



(51) International Patent Classification:

C12N 5/0735 (2010.01) C12N 5/10 (2006.01)
C12N 5/074 (2010.01)

(21) International Application Number:

PCT/SG2016/050190

(22) International Filing Date:

22 April 2016 (22.04.2016)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

10201503148P 22 April 2015 (22.04.2015) SG

(71) Applicant: NATIONAL UNIVERSITY OF SINGAPORE [SG/SG]; 21 Lower Kent Ridge Road, Singapore 119077 (SG).

(72) Inventors: **LI, Shang**; c/o National University of Singapore, Duke-NUS Graduate Medical School Singapore, 21 Lower Kent Ridge Road, Singapore 119077 (SG). **LIU, Chang Ching**; c/o National University of Singapore,

Duke-NUS Graduate Medical School Singapore, 21 Lower Kent Ridge Road, Singapore 119077 (SG). **TAN, Patrick Boon Ooi**; c/o National University of Singapore, Duke-NUS Graduate Medical School Singapore, 21 Lower Kent Ridge Road, Singapore 119077 (SG). **GOH, Eyleen Lay Keow**; c/o National University of Singapore, Duke-NUS Graduate Medical School Singapore, 21 Lower Kent Ridge Road, Singapore 119077 (SG). **MA, Dongliang**; c/o National University of Singapore, Duke-NUS Graduate Medical School Singapore, 21 Lower Kent Ridge Road, Singapore 119077 (SG).

(74) Agent: **MATTEUCCI, Gianfranco**; Marks & Clerk Singapore LLP, Tanjong Pagar, P.O. Box 636, Singapore 910816 (SG).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG,

[Continued on next page]

(54) Title: TARGETING TELOMERASE FOR CELL THERAPY

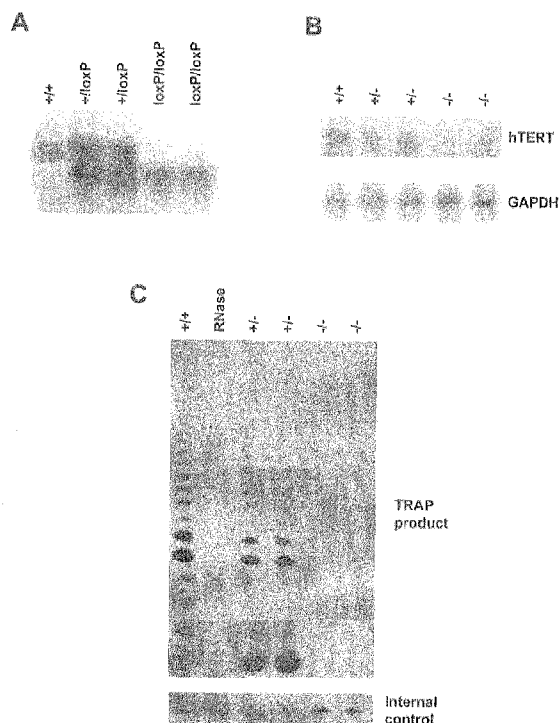


Figure 5

(57) Abstract: The present invention relates to stem cell technology. More particularly, the present invention relates to a method for engineering telomerase null pluripotent stem cells. These telomerase null stem cells remain pluripotent and have a limited life span and reduced tumor formation potential in vivo, which suggests they are useful for cell therapy.



MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE,

Published:

- with international search report (Art. 21(3))
- with sequence listing part of description (Rule 5.2(a))

TARGETING TELOMERASE FOR CELL THERAPY

FIELD OF THE INVENTION

The present invention relates to stem cell technology. More particularly, the present invention relates to inducing telomerase knockout in stem cells to limit their life span whilst retaining pluripotency until differentiated. As a result they can be banked with a predetermined lifespan and have reduced ability to cause tumor formation if used in therapy.

BACKGROUND OF THE INVENTION

Since the isolation of human embryonic stem (ES) cells, the potential of stem cells' unlimited renewal capacity and their pluripotency to differentiate into any cell type have opened up the possibility of cell therapy and disease modelling (Thomson, J. A. *et al. Science* 282, 1145-1147 (1998)). The development of human induced pluripotent stem cells (iPSCs) further established personalized cell therapy by providing a solution to overcome the immune rejection of cells transplanted into unmatched patients (Takahashi, K. *et al. Cell* 131, 861-872 (2007); Takahashi, K. & Yamanaka, S. *Cell* 126, 663-676 (2006); Yu, J. *et al. Science* 318, 1917-1920 (2007)).

However, stem cell-based therapy also brings new safety challenges. One major concern is the tumorigenic potential of stem cells. Pluripotent stem cells share cellular and genetic similarity with tumor cells; such as unlimited potential for cell proliferation, rapid cell proliferation rate and a propensity for genomic instability when cultured *in vitro* (Baker, D. E. *et al. Nat Biotechnol* 25, 207-215 (2007)). Such pluripotent stem cells can form teratomas when injected into immunodeficient mice (Blum, B. & Benvenisty, N. *Stem Cells* 25, 1924-1930 (2007)), and as little as a few hundred pluripotent stem cells are sufficient (Lee, A. S. *et al. Cell Cycle* 8, 2608-2612 (2009); Hentze, H. *et al. Stem cell research* 2, 198-210 (2009)). Furthermore, if the stem cells contain genetic mutations they may potentially develop into more malignant teratocarcinomas (Blum, B. & Benvenisty, N. *Advances in cancer research* 100, 133-158 (2008)), raising the legitimate concern of safety in clinical applications. A recent report indicates the possibility of donor-derived tumor development

following transplantation of undifferentiated neural stem cells (Amariglio, N. *et al. PLoS medicine* 6 (2009)).

In addition to tumorigenicity, undifferentiated stem cells show a high propensity to accumulate chromosome aberrations during *in vitro* culture. The differentiated cells derived from stem cells may themselves also acquire genetic mutations and tumorigenicity during *in vitro* culture. Therefore, the technical burden of separating differentiated cells from undifferentiated stem cells, as well as ensuring the genetic stability of differentiated cells, remain major challenges for the clinical application of cell therapy.

An unlimited proliferation potential is a hallmark of cancer and is shared by stem cells. In order to proliferate continuously, cells need to find a way to maintain their telomere, a special nucleoprotein complex found at the ends of human linear chromosomes (Blackburn, E. H. *Nature* 408, 53-56 (2000)). Human chromosome ends are capped by telomeres that contain long six-nucleotide DNA repeats 5'-TTAGGG-3' with single stranded 3' G-rich overhangs. The telomeric DNA repeats are bound by shelterin protein complexes consisting of TRF1, TRF2, RAP1, TIN2, TPP1 and POT1 that distinguish naturally occurring chromosomal ends from DNA double-strand breaks. Therefore, telomeres are essential for cell genomic stability. Telomeres are synthesized by telomerase, a reverse transcriptase that contains two core components: a catalytic protein, hTERT, and a RNA, hTER (Feng, J. *et al. Science* 269, 1236-1241 (1995); Nakamura, T. M. *et al. Science* 277, 955-959 (1997)). Although the telomerase RNA (hTER) is widely expressed, the catalytic protein hTERT and, consequently, telomerase activity are hardly detectable in the majority of adult human cells, with the exception of stem cells and germ cells (Feng, J. *et al. Science* 269, 1236-1241 (1995); Nakamura, T. M. *et al. Science* 277, 955-959 (1997); Kim, N. W. *et al. Science* 266, 2011-2015 (1994); Wright, W. E. *et al. Dev Genet* 18, 173-179 (1996)). Normal somatic cells only have a limited proliferation potential that is controlled by their telomere length (Hayflick, L. & Moorhead, P. S. *Exp Cell Res* 25, 585-621 (1961)), whereas pluripotent stem cells share unlimited proliferation capacity with cancer cells, expressing high telomerase activity for telomere maintenance (Thomson, J. A. *et al. Science* 282, 1145-1147 (1998); Takahashi, K. *et al. Cell* 131, 861-872 (2007); Takahashi, K. & Yamanaka, S. *Cell* 126, 663-676 (2006); Yu, J. *et al. Science* 318, 1917-1920 (2007)).

Previous studies in a mouse model have shown that inactivation of telomerase activity by inducible knockout of either mTERT or mTER does not result in dramatic phenotype in the first 2-3 generations, owing to the extremely long telomeres in laboratory mice (Lee, H. W. *et al. Nature* 392, 569-574 (1998); Strong, M. A. *et al. Mol Cell Biol* 31, 2369-2379 (2011)), but as the telomeres shortened over generations of breeding there was loss of tissue renewal in several proliferative organs.

It is desirable to provide a method that allows expansion of pluripotent stem cell numbers followed by inducible inhibition of telomerase activity to limit their proliferation capacity yet retain pluripotency until differentiated.

SUMMARY OF THE INVENTION

The novel strategy described herein involves engineering telomerase null stem cells with limited lifespan and reduced tumorigenic potential.

According to one aspect of the invention, there is provided at least one isolated pluripotent telomerase activity null stem cell, comprising two mutated alleles of a telomerase activity gene selected from the group comprising the human telomerase reverse transcriptase (hTERT) gene and the human telomerase RNA (hTER) gene, wherein said at least one telomerase activity null stem cell has limited lifespan and reduced tumorigenic potential.

One way of effecting the strategy described herein involves engineering inducible telomerase activity knockout stem cells, using a gene targeting approach which allows for an unlimited supply of pluripotent genetically-modified stem cells and the convenience to inactivate telomerase activity at the desired time in order to reduce the cells' life-span and reduce tumorigenicity.

A preferred embodiment of the invention provides at least one isolated pluripotent inducible telomerase activity knockout stem cell, comprising two mutated alleles of a telomerase activity gene selected from the group comprising the human telomerase reverse transcriptase (hTERT) gene and the human telomerase RNA (hTER) gene; wherein said alleles comprise an introduced and removable portion of said hTERT or hTER gene.

In a preferred embodiment of the invention the at least one isolated pluripotent inducible telomerase activity knockout stem cell has been subjected to transient over-expression of hTERT, hTER or both hTERT and hTER to increase the average telomere length.

5 Another embodiment of the invention provides at least one isolated pluripotent telomerase activity null stem cell, wherein the pluripotent inducible telomerase activity knockout stem cell described supra has been further contacted with a knockout inducer to remove said introduced removable gene portion thereby
10 resulting in at least one telomerase activity null stem cell with limited lifespan and reduced tumorigenic potential.

In a preferred embodiment of the invention there is provided the isolated pluripotent inducible telomerase activity knockout stem cell described supra, wherein said removable portion of the hTERT or hTER gene is replaced by homologous DNA flanked by LoxP sites, which retains gene function, and at a selected time transient
15 expression of Cre recombinase in the recombinant cell causes deletion of the homologous DNA resulting in loss of hTERT or hTER activity and, consequently, telomerase activity.

In another embodiment of the invention there is provided at least one isolated pluripotent telomerase activity null stem cell, wherein said telomerase activity null
20 stem cell has been produced by exposing at least one isolated pluripotent stem cell to a CRISPR-Cas or other synthetic nuclease system to knock out alleles of a telomerase activity gene selected from the group comprising the human telomerase reverse transcriptase (hTERT) gene and the human telomerase RNA subunit (hTER) gene, thereby resulting in at least one telomerase activity null stem cell with limited
25 lifespan and reduced tumorigenic potential.

In a preferred embodiment of the invention the at least one isolated pluripotent stem cell has been subjected to transient over-expression of hTERT, hTER or both hTERT and hTER to increase the average telomere length prior to exposure to CRISPR-Cas or other synthetic nuclease system.

Another aspect of the invention provides a method of producing one or more isolated pluripotent telomerase activity null stem cells with limited lifespan and reduced tumorigenicity compared to wild type cells, comprising the steps of:

(a)(i) engineering, by gene targeting, at least one isolated pluripotent stem cell to have inducible knockout alleles of a telomerase activity gene selected from the group comprising the human telomerase reverse transcriptase (hTERT) gene and the human telomerase RNA subunit (hTER) gene;

(a)(ii) selecting engineered pluripotent stem cell clones;

(a)(iii) contacting the engineered pluripotent stem cell clones with a gene knockout inducer; or

(b)(i) engineering, by gene editing, at least one isolated pluripotent stem cell having knocked out alleles of a telomerase activity gene selected from the group comprising the human telomerase reverse transcriptase (hTERT) gene and the human telomerase RNA subunit (hTER) gene; and

(c) selecting telomerase activity null cells with an average telomere length evaluated to be 5 kb or less and a reduced ability to form a teratoma in vivo; or

(d) selecting telomerase activity null cells and passaging them until the average telomere length is evaluated to be 5 kb or less and the cells having reduced ability to form a teratoma in vivo.

Another aspect of the invention provides a method of producing one or more isolated pluripotent telomerase activity null stem cells suitable for clinical use, comprising: evaluating the average telomere length and/or in vivo tumorigenicity of said pluripotent stem cells in which the human telomerase reverse transcriptase (hTERT) or the human telomerase RNA (hTER) activity has been inactivated; and identifying said pluripotent stem cells as suitable for clinical use if said evaluated average telomere length is about 5 kb or less and/or said cells have reduced ability to form a teratoma in vivo.

Another aspect of the invention provides the use of at least one isolated pluripotent telomerase activity null stem cell as described supra for the preparation of a therapeutic composition for cell therapy of a subject in need thereof.

In a preferred embodiment of the invention, the at least one isolated pluripotent telomerase activity null stem cell is differentiated into cells of at least one of ectodermal, endodermal or mesodermal lineage.

Preferably, the at least one isolated pluripotent telomerase activity null stem cell is differentiated into cells of at least one lineage selected from the group comprising neurons, astrocytes and/or glia and hematopoietic cells.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the engineering of ZFNs and TALENs for gene targeting. **A).** Binding sequences of synthetic ZFNs (underlined italics) and TALENs (bold). **B).** Cel-I assay showing the double strand breaks (DSBs) (indicated with “*”) resulting from the expression of ZFNs and TALENs in different cell lines.

Figure 2 shows a schematic design of the gene targeting strategy used to introduce loxP sites flanking the exon 1 and exon 2 of hTERT genomic locus.

Figure 3 demonstrates the increased stability of the targeting vector that can be achieved with mutated ZFNs binding sites. **A).** ZFNs recognition sequences in the targeting vector were mutated by site-directed mutagenesis. **B).** The stability of targeting vectors with or without ZFNs binding sequence *in vivo*. The relative amount of intact DNA as measured by P1 primer set is normalized to the total input DNA as measured by P2 primer set. WT: wild type; Δ ZFN: mutated ZFN binding sites.

Figure 4 is a schematic representation of the gene targeting procedure in human H1 ES cells, as described in Example 3.

Figure 5 A) is a Southern blot analysis, using genomic DNA samples that were digested with XbaI and HincII restriction enzymes, confirming the targeted alleles of hTERT. The probe used for Southern blotting analysis is shown on Figure 2, to the left of the first LoxP site. **B).** shows the expression of full length hTERT in hTERT^(+/+); hTERT^(+/-) and hTERT^(-/-) ES cells, as analyzed by RNA protection assay (RPA) using ³²P labeled radioactive RNA probe encompassing hTERT exon 2. **C).** provides the results of a TRAP assay, performed using whole cell extracts from independent hTERT^(+/+); hTERT^(+/-) and hTERT^(-/-) ES cell lines to measure the telomerase activity. The RNase-treated sample was used as negative control.

Figure 6 shows telomere shortening in telomerase null human ES cells. **A).** Genomic Southern analysis of telomere length in independent $hTERT^{+/+}$, $hTERT^{+/loxP}$, $hTERT^{loxP/loxP}$ and $hTERT^{-/-}$ ES cell lines. P2, P4, P6 and P8: indicate that the $hTERT^{-/-}$ ES cells have been passaged 2, 4, 6 and 8 times, respectively. **B).** The expression of senescence-associated β -galactosidase activity in young WI38 primary lung fibroblast cells (PD35) and senescent WI38 (PD67) cells. PD: population doubling. **C).** Expression of senescence-associated β -galactosidase activity in late passage (P8) $hTERT^{-/-}$ ES cells.

Figure 7 show loss of cell proliferation capacity and increased cell death in senescent $hTERT^{-/-}$ ES cells. **A).** Incorporation of 5-ethynyl-2'-deoxyuridine (EdU) during S phase in independent $hTERT^{+/+}$, $hTERT^{+/-}$, and $hTERT^{-/-}$ ES cell lines. **B).** Cell viability was measured using CellTiter-Glo® luminescent assay in independent $hTERT^{+/+}$, $hTERT^{+/-}$, and $hTERT^{-/-}$ ES cell lines. **C).** Cell cycle profile analysis of independent $hTERT^{+/+}$, $hTERT^{+/-}$, and $hTERT^{-/-}$ ES cell lines using FACS. **D).** Annexin V and 7-AAD staining in independent $hTERT^{+/+}$, $hTERT^{+/-}$, and $hTERT^{-/-}$ ES cell lines.

Figure 8 demonstrates that transient overexpression of $hTERT$ in $hTERT^{loxP/loxP}$ ES cells resets telomere length.

Figure 9 shows telomere shortening of $hTERT^{-/-}$ ES cells derived from $hTERT^{loxP/loxP}$ ES cells whose telomere length was reset by transient overexpression of $hTERT$. OE: overexpression; P1, P5 and P10: indicate the number of passages.

Figure 10 provides karyotyping of human ES cell lines, which shows that the cells maintained a normal karyotype.

Figures 11A-D show immunocytochemistry analysis of the expression of ES cell markers in $hTERT$ inducible knockout $hTERT^{+/+}$, $hTERT^{+/-}$ and $hTERT^{-/-}$ ES cells: **A).** TRA-1-60; **B).** SSEA-4; **C).** OCT-4; and **D).** NANOG. The cell nuclei were stained with DAPI.

Figure 11E sets out the expression of *OCT-4*, *SOX-2*, *KLF-4*, and *NANOG* in independent $hTERT^{+/+}$, $hTERT^{+/-}$ and $hTERT^{-/-}$ ES cell lines, as quantified by qRT-PCR and normalized to the expression of GAPDH. The expression level of *OCT-4*, *SOX-2*, *KLF-4*, and *NANOG* in independent $hTERT^{+/+}$ is indicated by the dotted line.

Figures 12A-12E demonstrate spontaneous differentiation of human hTERT^(+/+) and hTERT^(-/-) ES cells *in vitro* into cell lineages of all three germ layers. Representative confocal images showing pluripotency of ES cells in culture, capable of differentiating into cells of the 3 specific lineages, are provided: **A).** and **B).** ectoderm: markers Tuj1 and GFAP, respectively; **C).** and **D).** mesoderm: markers Desmin and α -SMA, respectively; and **E).** endoderm: marker AFP.

Figure 13 A). shows the different teratoma formation efficiency *in vivo* of independent hTERT^(+/+) (+/+); hTERT^(+/-) (+/-), and three different hTERT^(-/-) (-/-) ES cell lines. When the telomere of the hTERT^(-/-) ES cell line is 3.5 kb, the ability of the ES cells to form teratomas *in vivo* is completely suppressed. **B).** shows H&E staining of teratomas derived from independent hTERT^(+/+); hTERT^(+/-), and hTERT^(-/-) ES cell lines. Endoderm: respiratory epithelium (e); Mesoderm: cartilage (c) and skeletal muscle (m); Ectoderm: neural epithelium with rosettes (n). Scale bars, 200 μ m.

Figure 14 A). is a schematic diagram showing the schedule for induction of neural progenitor cells (NPC) from human ES cells.

Figures 14B-14E show the differentiation of telomerase null human ES cells to neural lineages *in vitro*. **B).** provides representative confocal images showing progenitor cells (identified by Nestin and Sox2 staining) induced from hTERT^(+/+); hTERT^(+/-), and hTERT^(-/-) ES cells. **C).** provides representative confocal images showing the differentiation of these 3 groups of ES cells to neurons, identified by DCX staining; HuN: human nuclei; **D).** is a graphical representation of the percentage of DCX positive neurons (total DAPI positive cells) that are differentiated from neuronal progenitor cells induced from the different groups of ES cells, as indicated. **E).** provides representative confocal images showing the differentiation of these different groups of ES cells to astrocytes, marked by GFAP staining.

Figures 15A-F shows effects of injection of hTERT^(+/+) (with mean telomere length around 13 kb) and hTERT^(-/-) ES cells (with mean telomere length around 3.5 kb) into mouse brain. Representative images showing immunocytochemistry of brain sections from mice injected with: **A)-B)** hTERT^(+/+) ES cells; **C)-D)** hTERT^(-/-) ES cells. **E).** shows the gross morphology of brains from mice injected with hTERT^(+/+) ES cells or hTERT^(-/-) ES cells. Tumor formation was visible in hTERT^(+/+) ES but not hTERT^(-/-) ES-injected mouse brains. n=5 is the number of mice injected with hTERT^(+/+) ES cells

or hTERT^{-/-} ES cells. **F).** provides representative confocal images showing human nuclei (HuN) staining of tumors in hTERT^(+/+) ES cells-injected mouse.

Figure 16 includes representative confocal images showing lack of tumor formation but, instead, *in vivo* differentiation of hTERT^(-/-) ES cells (with mean telomere length around 3.5 kb) into mature neurons. **A).** demonstrates that there is no tumor formation in immunodeficient mouse brain injected with hTERT^(-/-) ES cells at 8 and 16 weeks post-injection. **B).** shows the expression of mature neuronal marker, MAP2a, in hTERT^(-/-) ES cell-derived neuronal cells *in vivo*. **C).** shows the expression of TH, a marker for dopaminergic neurons, in hTERT^(-/-) ES cell-derived neuronal cells *in vivo* (marked by arrows).

Figure 17 shows the engineering of telomerase-null ES cells (WA018) using the CRISPR-Cas9 system. **A)** Shows sequences (S1-S5) of five independent CRISPR guide RNAs targeting hTERT exon 1. **B)** Cel-I assay showing DNA cleavage induced by the five independent CRISPR guide RNAs S1-S5. **C)** Schematic of paired CRISPR guide RNAs (S2 and S3) used for gene targeting. **D)** Shows Sanger sequencing results confirming DSB-induced insertion/deletion (indel) mutations in three independent ES cell clones derived from parental WA018 ES cells. **E)** TRAP assay showing complete loss of telomerase activity in three independent telomerase-null ES cell clones. Parental WA018 ES cells and one isogenic hTERT^{+/+} ES cell clones are positive controls. **F)** Shows the median telomere length in hTERT^{-/-} ES cells is very short compared to parental WA018 ES cells and isogenic hTERT^{+/+} ES cells.

Definitions

The term "induced pluripotent stem cell" (iPSC) refers to a pluripotent stem cell derived from a non-pluripotent cell (e.g. an adult somatic cell). Induced pluripotent stem cells are identical to embryonic stem cells in the ability to form any adult cell, but are not derived from an embryo.

As used herein, the term "pluripotent" refers to the potential of a stem cell to make any differentiated cell of an organism. Pluripotent stem cells can give rise to any foetal or adult cell type. However, alone they cannot develop into a foetal or

adult organism because they lack the potential to contribute to extraembryonic tissue, such as the placenta.

As used herein, the term "Cre" refers to Cre recombinase; a tyrosine recombinase enzyme derived from the P1 Bacteriophage. The enzyme uses a
5 topoisomerase I like mechanism to catalyse the site specific recombination event between two DNA recognition (LoxP) sites.

