

Retrovirally mediated telomerase immortalization of neural progenitor cells

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Traditional methods of generating immortalized lines of both somatic cells and their progenitors have relied on the use of oncogenes. However, the resulting lines are typically anaplastic *in vitro* and tumorigenic *in vivo*, and hence of limited utility. The overexpression of telomerase, as mediated by the induced overexpression of human telomerase reverse transcriptase (hTERT), has permitted the generation of stable, non-oncogenic lines of a variety of cell types. This strategy for immortalization has found special utility in the central nervous system, as few stable lines are available for the study of either human neural progenitor cells, or of neurons or glia of restricted phenotype. We describe the use of retroviral hTERT overexpression for generating lines of immortalized human neural progenitor cells, whose neuronal progeny are phenotypically restricted, post-mitotic and functionally competent. Although we focus here on telomerase immortalization of spinal neural progenitors, this is a broadly applicable protocol for using hTERT to immortalize human fetal neural progenitors of any pre-selected phenotype and for characterizing the cell lines thereby generated.

INTRODUCTION

The central nervous system (CNS) is composed of a tremendous diversity of neuronal and glial phenotypes, each with a unique ontogeny. Neurons in particular may be categorized by distinct permutations of positionally encoded transcription factors, expressed receptors, physiological properties and operative neurotransmitters, and patterns of connectivity that define both the anatomy and function of distinct neuronal subpopulations. Yet we know how to generate only a few of these neuronal phenotypes from human embryonic stem cells, motor neurons and dopaminergic neurons being the best described examples¹⁻³. For the overwhelming majority of neuronal and glial phenotypes of the brain and spinal cord, tissue remains the only predictable source of defined neuronal subpopulations or of the progenitors thereof. Thus, to establish homogeneous populations of phenotypically restricted neurons and glia, one needs to isolate and expand defined populations of progenitors, whether from the fetal or adult CNS.

A variety of methods, based on both expressed surface antigens^{4,5} and transcriptionally active promoters^{3,6,7}, have now been established that permit the isolation of lineage-restricted neural progenitors, using either magnetic-assisted or fluorescence activated cell sorting (reviewed in ref. 8). However, once acquired, these progenitors have a limited capacity for mitotic expansion, lacking the self-renewal competence of more broadly competent neuroepithelial stem cells. This is in part due to their loss of telomerase expression⁹. Telomerase activity permits the retention of mitotic competence, by maintaining the length of chromosomal telomeres. As telomerase levels decrease, telomeric erosion occurs with frank shortening of the telomeres, delimiting and ultimately preventing mitotic expansion^{10,11}. Human neural progenitors typically down-regulate telomerase activity to undetectable levels by 16 weeks of gestational age, or with early passage *in vitro*^{9,12,13}. The developmental decline in telomerase activity is largely due to the transcriptional inactivation of TERT, the rate-limiting component of the telomerase enzyme complex⁹. To circumvent the attenuated mitotic competence of somatic cells associated with TERT inactivation,

a number of groups have used human TERT (hTERT) overexpression as a means of immortalizing cells in a nominally non-oncogenic fashion. In particular, hTERT overexpression has been successfully employed in fibroblastic, dermal, retinal, epithelial, endothelial and pancreatic cell types, among others, to generate mitotically stable lines of defined lineage¹⁴⁻¹⁷. When used alone, hTERT overexpression does not induce oncogenesis; its principal actions are limited to extending the mitotic competence of cells, and hence the number of executed cell divisions^{15,18}. Indeed, hTERT-transduced lines typically remain both dependent upon and normally responsive to mitogens and growth control signals. However, by so increasing the number of divisions, telomerase overexpression may effectively provide cells a greater opportunity to accumulate sufficient mutations in native tumor suppressors to permit oncogenesis¹⁹. Thus, although not an oncoprotein, telomerase immortalization may predispose transduced lines to later oncogenesis²⁰, a possibility that needs to be assessed throughout the propagation of any telomerase-immortalized line.

In some phenotypes, hTERT alone has been found insufficient to immortalize the lineages of interest, and in answer to this combination strategies to immortalization have been developed that include hTERT transduction in tandem with cdk4 overexpression²¹ or RNAi-mediated knockdown of p53 (see ref. 22), Bmi-1 (see ref. 23) or retinoblastoma protein²⁴. Yet all these methods have focused on hTERT immortalization of mitotically competent somatic cells, rather than their mitotic progenitors. The CNS is unusual among solid organs in its preponderance of terminally post-mitotic cells, neurons and oligodendrocytes, which are generated by the concurrent expansion and progressive lineage restriction of mitotic progenitor cells, in a manner akin to that of the hematopoietic system (reviewed in refs. 25,26). Thus, to address the specific needs of neurobiologists to generate phenotypically restricted lines of neuronal and glial progenitors, we have established protocols for immortalizing position- and stage-defined populations of neural progenitor cells, as well as of the sorted isolates thereof.

This protocol describes a set of methods designed to generate, select and characterize mitotically competent but lineage-restricted progenitor cells from the human CNS. Although we will make special reference to a spinal cord (SC)-derived line of hTERT-immortalized spinal neural progenitors that we derived from 11-week gestational age spinal cords (hSC11V-TERT cells)²⁷, we have successfully used hTERT overexpression to immortalize a broad variety of neural progenitors of both neuronal and glial phenotype. In each of these cases, we used viral delivery of hTERT, carried by a genomically integrating vector, either retroviral or lentiviral, to stably introduce hTERT under the control of a constitutive promoter into the cell type of interest. We have used only hTERT overexpression, having chosen to avoid concurrent p53 or Rb knockdown so as to minimize the likelihood of oncogenic transformation, recognizing that our reliance on hTERT significantly diminishes the efficiency of immortalization, and hence our net yield of successfully generated lines.

