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(54) **Title:** SYNTHETIC LETHALITY SCREENING PLATFORM FOR CELLS UNDERGOING ALT

(57) **Abstract:** An in vitro culture system of human embryonic stem (hES) derived cells is used as a synthetic lethality screening platform for cells undergoing alternative lengthening of telomeres (ALT).

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Synthetic Lethality Screening Platform for Cells Undergoing ALT

This invention was made with government support under Grant Number R01CA196884 awarded by the National Institutes of Health. The government has certain rights in the invention.

[001] Introduction

[002] Cancer cells can immortalize by inducing the recombination-based alternative lengthening of telomeres (ALT) pathway. Current methods of producing *de novo* cell lines which utilize the ALT mechanism of telomere elongation require first transforming a mortal cell line (e.g. IMR90) with SV40 large T antigen followed by passaging the culture through weeks-to-months of telomere crisis, after which individual cell clones will grow out of the culture. The immortalization efficiency of this process is low. Genetic inactivation by genome editing (knockout) of ATRX in these fibroblast cell lines will increase the rate at which the cells will activate ALT rather than reactivate telomerase expression, but even in this case the culture will still go through an extended crisis phase before individual clones activate ALT and resume proliferation.

[003] Summary of the Invention

[004] In an aspect the invention provides an in vitro culture of human embryonic stem derived cells immortalized through the alternative lengthening of telomeres (ALT) pathway, wherein the cells comprise:

(a) a genetic disruption of *TERT* locus that is:

(i) a heterozygous genetic disruption of the *TERT* locus; or

(ii) a homozygous genetic disruption of the *TERT* locus and a heterologous *TERT* allele operable as a conditional allele;

(b) a genetic disruption of the *CDKN2A* locus; and

(c) a genetic disruption of the *ATRX* locus,

wherein the culture is configured and operative as a synthetic lethality screening platform to identify gene products or drugs that interfere with ALT or homologous recombination.

[005] In embodiments, the cells comprise:

[006] (i) a heterozygous genetic disruption of the *TERT* locus, wherein: *TERT* was heterozygously knocked out by targeted excision of the *TERT* promoter using CAS9 and two guide RNAs surrounding the *TERT* promoter;

[007] (ii) a homozygous genetic disruption of the *TERT* locus and a heterologous *TERT* allele operable as a conditional allele, wherein: a hygromycin selection cassette is integrated into the first exon of the TERT gene;

[008] (ii) a homozygous genetic disruption of the *TERT* locus and a heterologous *TERT* allele operable as a conditional allele, wherein: a floxed *TERT* allele cassette is integrated at the AAVS1 safe harbor locus, operable as a conditional allele that can be excised to remove the *TERT* gene;

[009] (b) a genetic disruption of the *CDKN2A* locus; and wherein the genetic disruption is of exon 2 the *CDKN2A* locus;

[010] (c) a genetic disruption of the *ATRX* locus, wherein the genetic disruption is of exon 1 of the *ATRX* locus;

[011] (d) a genetic disruption of the *TP53* gene;

[012] (d) a genetic disruption of the *TP53* gene, wherein: a puromycin cassette is integrated into exon 4 of the *TP53* gene;

[013] an activated ALT pathway; and/or

[014] an activated ALT pathway independent of *TERT* expression.

[015] In an aspect the invention provides a method of using a subject culture comprising: (a) contacting the cells with an agent; and (b) detecting an effect of the agent on ALT activity in the cells.

[016] In embodiments the method comprises: (i) screening for genetic interactors by genome-wide KO/siRNA libraries to determine novel targets; (ii) small molecule screening to identify novel chemotherapeutics for treating ALT cancers; or (iii) arrayed imaging screening to identify genetic interactors necessary for ALT activity;

[017] In an aspect the invention provides a method for making a subject culture comprising:

(a) making a genetic disruption of *TERT* locus that is:

(i) making a heterozygous genetic disruption of the *TERT* locus; or

(ii) making a homozygous genetic disruption of the *TERT* locus and a heterologous *TERT* allele operable as a conditional allele;

(b) making a genetic disruption of the *CDKN2A* locus; and

(c) making a genetic disruption of the *ATRX* locus.

[018] In embodiments the method comprises steps:

a) genetically disrupt the human TERT gene at the endogenous locus using zinc finger nuclease (ZFN) mediated integration of a hygromycin selection cassette into the first exon of TERT;

b) insert a floxed TERT allele at the AAVS1 safe harbor locus by ZFN-mediated integration, wherein this cassette functions as a conditional allele that can be excised to remove the TERT gene, and ensures that all cells immortalize through the ALT process;
c) genetically disrupt TP53 using ZFN mediated targeted integration of a puromycin cassette into the exon 4 of the TP53 gene;
d) genetically disrupt exon 2 of the CDKN2A locus by Cas9-mediated genome editing, wherein this locus encodes both p14 and p16 tumor suppressors which can be activated as a consequence of telomere shortening and telomere dysfunction; and
e) genetically disrupt ATRX by Cas9-mediated removal of the majority of the first coding exon of ATRX.

[019] In embodiments the method further comprises: inducing ALT, comprising: removing the TERT transgene from the AAVS1 locus by expressing Cre recombinase and culturing of the resulting TERT-, TP53-, CDKN2A-, ATRX- stem cells in a stem cell media with reduced growth factors to induce differentiation.

[020] The invention encompasses all combinations of the particular embodiments recited herein, as if each combination had been laboriously recited.