As used herein, the term "CRISPR-Cas" system refers to a microbial adaptive immune system that uses RNA-guided nucleases to cleave foreign genetic elements. It comprises clustered regularly interspaced short palindromic repeats (CRISPRs), a
10 CRISPR-associated (Cas) endonuclease and a synthetic guide RNA that can be programmed to identify and introduce a double strand break at a specific site within a targeted gene sequence. The palindromic repeats are interspaced by short variable sequences derived from exogenous DNA targets known as protospacers, and together they constitute the CRISPR RNA (crRNA) array. Within the DNA target,
15 each protospacer is always associated with a protospacer adjacent motif (PAM), which can vary depending on the specific CRISPR system. CRISPR-Cas9 is a specific version of the system referring to use of RNA-guided Cas9 nuclease, originally derived from *Streptococcus pyogenes*, whereby the target DNA must immediately precede a 5'-NGG PAM. Variations of the CRISPR-Cas9 system are
20 known [Ran FA, et al., *Nat. Protoc* 8, 2281-2308 (2013); Ran FA, et al., *Cell* 154, 1380-1389 (2013)] and although CRISPR-Cas9 has been used herein in the Examples, it is not intended that the present invention be limited to a particular CRISPR-Cas system.

As used herein, the term "FLP" refers to FLP recombinase; derived from the
25 baker's yeast *Saccharomyces cerevisiae*. The enzyme catalyses a site-specific recombination event between two DNA recognition (FRT) sites.

As used herein, the term "FRT" refers to a flippase recognition target (FRT) site. The 34bp minimal FRT site sequence has the sequence 5'GAAGTTCCTATTCTCTAGAAAGTATAGGAAGTTC3' (SEQ ID NO: 1), wherein
30 (FLP) binds to both 13-bp 5'-GAAGTTCCTATTC-3' arms flanking the 8 bp spacer.

As used herein, the term "LoxP" refers to locus of X-over P1; a site on the bacteriophage P1 consisting of 34 base pairs (bp). The site includes an asymmetric 8 bp sequence, variable except for the middle two bases, in between two sets of palindromic 13 bp sequences. The sequence is
5 ATAACTTCGTATANNNTANNNTATACGAAGTTAT (SEQ ID NO: 2), wherein 'N' indicates bases which may vary.

As used herein, the term "Telomerase reverse transcriptase" (abbreviated to TERT, or hTERT in humans) is a catalytic protein component (or subunit) of the ribonucleoprotein enzyme telomerase which, together with the telomerase RNA
10 component (hTER), comprises the most important unit of the telomerase complex.

As used herein, the term "Telomerase RNA" (abbreviated to TERC, TER, hTER, TRC3, TR and hTR) is an RNA, found in eukaryotes that is a component (or subunit) of telomerase, that contains a short segment that provides the template for telomere repeat synthesis.

As used herein, the term 'comprising' does not preclude the presence of
15 additional steps or substances in the methods and compositions, respectively, of the invention, and is understood to include within its scope the terms 'consisting of' and 'consisting essentially of' features defined in the claimed invention.

DETAILED DESCRIPTION OF THE INVENTION

According to one aspect of the invention, there is provided at least one
20 isolated pluripotent telomerase activity null stem cell, comprising two mutated alleles of a telomerase activity gene selected from the group comprising the human telomerase reverse transcriptase (hTERT) gene and the human telomerase RNA (hTER) gene, wherein said telomerase activity null stem cell has limited lifespan and
25 reduced tumorigenic potential.

It would be understood that the specific type of mutation or alteration of the hTERT or hTER gene for the purpose of the invention is not intended to be limited, other than the mutation effects loss of telomerase activity in said stem cell. The mutation may be a substitution, deletion, insertion or any modification of one or more
30 nucleotides, or combination thereof, providing it results in loss of activity.

One way of effecting the strategy described herein involves engineering inducible telomerase activity knockout stem cells, using a gene targeting approach, which allows for an unlimited supply of pluripotent genetically-modified stem cells and the convenience to inactivate telomerase activity at the desired time in order to reduce the cells' life-span and reduce tumorigenicity.

According to a preferred embodiment, there is provided at least one isolated inducible telomerase activity knockout pluripotent stem cell, comprising two mutated alleles of a telomerase activity gene selected from the group comprising the human telomerase reverse transcriptase (hTERT) gene and the human telomerase RNA (hTER) gene; wherein said alleles comprise an introduced and removable portion of said hTERT or hTER gene.

The generation of such inducible telomerase activity knockout pluripotent stem cells tends to result in the cells having a reduced average telomere length compared to wild type cells.

The inventors have found that mutating both alleles of the cells by introducing a knockout cassette may reduce the average telomere length to around 5 kb. Once the telomerase activity gene is knocked out the average telomere length may be around 4 kb which may equate to around 20-30 cell divisions remaining before senescence. It may be advantageous to increase the average telomere length in the mutated cells so that, once the activity of the said mutated hTERT or hTER gene is knocked out, the null cells have an increased lifespan so they can be expanded to derive enough cells for clinical use.

In a preferred embodiment of the invention, the at least one isolated inducible telomerase activity knockout stem cell has been further subjected to transient overexpression of hTERT, hTER or both hTERT and hTER to increase the average telomere length. The average telomere length may be increased to any size between about 6 kb and the approximately 12-14 kb or longer of the parental H1 cells. An advantage of increasing the average telomere length of the isolated inducible knockout cells is that it is then possible to generate telomerase activity null cells that can be passaged until they have a desired average telomere length and, consequently, a desired fixed number of cell divisions remaining. This allows for banking of cells from a single cell line that have different set lifespans.

A preferred embodiment relates to an isolated inducible telomerase activity knockout stem cell as described above, wherein after transient overexpression of hTERT or hTER the average telomere length in the stem cell is increased to at least 6 kb.

- 5 More preferably, the average telomere length in the isolated inducible telomerase activity knockout stem cell is increased to at least 9 kb.

Another aspect of the invention relates to at least one isolated pluripotent telomerase activity null stem cell, comprising:

- 10 at least one inducible telomerase activity knockout pluripotent stem cell comprising an introduced and removable portion of a hTERT or hTER gene which has been contacted with a knockout inducer to remove said introduced removable gene portion;

wherein said telomerase activity null stem cell has limited lifespan and reduced tumorigenic potential.

- 15 In a preferred embodiment of the invention, said portion of the hTERT or hTER gene is replaced by homologous DNA flanked by LoxP sites, which retains gene function, and at a selected time transient expression of Cre recombinase in the recombinant cell causes deletion of the homologous DNA resulting in loss of hTERT or hTER activity and, consequently, telomerase activity.

- 20 It would be understood by the person skilled in the art that other systems may be used instead of Cre/LoxP to create inducible knockout. For example, the LoxP sites could be substituted by FRT sites and the respective portion of the gene removed with FLP recombinase.

- 25 Another way of effecting the strategy described herein involves engineering telomerase activity knockout stem cells using a gene editing approach. An example of such an approach is use of a CRISPR-Cas system.

- 30 A CRISPR-Cas system used may be the CRISPR-Cas9 system described by Ran FA, et al., [*Nat. Protoc* 8: 2281-2308 (2013)] incorporated herein by reference. CRISPR-Cas9 can be engineered to target and insert double strand DNA breaks in the hTERT or hTER gene, as shown in Example 13 herein.

In another preferred embodiment of the invention there is provided at least one isolated pluripotent telomerase activity null stem cell, wherein said telomerase activity null stem cell has been produced by exposing at least one isolated pluripotent stem cell to a CRISPR-Cas or other synthetic nuclease system to knock out alleles of a telomerase activity gene selected from the group comprising the human telomerase reverse transcriptase (hTERT) gene and the human telomerase RNA subunit (hTER) gene, thereby resulting in at least one telomerase activity null stem cell with limited lifespan and reduced tumorigenic potential.

Preferably the CRISPR-Cas9 system is used. In a preferred embodiment, hTERT exon 1 is targeted by CRISPR-Cas using one or more guide RNAs having a nucleic acid sequence selected from the group comprising SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18 and SEQ ID NO: 19. More preferably, guide RNAs having the sequences SEQ ID NO: 16 and SEQ ID NO: 17 may be used.

If the CRISPR-Cas system is used the resulting null cells may have relatively short telomeres which could limit the remaining possible cell divisions to too few to provide sufficient cells for clinical use before the cells senesce. To increase the number of cell divisions, it may be necessary to transiently expose the stem cells to a telomerase inducer to increase telomere length prior to CRISPR-Cas treatment.

In a preferred embodiment of the invention the at least one isolated pluripotent stem cell has been subjected to transient over-expression of hTERT, hTER or both hTERT and hTER to increase the average telomere length prior to exposure to CRISPR-Cas or other synthetic nuclease system.

In a preferred embodiment, the isolated telomerase activity null stem cell has an average telomere length of 9 kb or less. More preferably, the average telomere length is 5 kb or less. More preferably, the average telomere length is 4 kb or less. Even more preferred, the average telomere length is 2 kb to 4 kb, or 3 to 4 kb. The person skilled in the art would understand that the telomere length has a direct relationship with the number of cell divisions remaining for a cell, and that the cells of the invention may be passaged until the desired average telomere length is obtained.

In another preferred embodiment, the isolated telomerase activity null stem cell described above retains expression of at least one pluripotency marker. Preferably, the at least one pluripotency marker is selected from the group comprising Oct4, Nanog, Sox2 and Klf4.

5 The method according to any aspect of the invention may be performed *in vitro*. Accordingly, the method is performed with isolated pluripotent stem cell(s).

 The pluripotent stem cell(s) for use in the invention may be from any animal. For example, the pluripotent stem cells may be human. The pluripotent stem cell(s) may comprise induced pluripotent stem cell(s) or embryonic stem cell(s). Any
10 method of preparing pluripotent stem cells or induced pluripotent stem cells is applicable for the invention.

 In another preferred embodiment of the invention, the stem cell is a human embryonic stem cell (hESC) or induced pluripotent stem cell (iPSC).

 Specific gene activity may be knocked out either directly or delayed until
15 desired and then knocked out using knockout inducers. Although direct knockout of telomerase activity using for example ZFN, TALEN or CRISPR can be achieved, the cells derived from direct knockout do not last very long before senescing. Moreover, knockout cells generated this way have to be replaced by newly generated knockouts, which is impractical and quality control becomes a problem.
20 Recombinant cells carrying an inducible knockout can be passaged and manipulated for longer before the target gene is knocked out. Alternatively, if a CRISPR-Cas system is to be used, a more practical telomere length to allow for expansion of telomerase knockout cells could be obtained by transiently increasing telomerase activity prior to CRISPR Cas-induced TERT or TER knockout.

25 Another aspect of the invention provides a method of producing one or more isolated pluripotent telomerase activity null stem cells with limited lifespan and reduced tumorigenicity compared to wild type cells, comprising the steps of:

 (a)(i) engineering, by gene targeting, at least one isolated pluripotent stem cell to have inducible knockout alleles of a telomerase activity gene selected from the group
30 comprising the human telomerase reverse transcriptase (hTERT) gene and the human telomerase RNA subunit (hTER) gene;

(a)(ii) selecting engineered pluripotent inducible telomerase activity knockout stem cell clones;

(a)(iii) contacting the engineered pluripotent inducible telomerase activity knockout stem cell clones with a gene knockout inducer; or

5 (b)(i) engineering, by gene editing, at least one isolated pluripotent stem cell having knocked out alleles of a telomerase activity gene selected from the group comprising the human telomerase reverse transcriptase (hTERT) gene and the human telomerase RNA subunit (hTER) gene; and

(c) selecting telomerase activity null cells with an average telomere length evaluated
10 to be 5 kb or less and a reduced ability to form a teratoma in vivo; or

(d) selecting telomerase activity null cells and passaging them until the average telomere length is evaluated to be 5 kb or less and the cells have reduced ability to form a teratoma in vivo.

In a preferred embodiment, the average telomere length is around 4 kb. In
15 other preferred embodiments, the average telomere length may be 2 kb to 4 kb or 3 kb to 4 kb. It would be understood that the optimum telomere length desired may depend on the particular use intended for the cells; for example a requirement for in vitro use or a requirement for expansion prior to clinical implantation.

In a preferred embodiment, in (a) a portion of the hTERT or hTER gene is
20 replaced by homologous DNA flanked by LoxP sites, which retains gene function, and at a selected time transient expression of Cre recombinase in the recombinant cells causes deletion of the homologous DNA and in (b) a portion of the hTERT or hTER gene is edited by CRISPR-Cas, resulting in loss of telomerase activity.

In a preferred embodiment, in (a) the recombinant cells are homozygous for
25 the homologous DNA insert.

In another preferred embodiment, in (a) the hTERT gene is replaced and the portion of the hTERT gene replaced includes exon 1 and/or exon 2.

The method may further comprise a step of inducing the telomerase activity null cells to differentiate.

In a preferred embodiment, the telomerase activity null cells are induced to differentiate into cells of at least one of ectodermal, endodermal or mesodermal lineage. Methods exist in the art to differentiate stem cells down particular lineages.

In a preferred embodiment, the telomerase activity null cells of the invention
5 are induced to differentiate into neurons. For example, the telomerase null cells may be induced to differentiate in dopaminergic neurons, GABAergic neurons, motor neurons, or glutamatergic neurons.

In another preferred embodiment, the telomerase activity null cells are induced to differentiate into astrocytes and/or glia.

10 In yet another preferred embodiment, the telomerase activity null cells are induced to differentiate into hematopoietic cells.

Another aspect of the invention provides a method of producing one or more isolated pluripotent telomerase activity null stem cells suitable for clinical use, comprising: evaluating the average telomere length and/or in vivo tumorigenicity of
15 said pluripotent stem cells in which the human telomerase reverse transcriptase (hTERT) or the human telomerase RNA subunit (hTER) activity has been inactivated; and identifying said pluripotent stem cells as suitable for clinical use if said evaluated average telomere length is 5 kb or less and/or said cells have reduced ability to form a teratoma in vivo.

20 In a preferred embodiment, the average telomere length is around 4 kb. In other preferred embodiments, the average telomere length may be 2 kb to 4 kb or 3 kb to 4 kb. It would be understood that the optimum telomere length desired may depend on the particular clinical use intended for the cells.

In a preferred embodiment, the hTERT or hTER inactivation is by virtue of an
25 induced, or CRISPR-Cas, knockout of hTERT or hTER gene activity.

In a preferred embodiment, (i) a portion of the hTERT or hTER gene is replaced by homologous DNA flanked by LoxP which retains gene function, and at a selected time transient expression of Cre recombinase in the recombinant cells causes deletion of the homologous DNA resulting in loss of hTERT or hTER activity
30 and, consequently, telomerase activity; or (ii) a portion of the hTERT or hTER gene

is edited by CRISPR-Cas resulting in loss of hTERT or hTER activity and, consequently, telomerase activity.

As stated supra, any suitable inducible knockout system could be used, such as FLP/FRT. However, consideration must be had as to the system used to remove, for example, the selection cassette. In Example 2 shown herein, the PGK-neo selection cassette was removed with FLP/FRT because the hTERT gene portion was cloned to be removable with Cre/LoxP.

In a preferred embodiment, the recombinant cells from the Cre-Lox method (i) are homozygous for the homologous DNA insert.

In a preferred embodiment, the stem cells have limited proliferation capacity.

In a preferred embodiment, the stem cells retain expression of at least one stem cell pluripotency marker. More preferably, the at least one stem cell marker is selected from the group comprising Oct4, Nanog, Sox2 and Klf4.

In a preferred embodiment, the pluripotent telomerase activity null stem cells are human embryonic stem cells (hESC), progenitor cells or induced pluripotent stem cells (iPSC).

The method may further comprise a further step of inducing said at least one isolated pluripotent telomerase activity null stem cells to differentiate. The stem cells may be partially differentiated into progenitor cells such as, for example, neural progenitor cells. Progenitor cells are described by some in the art to be a form of stem cell and will be considered as such for the purpose of the invention.

In a preferred embodiment, the at least one telomerase activity null cells are induced to differentiate into cells of at least one of ectodermal, endodermal or mesodermal lineage.

In a preferred embodiment, the at least one telomerase activity null cells are induced to differentiate into neurons.

In another preferred embodiment, the at least one telomerase activity null cells are induced to differentiate into astrocytes and/or glia.

In yet another preferred embodiment, the at least one telomerase activity null cells are induced to differentiate into hematopoietic cells.

In a preferred embodiment of the method of producing one or more isolated pluripotent telomerase activity null stem cells suitable for clinical use, said evaluating
5 step comprises performing one or more of: single telomere length analysis (STELA), fluorescence in-situ hybridization (FISH), flow-FISH and Southern blot analysis.

The method of producing one or more isolated pluripotent telomerase activity null stem cells suitable for clinical use may further comprise:

(a) assessing the genomic integrity of said pluripotent telomerase activity null stem
10 cells to obtain a genomic integrity result, wherein said assessing comprises one or more of: karyotyping; analysis of variable number tandem repeats (VNTRs), short tandem repeats (STRs), single nucleotide polymorphisms (SNPs), and/or copy number variations (CNVs); analysis of culture mosaicism; analysis of DNA sequences related to genetic diseases; and complete genome sequencing and
15 analysis; and

(b) identifying the pluripotent telomerase activity null stem cells as suitable for clinical use based on the genomic integrity result.

In a preferred embodiment, the genome integrity is assessed prior to gene inactivation.

20 In another aspect of the invention there is provided the use of at least one isolated pluripotent telomerase activity null stem cell herein defined for the preparation of a therapeutic composition for cell therapy of a subject in need thereof.

In a preferred embodiment, the at least one isolated pluripotent telomerase activity null stem cell is differentiated into cells of at least one of ectodermal,
25 endodermal or mesodermal lineage. More preferably, the at least one isolated pluripotent telomerase activity null stem cell is differentiated into cells of at least one lineage selected from the group comprising neurons, astrocytes and/or glia and hematopoietic cells.

In particular, the method according to any aspect of the invention may include
30 inducing differentiation of the at least one isolated pluripotent telomerase activity null

stem cells into endoderm, mesoderm or ectoderm lineage. More particularly, the isolated pluripotent telomerase activity null stem cells are induced to differentiate into neurons, glia or hematopoietic cells.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention.

EXAMPLE 1

Zinc Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs) expression vectors for generating double strand breaks in telomerase (hTERT) exon 1.

Given the low homologous recombination efficiency in human ES cells, synthetic nucleases were necessary to create site-specific double strand breaks (DSBs) to stimulate DNA recombination efficiency (Jasin M, *Trends Genet* 12, 224-228 (1996); Van der Oost, *Science* 339, 768-770 (2013)). The introduction of DSBs is made possible due to the recent advances in engineering of synthetic nucleases. Both Transcription Activator-Like Effector Nucleases (TALENs) and zinc finger nucleases (ZFNs) utilize the heterodimeric Fok-I nuclease and provide high specificity and low off-target effects in the generation of site-specific DSB. Paired ZFNs were purchase from Sigma (Cat# CSTZFN-1KT-hTERT). TALENs was engineered as previously described (Christian M., *et al. Genetics* 186, 757-761 (2010)). Paired ZFNs and TALENs were used to target distinct genomic DNA sequences in exon 1 of hTERT (refer to Figure 1).

We proceeded with ZFNs for gene targeting to create hTERT inducible knockout human ES cells.

EXAMPLE 2

Generation of a targeting vector for use in abolishing telomerase activity

The hTERT genomic locus was targeted to engineer telomerase inducible knockout in human ES cells. The gene targeting strategy relies on the replacement of endogenous gene locus by exogenous DNA sequence, mediated by homologous recombination. The targeting vector was engineered to introduce two LoxP sites

flanking hTERT exon 1 and exon 2, as seen in Figure 2. Upon expression of Cre recombinase, both exon 1 and exon 2 are deleted from the hTERT genomic locus, which encompasses almost 50% of hTERT N-terminal protein coding region.

To avoid the cutting of the targeting vector by the co-expressed ZFNs during the gene targeting process, the ZFNs binding sites on the targeting vector were mutated. A dramatic improvement in the stability of full-length targeting vector was thereby achieved (Figures 3A and 3B).

The targeting vector, pBSK-3kb-5'-LoxP-hTERT-FRT-PGKNeo-FRT-LoxP-3'-PGK-DTA (Δ ZFN) (refer to Figure 2), was constructed as follows:

Genomic DNA from H1 human embryonic stem cells was used as template for PCR amplification using platinum Taq HiFi. The amplified PCR fragment was first cloned into pBSK, and the sequence was verified by capillary DNA sequencing. Multiple polymorphisms were detected in the PCR amplified fragments. The PCR fragment was then used in stepwise construction of gene targeting vector, using specific restriction enzymes.

Step 1

pBSK-5'-hTERT

SL0094 and SL0095 primers were used to amplify the Exon 1+Intron 1+Exon 2+Intron 2 region with 5' Sall site (underlined) and 3' BamHI site (underlined) and cloned into pBSK (pBSK-5'-hTERT).

Primer SL0094

5'-TTTTGTCGACTGCTGCGCACGTGGGAAGCCCT-3' (SEQ ID NO: 3)

Primer SL0095

5'-TTTTGGATCCATGCAAAGTGGACAGGAGGGAGA-3' (SEQ ID NO: 4)

Step 2

pBSK-5'-LoxP-hTERT

Subsequently, the annealed double stranded oligonucleotides (SL0098 and SL0099) that encode a single LoxP site (underlined) were inserted in the 5' Sall site
5 using in-fusion ligation kit (Clontech) to create (pBSK-5'-LoxP-hTERT).

SL0098

5'-CAGGCAGCGCTGCGTCCATAACTTCGTATAATGTATGCTATACGAAGTTAT

CTGCTGCGCACGTGGG-3' (SEQ ID NO: 5)

SL0099

10 5'-CCCACGTGCGCAGCAGAATAACTTCGTATAGCATACATTATACGAAGTTATG

ACGCAGCGCTGCCTG-3' (SEQ ID NO: 6)

Step 3

pBSK-5'-LoxP-hTERT-3'

The 3' homologue arm of the targeting vector was amplified using SL0096
15 and SL0097 primer set and cloned into pBSK-5'-LoxP-hTERT to create pBSK-5'-LoxP-hTERT-3'. Restriction sites are underlined.

SL0096

5'-AAAAGCTAGCTTACGAGGTTACCTTCACGTTTTG-3' (SEQ ID NO: 7)

SL0097

20 5'- AAAACTCGAGAACAGCAATGACAGGCAGAGTCCT-3' (SEQ ID NO: 8)

Step 4

pBSK-5'-LoxP-hTERT-FRT-PGKNeo-FRT-LoxP-3'

The DNA fragment containing PGK-neo selection cassette and 3' LoxP site was cut out from pF2L2 vector using BamHI and NheI, and inserted into the BamHI

/NheI site in pBSK-5'-LoxP-hTERT-3' to derive pBSK-5'-LoxP-hTERT-FRT-PGKNeo-FRT-LoxP-3'.

Step 5

pBSK-5'-LoxP-hTERT-FRT-PGKNeo-FRT-LoxP-3'-PGK-DTA

- 5 The Sall/XhoI DNA fragment from pF2L2 vector that contains PGK-DTA was then cloned into pBSK-5'-LoxP-hTERT-FRT-PGKNeo-FRT-LoxP-3' to create pBSK-5'-LoxP-hTERT-FRT-PGKNeo-FRT-LoxP-3'-PGK-DTA.

Step 6

pBSK-5'-LoxP-hTERT-FRT-PGKNeo-FRT-LoxP-3'-PGK-DTA (Δ SacI)

- 10 Site-directed mutagenesis was performed to eliminate the SacI site in the PGK-Neo and PGK-DTA fragments in the pBSK-5'-LoxP-hTERT-FRT-PGKNeo-FRT-LoxP-3'-PGK-DTA vector, using oligonucleotide primers SL00171 and SL0172 (mutated site underlined).

