaceutical composition including the mesenchymal stem cells and the oncolytic virus to a subject.

[Technical Solution]

30 **[0020]** The three major difficulties posed by tumor treatment methods by a virus alone are as follows.

[0021] First, destruction by circulating antiviral antibodies against an oncolytic adenovirus, second, non-specific adsorption by other non-tumor tissues such as liver and spleen, and third, viral outflow from blood vessels to tumor sites rarely occurs.

35 **[0022]** In order to overcome these fundamental problems of virotherapy, mesenchymal stem cells were used as a cell carrier to facilitate the systemic blood transport of oncolytic viruses, and in addition to virus delivery, a genetically modified mesenchymal stem cell line (MSC) was developed for effective anti-tumor action caused by a large amount of virus produced during migration to a target tumor site.

40 **[0023]** The present invention relates to the preparation of mesenchymal stem cells, which are for the proliferation of viral vectors and viruses, and enable virus production and release timing control such that tumor targeting can be improved and viruses can be mass-produced.

[Advantageous Effects]

45 **[0024]** Since the majority of mortality in cancer patients is due to metastatic tumors, the migration to tumors relative to the administered virus and the existing adenoviral replication rate reduction in MSCs were remarkably improved such that a sufficient amount of an oncolytic virus could be delivered to tumors, and simultaneously, MSCs with improved tumor targeting were developed.

50 **[0025]** The MSCs according to the present invention can achieve innovative improvements in stability and anticancer efficacy while minimizing side effects caused by a remarkable improvement in tumor targeting and simultaneously a remarkable increase in infectious virus particle/virus particle (IVP/VP) using MSCs as virus production factories with efficiencies comparable to existing virus-producing cell lines. That is, it is possible to simultaneously achieve the efficacy maximization and toxicity minimization of MSCs loaded with an oncolytic virus by solving the most difficult problems of virus-based anticancer drugs, such as tumorigenesis, tumor tropism, tumor migration ability, and control of virus production/release at an appropriate time point.

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[Description of Drawings]

[0026]

FIG. 1 shows the confirmation of TERT expression of human adipose MSC-TERT by western blotting.

FIG. 2 shows the confirmation of whether the passage extension of human adipose MSC-TERT is effective even at passage 30 by western blotting.

FIG. 3 shows the confirmation of the adenovirus infection ability of human adipose MSC-TERT by fluorescence microscopy.

FIG. 4 shows that the reductions in p53 accumulation and survival-related signals upon induction of E1B55K expression in human bone marrow and adipose MSCs occur, as confirmed by western blotting.

FIG. 5 shows the confirmation of an increase in p53 promoter activity by the expression of E1B55K in the presence of doxycycline by a Tet-One inducible expression system by luminescence intensity.

FIG. 6 shows that the increase in p53 promoter activity by E1B55K is decreased by E4orf6, as confirmed by luminescence intensity.

FIG. 7 shows that an E4orf6 gene in an adenoviral backbone dl324-BstBI is amplified by PCR, transferred to a pFlag-CMV2 vector, and sequenced to confirm 100% matching.

FIG. 8 shows the insertion of an E1B55K gene into pCA14, which is an adenovirus shuttle vector, as confirmed by DNA electrophoresis.

FIG. 9 shows the confirmation of the E1B55K gene sequence of pCA14-E1B55K.

FIG. 10A confirms that E1B55K detection by a polyclonal antibody against E1B55K is normal.

FIG. 10B shows that the detection result of a replication-incompetent virus expressing purified E1B55K by MOI confirms that normal expression is achieved.

FIG. 11 shows the confirmation of the transfection of MSC-TERT with an E1B55K-expressing plasmid and the improvement in virus production during the infection of an oncolytic adenovirus by virus titration.

FIG. 12 shows the confirmation of the improvement in virus production according to the increase in MOI during the additional infection of an oncolytic adenovirus after infecting MSC-TERT with a replication-incompetent virus expressing purified E1B55K for each MOI by virus titration.

FIG. 13 illustrates the protocol for the Tet-One inducible expression system.

FIG. 14 illustrates PCR conditions during construction of pRetro-x-tetone-puro-E1B55K.

FIG. 15 shows the confirmation of a construct plasmid in which E1B55K is properly inserted into pRetro-x-Tetone by the in-fusion method of exogenous gene transfer.

FIG. 16 is a cleavage map of the pRetro-x-Tetone-puro-E1B55K vector used for construction.

FIG. 17 shows the confirmation of final introduction by sequence analysis of an E1B55K plasmid inserted into pRetro-x-Tetone.

FIG. 18 shows the confirmation of whether E1B55K, which is a gene inserted into pRetro-x-Tetone, is induced to be expressed as a protein by doxycycline.

FIG. 19 shows protein expression of E1B55K in pRetro-x-tetone-puro-E1B55K according to the amount of doxycycline during treatment with doxycycline.

FIG. 20 compares virus production with and without treatment with doxycycline after infection with an oncolytic adenovirus after transfection of human adipose MSC-TERT with pRetro-Tetone-E1B55K.

FIG. 21 shows the results of western blotting in which an MSC-TERT-tetoneE1B55K cell line is established from MSC-TERT.

FIG. 22 shows the results of confirming p53 accumulation by western blot in the presence of doxycycline (2.5 μ g/ml) from the MSC-TERT-tetoneE1B55K cell line.

FIG. 23 confirms the possibility of removing the tumorigenesis of the MSC-TERT-tetoneE1B55K cell line.

FIG. 24A confirms that the expression of all factors acting at each stage of MSC homing is increased by the increase in GRP78.

FIG. 24B shows the confirmation of an increase in the expression of GRP78 mRNA by introducing pcDNA3.1-GRP78 into human MSC-TERT in order to confirm that the expression of GRP78 is assured.

FIG. 25 shows the result of confirming whether a GRP78 gene is inserted into a pcDNA3.1-hygro vector [C: pcDNA3.1-hygro].

FIG. 26 shows that a liver cancer cell line is transfected with a plasmid obtained from a pcDNA3.1-GRP78 clone, and then an increase in protein expression of GRP78 is confirmed by western blotting to verify gene introduction.

FIG. 27 shows the result of analyzing the base sequence of the GRP78 gene introduced from a plasmid obtained from a pcDNA3.1-GRP78 clone.

FIG. 28 illustrates a cleavage map of an LNCXneo vector with increased restriction enzyme recognition sites in an LNCX vector.

FIG. 29 shows the results of screening plasmid clones in which the GRP78 gene is inserted into LNCXneo by restriction enzyme digestion.

FIG. 30 shows the results of screening GRP78-expressing clones by introducing a retrovirus expressing the GRP78 gene into an MSC-TERT cell line.

FIG. 31 shows the results of clearly confirming that an MSC-TERT-GRP78-arrival site gathers at tumor tissue sites without adsorption to other organs within a very short period of time.

FIG. 32 shows the results of confirming the tumor targeting verification (rapid and accurate targeting of tumors) of a final type of MSC-TERT-tetoneE1B55K-GRP78 whose gene transfer is completed in lung cancer (a), liver cancer (b), and pancreatic cancer (c) cell lines.

FIG. 33 shows the results of confirming the enhanced intratumoral infiltration of the final type MSC-TERT-tetoneE1B55K-GRP78 at 24 hours (a), 48 hours (b) and 72 hours (c) after injection.

FIG. 34 shows the expression of adenovirus-related proteins in tumor tissue in the presence of doxycycline when the final type MSC-TERT-tetoneE1B55K-GRP78 is infected with oncolytic viruses.

FIG. 35 shows the results of establishing final type MSC-TERT-tetone E1B55K-GRP78 cell line clone 21, 24, 25 and 26 cell lines.

FIG. 36 shows the result of confirming that E1B55K induction by doxycycline in the final clone 21 is effectively and sufficiently induced without being leaked even at a very low concentration.

FIG. 37 shows the amount of virus produced when the final type MSC-TERT-tetoneE1B55K-GRP78 is infected with an oncolytic virus according to the presence of doxycycline or during expression of E4orf6.

FIG. 38 shows that the expression of tumor homing markers according to an increase in the passage of MSC-TERT is confirmed.

FIG. 39 shows the anti-tumor effect of MSC-TERT-tetoneE1B55K-GRP78 infected with an oncolytic adenovirus by confirming changes in tumor size in mice by a graph.

FIG. 40A confirms the anti-tumor effect of MSC-TERT-tetoneE1B55K-GRP78 infected with an oncolytic adenovirus by the change in size after removal of mouse tumors.

FIG. 40B shows the anti-tumor effect of MSC-TERT-tetoneE1B55K-GRP78 infected with an oncolytic adenovirus by confirming changes in tumor size in mice by photographs of mice.

FIG. 41 illustrates the ratio of virus adsorption to a tumor (A) and other organs [liver (B), lung (C), spleen (D), kidney (E) and heart (F)].

[Modes of the Invention]

[0027] Hereinafter, the present invention will be described in more detail.

[0028] The present invention includes mesenchymal stem cells for delivering an oncolytic virus into which a human glucose regulated protein 78 (GRP78) gene (GenBank NM_005347) is introduced.

[0029] Further, the present invention provides mesenchymal stem cells for delivering an oncolytic virus with improved tumor-targeting ability by introducing the GRP78 gene into mesenchymal stem cells used as a viral vector.

[0030] The GRP78 gene is a glucose regulated protein 78 gene, whose role of increasing cell infiltration by increasing cell motility is already known, and its gene sequence is as shown in GenBank NM_005347 SEQ NO: 1 (human). In the present invention, a tumor homing marker serves to improve tumor targeting ability.

[SEQ ID NO: 1]

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atg aagctctccc tggaggccgc gatgctgctg ctgctcagcg cggcgccgggc cgaggaggag
gacaagaagg aggacgtggg cacggtggtc ggcatcgacc tggggaccac ctactcctgc gtcggcgtgt
5 tcaagaacgg ccgctgggag atcatcgcca acgatcaggg caaccgcate acgccgtect atgtgcctt
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25 gtgtctcttc tcaccattga caatggtgtc ttcgaagttg tggccactaa tggagatact catctgggtg

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atggaagtgc agccctccc ccaactggtg aagaggatac agcagaaaaa gatgattgt ag
40

[0031] In the present invention, it was confirmed that the tumor tropism was improved by the GRP78 gene, and in particular, the virus was delivered to tumor tissue sites without adsorption to organs other than tumors.

[0032] Although the passage extension in the case of human mesenchymal stem cells in order to facilitate the introduction of such a specific gene is difficult, a telomerase reverse transcriptase (TERT) gene, which induces cell proliferation, may be additionally introduced to overcome this difficulty.

[0033] The TERT gene is a telomerase reverse transcriptase gene, and its gene sequence is as shown in GenBank NM_198253, SEQ ID NO: 2 (human).

[SEQ ID NO: 2]

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atgccgcgcgc tccccgctgc cgagccgtgc gtcacctgct gcgcagccac taccgcgagg
tgctgccgct ggccacgttc gtgcggcgcc tggggcccca gggctggcgg ctggtgcagc gcggggacc
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 acagcccaga cgcagctgag tcggaagctc ccggggacga cgctgactgc cctggaggcc gcagccaacc

cggcactgcc ctcagacttc aagaccatcc tggactga

5 **[0034]** In the present invention, an MSC-TERT-GRP78 cell line was established, and the improvement in the tumor targeting of mesenchymal stem cells for the proliferation of viral vectors and viruses was confirmed. Therefore, the mesenchymal stem cells may be used for both tumor diagnosis and treatment.

[0035] In addition, the present invention provides mesenchymal stem cells for delivering an oncolytic virus into which an E1B55K gene is introduced such that the expression thereof is induced by an expression inducer.

10 **[0036]** The E1B55K gene is a viral gene encoding the E1B55K protein that is expressed at the early stage in the life of an adenovirus. The gene sequence is as shown in GenBank AC_000008, SEQ ID NO: 3. (The underlined a in the first line was originally g, but the KpnI site is removed by substitution. The underlined t in the 14th line is one in which a was originally substituted with t to remove the HindIII site.)

15 [SEQ ID NO: 3]

atggagcgaaga aacctatctg agcggggagt acctgctgga tttctggcc atgcatctgt
 20 ggagagcggg tgtgagacac aagaatcgcc tgctactgtt gtcttccgtc cgcccggcga taataccgac
 ggaggagcag cagcagcagc aggaggaagc caggcggcgg cggcaggagc agagcccatg gaacccgaga
 25 gccggcctgg accctcggga atgaatgttg tacaggtggc tgaactgtat ccagaactga gacgcatttt
 gacaattaca gaggatgggc aggggctaaa gggggtaaag agggagcggg gggcttgtga ggctacagag
 30 gaggctagga atctagcttt tagcttaatg accagacacc gtcctgagtg tattactttt caacagatca aggataattg
 cgctaattgag ctgatctgc tggcgcagaa gtattccata gagcagctga ccacttactg gctgcagcca
 ggggatgatt ttgaggaggc tattagggta tatgcaaagg tggcacttag gccagattgc aagtacaaga
 35 tcagcaaact tgtaaatac aggaattgtt gctacatttc tgggaacggg gccgaggtgg agatagatac
 ggaggatagg gtggccttta gatgtagcat gataaatatg tggccggggg tgcttggcat ggacgggggtg
 40

45

50

55

gttattatga atgtaaggtt tactggcccc aattttagcg gtacggtttt cctggccaat accaacctta tcctacacgg
 tgttagcttc tatgggttta acaataacctg tgtggaagcc tggaccgatg taagggttcg gggctgtgcc tttactgct
 5 gctggaaggg ggtggtgtgt cgccccaaaa gcagggcttc aattaagaaa tgcctctttg aaaggtgtac
 ctggtgtatc ctgtctgagg gtaactccag ggtgcgccac aatgtggcct ccgactgtgg ttgcttcacg
 10 ctagtgaaaa gcgtggctgt gattaagcat aacatggtat gtggcaactg cgaggacagg gcctctcaga
 tgctgacctg ctcggacggc aactgtcacc tgctgaagac cattcacgta gccagccact ctcgcaaggg
 15 ctggccagtg tttgagcata acatactgac ccgctgttcc ttgcatttg gtaacaggag ggggggtgtc
 ctaccttacc aatgcaattt gagtcacact aagatattgc ttgagcccga gagcatgtcc aaggtgaacc
 20 tgaacgggggt gttgacatg accatgaaga tctggaaggt gctgaggtag gatgagacc gcaccaggtg
 cagaccctgc gagtgtggcg gtaaacatat taggaaccag cctgtgatgc tggatgtgac cgaggagctg
 25 aggcccgatc acttgggtgct ggctgcacc cgcgctgagt ttggctctag cgatgaagat acagattga

[0037] The present inventors established a human MSC-CAR-E1B55K cell line from bone marrow-derived human MSCs that have already been filed (Patent Document 1), and confirmed an increase in virus production, but confirmed that passage extension was not easy and the condition of cell lines deteriorates as the passage number increased, and confirmed that animal experiments and master cell banking were impossible for this reason, and it took a lot of time to figure out the cause. In particular, a new function of E1B55K was confirmed. This is the fact that E1B55K not only induces an increase in viral proliferation, but also induces a decrease in viability of cell lines at the same time.

[0038] The increase in tumorigenic potential by the TERT gene, which was inevitably introduced for mass production of MSC cell lines, could provide a solution by determining, for the first time, that E1B55K induces not only an increase in viral replication, but also an overall decrease in viability of MSC cells. That is, the time difference induction of the expression time point of the E1B55K gene enabled the passage extension of MSCs, thereby enabling mass production through cell banking, and at the same time, fundamentally blocking the tumorigenesis of MSCs.

[0039] In the examples, the Tet-on system was applied in order to induce the expression time point of the E1B55K gene with a time difference, that is, to allow for expression after introduction of mesenchymal stem cells into the body, but any other time difference induction system is also possible. Furthermore, an expression inducer is preferably doxycycline, tetracycline, or the like.

[0040] In the present invention, an MSC-TERT-tetoneE1B55K cell line was established, and E1B55K was expressed (oncolysis) at desired time points through a time difference to fundamentally block the mass production of viruses and the tumorigenesis of MSCs.

[0041] Further, the present invention includes mesenchymal stem cells for delivering an oncolytic virus, including a glucose regulated protein 78 (GRP78) gene and an E1B55K gene, wherein the E1B55K gene is expressed by an expression inducer.

[0042] In the present invention, an MSC-TERT-tetoneE1B55K-GRP78 cell line was established, and it was confirmed that it is possible to control virus production and release timing such that tumor targeting can be improved and viruses can be mass-produced.

[0043] The mesenchymal stem cell line used in the present invention is preferably human-derived. The mesenchymal stem cell line is preferably derived from bone marrow, cord blood or fat.

[0044] The oncolytic virus used in the present invention may be an oncolytic adenovirus, an oncolytic adeno-associated virus (AAV), an oncolytic retrovirus, an oncolytic lentivirus, an oncolytic herpes simplex virus or an oncolytic vaccinia virus.

[0045] In addition, the oncolytic adenovirus may be an oncolytic adenovirus described in Korean Patent Application No. 2016-0166171 developed by the present inventors.

[0046] The present invention also includes a composition for delivering an anticancer gene, including the mesenchymal stem cells for delivering an oncolytic virus and the oncolytic virus.

[0047] The composition for delivering a gene of the present invention may be for systemic administration.

[0048] Stem cells (Ad-MSCs) loaded with the virus of the present invention, in addition to having cancer-specific characteristics, have a low or no immune response, and thus, can be systemically administered, and are highly targeted to tumor cells, have an effect of not inducing toxicity, and thus can remarkably enhance gene delivery efficiency.

[0049] The mesenchymal stem cells may be isolated from bone marrow, cord blood, fat, and the like, and allogeneic mesenchymal stem cells may be used using the patient's own or blood bank databases. Therefore, when the adenovirus-loaded stem cells of the present invention are used as a gene carrier, not only self-treatment but also allogeneic treatment is possible, so the unit cost of gene therapy may be further reduced.

[0050] Methods for introducing the adenovirus including the gene of the present invention into mesenchymal stem cells may be carried out by various methods known in the art. Specifically, the gene may be introduced by a virus infection method known in the art.

[0051] As used herein, the term "anticancer pharmaceutical composition" refers to a "pharmaceutical composition for use in treating cancer".

[0052] Since the mesenchymal stem cells included as an active ingredient in the anticancer pharmaceutical composition of the present invention are the same as the mesenchymal stem cells of the present invention described above, the detailed description on mesenchymal stem cells is equally applied to the compositions of the present invention. Therefore, the description on common matters is omitted to avoid undue complication due to unnecessary repeated descriptions of the present specification.

[0053] Since the oncolytic adenovirus included in the compositions of the present invention exhibits killing efficacy against various tumor cells as described above, the pharmaceutical composition of the present invention may be used for the treatment of cancer such as gastric cancer, lung cancer, breast cancer, ovarian cancer, liver cancer, bronchial cancer, nasopharyngeal cancer, laryngeal cancer, pancreatic cancer, biliary tract cancer, bladder cancer, colorectal cancer, colon cancer, cervical cancer, brain cancer, prostate cancer, bone cancer, head and neck cancer, skin cancer, kidney cancer, polyploid carcinoma, thyroid cancer, parathyroid cancer or ureteral cancer. As used herein, the term "treatment" includes: (i) prevention of tumor cell formation; (ii) inhibition of tumor-related diseases or disorders upon removal of tumor cells; and (iii) relief of tumor-related diseases or disorders upon removal of tumor cells. Therefore, as used herein, the term "therapeutically effective amount" refers to an amount sufficient to achieve the aforementioned pharmacological effect.

[0054] A pharmaceutically acceptable carrier included in the composition of the present invention is a pharmaceutically acceptable carrier typically used in formulation, and includes lactose, dextrose, sucrose, sorbitol, mannitol, starch, gum acacia, calcium phosphate, alginate, gelatin, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, water, syrup, methyl cellulose, methyl hydroxybenzoate, propyl hydroxybenzoate, talc, magnesium stearate, mineral oil, and the like, but is not limited thereto.

[0055] The pharmaceutical composition of the present invention may additionally include a lubricant, a wetting agent, a sweetening agent, a flavoring agent, an emulsifier, a suspending agent, a preservative, and the like, in addition to the aforementioned ingredients.

[0056] The pharmaceutical composition of the present invention is preferably parenterally administered, and may be administered, for example, using intravenous administration, intraperitoneal administration, intramuscular administration, subcutaneous administration or topical administration. The pharmaceutical composition of the present invention is administered intraperitoneally to treat ovarian cancer and administered through the hepatic portal vein to treat liver cancer, by an injection method, and in the case of liver cancer, melanoma and breast cancer, the pharmaceutical composition may be administered by direct injection into a tumor mass, and in the case of colon cancer, the pharmaceutical composition may be administered by direct injection into an enema, and in the case of bladder cancer, the pharmaceutical composition may be administered by direct injection into a catheter.

[0057] A suitable administration amount of the pharmaceutical composition of the present invention may vary depending on factors, such as formulation method, administration method, age, body weight, sex or disease condition of a patient, diet, administration time, administration route, excretion rate and response sensitivity, and a doctor with ordinary skill may readily determine and prescribe an effective dosage for a desired treatment. In general, the pharmaceutical composition of the present invention includes 1×10^5 to 1×10^{15} pfu/ml of oncolytic virus, and typically, the amount of virus that infects MSCs (based on 1×10^6 cells) at one injection is 1×10^7 to 1×10^8 pfu.

[0058] The pharmaceutical composition of the present invention may be prepared in the form of a unit dose or by being contained in a multi-dose container by being formulated using a pharmaceutically acceptable carrier and/or excipient according to a method that can be readily implemented by a person with ordinary skill in the art to which the present invention pertains. In this case, the formulation may also be in the form of a solution in an oil or aqueous medium, a suspension or in the form of an emulsion, an extract, a powder, a granule, a tablet or a capsule, and the pharmaceutical composition of the present invention may additionally include a dispersant or a stabilizer.

[0059] The pharmaceutical composition of the present invention may be used as a single therapy, but may also be used in combination with a common type of chemotherapy or radiation therapy, and when such combination therapy is

implemented, cancer may be more effectively treated. A chemotherapeutic agent that can be used together with the composition of the present invention includes gemcitabine, sorafenib, cisplatin, carboplatin, procarbazine, mechlorethamine, cyclophosphamide, ifosfamide, melphalan, chlorambucil, bisulfan, nitrosourea, dactinomycin, daunorubicin, doxorubicin, bleomycin, plicomycin, mitomycin, etoposide, tamoxifen, taxol, transplatinum, 5-fluorouracil, vincristin, vinblastin, methotrexate, and the like. A radiation therapy that can be used together with the composition of the present invention includes X-ray radiation, γ -ray radiation, and the like.

[0060] The mesenchymal stem cells according to the present invention may exhibit anticancer effects against gastric cancer, lung cancer, breast cancer, ovarian cancer, liver cancer, bronchial cancer, nasopharyngeal cancer, laryngeal cancer, pancreatic cancer, biliary tract cancer, bladder cancer, colorectal cancer, colon cancer, cervical cancer, brain cancer, prostate cancer, bone cancer, head and neck cancer, skin cancer, kidney cancer, polyploid carcinoma, thyroid cancer, parathyroid cancer or ureteral cancer.

[0061] Furthermore, the present invention may include a composition for diagnosing cancer, including a virus/stem cell complex including the mesenchymal stem cells and the oncolytic virus.

[0062] As used herein, the term "diagnosis" means confirming the presence or characteristics of the pathological state. Diagnosis in the present invention is to confirm the presence or absence of cancer and its progression.

[0063] The virus/stem cell complex refers to a stem cell infected or loaded with a virus.

[0064] The virus/stem cell complex may be combined with a luminescent material, a fluorophore, or an isotope to diagnose cancer through imaging.

[0065] The luminescent material refers to any material that emits light.

[0066] In the examples of the present invention, it was confirmed that after MSCs were infected with a luciferase-expressing adenovirus, luminescence was captured as images by intraperitoneally administering luciferin to mice.

[0067] The fluorophore is preferably a phosphor, a fluorescent protein, or other imaging materials capable of binding to the peptide that specifically binds to NRP1, but is not limited thereto.

[0068] The fluorophore is preferably fluorescein, BODIPY, tetramethylrhodamine, Alexa, Cyanine, allopicocyanine or derivatives thereof, but is not limited thereto.

[0069] The fluorescent protein is preferably Dronpa protein, fluorescent chromogenic gene (EGFP), red fluorescent protein (DsRFP), Cy5.5, which is a cyanine fluorophore exhibiting near-infrared fluorescence, or other fluorescent proteins, but is not limited thereto. Other imaging materials are preferably iron oxide, radioactive isotopes, and the like, but are not limited thereto, and may be applied to imaging equipment such as MR and PET.

[0070] The present invention also includes a method for treating cancer, the method including: administering a therapeutically effective amount of a pharmaceutical composition including the mesenchymal stem cells and the oncolytic virus to a subject.

[0071] All of the above-described contents in relation to the method for treating cancer can be applied to the above-described mesenchymal stem cells of the present invention and the composition including the same as they are or applied correspondingly.

[0072] The cancer may be specifically gastric cancer, lung cancer, breast cancer, ovarian cancer, liver cancer, bronchial cancer, nasopharyngeal cancer, laryngeal cancer, pancreatic cancer, biliary tract cancer, bladder cancer, colorectal cancer, colon cancer, cervical cancer, brain cancer, prostate cancer, bone cancer, head and neck cancer, skin cancer, kidney cancer, polyploid carcinoma, thyroid cancer, parathyroid cancer or ureteral cancer, but is not limited thereto.

[0073] As used herein, the term "subject" refers to a mammal that is the subject of treatment, observation or experimentation, preferably a human.

[0074] As used herein, the term "therapeutically effective amount" refers to an amount of active ingredient or pharmaceutical composition which induces a biological or medical response in a tissue system, animal or human considered by a researcher, veterinarian, physician or other clinicians, and this includes an amount that induces alleviation of the symptoms of a disease or disorder being treated. It is obvious to those skilled in the art that the therapeutically effective amount and frequency of administration of the active ingredient of the present invention will vary according to the desired effect. Therefore, the optimal dosage to be administered can be easily determined by those skilled in the art, and the range thereof varies according to the type of disease, the severity of disease, the content of active ingredient and other ingredients contained in the composition, the type of formulation, the body weight, age, sex, health condition, and diet of a patient, the administration time, the administration method, the excretion rate, and the like. In the treatment method of the present invention, 1×10^5 to 1×10^{15} pfu/ml of oncolytic virus is included, and typically, the amount of virus that infects MSCs (based on 1×10^6 cells) at one injection is 1×10^7 to 1×10^8 pfu.

[0075] In the treatment method of the present invention, the pharmaceutical composition of the present invention may be administered orally or parenterally (for example, applied intravenously, subcutaneously, intraperitoneally or topically) according to the desired method.

[Mode for Invention]

[0076] Hereinafter, the present invention will be described in more detail through the Examples according to the present invention, but the scope of the present invention is not limited by the Examples suggested below.

[Examples]

Reference Example 1: Construction of human oncolytic adenovirus YSC-02

[0077] YSC-02 was constructed as described in Preparation Example 19 of Korean Patent Application No. 2016-0166171.

Reference Example 2: Construction of human oncolytic adenovirus dl324-3484-H1-shHSP27-U6-shTGF β 1

[0078] dl324-3484-H1-shHSP27-U6-shTGF β 1 was constructed as described in Preparation Example 9 of Korean Patent Application No. 2016-0166171.