The protocol described here concentrates on the use of retroviral hTERT to overexpress telomerase in neuroepithelial cells derived from the human fetal spinal cord. By this means, we have established hTERT-immortalized lines of phenotypically restricted neural progenitor cells that give rise to neurons whose phenotypes reflect the region of the spinal cord from which they were derived. Our prototypic line, hSC11V-TERT, generates ventralized spinal neurons that mature predominantly as ventral spinal interneurons and less frequently as motor neurons²⁷. This largely neurogenic line has exhibited an extraordinary degree of expansion without evident replicative senescence. It is karyotypically and phenotypically stable, has exhibited no evidence of anaplastic transformation and can give rise to mature post-mitotic neurons of a variety of spinal phenotypes.

This protocol presents a generic strategy and detailed methods for establishing hTERT-immortalized lines of phenotypically restricted, tissue-derived neural progenitor cells. It presents algorithmically the methods necessary for fully characterizing the propagability and antigenic phenotype (Fig. 1), telomerase expression (Fig. 2), and karyotypic stability and cell cycle control (Fig. 3) of neurons generated from telomerase-immortalized neural progenitor cells. When combined with antigen or promoter-directed

fluorescence-activated cell sorting, this hTERT overexpression strategy may be used to establish relatively homogenous and expandable populations of any neural progenitor cell phenotype of interest, in particular those derived from the fetal brain and spinal cord. Although the methods that we describe here specify the immortalization of spinal neural progenitor cells²⁷, this strategy may be used to immortalize progenitor cells from throughout the developing human CNS. We have already described the functional competence of neurons generated from such hTERT-immortalized progenitors, as well as their *in vivo* integration and lack of anaplasia or tumorigenesis after orthotopic transplantation, all of which suggest the potential utility of hTERT transduction for producing phenotypically restricted progenitor cell lines of interest.

However, it is important to note that we have thus far been less successful in using this strategy to immortalize neural and glial progenitors derived from the adult human brain; in adult-derived neural cells, hTERT overexpression alone may be insufficient to generate telomerase-immortalized lines. In contrast, we have found that hTERT overexpression alone has proven sufficient to generate immortal lines of both multipotential and restricted neural progenitors, including phenotypically restricted neuronal progenitors, as well as glial progenitors, from the forebrain, brainstem and spinal cord, over a wide range of gestational ages. The phenotypic stability and general lack of anaplastic transformation of the resultant lines suggest that hTERT-immortalized neural progenitor cells may comprise important reagents for studying the signal pathways and responses of human neurons and glia, both *in vitro* and *in vivo*; indeed, these cells may ultimately prove to be effective vectors for cell-based therapy of the injured human CNS, for which we can now anticipate generating lines of cells dedicated to the production of pre-selected, lineage-restricted neuronal and glial cell types of therapeutic need.

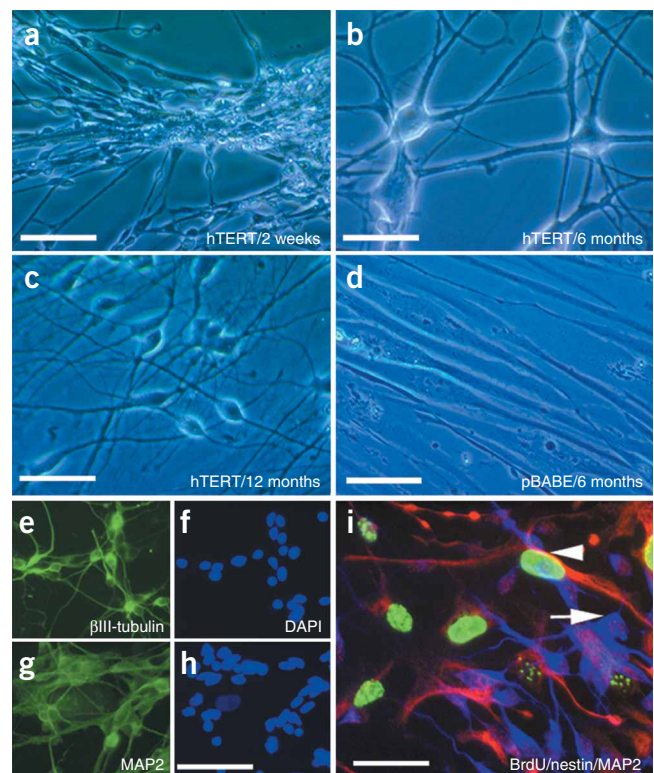
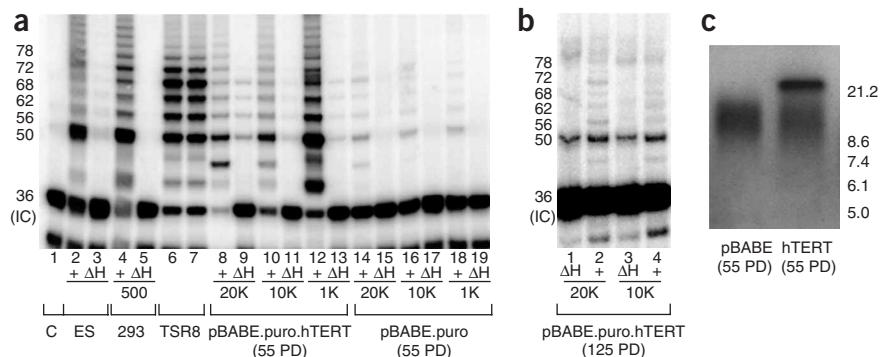