SL0171

- 15 5'-GAGCAGTGGTGGGAATGCAGATCCTAGCAGCTCGCTGATCAGCCTCGACTG
TGC-3' (SEQ ID NO: 9)

SL0172

5'-CTGAGGGGATCAATTCTCTAGCAGCTCGCTGATCAGCCTCGAC-3' (SEQ ID NO: 10)

Step 7

pBSK-3kb-5'-LoxP-hTERT-FRT-PGKNeo-FRT-LoxP-3'-PGK-DTA

- 25 To extend the homology arm of the targeting vector, 3 kb DNA fragment upstream of hTERT 5' (-1000—4000) was amplified from genomic DNA using SL0232 and SL0233. The PCR fragment was then cloned into SacI-linearized pBSK-5'-LoxP-hTERT-FRT-PGKNeo-FRT-LoxP-3'-PGK-DTA (Δ SacI) using In-Fusion® cloning kit (Clontech Laboratories Inc.).

SL0232

5'- AAGGGAACAAAAGCTGGAGCTCG-3' (SEQ ID NO: 11)

SL0233

5'- CAGTAGAAGCCACACGGCCACTG-3' (SEQ ID NO: 12)

5 **Step 8**

pBSK-3kb-5'-LoxP-hTERT-FRT-PGKNeo-FRT-LoxP-3'-PGK-DTA (Δ ZFN)

To eliminate the ZFN binding sites on the targeting vector, site-directed mutagenesis was performed using SL0259/SL0259 antisense primer pairs.

SL0259

10 5'-GAGCCGTGCGCTCCCTGCTGAGATCTCACTACAGAGAGGTGCTGCCGCT
GGCCACGT-3' (SEQ ID NO: 13)

SL0259 antisense

5'-ACGTGGCCAGCGGCAGCACCTCTCTGTAGTGAGATCTCAGCAGGGAGCG
CACGGCTC-3' (SEQ ID NO: 14)

15 **EXAMPLE 3**

Engineering of hTERT inducible knockout human embryonic stem (hES) cells

Feeder-independent human H1 embryonic stem cells from WiCell (WiCell Research Institute, Madison, WI, USA) were grown on Matrigel™ (BD biosciences)-coated cell culture dishes using mTeSR™1 culture medium (STEMCELL
20 Technologies). When the cells were 80-90% confluent, the cells were passaged using Dispase, and split 1:6 to 1:12 onto new Matrigel™-coated cell culture dishes.

Two rounds of gene targeting were performed to introduce the loxP sites to flank both alleles of hTERT genomic loci. This is shown diagrammatically in Figure 4.

The Neon® transfection system (Life Technologies) was used for targeting the
25 first allele of hTERT. Briefly, the cells were harvested using trypsin and counted and

the cells washed once with 1xPBS before being re-suspended in Neon® Re-suspension buffer at 1×10^7 /ml final concentration. For 1×10^7 cells in 1ml of Re-suspension buffer, 6.25 µg of pZFN1 and 6.25 µg of pZFN2 and 37.5 µg of SacI-linearized and purified pBSK-3kb-5'-LoxP-hTERT-FRT-PGKNeo-FRT-LoxP-3'-PGK-DTA(Δ ZFN) targeting plasmid was added and mixed well before electroporation. For electroporation, 100µl of cells and DNA mixture in Re-suspension buffer was electroporated using 100µl Neon pipette in Neon tube with 3ml of Neon® Electrolytic Buffer. The electroporation condition: Pulse, V 1050, MS 30, Number 2. After the electroporation, the cells were plated onto Matrigel™-coated 10cm dishes in the presence of 6ml of mTeSR™1 with 10µM Y-27632 (Rock inhibitor). About $5-6 \times 10^6$ cells were plated into one 10cm dish. The cells were maintained in mTeSR™1 and the medium changed every day.

At 48 hour after electroporation, mTeSR™1 with 50 µg/ml G418 (neomycin) was then added for selection of G418-resistant clones. The cells need to be maintained in mTeSR™1 with 50 µg/ml G418 for about 12 days before the colonies are big enough for picking. When the G418-resistant colonies were about 3-4 mm in size, the individual G418-resistant clone on the 10cm dish was picked up using sterile p200 pipette tip and transferred into two new wells on a Matrigel™-coated 96 well plate. The colonies were allowed to grow 2-3 days before being ready for screening, using PCR as previously described (Zhang *et al. Nat Methods* 5, 163-165 (2008)).

For targeting the second allele of hTERT, the PGK-Neo cassette was first removed by FLP. The cells were transiently transfected with pCAG-Flpe:GFP plasmid using Neon® transfection system (as described above). The cells were maintained in mTeSR™1 with 10µM Y-27632 (Rock inhibitor) for 24 hours, and the GFP-positive cells then sorted out and collected using FACS. The cells were then seeded at low density (200-500 cell/well in 6-well dishes) to allow the colonies derived from single ES cells to emerge. When the colonies grew to about 3-4 mm size, the colonies were transferred to 96-well dishes and used for PCR diagnosis for the loop out of PGK-Neo cassette. The second allele was targeted exactly as described above for the first allele.

The targeted alleles of hTERT were confirmed by Southern blotting analysis (shown on Figure 5A).

The genomic DNA was extracted using Gentra® Puregene® genomic DNA purification kit (Qiagen). Southern blots for detection of targeted genomic insertions were performed as previously described (Liu C.Y. *et al. Genes Dev* 10, 1835-1843 (1996)). For telomerase activity analysis, telomeric repeat amplification protocol (TRAP) assay was performed as previously described (Kim N.W., *et al. Science* 266, 2011-2015 (1994)). For telomere length measurement, the genomic DNA was digested with HphI and MnlI at 37°C for 16 hours. The DNA blot was hybridized to ³²P-labeled (TTAGGG)₆ oligonucleotide, as previously described (Li S. *et al., Cancer Res* 64, 4833-4840 (2004)).

EXAMPLE 4

Engineering of hTERT null ES cells

To create hTERT null ES cells, pCre-IRES-mCherry plasmid (pmCherry-C1 vector from Clontech in which Cre-IRES was inserted into the multiple cloning site) was transiently transfected into ES cells that have both hTERT genomic alleles flanked by a LoxP site (as generated in Example 3), using Neon® transfection system (as described above). The cells were maintained in mTeSR™1 with 10μM Y-27632 (Rock inhibitor) for 24 hours, and the mCherry-positive cells then sorted out and collected using FACS. The cells were then seeded at a low density of 200-500 cell/well in 6-well dishes to allow the colonies derived from single ES cells to emerge. When the colonies grew to about 3-4 mm size, the colonies were transferred to 96-well dishes and used for PCR diagnosis for the loop out of hTERT Exons 1 and 2.

As shown by RNA protection assay (using Ambion RPA III™ Ribonuclease Protection Assay Kit; refer to Figure 5B), deletion of one copy of hTERT (hTERT^{+/-}) results in about 50% loss of hTERT mRNA expression, while deletion of both copies of hTERT (hTERT^{-/-}) results in the complete loss of full-length hTERT mRNA expression in ES cells. Consistent with the hTERT mRNA expression results, Figure 5C shows that deletion of one allele of hTERT results in about 50% reduction of telomerase activity, whereas deletion of both alleles of hTERT results in the complete loss of telomerase activity, as shown by TRAP assay.

EXAMPLE 5Progressive loss of telomere length in engineered hES cells

The telomere length in the parental ES cells (hTERT^{+/+}) is about 13-14 kb as shown by genomic Southern blotting analysis (Figure 6A). ES cell colonies with LoxP sites flanking the only one allele of hTERT (hTERT^{+/loxP}) showed slight shortening of telomere compared to the parental ES cells. This is probably due to the disruption of hTERT expression by the neomycin cassette in the targeting vector during the gene targeting process (Figure 4). Introduction of LoxP sites flanking the exon 1 and exon 2 of hTERT can potentially inhibit hTERT mRNA expression as well. Consistent with that, further telomere attrition was observed in ES cell colonies with LoxP sites flanking both allele of hTERT (hTERT^{loxP/loxP}).

As predicted, transient expression of Cre recombinase in hTERT^{loxP/loxP} ES cells resulted in the loss of telomerase activity (Figure 5C) and gradual shortening of telomere length (Figure 6A) in hTERT^{-/-} ES cells.

EXAMPLE 6Telomerase null ES cells have limited life span

Since the hTERT^{loxP/loxP} ES cells have relatively short telomere, around 5 kb, the hTERT^{-/-} ES cell colonies derived from hTERT^{loxP/loxP} ES cells by transient expression of Cre recombinase have even shorter telomere length, and can only be passaged for approximately 8-9 times before they lose the capacity to divide continuously (as can be seen in Figure 6A).

Previous studies have shown that when primary fibroblast cells (WI38) are cultured *in vitro*, they eventually lost their proliferation capacity and entered a senescent state (Hayflick and Moorhead, *Exp Cell Res* 25, 585-621 (1961)).

Cellular senescence was analyzed using senescence β -galactosidase staining kit (Cell Signaling) according to the protocol provided. As shown in Figure 6B, the senescent primary fibroblast (WI38 PD67; ATCC, USA) cells have enlarged cell morphology and stained positive for senescence-associated β -galactosidase activity (Dimri G.P., *et al. Proc Natl Acad Sci USA* 92, 9363-9367 (1995)), which is distinct from young fibroblasts (WI38 PD35; ATCC, USA). Similar to the primary

fibroblasts, gradual loss of cell proliferation capacity and increased cell death of the hTERT^{-/-} ES cells was observed around passage 8-9. Moreover, again similar to senescent primary fibroblast cells, these hTERT^{-/-} ES cells also express senescence-associated β -galactosidase activity (Figure 6C).

5 To further address whether the loss of hTERT expression affects the proliferation capacity of ES cells, hTERT^{+/+}, hTERT^{+/-} and hTERT^{-/-} ES cells were analyzed for their incorporation of 5-ethynyl-2'-deoxyuridine (EdU) which marks dividing cells.

10 Cell proliferation was detected through the incorporation of 10 μ M EdU within an hour, using Click-iT® EdU Alexa Fluor® 488 Imaging Kit (Life Technologies). Immunocytochemistry was done in accordance with the protocol provided by the manufacturer.

As shown in Figure 7A, cellular incorporation of EdU during the S phase is comparable in hTERT^{+/+}, hTERT^{+/-} and hTERT^{-/-} ES cells.

15 In addition, Celltiter-Glo® luminescent cell viability assay showed that hTERT^{-/-} ES cells at early passage (P2) (refer to Figure 6A), have similar cell viability and cell proliferation rate as compared to TERT^{+/+} and hTERT^{+/-} ES cells. These results can be seen on Figure 7B. For this assay, cells were seeded into 96-well plates at a density of 1,000 cells/well/96-well plate, and cultured over a period of 7 days to
20 monitor cell proliferation using ATP assay (CellTiter-Glo® Luminescent Cell Viability Assay, Promega). Luminescence was measured on a microplate reader (Infinite 200, Tecan) every 24 hours.

Cell cycle profile analysis was also undertaken. Cells were harvested by trypsinization, washed and fixed in ice-cold 70% ethanol followed by staining with
25 propidium iodide (PI) with RNase A. Cell cycle profiles were acquired on flow cytometer (MACSQuant VYB, Miltenyi Biotec) and analyzed using ModFit LT. This analysis also showed comparable G1, S and G2/M cell cycle distribution in hTERT^{-/-} (P2) and hTERT^{+/+} and hTERT^{+/-} ES cells. However, the hTERT^{-/-} ES cells at late passage – hTERT^{-/-} (P8; refer to Figure 6A) – lost proliferation potential and entered
30 a senescence state. Increased cell population at G1 and decreased cell population

at G2/M phase were also observed in the hTERT^{-/-} (P8) cells. These results can be seen in Figure 7C.

In addition to loss of cell proliferation, hTERT^{-/-} (P8) cells also showed increased cell death. Annexin V and 7-AAD staining showed increased apoptosis in hTERT^{-/-} (P8) ES cells (29.78%) compared to hTERT^{+/+}, hTERT^{+/-} and hTERT^{-/-} (P2) ES cells (4.15%) (Figure 7D). Cells were harvested by trypsinization, washed once with PBS and stained with 7-AAD (BD Biosciences) and Annexin V (BD Biosciences) for 15 minutes at room temperature in the dark. Samples were acquired on flow cytometer (BD LSRFortessa, BD Biosciences) and data acquired were analyzed using FlowJo (Tree Star).

These results indicate that the cell proliferation capacity of the ES cells is limited by their telomere length in the absence of telomerase activity.

EXAMPLE 7

Transient overexpression of hTERT resets telomere length

The telomere length in hTERT^{loxP/loxP} ES cells is maintained at a short (about 5 kb) and stable state as compared to parental cells. This indicates that the telomerase activity in hTERT^{loxP/loxP} ES cells is sufficient for telomere maintenance. These results also raised a possibility that transient overexpression of hTERT, boosting the telomerase activity in hTERT^{loxP/loxP} ES cells, may be able to reset their telomere length.

To test this, a mammalian expression vector overexpressing hTERT-IRES-GFP was transiently transfected into the hTERT^{loxP/loxP} ES cells, following which the GFP positive cells were FACS sorted and the single cell colonies were isolated. As shown in Figure 8, several of the single cell colonies (those numbered 1, 2 and 5), derived from transient overexpression of hTERT, have elongated telomeres of similar length as, or even longer than, the parental hTERT^{loxP/loxP} H1 ES cells. No integration of the hTERT-IRES-GFP expressing vector was detectable in the newly derived hTERT^{loxP/loxP} ES cell colonies.

Accordingly, the hTERT^{-/-} ES cell colonies derived from the new hTERT^{loxP/loxP} ES cells also have longer telomeres to start with; about 9-10 kb, as shown in Figure

9; and can be passaged much longer than the hTERT^{-/-} ES cell with short telomeres of about 4 kb as was seen in Figure 6A.

EXAMPLE 8

Engineered hES cells maintain normal karyotype

5 Given the propensity of human ES cells to accumulate genetic aberrations during *in vitro* culture, karyotyping was done during each step of gene targeting to ensure that the clonally-derived human ES cell lines maintained a normal karyotype.

 For karyotyping, the cells were grown to 50-60% confluent, and colcemid was then added to the culture at final concentration of 10 µg/ml and incubation continued
10 for 4 hours. The cells were harvested by trypsin-EDTA and washed with HEPES buffered saline solution (HBSS). The cells were then re-suspended in hypotonic solution (2 parts of 0.6% sodium citrate tribasic dihydrate + 1 part of 75 mM KCl) and incubated in 37°C water bath for 20 minutes. Subsequently, 1ml of fixative (3 parts of
15 methanol + 1 part of glacial acetic acid) was added to the tube and mixed by pipetting up/down. Cells were spun down and re-suspended in 4ml of fixative and kept at -20°C overnight. The next day, the cells were spun down and re-suspended in fresh fixative buffer and dropped onto slides. The slides were baked at 56°C overnight, and stained with Giemsa stain (Sigma GS-500) before being analysed using Metafer slide scanning platform.

20 As Figure 10 demonstrates, the karyotype was always normal and no gross abnormalities were detected.

EXAMPLE 9

Demonstration of continued pluripotency of telomerase null hES cells *in vitro*

 Like their parental ES cells (hTERT^{+/+}), the hTERT^{+/-} and hTERT^{-/-} ES cells
25 express ES cell-specific surface antigens (Adewumi O., *et al. Nat Biotechnol* 25, 803-816 (2007)), such as TRA-1-60 and SSEA-4, as well as genes involved in the maintenance of undifferentiated ES cell state such as OCT4 and NANOG. This can be seen using immunocytochemistry assays, as set out in Figures 11A-11D.

Figure 11E provides qRT-PCR results showing that the expression levels of ES cell marker genes in the hTERT^{+/-} and hTERT^{-/-} ES cells, such as OCT4, SOX2, KLF4 and NANOG, were comparable to the parental ES cells (hTERT^{+/+}), although there were small variations in different independent hTERT^{+/-} and hTERT^{-/-} ES cell lines.

To determine the differentiation potential of telomerase null ES cells *in vitro*, these cells were grown in suspension to induce the formation of embryoid bodies (EBs) (Itskovitz-Eldor J., *et al. Mol Med* 6, 88-95 (2000)).

Embryoid bodies (EBs) were formed by trypsinization to a single-cell suspension and plating into low-adherence dishes in human ES cell mTeSR™1 medium. For spontaneous differentiation, 7-10 day old EBs were used according to the previously established protocol (Dimos J.T., *et al. Science* 321, 1218-1221 (2008)). In brief, the EBs (about 5-10) from human ES cells were transferred onto gelatin-coated 24-well plates and allowed to differentiate in DMEM+10% fetal bovine serum for 1-2 weeks.

The hTERT^{+/+}, hTERT^{+/-} and hTERT^{-/-} EBs were then plated on gelatin-coated plates for 2 weeks. The attached cells spontaneously differentiated into cell types representative of the three germ layers (refer to Figures 12A – 12E), which express early differentiation markers for ectoderm (Tuj1 and GFAP); mesoderm (SMA and Desmin) and endoderm (AFP). These data indicate that the hTERT^{-/-} ES cells remain pluripotent.

For immunofluorescence assays, the cells were fixed in 4% paraformaldehyde for 10 minutes. The fixed cells were washed three times with 0.1M Tris buffered saline containing 0.1% Triton-X 100 (TBS-TX) and incubated in primary antibody including: mouse anti-Oct3/4 (1:500, Santa Cruz Biotechnology), mouse anti-Tra-1-60 (1:100, Santa Cruz Biotechnology), rabbit anti-Nanog (1:100, Cell Signaling Technology), mouse anti-SSEA-4 (1:500, Millipore), rabbit anti-AFP (1:400, Dako), mouse anti-Desmin (1:100, Abcam); goat anti-SOX2 (1: 500, Santa Cruz Biotechnology), mouse anti-SMA (1:200, Sigma), mouse anti-Tuj1 (1:1000, Covance), mouse anti-GFAP (1:1000, Millipore), mouse anti-Human Nuclei (1:500, Abcam), mouse anti-Nestin (1:1000, Millipore), or goat anti-DCX (1:500, Santa Cruz Biotechnology) overnight at 4°C in blocking buffer. The following day, cells were

washed with TBS-TX and incubated with Alexa Fluor-conjugated secondary antibodies (Invitrogen, 500×) in TBS-TX for two hours at room temperature. Nuclei were visualized by DAPI staining (Sigma-Aldrich). Images were acquired on a Zeiss LSM 710 confocal microscope or LSM 7 ELYRA PS.1 system (Carl Zeiss, Pte. Ltd., Singapore).

EXAMPLE 10

Telomerase null hES cells do not form teratomas when telomeres are sufficiently short

hTERT^{+/+}, hTERT^{+/-} and hTERT^{-/-} ES cells were injected subcutaneously into the dorsal-lateral area of immunodeficient (NSG) mice, as described previously (Prokhorova T.A., *et al. Stem Cells Dev* 18, 47-54 (2009); incorporated herein by reference). Eight weeks following the injection, the formation of teratoma was evaluated. Similar to hTERT^{+/+} and hTERT^{+/-} ES cells, hTERT^{-/-} ES cells with long telomere (9 kb) form teratomas in immunodeficient mice with high frequency, as can be seen in Figure 13A. However, when hTERT^{-/-} ESCs with shorter telomere (4 kb, P2 in Figure 6A) were injected into immunodeficient mice, only one out of 20 injections resulted in the formation of teratoma. When hTERT^{-/-} ES cells with very short telomere (3.5 kb, P4 in Figure 6A) were injected into the immunodeficient mice, none out of 60 injections resulted in the formation of teratoma *in vivo*.

For the teratoma assay, ES cells were harvested using Dispase, washed with 1xPBS and re-suspended in 30% Matrigel™ (BD Science). About 1x10⁶ cells (100μl) were injected subcutaneously into NSG mice (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) in the dorso-lateral area on both sides, as previously described (Prokhorova T.A., *et al. Stem Cells Dev* 18, 47-54 (2009)). The mice were sacrificed and the tumors were harvested 8 weeks after injection. The tumors were dissected and fixed in PBS with 4% paraformaldehyde. Paraffin-embedded tissue was sliced and stained with hematoxylin and eosin.

Consistent with these results, the hTERT^{-/-} ES cells with very short telomeres (3.5 kb) can only be passaged *in vitro* for another 4-5 passages (as seen in Figure 6A). Histological examination of the tumors derived from hTERT^{+/+}, hTERT^{+/-} and TERT^{-/-} ES cells showed that they contained various tissues derived from all three

germ layers, including respiratory epithelium (endoderm); striated muscle and cartilage (mesoderm), and neural epithelium with rosettes (ectoderm). The results are shown in Figure 13B.

These results indicate that inactivation of telomerase activity does not affect the pluripotency of ES cells. However, the proliferation capacity of hTERT^{-/-} ES cells and their ability to form teratoma *in vivo* is limited by the telomere length, which presets their proliferation potential.

EXAMPLE 11

Telomerase null hES cells differentiate into neurons *in vitro*

To test whether hTERT^{-/-} ES cells could be differentiated into specific cell types, neural induction of hTERT^(+/+), hTERT^(+/-) and hTERT^(-/-) ES cells was carried out as previously reported (Li et al., 2011) (see Figure 14A).

Briefly, hTERT^(+/+) and hTERT^(-/-) ES cells were cultured in mTeSRTM1 medium. When the hESCs cultures reached ~20% confluence, mTeSRTM1 medium was removed and replaced with neural induction media containing DMEM/F12: Neurobasal (1:1), 1xN2, 1xB27, 1% Glutmax, 5 µg/mL BSA, 4 µM CHIR99021 (Cellagentech), 3 µM SB431542 (Cellagentech), 0.1 µM Compound E (γ-Secretase Inhibitor XXI, EMD Chemicals Inc.), 10 ng/mL hLIF (Millipore) for 7 days. The culture was then split 1:3 for the next six passages using AccutaseTM, and cells were cultured in human neural progenitor cells (NPCs) maintenance media containing DMEM/F12: Neurobasal (1:1), 1xN2, 1xB27, 1% Glutmax, 5 µg/mL BSA, 3 µM CHIR99021, 2 µM SB431542, 10 ng/mL hLIF on MatrigelTM-coated plates. After six passages, the cells were split 1:10 regularly.

The human NPCs neural differentiation assay was performed by plating 5x10⁴ cells/well on laminin-coated (37°C, 4 hours) 24-well plates in neural differentiation media containing DMEM/F12: Neurobasal (1:1), 1xN2, 1xB27, 1% Glutmax on polyL-Lysine (4°C, overnight). After 3 days, 10 ng/mL BDNF and 10 ng/mL GDNF (both from R&D Systems, MN, USA) were added to the media every other day and culturing was continued for another 14 days.

The human NPCs astrocytes differentiation assay was performed by plating 8×10^4 cells/well on 1% Glutamax on Matrigel™-coated 24-well plates in neural differentiation media containing DMEM/F12: Neurobasal (1:1), 1xN2, 1% Fetal Bovine Serum (FBS).