[021] Description of Particular Embodiments of the Invention

[022] Unless contraindicated or noted otherwise, in these descriptions and throughout this specification, the terms “a” and “an” mean one or more, the term “or” means and/or. The examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein, including citations therein, are hereby incorporated by reference in their entirety for all purposes.

[023] We disclose a method to generate new ALT cell lines at a high rate that provides a platform for discovery. We demonstrate across multiple human ES cell lines the capability of activating the ALT process upon differentiation.

[024] We have established and disclose a cell system in which ALT is induced with high frequency and without cells going through cellular crisis. The invention provides methods and compositions related to ALT-screening platforms, including novel methods of robustly generating ALT+ cells from genomically normal cells, and related ALT screening platforms. The ALT+ cells are early stage ALT, still exhibit significant genomic instability and exhibit features of ALT cancer cells and have wild-type isogenic control line that does not. Synthetic lethality/ALT inhibition by loss of other gene product can be assayed for by multiple

methodologies.

[025] We also show that excising the floxed TERT allele in the AAVS1 is not necessary to activate ALT and that ALT activity is fully compatible with TERT expression.

[026] In embodiments we demonstrate that TERT expression has no bearing on the ability of the cell to activate the ALT pathway; hence our claims encompass ALT utilization in the heterozygous TERT line, the conditional TERT line with TERT removed, and in the conditional TERT line with TERT still remaining.

[027] Our system can act as a platform not only for synthetic lethality for ALT screening, but as a platform to screen for novel recombination factors and chemical interventions that interfere with recombination. Chemical intervention in the recombination pathway is way to induce synthetic lethality in DNA repair deficient cancer cells.

[028] Applications of the invention include identification of cancer therapeutics by means of: Screening for genetic interactors by genome-wide KO/siRNA libraries to determine novel targets; small molecule screening to identify novel chemotherapeutics for treating ALT cancers; and arrayed imaging screening to identify genetic interactors necessary for ALT activity.

[029] In embodiments our cells are genomically normal until the point that they activate the ALT mechanism, and due to the population-wide nature of the immortalization process, they do not carry the genomic idiosyncrasies of a clonally derived cancer cell line. Because they can immortalize and exhibit features of ALT by a variety of differentiation paradigms in tissues of multiple germ layers, the features they exhibit are those general to ALT and not specific to the cell line. Therefore, they provide a platform for screening to produce targets for ALT therapeutics.

[030] One such screening method is genome wide CAS9 screens. Since the development of CAS9 as a genome editing tool and its simplicity of targeting, it has been possible to generate genome wide libraries for CRISPR knockout as well as those for knockdown and overexpression. In brief, these tools allow for near-comprehensive analysis of genetic interactions by either knocking out, reducing expression of, or increasing expression of every gene within a large population of cells.

[031] Another screen is for synthetic lethality with ALT, in which a CRISPR knockout library is delivered to a population of recently immortalized ALT cells and isogenic wildtype controls. Timepoints can be taken immediately after transduction to determine the initial complexity, and then days-to-weeks later to allow for time for synthetic lethality to occur. Guides which deplete within ALT cells but not wildtype cells are potential targets for therapeutics, as it indicates that those are genes which ALT cells specifically require for continued proliferation. Likewise, complementary CRISPRa and CRISPRi libraries can be utilized to discover other pathways

which interact with alternative telomere lengthening and proliferation of ALT cells.

[032] Furthermore, applications are not limited to assaying for cell viability in genome wide screens. In addition to screening using depletion and enrichment of guides determined by sequencing, it is also an option to perform genome wide imaging-based screening within the ALT cells our immortalization method produces (Wang et al., 2019, PNAS USA 116, 10842–10851). ALT cells have several phenotypes assayable by simple imaging readout. For instance, ALT cells exhibit colocalization of PML protein with telomeres (ALT-associated PML bodies, or APBs) as well as novel nucleotide incorporation at telomeres outside of S-phase, visualizable through treatment with EdU. We examined these phenotypes: they are simple, readily automatable staining assays, and the arrayed imaging is readily scaled to a genome wide knockout library to investigate the interactions between genes that can lead to the abrogation of these ALT phenotypes.

[033] Additionally, this imaging-based screening provides more meaningful information than a cell viability screen. While a standard synthetic lethal screen will only inform us of the genes necessary for proliferation, the imaging-based screen provides for the identification of factors necessary for the formation of ALT-specific phenotypes; for example, targeting a factor necessary for the agglomeration of telomeres into an APB may be less toxic to normal cells than a factor necessary for general cell proliferation.

[034] Example

[035] We genetically engineered human embryonic stem cells (e.g. WIBR#3, NIH stem cell registry 0079), followed by growth factor withdrawal induced differentiation. The WIBR#3 cells carry the following genetic modifications:

[036] Genetic disruption of the human *TERT* gene at the endogenous locus using zinc finger nuclease (ZFN) mediated integration of a hygromycin selection cassette into the first exon of TERT.

[037] Expression of a floxed TERT allele at the *AAVS1* safe harbor locus, inserted by ZFN-mediated integration. This cassette functions as a conditional allele that can be excised to remove the *TERT* gene. This will ensure that all cells immortalize through the ALT process.

[038] Genetic disruption of TP53 using ZFN mediated targeted integration of a puromycin cassette into the exon 4 of the *TP53* gene. Note: TP53 is mutated in the majority of ALT cancers and this genetic alteration recapitulates this state.