Figure 1 | hTERT transduction of progenitor cells can yield phenotypically restricted lines. These images show a line of hTERT-immortalized spinal cord progenitor cells, designated hSC11V-TERT. This neuronal line was derived from an 11-week gestational age human ventral spinal neuroepithelium. Phase images taken (a) 14 d after the first passage (at 1 month), (b) at 6 months and (c) at 12 months. Each line is passaged once per month, with 6–7 cell doublings, so that the images of a, b and c represent approximately 3, 39 and 75 PDs. (d) A control line infected with pBABE-puro at 6 months. Immunocytochemistry of hSC11V-TERT cells showed that the line generated neurons exclusively, as indicated by the expression of βIII-tubulin (e) and MAP2 (g). The corresponding Hoechst-labeled nuclei are shown in f and h. (i) hSC11V-TERT spinal progenitors, after *in vitro* exposure to BrdU. The culture was exposed to BrdU for 6 h, then fixed and stained for BrdU (green), nestin (red, arrowhead) and neuronal MAP-2 (blue, arrow). An abundance of BrdU⁺/nestin⁺ cells represents dividing progenitors. A large number of MAP2⁺ neurons are present, but these uniformly failed to incorporate BrdU. Thus, neurons arising in TERT-immortalized cultures derive from mitotic neuronal progenitor cells, but are themselves post-mitotic; they do not arise from aberrant replication of established neurons. Reproduced from ref. 27. Scale bars: a, 60 μm; b, 15 μm; c, 30 μm; d, 15 μm; e–h, 60 μm; i, 30 μm.

Figure 2 | Immortalized lines sustain high levels of telomerase and long telomeres. **(a,b)** The TRAP assay was used to assess telomerase enzyme activity in hSC11V-TERT cells after 55 **(a)** and 125 PDs **(b)**, as well as in pBABE-puro-transduced controls after 45 PDs **(a)**. **(a)** Lane 1: a cell-free negative control. Lanes 2 and 4: human embryonic stem cells and 293 cells, respectively; both positive control lines express high levels of telomerase, indicated by the ladder of bands 6 nucleotides apart, indicating hexameric nucleotide addition to the test substrate by cellular telomerase. Lanes 3 and 5: heat-inactivated negative controls. Lanes 6 and 7: TSR8 (telomeric substrate, 8 repeats), a calibration standard for assessing the relative activity of the test cell lines. Lanes 8, 10 and 12: hSC11V-TERT spinal neuronal progenitor cells, with 20,000, 10,000 and 1,000 extracted cells per lane, respectively. Lanes 9, 11 and 13 show their negative controls, whereas lanes 14–19 show pBABE transfection controls. The latter cells were transfected with a plasmid that expressed a puro selection cassette without hTERT. They exhibited scarcely measurable telomerase activity, which was estimated by densitometry to be less than one-thirtieth that of hSC11V-TERT cells (see text). **(b)** TRAP analysis of hSC11V-TERT cells after 125 PDs. Lanes 2 and 4 show telomerase activity of 20,000 and 10,000 cells, respectively. Lanes 1 and 3 are their heat-inactivated controls. Telomerase activity persisted, although it was much attenuated at 125 PDs relative to the same line's activity at 55 PDs. **(c)** Telomere length analysis, as assessed by Southern hybridization. The telomeric length of pBABE-control cells after 55 PDs was estimated at 15.9 kb, whereas at 55 PDs the hSC11V-TERT cells exhibited two bands, corresponding to respective telomeric lengths of 15.6 and 22.4 kb. Reproduced from ref. 27.