Immunocytochemistry, carried out as described in Example 9, above, showed that the (NPCs) derived from hTERT^(+/+); hTERT^(+/-) and hTERT^(-/-)(3.5 kb) ES cells were positive for Nestin and Sox2 (Figure 14B). When the NPCs were further induced to differentiate into either immature neurons or astrocytes, NPCs derived from hTERT^(+/+); hTERT^(+/-) and hTERT^(-/-) ES cells showed similar efficiency to differentiate into DCX-positive immature neurons as well as GFAP-positive glial cells (Figures 14C-14E).

hTERT^(-/-) hES cells differentiate into four different neuronal types. 1) dopaminergic neurons expressing TH; 2) GABAergic neurons expressing GAD65; 3) glutamatergic neurons expressing vGluT1; 4) motor neurons expressing ChAT (data not shown). The specific differentiation of these four types of neurons was performed as follows:

- 1) For dopaminergic neurons expressing TH, hTERT^(-/-) cells were first treated with 100 ng/mL Sonic hedgehog (SHH) and 100 ng/mL FGF8b in neural differentiation media for 10 days, and then with 10 ng/mL BDNF, 10 ng/mL GDNF, 10 ng/mL IGF1, 1 ng/mL TGF-β3 and 0.5 mM db-cAMP (Sigma-Aldrich) for another 14–21 days in neural differentiation media.
- 2) For GABAergic neurons expressing GAD65, hTERT^(-/-) cells were treated with SHH (50–500 ng/ml) or its small molecular agonist purmorphamine (0.1–1.5 mM; Calbiochem, San Diego, CA) at days 12–26 to induce ventral progenitors. Retinoic acid (RA, 0.1 mM) was added from day 10 to 23. At day 26, cell clusters were dissociated with Accutase (1 unit/ml, Invitrogen) at 37°C for 5 minutes and placed onto polyornithine/laminin-coated coverslips in Neurobasal medium in the presence of valproic acid (VPA, 10 mM, Sigma) for 1 week, followed by a set of trophic factors, including brain-derived neurotrophic factor (BDNF, 20 ng/ml), glial-derived neurotrophic factor (GDNF, 10 ng/ml), insulin-like growth factor 1 (IGF1, 10 ng/ml), and cAMP (1 mM).

- 3) For glutamatergic neurons expressing vGluT1, hTERT^(-/-) cells were treated with 200 ng/mL BMP2/4, 100 ng/mL FGF8b and 1 μ M RA (Sigma-Aldrich) in neural differentiation media for 10 days, and then with 10 ng/mL BDNF, 10 ng/mL GDNF, 1 ng/mL TGF- β 3 and 0.5 mM db-cAMP (Sigma-Aldrich) for another 14–21 days in neural differentiation media.
- 4) For motor neurons expressing ChAT, hTERT^(-/-) cells were sequentially treated with 1 μ M RA (Sigma-Aldrich) in neural differentiation media for 7 days, then with 100 ng/mL SHH and 0.1 μ M RA for additional 7 days, and finally with 50 ng/mL SHH and 0.1 μ M RA for another 7 days. The cells were terminally differentiated in the presence of 10 ng/mL BDNF and 10 ng/mL GDNF in the neural differentiation media for about 7 days. All growth factors were from R&D Systems. All tissue culture products were obtained from Invitrogen except where mentioned.

EXAMPLE 12

Telomerase null hES cells with short telomeres differentiate *in vivo* without forming tumors

To test whether the newly engineered hTERT^(-/-) ES cells can offer additional advantages by reducing safety risks, we injected hTERT^(+/+) and hTERT^(-/-)(3.5 kb) ES cells directly into the midbrain of immunodeficient mice.

Immunodeficient mice (SCID, NOD.CB17-*Prkdc*^{scid}/NcrCrI) (n=5) were anaesthetized with Ketamine (85 mg/kg) plus Xylazine (10 mg/kg) and fixed in a Stoelting stereotaxic apparatus. A small hole was drilled in the skull above the intended injection sites and a syringe (1 μ l, Hamilton) containing 1 μ l of human Accutase-dissociated ES cells (5×10^4 cells per μ l) in PBS (pH 7.4) was lowered into the striatum at -0.5 mm posterior to bregma, 2.0 mm lateral to midline, 3.2 mm ventral to dura. Four, 8 or 12 weeks after transplantation, animals were deeply anaesthetized, and perfused trans-cardially with 50 ml of saline, followed by 100 ml of 4% paraformaldehyde for 30 minutes. Coronal sections at 40 μ m thickness were cut using a cryostat, and serial sections were transferred to individual wells of a 24-well tissue culture dish. Sections were incubated in blocking solution (5% normal goat serum and 0.1% Triton X-100 in TBS) for 1 hour. Subsequently, primary antibody (Mouse anti-Human Nuclei, 1:500, Abcam; goat anti-DCX, 1: 500, Santa

Cruz; mouse anti-MAP2, 1:1:1,000, Sigma; mouse anti-NeuN, 1:500, Abcam; mouse anti-GFAP, 1:1000, Millipore) in blocking solution was added to the sections and they were incubated overnight at 4°C. After three TBS washes, sections were incubated with Alexa Fluor-conjugated secondary antibody solution for 2 hours at room temperature. Nuclei were visualized by DAPI staining (Sigma-Aldrich). Sections were then mounted to the glass slide. Images were acquired on a Zeiss LSM 710 confocal microscope or LSM 7 ELYRA PS.1 system (Carl Zeiss, Pte. Ltd., Singapore).

As shown in Figures 15A-15D, DCX-positive human cells were detected in mouse brain that received either hTERT^(+/+) or hTERT^(-/-) ES cells four weeks after the injection. However, mice that received the injection of hTERT^(+/+) ES cells also showed local proliferation and expansion of cell mass positive for human nuclei (HuN) staining. These data indicate continuous proliferation of hTERT^(+/+) ES cells-derived cells following injection. At 16 weeks after injection, large tumors could be observed in all five mice injected with hTERT^(+/+) ES cells (Figure 15E).

In contrast, no tumors were found in mice that were injected with hTERT^(-/-) ES cells with mean telomere length around 3.5 kb; these failed to form teratoma *in vivo* even after 16 weeks (Figure 16A). In addition, MAP2a-positive cells, a neuronal marker, and tyrosine hydroxylase (TH)-positive cells, a marker for mature dopaminergic neurons, differentiated from hTERT^(-/-) ES cells, can be detected at 16 weeks after injection (see Figures 16B and 16C, respectively).

These data establish the improved safety that can be achieved by using telomerase null human ES cells for cell therapy *in vivo*.

EXAMPLE 13

Engineering of telomerase-null ES cells (WA018) using CRISPR-Cas9

The exon 1 of human hTERT gene was subjected to potential sgRNA target search using the online software created by Feng Zhang's group [Ran FA, et al., *Nat. Protoc* 8: 2281-2308 (2013)]. The very top hits were chosen and used for the experiments hence described.

As shown in Figures 17A and 17B, DNA sequences encoding five CRISPR guide RNAs (S1-S5; SEQ ID NOs: 15-19) targeting hTERT exon 1 were designed,

and all resulted in efficient, site-specific double strand breaks (DSBs). The protospacer adjacent motif (PAM) in each sgRNA is at the 3' end and is shaded or underlined.

	S1	5-GGGGGCGGCC GTGCGTCCCA <u>GGG</u> -3	SEQ ID NO: 15
5	S2	5-GCTGCGCAGC CACTACCGCG <u>AGG</u> -3	SEQ ID NO: 16
	S3	5-GCAGCAGGGA GCGCACGGCT <u>CGG</u> -3	SEQ ID NO: 17
	S4	5-GGGGAGCGCG CGGCATCGCG <u>GGG</u> -3	SEQ ID NO: 18
	S5	5-GGGAGCGCGC GGCATCGCGG <u>GGG</u> -3	SEQ ID NO: 19

Feeder-independent human WA018 embryonic stem cells from WiCell (WiCell Research Institute, Madison, WN, USA) were grown on Matrigel™ (BD biosciences)-coated cell culture dishes using mTeSR™1 culture medium (Stemcell Technologies). When the cells were 80-90% confluent, the cells were passaged using Dispase, and split 1:6 to 1:12 onto new Matrigel™-coated cell culture dishes.

For engineering of telomerase-null WA018 cells using CRISPR/Cas9 system a Cas9 nickase expression vector, (pSPCas9 D10A_GFP S2/S3-derived from pSPCas9 D10A_GFP expression plasmid-Addgene Plasmid #44720 that co-express sgRNAs S2 and S3 as shown in Figure 17C), was transiently transfected into WA018 human ES cells using a Neon® transfection system to introduce targeted DSBs according to the methods in Ran FA, et al., *Cell* 154: 1380-1389 (2013) and illustrated in Figure 17C.

Briefly, the ES cells grown on Matrigel™ were harvested using Accutase™ and counted. The cells were washed once with 1xPBS before being re-suspended in Neon® Re-suspension buffer at 1×10^7 /ml final concentration. For 1×10^7 cells in 1 ml of Re-suspension buffer, 50 µg of pSPCas9 D10A_GFP S2/S3 vector was added and mixed well before electroporation. For electroporation, 100 µl of cells and DNA mixture in Re-suspension buffer was electroporated using 100 µl Neon® pipette in Neon® tube with 3 ml of Neon® Electrolytic Buffer. The electroporation condition: Pulse, V 1050, MS 30, Number 2. After the electroporation, the cells were plated onto Matrigel™-coated 10cm dishes in the presence of 6ml of mTeSR™1 with 10 µM

Y-27632 (Rock inhibitor). About $5\text{-}6 \times 10^6$ cells were plated into one 10 cm dish. The cells were maintained in mTeSR™1 with 10 μM Y-27632.

At 48 hours after electroporation, GFP positive ES cells were sorted out and collected using FACS. The cells were seeded at low density (800-2000 cells/ 10 cm dish) on Matrigel™-coated cell culture dish in mTeSR™1 with 10 μM Y-27632 for the first 48 hours, then cultured in mTeSR™1 for about 12 days before the single cell-derived colonies were big enough for picking. When the colonies were about 3-4 mm in size, the individual clone on the 10 cm dish was picked up using sterile p200 pipette tip and transferred into two new wells on a Matrigel™-coated 96 well plate. The colonies were allowed to grow 2-3 days before being ready for screening, using PCR as previously described [Ramlee MK, *Sci. Rep.* 5: 15587 (2015)]. No GFP expression was detected in the isolated clones, indicating that there was no integration of the pSPCas9 D10A_GFP S2/S3 targeting vector.

Six independent ES cell clones derived from the WA018 ES cell line with a deletion in hTERT exon 1 were isolated.

DSB-induced insertion/deletion (indel) mutations in three clones were confirmed by Sanger sequencing (Figure 17D). As shown in Figure 17E, the DSB-induced indels resulted in complete loss of telomerase activity in the three ES cell clones. All three telomerase-null ES cell clones maintained normal karyotype (data not shown). However, these telomerase null ES cell clones had short median telomeres, approximately 5-6 kb in length (Figure 17F, lanes 2-4). As a result, they could only be cultured for another 5-6 passages before loss of cell proliferation and induction of cell death, which is consistent with previous CRISPR-Cas work [Sexton AN, *Genes Dev.* 28: 1885-1899 (2014)] and with what is seen with the Cre-Lox approach described in Example 7.

These data show an alternative approach for generating telomerase deficient cells with the same characteristics as those generated using the Cre-LoxP type system. An advantage of the CRISPR-Cas 9 system is that it is simpler and much more time-efficient, generating telomerase deficient cells in a few months compared to around 12 months using the Cre-LoxP method described herein. As was the situation using the Cre-Lox approach, in order to engineer cells that retain a practical number of remaining cell divisions to enable expansion to sufficient numbers for clinical use

before senescence, the telomeres may need to be increased in length prior to CRISPR-Cas treatment by transient increased expression of telomerase.

SUMMARY

Pluripotent stem cells, such as human embryonic stem cells and induced pluripotent stem cells, hold great promise for cell therapy. However, stem cell-based therapy also brings concern due to the tumorigenic potential of stem cells. Such pluripotent stem cells form teratomas when injected into immunodeficient mice. If the stem cell contains genetic mutations, it may potentially develop into more malignant teratocarcinomas.

Current approaches to reduce the risk of tumorigenicity of stem cells have focused on separating differentiated cells from undifferentiated stem cells. The technical burden of such application is enormous. In addition, the high propensity of stem cells to accumulate chromosome aberrations during *in vitro* culture may result in the accumulation of genetic mutations in differentiated cells as well, which may result in tumorigenicity *in vivo*. It is close to impossible to check differentiated cells for their genetic variation and therefore eliminate their tumorigenicity *in vivo*.

The present invention provides telomerase knockout human embryonic stem cells, engineered by gene targeting. The data herein indicate that by inactivating telomerase in stem cells, the two unique properties of stem cell can be functionally separated: the ability to proliferate indefinitely and the ability to differentiate into various cell types.

Such transgenic stem cells constitute an unlimited source for cell engineering but provide, in addition, the concomitant advantage that by limiting their proliferation capacity at a desired time, the risk of their developing tumors *in vivo* is significantly reduced.

References:

- (Adewumi O., et al., Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. *Nat Biotechnol* 25, 803-816 (2007).
- Amariglio, N. et al. Donor-derived brain tumor following neural stem cell transplantation in an ataxia telangiectasia patient. *PLoS medicine* 6 (2009).
- Baker, D. E. et al. Adaptation to culture of human embryonic stem cells and oncogenesis in vivo. *Nat Biotechnol* 25, 207-215 (2007).
- Blackburn, E. H. Telomere states and cell fates. *Nature* 408, 53-56 (2000).
- Blum, B. & Benvenisty, N. Clonal analysis of human embryonic stem cell differentiation into teratomas. *Stem Cells* 25, 1924-1930 (2007).
- Blum, B. & Benvenisty, N. The tumorigenicity of human embryonic stem cells. *Advances in cancer research* 100, 133-158 (2008).
- Christian, M., et al. Targeting DNA double-strand breaks with TAL effector nucleases. *Genetics* 186, 757-761 (2010).
- Dimos, J.T., et al. Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. *Science* 321, 1218-1221 (2008).
- Dimri, G.P., et al. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci U S A* 92, 9363-9367 (1995).
- Feng, J. et al. The RNA component of human telomerase. *Science* 269, 1236-1241 (1995).
- Hayflick, L., and Moorhead, P.S. The serial cultivation of human diploid cell strains. *Exp Cell Res* 25, 585-621 (1961).
- Hentze, H. et al. Teratoma formation by human embryonic stem cells: evaluation of essential parameters for future safety studies. *Stem cell research* 2, 198-210 (2009).
- Itskovitz-Eldor, J., et al. Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. *Mol Med* 6, 88-95 (2000).

Jasin, M. Genetic manipulation of genomes with rare-cutting endonucleases. *Trends Genet* 12, 224-228 (1996).

Kim, N.W., *et al.* Specific association of human telomerase activity with immortal cells and cancer [see comments]. *Science* 266, 2011-2015 (1994).

- 5 Lee, A. S. *et al.* Effects of cell number on teratoma formation by human embryonic stem cells. *Cell Cycle* 8, 2608-2612 (2009).

Li, S., *et al.* Rapid inhibition of cancer cell growth induced by lentiviral delivery and expression of mutant-template telomerase RNA and anti-telomerase short-interfering RNA. *Cancer Res* 64, 4833-4840 (2004).

- 10 Li, W., *et al.* Rapid induction and long-term self-renewal of primitive neural precursors from human embryonic stem cells by small molecule inhibitors. *Proc Natl Acad Sci U S A* 108, 8299-8304 (2011).

- Liu, C.Y., *et al.* Inactivation of the mouse Brca1 gene leads to failure in the morphogenesis of the egg cylinder in early postimplantation development. *Genes Dev* 10, 1835-1843 (1996).

15 Nakamura, T. M. *et al.* Telomerase catalytic subunit homologs from fission yeast and human. *Science* 277, 955-959 (1997).

- Prokhorova, T.A., *et al.* Teratoma formation by human embryonic stem cells is site dependent and enhanced by the presence of Matrigel. *Stem Cells Dev* 18, 47-54 (2009).

Ramlee MK, *et al.* High-throughput genotyping of CRISPR/Cas9-mediated mutants using fluorescent PCR-capillary gel electrophoresis. *Sci. Rep.* 5: 15587 (2015).

Ran FA, *et al.* Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc* 8, 2281-2308 (2013).

- 25 Ran FA, Hsu PD, Lin CY *et al.* Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell* 154:1380-1389 (2013).

Sexton AN, et al. Genetic and molecular identification of three human TPP1 functions in telomerase action: recruitment, activation, and homeostasis set point regulation. *Genes Dev* 28: 1885-1899 (2014).

Strong, M. A. *et al.* Phenotypes in mTERT(+)/(-) and mTERT(-)/(-) mice are due to short telomeres, not telomere-independent functions of telomerase reverse transcriptase. *Mol Cell Biol* **31**, 2369-2379 (2011).

Takahashi, K. *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861-872 (2007).

Takahashi, K. & Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663-676 (2006).

Thomson, J.A. *et al.* Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145-1147 (1998).

Van der Oost, J. Molecular biology. New tool for genome surgery. *Science* 339, 768-770 (2013).

Wright, W. E. *et al.* Telomerase activity in human germline and embryonic tissues and cells. *Dev Genet* **18**, 173-179 (1996).

Yu, J. *et al.* Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318**, 1917-1920 (2007).

Zhang, X., *et al.* Epitope tagging of endogenous proteins for genome-wide ChIP-chip studies. *Nat Methods* 5, 163-165 (2008).

Claims:

1. An isolated pluripotent stem cell, comprising two mutated alleles of a telomerase activity gene selected from the group comprising the human telomerase reverse transcriptase (hTERT) gene and the human telomerase RNA (hTER) gene; wherein
5 said alleles comprise an introduced and removable portion of said hTERT or hTER gene.
2. The isolated stem cell of claim 1, wherein the cell has been further subjected to transient overexpression of hTERT or hTER to increase the average telomere length.
- 10 3. The isolated stem cell of claim 2, wherein after transient overexpression of hTERT or hTER the average telomere length in the stem cell is increased to at least 6 kb.
4. The isolated stem cell of claim 3, wherein the average telomere length in the stem cell is increased to at least 9 kb.
5. An isolated pluripotent telomerase activity null stem cell, comprising two mutated
15 alleles of a telomerase activity gene selected from the group comprising the human telomerase reverse transcriptase (hTERT) gene and the human telomerase RNA (hTER) gene; wherein said telomerase activity null stem cell has limited lifespan and reduced tumorigenic potential.
6. The isolated pluripotent telomerase activity null stem cell of claim 5, comprising:
20 a) the pluripotent stem cell of any one of claims 1 to 4 which has been contacted with a knockout inducer to remove said introduced removable gene portion; or
b) a pluripotent stem cell which has been contacted with CRISPR-Cas to knock out alleles of a telomerase activity gene selected from the group comprising the human telomerase reverse transcriptase (hTERT) gene and the human telomerase RNA
25 subunit (hTER) gene;
wherein said telomerase activity null stem cell has limited lifespan and reduced tumorigenic potential.
7. The isolated telomerase activity null stem cell of claim 5 or 6, wherein the average telomere length is 9 kb or less.

8. The isolated telomerase activity null stem cell of claim 7, wherein the average telomere length is in a range selected from the group comprising 5 kb or less, 4 kb or less, 2kb to 4 kb, 3kb to 4 kb and about 4 kb.

9. The isolated pluripotent stem cell of any one of claims 1 to 4, wherein said portion of the hTERT or hTER gene is replaced by homologous DNA flanked by LoxP sites, which retains gene function, and at a selected time transient expression of Cre recombinase in the recombinant cell causes deletion of the homologous DNA resulting in loss of hTERT or hTER activity and, consequently, telomerase activity.

10. The isolated stem cell of any one of the previous claims, wherein the stem cell retains expression of at least one pluripotency marker selected from the group comprising Oct4, Nanog, Sox2 and Klf4.

11. The isolated stem cell of any one of claims 1 to 10, wherein the stem cell is a human embryonic stem cell (hESC) or induced pluripotent stem cell (iPSC).

12. A method of producing one or more isolated pluripotent stem cells with limited lifespan and reduced tumorigenicity compared to wild type cells, comprising the steps of:

(a)(i) engineering, by gene targeting, at least one isolated pluripotent stem cell to have inducible knockout alleles of a telomerase activity gene selected from the group comprising the human telomerase reverse transcriptase (hTERT) gene and the human telomerase RNA subunit (hTER) gene;

(a)(ii) selecting engineered pluripotent stem cell clones;

(a)(iii) contacting the engineered pluripotent stem cell clones with a gene knockout inducer; or

(b)(i) engineering, by gene editing, at least one isolated pluripotent stem cell having knocked out alleles of a telomerase activity gene selected from the group comprising the human telomerase reverse transcriptase (hTERT) gene and the human telomerase RNA subunit (hTER) gene; and

(c) selecting telomerase activity null cells with an average telomere length evaluated to be 5 kb or less and a reduced ability to form a teratoma in vivo; or

(d) selecting telomerase activity null cells and passaging them until the average telomere length is evaluated to be 5 kb or less and the cells having reduced ability to form a teratoma in vivo.

13. The method of claim 12, wherein the average telomere length is around 4 kb.

5 14. The method of claim 12 or 13, wherein in (a) a portion of the hTERT or hTER gene is replaced by homologous DNA flanked by LoxP sites, which retains gene function, and at a selected time transient expression of Cre recombinase, in the recombinant cells causes deletion of the homologous DNA, and wherein in (b) a portion of the hTERT or hTER gene is edited by CRISPR-Cas, resulting in loss of
10 telomerase activity.

15. The method of claim 14, wherein in (a) the recombinant cells are homozygous for the homologous DNA insert.

16. The method of claim 14 or 15, wherein in (a) the hTERT gene is replaced and the portion of the hTERT gene replaced includes exon 1 and/or exon 2.

15 17. The method of any one of claims 11 to 16, further comprising a step of inducing the telomerase activity null cells to differentiate into cells of at least one of ectodermal, endodermal or mesodermal lineage.

18. The method of claim 17, wherein the telomerase null cells are induced to differentiate into at least one of the group comprising neurons, astrocytes, glia and
20 hematopoietic cells.

19. A method of producing one or more isolated pluripotent telomerase activity null stem cells suitable for clinical use, comprising: evaluating the average telomere length and/or in vivo tumorigenicity of said pluripotent stem cells in which the human telomerase reverse transcriptase (hTERT) or the human telomerase RNA subunit
25 (hTER) activity has been inactivated; and identifying said pluripotent stem cells as suitable for clinical use if said evaluated average telomere length is 5 kb or less and/or said cells have reduced ability to form a teratoma in vivo.

20. The method of claim 19, wherein the evaluated telomere length is around 4 kb.

21. The method of claim 19 or 20, wherein the hTERT or hTER inactivation is by virtue of an induced, or CRISPR-Cas, knockout of hTERT or hTER gene activity.

22. The method of claim 21, wherein:

i) a portion of the hTERT or hTER gene is replaced by homologous DNA flanked by LoxP sites, which retains gene function, and at a selected time transient expression of Cre recombinase in the recombinant cells causes deletion of the homologous DNA resulting in loss of hTERT or hTER activity and, consequently, telomerase activity; or

ii) a portion of the hTERT or hTER gene is edited by CRISPR-Cas resulting in loss of hTERT or hTER activity and, consequently, telomerase activity.

23. The method of claim 22, whereby in i) the recombinant cells are homozygous for the homologous DNA insert.

24. The method of any one of claims 19 to 23, wherein the stem cells have limited proliferation capacity.

25. The method of claim 24, wherein the stem cells retain expression of at least one stem cell pluripotency marker selected from the group comprising Oct4, Nanog, Sox2 and Klf4.

26. The method of any one of claims 19 to 25, wherein the stem cells are human embryonic stem cells (hESC) or induced pluripotent stem cells (iPSC).

27. The method of any preceding claim, comprising a further step of inducing said stem cells to differentiate into cells of at least one of ectodermal, endodermal or mesodermal lineage.

28. The method of claim 27, wherein the stem cells are induced to differentiate into at least one of the group comprising neurons, astrocytes, glia and hematopoietic cells.

29. The method of any preceding claim, wherein said evaluating step comprises performing one or more of: single telomere length analysis (STELA), fluorescence in-situ hybridization (FISH), flow-FISH and Southern blot analysis.

30. The method of any preceding claim, further comprising:

(a) assessing the genomic integrity of said pluripotent stem cells to obtain a genomic integrity result, wherein said assessing comprises one or more of: karyotyping; analysis of variable number tandem repeats (VNTRs), short tandem repeats (STRs),
5 single nucleotide polymorphisms (SNPs), and/or copy number variations (CNVs); analysis of culture mosaicism; analysis of DNA sequences related to genetic diseases; and complete genome sequencing and analysis; and

(b) identifying the pluripotent stem cells as suitable for clinical use based on the genomic integrity result.