[039] Genetic deletion of exon 2 of the CDKN2A locus by Cas9-mediated genome editing. This locus encodes both p14 and p16 tumor suppressors which can be activated as a consequence of telomere shortening and telomere dysfunction.

[040] Genetic disruption of ATRX by Cas9-mediated removal of the majority of the first

coding exon of ATRX.

[041] To induce ALT, we removed the TERT transgene from the AAVS1 locus by expressing Cre recombinase and culture of the resulting TERT-, TP53-, CDKN2A-, ATRX- stem cells in a stem cell media with reduced growth factors to induce differentiation. These differentiated cells acquire ALT characteristics (e.g. long, heterogeneous telomeres, ALT-associated colocalizations of PML protein and telomeres [APBs], circular extrachromosomal telomeric repeats [C-circles]) and exhibit continuous proliferation long after the matched hESC cultures go through telomere shortening-induced crisis.

[042] Our method causes cell cultures to experience about a week of slower proliferation, but recovery of the majority of cells which activate ALT and contribute to the culture's growth. Generally more that 25% of the cells effectively induce ALT and have not undergone cellular crisis. Thus, the ALT cells produced are polyclonal rather than monoclonal.

[043] Cell culture

[044] Genome editing was performed in WIBR3 hESCs, NIH stem cell registry #0079 (Lengner et al., 2010, Cell 141, 872–883). Cell culture was carried out as previously described (Soldner et al., 2009, Cell 136, 964–977). Briefly, hESC lines were maintained on a monolayer of CD-1 strain mouse embryonic fibroblasts (MEFs) [Charles River] inactivated by 35 Gy of γ-irradiation. hESCs were grown in hESC medium (DMEM/F12 [Gibco] supplemented with 20% KnockOut Serum Replacement [Gibco], 1 mM glutamine [Sigma-Aldrich], 1% non-essential amino acids [Gibco], 0.1 mM β-mercaptoethanol [Sigma-Aldrich], 100 U/mL Penicillin-Streptomycin [Gibco], and 4 ng/mL FGF-Basic (AA 1-155) [Gibco]). Cultures were passaged every 5-7 days either manually or enzymatically with 1.5 mg/mL collagenase type IV [Gibco] by sedimentation and washing 3 times in wash medium (DMEM [Gibco] supplemented with 5% newborn calf serum [Sigma-Aldrich] and 100 U/mL Penicillin-Streptomycin [Gibco]).

[045] HeLa 1.3 cervical carcinoma cells (The Rockefeller University, New York, NY) (Takai et al., 2009, JBC 285, 1457–1467). U2OS osteosarcoma cells were obtained from the UC Berkeley Cell Culture Facility. HeLa 1.3 and U2OS were maintained in fibroblast medium (DMEM [Gibco] supplemented with 15% FB Essence [Seradigm], 1 mM glutamine [Sigma-Aldrich], 1% non-essential amino acids [Gibco], and 100 U/mL Penicillin-Streptomycin [Gibco]) and passaged every 3-5 days enzymatically with Trypsin-EDTA (0.25%) [Gibco]. Trypsin was inactivated by either wash medium or fibroblast medium.

[046] Transient feeder-free hESC and E7-differentiated cultures were maintained in "E7" medium (DMEM/F12 [Gibco] supplemented with 10% FB Essence [Seradigm], 64 mg/L L-ascorbic acid [Sigma-Aldrich], 14 μg/L sodium selenium [Sigma-Aldrich], 100 μg/L FGF-Basic (AA 1-155) [Gibco], 19.4 mg/L insulin [Sigma-Aldrich], 543 mg/L $NaHCO_3$ [Sigma-Aldrich],

and 10.7 mg/L transferrin [Sigma-Aldrich]. Cells were grown on tissue culture plates treated with Matrigel matrix [Corning].

[047] Genome editing in hESCs

[048] All targeting was performed as previously described (Hockemeyer et al., 2009 Nat Biotechnol 27, 851–857; Hockemeyer et al., 2011 Nat Biotechnol *29*, 731–734; Chiba and Hockemeyer, 2014). ZFNs targeting *TERT* and *TP53* were obtained from Sangamo Therapeutics, Inc. ZFN and TALEN targeting was performed as previously described.. CAS9 and sgRNAs were expressed using the pX330 plasmid (Le Cong et al., 2013 Science (New York, NY) 339, 819–823). Each targeting step was performed by co-electroporation of 1-2 $\times$ 10^7 hESCs with 15 µg of each pX330 plasmid and 7.5 µg of GFP expression plasmid. 48-72 hours later cells were sorted for GFP fluorescence and single cell-derived hESC colonies were isolated and genotyped by Southern blotting or PCR followed by Sanger sequencing.

[049] Cre-mediated and Flp-mediated recombination was performed by co-transfection of StemMACS Cre recombinase mRNA [Milltenyi Biotec] or StemMACS Flp recombinase mRNA [Milltenyi Biotec] with Stemgent eGFP mRNA [Milltenyi Biotec] into hESCs using StemFect RNA Transfection Kit [ReproCELL] according to manufacturer instructions. 24-72 hours later cells were sorted for GFP fluorescence and single cell-derived hESC colonies were isolated and genotyped by PCR.