Preparation Example 1: Introduction of TERT gene for mass production of MSC cell line

1) Confirmation of human adipose MSC-TERT single clone expression

[0079] A TERT gene was introduced to induce the expression of a specific gene that overcomes the short supply and lifespan of MSCs. In the case of human MSCs, passage extension is not easy. To overcome this problem, a telomerase reverse transcriptase (TERT) gene, which induces cell proliferation, was inserted and clones highly expressing TERT were selected.

[0080] The method for introducing the TERT gene is as follows.

[0081] A retrovirus vector pBABE-hygro-hTERT was purchased from Addgene, and cell clones resistant to 200 μ g/ml hygromycin B Gold were obtained by infecting human adipose MSCs with a filtered medium obtained by transfecting platinum-A packaging cells (Cell Biolabs) with 1 μ g of the retrovirus vector pBABE-hygro-hTERT, and then screened and selected as in FIG. 1. MSC clone 22 was finally selected, and clone 22 was used for human adipose MSC-TERT used in subsequent experiments.

2) Confirmation of passage extension

[0082] FIG. 2 confirms the passage extension of human MSCs, which was proliferated to 30 passages. It was confirmed that the increase in expression level of TERT from the MSCs extensively passaged to 30 passages was still very clearly increased and the expression of other MSC-related major markers was also maintained or increased compared to the wild type.

3) Confirmation of adenovirus infection ability

[0083] A TERT-expressing MSC clone (No. 22) was infected with a replication-incompetent adenovirus expressing red fluorescence protein (RFP) at 20 MOI and 100 MOI, and 48 hours later, fluorescence-stained cells were observed under a fluorescence microscope. As a result of comparison with a wild type MSC, which is a control, it was confirmed that the infection rate of human MSC-TERT was improved compared to the control, and at least a two-fold increase in infection rate at (20 MOI) and a 90% or more infection rate at 100 MOI were met (FIG. 3).

Example 1: Confirmation of new function of adenoviral gene E1B55K

[0084] When MSCs are to be used as a cell line for clinical mass production, master cell banking needs to be conducted. For this purpose, it is essential that the passage extension of MSCs be possible above all else. However, on the other hand, a principle that extension induction must not contribute to an increase in tumorigenic potential also needs to be maintained. Although the dilemma of simultaneously satisfying these two conditions was considered impossible at first, a solution was suggested for the first time in the present inventor's laboratory while constructing a stable MSC cell line in which CAR and E1B55K genes were introduced into MSCs. A human MSC-CAR-E1B55K cell line was established from bone marrow-derived human MSCs that had been filed, and an increased virus production was confirmed [Patent Document 1]. However, it was confirmed several times that passage extension was not easy and the condition of the cell line deteriorated as the passage number increased. Accordingly, it was confirmed that in the case of animal exper-

iments, master cell banking was impossible, and as a result of finding out the cause, it was confirmed that E1B55K induces increased viral proliferation and reduced viability of cell lines at the same time (FIG. 4). That is, it was confirmed that not only p53, which is well known as a tumor suppressor gene and a cytostatic factor, is distinctly accumulated, but also the expression of factors associated with cell survival (HSP27, HSP70, Daxx, phospho-p65, NF- κ B, c-Me) was decreased by E1B55K.

[0085] The experimental process for this is as follows.

[0086] First, E1B55K was cloned into pcDNA3.1 hygro (+). To extract an E1B55K gene from pBSK-3484 including the E1B55K gene, after PCR (initial denaturing: 95°C for 2 minutes, denaturing: 95°C for 30 seconds, annealing: 58°C for 30 seconds, extension: 72°C for 2 minutes; 30 cycles) and BamHI/NotI digestion with primers each having BamHI and NotI (forward, 5'-GCGGATCCATGGAGCGAAGAAACCCATCT-3': SEQ ID NO: 4, reverse, 5'-GAG CGGCCGCTCAATCTGTATCTTCATCGCT-3': SEQ ID NO: 5), a plasmid was constructed by introducing the E1B55K gene into pcDNA3.1 hygro (+) (Invitrogen), which had been previously digested with BamHI/NotI.

[0087] It was confirmed by transfecting the plasmid expressing E1B55K into bone marrow-derived MSCs and adipose-derived MSCs that the cell line long-term expression of E1B55K reduced the viability of the cell line. That is, the accumulation of p53 and the reduction of various survival-related signals were observed.

[0088] In order to confirm how the accumulation of p53, reduction of cell survival signals and induction of cell death were caused by E1B55K expression, the following assumption was made. That is, it was known that E1B55K maintains cell survival by well-known p53 degradation in the related art only through a complex with an E4orf6 protein. Therefore, a hypothesis was made, in which when the expression of E1B55K alone is induced in MSC cells, (stoichiometrically) free E1B55K alone acts as a transcriptional activator that promotes p53 transcription to reduce cell viability due to the quantitative comparative advantage of E1B55K over E4orf6, and experiments were carried out as follows.

[0089] After the MSC-TERT-tetoneE1B55K cell line was co-transfected with 1 μ g of pFlag-CMV2-E4orf6 subcloned with a pGL2-p53 promoter (pGL2-356bp, Addgene) constructed such that a constant portion (-344 to +12) of the p53 promoter was positioned in front of a site where a luciferase coding gene was inserted and the E4orf6 gene or 1 μ g of pFlag-CMV2 (Sigma) as a control, luciferase activity with and without doxycycline (1.25 μ g/ml for 48 hours) was confirmed by luminescence intensity.

[0090] The analytical method used in this case was a Dual-Luciferase Reporter Assay System from Promega (catalog number #E1910, FIG. 5).

[0091] E1B55K, whose expression was induced in the MSC-TERT-tetoneE1B55K cell line by doxycycline treatment, was directly or indirectly bound to the p53 promoter to activate transcription, resulting in a 4-fold increase in luminescence intensity due to luciferase activity (FIG. 5). Conversely, it was confirmed that when the expression of E4orf6 occurred as in E1B55K-expressing MSCs, the increase in luminescence intensity did not exceed 1.5-fold. These results show that E4orf6 interferes with the action of E1B55K alone due to the formation of a complex with E1B55K, and at the same time, more actively fulfills its already known role in promoting the degradation of the p53 protein (FIG. 6).

[0092] Here, subcloning of the E4orf6 (GenBank AC_000008; also called E434K) gene was performed to transfer the E4orf6 gene from an adenoviral backbone dl324-BstBI into a pFlag-CMV2 vector. For this purpose, an E4orf6 coding gene site was amplified by PCR (initial denaturing: 95°C for 2 minutes, denaturing: 95°C for 30 seconds, annealing: 58°C for 30 seconds, extension: 72°C for 1 minute and 30 seconds; 30 cycles) in dl324-BstBI using a primer for E4orf6 PCR (forward 5'-GTACAAGCTTATGACTAC GTCCGGCGTTCC-3' including HindIII: SEQ ID NO: 6 and reverse 5'-CACCTCTAGACTACA TGGGGGTAGAGTCAT-3' including XbaI: SEQ ID NO: 7), and then doubly digested with HindIII/XbaI and ligated to pFlag-CMV2 digested with HindIII and XbaI. 100% matching was confirmed by performing sequencing using CMV30, one of the universal primers from Cosmogenetech Co. Ltd., to confirm whether subcloning was properly performed (FIG. 7).

[0093] From the above experimental results, the induction of overexpression of E1B55K acts directly or indirectly on the p53 promoter, which is contrary to the main action of E1B55K in adenovirus wild type (forms a complex with E4orf6 to induce degradation of p53 protein), so a new action that induces the accumulation of p53 was discovered through transcriptional induction. In addition, in order to confirm how the increase in MSC cell death and the decrease in viability signal depending on the increase in expression of E1B55K affect the virus production ability in MSCs, it was intended to again confirm whether the virus production ability in MSCs increased by externally supplying E1B55K using an adenoviral vector. For this purpose, a replication-incompetent adenovirus expressing E1B55K was constructed as follows.

1) Construction of replication-incompetent adenovirus (pCA14-E1B55K) expressing E1B55K

[0094] The E1B55K gene was cloned into pCA14 which is an adenovirus shuttle vector. E1B55K was included in pBSK-3484, and a primer including XbaI and HindIII at both ends thereof (sense 5'-TTCATCTAGAATGGAGCGAA-GAAACCCATC-3' (XbaI): SEQ ID NO: 9, antisense 5'-GACGAAGCTTCAATCTGTATCTTCATCGC-3' (HindIII): SEQ ID NO: 10) was constructed and amplified by PCR (initial denaturing: 95°C for 2 minutes, denaturing: 95°C for 30 seconds, annealing: 58°C for 30 seconds, extension: 72°C for 2 minutes; 30 cycles) and ligated into pCA14 (Microbix, Canada)

digested in advance with XbaI/HindIII. As a result, it was confirmed that all E1B55K PCR fragments were inserted into PCA14 (FIG. 8). A confirmed adenoviral shuttle vector, pCA14-E1B55K, was linearized again with XmnI digestion after sequence confirmation (FIG. 9), an adenoviral vector, dl324-BstBI, was linearized with Bsp119I digestion, these two were cotransformed into *E. coli* BJ5183 to induce homologous recombination to construct an adenoviral vector expressing E1B55K, and then the vector was transfected into 293A, and then virus production was induced to expand production, and after isolation and purification, the titer was calculated.

[0095] It was confirmed that 1, 2, and 3 out of pc14-E1B55K plasmid clones 1, 2, 3, and 4 read in the forward and reverse directions with a primer dedicated to pCA14 sequence analysis (self-constructed forward (hCMV): 5'-GGG AGGTCTATATAAGCAGAGCTCG-3': SEQ ID NO: 11, reverse (pCA14 SV40): 5'-CATGATCGATGCTAG ACGATCCAGA-3': SEQ ID NO: 12) matched, and here, FIG. 9 shows the results of sequence analysis of sample #3 clone plasmid DNA. In the forward non-matching two (red) sequences shown in FIG. 9, the first base of E1B55K of the existing wild adenovirus type 5, a, is the 2019th base of AC_000008 in GenBank, and the 2048th base, g, in the forward primer was intentionally substituted with a to remove a KpnI site (GGTACC→AGTACC) and the 2084th base, a, in the forward primer (overlapped with red of the reverse primer) was substituted with t to intentionally remove a HindIII site (AAGCTT→TAGCTT) (100% homology).

2) Confirmation of E1B55K expression in MSC lysate infected with virus expressing E1B55K

[0096] After it was confirmed that the detection of E1B55K by a polyclonal antibody secured by commissioning the construction of a specific antibody against E1B55K for E1B55K expression was not abnormal (FIG. 10A), and as a result of detecting the produced and purified virus at each MOI, normal expression was confirmed by western blotting (FIG. 10B). The drawing on the right side of FIG. 10A is attached as raw data for the drawing on the left side in order to confirm the correct size and band of a polyclonal antibody E1B55K. In Lane 3 of FIG. 10A, 3 μg of adenoviral vector (dl324-E1B55K) DNA expressing E1B55K was digested with PaeI, then transfected into 293A, and after confirmation of virus production, human adipocyte-derived MSC-TERT was infected with the harvested soup, in Lane 4, a final soup was infected by freezing and thawing the soup and plus cells obtained in the same manner as Lane 3, and in Lane 5, the starting amount of DNA during the first transfection is 2.5 μg. In FIG. 10B, it was confirmed that the polyclonal antibody against E1B55K is working normally by confirming that after a viral soup obtained under the conditions of Lane 5 was amplified and purified, the intensity of a band corresponding to E1B55K is also increasing as the MOI increases when MSC-TERT is infected with a virus expressing the replication-incompetent E1B55K at each MOI by calculating virus infectivity particles.

[0097] The process of constructing a polyclonal antibody specific to E1B55K is as follows.

[0098] First, a peptide sequence located at the N-terminal site of E1B55K (MERRNPSEKGVPAFGSGHASVESGC: SEQ ID NO: 13) was synthesized, bovine serum albumin (BSA) was conjugated to an immunogen carrier, and then New Zealand White rabbits were immunized and serum was purified to obtain the polyclonal antibody (GW Vitek, Korea).

3) Comparison of virus production according to E1B55K expression level in human adipose MSC-TERT → validation of innovative expansion of virus production ability of MSCs according to gene transfer

[0099] First, human adipocyte-derived MSC-TERT was transfected with 1 μg of an E1B55K-expressing plasmid (pcDNA3.1-E1B55K) (FIG. 11) or infected with a replication-incompetent adenovirus expressing E1B55K at each MOI (FIG 12), then the medium was replaced after 4 hours. And after infection with an oncolytic adenovirus (YSC-02) at 100 MOI, all of the medium and cells were harvested 48 hours later, and then frozen and thawed to excite the virus as much as possible, and then the results of performing the titration of an infectious virus in a supernatant obtained by centrifugation are shown. As a result, it was confirmed that virus production was clearly increased compared to the control after infection with the oncolytic adenovirus in the E1B55K-expressing MSC-TERT (FIG. 12).

Example 2: Innovative viral production increase induction, regulation of expression time point of loaded gene, and intrinsic tumorigenesis control technique can be simultaneously realized by introducing time-difference expression regulation system of E1B55K

[0100] The increase in control of tumorigenic potential by the TERT gene, which was inevitably introduced for mass production of MSC cell lines, could provide a solution by determining, for the first time, that E1B55K induces not only an increase in viral replication, but also an overall decrease in viability of MSC cells. That is, a control technique, in which the time difference induction of the expression time point of the E1B55K gene enabled the passage extension of MSCs, thereby enabling mass production through cell banking, and at the same time, fundamentally blocking the tumorigenesis of MSCs, was developed. It is of great significance that this has provided an innovative turning point for virus/MSK therapeutic agents.

[0101] The protocol in FIG. 13 was referenced for the Retro-X™Tet-One™ induced expression system (TAKARA cat# 634307).

1) Construction of pRetro-X-Tet-one-puro-E1B55K

[0102] In order to insert the E1B55K gene into a Retro-X-Tet-one-purovector plasmid, both ends need to have EcoRI and BamHI recognition sites, respectively. pCA14-E1B55K was used as a template for the construction of E1B55K having recognition sites, an E1B55K In-fusion EcoRI sense primer is 5'-CCCTCGTAAAGAATTCATGGAGCGAA-GAAACCCATCTGAG-3' (SEQ ID NO: 14), and an E1B55K In-fusion BamHI antisense primer is 5'-GAGGTGGTCT-GGATCCTCAATCTGTATCTTCATCGCTAGA-3' (SEQ ID NO: 15).

[0103] PCR conditions are as follows (FIG. 14).

[0104] After initial denaturing at 95°C for 2 minutes, 30 cycles of denaturing at 95°C for 40 seconds, annealing at 61°C for 40 seconds, and extension at 72°C for 1 minute and 50 seconds were performed.

[0105] APromega (#M750B) product was used to perform PCR.

[0106] After a construct plasmid (sample pRetro-X-Tetone-E1B55K #13 in FIG. 15), in which E1B55K was properly inserted into pRetro-X-Tetone by the in-fusion method of exogenous gene introduction, was secured, the introduction was finally confirmed by performing sequence analysis (FIG. 17). Next, it was confirmed whether the expression of the E1B55K protein, which is an actually inserted gene, was induced by doxycycline (FIG. 18). As in Lane 5 in FIG. 18, it was confirmed that the expression of E1B55K was induced when A549 was transfected with the pRetro-X-Tetone-E1B55K plasmid only in the presence of doxycycline (2.5 µg/ml), and the expression of E1B55K was not induced when A549 was transfected with the same plasmid in the absence of doxycycline. That is, no leakage phenomenon occurred. The rightmost lane is a positive condition under which E1B55K was constitutively expressed and thus E1B55K expression was confirmed.

[0107] The pRetro-X-tetone-puro-E1B55K vector used for construction is shown in FIG. 16.

2) Confirmation of pRetro-X-tetone-puro-E1B55K expression

[0108] After transfection with the pRetro-X-Tetone-puro-E1B55K plasmid, the expression of E1B55K was confirmed in pRetro-X-tetone-puro-E1B55K according to the amount of doxycycline during treatment with doxycycline. When a tet one system was introduced to investigate whether the time difference expression regulation of E1B55K could be controlled with the tet one system, it was confirmed that no E1B55K expression occurred at all before the treatment with doxycycline (FIG. 19). That is, it was confirmed that no leakage occurs. Conversely, even treatment with a very low concentration of 0.05 µg/ml of doxycycline immediately induced the expression of E1B55K. This means that cell survival can be maintained before treatment with doxycycline and expression can be induced immediately after treatment with doxycycline after reaching tumors.

[0109] The time difference virus release was secondarily verified as follows.

[0110] After human adipose MSC-TERT was transfected with a pRetro-tetone-E1B55K plasmid (4 hours) and then infected with an oncolytic adenovirus expressing a red fluorescence protein (RFP) (4 hours), virus production was compared with and without treatment with doxycycline 48 hours later.

[0111] As a result, it was confirmed that viral production was clearly increased 10-fold or more upon treatment with doxycycline (FIG. 20).

3) Establishment of MSC-TERT-tetoneE1B55K cell line

[0112] After a gene encoding E1B55K, which is an early region 1B-55kDa of an adenovirus, was introduced into a Retro-X-Tet-One inducible expression system (TAKARA Bio Inc.), MSC-TERT was infected with the same to secure clones expressing E1B55K in the presence of doxycycline. For this purpose, GP2-293 packaging cells were co-transfected with pRetroX-TetOne-E1B55K and a pAmpho vector encoding the membrane protein (included in the TAKARA kit). After 48 hours, MSC-TERT, which is a target cell, was infected with the filtered retroviral supernatant. All clones selected with puromycin during the culture process were subjected to western blotting again to confirm that the induction of E1B55K expression (FIG. 21) and p53 accumulation (Fig. 22) proceeded simultaneously in the presence of doxycycline (2.5 µg/ml).

[0113] These results indicated that the induction of viral production could be increased at desired time points by regulating the expression time point of E1B55K, which is a loaded gene, according to the treatment with doxycycline. (Since the gene expression of E1B55K must be induced, the virus appears to begin to increase at least 24 hours after treatment with doxycycline.)

4) Realization of intrinsic tumorigenesis control technique

[0114] It was confirmed whether clone 2 or 10 in FIG. 21 could control tumorigenesis. For this purpose, long-term (about 14 days) culture was performed after 6-wells in which cloned cell lines were aliquoted were treated with doxycycline.

[0115] As a result, it was confirmed that after 2 weeks of culture with doxycycline treatment (1.25 $\mu\text{g/ml}$), most of the cells lost their viability (1.25 $\mu\text{g/ml}$) (FIG. 23). Accordingly, even for uninfected MSCs other than by lysis caused by infection with an oncolytic virus, the used cell lines infected and selected by the E1B55K-expressing retrovirus eventually leads to cell death, thereby fundamentally ruling out the possibility of tumorigenesis.

Example 3: Establishment of innovative increase in tumor targeting - Discovery of tumor homing improvement factor

[0116] By inducing the additional expression of GRP78 in human adipose MSC-TERT, the expression of tumor tropism-related markers was confirmed.

[0117] It was confirmed that the expression of all factors acting at each stage of MSC homing is increased by the increase in GRP78 (FIG. 24A).

[0118] In order to confirm that GRP78 was certainly expressed, an increase in mRNA expression of GRP78 was confirmed by transfecting human MSC-TERT with pcDNA3.1-GRP78 (2 μg) (FIG. 24B).

1) GRP78 cloning into pcDNA3.1 hygro (+)

[0119] RNA was extracted from SNU449, a liver cancer cell line with a high expression level of GRP78, with Trizol. The extracted RNA was reverse transcribed using SuperScript™ First-Strand Synthesis System for RT-PCR kit from Invitrogen. The primers used to perform PCR were then sequenced by adding an XhoI site in the forward direction and an XbaI site in the reverse direction. PCR (initial denaturing: 95°C for 2 minutes; denaturing: 95°C for 40 seconds; annealing: 58°C for 40 seconds; extension: 72°C for 2 minutes and 30 seconds; 30 cycles) cloning was performed using Forward (5'-GATTCTCGAGATGAAGCTCTCCCTGG-3': SEQ ID NO: 16) and Reverse (5'-GGCCTCTAGACTACAACATCATCTTTT-3': SEQ ID NO: 17) primers. After PCR and digestion with XhoI/XbaI, the backbone pcDNA3.1-hygro vector was also digested with XhoI/XbaI and ligated. Colonies obtained after transformation into bacteria were cultured. Each of the plasmids thus obtained was digested simultaneously with XhoI/XbaI to confirm a size-matched insertion only in plasmid 5 by confirming insertion (FIG. 25). After transfection of a liver cancer cell line SNU449 with No. 5 pcDNA3.1-GRP78, an increase in protein expression of GRP78 was confirmed by western blotting (FIG. 26). Also, sequence analysis of the sample 5 plasmid showed 100% homology with NCBINM_005347 (FIG. 27).

2) GRP78 cloning into PLNCX neo

[0120] The following procedure was performed to introduce the GRP78 gene into MSC-TERT or MSC-TERT-tetonE1B55K.

[0121] In order to introduce GRP78 into a retroviral vector pLNCXneo, a pcDNA3.1-GRP78 vector was digested with XhoI/PmeI to extract a GRP78 site, and the GRP78 site was ligated and inserted into PLNCXneo previously digested with XhoI/PmeI.

[0122] pLNCX neo was originally constructed by increasing the restriction enzyme recognition site from HindIII-HpaI-ClaI, which is a cloning site in existing LNCX, to HindIII-PmlI-BstXI-NotI-XhoI-Sall-ApaI-PmeI-HpaI-ClaI (FIG. 28).

[0123] In this case, a strand sequence is as follows.

Top

5'-

AGCTTCACGTGCCAGCACAGTGGCGGCCGCTCGAGTCGACGGGCCCGTTT

AAACGTTAACAT-3', (SEQ ID NO: 18)

Bottom

5'-

CGATGTTAACGTTTAAACGGGCCCGTCGACTCGAGCGGCCCGCCACTGTGC

TGGCACGTGA-3 (SEQ ID NO: 19).

[0124] To confirm whether LNCXneo-GRP78 was inserted, pLNCXneo (control) and pcDNA3.1-GRP78 (GRP78 gene-providing plasmid) as controls were digested with HindIII/PmeI, respectively, and the GRP78 fragments derived from linearized pLNCXneo and pcDNA3.1-GRP78 were ligated and transformed into DH5 α competent cells, and then it was confirmed that pLNCXneo-GRP78 candidate constructs (Samples 1 to 24) were cleaved at the same time and inserted into Samples 2, 5, 9, 11, 12, 20 and 21 by obtaining plasmids (FIG. 29).

3) Construction of MSC-TERT-GRP78 cell line

[0125] Platinum-A packaging cells (Cell Biolabs) were transfected with a retroviral vector pLNCXneo-GRP78, and after 48 hours, the medium was filtered. Cell clones resistant to G418 (500 μ g to 600 μ g/ml) were obtained by infecting MSC-TERT with a medium solution containing the retrovirus obtained by filtration, and then screened to select clones 2 and 4 clearly expressing GRP78 and MMP2 (FIG. 30).

4) Tumor targeting of MSCs with gene transfer: confirmation of conditions to specifically reach only the tumor site within very short period of time

① Verification of tumor targeting of MSC-TERT-GRP78

[0126] 2×10^6 SNU398 cells (a liver cancer cell line) were subcutaneously transplanted into the shoulder regions of 6-week-old BALB/c thymic nude mice, and 7 days later, 1×10^6 MSC-TERT-GRP78 cells infected with a replication-incompetent adenovirus expressing firefly luciferase were injected into the tail veins. 150 mg of D-luciferin per kg of mouse was injected intraperitoneally into the mice in order to use an *in vivo* imaging system (IVIS) device that recognizes luminescence. The biodistribution of MSC-TERT-GRP78 infected with an adenovirus expressing luciferase *in vivo* was observed 6 hours after injection into the tail vein by imaging using an IVIS Spectrum System (PerkinElmer). A feature of this experiment is that MSC-TERT-GRP78 with GRP78 gene transfer is allowed to form tumors in the shoulder to distinguish them from internal organs such as the liver and lungs in order to accurately distinguish how much tumor migration ability the MSC-TERT-GRP78 has, clearly confirming that the arrival sites of MSC-TERT-GRP78 gathered at tumor tissue sites within a very short time without adsorption to other organs (FIG. 31).

(2) Verification of tumor targeting of gene-transferred final type MSC-TERT-tetoneE 1B55K-GRP78

[0127] 8×10^6 A549 cells (a lung cancer cell line), 2×10^6 SNU398 cells (a liver cancer cell line) or 2×10^6 MiaPaCa-2 cells (a pancreatic cancer cell line) were subcutaneously transplanted into the shoulder regions of 6-week-old BALB/c thymic nude mice, and 7 days later, 1×10^6 MSC-TERT-tetoneE1B55K-GRP78 cells infected with a replication-incompetent adenovirus expressing firefly luciferase were injected into the tail veins. 150 mg of D-luciferin per kg of mouse was injected intraperitoneally into the mice in order to use an IVIS device that recognizes luminescence. The biodistribution of MSCs infected with an adenovirus expressing luciferase *in vivo* was observed 6 hours or 24 hours later using the IVIS Spectrum System (PerkinElmer).

[0128] As a result, similarly to the previous targeting experiment ①, tumors were formed on the shoulder side in order to distinguish them from internal organs such as the liver and lungs, thereby clearly confirming that the arrival sites of MSC gathered at tumor tissue sites within a very short time without adsorption to other organs (FIG. 32). As can be seen here, it can be observed that a final type of MSC-TERT-tetoneE1B55K-GRP78 including GRP78 migrated to the tumor site within a very short time, as compared to MSCs without GRP78, regardless of cancer type, and it can be confirmed that there is a certain intensity difference when compared to the luciferase activity intensity values (minimally 10-fold up to maximally 100-fold depending on the luminescence intensity in tumors). This shows that tumor targeting occurs very quickly and accurately. Here, E1B55K is not expressed because the diet does not include doxycycline.

[0129] Such surprisingly rapid and accurate trafficking of luciferase-expressing MSC-GRP78 to tumors and targeting to various tumor types greatly enhances the possibility of utilizing MSC-GRP78 for tumor diagnosis. In a primary mouse model, solid cancer was artificially transplanted subcutaneously, and the tumor site was confirmed by allowing MSC-GRP78 to migrate, but when there is a tumor in the same manner as in the primary mouse model, even in a metastatic

model present in the body, MSC-GRP78 can be delivered to the tumor site to emit light, or tumors can be detected by labeling the tumors with a higher resolution isotope. This can be applied to tumor diagnosis, and it will be possible to construct an OV/MSK complex simultaneously capable of tumor diagnosis and treatment in the long term.

5 ③ Confirmation of infiltration of gene-transferred final type MSC-TERT-tetoneE1B55K-GRP78 into tumors

[0130] It was confirmed that the final type of MSC-TERT-tetoneE1B55K-GRP78 reached tumor sites, and the following experiment was performed to confirm whether the MSCs that actually reached the tumor sites infiltrated into tumor tissue.

10 [0131] First, A549 cells were transplanted into the subcutaneous tissue of mice to form tumors. Then, the final type of MSC cells (MSC-TERT-tetoneE1B55K-GRP78) was labeled with a fluorescent cell tracker probe (Invitrogen, C34565) and then injected into the mouse tail vein. At 24 hours, 48 hours and 72 hours after the injection, the tumor tissue was removed and tissue-sectioned, and then the fluorescent site was confirmed under a fluorescence microscope. As shown in FIG. 33, it could be confirmed that an excessively large number of MSCs with GRP78 were present in the tumor. In the case of MSCs lacking GRP78, it was observed that the MSCs remained predominantly at the tumor interface with low fluorescence intensity.