10 31. The method of claim 30, wherein the genome integrity is assessed prior to gene inactivation.

32. Use of at least one isolated telomerase activity null stem cell of any one of claims 5 to 11 for the preparation of a therapeutic composition for cell therapy of a subject in need thereof.

15 33. The use according to claim 32, wherein the at least one cell is differentiated into cells of at least one of ectodermal, endodermal or mesodermal lineage.

34. The use according to claim 33, wherein the at least one cell is differentiated into cells of at least one lineage selected from the group comprising neurons, astrocytes, glia and hematopoietic cells.

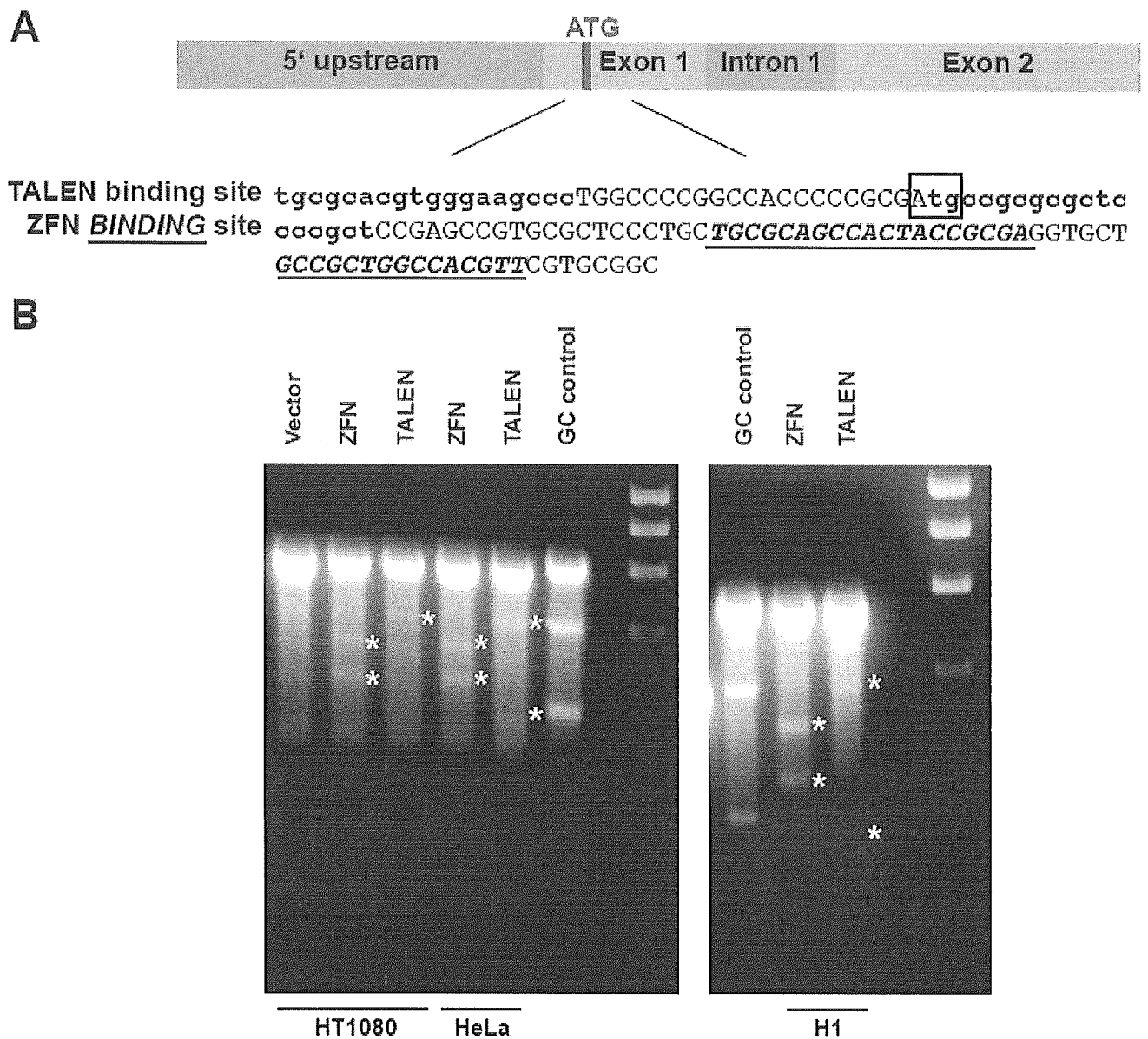


Figure 1

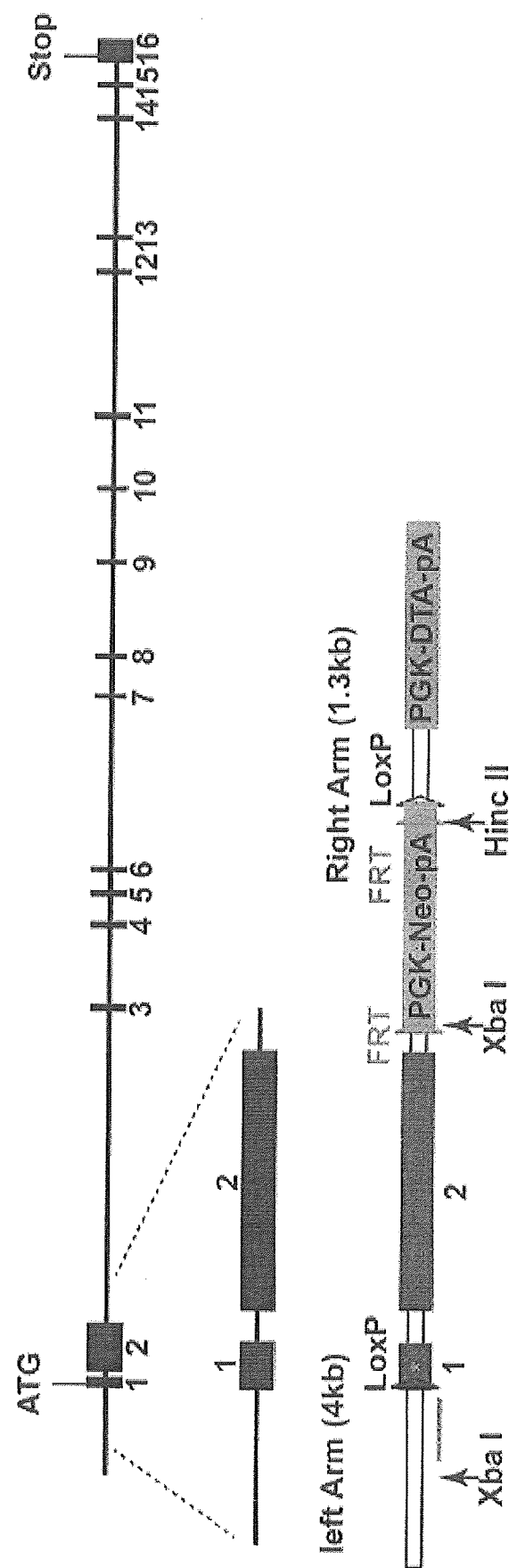
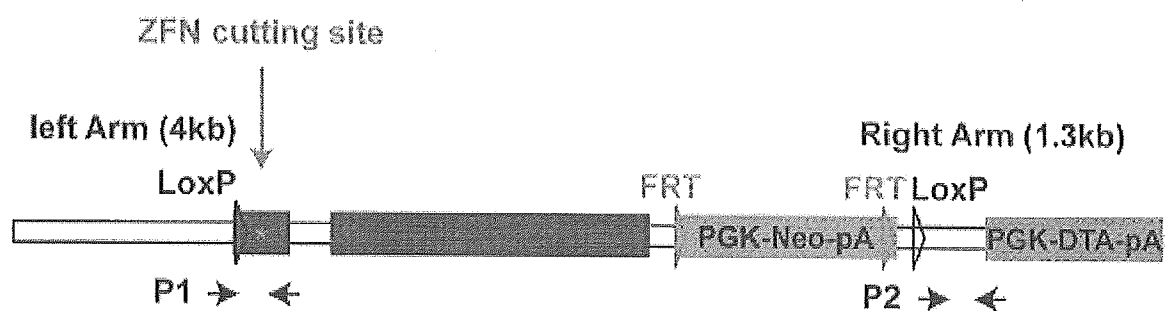
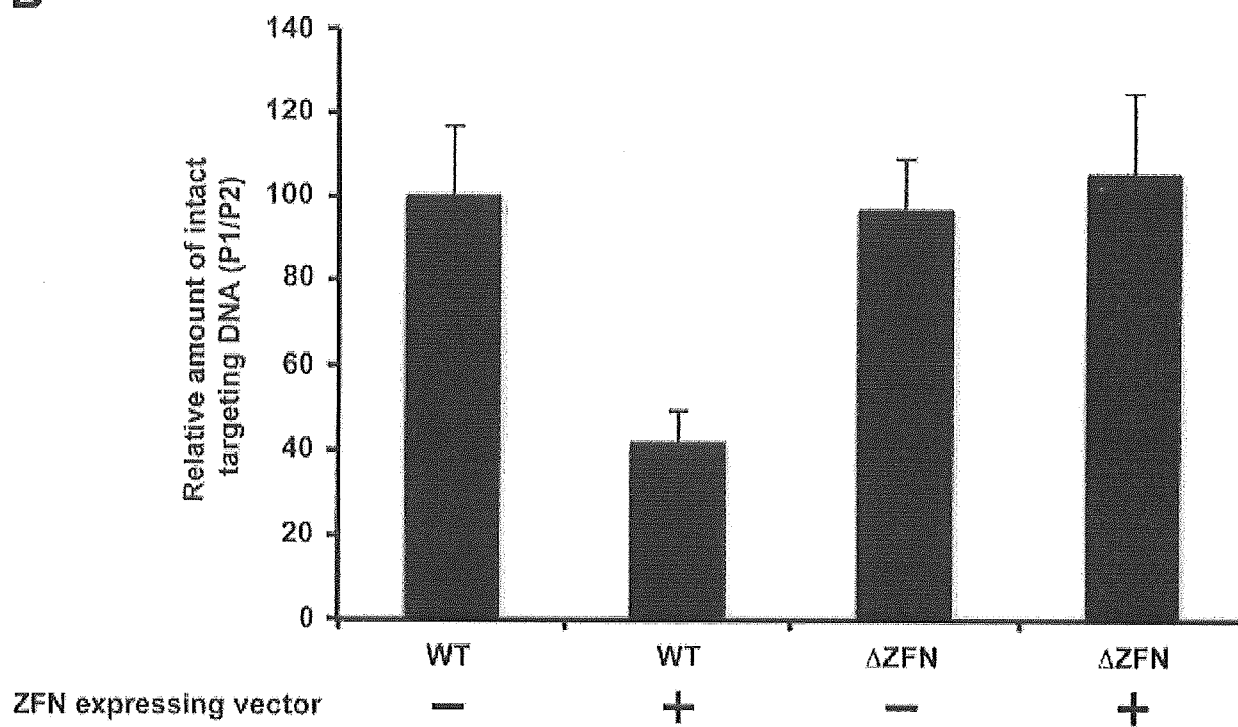


Figure 2

A**B****Figure 3**

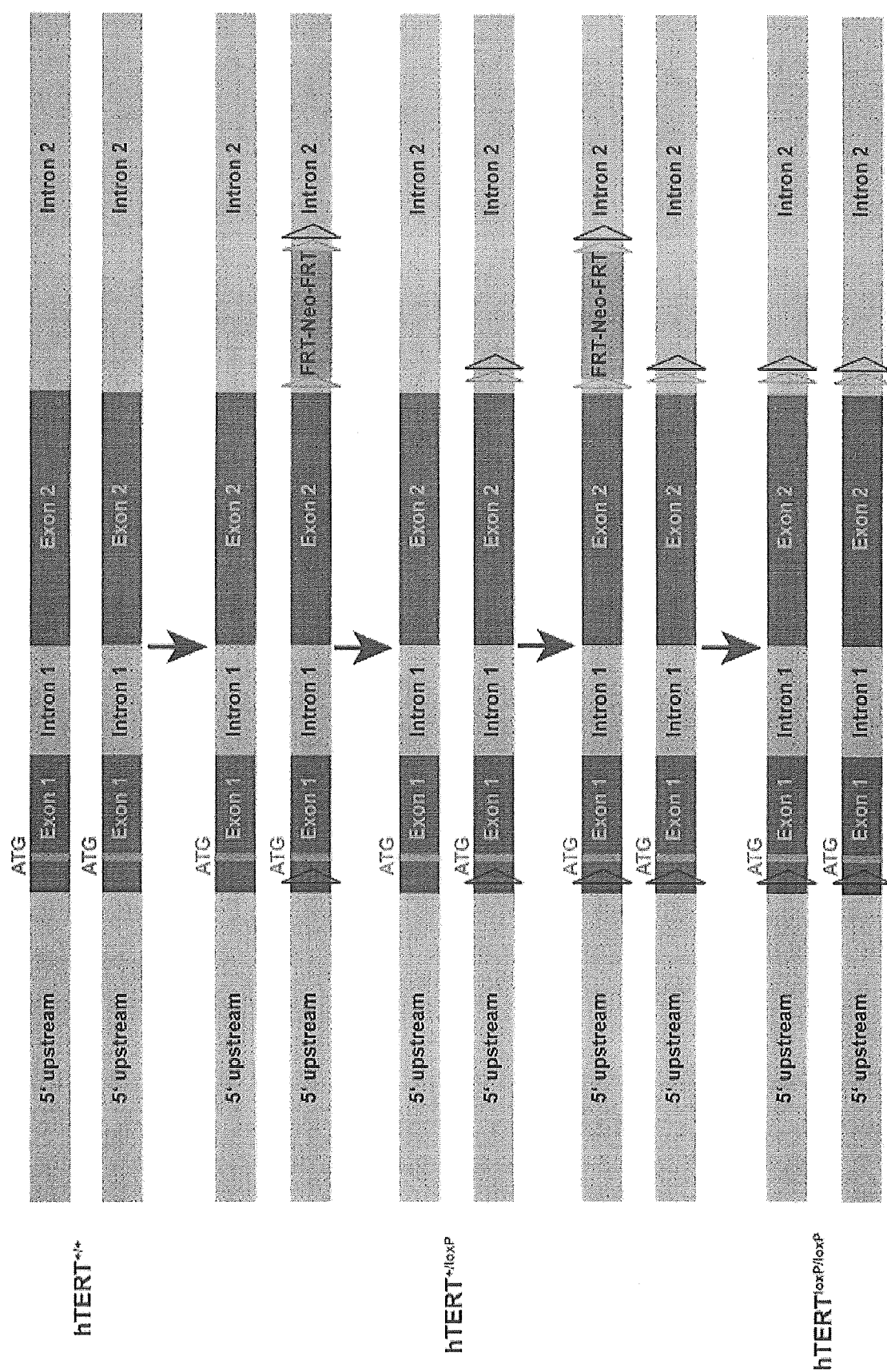


Figure 4

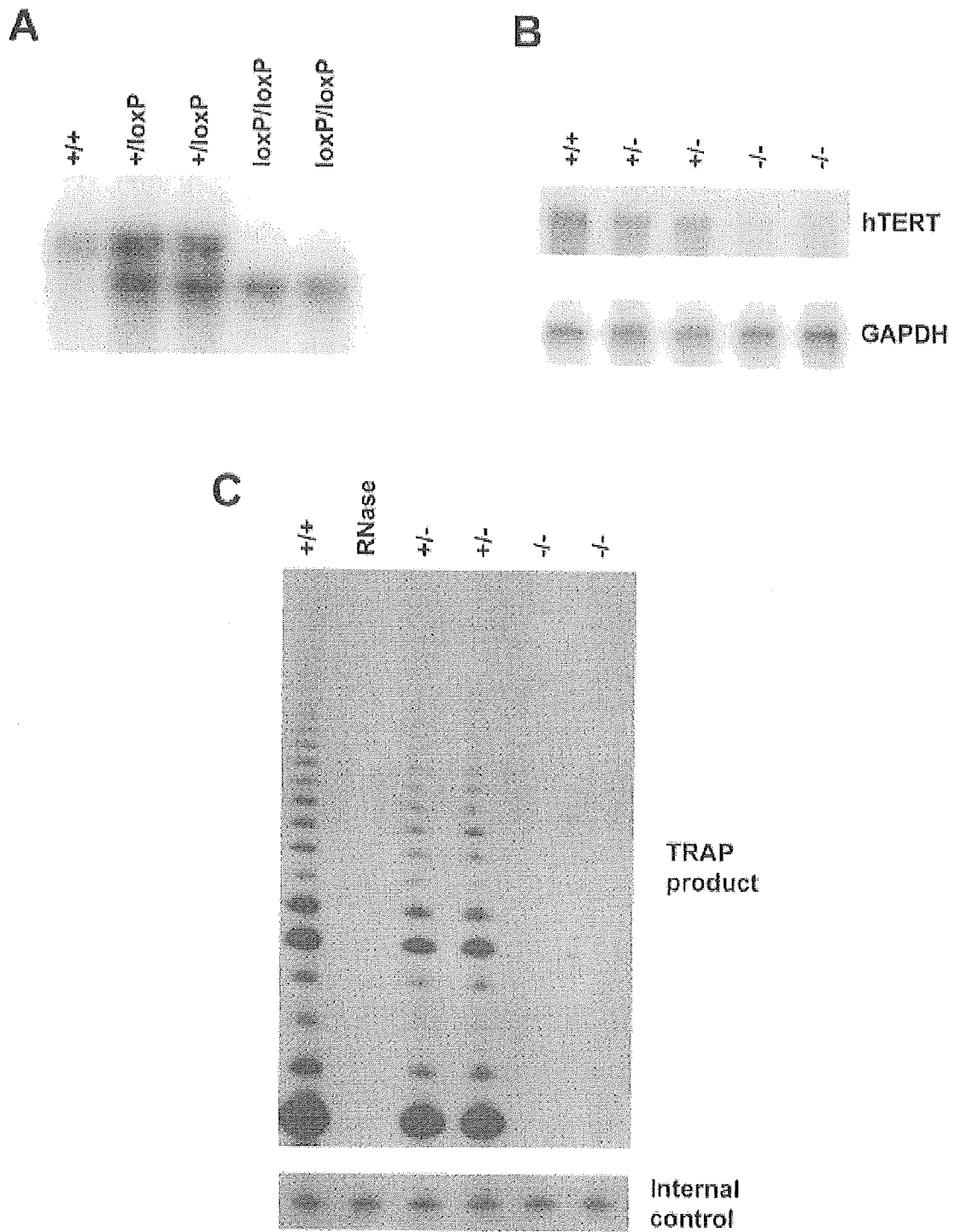
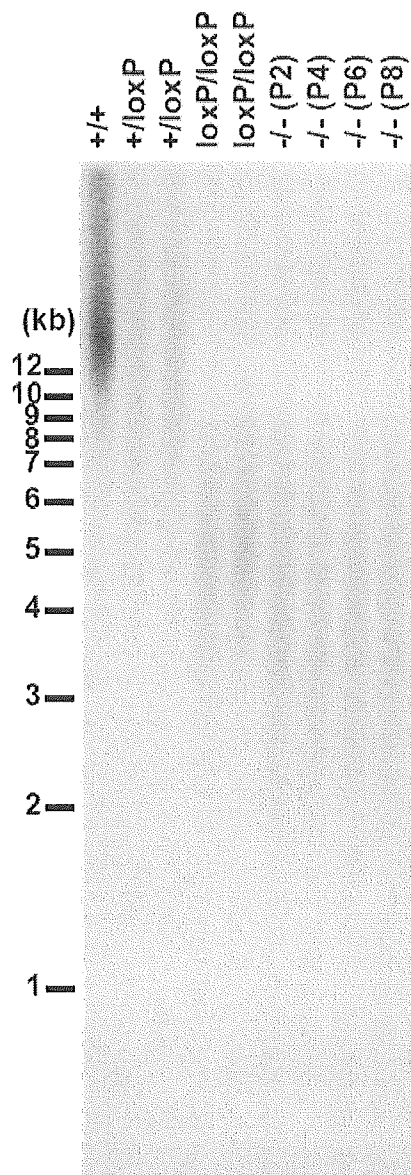
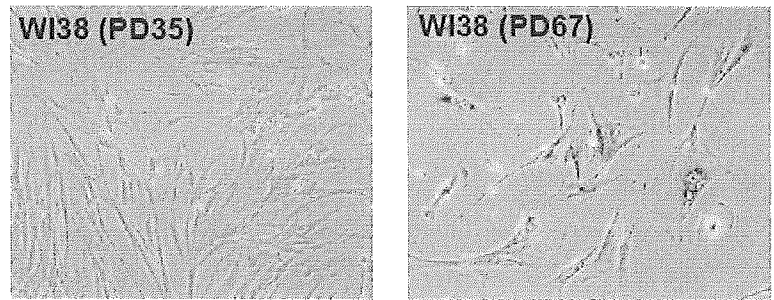
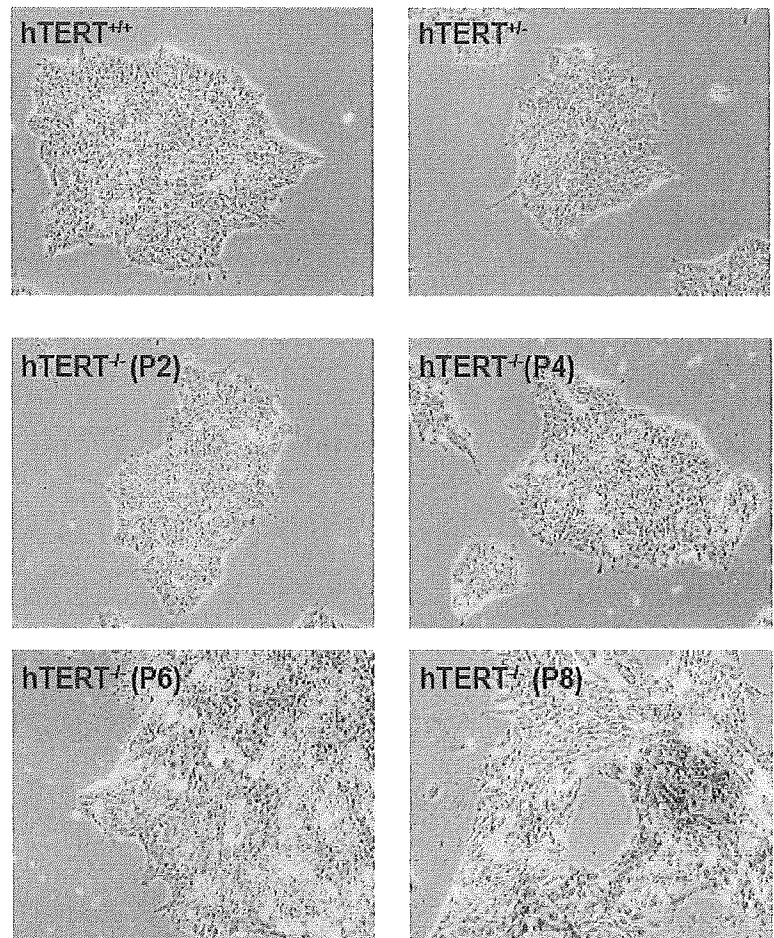