[050] Southern blotting and PCR genotyping

[051] Southern blot analysis was performed as previously described (Hockemeyer et al., 2009 Nat Biotechnol 27, 851–857; Hockemeyer et al., 2011 Nat Biotechnol 29, 731–734; Sexton et al., 2014 Genes Dev 28, 1885–1899). *TP53* deletion was confirmed using an external 5' probe amplified from genomic DNA with primers. *CDKN2A* deletion was confirmed using a probe 5' to the excision site amplified from genomic DNA with primers. ATRX deletion was confirmed using PCR primers. ATRX conditional reintroduction was confirmed using PCR primers. Cre-mediated loopout of TERT from *AAVS1* was confirmed using PCR primers.

[052] Fibroblast differentiation

[053] hESC colonies were lifted from the MEF feeder layer enzymatically with 1.5 mg/mL collagenase type IV [Gibco] and isolated by sedimentation and washing 3 times with wash medium. Colonies were suspended in fibroblast medium and grown in Ultra-Low Attachment Culture Dishes [Corning] for formation of embryoid bodies (EBs). Medium was replenished every 3 days by sedimentation and resuspension of EBs. After 9 days EBs were transferred to tissue culture dishes to attach. 7 days later, EBs and fibroblast-like cells were passaged using Trypsin-EDTA (0.25%) [Gibco], triturated to single-cell suspension, and plated on tissue culture dishes. Cultures were maintained in fibroblast medium on plates treated with gelatin [Sigma-

Aldrich] and were passaged every 5-7 days.

[054] E7 differentiation

[055] hESC colonies were lifted from the MEF feeder layer enzymatically with 1.5 mg/mL collagenase type IV [Gibco] and isolated by sedimentation and washing 3 times with wash medium. Colonies were suspended in E7 medium and transferred to tissue culture dishes treated with Matrigel [Corning]. After 7 days, cultures were passaged using Trypsin-EDTA (0.25%) [Gibco], triturated to single-cell suspension, and plated on Matrigel-coated tissue culture dishes. Cultures were maintained in E7 medium and were passaged every 5-7 days.

[056] Neural precursor cell differentiation

[057] Single-cell dissociated hESCs were cultured on Matrigel-coated plates at a density of 5×10^4 cells/cm^2 and maintained in complete conditioned hESC medium until >90% confluent. A modified dual-SMAD inhibition protocol was performed to differentiate hESCs into NPCs as described previously (Blair et al., 2018 Nat Med 24, 1568–1578. Cells were passaged by dissociation with StemPro Accutase, split 1:3 every 5 days, and maintained in N2 medium (50% DMEM/F12 [Gibco] and 50% Neurobasal Medium [Gibco] supplemented with N-2 Supplement [Gibco], GlutaMAX [Gibco], 100 U/mL Penicillin-Streptomycin [Gibco], 0.2% insulin [Sigma-Aldrich], and 0.075% (w/v) bovine serum albumin [Sigma-Aldrich].

[058] Teratoma formation

[059] hESCs were collected by collagenase type IV (1.5 mg/mL) treatment and separated from MEF feeder cells by sedimentation. Cells were resuspended in 250 µL of hESC medium and injected subcutaneously into NOD-SCID mice [Taconic Biosciences]. Tumors which grew to the maximum size of 2.5 cm were explanted, measured, and divided for frozen sections and formalin fixation.

[060] Immunocytochemistry (ICC) and EdU staining

[061] Cells were plated on to glass coverslips and fixed in 4% paraformaldehyde solution in PBS [Sigma-Aldrich]. Samples were permeabilized in permeabilization solution (PBS with 3% horse serum [Sigma-Aldrich] and 0.1% Triton X-100 [Sigma-Aldrich]). Primary antibody incubation was performed overnight in permeabilization solution at 4°C. Samples were then washed 3 times in PBS, 5 minutes each wash. Secondary antibody incubation was performed for 1 hour in permeabilization solution at 25°C protected from light. Samples were washed 3 times in PBS, 5 minutes each wash, with 1 µg/mL DAPI [KPL] added to the second wash. Coverslips were then mounted in ProLong Gold Antifade mountant on glass slides and imaged.

[062] For EdU staining, cells were plated on glass coverslips. 24 hours prior to fixation, cells were treated with 10 µM RO-3306 [Sigma-Aldrich]. 2 hours prior to fixation, 10 µM EdU was added to cell culture media. Samples were then fixed in 4% paraformaldehyde solution in PBS.

EdU detection was then performed using the Click-iT Plus EdU Alexa Fluor 647 Imaging Kit [Thermo Fisher]. For further ICC steps, samples were subsequently treated as described above.

[063] C-circle assay.

[064] The C-circle assay was performed as previously described (Henson et al., 2009 Nat Biotechnol *27*, 1181–1185). Briefly, extracted genomic DNA was digested in *Eco*RI [New England BioLabs], precipitated and extracted by phenol-chloroform, resuspended, and quantified by a Qubit™ 2.0 Fluorometer [Life Technologies]. 20 ng of each sample was incubated with φ29 polymerase as previously described. Samples were attached to an Amersham Hybond-XL membrane [Fisher Scientific] by dot blot and probed with a ^{32}P-end-labeled oligonucleotide. Parallel membranes were probed with a 5'-gtaatcccagcactttgg-3' end-labeled oligonucleotide which binds to the Alu consensus sequence to normalize for genomic DNA content.