④ Confirmation of expression of adenovirus-related proteins in tumor tissue

20 [0132] Viruses were released from MSC-TERT-tetoneE1B55K-GRP78 that had infiltrated into tumor tissue to infect tumor cells, and the following experiments were performed to confirm whether the virus was replicated and maintained for a certain period of time after virus production.

25 [0133] 8×10^6 A549 cells (a lung cancer cell line) were subcutaneously transplanted into the flank regions of 6-week-old BALB/c thymic nude mice, and 1×10^6 MSC-TERT-tetoneE1B55K-GRP78 cells infected with an oncolytic adenovirus expressing shHSP27-shTGF β were injected into the tail veins of each of every 2 mice whose tumor size reached an average of 150 mm³. Then, 3 days later, 1×10^6 MSC-TERT-tetoneE1B55K-GRP78 cells infected with an oncolytic adenovirus expressing shHSP27-shTGF β 1 were additionally injected. Then, 7 days after the second injection, tumors were removed from the mice in each group, and tumor-sectioned samples were reacted with an adenovirus type 5-specific antibody (Abcam, Cambridge, UK) to perform a DAB coloring reaction with a secondary antibody, and control staining for immunohistochemistry was performed with hematoxylin nuclear stain. In this case, a diet containing doxycycline (625 mg/kg) as feed was fed to all groups.

30 [0134] As a result, adenovirus-specific proteins were confirmed in most confirmed areas (FIG. 34). An adenovirus was released from MSCs in the tumor tissue until at least 7 days after tail vein injection, indicating that replication, proliferation and spreading occurred repeatedly and confirming the possibility that the virus remained until around 20 days, when the size of the tumor had decreased to a level of almost zero.

35 5) Establishment of human adipose MSC-TERT-tetoneE1B55K-GRP78 cell line - Confirmation of E1B55K expression after 1.25 μ g/mL doxycycline treatment for 48 hours

40 [0135] After infection with a retroviral soup obtained by co-transfecting GP2-293 packaging cells with pRetroX-TetOne-E1B55K and a pAmpho vector encoding a membrane protein, puromycin selection (0.8 μ g/ml to 1.0 μ g/ml) was performed, and after infection with a retroviral soup obtained by transfecting platinum-A packaging cells again with a retroviral vector pLNCXneo-GRP78, G418 selection (500 μ g/ml to 600 μ g/ml) was performed, and final clones with clear E1B55K and MMP2 protein expression were selected in the presence of doxycycline (1.25 μ g/ml) (FIG. 35, final clones 21, 24, 25, and 26).

45 [0136] In final clone 21, it is shown that the E1B55K induction concentration by doxycycline was effectively and sufficiently induced without any leakage even at a very low concentration, less than 1/10 of the 1.25 μ g/ml used (FIG. 36). This suggests that it can be reliably turned on/off *in vivo* and thus viral production can be induced at desired time points because it is not greatly affected by the treatment amount of doxycycline, which is an expression inducer *in vivo*, and responds very sensitively to the presence or absence of doxycycline.

50 [0137] For the ability of human adipose MSC-TERT-tetoneE1B55K-GRP78 (Clone 21) to produce viruses, after infection with an oncolytic adenovirus expressing shHSP27/shTGF β 1, the increased rate of virus production was confirmed after 48 hours in MSCs during treatment with doxycycline (1.25 μ g/ml), and in this case, the difference in virus production according to the expression of the E4orf6 gene was also confirmed. For this experiment, Clone 21 MSCs were transfected with 1 μ g of pFlag-CMV2-E4orf6 in which the E4orf6 gene was subcloned or 1 μ g of pFlag-CMV2 as a control, and after additional infection with an oncolytic adenovirus expressing shHSP27/shTGF β 1, the cells were treated with doxycycline (1.25 μ g/ml for 48 hours), and then the total amount of virus in the harvested soup and remaining cells was determined by titration. As a result, in final clone 21, a nearly 10-fold increase in virus production upon treatment with doxycycline was confirmed, and differences in virus production were observed depending on the presence or absence of transfection

with the E4orf6 gene (FIG. 37). That is, the amount of virus production was decreased according to the E4orf6 external expression induction. This seems that an increase in virus production by the action of E1B55K alone acts as a virus production inhibitory factor caused by complex formation by E4ORF6. (It was confirmed that induction of cell death process of MSCs by E1B55K alone contributes to virus production. The E1B55K/E4orf6 complex interferes with the cell death of MSCs, resulting in missing the optimal time for virus production.)

6) Comparison of efficacy maintenance in human adipose MSC-TERT low passage and high passage

[0138] It is essential to confirm that MSC efficacy is maintained during mass production of 200 vials or more of master cell bank cell lines. That is, it was confirmed, by western blotting, from samples in which each cell acquired at low passage (p13) and high passage (p23) was lysed whether biomarkers known as markers related to tumor homing, which is an MSC characteristic caused by differences in passage of final MSC-TERT-tetoneE1B55K-GRP78, were maintained.

[0139] As a result, it was confirmed that even when the passage was increased to 20, there was no difference in the expression of proteins known as a tumor homing marker (FIG. 38).

Example 4: Anticancer efficacy of MSC-TERT-tetoneE1B55K-GRP78 infected with oncolytic adenovirus

[0140] 8×10^6 A549 cells (a lung cancer cell line) were subcutaneously transplanted into the flanks of 6-weeks-old BALB/c thymic nude mice, and mice with tumors reaching an average size of 150 mm^3 were divided into groups of 10 mice each. 1×10^6 MSC-TERT-tetoneE1B55K-GRP78 cells infected with an oncolytic adenovirus expressing shHSP27-shTGF β 1 were injected into the mouse tail veins. Then, 3 days later, 1×10^6 MSC-TERT-tetoneE1B55K-GRP78 cells infected with an oncolytic adenovirus expressing shHSP27-shTGF β 1 were additionally injected intravenously. As a control, a group transplanted with tumor cells alone (no treat) and a group injected with the final MSC-TERT-tetoneE1B55K-GRP78 (MSC only) were used. In this case, a diet containing doxycycline (625 mg/kg) as feed was fed to all groups.

[0141] As a result, it was observed that when an antitumor effect obtained by direct intratumoral injection of the oncolytic virus (Ad-3484-shHSP27-shTGF β 1) (a state in which the growth rate of the tumor size was delayed compared to the control, but the tumor size itself decreased and thus did not regress) was compared, the tumor size was significantly reduced and the tumor almost disappeared within a few days when an oncolytic virus (Ad-3484-shHSP27-shTGF β 1) in the final MSC-TERT-tetoneE1B55K-GRP78 was injected into the tail veins compared to untreated tumors or the control in which the final type of MSC alone was injected into the tail vein as observed in FIG. 39, and it could be seen that for 20 days after injection, the tumors almost disappeared, and then tumors were again grew slightly in several individuals. Through individual photographs of mice in each group and photographs of tumors taken for each group after removal of tumors one month after the first injection of OV/MSC, the antitumor effect of the oncolytic virus (Ad-3484-shHSP27-shTGF β 1) was confirmed more reliably in the final MSC-TERT-tetoneE1B55K-GRP78 (FIG. 40).

Example 5: Confirmation of adsorption to organs other than tumors

[0142] After it was confirmed that tumor targeting occurs very efficiently, the copy number of adenoviral genes was measured for each time period for the amount of adenovirus in each major organ including tumors in order to confirm the amount captured in major organs other than tumors (lungs, liver, spleen, and the like), the migration to the tumor site, the elapsed time, and the like in more detail. The viral genomic plasmid DNA was defined as standard known DNA, and confirmed by drawing a standard curve (in the case of the viral DNA of the currently used oncolytic adenovirus, 1 copy is 10^3 pg).

[0143] For this purpose, after infection with a replication-incompetent defective E1B55K virus, a single dose of MSC-TERT-GRP78 (1×10^6) infected with the YSC-02 oncolytic virus of Reference Example 1 was administered IV through the tail once, and samples were collected by date (day 0, 1, 2, 3, 4, 5, 10, and 15). The collected tissues (tumors, lungs, liver, spleen, kidneys, heart) were immediately stored in liquid nitrogen, and on the day when tissue sampling was completed, genomic DNA was extracted from each tissue under the same conditions. To confirm whether the gene was expressed using the extracted genomic DNA, real-time PCR was performed using primers for the E4ORF gene. The primers for screening E4ORF used at this time are as follows.

Forward 5'-CGTGGTCAAACCTCTACAGCC-3': SEQ ID NO: 20

Reverse 5'-GCATGAGCATGACTACGATG-3': SEQ ID NO: 21

[0144] As can be seen in FIG. 41, although the variation between individuals is severe, it can be seen that the range in individual tissues when the oncolytic virus enters the tumor tissue is 10^4 to 10^{10} copies, and almost all viruses are adsorbed and absorbed in tumor tissue compared to several to 10 copies found in all other organs.

Claims

1. Mesenchymal stem cells for delivering an oncolytic virus, having improved tumor-targeting ability, into which a glucose regulated protein 78 (GRP78) gene is introduced.
2. Mesenchymal stem cells for delivering an oncolytic virus into which an E1B55K gene is introduced such that the expression thereof is induced by an expression inducer.
3. Mesenchymal stem cells for delivering an oncolytic virus, comprising a glucose regulated protein 78 (GRP78) gene and an E1B55K gene, wherein the E1B55K gene is expressed by an expression inducer.
4. The mesenchymal stem cells of any one of claims 1 to 3, wherein a telomerase reverse transcriptase (TERT) gene is additionally introduced.
5. The mesenchymal stem cells of any one of claims 1 to 3, wherein the oncolytic virus is an oncolytic adenovirus, an oncolytic adeno-associated virus (AAV), an oncolytic retrovirus, an oncolytic lentivirus, an oncolytic herpes simplex virus or an oncolytic vaccinia virus.
6. The mesenchymal stem cells of any one of claims 1 to 3, wherein the oncolytic virus is an oncolytic adenovirus.
7. The mesenchymal stem cells of claim 2 or 3, wherein the E1B55K gene is expressed after the mesenchymal stem cells are introduced into the body.
8. The mesenchymal stem cells of claim 2 or 3, wherein the expression inducer is doxycycline or tetracycline.
9. The mesenchymal stem cells of any one of claims 1 to 3, wherein the mesenchymal stem cells are human-derived.
10. The mesenchymal stem cells of any one of claims 1 to 3, wherein the mesenchymal stem cells are derived from cord blood, bone marrow or fat.
11. A composition for delivering an anticancer gene, comprising the mesenchymal stem cells and the oncolytic virus of any one of claims 1 to 3.
12. The composition of claim 11, wherein the cancer is gastric cancer, lung cancer, breast cancer, ovarian cancer, liver cancer, bronchial cancer, nasopharyngeal cancer, laryngeal cancer, pancreatic cancer, biliary tract cancer, bladder cancer, colorectal cancer, colon cancer, cervical cancer, brain cancer, prostate cancer, bone cancer, head and neck cancer, skin cancer, kidney cancer, polyploid carcinoma, thyroid cancer, parathyroid cancer or ureteral cancer.
13. An anticancer pharmaceutical composition comprising the mesenchymal stem cells and the oncolytic virus of any one of claims 1 to 3.
14. The pharmaceutical composition of claim 13, wherein the cancer is gastric cancer, lung cancer, breast cancer, ovarian cancer, liver cancer, bronchial cancer, nasopharyngeal cancer, laryngeal cancer, pancreatic cancer, biliary tract cancer, bladder cancer, colorectal cancer, colon cancer, cervical cancer, brain cancer, prostate cancer, bone cancer, head and neck cancer, skin cancer, kidney cancer, polyploid carcinoma, thyroid cancer, parathyroid cancer or ureteral cancer.
15. A composition for diagnosing cancer, comprising the mesenchymal stem cells and the oncolytic virus of any one of claims 1 to 3.
16. The composition of claim 15, wherein the cancer is gastric cancer, lung cancer, breast cancer, ovarian cancer, liver cancer, bronchial cancer, nasopharyngeal cancer, laryngeal cancer, pancreatic cancer, biliary tract cancer, bladder cancer, colorectal cancer, colon cancer, cervical cancer, brain cancer, prostate cancer, bone cancer, head and neck cancer, skin cancer, kidney cancer, polyploid carcinoma, thyroid cancer, parathyroid cancer or ureteral cancer.
17. A method for treating cancer, the method comprising administering, to a subject, a therapeutically effective amount of a pharmaceutical composition comprising the mesenchymal stem cells and the oncolytic virus of any one of claims 1 to 3.

18. The method of claim 17, wherein the cancer is gastric cancer, lung cancer, breast cancer, ovarian cancer, liver cancer, bronchial cancer, nasopharyngeal cancer, laryngeal cancer, pancreatic cancer, biliary tract cancer, bladder cancer, colorectal cancer, colon cancer, cervical cancer, brain cancer, prostate cancer, bone cancer, head and neck cancer, skin cancer, kidney cancer, polyploid carcinoma, thyroid cancer, parathyroid cancer or ureteral cancer.

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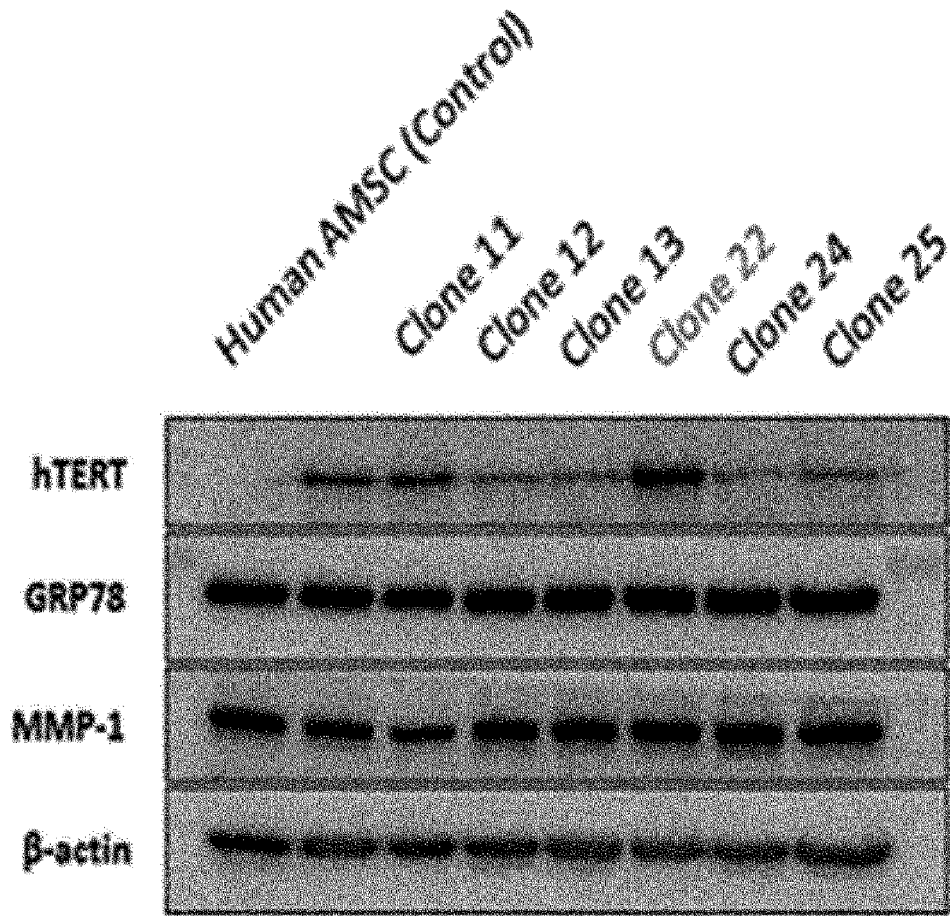
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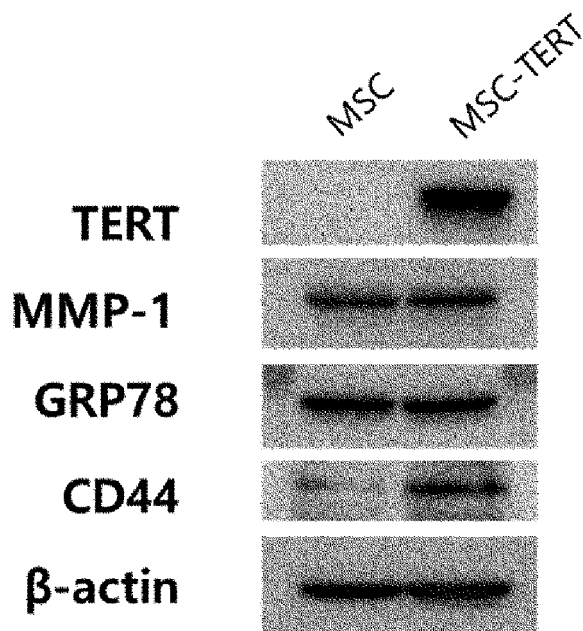
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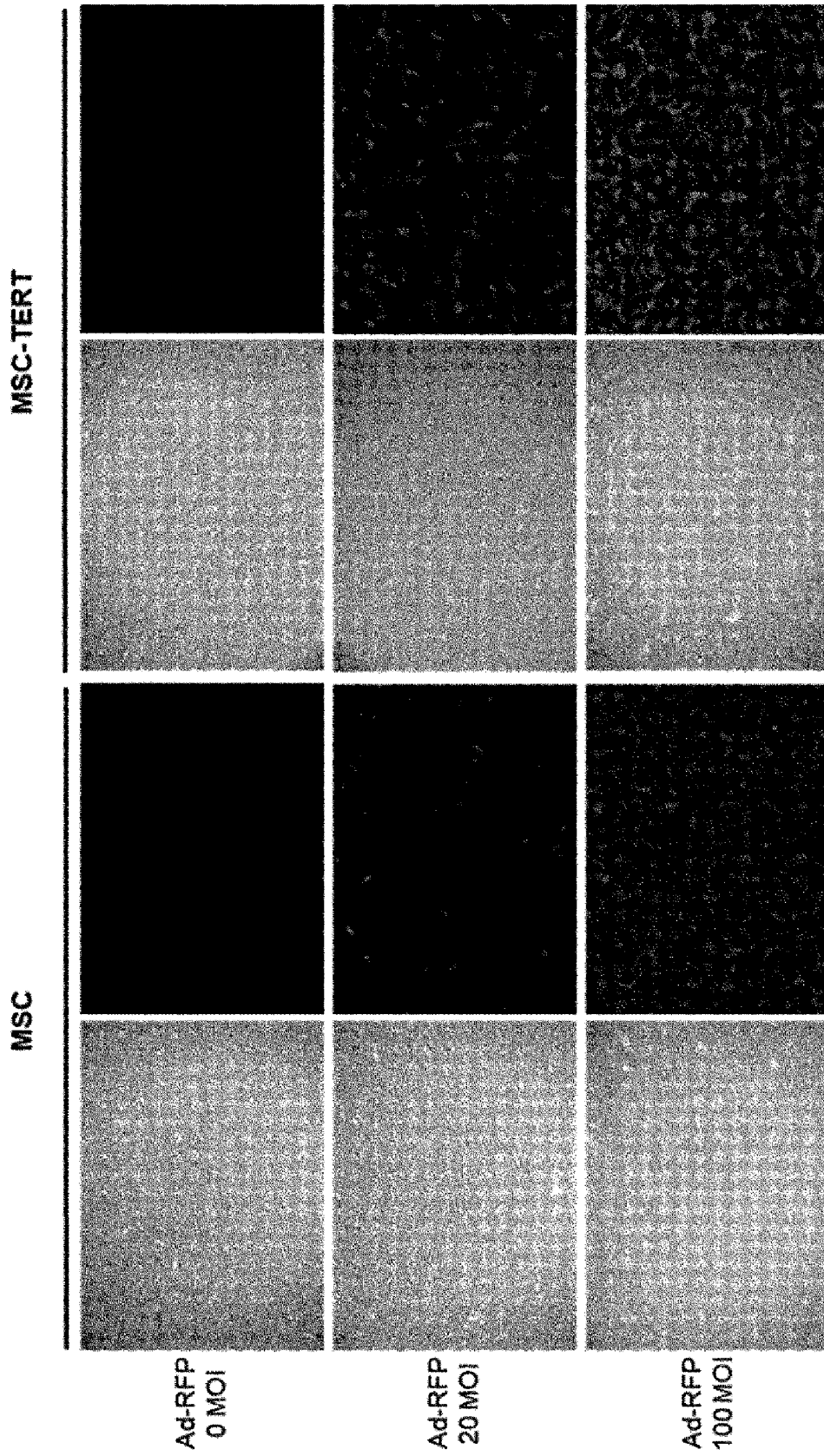
【FIG.1】



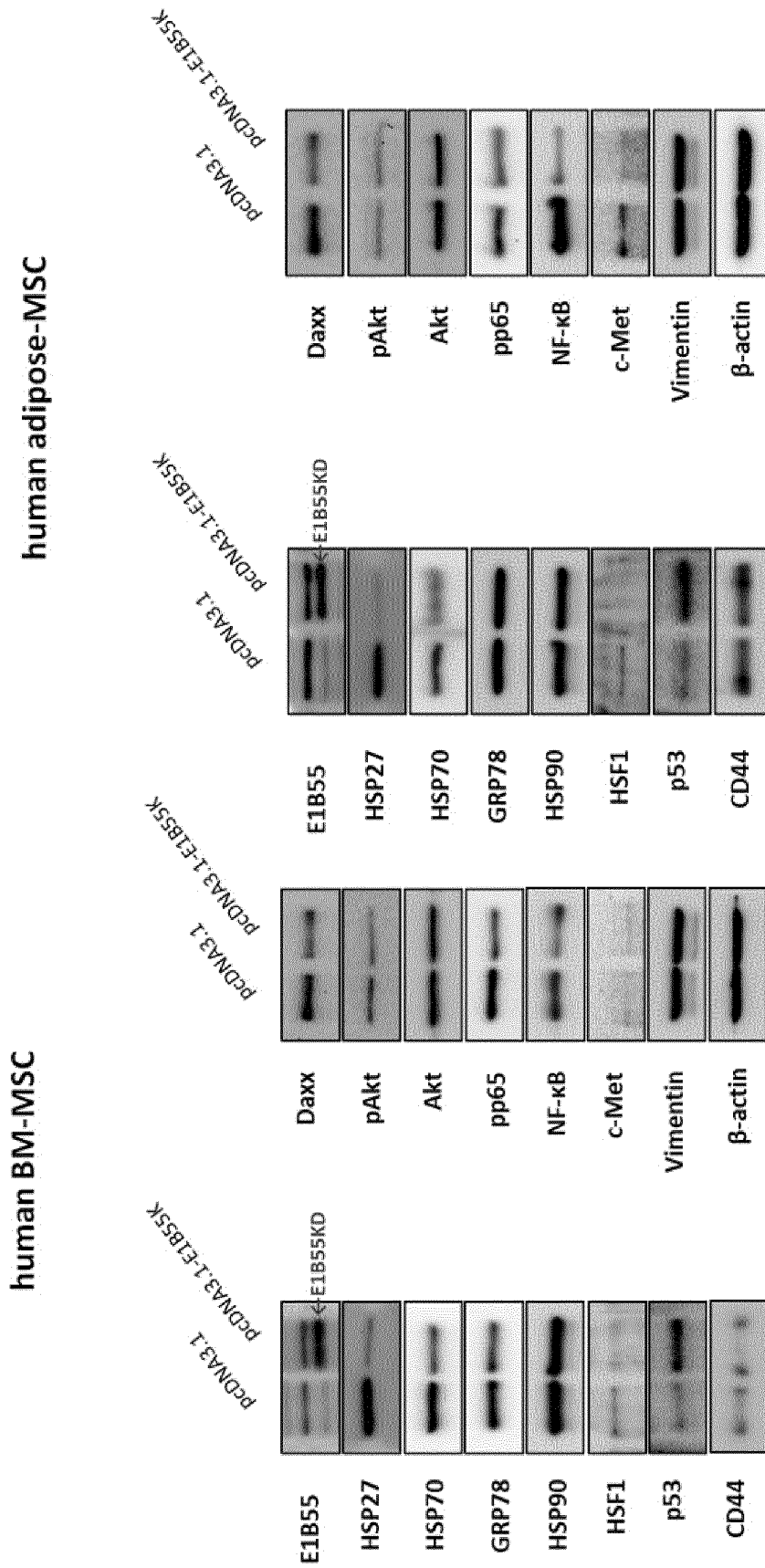
【FIG.2】



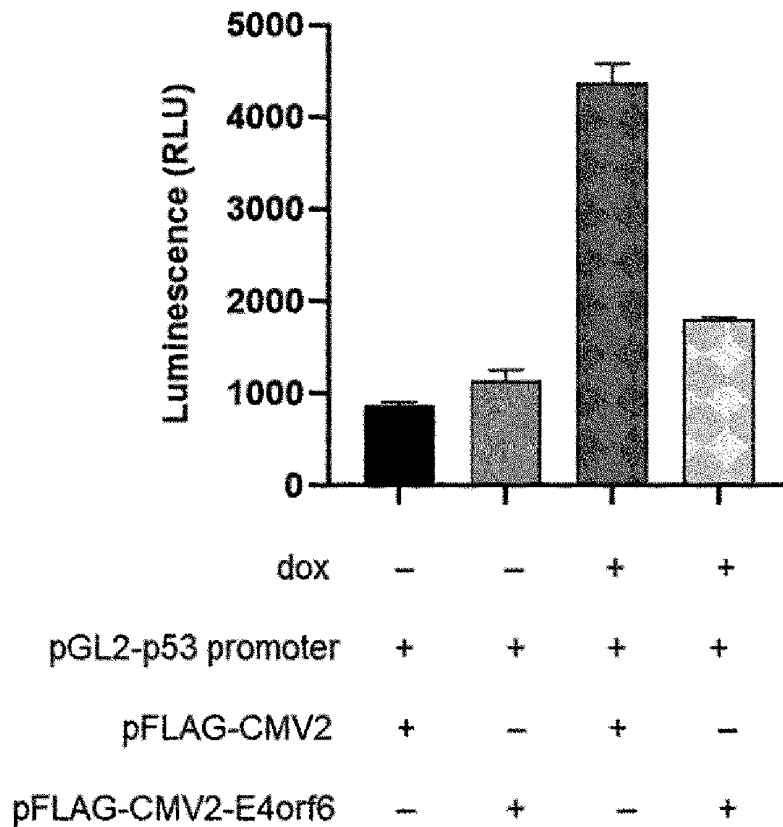
【FIG.3】



[FIG. 4]