Figure 5

A**B****C****Figure 6**

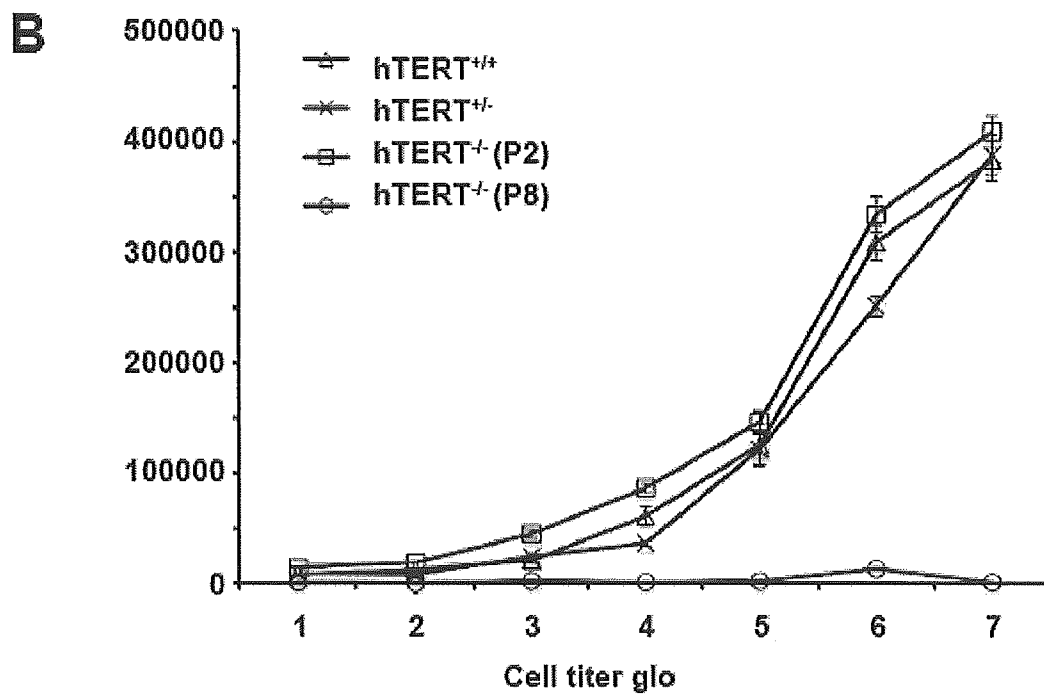
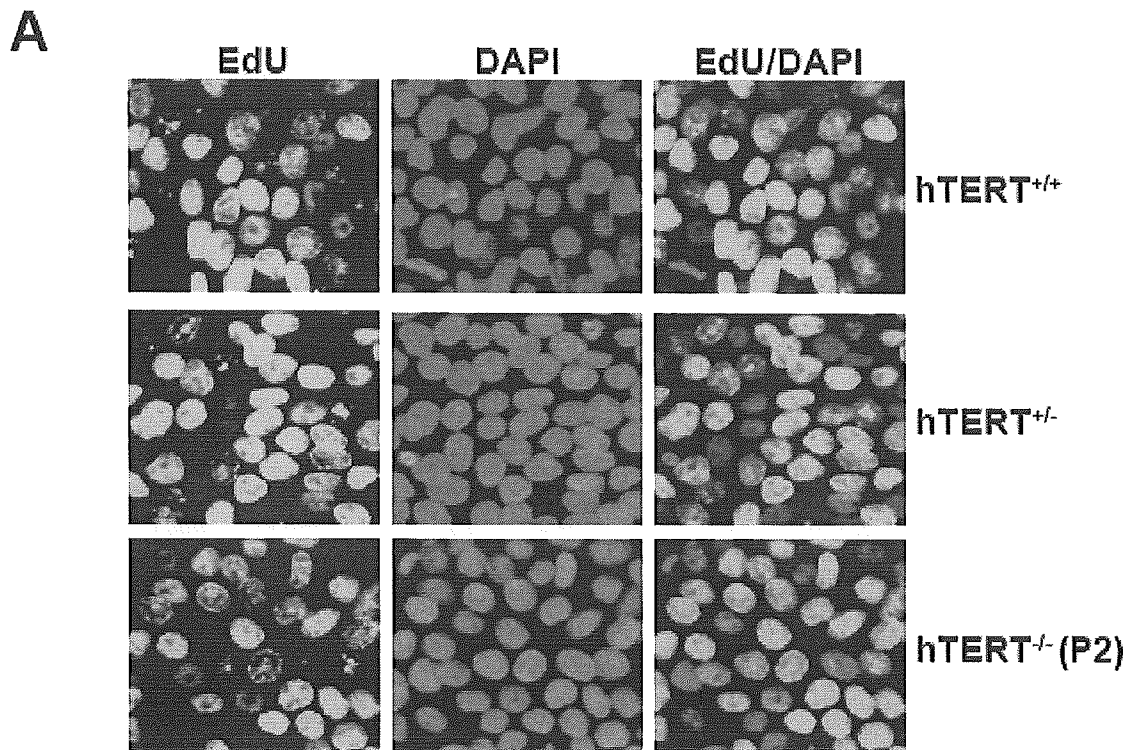
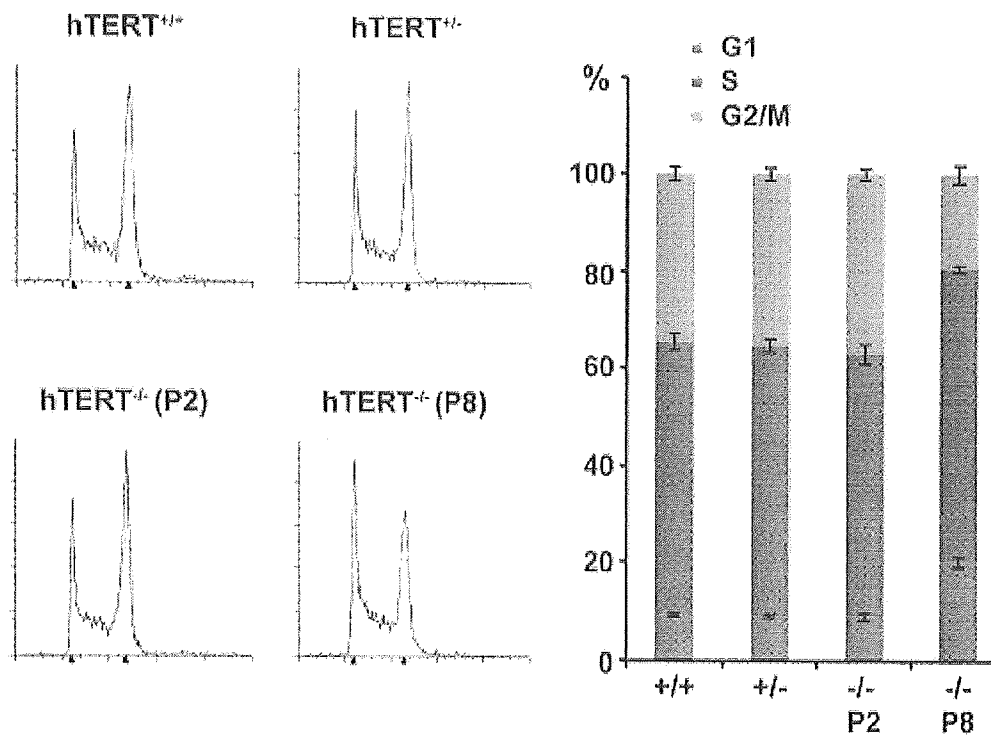


Figure 7 A-B

C



D

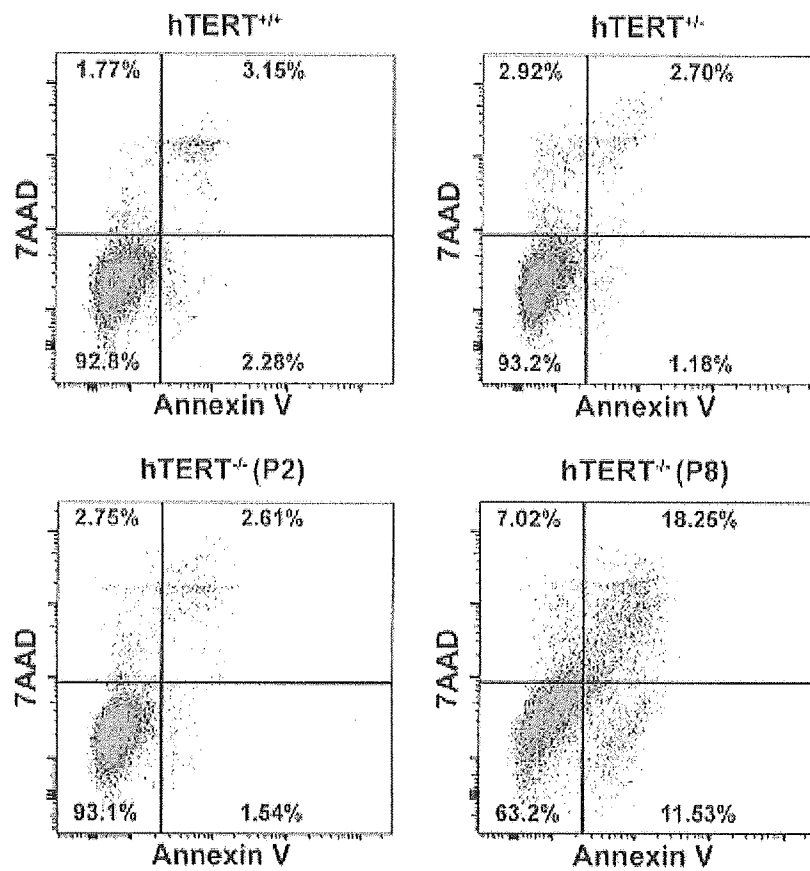


Figure 7 C-D

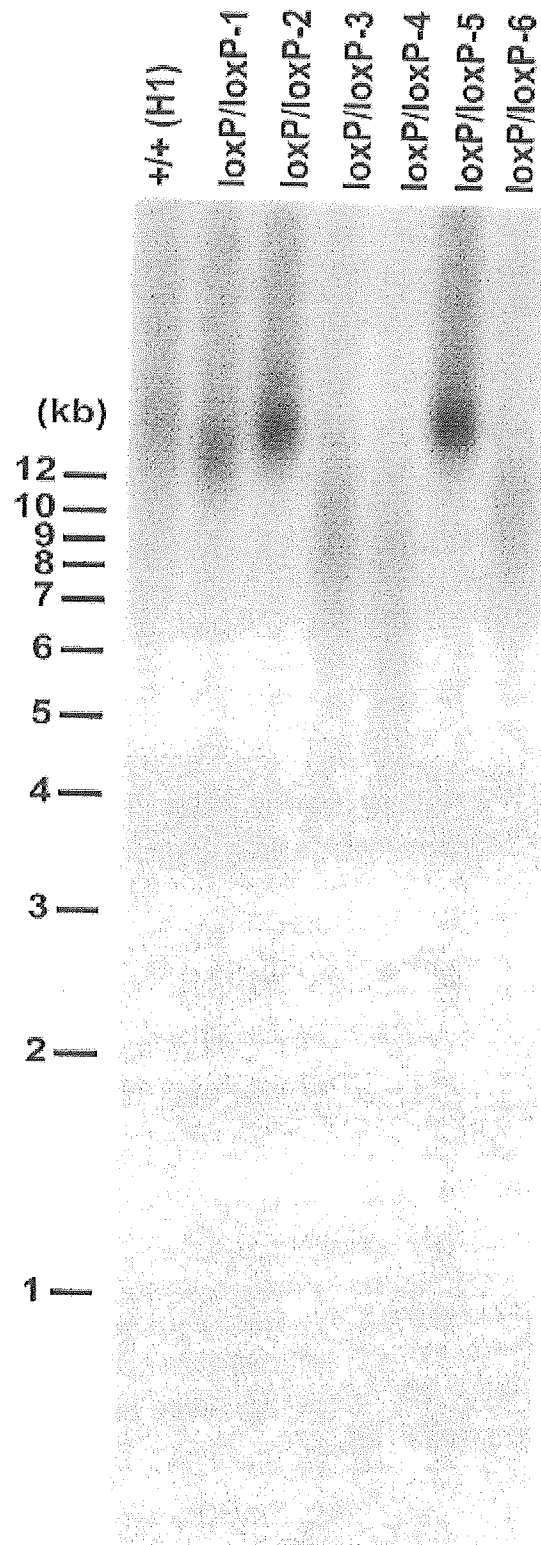


Figure 8

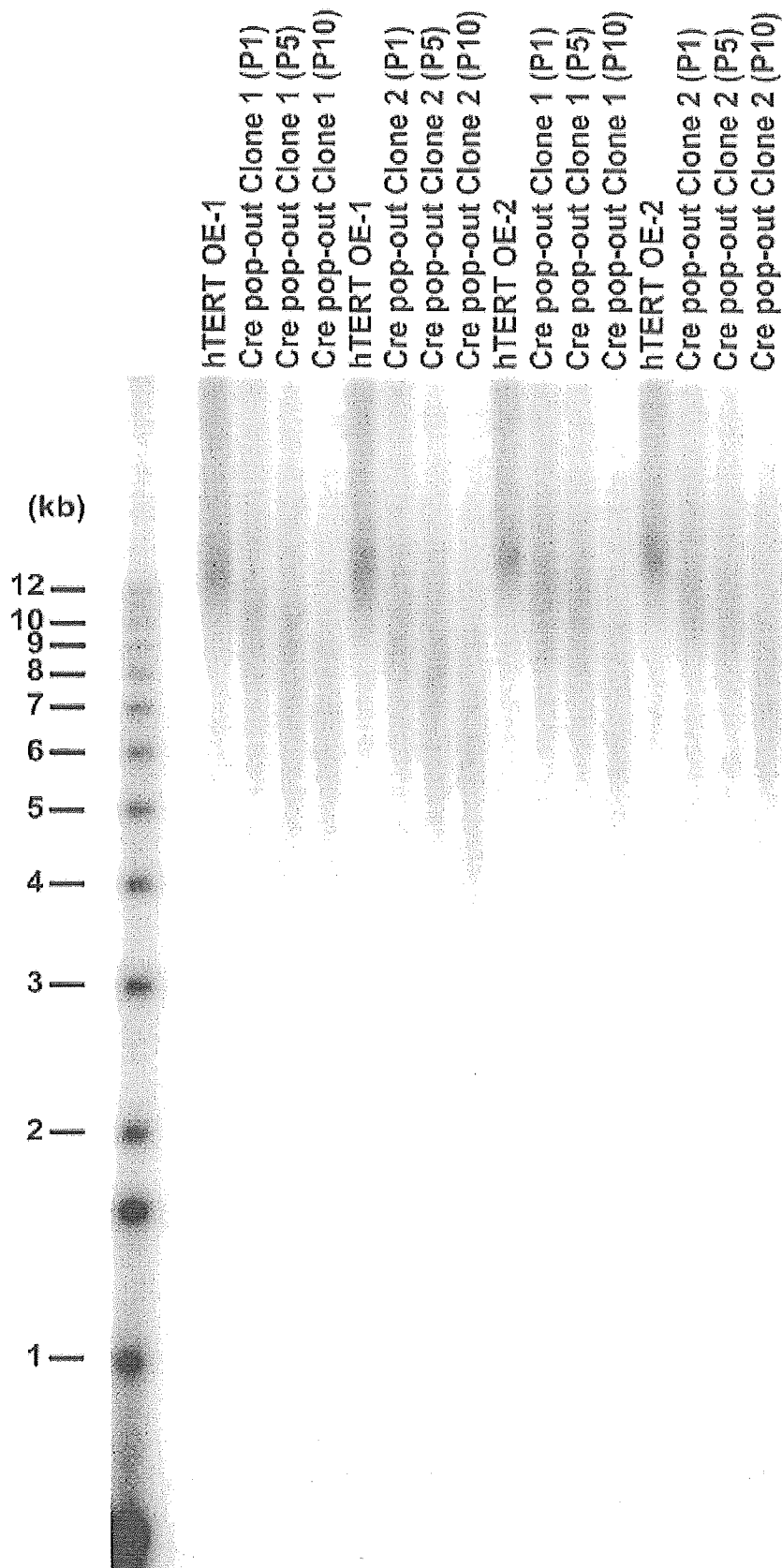
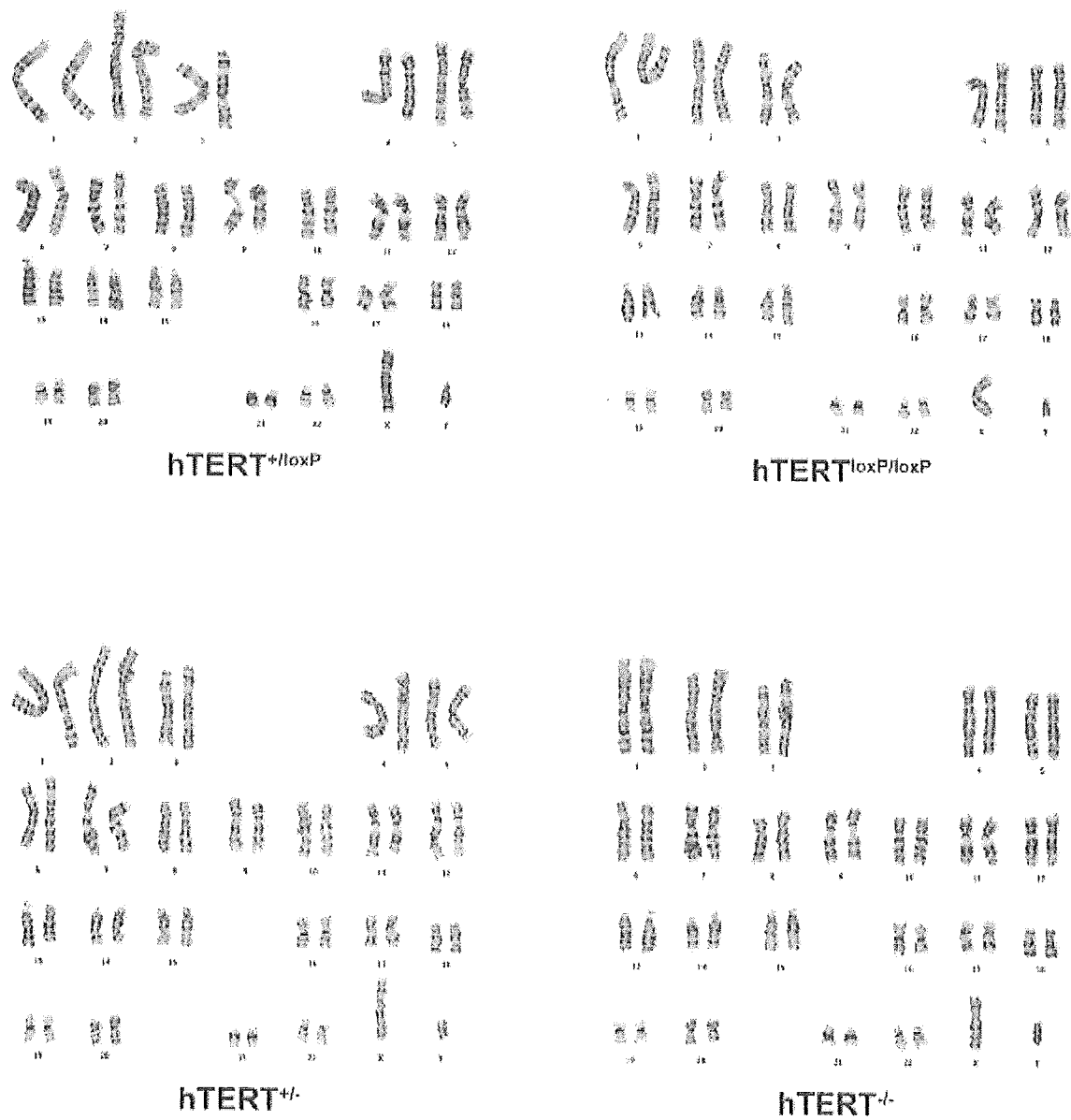


Figure 9

**Figure 10**

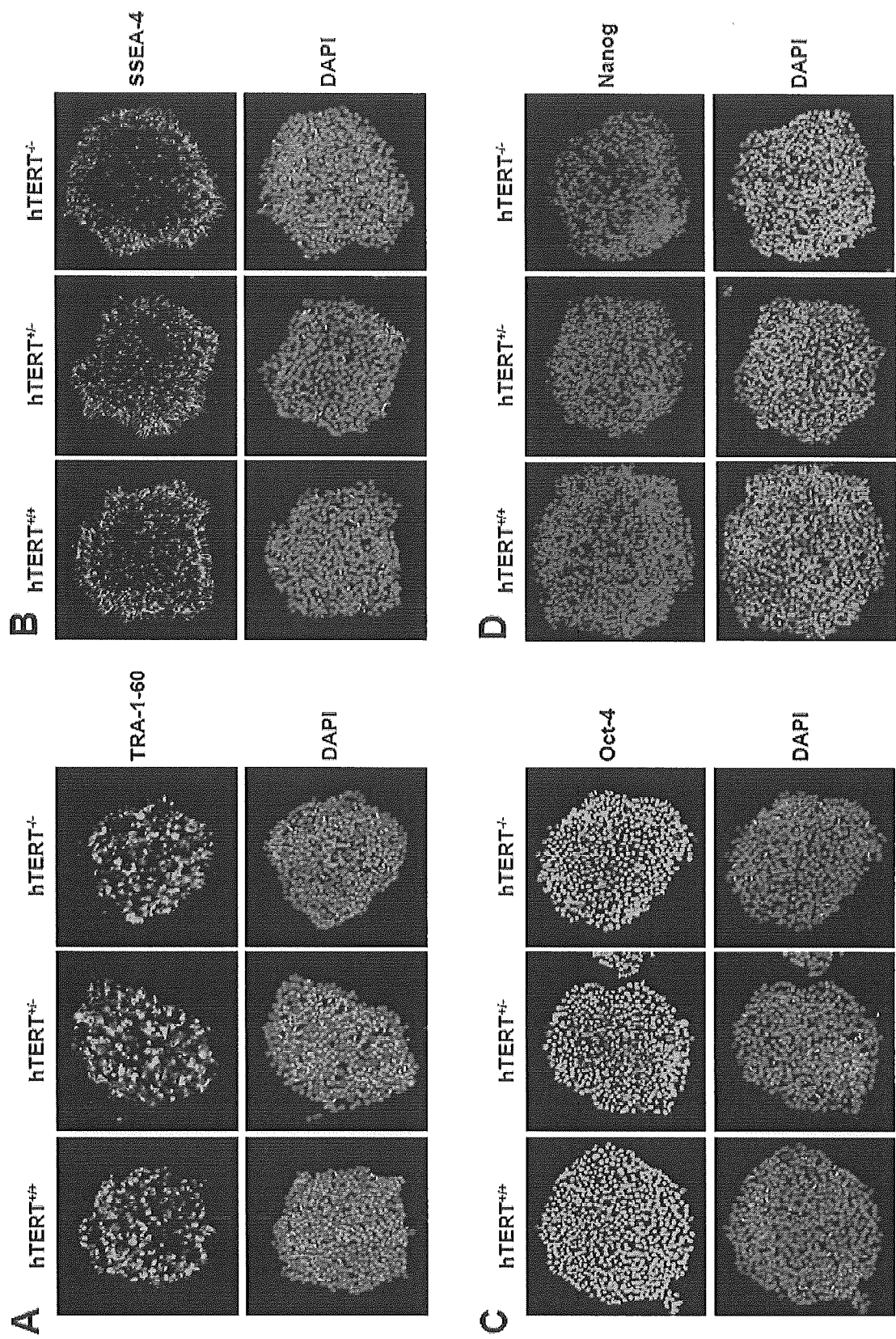
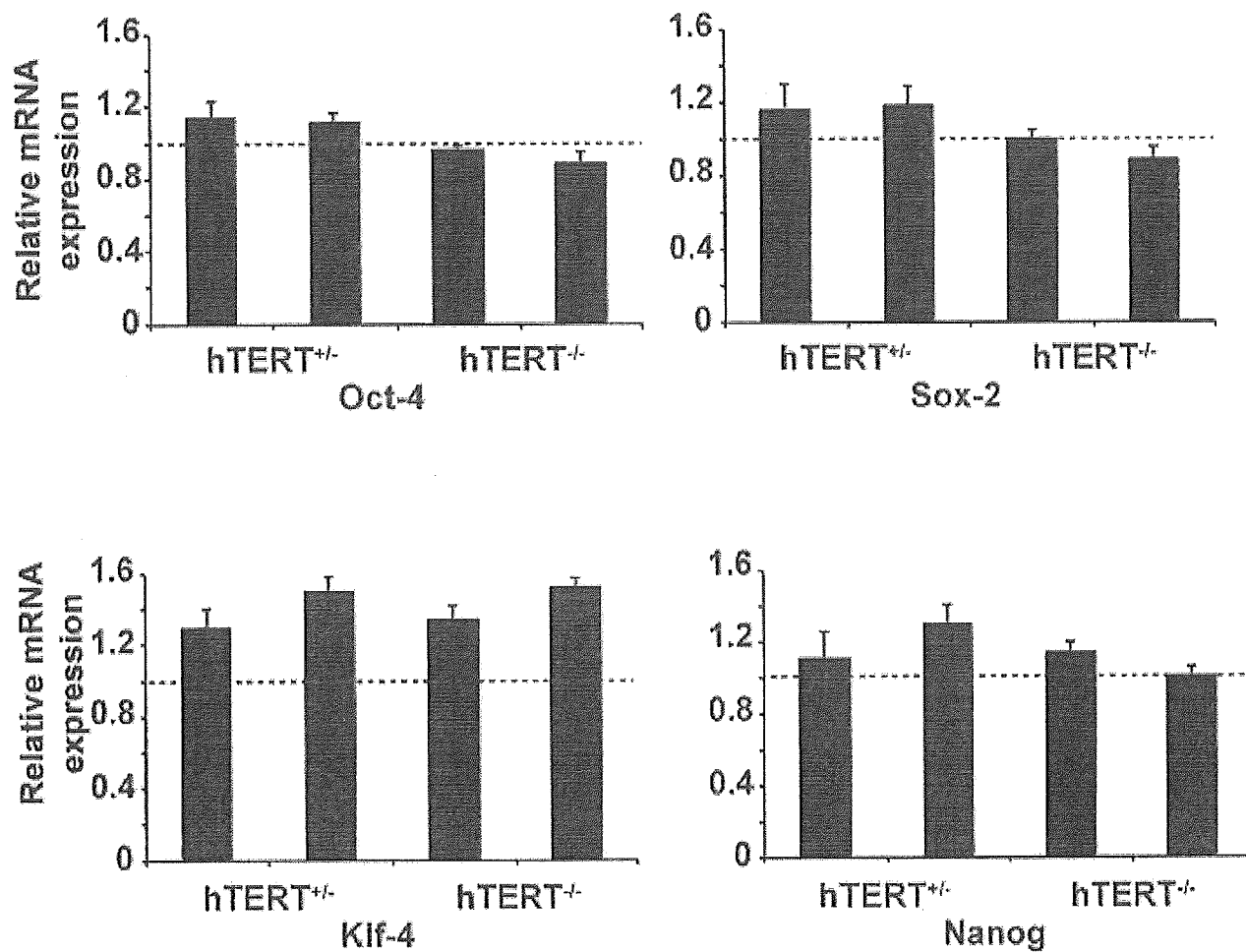