[065] Chromosome-orientation fluorescence *in situ* hybridization (CO-FISH)

[066] CO-FISH analysis was performed as previously described (Williams and Bailey, 2010 Cold Spring Harb Protoc 2009, pdb.prot5269). Briefly, 24 hours prior to fixation, cells were cultured in growth medium containing 10 μM bromodeoxyuridine (BrdU) [Invitrogen]. 2 hours prior to fixation, 0.2 μg/mL colcemid [Roche] was added to medium. Cells were dissociated by Trypsin-EDTA (0.25%) [Gibco], centrifuged and gently resuspended for 5 minutes in 75 mM KCl. Cells were then centrifuged, supernatant was aspirated, and cells were gently resuspended in residual supernatant before fixation in 3:1 methanol:acetic acid. Metaphase spreads were made according to standard techniques and BrdU-containing strands were digested according to the Williams and Bailey 2010 protocol cited above. Telomeres were hybridized sequentially with TelG-Alexa488 and TelC-Cy3 (PNA Bio). After dehydration, slides were stained with 1 μg/mL DAPI [KPL] and mounted with ProLong Gold mountant before imaging.

[067] Image acquisition and analysis

[068] All ICC and CO-FISH micrographs were taken on a Nikon Eclipse TE2000-E with a 100× objective using an Andor Zyla 4.2 sCMOS camera. Images were acquired using Nikon NIS-Elements. For experiments in which two or more conditions were quantitatively compared the same exposure and acquisition settings were used for each image. APB, TIF, and EdU colocalization with telomeres were automatically processed using ImageJ with ComDet plugin.

[069] Strand-seq, library preparation, Illumina sequencing, and bioinformatics

[070] Cells were prepared for Strand-seq analysis as previously described (Sanders et al., 2017 Nat Protoc 12, 1151–1176). Briefly, hESCs were collected after BrdU pulse and resuspended in nuclei staining buffer (100 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% NP-40, and 2% bovine serum albumin [Sigma-Aldrich] supplemented with 10 μg/mL

Hoechst 33258 [Enzo] and 10 µg/mL propidium iodide [Sigma-Aldrich]. Single nuclei were sorted into 5 µL ProFreeze-CDM freeze medium [Lonza] + 7.5% dimethyl sulfoxide in 96-well skirted PCR plates [4titude], based on low propidium iodide and low Hoechst fluorescence using an Influx cell sorter [BD Biosciences]. For each experiment, 96 libraries were pooled and 250-450 bp-sized fragments were isolated and purified. DNA was processed for Strand-seq (Sanders et al., 2017 Nat Protoc 12, 1151–1176). DNA quality and concentrations were assessed using the High Sensitivity dsDNA kit [Agilent] on the Agilent 2100 Bio-Analyzer and on a Qubit 2.0 Fluorometer [Life Technologies].

[071] Clusters were generated on the cBot (HiSeq2500) and single-end 50 bp reads were generated using the HiSeq2500 sequencing platform [Illumina]. Indexed bam files were aligned to human (GRCh37) using Bowtie2 (Langmead and Salzberg, 2012). SCEs were identified and mapped with the BAIT software package using standard settings.

[072] Telomere length assessment

[073] To collect genomic DNA, hESC lines were enzymatically released from the MEF feeder layer by treatment with 1.5 mg/mL collagenase type IV and washed 3 times in wash medium and gravitational sedimentation to minimize contaminating MEF cells. Genomic DNA was prepared as described previously (Hockemeyer et al., 2005 EMBO J *24*, 2667–2678). MEF telomeres are resolved by size from hESC telomeres and do not interfere with analysis of telomere length. Genomic DNA was digested with *Mbo*I and *Alu*I overnight at 37°C. Digested DNA was normalized and run on a 0.75% Seakem ME Agarose [Lonza] gel and dried under vacuum for 2 hours at 50°C. The dry gel was denatured in 0.5 M NaOH, 1.5 M NaCl for 30 minutes at 25°C, then neutralized with 1 M Tris-HCl pH 6.0, 2.5 M NaCl, 2x for 15 minutes. The gel was then pre-hybridized in Church's buffer (1% BSA, 1 mM EDTA, 0.5 M $NaPO_4$, 7% SDS, pH 7.2) for 1 hour at 55°C before adding ^{32}P-end-labeled $(CCCTAA)_3$ probe. The gel was washed in 4X SSC buffer 3 times for 15 minutes at 50°C and once in 4X SSC + 0.1% SDS at 25°C before exposing on a phosphorimager screen.

[074] Single telomere length analysis (STELA) was performed as previously described (Baird et al., 2003 Nat Genet 33, 203–207). hESC colonies were separated from the MEF layer by treatment with 1.5 mg/mL collagenase type IV and washed 3x in wash medium, collecting by sedimentation to minimize contaminating MEF cells. DNA was extracted from cell pellets using the Norgen Cells and Tissue DNA Isolation Micro Kit. DNA was solubilized by digestion with *Eco*RI and quantified on a Qubit 2.0 Fluorometer, then diluted to 10 ng/µL in 10 mM Tris-HCl (pH 7.5). DNA was ligated at 35°C for 12 hours in a volume of 10 µL containing 10 ng genomic DNA, 0.9 µM telorette linker, and 0.5 U T4 DNA ligase [New England Biolabs]. Ligated DNA was diluted to 250 pg/µL in water and multiple PCRs were performed in volumes of 15 µL

containing 200 pg ligated DNA, 0.25 μM XpYpE2+G and teltail primers, 0.3 mM dNTPs, 7.4 mM $MgCl_2$, 1x Taq Buffer with $(NH_4)_2SO_4$, and 1 U of a 10:1 mix of *Taq* [New England Biolabs] and *Pwo* [Sigma-Aldrich] polymerase. Reactions were cycled on a Bio-Rad C1000 Touch Thermal Cycler: 25 cycles of 94°C for 15 seconds, 65° for 30 seconds, 68° for 10 minutes. DNA fragments were resolved on a 0.5% agarose gel and detected by Southern blot with a random-primed α-^{32}P-labeled XpYp probe generated by PCR using primers (XpYpE2; XpYpB2). Telomere lengths were estimated using TeSLA-QUANT software (Lai et al., 2017 Nat Commun 8, 1356).