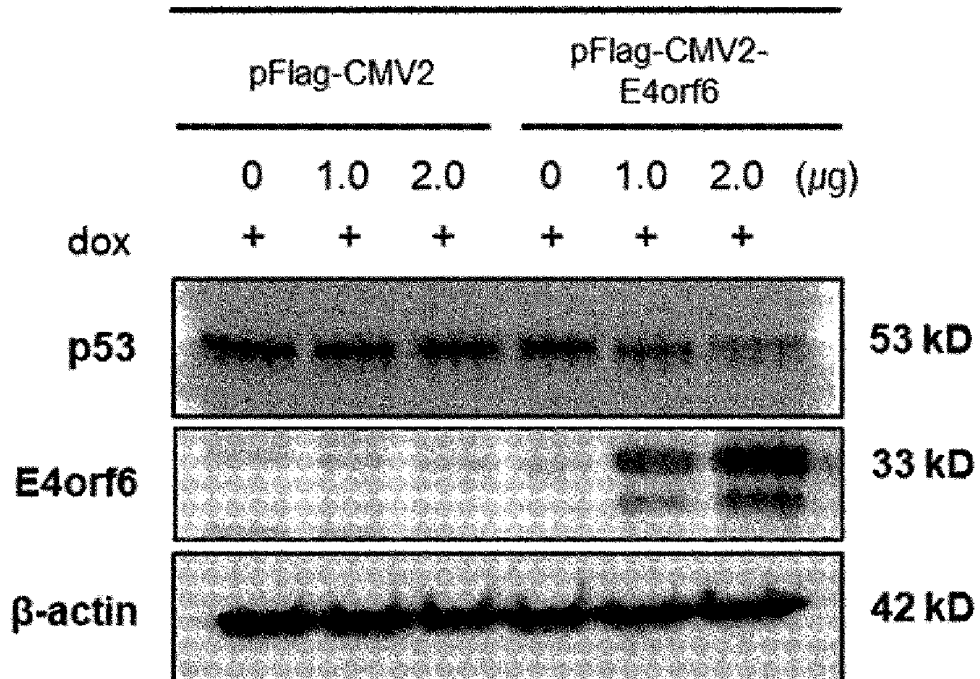


【FIG.5】



【FIG.6】

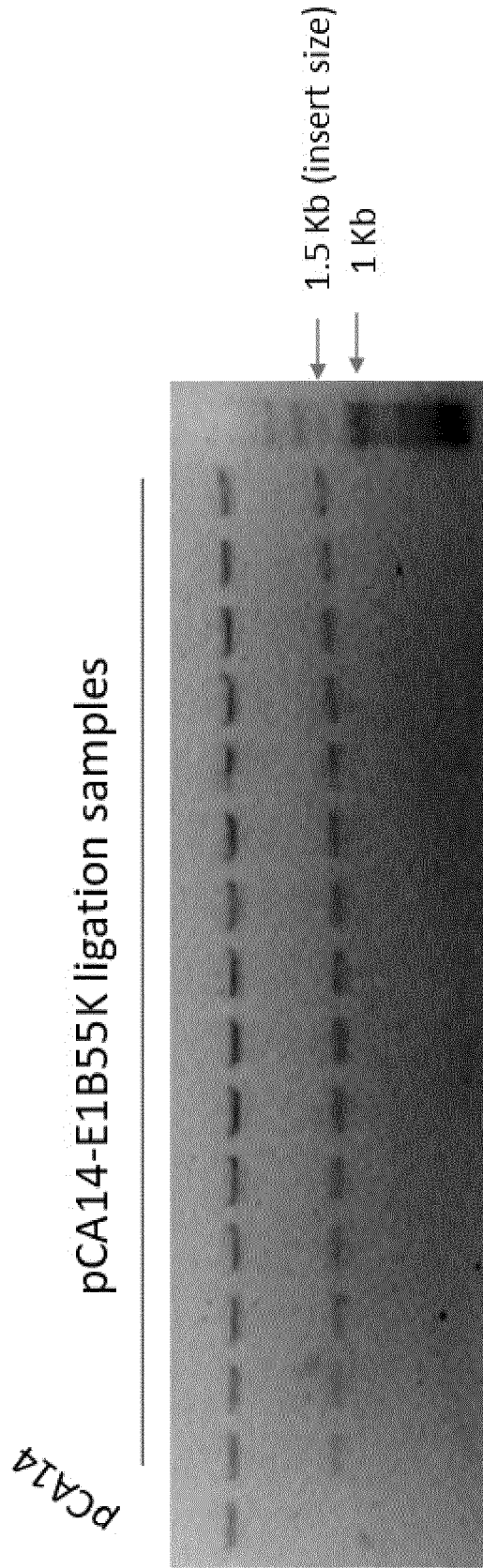
MSC-TERT-tetoneE1B55K



[FIG. 7]



[FIG.8]



[FIG.9b]

E1B55K reverse

```

E1B55K          tttactggccccaatttttagogggtacgggttttcctggccaataccaaccttatcctacac
pCA14-E1B55K   TTTACTGGCCCCAATTTTAGCGGTACGGTTTTTCCTGGCCAATACCAACCTTATCCTACAC
*****

E1B55K          ggtgtaagcttctatgggtttaacaatacctgtgtggaagcctggaccgatgtaagggtt
pCA14-E1B55K   GGTGTTAGCTTCTATGGGTTTAAACAATACCTGTGTGGAGCCTGGACCGATGTAAGGGTT
*****

E1B55K          cggggctgtgccttttactgctgctggaaggggggtggtgtgtcgcccaaaagcagggtt
pCA14-E1B55K   CGGGCTGTGCCITTTACTGCTGCTGGAAGGGGGTGGTGTGTGCCCCAAAAGCAGGGCT
*****

E1B55K          tcaattaagaaatgectctttgaaaggtgtaccttgggtatcctgtctgagggttaactcc
pCA14-E1B55K   TCAATTAAGAAATGECTCTTTGAAAGGTGTACCTTGGGTATCCTGTCTGAGGGTAACTCC
*****

E1B55K          aggtgcgccacaatgtggcctccgactgtggttgcttcatgctagtgaagcgtggct
pCA14-E1B55K   AGGTGCGCCACAATGTGGCCTCCGACTGTGGTTGCTTCATGCTAGTGAAGCGTGGCT
*****

E1B55K          gtgattaagcataacatggatgtggcaactgagaggacagggcctctcagatgctgacc
pCA14-E1B55K   GTGATTAAGCATAACATGGTATGTGGCAACTGAGAGGACAGGGCCTCTCAGATGCTGACC
*****

E1B55K          tgctcggacggcaactgtcacctgctgaagaccattcacgtagccagccactctcgcaag
pCA14-E1B55K   TGCTCGGACGGCAACTGTCACCTGCTGAAGACCATTACGTTAGCCAGCCACTCTCGCAAG
*****

E1B55K          gcctggccagtgtttgagcataacatactgacccgctgttccttgcatgtggtaacagg
pCA14-E1B55K   GCCTGGCCAGTGTITGAGCATAACATACTGACCCGCTGTTCCTTGCAATTTGGGTAACAGG
*****

E1B55K          aggggggtgttcctaccttaccaatgcaatttgagtcacactaagatattgcttgagccc
pCA14-E1B55K   AGGGGGGTGTTCCCTACCTTACCAATGCAATTTGAGTCACACTAAGATATTGCTTGAGCCC
*****

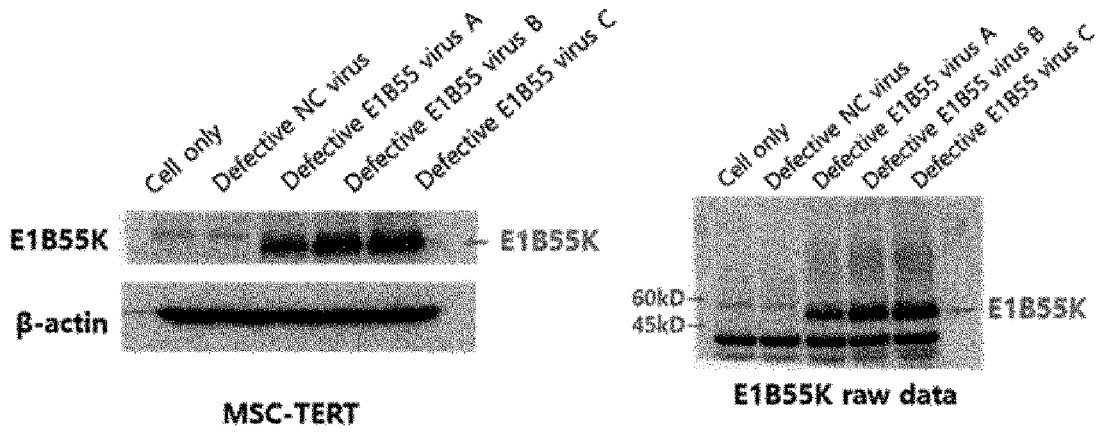
E1B55K          gagagcatgtccaaggtgaacctgaacgggggtgtttgacatgaccatgaagatctggaag
pCA14-E1B55K   GAGAGCATGTCCAAGGTGAACCTGAACGGGGTGTTTGACATGACCATGAAGATCTGGAAG
*****

E1B55K          gtgctgaggtacgatgagaccgcaaccaggtgcagaccctgcgagtggtggcggtaaacat
pCA14-E1B55K   GTGCTGAGGTACGATGAGACCCGCACCAGGTGCAGACCCTGCAGTGTGGCGGTAACAT
*****

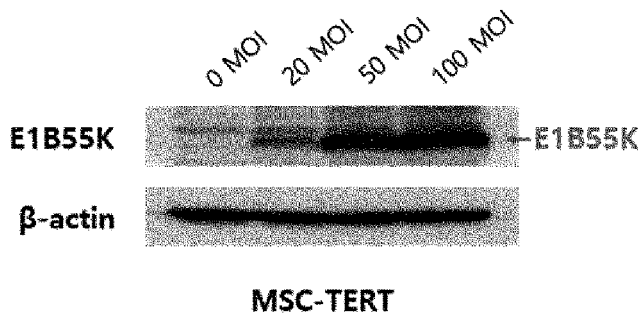
E1B55K          attaggaaccagcctgtgatgctggatgtgaccgaggagctgaggcccgatcacttgggt
pCA14-E1B55K   ATTAGGAACCAGCCTGTGATGCTGGATGTGACCGAGGAGCTGAGGCCCGATCACTTGGGT
*****

E1B55K          ctggcctgcacccgcgctgagtttggctctagcgatgaagatacagattga-----
pCA14-E1B55K   CTGGCCTGCACCCGCCTGAGTTTGGCTCTAGCGATGAAGATACAGATTGAAAGCTTGTC
*****
    
```

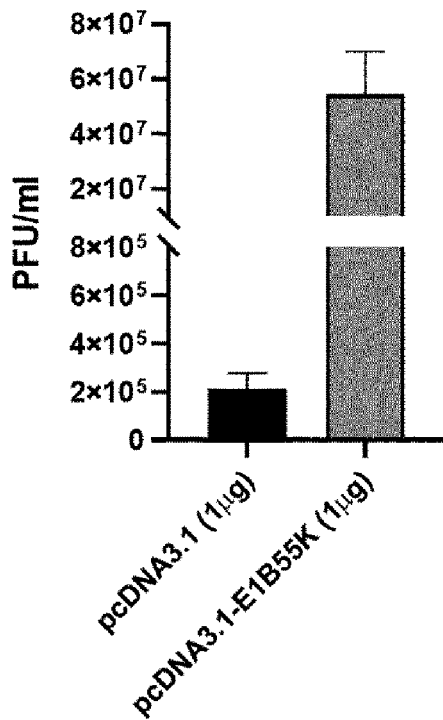
【FIG.10a】



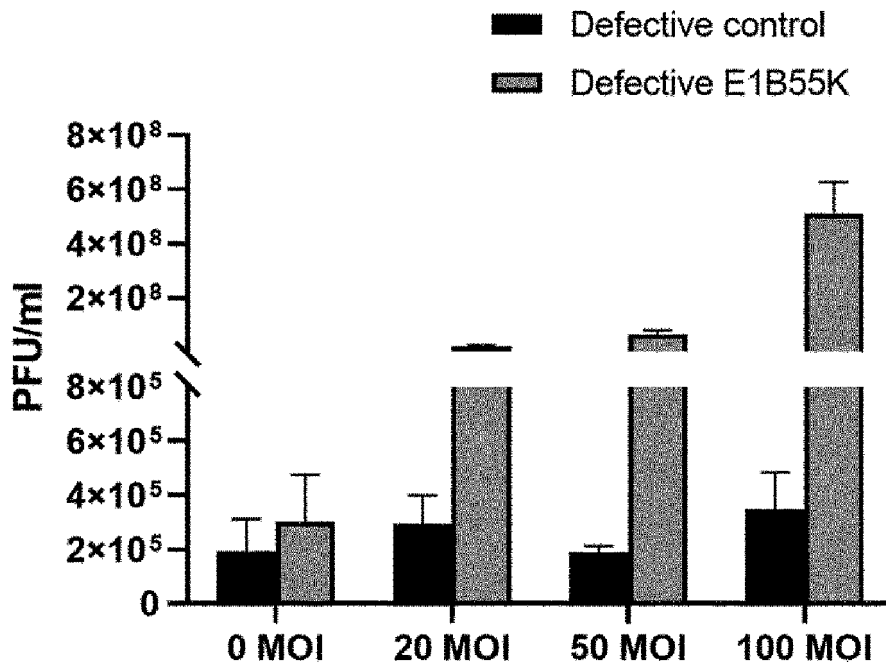
【FIG.10b】



【FIG.11】



【FIG.12】

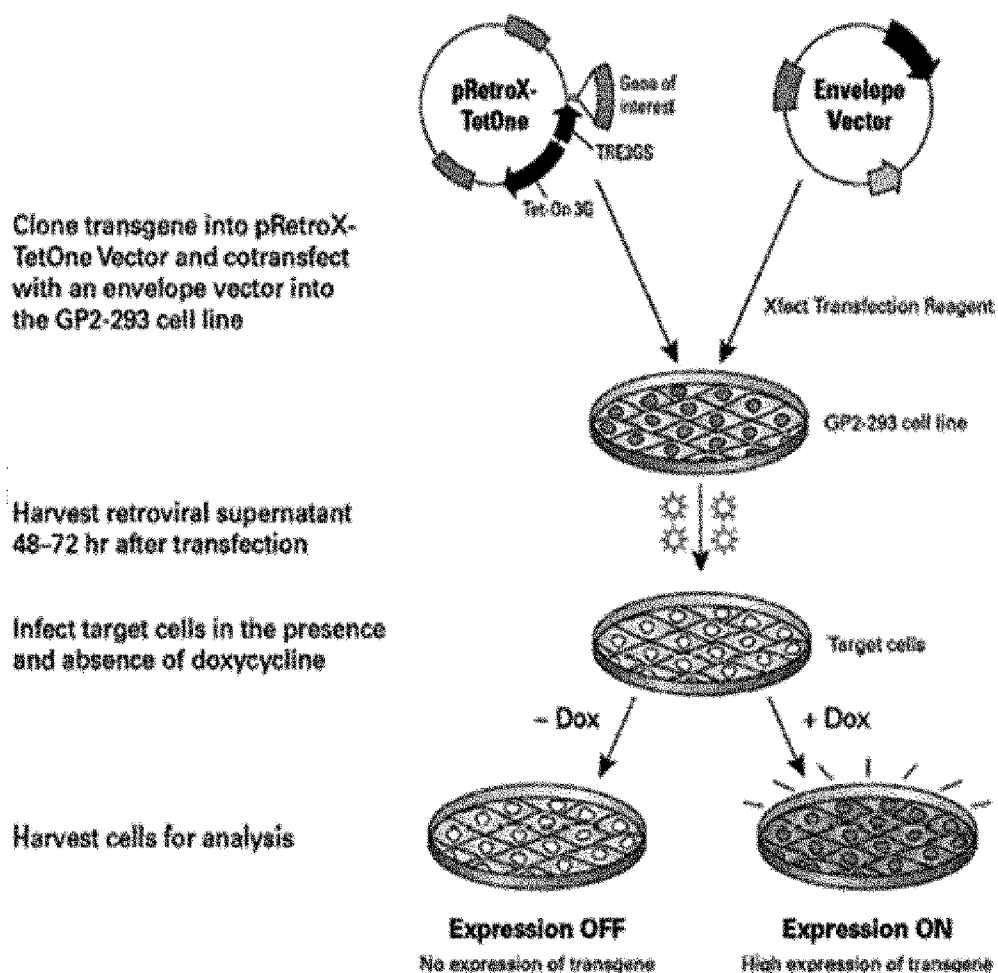


【FIG.13】

Protocol Summary

The following are the steps required to create a doxycycline-inducible expression system using retrovirus (see Figure 2).

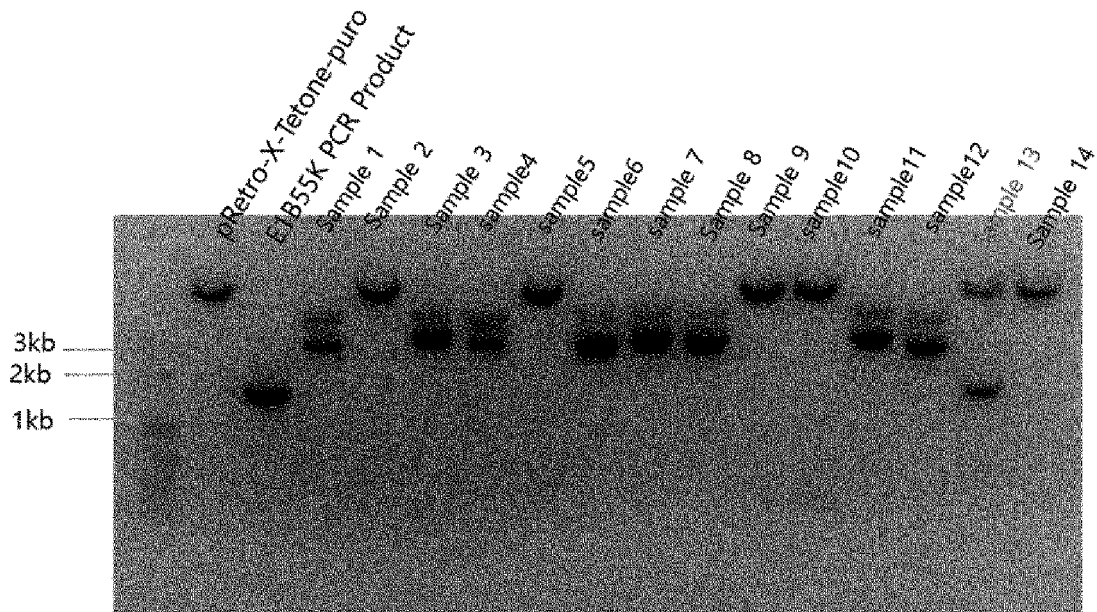
1. Clone your gene of interest into the pRetroX-TetOne Vector using In-Fusion HD (Section V).
2. Pilot test Tet-based induction of your construct using transient transfection (Section VI).
3. Produce retroviral supernatants using the Retro-X Universal Packaging System (Section VII).
4. Transduce your target cells with TetOne virus (Section IX).



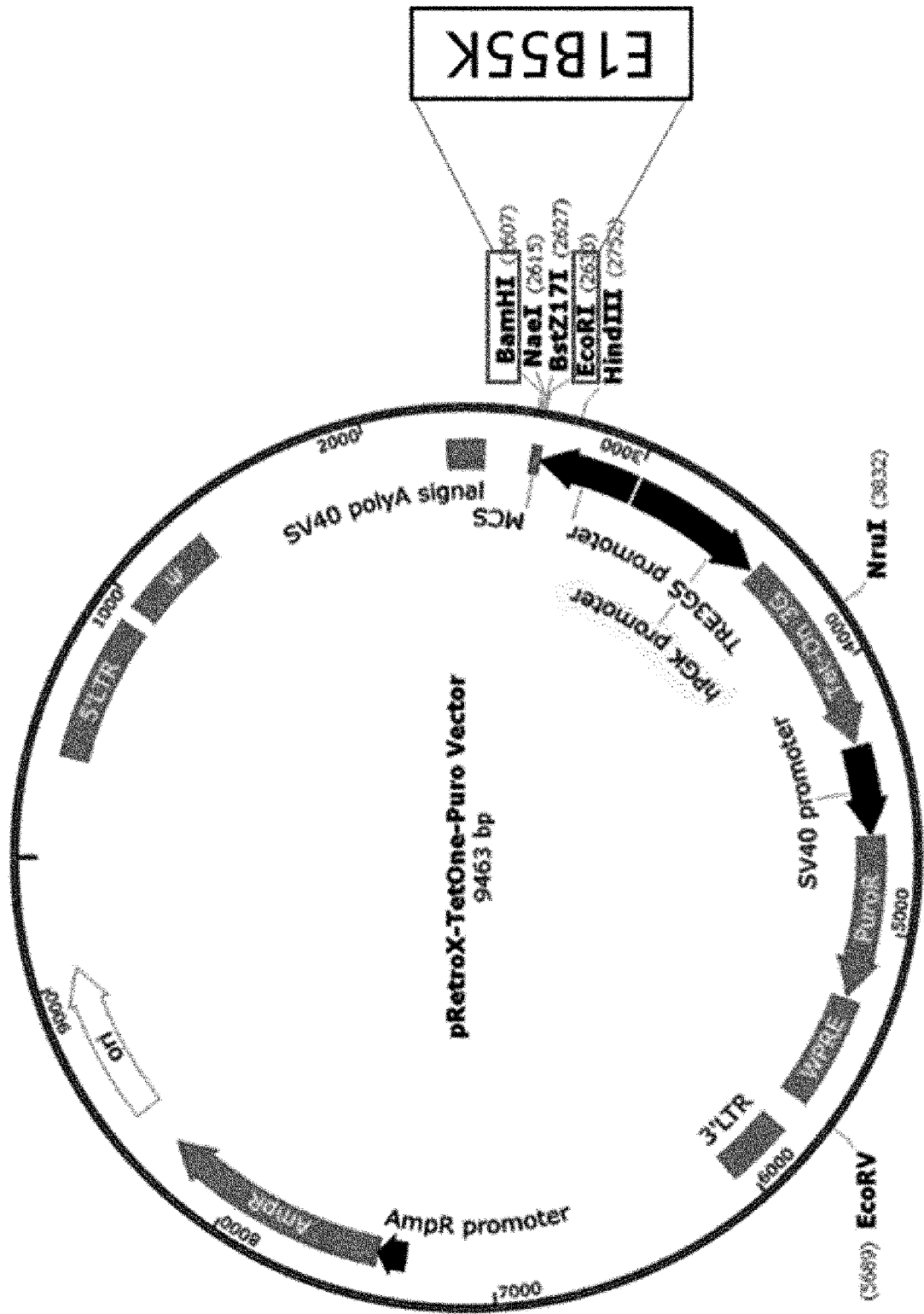
【FIG.14】

STEP	TEMP	TIME		Actual amount
Initial Denaturation	95°C	2min	2x Master Mix	25 μ l
30 cycle	95°C	40 seconds	10 μ M Forward primer	2 μ l
	61°C	40 seconds	10 μ M Reverse primer	2 μ l
	72°C	1min 50seconds	DNA template	1 μ l
Final Extension	72°C	5minutes	DW	20 μ l
			Sum	50 μ l

【FIG.15】



【FIG.16】



[FIG. 17a Continued]

```

E1B55K_sequence      tctgggaaacggggccgaggtggagatagatacggaggataggggtggcctttagatgtagc
sample_forward_primer TCTGGGAACGGGGCCGAGGTGGAGATAGATACGGAGGATAGGGGTGGCCTTTAGATGTAGC
*****

E1B55K_sequence      atgataaatatgtggccgggggtgcttgccatggacgggggtggttattatgaatgtaagg
sample_forward_primer ATGATAAATATGTGGCCGGGGGTGCTTGGCATGGACGGGGTGGTTATTATGAATGTAAGG
*****

E1B55K_sequence      tttactggcccaatttttagcggtagcgttttcctggccaataccaaccttatcctacac
sample_forward_primer TTTACTGGCCCAATTTTAGCGGTACGGTTTCCTGGCCAATACCAACCTTATCCTACAC
*****

E1B55K_sequence      ggtgtagcttctatgggtttaacaatacctgtgtggaagcctggaccgatgtaagggtt
sample_forward_primer GGTGTAGCTTCTATGGGTTTAAACAATACCTGTGTGGAAGCCTGGACCGATGTAAGGGTT
*****

E1B55K_sequence      cggggctgtgccttttactgctgctggaagggggtggtgtgtcgcccaaaagcagggct
sample_forward_primer CGGGGCTGTGCCTTTTACTGCTGCTGGAAGGGGGTGGTGTGTGCGCCCAAAGCAGGGCT
*****

E1B55K_sequence      tcaattaagaaatgcctcctttgaaaggtgtaccttgggtatcctgtctgagggttaactcc
sample_forward_primer TCAATTAAGAAATGCCTCCTTTGAAAGGTGTACCTTGGGTATCCTGTCTGAGGGTAACTCC
*****

E1B55K_sequence      aggggtgcgccacaatgtggcctccgactgtggttgcttcattgctagtgaaaagcgtggct
sample_forward_primer AGGGTGCGCCACAATGTGGCCTCCGACTGTGTTGCTTCATGCTAGTAAAAGCAGGTGGCT
*****

E1B55K_sequence      gtgattaagcataacatggtatgtggcaactgcgaggacagggcctctcagatgctgacc
sample_forward_primer GTGATTAAGCATAACATGGTATGTGGCAACTGCGAGGACAGGGCCTCTCAGATGCTGACC
*****

E1B55K_sequence      tgctcggacggcaactgtcacctgctgaagaccattcacgtagccagccactctcgcaag
sample_forward_primer TGCTCGGACGGCAACTGTACCTGCTGAAGACATTACGTAGCCAGCCCTTTCGCAA--G
***** * * * * *

E1B55K_sequence      gcctggccagtggttgagcataacatactgaccgctgttccttgcatctgggtaacagg
sample_forward_primer GCTG-GCCAGG--GTTGAGCATACCTACTGACCCCTGTTCTTGTCTTGGGAAAAA---
** * * * * * * * * * *
    
```

[FIG. 17b]

Primer: E1B55K internal primer

```

E1B55K_sequence      ggtggttagcttctatgggtttaacaatacctgtgtggaagcctggaccgatgtaagggtt
sample_internal_primer -----ACCTGTAATTTGTTTATTTGGGCGTA
                                     *      * * * * *

E1B55K_sequence      cggggctgtgccttttactgctgctggaagggggtggtgtgtcgcccaaaagcagggct
sample_internal_primer GGTTCGGGCGTGCTTTTACTGCTGCTGGAAGGGGTGGTGTGTCGCCCAAAGCAGGGCT
                                     * *      *

E1B55K_sequence      tcaattaagaaatgcctctttgaaaggtgtaccttgggtatcctgtctgagggtaactcc
sample_internal_primer TCAATTAAGAAATGCCTCTTTGAAAGGTGTACCTTGGGTATCCTGTCTGAGGGTAACTCC
                                     *

E1B55K_sequence      aggggtgcccacaatgtggcctccgactgtggttgcctcatgctagtgaaaagcgtggct
sample_internal_primer AGGGTGCGCCACAATGTGGCCTCCGACTGTGTTGCTTCATGCTAGTGAAAAGCGTGGCT
                                     *

E1B55K_sequence      gtgattaagcataacatggtatgtggcaactgagggacagggcctctcagatgctgacc
sample_internal_primer GTGATTAAGCATAACATGGTATGTGGCAACTGCGAGGACAGGGCCTCTCAGATGCTGACC
                                     *

E1B55K_sequence      tgctcggacggcaactgtcacctgctgaagaccattcacgtagccagccactctcgcaag
sample_internal_primer TGCTCGGACGGCAACTGTCACTGCTGAAGACCATTACGTAGCCAGCCACTCTCGCAAG
                                     *

E1B55K_sequence      gcctggccagtgtttgagcataacatactgaccgctgttccttgcaattgggtaacagg
sample_internal_primer GCCTGGCCAGTGTTTGAGCATAACATACTGACCCGCTGTTCCTTGCAATTTGGGTAACAGG
                                     *

E1B55K_sequence      aggggggtgttcctaccttaccaatgcaatttgagtcacactaagatattgcttgagccc
sample_internal_primer AGGGGGGTGTTCCTACCTTACCAATGCAATTTGAGTCACACTAAGATATTGCTTGAGCCC
                                     *

E1B55K_sequence      gagagcatgtccaaggtgaacctgaacggggtgtttgacatgaccatgaagatctggaag
sample_internal_primer GAGAGCATGTCCAAGGTGAACCTGAACGGGGTGTTTGACATGACCATGAAGATCTGGAAG
                                     *

E1B55K_sequence      gtgctgaggtacgatgagaccgcaccaggtgcagaccctgagagtggtggcggtaaacat
sample_internal_primer GTGCTGAGGTACGATGAGACCCGCACCAGGTGCAGACCCTGCGAGTGTGGCGGTAAACAT
                                     *

E1B55K_sequence      attaggaaccagcctgtgatgctggatgtgaccgaggagctgaggccogatcacttggtg
sample_internal_primer ATTAGGAACCAGCCTGTGATGCTGGATGTGACCAGGAGCTGAGGCCGATCACTTGGTG
                                     *

E1B55K_sequence      ctggcctgcacccgcgctgagtttggtctctagcgatgaagatacagattga-----
sample_internal_primer CTGGCCTGCACCCGCCTGAGTTTGGCTCTAGCGATGAAGATACAGATTGAGGATCCAGA
                                     *