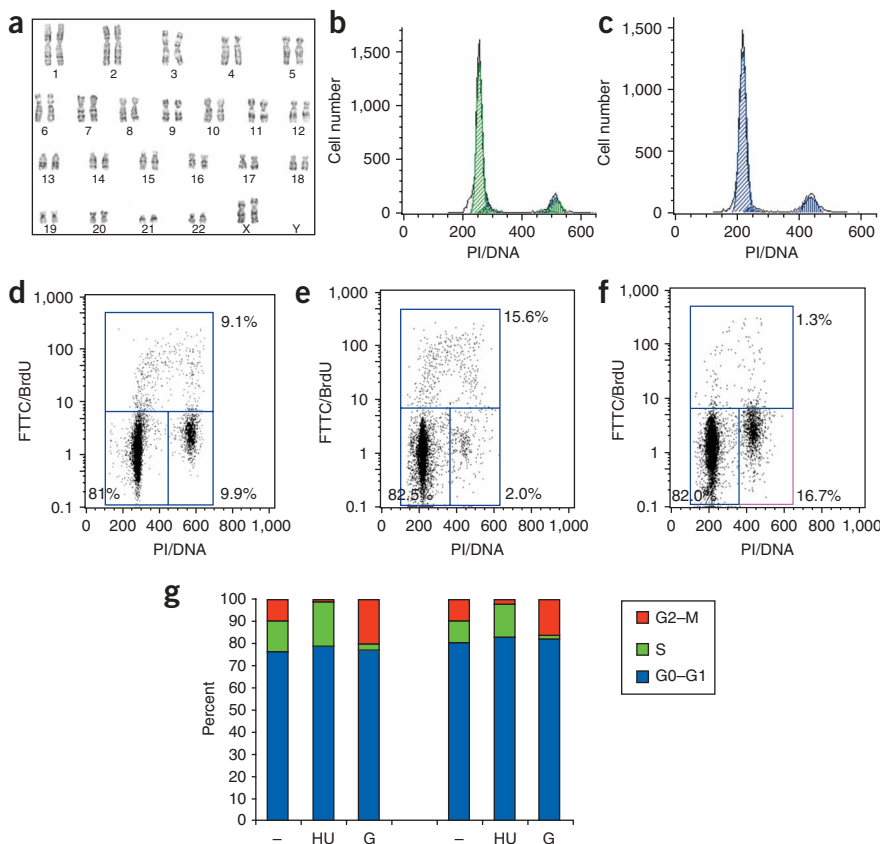
MATERIALS

REAGENTS

- Human fetal spinal cords derived from 9- to 23-week gestational age abortuses, obtained at surgery **! CAUTION** Acquisition and use of human fetal tissue must be done with the approval of the institutional review board and according to all applicable guidelines for working with human tissues and biohazards. The use must also conform to national regulations.
- VSVg-pseudotyped retrovirus encoding hTERT in pBABE-puro under CMV control (Geron Corp.)
- Control retrovirus encoding pBABE-puro without hTERT (Geron Corp.)
- PIPES (Sigma-Aldrich, cat. no. P1851)
- Potassium chloride (KCl) (Sigma-Aldrich, cat. no. P5405)
- Sodium chloride (NaCl) (Sigma-Aldrich, cat. no. S5886)

- 45% (wt/vol) glucose (Sigma-Aldrich, cat. no. G8769)
- Phenol red (Sigma-Aldrich, cat. no. P0290)
- Distilled water (Invitrogen, cat. no. 15230-204)
- L-Cysteine-HCl (Sigma-Aldrich, cat. no. C7477)
- 0.5 M EDTA pH 8.0 UltraPure (Invitrogen, cat. no. 15575020)
- Papain (Worthington, cat. no. LS003126)
- DNase-1 from bovine pancreas (Sigma-Aldrich, cat. no. D4263)
- Hank's balanced salt solution (without Mg and Ca, HBSS; Invitrogen, cat. no. 14175103)

Figure 3 | hSC11V-TERT cells retain mitotic and karyotypic stability. **(a)** Karyotypic analysis of hSC11v-TERT cells ($n = 20$ cells) revealed that all of the analyzed cells had a normal diploid karyotype. **(b,c)** Flow cytometric analysis of propidium iodide (PI)-labeled cells revealed that cells from both early (55 PDs) **(b)** and late (120 PDs) **(c)** passages had normal DNA complements with no hyperploidy. **(d-f)** Cells at 120 PDs responded to the S-phase blocker, hydroxyurea, by an increase in percentage of cells in the S phase **(e)**. They responded to γ -irradiation, a G1-phase blocker, by an increase in the G1/S ratio **(f)**. **(d)** Distribution of control cells (unexposed to cell cycle blockers) in the G0–G1, S and G2–M phases. **(g)** hTERT overexpression did not affect the ability of cells to respond to cell cycle blockers. This graph shows the relative percentage of cells in G0–G1, S and G2–M in response to hydroxyurea (HU) or γ -irradiation (G), relative to untreated control cultures (–), at both 55 and 120 PDs. These data indicate that hTERT did not suppress the cellular response to cell cycle inhibitors, even after prolonged *in vitro* expansion. Reproduced from ref. 27.