[075] Immunoblotting

[076] Cells were collected RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0) with 1 mM phenylmethanesulfonyl fluoride and cOmplete ULTRA protease inhibitor [Roche] and Halt Phosphatase inhibitor [Thermo Scientific]. Protein concentration was determined by Bio-Rad Protein Assay colorimetric dye quantified by a Bio-Rad xMark microplate reader. 15-20 μg protein in Laemmli sample buffer was loaded onto 5% (ATRX) or 10% (DDR proteins) polyacrylamide gels. Proteins were transferred to nitrocellulose membranes [Bio-Rad], blocked in 5% BSA in tris-buffered saline (TBS)-Tween 20 for 1 hour at 25°C, then incubated with primary antibodies diluted in 5% BSA in TBS-T overnight at 4°C. Membranes were then washed 3x 15 minutes in TBS-T and incubated in horseradish peroxidase-conjugated secondary antibodies [Bio-Rad] for 1 hour at 25°C, washed, incubated with Clarity Western ECL substrate [Bio-Rad] before imaging on a Bio-Rad ChemiDoc XRS+. Membranes were stripped by 2x 10 minute incubation at 25°C in stripping buffer (200 mM glycine, 0.1% SDS, 1% Tween 20, pH 2.2) before re-blocking and incubation with subsequent primary antibodies.

[077] qRT-PCR analysis

[078] RNA was isolated using Trizol [Invitrogen] extraction followed by ethanol precipitation. Reverse transcription was performed on 1000 ng of total RNA by oligo(dT) and random priming using the iScript cDNA Synthesis Kit [Bio-Rad]. qRT-PCR was performed in a CFX96 [Bio-Rad] with KAPA SYBR FAST master mix ROX low [Roche].

[079] Telomerase catalytic activity assay

[080] PCR-based telomeric repeat amplification protocol (TRAP) was performed as previously described (Xin, 2011 Methods Mol Biol 735, 107–111). Protein extracts were generated by repeated freeze-thaw cycles in hypotonic lysis buffer (HLB) (20 mM HEPES, 2 mM $MgCl_2$, 0.2 mM EGTA, 10% glycerol, 1 mM dithiothreitol, 0.1 mM PMSF, 0.5% CHAPS). Protein concentrations were determined by Bio-Rad Protein Assay colorimetric dye quantified by a Bio-Rad xMark microplate reader. 200 ng of total protein were used for input into ^{32}P-

dGTP PCR. TRAP products were resolved on a 10% polyacrylamide in 1X TAE gel. Dried gels were visualized by exposure on a phosphorimager screen.

[081] hESC colony cell counting

[082] To measure hESC population doubling, hESC colonies were grown feeder-free in E7 medium supplemented with ROCK inhibitor (Y-27632) [Chemdea] for 24 hours, then treated with Trypsin-EDTA (0.25%) for single-cell suspension. hESCs were plated at low density (1000 cells/10 cm plate) on tissue culture plates coated with Matrigel. After 72 hours, cells were washed with PBS and fixed with 4% paraformaldehyde in PBS. Nuclei were counterstained with 1 µg/mL DAPI. Distinct clonal colonies were imaged and nuclei counted. Population doublings were calculated assuming colonies were founded by single cells.

CLAIMS:

1. An in vitro culture of human embryonic stem derived cells immortalized through the alternative lengthening of telomeres (ALT) pathway, wherein the cells comprise:

(a) a genetic disruption of *TERT* locus that is:

(i) a heterozygous genetic disruption of the *TERT* locus; or

(ii) a homozygous genetic disruption of the *TERT* locus and a heterologous *TERT* allele operable as a conditional allele;

(b) a genetic disruption of the *CDKN2A* locus; and

(c) a genetic disruption of the *ATRX* locus,

wherein the culture is configured and operative as a synthetic lethality screening platform to identify gene products or drugs that interfere with ALT or recombination.

2. The in vitro culture of claim 1, wherein the cells comprise:

(i) a heterozygous genetic disruption of the *TERT* locus, wherein:

TERT was heterozygously knocked out by targeted excision of the *TERT* promoter using CAS9 and two guide RNAs surrounding the *TERT* promoter.

3. The in vitro culture of claim 1, wherein the cells comprise:

(ii) a homozygous genetic disruption of the *TERT* locus and a heterologous *TERT* allele operable as a conditional allele, wherein:

a hygromycin selection cassette is integrated into the first exon of the TERT gene.

4. The in vitro culture of claim 1, wherein the cells comprise:

(ii) a homozygous genetic disruption of the *TERT* locus and a heterologous *TERT* allele operable as a conditional allele, wherein:

a floxed *TERT* allele cassette is integrated at the AAVS1 safe harbor locus, operable as a conditional allele that can be excised to remove the *TERT* gene;

5. The in vitro culture of claim 1, 2, 3 or 4 wherein the cells comprise:

(b) a genetic disruption of the *CDKN2A* locus; and

wherein the genetic disruption is of exon 2 the *CDKN2A* locus.