```

【FIG. 17c】

		TTT→TTC Phe(Phenylalanine)
	Primer: E1B55K 5' near internal reverse primer	
E1B55_sequence	-----atggagcgaagaaacccatctgagcggggagtagcctgctggatcttctggc	
sample_5'_near_reverse	AAAGAATTCATGGAGCGAAGAAACCCATCTGAGCGGGGAGTACCTGCTGGATTCTCTGGC	

E1B55_sequence	catgcatctgtggagagcgttgtgagacacaagaatcgccctgctactgttgtcttccgt	
sample_5'_near_reverse	CATGCATCTGTGGAGAGCGTGTGTGAGACACAAGAATCGCCCTGCTACTGTTGTCTTCCGT	

E1B55_sequence	cggccggcgataataaccgacggaggagcagcagcagcagcaggaggaagccaggcggcg	
sample_5'_near_reverse	CGGCCGGCGATAATAACCGACGGAGGAGCAGCAGCAGCAGCAGGAGGAAGCCAGGCGCG	

E1B55_sequence	gggcaggagcagagcccatggaacccgagagccggcctggaccctcggaatgaatggt	
sample_5'_near_reverse	GCGGCAGGAGCAGAGCCCATGGAACCCGAGAGCCGCCCTGGACCCTCGGAATGAATGTT	

E1B55_sequence	gtacaggtggctgaactgtatccagaactgagacgcatctttgacaattacagaggatggg	
sample_5'_near_reverse	GTACAGGTGGCTGAACTGTATCCAGAACTGAGACGCATCTTTGACAATTACAGAGGATGGG	

E1B55_sequence	caggggctaagggggtaagaggagcgggggcttgtgaggctacagaggaggctagg	
sample_5'_near_reverse	CAGGGGCTAAGGGGTAAGAGGAGCGGGGGCTTGTGAGGCTACAGAGGAGGCTAGG	

E1B55_sequence	aatctagcttttagcttaatgaccagacaccgtcctgagtgtattactttcaacagatc	
sample_5'_near_reverse	AATCTAGCTTTTAGCTTAATGACCAGACACCGTCTGAGTGTATTACTTTCAACAGATC	

E1B55_sequence	aaggataattgcgctaataagacttgatctgctggcgagaagtattccatagagcagctg	
sample_5'_near_reverse	AAGGATAATTGCGCTAATGAGCTGATCTGCGCGCAGACCGCCACAAAACAGAGTCCA	

		* * * * *

[FIG.17d]

Primer:E1B55K In-fusion BamHI antisense

```

E1B55K_sequence      gctgaactgtatccagaactgagacgcattttgacaattacagaggatgggcaggggcta
sample_reverse_primer -----AAAGGAT--GGCAGGGCT-
                        * * * * *
E1B55K_sequence      aagggggtaagaggggagcggggggcttgtgaggctacagaggaggctaggaatctagct
sample_reverse_primer ---AAAGGGGTAAAGAGGAGCGGGGGCTTGGAGGCTACAGAGGAGGCTAGGAATCTAGCT
                        * * * * *
E1B55K_sequence      tttagcttaatgaccagacaccgtcctgagtgtattacttttcaacagatcaaggataat
sample_reverse_primer TTAGCTTAAAGACCAGACACCGTCCTGAGTGTATTACTTTTCAACAGATCAAGGATAAT
                        * * * * *
E1B55K_sequence      tgcgctaataagccttgatctgctggcgcagaagtattccatagagcagctgaccacttac
sample_reverse_primer TCGCTAATGAGCTTIGATCTGCTGGCGCAGAAGTATCCATAGAGCAGCTGACCACTTAC
                        * * * * *
E1B55K_sequence      tggctgcagccaggggatgattttgaggaggctattagggtatatgcaaaggtggcactt
sample_reverse_primer TGGCTGCAGCCAGGGGATGATTTTGGAGGAGGCTATTAGGTATATGCAAAGGTGGCACTT
                        * * * * *
E1B55K_sequence      aggccagattgcaagtacaagatcagcaacttgtaaatatcaggaattggtgctacatt
sample_reverse_primer AGGCCAGATTGCAAGTACAAGATCAGCAAACCTTGTAATATCAGGAATTGTTGCTACATC
                        * * * * *
E1B55K_sequence      tctgggaacggggccgaggtggagatagatacggaggatagggtggccttttagatgtagc
sample_reverse_primer TCTGGGAACGGGGCCGAGGTGGAGATAGATACGGAGGATAGGGTGGCCTTTAGATGTAGC
                        * * * * *
E1B55K_sequence      atgataaatatgtggccgggggtgcttggcatggacgggggtggttattatgaatgtaagg
sample_reverse_primer ATGATAAATATGTGGCCGGGGTGCTTGGCATGGACGGGGTGGTTATTATGAATGTAAGG
                        * * * * *
E1B55K_sequence      ttactggcccaatttttagcgggtacggttttcctggccaataccaaccttatcctacac
sample_reverse_primer TTACTGGCCCAATTTTtagcgggtacggttttcctggccaataccaaccttatcctacac
                        * * * * *
E1B55K_sequence      ggtgtagcttctatgggtttaacaatacctgtgtggaagcctggaccgatgtaaggggt
sample_reverse_primer GGIGTAGCTTCTATGGGTTTAAACAATACCTGTGTGGAAGCCTGGACCGATGTAAGGGTT
                        * * * * *

```

[FIG. 17d Continued]

```

E1B55K_sequence      cggggctgtgccttttactgctgctggaagggggtggtgtgtcgccccaaaagcagggct
sample_reverse_primer CGGGGCTGTGCCITTTACTGCTGCTGGAAGGGGGTGGTGTGTGCGCCCCAAAAGCAGGGCT
*****

E1B55K_sequence      tcaattaagaaatgcctcctttgaaaggtgtaccttgggtatcctgtctgagggtaactcc
sample_reverse_primer TCAATTAAGAAATGCCTCCTTTGAAAGGTGTACCTTGGGTATCCTGTCTGAGGGTAACTCC
*****

E1B55K_sequence      aggggtgcgccacaatgtggcctccgactgtggttgcttcatgctagtgaaaagcgtggct
sample_reverse_primer AGGGTGCGCCACAATGTGGCCTCCGACTGTGGTTGCTTCATGCTAGTAAAAGCAGTGGCT
*****

E1B55K_sequence      gtgattaagcataacatggtatgtggcaactgcgaggacagggcctctcagatgctgacc
sample_reverse_primer GTGATTAAGCATAACATGGTATGTGGCAACTGCGAGGACAGGGCCTCTCAGATGCTGACC
*****

E1B55K_sequence      tgctcggacggcaactgtcacctgctgaagaccattcacgtagccagccactctcgaag
sample_reverse_primer TGCTCGGACGGCAACTGTCACCTGCTGAAGACCATTACGTAGCCAGCCACTCTCGAAG
*****

E1B55K_sequence      gcctggccagtgtttgagcataacatactgaccgctgttccttgcaattgggtaacagg
sample_reverse_primer GCCTGGCCAGTGTTTGAGCATAACATACTGACCCGCTGTTCCCTTGCAATTTGGGTAACAGG
*****

E1B55K_sequence      aggggggtgttcctaccttaccaatgcaatttgagtcacactaagatattgcttgagccc
sample_reverse_primer AGGGGGGTGTTCCCTACCTTACCAATGCAATTTGAGTCACACTAAGATATTGCTTGAGCCC
*****

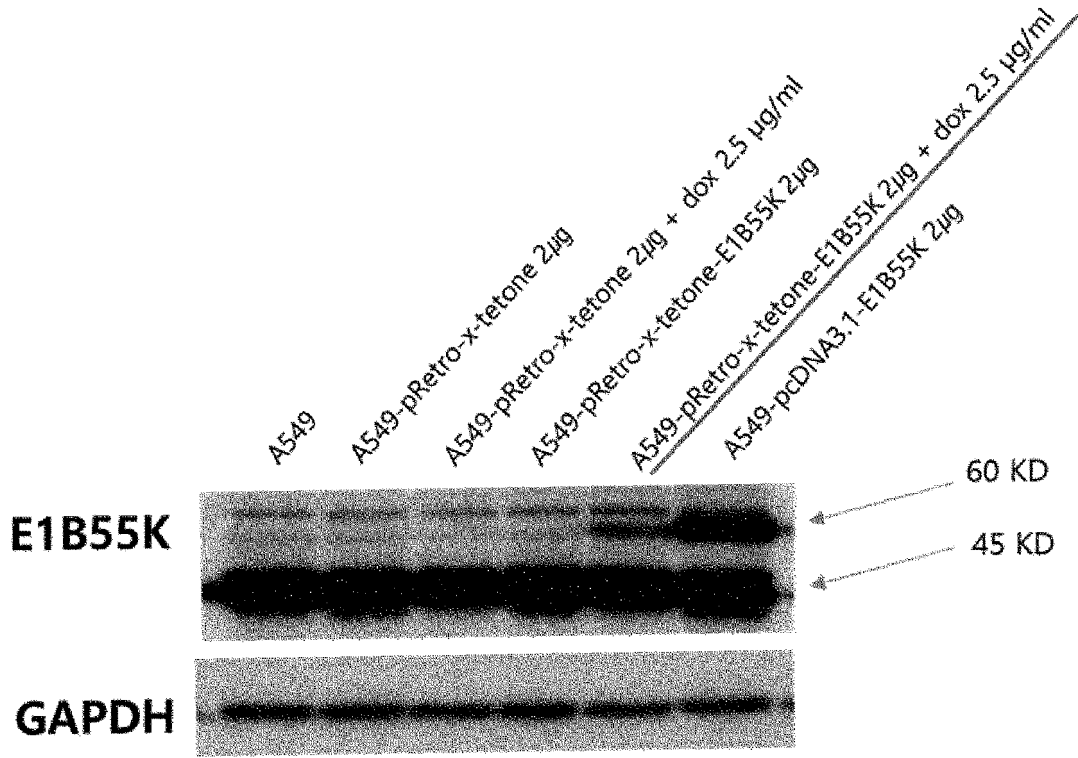
E1B55K_sequence      gagagcatgtccaaggtgaacctgaacggggtgtttgacatgaccatgaagatctggaag
sample_reverse_primer GAGAGCATGTCCAAGGTGAACCTGAACGGGGTGTTTGACATGACCATGAAGATCTGGAAG
*****

E1B55K_sequence      gtgctgaggtacgatgagaccgcaccaggtgcagaccctgcgagtggtggcgtaaacat
sample_reverse_primer GTGCTGAGGTACGATGAGACCCGACCAGGTGCAGACCCTGCGAGTGTGGCGGTAACAT
*****

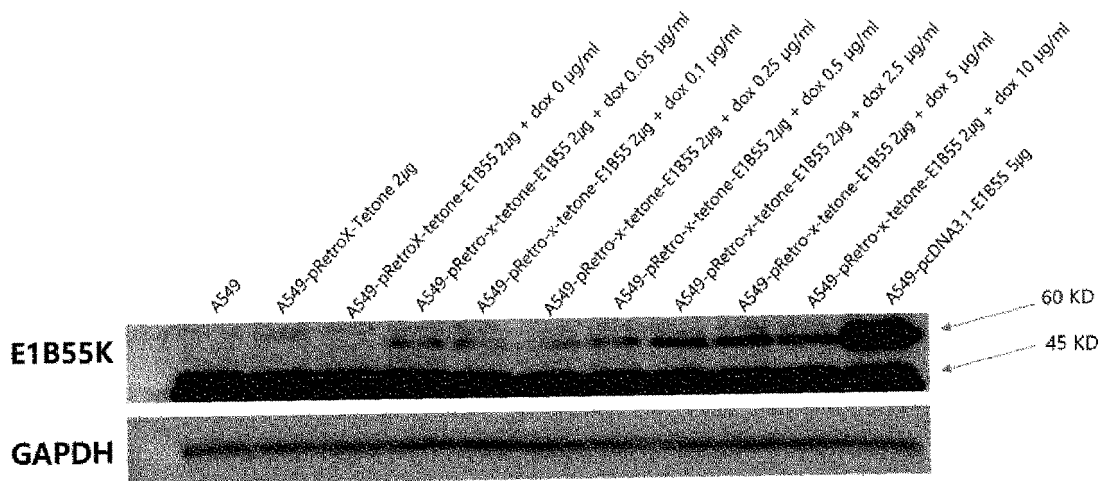
E1B55K_sequence      attaggaaccagcctgtgatgctggatgtgaccgaggagctgaggcccgatcacttggtg
sample_reverse_primer ATTAGGAACCAGCCTGTGATGCTGGATGTGACCGAGGAGCTGAGGCCCGATCACTTGGTG
*****

E1B55K_sequence      ctggcctgcacccgcgctgagtttgctctagcgtgaagatacagattga
sample_reverse_primer CTGGCCTGCACCCGCGCTGAGTTTGGCCCCCGT-----
***** * *
    
```

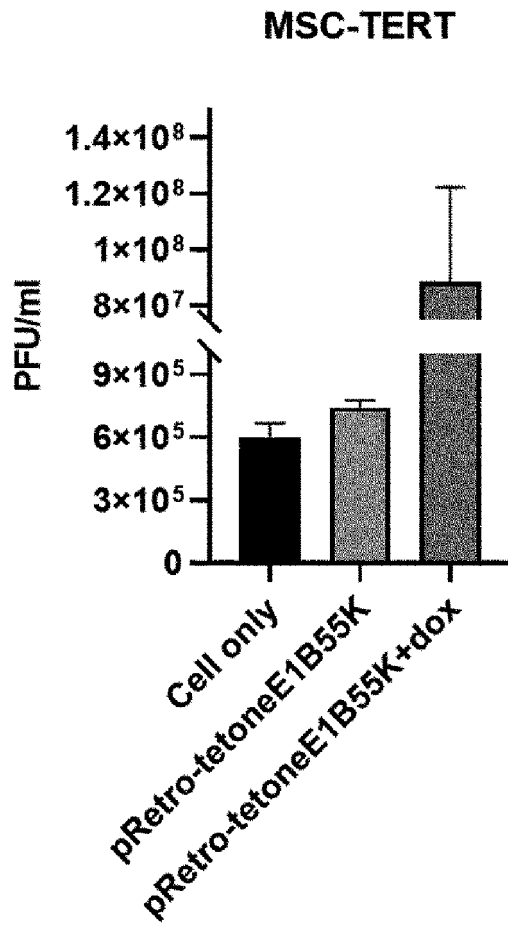
【FIG.18】



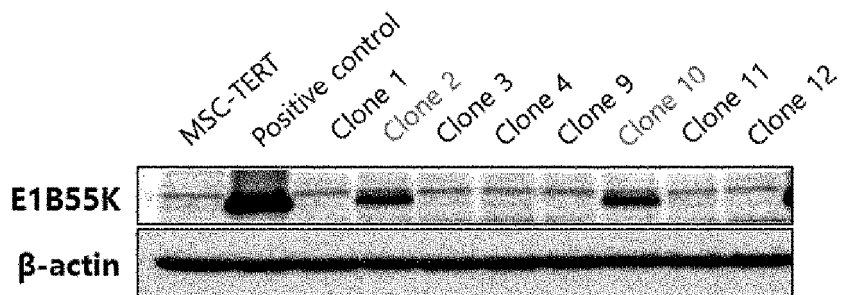
【FIG.19】



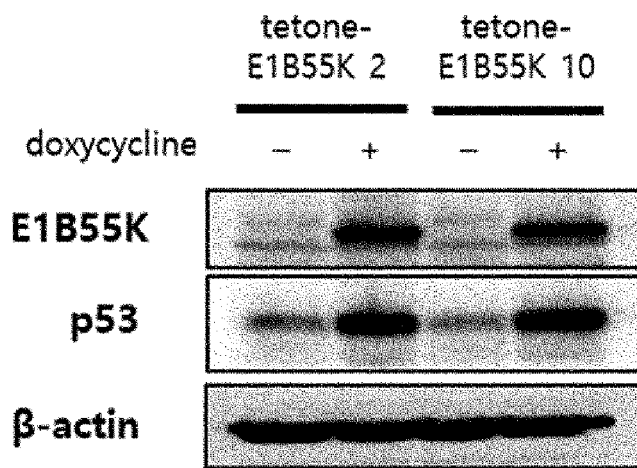
【FIG.20】



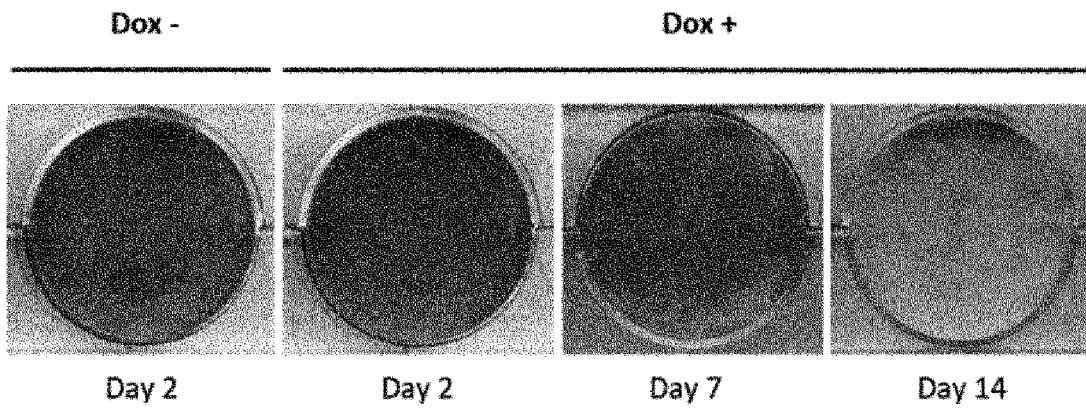
【FIG.21】



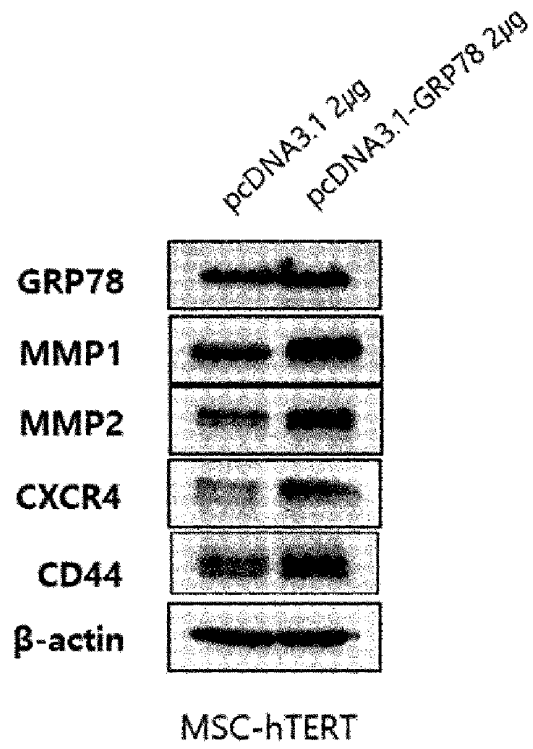
【FIG.22】



【FIG.23】

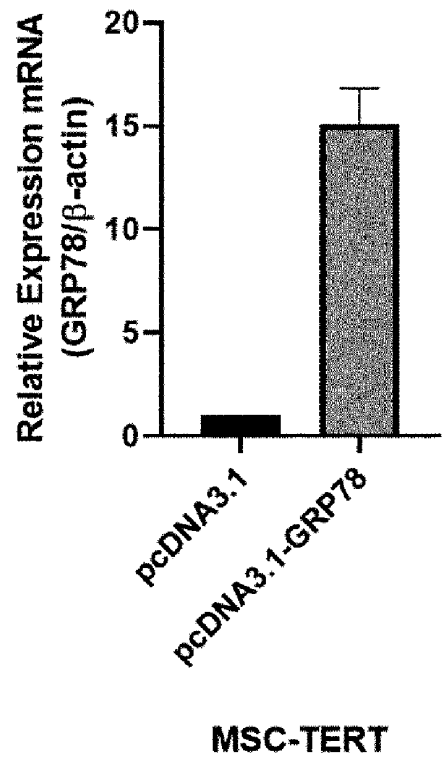


【FIG.24a】

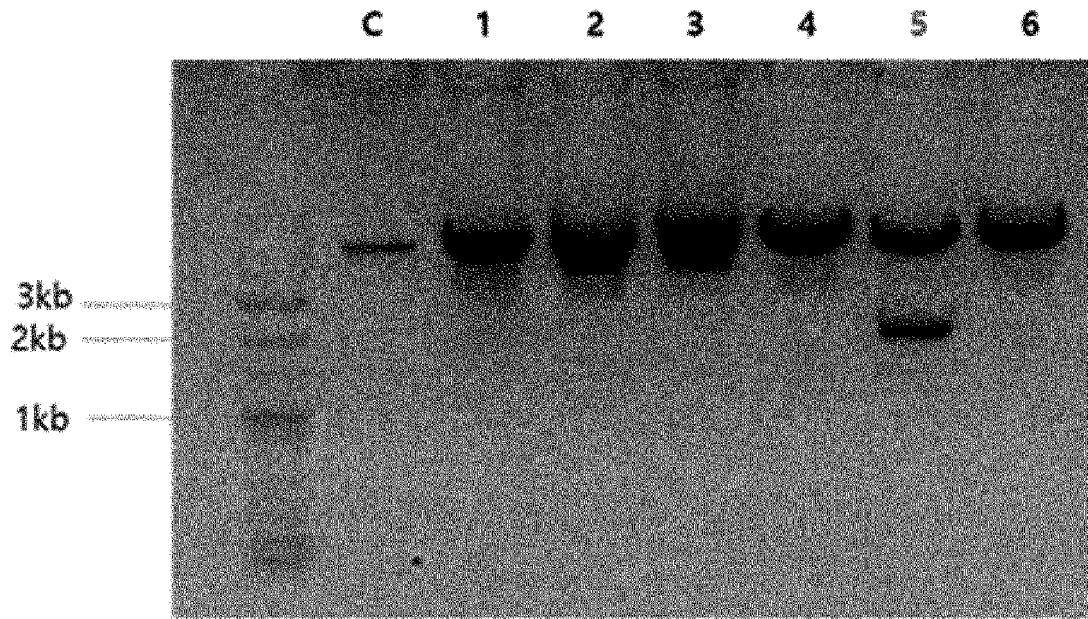


【FIG.24b】

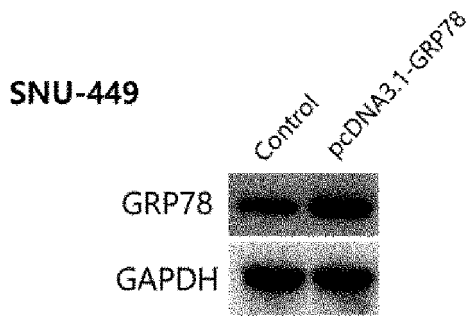
48 hours post-transfection



【FIG.25】



【FIG.26】



[FIG.27a]

pcDNA3.1 GRP78 forward

```

NM_005347_GRP78 -----atgaagctctccctgg
pcDNA3.1GRP78 TGGTGGAAATCTGCAGATATCCAGCACAGTGGCGGGCCGCTCGAGATGAAGCTCTCCCTGG
*****

NM_005347_GRP78 tggccgcgatgctgctgctgctcagcgcggcggcggccgaggaggaggaagaaggagg
pcDNA3.1GRP78 TGGCCGCGATGCTGCTGCTGCTCAGCGCGGGCCGAGGAGGAGACAAGAAGGAGG
*****

NM_005347_GRP78 acgtgggcaoggtggctggcatogacctggggaccacctactcctgctgctggcgtgttca
pcDNA3.1GRP78 ACGTGGGCACGGTGGTTCGGCATCGACCTGGGGACCACCTACTCCTGCGTGGCGGTGTTCA
*****

NM_005347_GRP78 agaacggccgctggagatcatcgccaacgatcagggcaaccgcatcagccgctcctatg
pcDNA3.1GRP78 AGAACGGCCGCTGGAGATCATCGCCAACGATCAGGGCAACCGCATCACGCCGCTCCTATG
*****

NM_005347_GRP78 tcgccttcaactcctgaaggggaaogtctgatggcgatgcccgcaagaaccagctcacct
pcDNA3.1GRP78 TCGCCTTCACTCCTGAAGGGGAAOGTCTGATTGGCGATGCCGCCAAGAACCAGCTCACCT
*****

NM_005347_GRP78 ccaaccccgagaacaacgggtctttgacgccaagcgggctcatcgccgacgctggaatgacc
pcDNA3.1GRP78 CCAACCCCGAGAACAACGGGTCTTTGACGCCAAGCGGCTCATCGCCGACGCTGGAATGACC
*****

NM_005347_GRP78 cgtctgtgcagcaggacatcaagttcttgccgttcaaggtggtgaaaagaaaactaaac
pcDNA3.1GRP78 CGTCTGTGCAGCAGGACATCAAGTTCCTTGCCGTTCAAGGTGGTTGAAAAGAAAACATAAAC
*****

NM_005347_GRP78 catacattcaagttgatattggaggtgggcaacaaagacatttgcctcctgaagaaattt
pcDNA3.1GRP78 CATACATTC AAGTTGATATTGGAGGTGGGCAAACAAAGACATTTGCTCCTGAAGAAATTT
*****

NM_005347_GRP78 ctgccatggttctcactaaaaatgaaagaaaccgctgaggcttatttgggaaagaaggtta
pcDNA3.1GRP78 CTGCCATGGTTCTCACTAAAATGAAAGAAACCGCTGAGGCTTATTTGGGAAAGAAGTTA
*****

NM_005347_GRP78 cccatgcagttgttactgtaccagcctatttaatgatgcccaacgccaagcaaccaag
pcDNA3.1GRP78 CCCATGCAGTTGTTACTGTACCAGCCTATTTAATGATGCCCAACGCCAAGCAACCAAG
*****

NM_005347_GRP78 acgctggaactattgctggcctaaatgttatgaggatcatcaacgagcctacggcagctg
pcDNA3.1GRP78 ACGCTGGAACTATTGCTGGCCTAAATGTATGAGGATCATCAACGAGCCTACGGCAGCTG
*****

NM_005347_GRP78 ctattgcttatggcctggataagagggagggggagaagaacatcctggtgtttgacctgg
pcDNA3.1GRP78 CTATTGCTTATGGCCTGGATAAGAGGGAGGGGGAGAAGAACATCCTGGTGTTTGACCTGG
*****

NM_005347_GRP78 gtggcggaaaccttcgatgtgtctctctcaccattgacaatggtgtcttogaagttgtgg
pcDNA3.1GRP78 GTGGCGGAACCTTCGATGTGTCTCTCTCACCATTGACAATGSGTGTCTTGAAGTTGTGG
*****

NM_005347_GRP78 ccaactaatggagatactcatctgggtggagaagactttgaccagcgtgtcatggaacct
pcDNA3.1GRP78 CCACTAATGGAGATACTCATCTGGGTGGAGAAGACTTTGACCAGCGTGTGATGGAACACT
*****

NM_005347_GRP78 tcatcaaaactgtacaaaaagaagacgggcaaaagatgtcaggaaagacaaatagagctgtgc
pcDNA3.1GRP78 TCATCAAACCTGTACAAAAAGAAGACGGGCAAAGATGTCAGGAAAGACAATAGAGCTGTGC
*****

NM_005347_GRP78 agaaactccggcgcgaggttagaaaaggccaaacggggccctgtcttctcagcatcaagcaa
pcDNA3.1GRP78 AGAAACTCCGGCGCGAGGTAGAAAAGGCCAAACGGGGCCCTGTCTTCTCAGCATCAAGCAA
*****

NM_005347_GRP78 gaattgaaattgagtccttctatgaaggagaagacttttctgagaccctgactcgggcca
pcDNA3.1GRP78 GAATTGAAATTGAGTCCTTCTATGAAGGAGAAGAC-----
*****