PROTOCOL

- D-MEM/F-12 (1×) liquid 1:1 (Invitrogen, cat. no. 11330032)
- GIBCO L-glutamine-200 mM (100×) (Invitrogen, cat. no. 25030081)
- GIBCO antibiotic-antimycotic (100×) liquid (Invitrogen, cat. no. 15240096)
- Bovine Albumin Fraction V Solution (7.5% (wt/vol) BSA) (Invitrogen, cat. no. 15260037)
- GIBCO MEM non-essential amino acids solution 10 mM (100×) (Invitrogen, cat. no. 11140050)
- N1 medium supplement (100×) (Sigma-Aldrich, cat. no. N6530)
- GIBCO MEM sodium pyruvate solution 100 mM (100×) (Invitrogen, cat. no. 11360070)
- Hydrocortisone (Sigma-Aldrich, cat. no. H0396)
- 3,3',5-Triiodo-L-thyronine sodium salt (Sigma-Aldrich, cat. no. T6397)
- FGF basic recombinant human (FGF2) (Sigma-Aldrich, cat. no. F0291)
- Dimethylsulfoxide (Sigma-Aldrich, cat. no. D2650)
- Platelet-depleted fetal bovine serum (PD-FBS) (Cocalico, cat. no. 540115)
- UltraPure 1 M Tris-HCl pH 7.5 (Invitrogen, cat. no. 15567027)
- MgCl₂ (Sigma-Aldrich, cat. no. M1028)
- EGTA (Sigma-Aldrich, cat. no. E3889)
- Benzamidine (Sigma-Aldrich, cat. no. 12072)
- β-Mercaptoethanol (Calbiochem, cat. no. 444203)
- CHAPS (Sigma-Aldrich, cat. no. C9426)
- UltraPure glycerol (Invitrogen, cat. no. 15514029)
- Tween 20 (Bio-Rad, cat. no. 170-6531)
- TRAPeze telomerase detection kit (Chemicon, cat. no. S7700)
- 1× PBS pH 7.4 (Invitrogen, cat. no. 10010049)
- RNaseOUT (Invitrogen, cat. no. 10777019)
- Titanium *Taq* polymerase (Clontech, cat. no. 639208)
- UltraPure 10× TBE buffer (Invitrogen, cat. no. 15581044)
- SYBR Green I nucleic acid stain (Invitrogen, cat. no. S7563)
- Poly-L-ornithine (Sigma-Aldrich, cat. no. P4957)
- Fibronectin (Sigma-Aldrich, cat. no. F4759)
- Calcein AM (Molecular Probes, cat. no. C1430)
- Hexadimethrine bromide (polybrene) (Sigma-Aldrich, cat. no. H9268)
- Puromycin dihydrochloride (Sigma-Aldrich, cat. no. 82595)
- TrypLE Select (Tryple X) (Invitrogen, cat. no. 12563-011)
- Wizard SV genomic DNA purification system (Promega, cat. no. A2631)
- UltraPure 10× Tris-acetate-EDTA buffer (Invitrogen, cat. no. 15558042)
- 20× SSC buffer (Roche Applied Science, cat. no. 11666681001)
- TeloTAGGG Telomere Length Assay (Roche Applied Science, cat. no. 12209136001)
- KaryoMAX colcemid solution (Invitrogen, cat. no. 15212-012)
- Methyl alcohol (Sigma-Aldrich, cat. no. M3641)
- KaryoMAX Giemsa stain stock solution (Invitrogen, cat. no. 10092013)
- Hydroxyurea (Sigma-Aldrich, H8627)
- 5-Bromo-2'-deoxyuridine (BrdU) (Sigma-Aldrich, cat. no. B9285)
- Rat anti-BrdU antibody, clone BU1/75 (Abcam, cat. no. ab6326)
- FITC-conjugated goat anti-rat IgG (Jackson ImmunoResearch Laboratories, cat. no. 112-095-143)
- Propidium iodide (Molecular Probes, cat. no. P-3566)
- RNase A (Worthington, cat. no. LS005649)
- Characterized fetal bovine serum (HyClone, cat. no. SH30071.03)
- Brain-derived neurotrophic factor recombinant human (BDNF) (R&D Systems, cat. no. 248-BD)
- Glial cell line-derived neurotrophic factor recombinant human (GDNF) (R&D Systems, cat. no. 212-GD)
- Saponin (Sigma-Aldrich, cat. no. S4521)
- Normal goat serum (BioSource) (NGS) (Invitrogen, cat. no. PCN5000)
- Anti-neuronal class IIIβ-tubulin monoclonal antibody, clone TUJ1 (Covance, cat. no. MMS-435P-100)
- Mouse anti-MAP2 (2a + 2b) monoclonal antibody, clone AP-20 (Sigma-Aldrich, cat. no. M1406)
- Mouse anti-Islet-1 antibody (DSHB, clone 39.4D5)
- Rabbit anti-human nestin antibody (Chemicon, cat. no. AB5922)
- Rabbit anti-choline acetyltransferase (ChAT) antibody (Chemicon, cat. no. AB5964)
- Rabbit anti-NMDA-NR1 antibody (Chemicon, cat. no. AB1516)

EQUIPMENT

- ABI Prism 7000 sequence detection system (Applied Biosystems)
- NALGENE TM Cryo 1 °C freezing containers
- CCD gel imaging system with 535 nm filter for SYBR Green
- Hybridization oven or water bath with adjustable temperature settings
- ImageQuant software (Molecular Dynamics)

REAGENT SETUP

PIPES buffer 2 ml PIPES buffer stock (0.5 M, 6.048 g PIPES in 40 ml of 1 M NaOH, adjust the pH to 7.4, filter-sterilize), 5 ml of 10× stock of 1.2 M NaCl/50 mM KCl solution, 0.5 ml of 45% glucose, 50 μl of Phenol red and dH₂O to make up volume to 50 ml. Filter through a 0.2 μm filter unit.

Dissociation enzyme mixture (5 ml) 0.5 ml of 10× EDTA (11 mM)/L-cysteine-HCl (55 mM) solution, 4.5 ml of PIPES buffer and 100 U papain. Activate at 37 °C for 5 min. Filter through a 0.22 μm syringe filter. Add 80 μl of 20,000 U DNase I reconstituted in 1 ml of HBSS. **! CAUTION** Different lots of papain from Worthington can have different units per mg per ml. Recalculate the amount to be added with each new bottle. The enzyme mixture (with PIPES and cysteine-HCl) should contain a final concentration of 20 U per ml of papain. **▲ CRITICAL** Enzyme mixture should be prepared fresh just before addition to tissue.

Culture medium (500 ml) 470 ml DMEM/F12, 10 ml glutamine (200 mM), 6 ml glucose (45%), 4 ml antibiotic-antimycotic (100×), 5 ml non-essential amino acids (10 mM), 660 μl BSA (7.5%), 5 ml N2 supplement (100×), 5 ml sodium pyruvate (100 mM), 1.9 ml HEPES (1 M), 150 μl hydrocortisone (1 μg μl⁻¹ stock) and 150 μl triiodothyronine (100 μg μl⁻¹ stock). This can be prepared in bulk and frozen in 50 ml aliquots. Thaw as needed and supplement with fresh 10 ng ml⁻¹ FGF2.

Freezing medium (10 ml) 1 ml DMSO, 2 ml PD-FBS and 7 ml supplemented culture medium. **▲ CRITICAL** Freezing medium should be mixed up fresh just before use and should be at 4 °C.