6. The in vitro culture of claim 1, 2, 3 or 4, wherein the cells comprise:

(c) a genetic disruption of the *ATRX* locus,

wherein the genetic disruption is of exon 1 of the *ATRX* locus.

7. The in vitro culture of claim 1, 2, 3 or 4, wherein the cells comprise:
(d) a genetic disruption of the *TP53* gene.

8 The in vitro culture of claim 1, 2, 3 or 4, wherein the cells comprise:
(d) a genetic disruption of the *TP53* gene, wherein:
a puromycin cassette is integrated into exon 4 of the *TP53* gene.

9. The in vitro culture of claim 1, 2, 3 or 4, wherein the cells comprise an activated ALT pathway.

10. The in vitro culture of claim 1, 2, 3 or 4, wherein the cells comprise an activated ALT pathway independent of *TERT* expression.

11. A method of using the culture of claim 1, 2, 3 or 4, comprising:
(a) contacting the cells with an agent; and
(b) detecting an effect of the agent on ALT activity in the cells.

12. The method of claim 1, 2, 3 or 4, comprising:
(a) contacting the cells with an agent; and
(b) detecting an effect of the agent on ALT activity in the cells; wherein the method comprises:
(i) screening for genetic interactors by genome-wide KO/siRNA libraries to determine novel targets;
(ii) small molecule screening to identify novel chemotherapeutics for treating ALT cancers; or
(iii) arrayed imaging screening to identify genetic interactors necessary for ALT activity.

13. A method for making the culture of claim 1, 2, 3 or 4, comprising:
(a) making a genetic disruption of *TERT* locus that is:
(i) a heterozygous genetic disruption of the *TERT* locus; or
(ii) a homozygous genetic disruption of the *TERT* locus and a heterologous *TERT* allele operable as a conditional allele;
(b) making a genetic disruption of the *CDKN2A* locus; and
(c) making a genetic disruption of the *ATRX* locus.

14. The method of claim 13, comprising:

a) genetically disrupt the human TERT gene at the endogenous locus using zinc finger nuclease (ZFN) mediated integration of a hygromycin selection cassette into the first exon of TERT;
b) insert a floxed TERT allele at the AAVS1 safe harbor locus by ZFN-mediated integration, wherein this cassette functions as a conditional allele that can be excised to remove the TERT gene, and ensures that all cells immortalize through the ALT process;
c) genetically disrupt TP53 using ZFN mediated targeted integration of a puromycin cassette into the exon 4 of the TP53 gene;
d) genetically disrupt exon 2 of the CDKN2A locus by Cas9-mediated genome editing, wherein this locus encodes both p14 and p16 tumor suppressors which can be activated as a consequence of telomere shortening and telomere dysfunction; and
e) genetically disrupt ATRX by Cas9-mediated removal of the majority of the first coding exon of ATRX.

15. The method of claim 14 further comprising: inducing ALT, comprising: removing the TERT transgene from the AAVS1 locus by expressing Cre recombinase and culturing of the resulting TERT-, TP53-, CDKN2A-, ATRX- stem cells in a stem cell media with reduced growth factors to induce differentiation.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 20/49616

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A01K 67/027; C12N 15/85; C12N 15/90 (2020.01)

CPC - A01K 67/0276; C07K 14/4705; C12N 15/85; C12N 15/8509

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)
See Search History document

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2014/0079836 A1 (LifeSpan Extension, LLC) 20 March 2014 (20.03.2014), entire document esp para [0014], [0354]-[0356], claim, 1-19	1-10
Y	WO 2014/089290 A1 (Sigma-Aldrich CO. LLC) 12 June 2014 (12.06.2014), entire document esp para [0001], [0115]-[0121]	1-10
A	US 2005/0144655 A1 (Economides et al) 30 June 2005 (30.06.2005), entire document esp abstract, para [0055], [0083]	1-10
A	https://en.wikipedia.org/w/index.php?title=Puromycin&oldid=886373289 'Puromycin' 5 March 2019 (05.03.2019), entire document esp pg 2 para 3	1-10

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

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

Date of the actual completion of the international search	Date of mailing of the international search report
6 November 2020	04 FEB 2021
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Lee Young Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 20/49616

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

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

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

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

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I: Claims 1-10 directed to an in vitro culture of human embryonic stem derived cells immortalized alternative lengthening of telomeres (ALT) pathway.

Group II: Claims 11-12 directed to a method of using a culture comprising: (a) contacting the cells with an agent.

Group III: Claims 13-15 directed to a method for making a culture comprising making a genetic disruptions.

The group of inventions listed above do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:
**********See supplemental box below**********

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

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

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

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-10

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US 20/49616

Box III Lack of Unity

Special Technical Features:

Group I requires the technical feature of an in vitro culture of human embryonic stem derived cells immortalized alternative lengthening of telomeres (ALT) pathway, not required by Group II-III.

Group II requires the technical feature of a method of using a culture comprising: (a) contacting the cells with an agent, not required by Group I or III.

Group III requires the technical feature of a method for making a culture comprising making a genetic disruptions, not required by Groups I-II.