```

[FIG.27b]

pcDNA3.1 GRP78 reverse

```

NM_005347_GRP78      tctcagcatcaagcaagaattgaaattgagtccttctatgaaggagaagacttttctgag
pcDNA3.1GRP78      -----TCTATGAAGGAGAAGACTTTTCTGAG
                      *****

NM_005347_GRP78      accctgactcgggccaaatttgaagagctcaacatggatctggtccgggtctactatgaag
pcDNA3.1GRP78      ACCCTGACTCGGGCCAAATTTGAAGAGCTCAACATGGATCTGTTCCGGTCTACTATGAAG
                      *****

NM_005347_GRP78      cccgtccagaagtgttgaagattctgatttgaagaagtctgatattgatgaaattggt
pcDNA3.1GRP78      CCCGTCCAGAAAGTGTTGGAAGATTCTGATTTGAAGAAGTCTGATATTGATGAAATTGTT
                      *****

NM_005347_GRP78      ctgtgtggtggtctgactcgaattccaaagattcagcaactgggttaaagagttcttcaat
pcDNA3.1GRP78      CTTGTTGGTGGCTCGACTCGAATTCCAAAGATTCAGCAACTGGTTAAAGAGTCTCTCAAT
                      *****

NM_005347_GRP78      ggcaaggaaccatcccgtggcataaaaccagatgaagctgtagcgtatggtgctgctgct
pcDNA3.1GRP78      GGCAAGGAACCATCCC GTGGCATAAAACCCAGATGAAGCTGTAGC GTATGGTGTCTGCTGTC
                      *****

NM_005347_GRP78      caggctggtgtgctctctggtgatcaagatacaggtgacctgggtactgcttgatgtatgt
pcDNA3.1GRP78      CAGGCTGGTGTGCTCTCTGGTGATCAAGATACAGGTGACCTGGTACTGCTTGATGTATGT
                      *****

NM_005347_GRP78      ccccttacacttggatttgaactgtggggaggtgtcatgaccaaactgattccaaggaac
pcDNA3.1GRP78      CCCCTTACACTTGGTATTGAACTGTGGGAGGTGTCTATGACCAAACCTGATTC AAGGAAC
                      *****

NM_005347_GRP78      acagtgtgctctaccaagaagtctcagatcttttctacagcttctgataatcaaccaact
pcDNA3.1GRP78      ACAGTGGTGCCTACCAAGAAGTCTCAGATCTTTTCTACAGCTTCTGATAATCAACCAACT
                      *****

NM_005347_GRP78      gttacaatcaaggtctatgaagtgaaagaccctgacaaaagacaatcatcttctgggt
pcDNA3.1GRP78      GTTACAATCAAGGTCTATGAAGTGAAAGACCCTGACAAAAGACAATCATCTTCTGGGT
                      *****

NM_005347_GRP78      acatttgatctgactggaattctcctcctgctcctcgtgggtcccacagattgaagtcacc
pcDNA3.1GRP78      ACATTTGATCTGACTGGAATTCCTCCTGCTCCTCGTGGGTCCCACAGATTGAAGTCACC
                      *****

NM_005347_GRP78      tttgagatagatgtgaatggtattcttctcgagtgacagctgaagacaaggttacagggaac
pcDNA3.1GRP78      TTTGAGATAGATGTGAATGGTATTCTTCTCGAGTGACAGCTGAAGACAAGGTACAGGGAAC
                      *****

NM_005347_GRP78      aaaaataagatcacaatcaccaatgaccagaatcgctgacacctgaagaaatcgaaagg
pcDNA3.1GRP78      AAAAATAAGATCACAATCACCAATGACCAGAATCGCTGACACCTGAAGAAATCGAAAGG
                      *****

NM_005347_GRP78      atggttaatgatgctgagaagtttctgctgaggaagacaaaaagctcaaggagcgcattgat
pcDNA3.1GRP78      ATGGTTAATGATGCTGAGAAGTTTCTGCTGAGGAAGACAAAAAGCTCAAGGAGCGCATTGAT
                      *****

NM_005347_GRP78      actagaaatgagttgaaagctatgcctattctctaaagaatcagattggagataaagaa
pcDNA3.1GRP78      ACTAGAAATGAGTTGAAAGCTATGCCTATTCTCTAAAGAATCAGATTGGAGATAAAGAA
                      *****

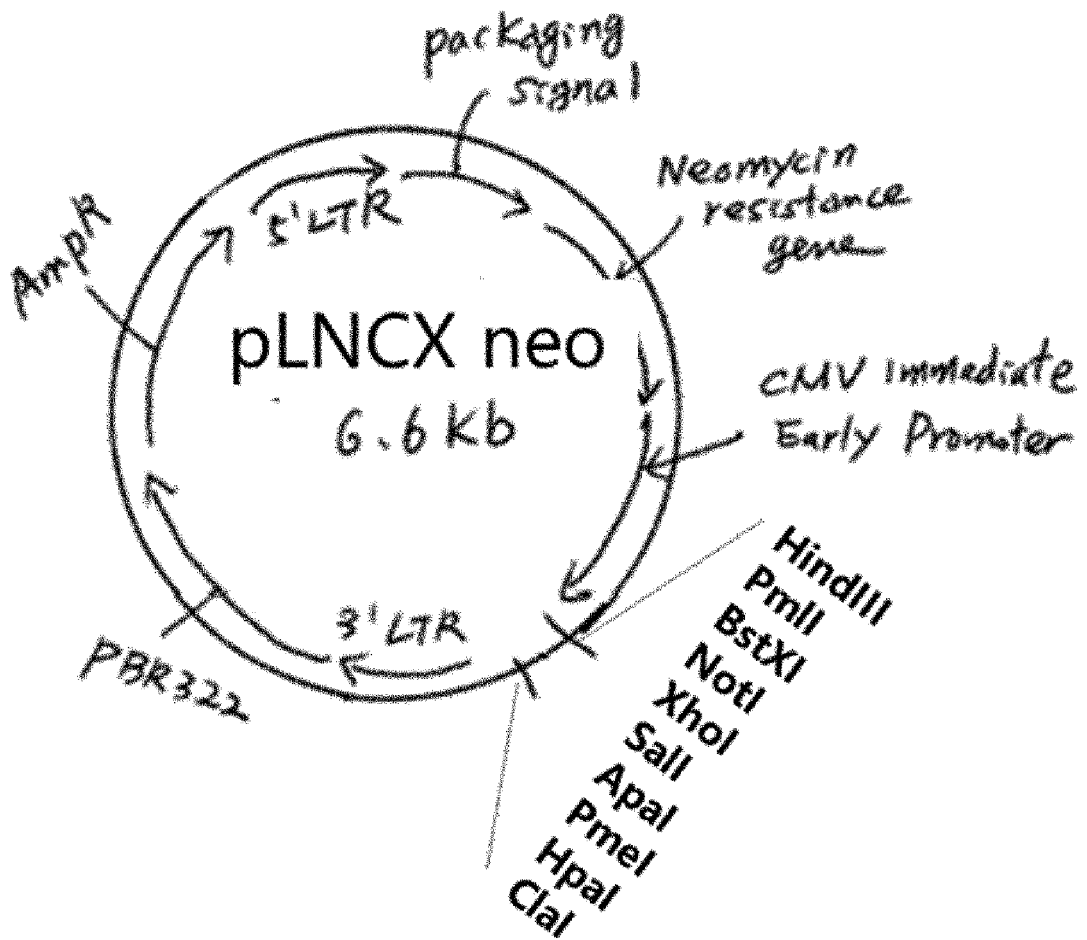
NM_005347_GRP78      aagctgggaggtaaactttcctctgaagataaggagaccatggaaaaagctgtagaagaa
pcDNA3.1GRP78      AAGCTGGGAGGTAAACTTTCTCTGAAAGATAAGGAGACCATGGAAAAAGCTGTAGAAGAA
                      *****

NM_005347_GRP78      aagattgaaatggtggaagccaccaagatgctgacattgaagacttcaagctaaagaag
pcDNA3.1GRP78      AAGATTGAAATGGCTGGAAAGCCACCAAGATGCTGACATTGAAGACTTCAAAGCTAAGAAG
                      *****

NM_005347_GRP78      aaggaactggaagaaattgttcaaccaattatcagcaaactctatggaagtgcaggccct
pcDNA3.1GRP78      AAGGAACTGGAAGAAATTGTTCAACCAATTATCAGCAAACCTCTATGGAAGTGCAGGCCCT
                      *****

NM_005347_GRP78      cccccaactggtgaagaggatacagcagaaaaagatgagttgtag-----
pcDNA3.1GRP78      CCCCCAACTGGTGAAGAGGATACAGCAGAAAAAGATGAGTTGTAGTCTAGAGTCCCGTAA
                      *****
    
```