Figure 11 A-D

E**Figure 11 E**

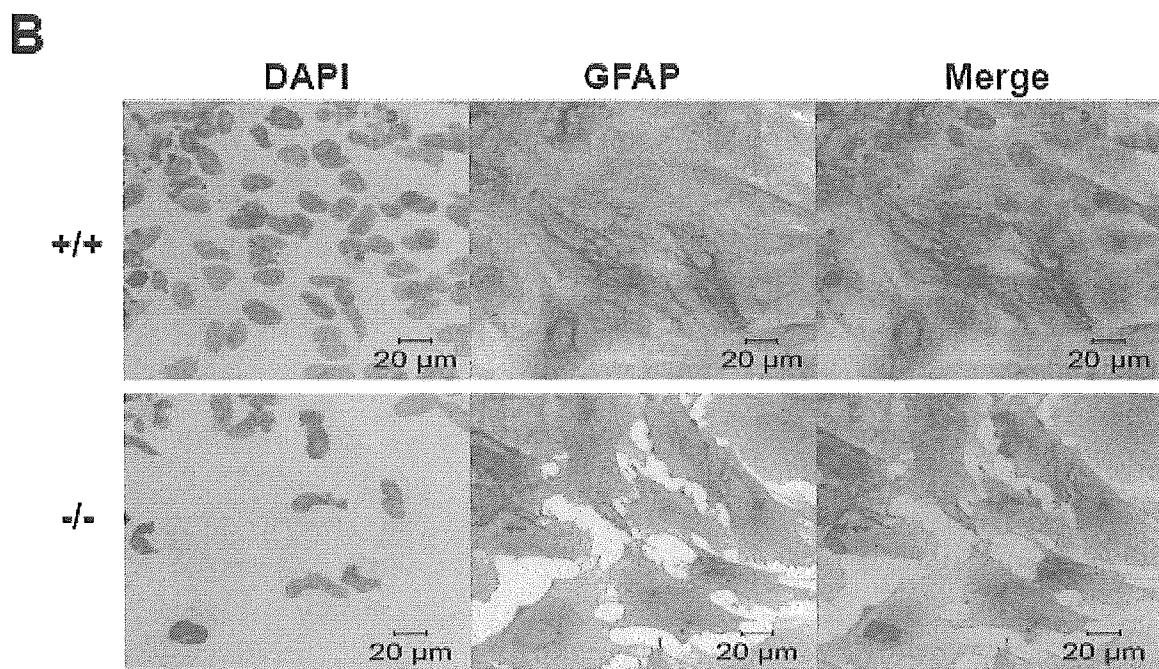
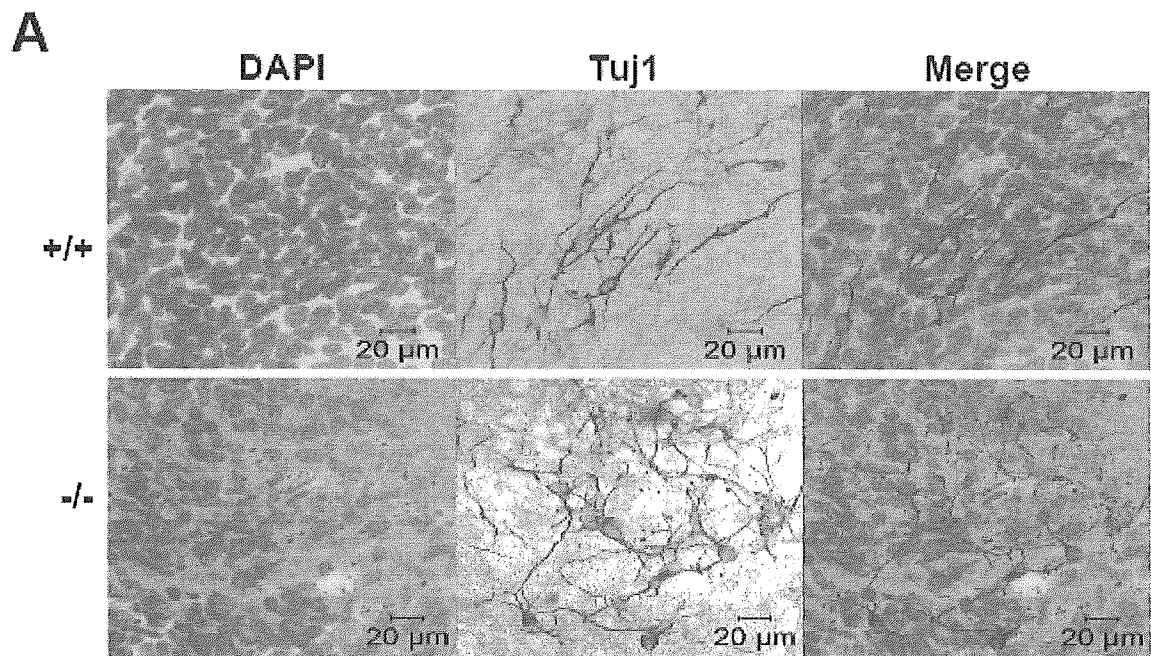


Figure 12 A-B

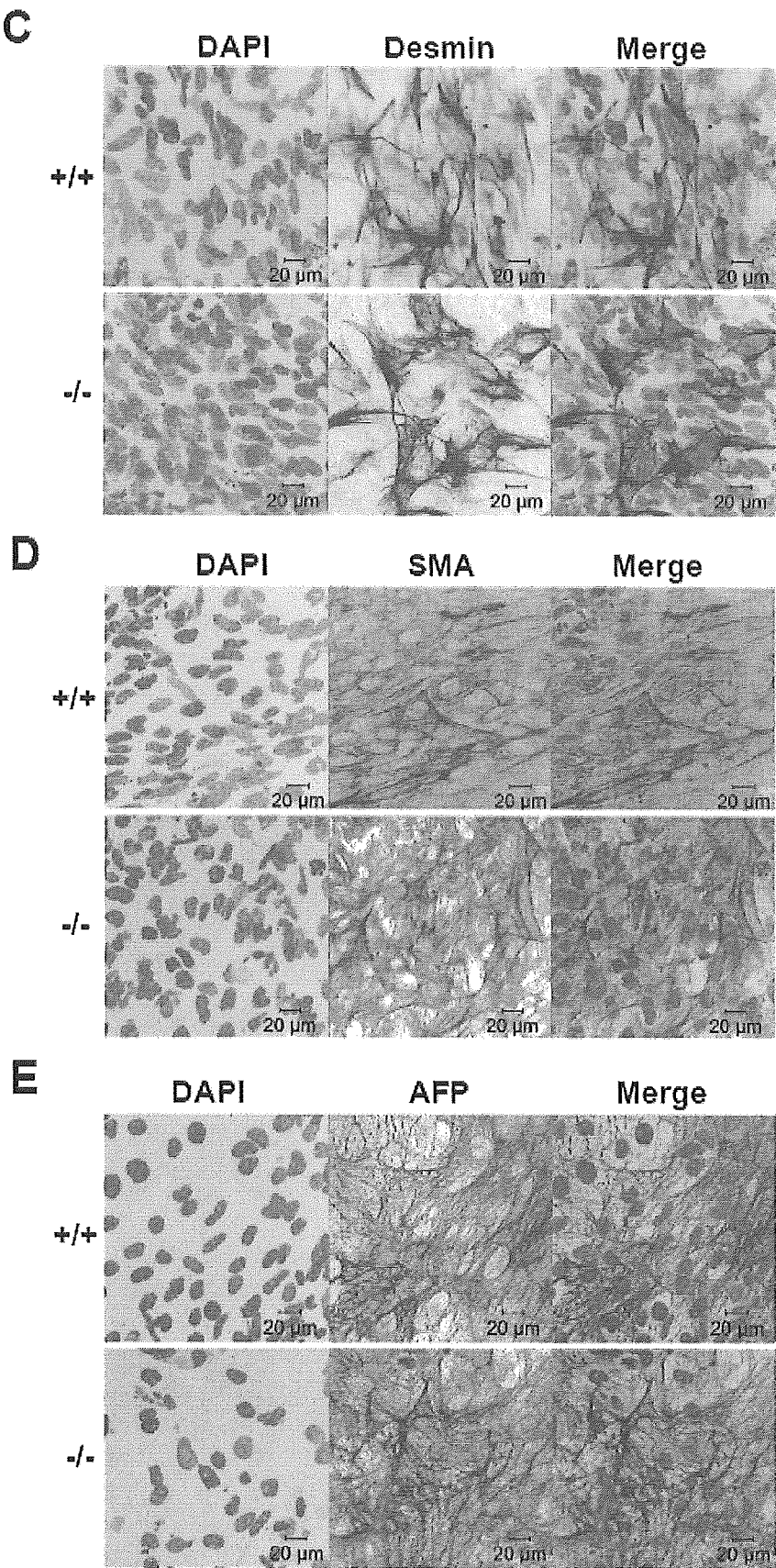


Figure 12 C-E

A

	$+ / +$	$+ / -$	$- / -$ (9kb)	$- / -$ (P2, 4kb)	$- / -$ (P4, 3.5kb)
Teratoma/Injection	17/20	18/20	17/20	1/20	0/60

B

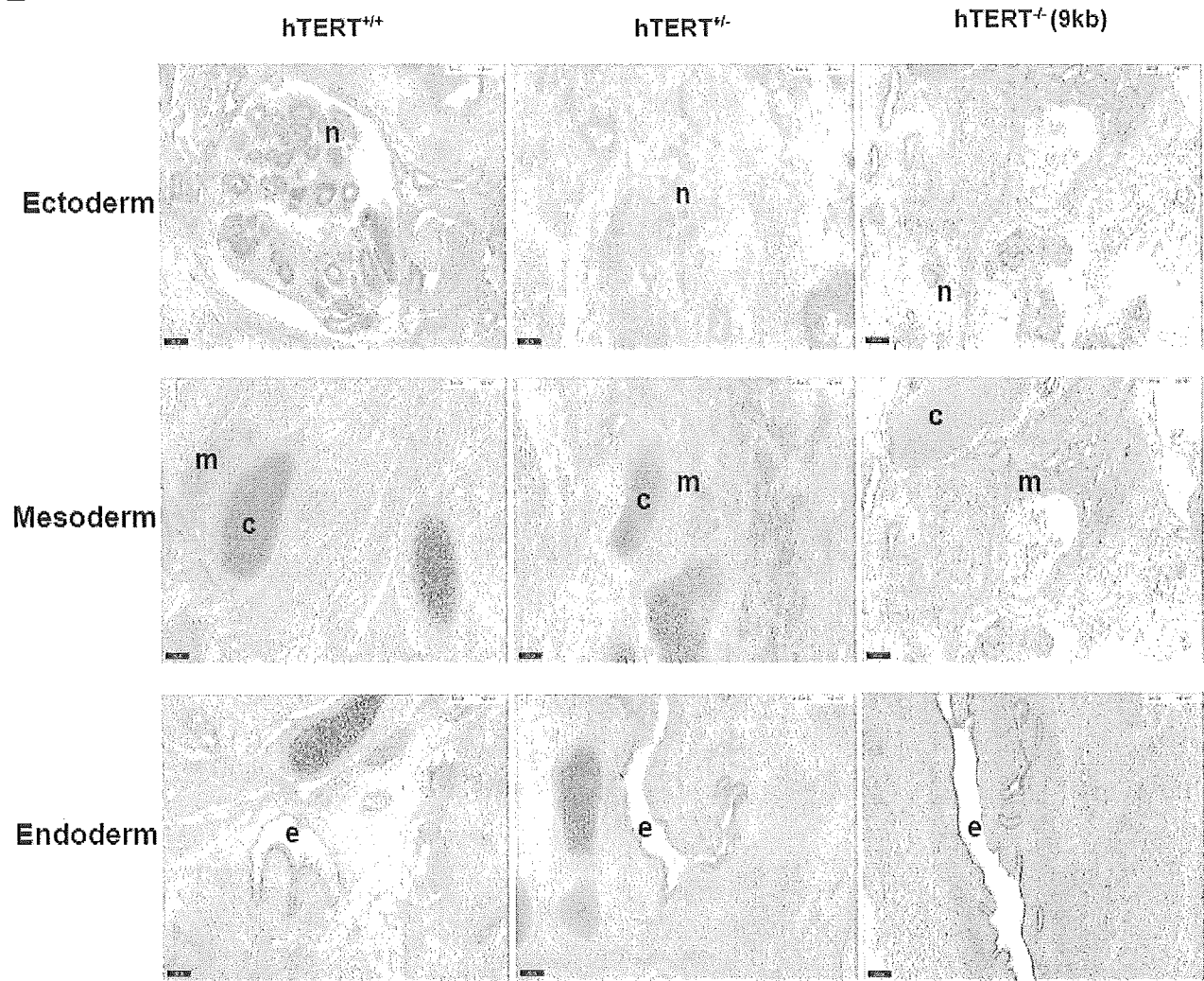


Figure 13

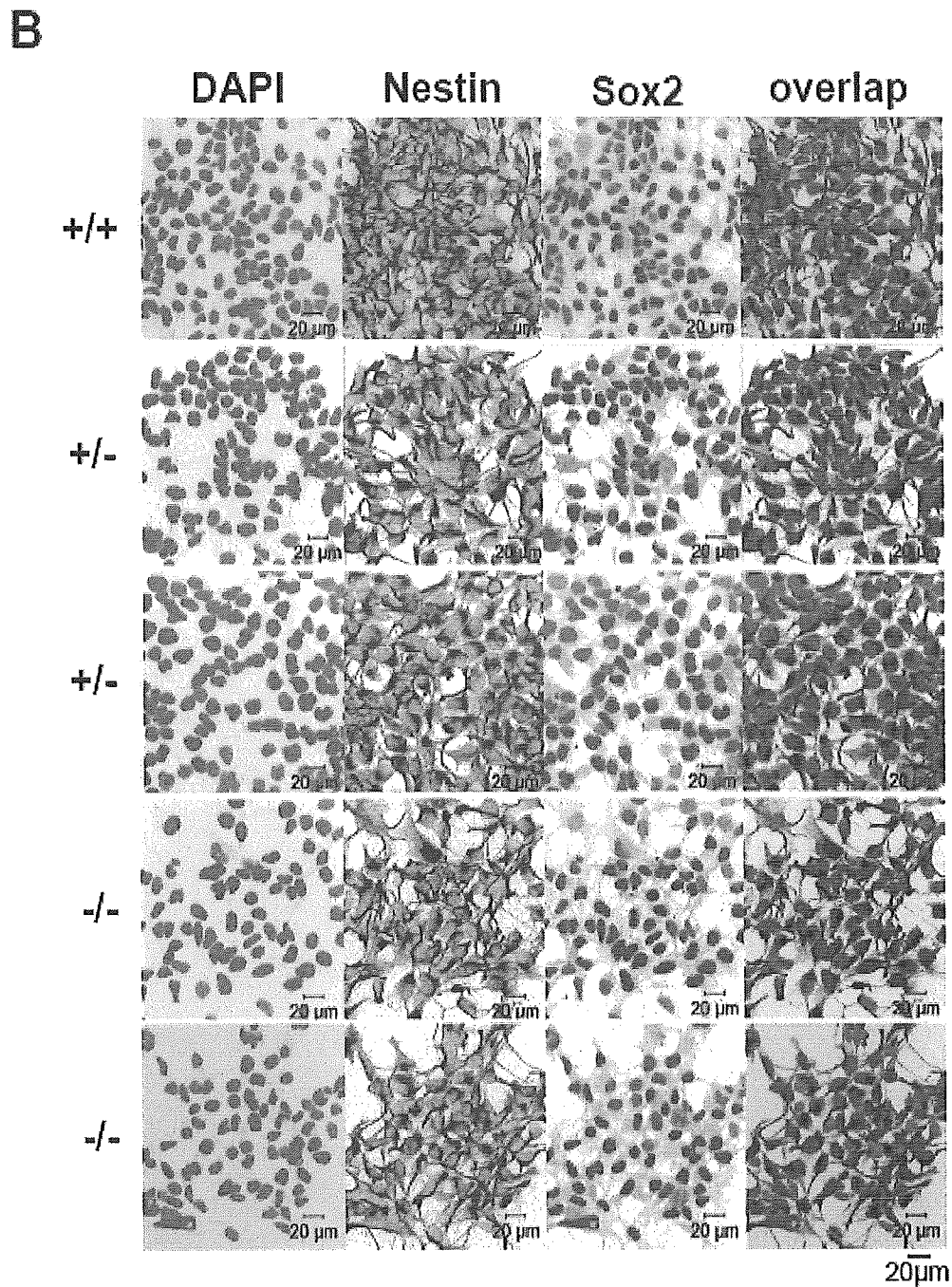
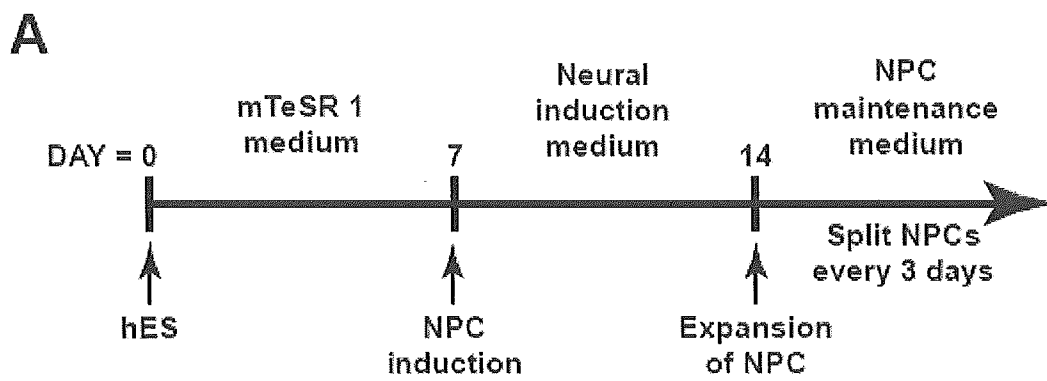
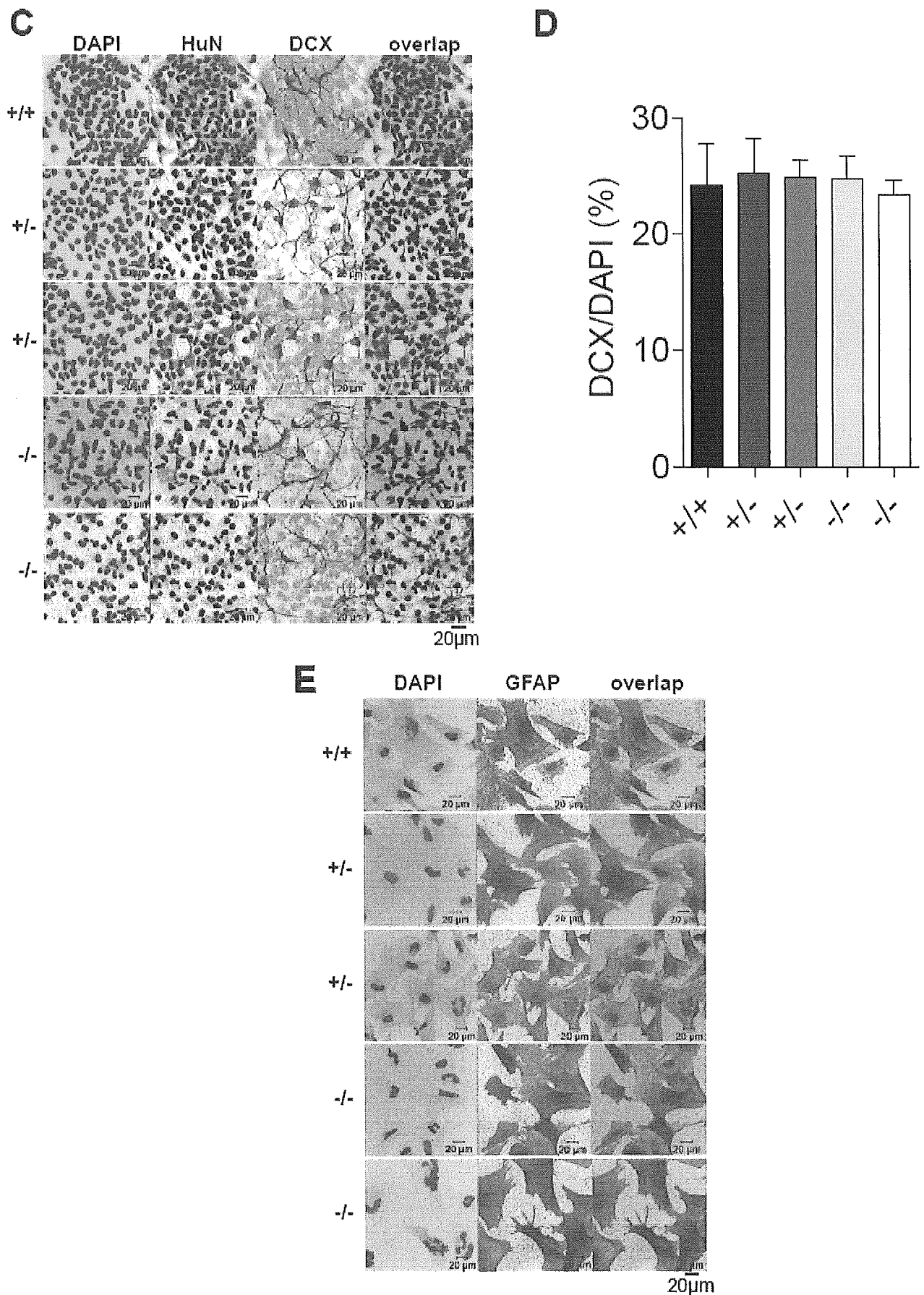