1× CHAPS lysis buffer 10 mM Tris-HCl pH 7.5, 1 mM MgCl₂, 1 mM EGTA, 0.1 mM benzamidine, 5 mM β-mercaptoethanol, 0.5% CHAPS and 10% glycerol. Add RNaseOUT (100 U ml⁻¹) to the lysis buffer just before use.

10× telomerase repeat amplification protocol (TRAP) reaction buffer 200 mM Tris-HCl pH 8.3, 15 mM MgCl₂, 630 mM KCl, 0.5% Tween 20 and 10 mM EGTA.

TRAP assay mixture (for 20 assays) 100 μl of 10× TRAP reaction buffer, 20 μl of 50× dNTP mix, 20 μl of TRAP primer mix, 20 μl of TS primer, 8 μl of titanium *Taq* polymerase and add dH₂O to make up a final volume of 480 μl.

TRAP assay loading dye 0.25% bromophenol blue and xylene cyanol in 50% glycerol/50 mM EDTA.

Coating plates with poly-L-ornithine and fibronectin Dilute poly-L-ornithine with HBSS (1:10) and add enough to cover the surface of culture plate. Coat for a minimum of 2 h at room temperature (RT; 25 °C). Wash three times with HBSS and add enough fibronectin (5 μg ml⁻¹ in HBSS) to coat surface. Incubate for 2 h at 37 °C. If not used right away, these plates can be stored at 4 °C for a week.

PROCEDURE

Tissue dissection, dissociation and culture ● TIMING 3–4 h

1| Separate different regions of the spinal cord from 9–11 week gestational age (g.a.) human fetuses, including the cervical, thoracic and lumbar segments, by dissection in HBSS (Ca²⁺/Mg²⁺-free). For later fetuses (at and beyond 11 weeks gestational age), divide the spinal cord longitudinally into ventral and rostral strips before rostral-caudal segmentation. Alternatively, discrete brain regions of analogous developmental stage may be substituted for the spinal cord, depending on the target phenotypes of interest.

2| Chop the dissected tissue finely and collect in a 15 ml tube in HBSS (Ca²⁺/Mg²⁺-free). Never allow the volume of tissue to exceed 1.5 ml. Raise the total volume of HBSS to 8 ml. Quickly spin for 20 s at 200g at RT. Discard the supernatant and repeat the step with fresh HBSS.

3| Add PIPES buffer to the 2.5 ml mark, then add 2.5 ml of the dissociation enzyme mixture. Incubate for 1 h at 37 °C on a shaker.

▲ **CRITICAL STEP** At the end of 1 h, the liquid in the tube should become turbid upon shaking. If not, then incubate for an additional 30 min.

4| Centrifuge for 5 min at 200g at RT. Remove 2.5 ml of the supernatant and add 2.5 ml of culture medium. Add 40 µl of DNase I solution. Incubate for 15 min at 37 °C on a shaker.

▲ **CRITICAL STEP** Shake the tube after adding DNase I. If tissue pieces appear to be trapped in a clump, add another 40 µl of DNase I solution. High cell death (e.g., due to a large lag time between retrieval of tissue and addition of dissociation enzyme) may result in higher quantities of DNA being released.

5| Centrifuge for 5 min at 200g at RT. Remove 3 ml of the supernatant. Triturate with graduated pipettes made by fire polishing glass Pasteur pipettes (large, medium and small bore sizes).

▲ **CRITICAL STEP** Wet pipette with culture medium before trituration to prevent tissue from sticking to the glass and resulting in lower cell yields. Tissue can be triturated 10–15 times with the large and medium pipettes till you get no resistance. The suspension should not be triturated more than three times with the smallest bore sized pipette as this will result in too much cell death.

6| Add PD-FBS (to amount to 5%) to each tube to stop the enzymatic dissociation. Wait for a minute and then add 5 ml of culture medium. Filter the dissociated cells through a 70 µm nylon mesh filter in a 50 ml tube. Pool the cells in one tube if more than one tube of tissue from the same region is involved. Wash the filter with 2 ml of culture medium to recover all the cells.

▲ **CRITICAL STEP** Wet the filter with culture medium before filtering to prevent cells from sticking to the mesh and resulting in lower cell yields.

7| Centrifuge for 5 min at 200g at RT. Discard the supernatant and resuspend in 20 ml of culture medium. Repeat centrifugation and finally suspend cells in a small amount of culture medium supplemented with 10 ng ml⁻¹ FGF2. Take viable cells count by adding 1 µl of calcein AM (500 µM) to a 100 µl aliquot of cell suspension and incubating for 15 min at 37 °C. The cell count can be determined on a hemocytometer under a fluorescent microscope using a 489 nm emission filter. Viable cells will show green fluorescence.

? TROUBLESHOOTING

8| Add 2,000 cells per 2 ml per well in a six-well tissue culture plate for infection with hTERT retrovirus. Plate 100,000 cells per 0.75 ml per well of a 12-well plate to determine the concentration of puromycin needed to select transfected lines. Maintain cells in culture medium supplemented with 10 ng ml⁻¹ FGF2.

Determination of optimal puromycin concentration for selection ● TIMING 1–2 weeks

9| Add puromycin at concentrations of 0, 0.2, 0.4, 1.0 and 2 µg ml⁻¹, in triplicate, to cells plated in 12-well plates in Step 8 at 72 h post-dissociation.