【FIG.28】

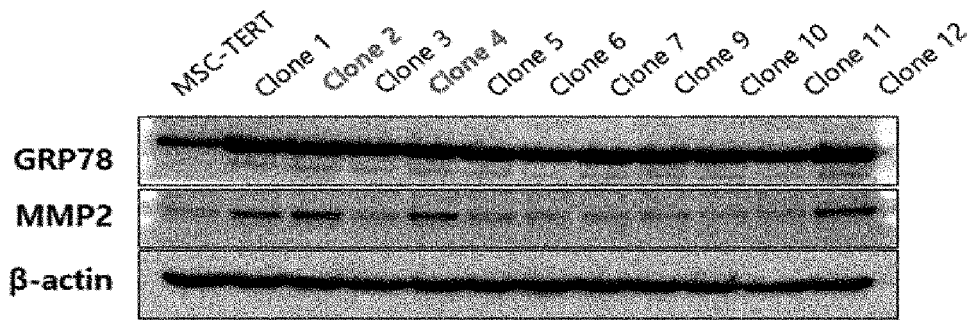


【FIG.29】

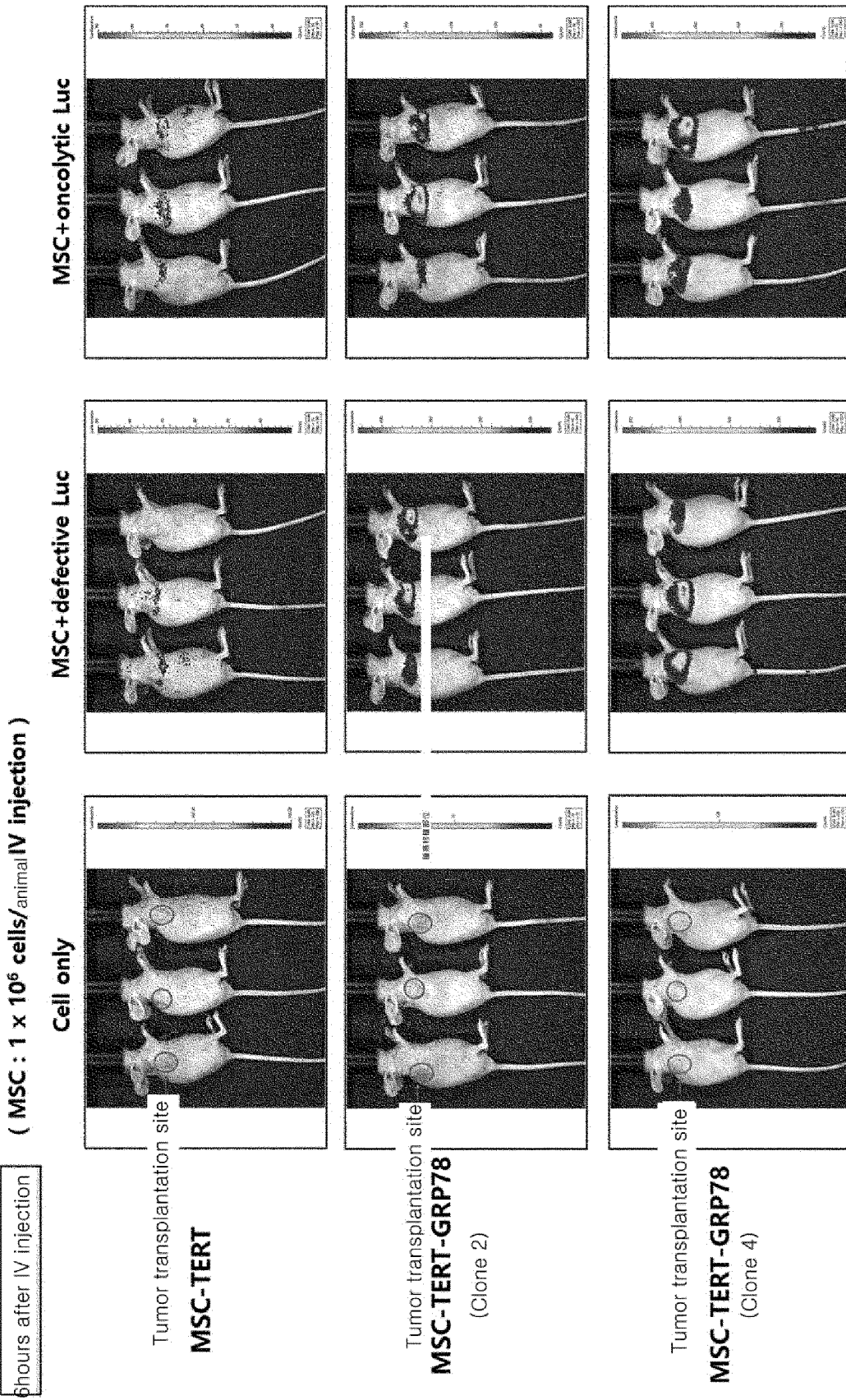


C : pLNCX Neo
2,5,9,11,12,20,21번 right construct: pLNCXNeo-GRP78

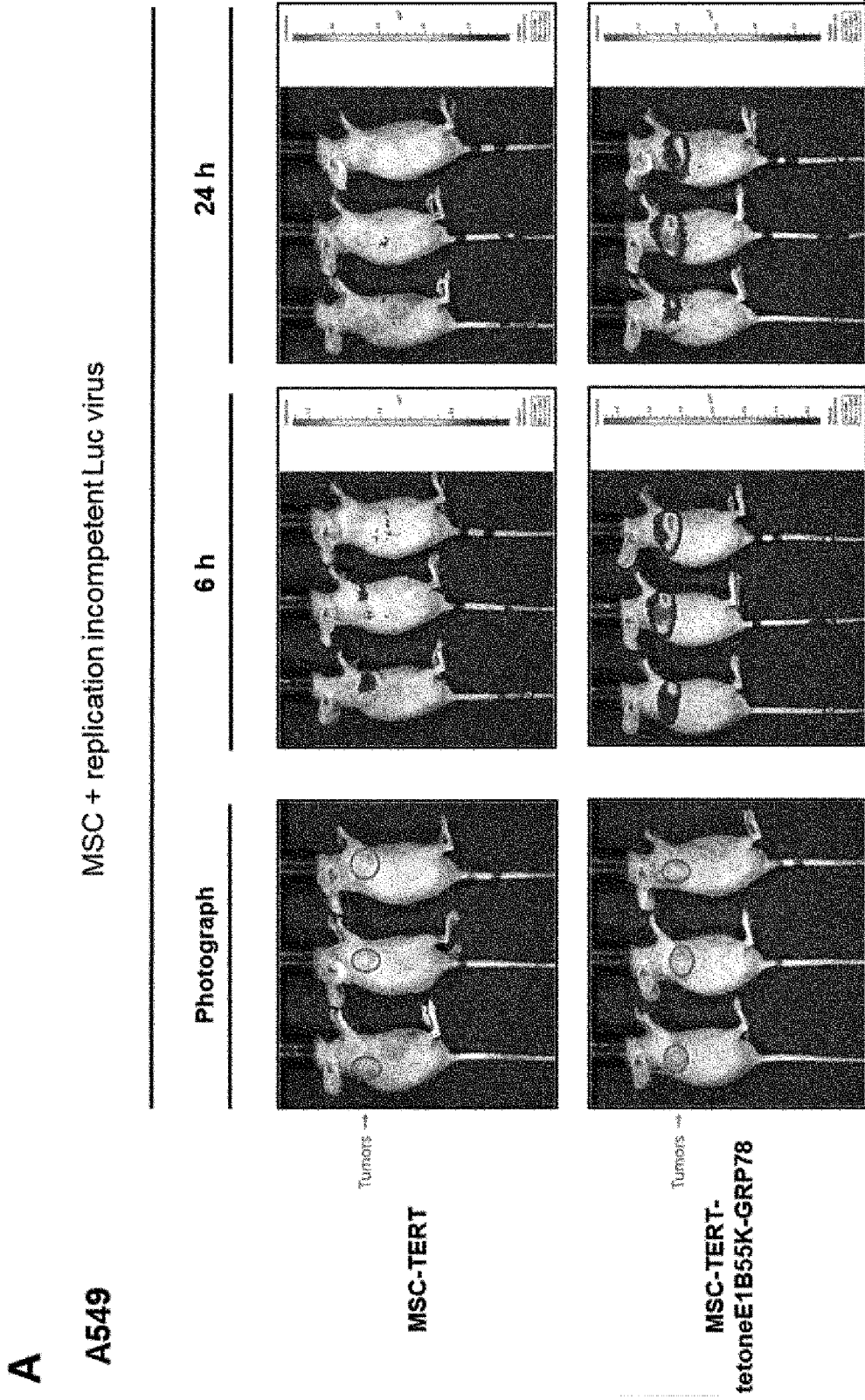
【FIG.30】



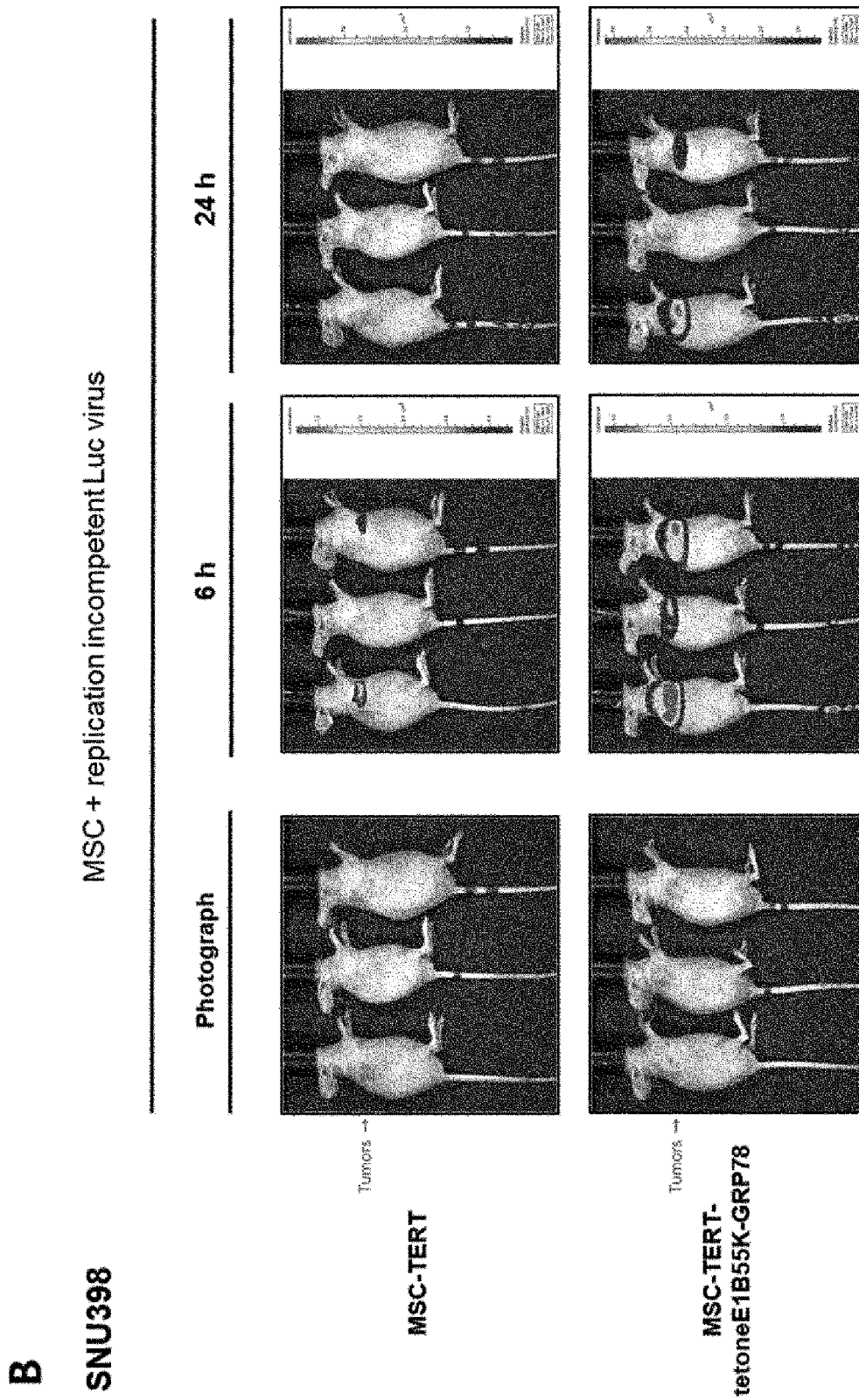
【FIG.31】



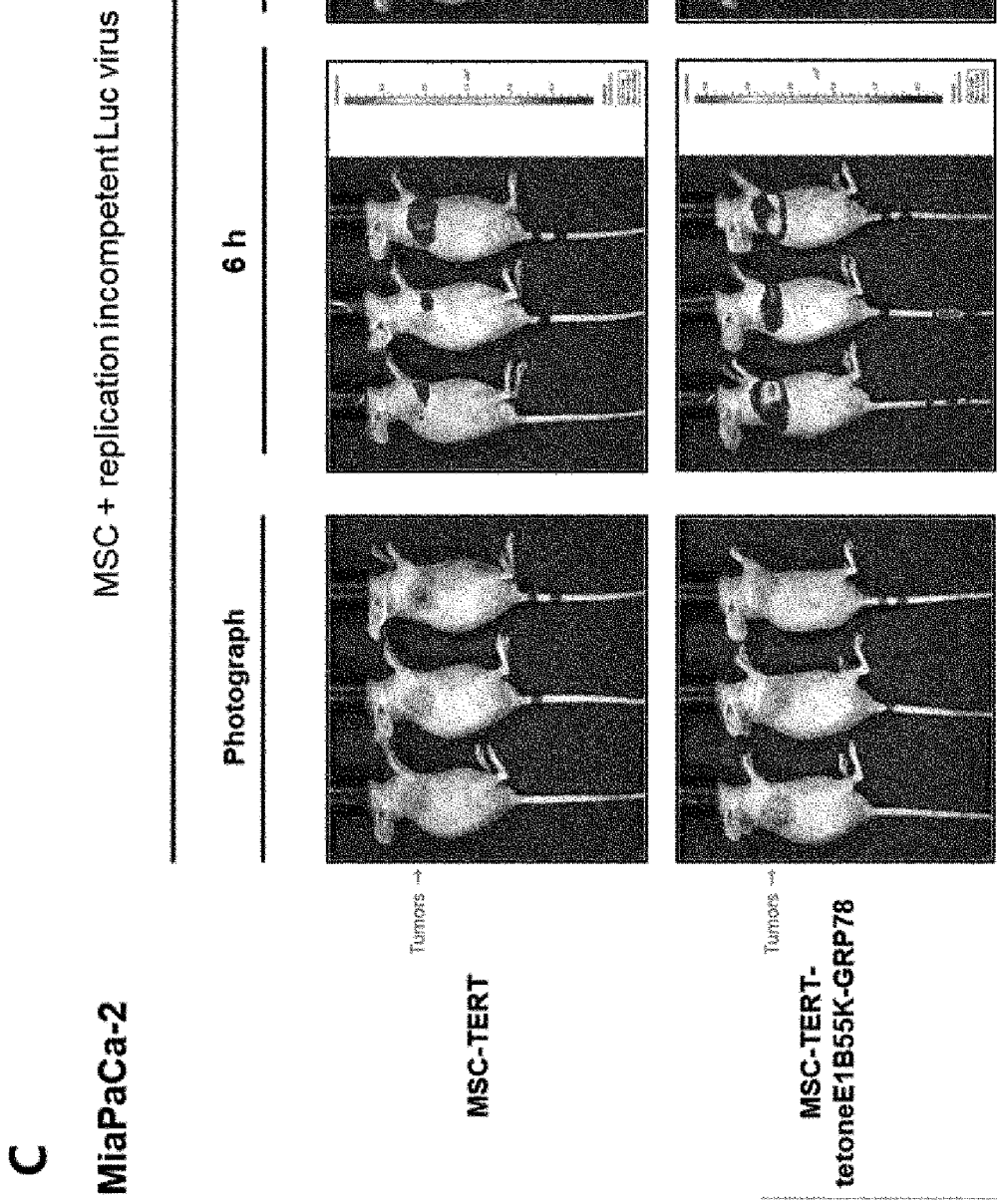
【FIG.32a】



【FIG.32b】

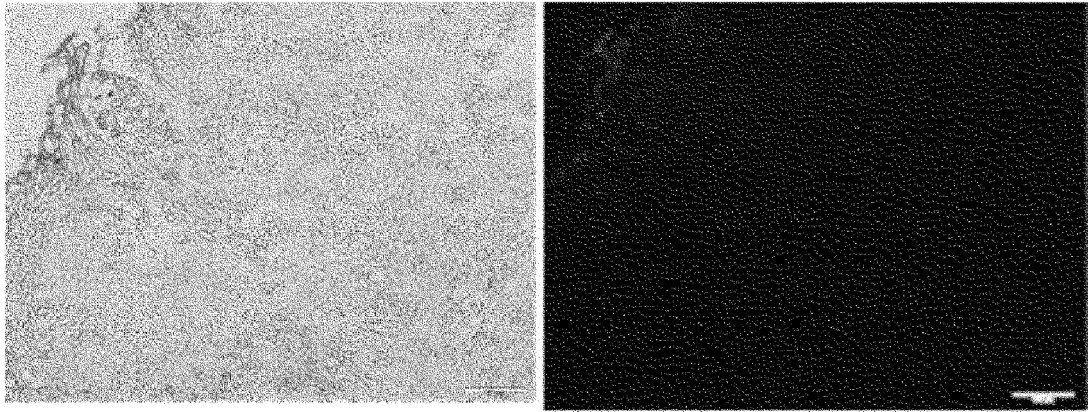


【FIG.32c】

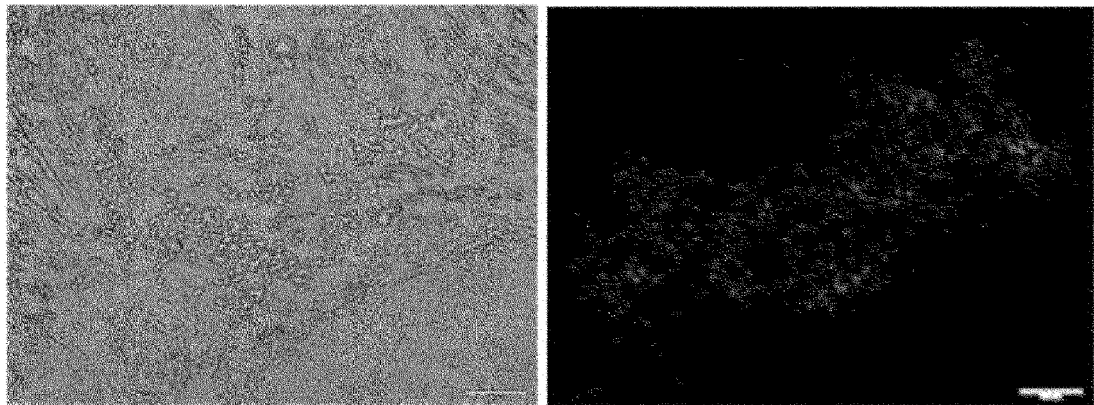


【FIG.33a】

24 hours after IV injection



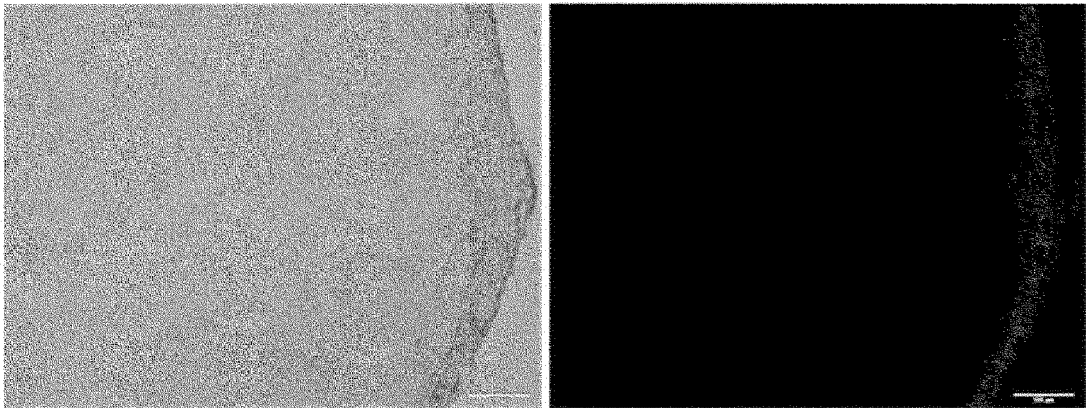
MSC-TERT



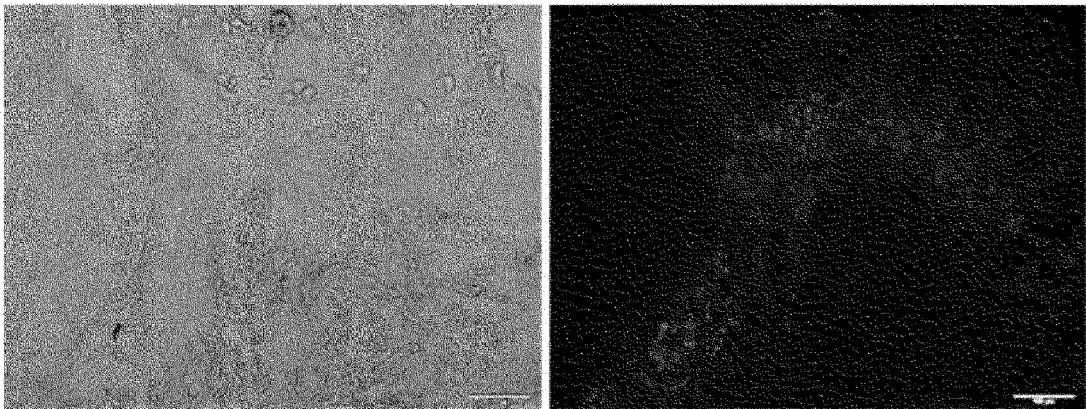
MSC-TERT-tetoneE1B55K-GRP78

【FIG.33b】

48 hours after IV injection



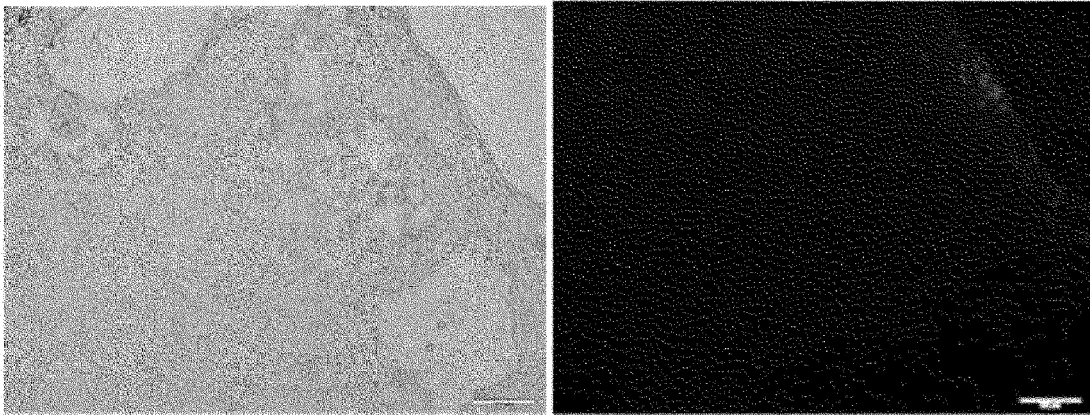
MSC-TERT



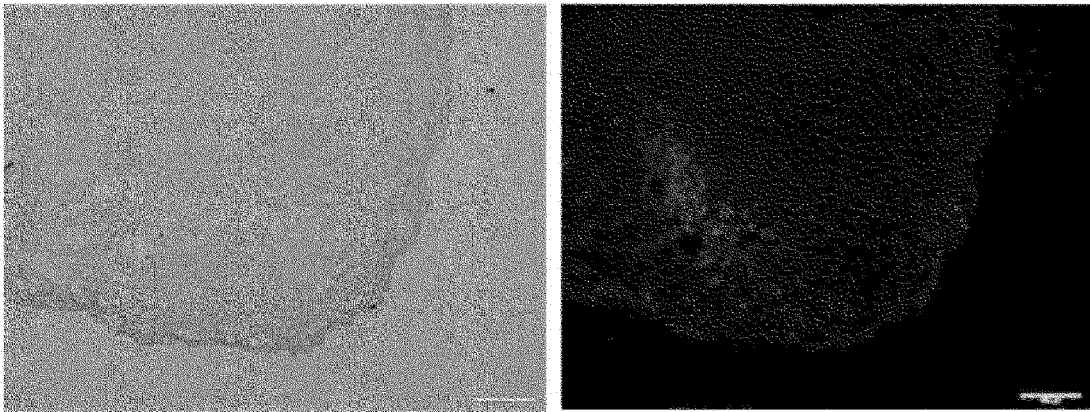
MSC-TERT-tetoneE1B55K-GRP78

【FIG.33c】

72 hours after IV injection

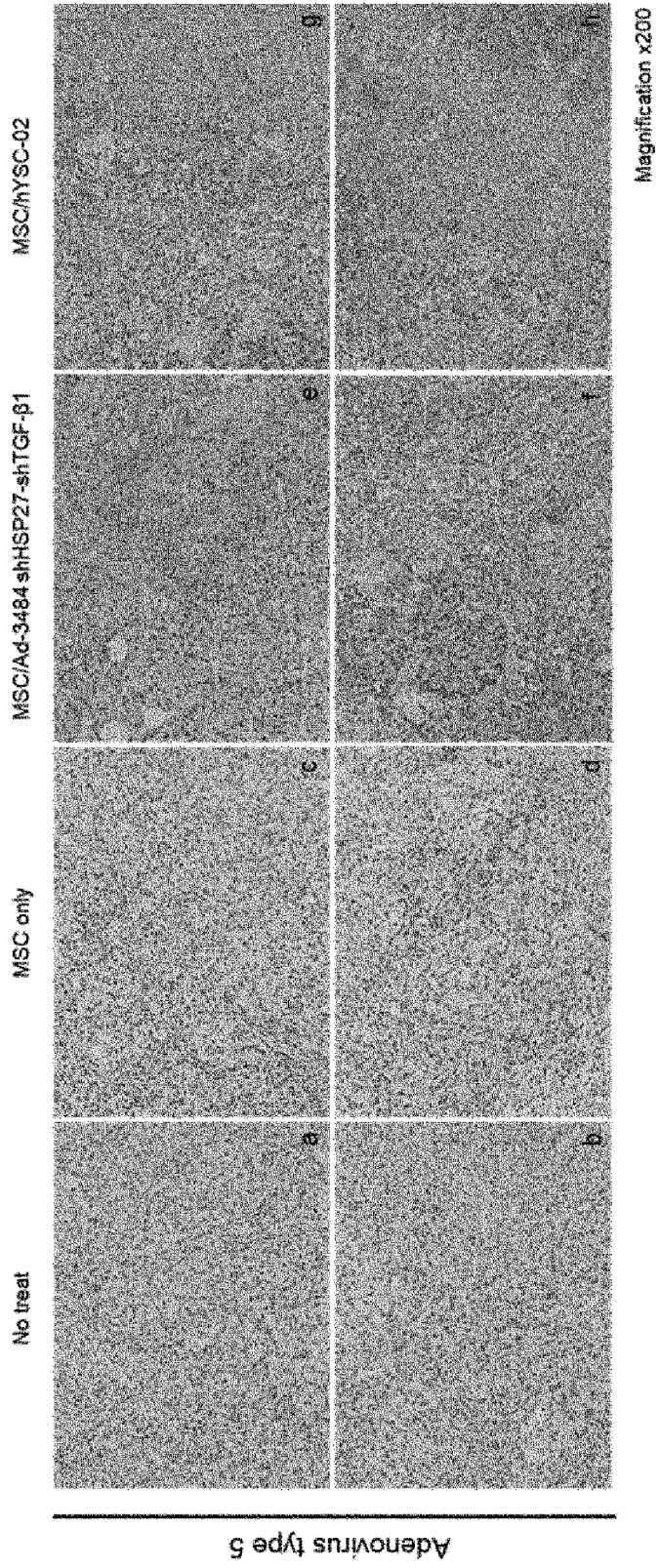


MSC-TERT

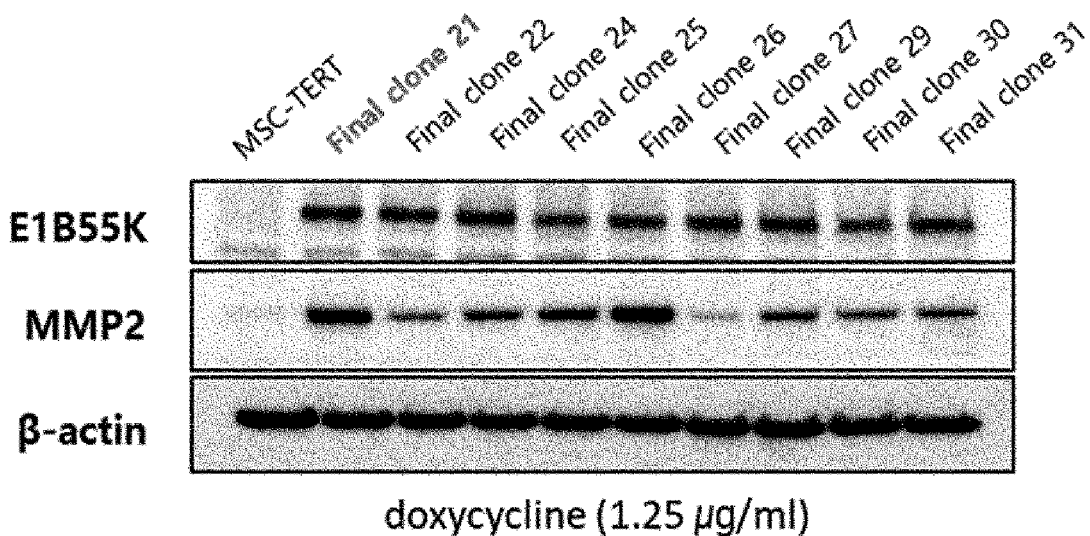


MSC-TERT-tetoneE1B55K-GRP78

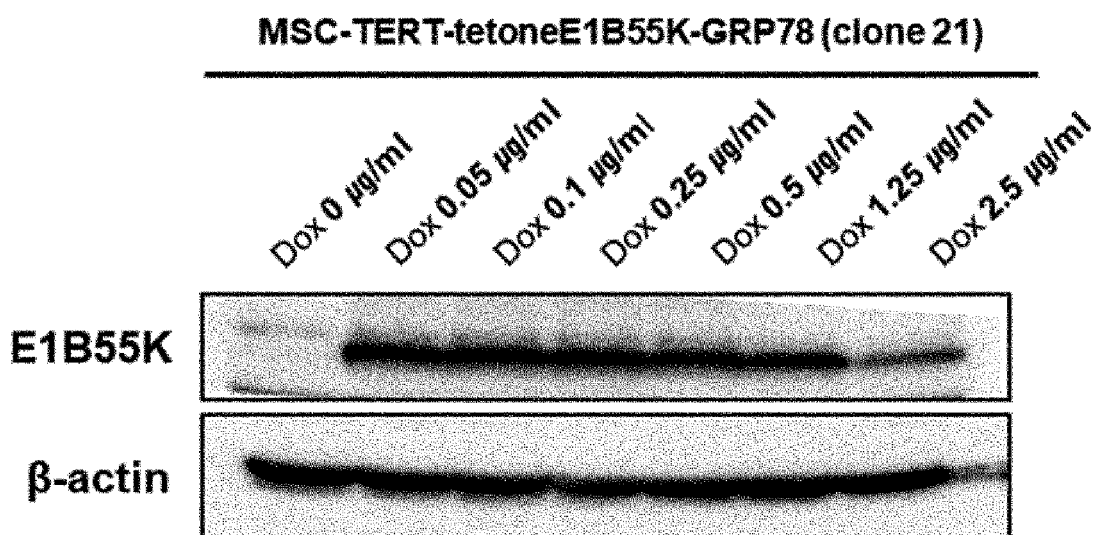
【FIG.34】



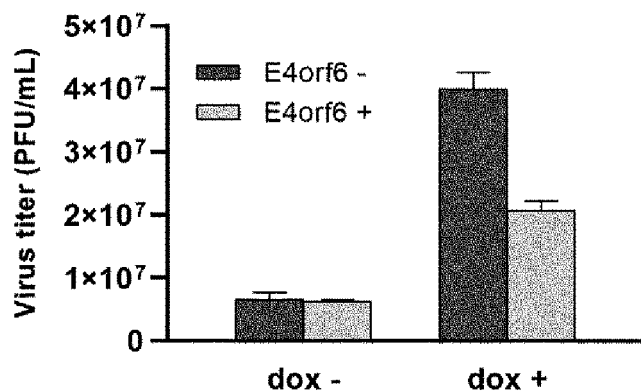
【FIG.35】



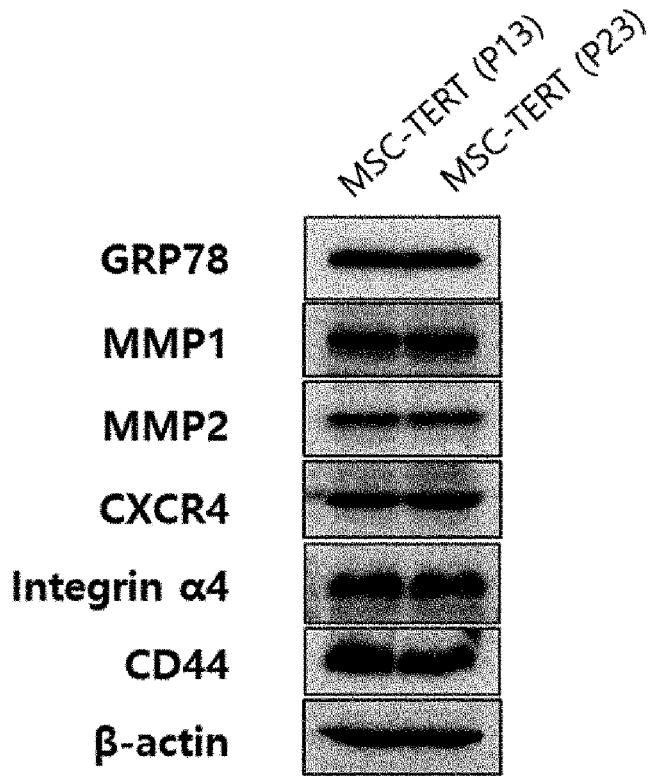
【FIG.36】



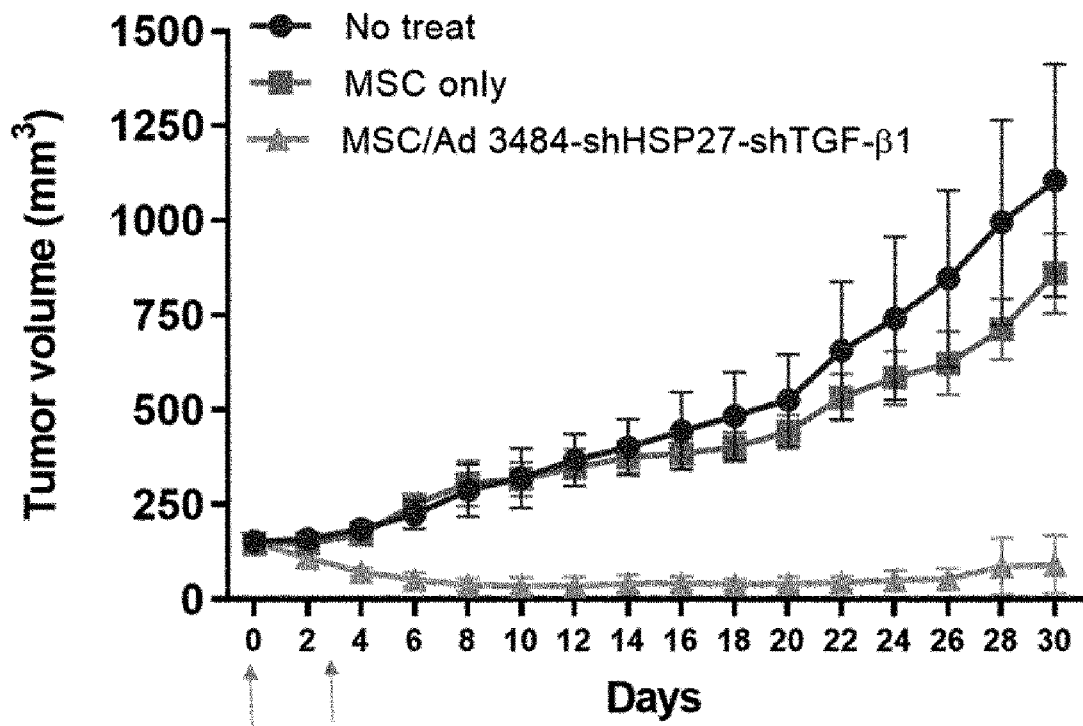
【FIG.37】



【FIG.38】

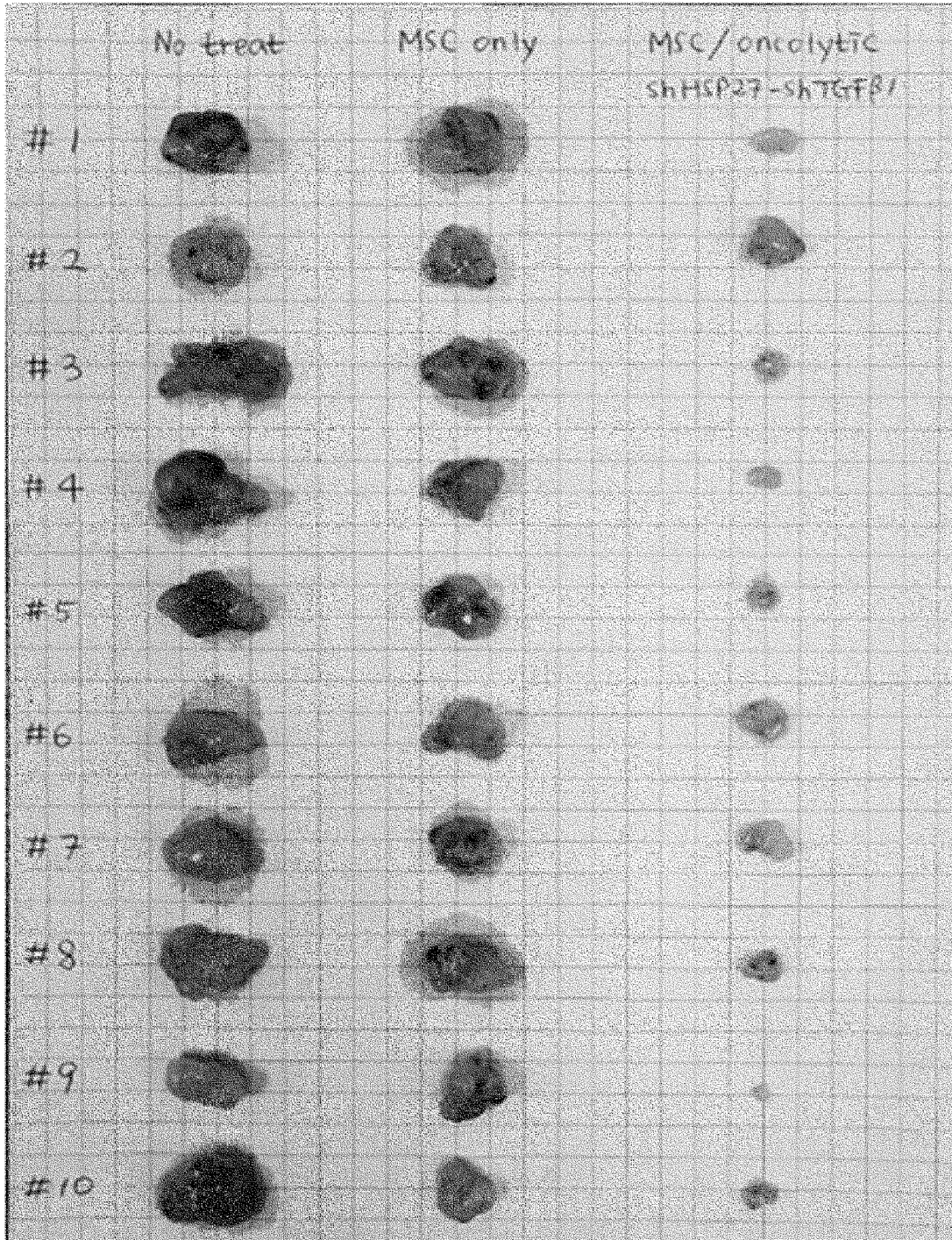


【FIG.39】



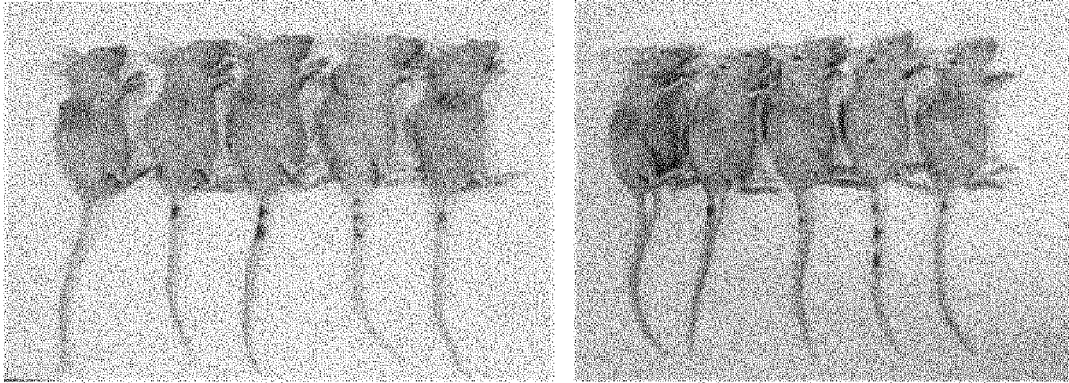
【FIG.40a】

Size of tumor removed 30 days after OV/MS
after 10 animals per group were separated into groups