Common technical features:

Groups I-III share the technical feature of an in vitro culture of human embryonic stem derived cells immortalized through the alternative lengthening of telomeres (ALT) pathway, wherein the cells comprise: (a) a genetic disruption of TERT locus that is: (i) a heterozygous genetic disruption of the TERT locus; or (ii) a homozygous genetic disruption of the TERT locus and a heterologous TERT allele operable as a conditional allele; (b) a genetic disruption of the CDKN2A locus; and (c) a genetic disruption of the ATRX locus, wherein the culture is configured and operative as a synthetic lethality screening platform to identify gene products or drugs that interfere with ALT or recombination.

These shared technical features, however, do not provide a contribution over the prior art, as being obvious over US 2014/0079836 A1 LifeSpan Extension, LLC (hereinafter LEL) in view of WO 2014/089290 A1 Sigma-Aldrich CO. LLC (hereinafter Sigma).

LEL discloses an in vitro culture of human embryonic stem derived cells immortalized (para [0014] .. Provided herein are methods and compositions that can be employed to increase telomerase activity, and/or modulate the activity of other telomere maintenance genes so as to repair, maintain or lengthen telomere structure to lengthen the lifespan of healthy cells. Decreasing telomerase activity in cancer cells, thus making cancer cells mortal and healthy cells longer lasting if not immortal is another method to increase longevity.. para [0354] .. In vitro fertilization and embryo and stem cell research are yet other embodiments wherein the modulating agent may be used to extend the lifespan of cells.. para [0355] .. n another embodiment the modulating agent may be used to extend the lifespan of the progeny or a cloned derivative of an organism. It is known that somatic and embryonic cloning may produce cloned organisms with shorter lifespan than the original organism that was cloned.. method can be used on a variety of cell including embryonic stem cells) through the alternative lengthening of telomeres (ALT) pathway (para [0056] .. A study in human cells demonstrated that the action of telomerase can effectively inhibit the alternate recombination pathway by maintaining genomic stability. Cells relying on the recombination based pathway for telomere maintenance were forced to express telomerase. These cells never demonstrated the shortened telomeres required for initiation of recombination, due to the expressed telomerase preventing such an occurrence. Other alternate pathways or mechanisms may also exist.. claim 4 .. A method for modulating response or resistance to stress of a cell, tissue, organ or organism, comprising modulating the level and/or activity of at least one gene selected from the group consisting of those listed in Data Table 7 and those listed as part of Array 2.. Claim 5 ..The method of claim 4, wherein modulating comprises modulating the level and/or activity of: .. four or more of TERT.. modulating TERT, telomerase catalytic subunit, can cause activation of ALT), wherein the cells comprise: (a) a disruption of TERT locus (claim 2 .. wherein modulating comprises modulating the level and/or activity of:.. TERT.. modulation may be increase, or decrease such as disruption): (b) a disruption of the CDKN2A locus (claim 2 .. CDKN2A..); and (c) a disruption of the ATRX locus (claim 2 .. The method of claim 1, wherein modulating comprises modulating the level and/or activity of.. (o) another list of genes described herein; or (p) a combination of two or more of (a) through (o).. para [0294] ..ATRX..). LEL does not disclose wherein the culture is configured and operative as a synthetic lethality screening platform to identify gene products or drugs that interfere with ALT or recombination. It would have been obvious to one skilled in the art to use the stem cells which are immortalized via reliance on ALT to screen for drugs which interfere with ALT because this interference would remove their immortality. LEL does not disclose wherein the disruptions are genetic and heterozygous or a homozygous genetic disruption of the TERT locus and a heterologous TERT allele operable as a conditional allele. LEL instead uses molecular inhibitors (claim 1 .. method for modulating the lifespan of a cell, tissue, organ or organism, or of increasing or decreasing cellular respiration and/or capacity and/or biogenesis of mitochondria in a cell, tissue, organ or organism, comprising contacting the cell, tissue, organ or organism with at least one lifespan modulating agent..). Sigma discloses a method of manipulating gene expression comprising disruptions that are genetic and can be heterozygous or a homozygous genetic disruption with a heterologous allele operable as a conditional allele (para [0001] .. The present disclosure relates targeted genome modification. In particular, the disclosure relates to RNA-guided endonucleases or fusion proteins comprising CRISPR/Cas-like protein and methods of using said proteins to modify or regulate targeted chromosomal sequences.. para [0121] .. In any of these embodiments, the genetically modified animal disclosed herein can be heterozygous for the modified chromosomal sequence. Alternatively, the genetically modified animal can be homozygous for the modified chromosomal sequence.. para [0115] .. he modified chromosomal sequence may be inactivated such that the sequence is not transcribed and/or a functional protein product is not produced. Thus, a genetically modified animal comprising an inactivated chromosomal sequence may be termed a knock out or a conditional knock out.. para [0117] .. In another embodiment, the genetically modified animal can comprise at least one chromosomally integrated sequence. A genetically modified animal comprising an integrated sequence may be termed a knock in or a conditional knock in..). It would have been obvious to one skilled in the art to combine the method of inducing immortality via gene manipulations disclosed by LEL with a specific method of genetic engineering disclosed by Sigma in order to do more specific engineering of the desired genetic effects.

As the technical features were known in the art at the time of the invention, this cannot be considered a special technical feature that would otherwise unify the groups. Groups I-III therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.

Note: Claim 12 improperly depends on claim 1, refering to 'the method of claim 1', but claim 1 is 'An in vitro culture of human embryonic stem derived cells'. For the purposes of completing this ISR, claim 12 will be assumed to depend on claim 11.