10| Feed the cells every 72 h with fresh FGF-supplemented medium with puromycin. Continue to feed and monitor cells till a point where no viable cells are seen at a given concentration. Untransfected human cells, depending on cell type and puromycin batch, are generally vulnerable to a concentration range of 0.4–1.0 µg ml⁻¹. The lowest concentration needed to kill all untransfected cells is the working concentration needed for selection of transduced cells.

Infection of dissociates with retroviral hTERT and selection of transfected lines ● TIMING 3–4 weeks

11| Infect freshly dissociated cells (preferably at the end of the day) with the virus (VSVg-pseudotyped retrovirus encoding hTERT in pBABE-puro under CMV control or control retrovirus encoding pBABE-puro without hTERT). For infection, either viral supernatant or partially purified virus (viral supernatant centrifuged at 25,000 r.p.m./64,300g for 2 h at 10 °C) can be used.

▲ **CRITICAL STEP** When viral supernatant is used for infection, reduce the culture medium in the dishes to 1 ml and add 1 ml of the supernatant. When partially purified virus is used, the culture medium can be left at 2 ml. Add polybrene (8 µg ml⁻¹) to each culture well.

! **CAUTION** All steps from this point onward should be performed under BSL2 containment, optimally with BSL3 compatible procedures. All plasticware and consumables should be immersed in 1% sodium hypochlorite before discarding.

12| Repeat viral infection a total of three times at 8 h intervals. For this, remove spent culture medium and replace with fresh medium and viral suspension as described in Step 11.

PROTOCOL

13| After three infections, feed the cells with FGF2-supplemented culture medium. Feed the cells every 72 h with a 2/3 fresh culture medium change.

14| After 1–2 weeks (depending on the density of the cells in the well), switch the transduced cells to media containing 0.4–1 $\mu\text{g ml}^{-1}$ puromycin.

▲ CRITICAL STEP Determination of puromycin concentration should have been done by this time point as described in Steps 8–10.

15| Maintain cells in puromycin until colony(ies) of cells appear (**Fig. 1a**).

▲ CRITICAL STEP Usually no more than one colony is seen in a well. In case more than one colony is seen, they should be segregated using a sterile cloning ring with a silicone grease coating at one end of the ring. Lower the cloning ring on the grease-coated side to surround the colony. Using a plastic fine-tipped pipette, aspirate out the medium and replace with 20 μl of Tryple X solution for 1 min. Aspirate out the Tryple X solution without disturbing the cells and replace with 100 μl of culture medium. Triturate a few times and transfer cells to one well of a 12-well plate. Add an additional 0.7 ml culture medium.

? TROUBLESHOOTING

16| Transfer all selectants, both retroviral pBABE-puro- and pBABE:hTERT-puro-infected cells, to one well each of a 12-well plate. Culture medium should be replaced with 2/3 of fresh medium every 3–4 d.

▲ CRITICAL STEP At this point, puromycin should no longer be added.

Maintenance and characterization of selected lines ● TIMING As per experimental requirement

17| The following options describe how selected lines should be maintained and characterized. Usually selected lines are maintained in two parallel sets of cultures. One set of cells is maintained and passaged regularly, once at 70% confluency, to be used for ongoing experiments; monthly or every 4–7 passages, cells are also frozen for future reference or use (see option B). The second set of cells is passaged every 4–5 d (see option A), so as to precisely track their estimated number of population doublings (PDs). Successfully immortalized lines are expected to replicate for additional generations beyond those achieved by non-immortalized controls. This is assessed by estimating the number of PDs exhibited by the cells. PDs are determined by using a modified 3T3 protocol^{28,29} (see option C). Briefly, the lines are split every 4–5 d at 500,000 cells per ml (1×10^6 cells per well, in a six-well plate). The time between splits depends on the doubling time, which can vary not only by line, but also within a line over time, so that it must be estimated for each line generated and reassessed periodically during prolonged passage. (For example, hSC11V-TERT cells have a cell cycle time between 72 and 96 h, so that we split the cells every 4–5 d rather than every 3 d, so as not to diminish the total pool over time.) Each resulting line should be checked for telomerase activity, both following initial selection and periodically thereafter (see option D). TRAP is a highly sensitive *in vitro* assay for detecting telomerase activity^{30,31} (option D). In the TRAP assay, telomerase adds telomeric repeats onto substrate oligonucleotides, the extended product of which is amplified by PCR. Increases in telomerase activity are usually accompanied by increments in telomere length relative to control lines. To correlate these outcome measures, every TRAP assay should also be accompanied by telomere length estimation (option E). Telomere length is determined by Southern blot analysis of terminal restriction fragments obtained by digestion of genomic DNA. A distinctive feature of hTERT-based immortalization of human cells is the lack of associated karyotypic abnormalities, even after many PDs. To validate the stable diploid genotype of hTERT-transduced cells, the lines should be subjected to karyotypic analysis (see option F), preferably after 100 PDs have been achieved. Another characteristic feature of hTERT-immortalized human cells is their retained ability to respond appropriately to cell cycle checkpoints. In this regard, an effective means of assessment is to determine how the cells respond to S-phase blockade (see option G). Analysis of the cell lines must include an estimation of their differentiation potential (see option H), with consideration of both anticipated and unanticipated phenotypes. The cells should be exposed to culture conditions broadly able to induce glial (oligodendrocyte and astrocyte) or neuronal differentiation. In some instances, such as when using cells already selected for a specific progenitor phenotype before hTERT transduction, a given lineage restriction or limited differentiation potential might be anticipated, but even then one must proceed with a complete assessment of the antigenic phenotypes generated by a given line.