Figure 14 A-B



STITUTE SHEET (RULE 2)
Figure 14 C-E

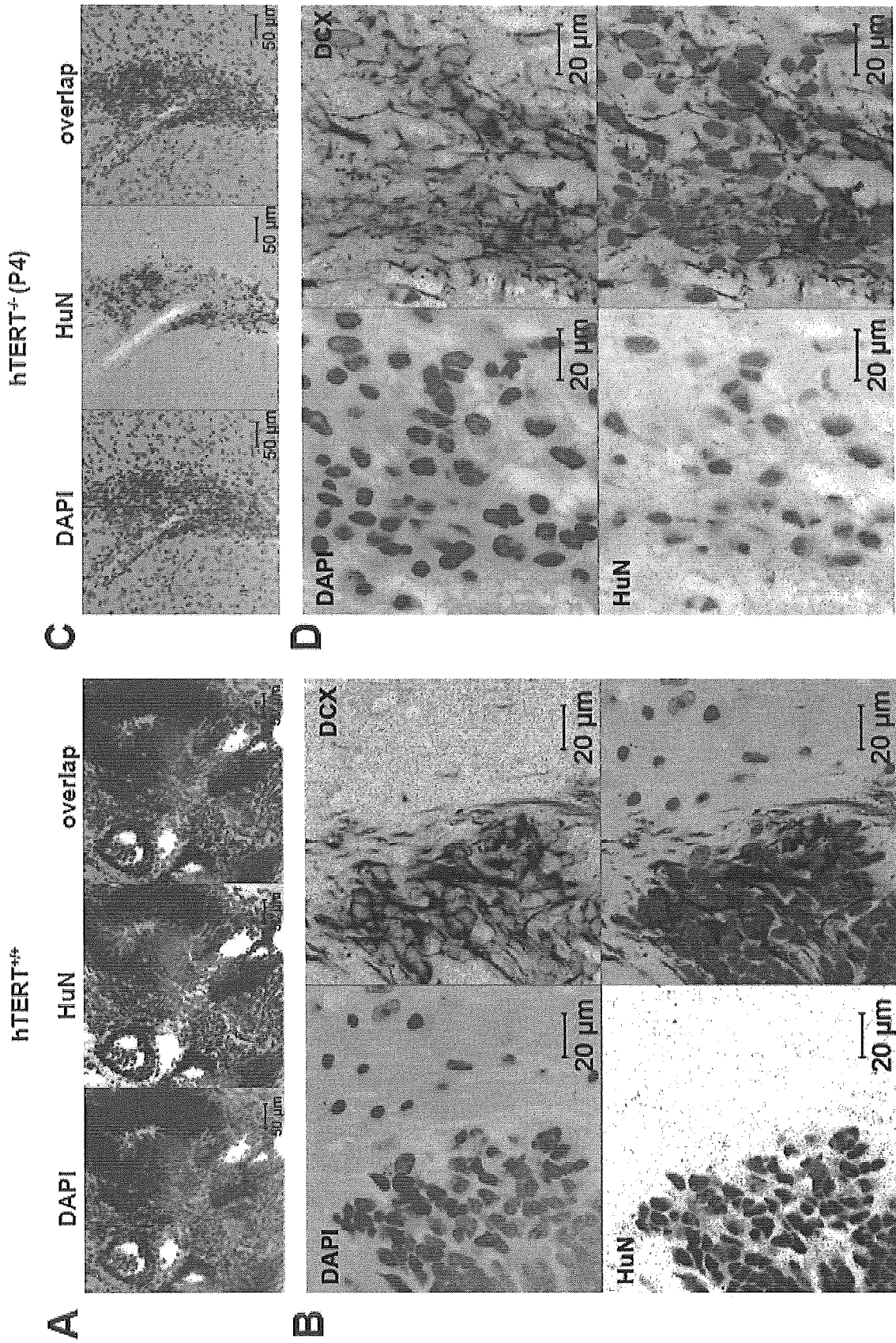
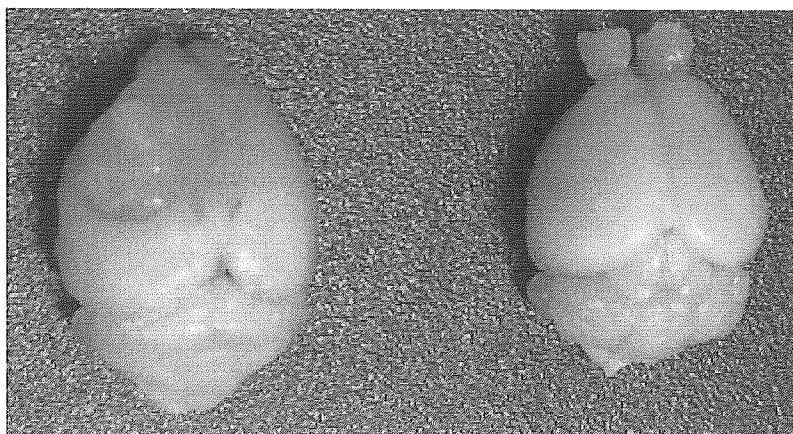
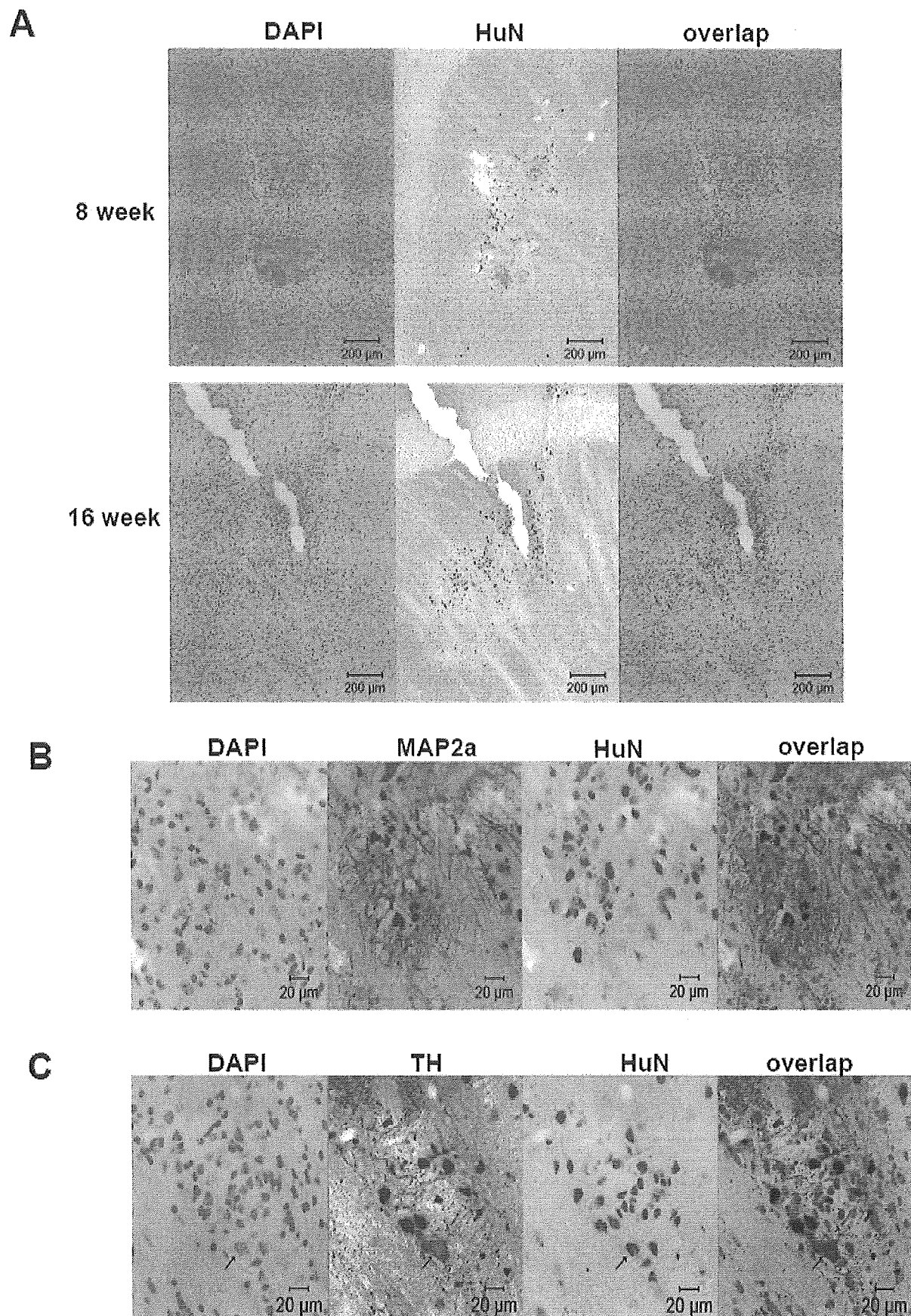


Figure 15 A-D

E $hTERT^{+/+}$ $hTERT^{-/-}$ (P4)

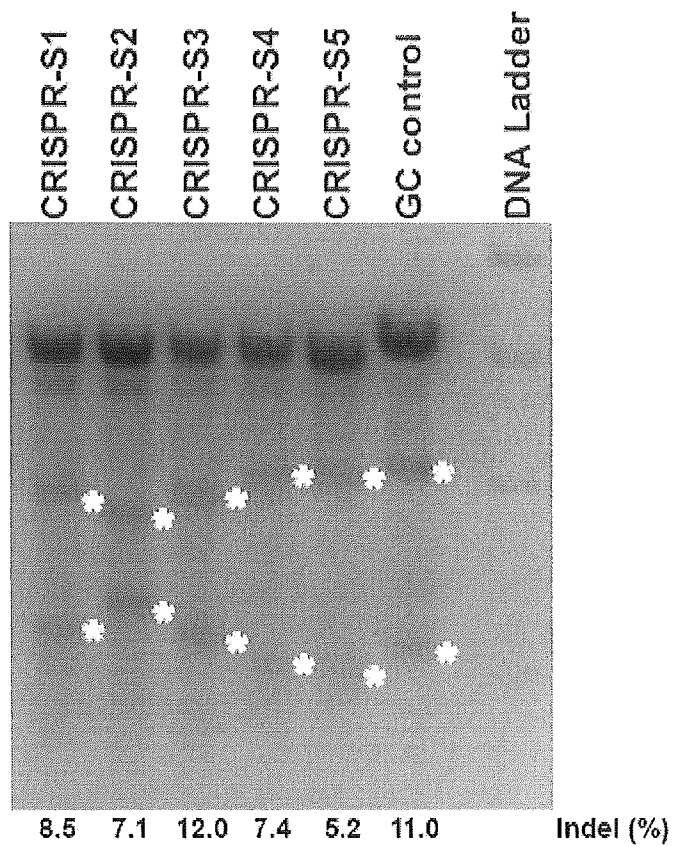
n=5

F $hTERT^{+/+}$ **Figure 15 E-F**

**Figure 16**

A

Protospacers	Sequence (<u>PAM</u>)
1	GGGGGCGGCCGTGCGTCCCAGGG
2	GCTGCGCAGCCACTACCGCGAGG
3	GCAGCAGGGAGCGCACGGCTCGG
4	GGGGAGCGCGCGGCATCGCGGGG
5	GGGAGCGCGCGGCATCGCGGGG

B**Figure 17 A-B**

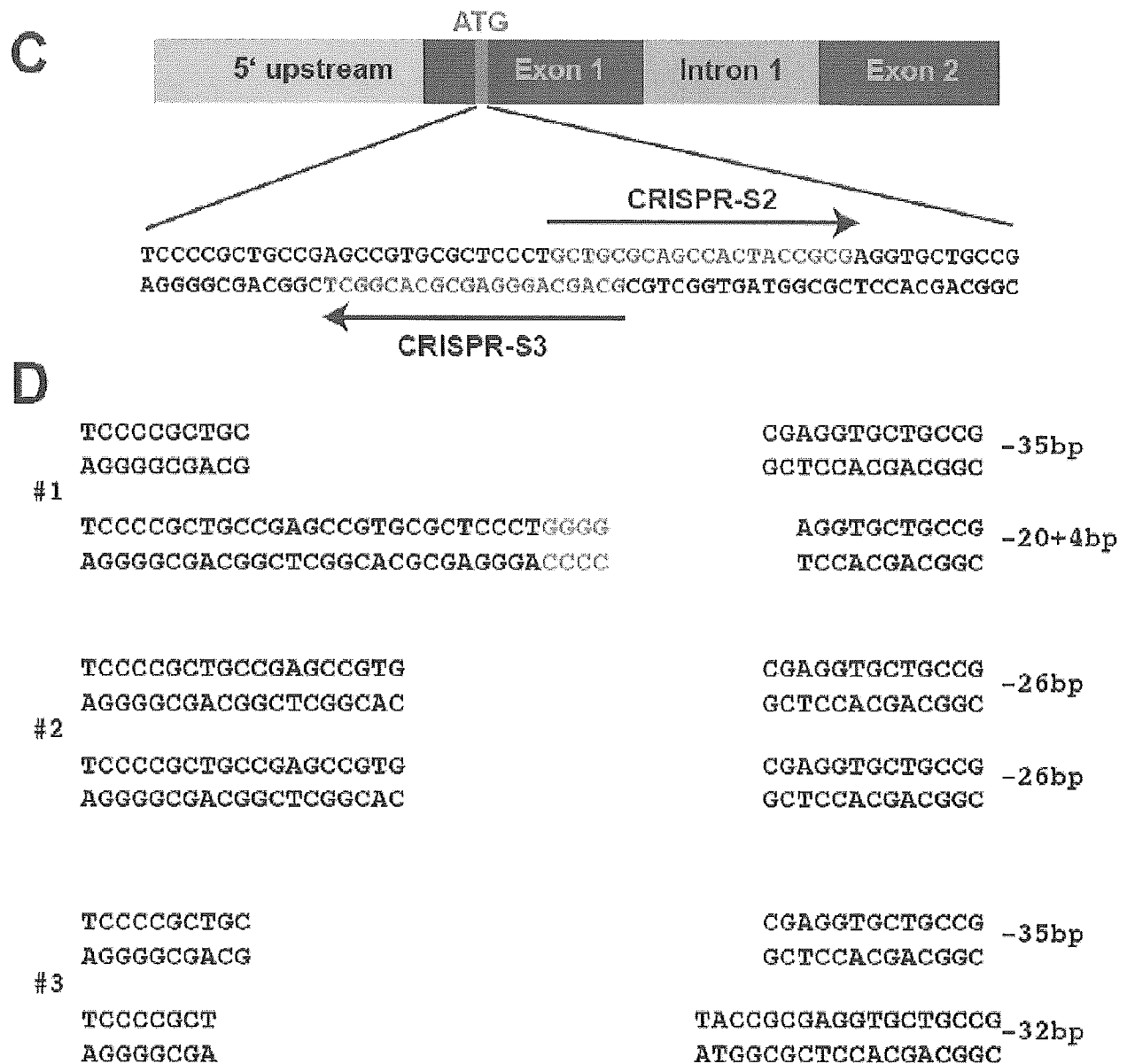


Figure 17 C-D

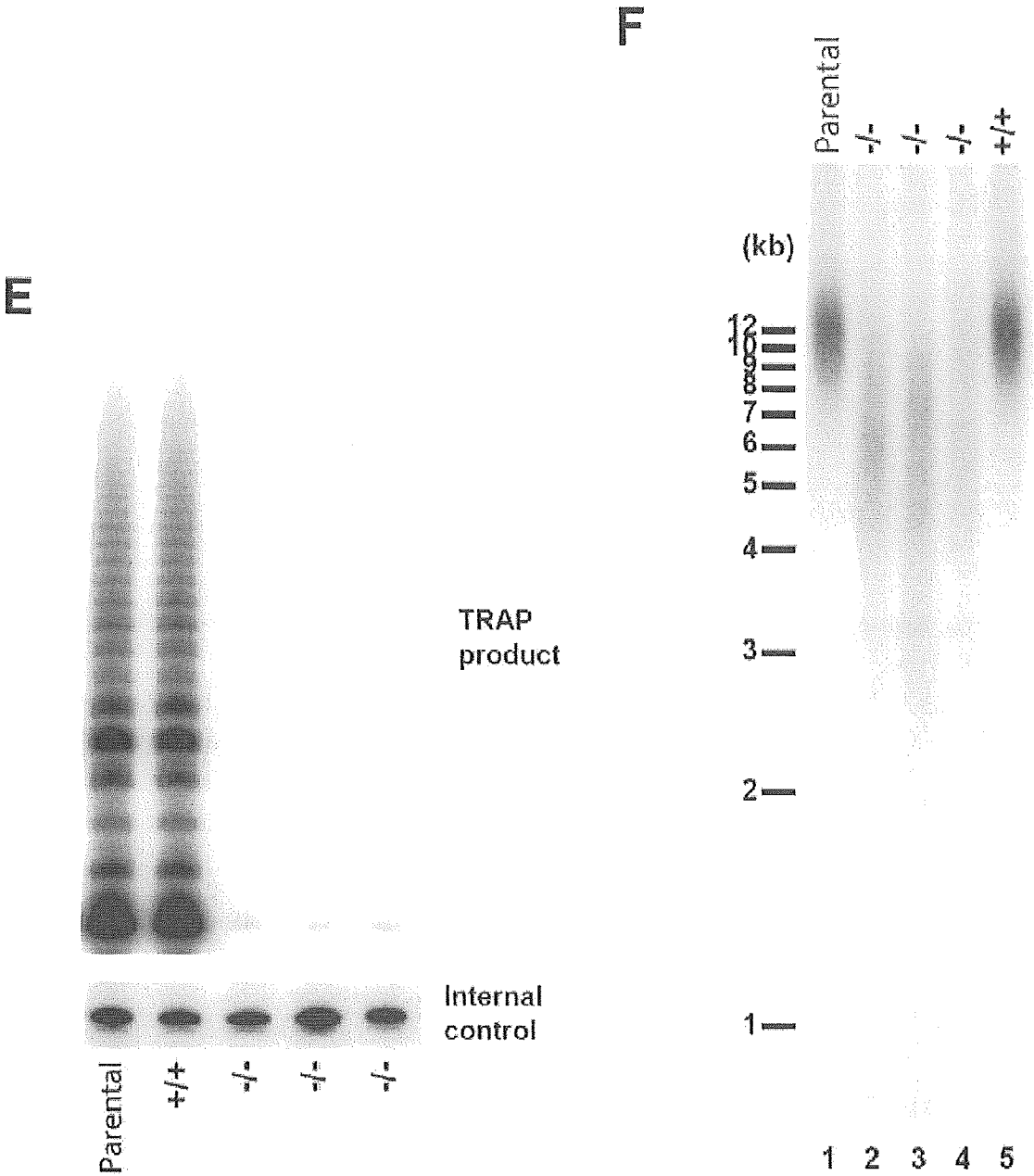


Figure 17 E-F

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SG2016/050190

A. CLASSIFICATION OF SUBJECT MATTER

C12N 5/0735 (2010.01) C12N 5/074 (2010.01) C12N 5/10 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Databases: WPIAP, EPODOC, MEDLINE, CAPLUS, BIOSIS, EMBASE; Keywords: telomerase, hTER, hTERT, null, knockout, inactivated, pluripotent, embryonic stem cell, ESC, iPSC, tumour, teratoma, cancer, gene editing, crispr, loxp, reversible expression, telomere length, lifespan, senescence, and similar terms

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	



Further documents are listed in the continuation of Box C



See patent family annex

* "A"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	"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
"E"	earlier application or patent but published on or after the international filing date	"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
"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)	"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
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 1 July 2016	Date of mailing of the international search report 01 July 2016
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustalia.gov.au	Authorised officer Daniel Sheahan AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. +61 2 62837969

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/SG2016/050190
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2012/010976 A2 (CELLECTIS) 26 January 2012 Abstract; page 2, lines 9-25; page 15, line 30 to page 17, line 5; page 26, line 11 to page 28, line 8; page 36, lines 1-17; page 39, lines 16-28; Figure 3	1, 5-8, 10, 11, 32-34
A	WINKLER, T. et al., "Defective telomere elongation and hematopoiesis from telomerase-mutant aplastic anemia iPSCs", The Journal of Clinical Investigation, 2013, Vol. 123, No. 5, pages 1952-1963 Abstract	
A	SCARIA, G. et al., "Increased telomere length in natural killer cells generated from human induced pluripotent stem cells", Blood, 2014, Vol. 124:5816 see whole document	
X	WO 2009/061442 A1 (CHILDREN'S MEDICAL CENTER CORPORATION) 14 May 2009 page 2, line 5 to page 3, line 17; page 24, lines 4-22; page 26, lines 1-27; page 48, lines 21-22; Examples 1, 2 and 4	1-4, 9-11
X	PUCCI, F. et al., "Short telomeres in ESCs lead to unstable differentiation", Cell Stem Cell, 2013, Vol. 12, pages 479-486 Abstract; page 481, paragraphs 2 and 3	5-8, 10, 11
X	WANG, F. et al., "Molecular insights into the heterogeneity of telomere reprogramming in induced pluripotent stem cells", Cell Research, 2012, Vol. 22, pages 757-768 Abstract; page 766, paragraph 2	5-8, 10, 11
X	KINOSHITA, T. et al., "Telomerase reverse transcriptase has an extratelomeric function in somatic cell reprogramming", The Journal of Biological Chemistry, 2014, Vol. 289, No. 2, pages 15776-15787 Abstract; page 15781, paragraph 3; page 15786, paragraph 1; Figures 3 and 4	5-8, 10, 11
X	ZENG, S. et al., "Telomerase-mediated telomere elongation from human blastocysts to embryonic stem cells", Journal of Cell Science, 2014, Vol. 127, pages 752-762 pages 754 and 755; page 758, paragraph 2 to page 759, paragraph 1; Figure 2	5-8, 10, 11

INTERNATIONAL SEARCH REPORT Information on patent family members		International application No. PCT/SG2016/050190	
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.			
Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
WO 2012/010976 A2	26 January 2012	WO 2012010976 A2	26 Jan 2012
WO 2009/061442 A1	14 May 2009	WO 2009061442 A1	14 May 2009
		US 2011151447 A1	23 Jun 2011
End of Annex			
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001. Form PCT/ISA/210 (Family Annex)(July 2009)			