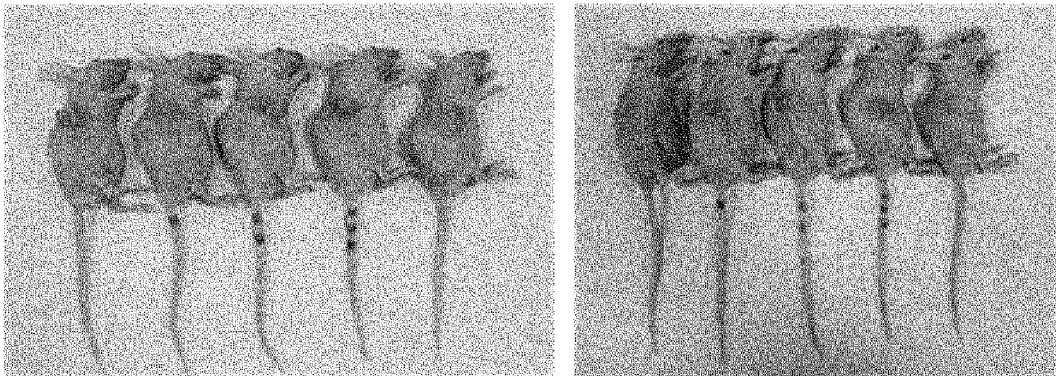


【FIG.40b】

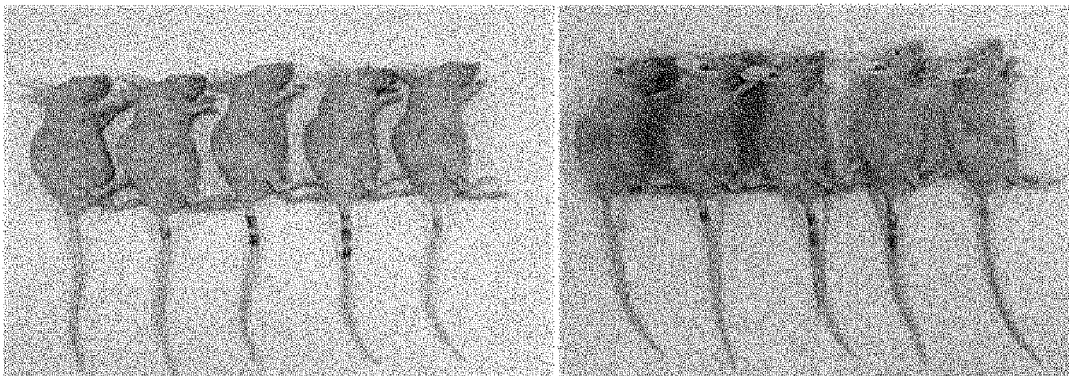
No treat



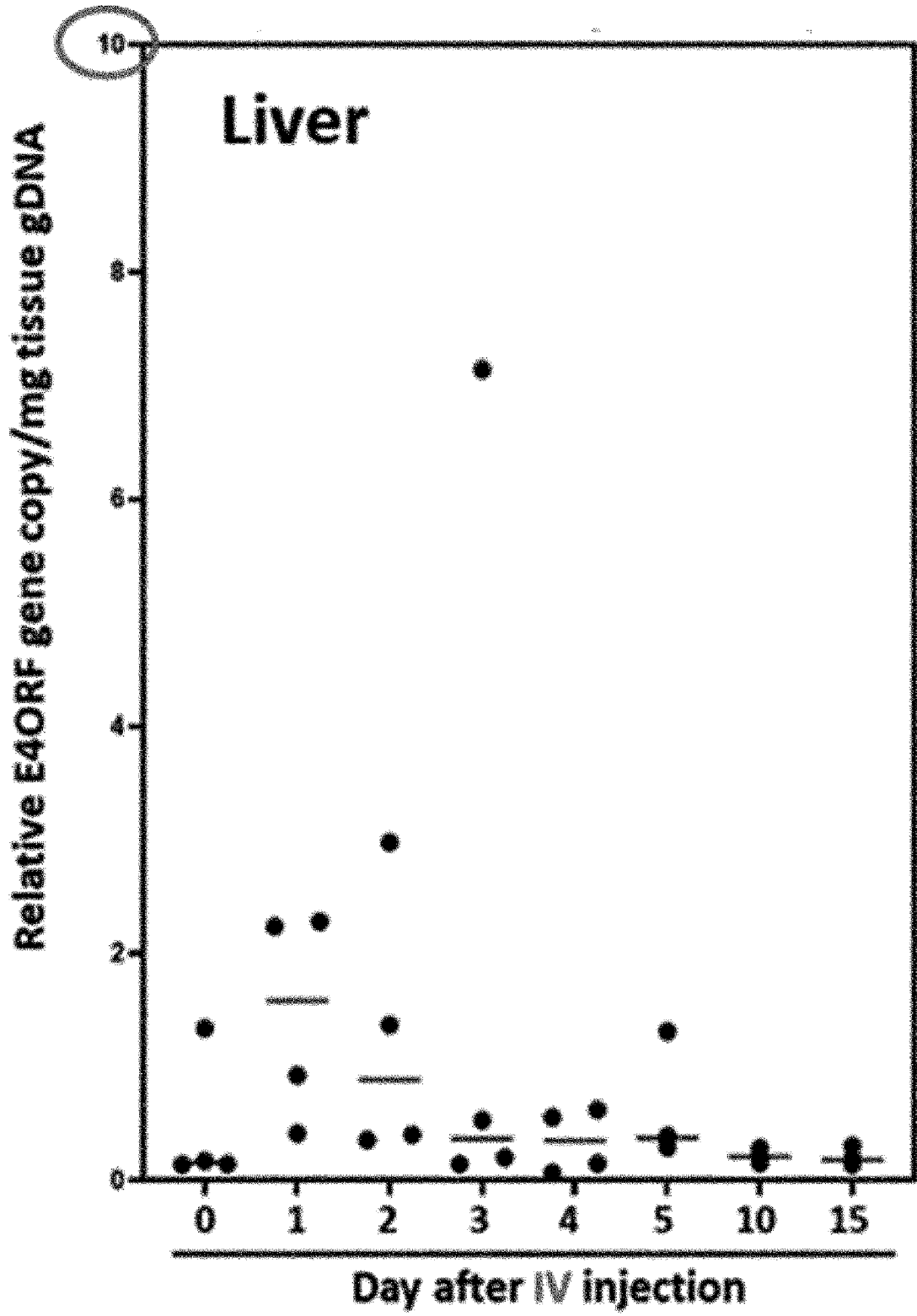
MSC only



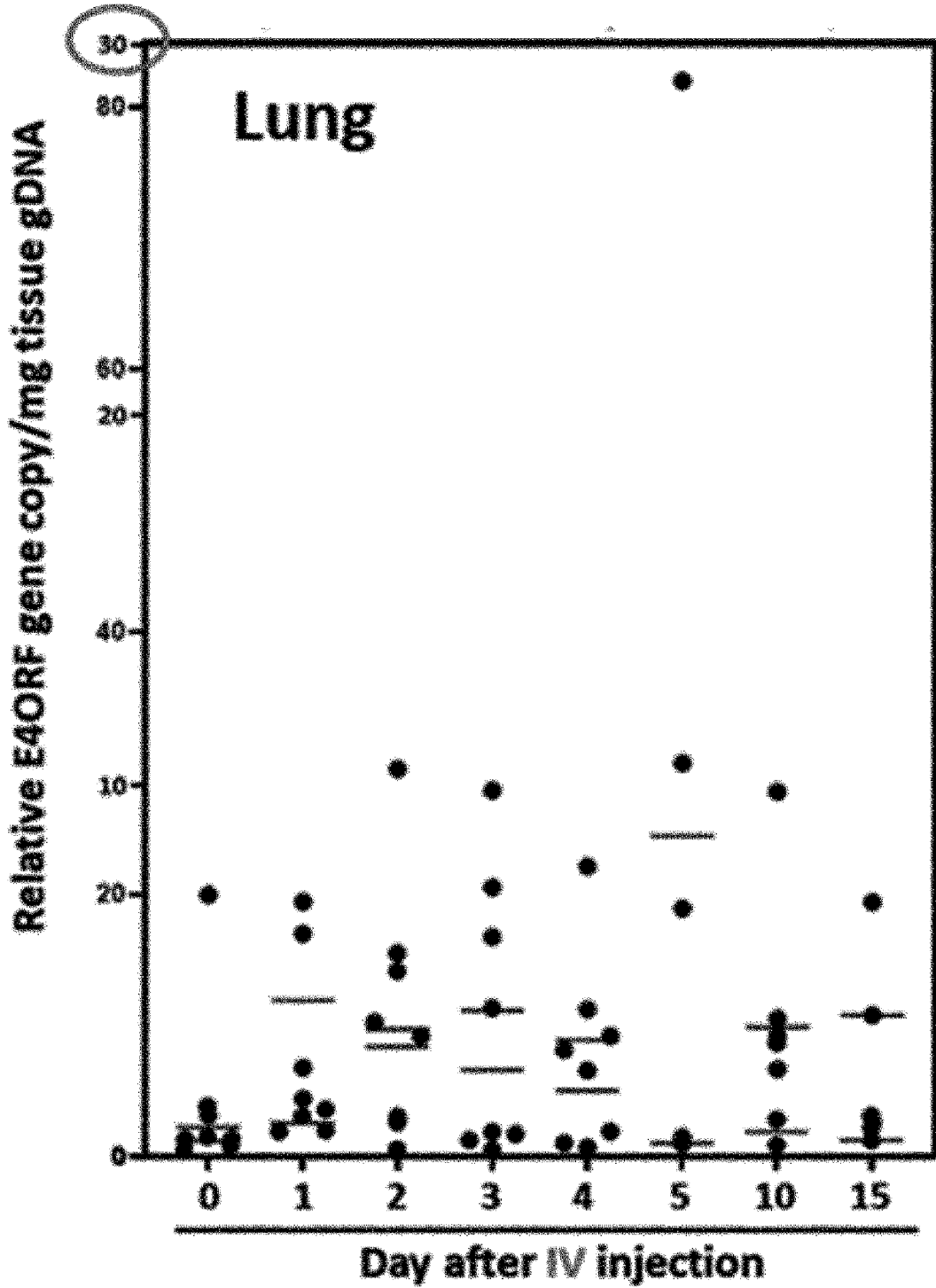
MSC/Ad-3484 shHSP27-shTGF-β1



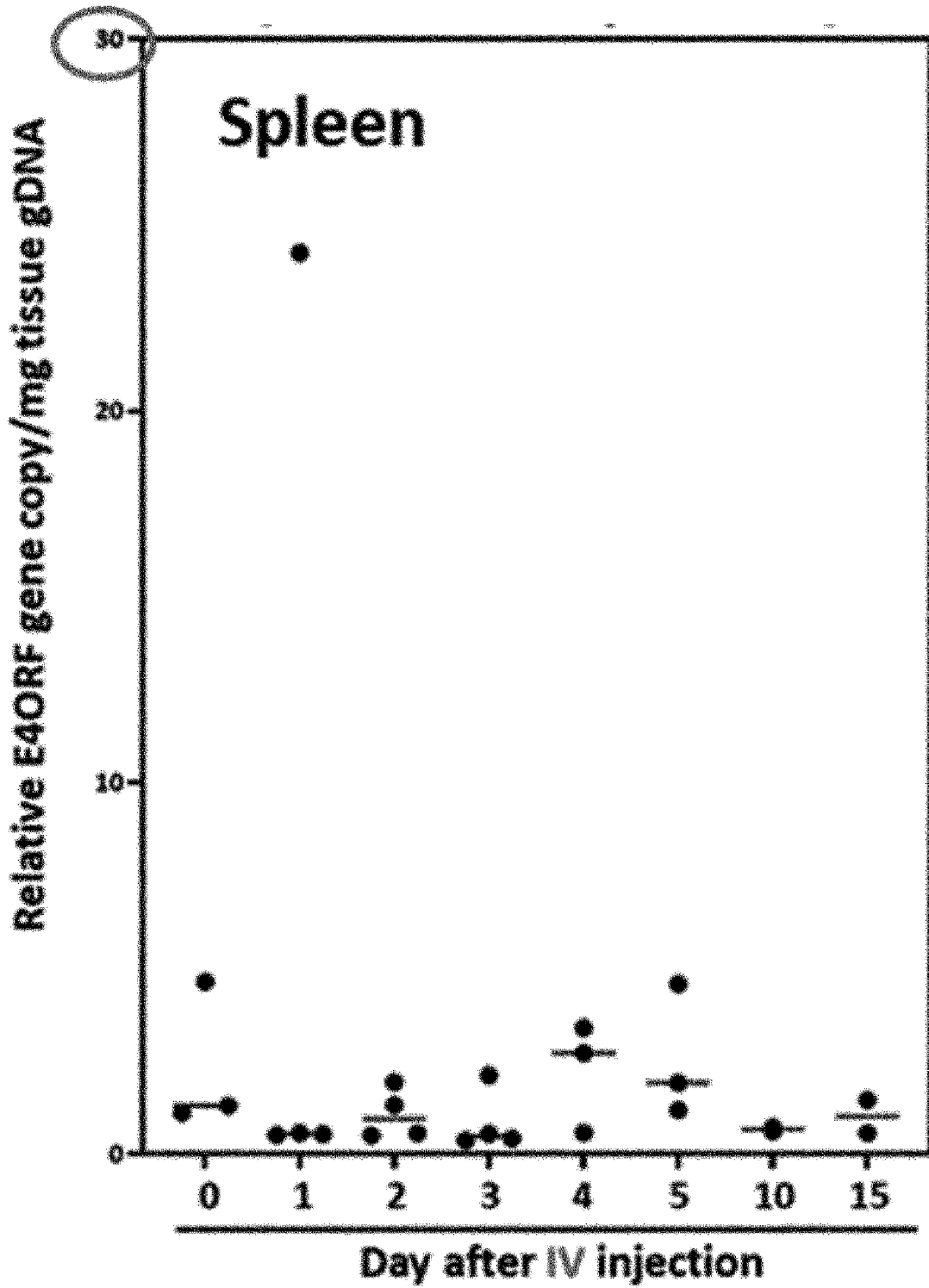
【FIG.41b】



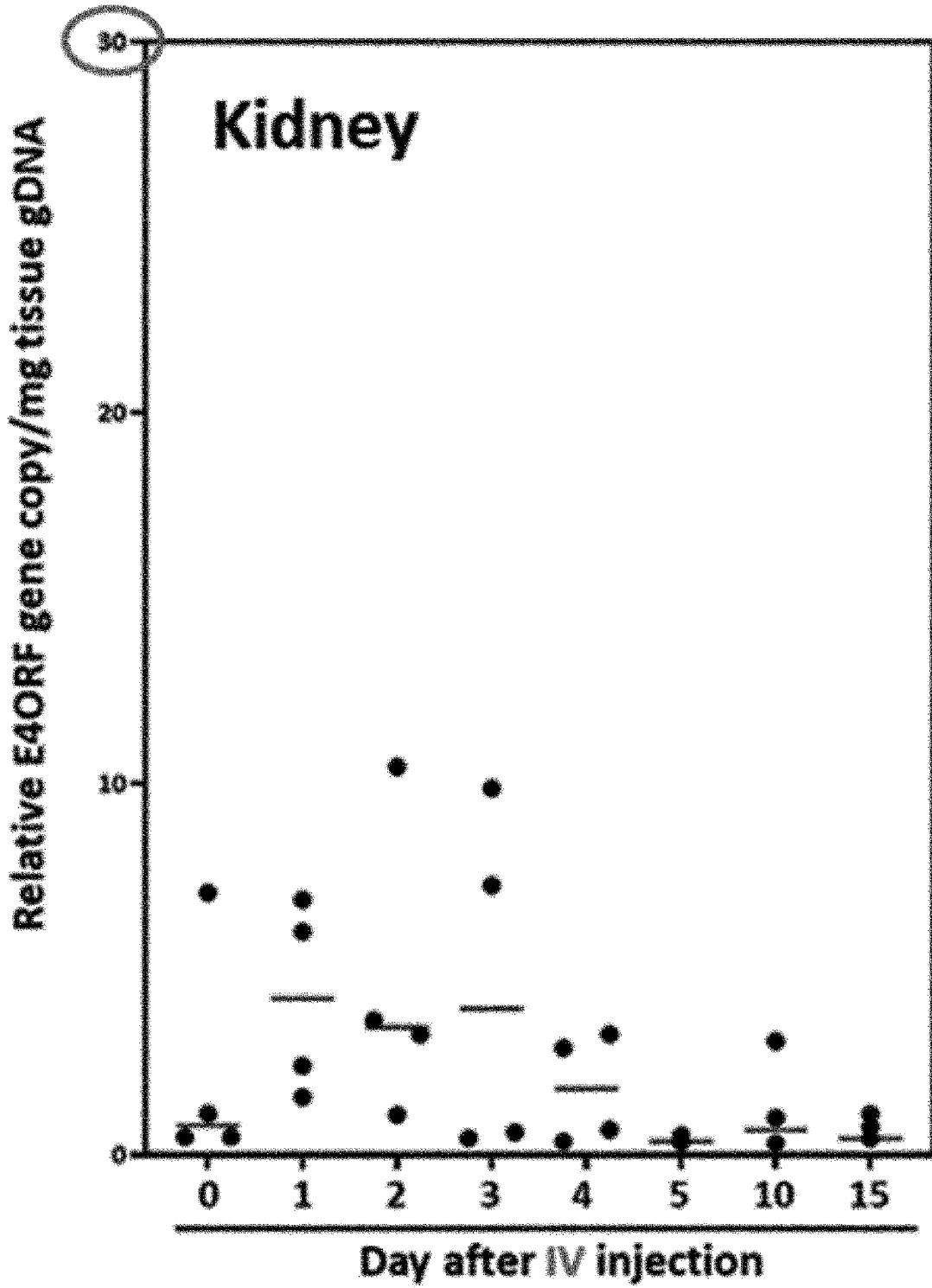
【FIG.41c】



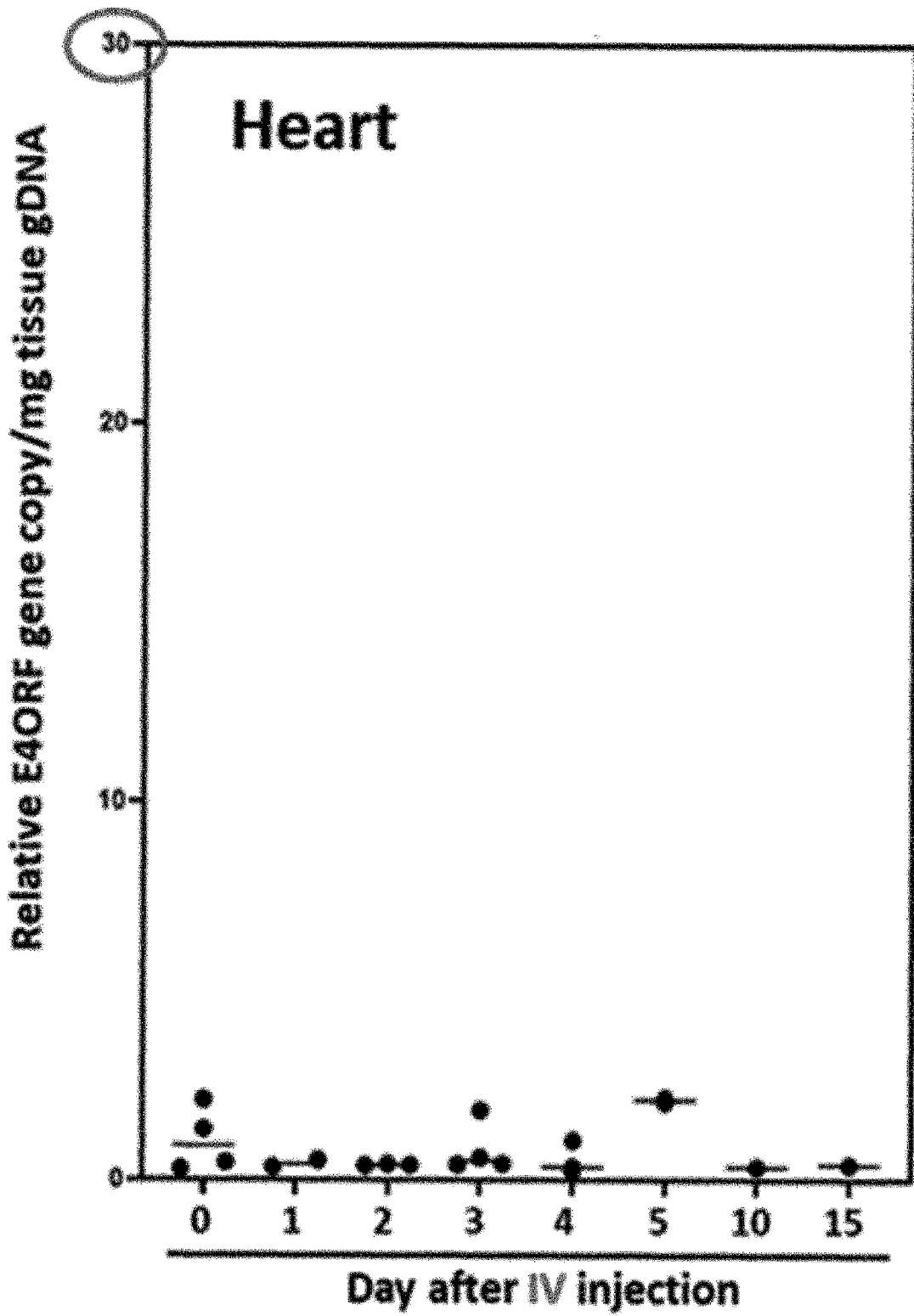
【FIG.41d】



[FIG.41e]



[FIG.41f]



INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2021/015179

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A. CLASSIFICATION OF SUBJECT MATTER
C12N 5/0775(2010.01)i; **C12N 7/00**(2006.01)i; **C12N 9/12**(2006.01)i; **A61K 35/761**(2014.01)i; **A61K 35/28**(2006.01)i; **A61P 35/00**(2006.01)i
 According to International Patent Classification (IPC) or to both national classification and IPC

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B. FIELDS SEARCHED
 Minimum documentation searched (classification system followed by classification symbols)
 C12N 5/0775(2010.01); A61K 35/28(2006.01); C12N 15/113(2010.01); C12N 5/0784(2010.01)

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
 Korean utility models and applications for utility models: IPC as above
 Japanese utility models and applications for utility models: IPC as above

20

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
 eKOMPASS (KIPO internal) & keywords: 중간엽 줄기세포 (mesenchymal stem cells), 유전자 전달 (gene delivery), 아데노 바이러스 (adenovirus), 종양 친화성 (tumor tropism), 글루코오스 조절 단백질 78 (glucose regulated protein 78, GRP78), E1B55K, 텔로머라제 역전사효소 (telomerase reverse transcriptase, TERT)

25

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y A	KR 10-2019-0139763 A (INDUSTRY-ACADEMIC COOPERATION FOUNDATION, YONSEI UNIVERSITY) 18 December 2019 (2019-12-18) See claims 1-9 and paragraphs [0016]-[0168].	2,4-16 1,3
Y	CHEN, H. et al. A novel adenoviral vector carrying an all-in-one Tet-On system with an autoregulatory loop for tight, inducible transgene expression. BMC Biotechnology. 2015, vol. 15, article no. 4, inner pp. 1-8. See abstract and inner page 1.	2,4-16
Y	SIMONSEN, J. L. et al. Telomerase expression extends the proliferative life-span and maintains the osteogenic potential of human bone marrow stromal cells. Nature Biotechnology. 2002, vol. 20, pp. 592-596. See abstract, page 593, left column; and figure 2.	4

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Further documents are listed in the continuation of Box C. See patent family annex.

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* Special categories of cited documents:
 "A" document defining the general state of the art which is not considered to be of particular relevance
 "D" document cited by the applicant in the international application
 "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

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Date of the actual completion of the international search **28 January 2022**
 Date of mailing of the international search report **28 January 2022**

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Name and mailing address of the ISA/KR
Korean Intellectual Property Office
Government Complex-Daejeon Building 4, 189 Cheongsaro, Seo-gu, Daejeon 35208
 Facsimile No. **+82-42-481-8578**
 Authorized officer
 Telephone No.

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2021/015179

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C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KIA, A. et al. Dual Systemic Tumor Targeting with Ligand-Directed Phage and Grp78 Promoter Induces Tumor Regression. <i>Molecular Cancer Therapy</i> . 2012, vol. 11, no. 12, pp. 2566-2577. See abstract, page 2571, right column, page 2572, left column; and figures 4 and 5.	1-16
A	KR 10-2017-0067667 A (INDUSTRY-ACADEMIC COOPERATION FOUNDATION, YONSEI UNIVERSITY) 16 June 2017 (2017-06-16) See claims 1-13.	1-16
A	KR 10-2019-0070890 A (IUCF-HYU (INDUSTRY-UNIVERSITY COOPERATION FOUNDATION HANYANG UNIVERSITY)) 21 June 2019 (2019-06-21) See claim 3 and paragraph [0016].	1-16

INTERNATIONAL SEARCH REPORT

International application No.

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Box No. I	Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)
10	<p>1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:</p> <p>a. <input checked="" type="checkbox"/> forming part of the international application as filed:</p> <p style="padding-left: 20px;"><input checked="" type="checkbox"/> in the form of an Annex C/ST.25 text file.</p> <p style="padding-left: 20px;"><input type="checkbox"/> on paper or in the form of an image file.</p> <p>b. <input type="checkbox"/> furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.</p> <p>c. <input type="checkbox"/> furnished subsequent to the international filing date for the purposes of international search only:</p> <p style="padding-left: 20px;"><input type="checkbox"/> in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).</p> <p style="padding-left: 20px;"><input type="checkbox"/> on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).</p> <p>2. <input type="checkbox"/> In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.</p> <p>3. Additional comments:</p>
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Form PCT/ISA/210 (continuation of first sheet) (July 2019)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2021/015179

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

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

10

1. Claims Nos.: **17,18**
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 17 and 18 pertain to a method for treatment of the human body by surgery or therapy, as well as a diagnostic method (PCT Article 17(2)(a)(i) and PCT Rule 39.1(iv)).
2. Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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This International Searching Authority found multiple inventions in this international application, as follows:

Claims 1, 4-6 and 9-16 pertain to a mesenchymal stem cell for delivering an oncolytic virus, into which a GRP78 gene is introduced, having improved tumor targeting ability,
Claims 2 and 4-16 pertain to a mesenchymal stem cell for delivering an oncolytic virus, into which an E1B55K gene is introduced, to induce the expression thereof by means of an expression-inducing substance,
Claims 3-16 pertain to a mesenchymal stem cell for delivering an oncolytic virus comprising a GRP78 gene and an E1B55K gene of which the expression is induced by means of an expression-inducing substance.

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1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

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- Remark on Protest**
- The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
 - The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
 - No protest accompanied the payment of additional search fees.

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INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/KR2021/015179

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Patent document cited in search report	Publication date (day/month/year)	Patent family member(s)	Publication date (day/month/year)
KR 10-2019-0139763 A	18 December 2019	KR 10-2169798 B1 WO 2019-235771 A1 WO 2019-235771 A9	26 October 2020 12 December 2019 12 December 2019
KR 10-2017-0067667 A	16 June 2017	CN 109072253 A EP 3388522 A1 EP 3388522 B1 JP 2019-502375 A JP 6898325 B2 KR 10-1860233 B1 US 2018-0360996 A1 US 2021-0154329 A1 WO 2017-099474 A1	21 December 2018 17 October 2018 12 August 2020 31 January 2019 07 July 2021 21 May 2018 20 December 2018 27 May 2021 15 June 2017
KR 10-2019-0070890 A	21 June 2019	CN 111630159 A EP 3725875 A1 JP 2021-506267 A KR 10-2167934 B1 SG 11202005509 A US 2021-0069253 A1 WO 2019-117632 A1	04 September 2020 21 October 2020 22 February 2021 20 October 2020 29 July 2020 11 March 2021 20 June 2019

Form PCT/ISA/210 (patent family annex) (July 2019)

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- KR 102169798 [0010]
- KR 20160166171 [0045] [0077] [0078]

Non-patent literature cited in the description

- **SILVA, N et al.** Double trouble for tumors: Exploiting the tumor microenvironment to enhance anticancer effect of oncolytic viruses. *Cytokine & Growth Factor Reviews*, 2010, vol. 21, 135 [0011]
- **XIA, X et al.** Mesenchymal stem cells as carriers and amplifiers in CRAd delivery to tumors. *Molecular Cancer*, 2011, vol. 10, 134 [0011]
- **KERRIGAN BCP ; SHIMIZU Y ; ANDREEFF M ; LANG FF.** Mesenchymal stromal cells for the delivery of oncolytic viruses in gliomas. *Cytotherapy*, 2017, vol. 19, 445-457 [0011]
- **NAKASHIMA H ; KAUR B ; CHIOCCA EA.** Directing systemic oncolytic viral delivery to tumors via carrier cells. *Cytokine Growth Factor Rev*, 2010, vol. 21, 119-126 [0011]