(A) Passaging of cells

(i) Once cells reach 70% confluency, split each line at a ratio of 1:4 in a 12-well plate. For this, treat cells in a culture dish with a small amount of Tryple X for 1 min.

(ii) Discard Tryple X and replace with FGF2-supplemented culture medium (1 ml if six-well plate, 0.5 ml if 12-well plate) and triturate with a fine-tipped plastic pipette. Transfer at a ratio of 1:4 into a new culture dish. Wash the well with additional culture medium and transfer to same culture dishes. Add additional medium to each new well to make up an appropriate volume.

▲ CRITICAL STEP Once the wells are ready for the next split, freeze half of the cells. Viable freezing of cells should be done periodically (described in option B).

(iii) Continue to maintain the cells by splitting at 70% confluency. Once a large number of cells have been generated, cells can be cultured in six-well plates.

▲ **CRITICAL STEP** Routine cell maintenance should involve 2/3 replacement with fresh FGF2-supplemented medium every 3–4 d.

(B) Cryopreservation of selected lines ● TIMING 2 h

(i) Treat cells in culture dish with Tryple X as described in option A. Collect the cells in a 15 ml tube. Take a count on hemocytometer. Optimum cryopreservation is achieved at cell densities of 500,000 cells per ml and above.

(ii) Centrifuge the cells at 300g for 5 min at RT.

(iii) Discard the supernatant and resuspend in an appropriate amount of cold freezing medium. Mix very gently and transfer 500 µl of cell suspension to pre-labeled cryovials.

(iv) Transfer the vials to the freezing container (stored at RT and filled with isopropanol) and store in a –70 °C freezer overnight.

▲ **CRITICAL STEP** Remove cryovials the next day and place in liquid nitrogen. Leaving cells in a –70 °C freezer for long periods will diminish viability.

(C) Estimation of PDs ● TIMING As per experiment

(i) Plate 1 million cells per 2 ml of medium per well of a six-well plate. In 4–5 d (or less, depending on the cell line), replace conditioned medium with 0.5 ml Tryple X.

▲ **CRITICAL STEP** Save the conditioned medium for feeding cells for the next passage.

(ii) After 1 min, replace Tryple X with 1 ml of culture medium. Triturate the well thoroughly without introducing air bubbles and transfer cells to a sterile 2 ml Eppendorf tube. Wash the well with 1 ml medium and transfer to the same Eppendorf tube.

(iii) Spin at 300g for 2 min and resuspend cells in media containing conditioned medium and fresh FGF2-supplemented medium in a ratio of 1:2.

▲ **CRITICAL STEP** Continue following and obtaining PD estimates at least until cells exceed 120 PDs.

(D) Determination of telomerase activity ● TIMING 2 d

(i) Collect the cells by treating with Tryple X as described in option A. After spinning at 300g for 5 min, discard the supernatant without disturbing the pellet and resuspend in 1 ml of DMEM/F12. Count by hemocytometer and then transfer an aliquot of 100,000 cells to an Eppendorf tube. Spin (200g for 10 min) and discard the supernatant. Resuspend the cells in 1 ml of 1× PBS, repellet and carefully remove the supernatant. If the cells will be extracted immediately, proceed to next step.

▲ **CRITICAL STEP** Telomerase is an RNase-sensitive ribonucleoprotein. To detect telomerase activity, RNase contamination must be avoided. The work area and labware must be free of contaminating ribonucleases and amplified PCR products.

■ **PAUSE POINT** If the TRAP assay will not be performed immediately, flash-freeze cells on dry ice and store at –80 °C. Telomerase activity is stable in frozen cells for at least 1 year.

(ii) Solubilize the cells in 200 µl of cold 1× CHAPS lysis buffer (per 10⁵ cells; supplemented with 100 U ml⁻¹ RNaseOUT) by triturating the pellet a few times. Transfer to an Eppendorf tube and leave on ice for 30 min. As positive control, extract telomerase from 10⁵ 293 cells by adding 200 µl of 1× CHAPS to the cell pellet.

(iii) Centrifuge at 12,000g for 20 min at 4 °C. Collect the supernatant and aliquot to new Eppendorf tubes.

■ **PAUSE POINT** If the TRAP assay will not be performed immediately, flash-freeze the protein extracts on dry ice and store at –80 °C. However, TRAP assay should be done as soon as possible after extraction.

(iv) Add 48 µl of TRAP assay mixture to each PCR tube. Add sample extracts corresponding to 1,000 cells (2 µl of extract). Also include the following as controls: (a) as a primer/dimer/PCR contamination control, use 2 µl CHAPS lysis buffer, (b) as a positive control for telomerase activity, use the telomerase-positive cell extract corresponding to 500 cells, (c) use TSR8 (telomeric substrate, 8 repeats), a calibration standard showing characteristic TRAP product bands, for assessing the relative activity of the test cell lines and (d) as a negative control, pre-incubate 2 µl of each cell extract at 85 °C for 20 min and then add to the TRAP assay mixture to ensure heat-inactivation of telomerase.

! **CAUTION** TSR8 is a potent contaminant of the TRAP assay. If contamination occurs, all TRAP reactions will show false positive bands that are resistant to RNase and heat treatments. Therefore, aliquot TSR8 in an area separate from where the TRAP assay is being carried out.

▲ **CRITICAL STEP** Dilute the telomerase-positive extract (1:20) with 1× CHAPS lysis buffer (supplemented with 100 U ml⁻¹ RNaseOUT) before use (250 cells per µl of extract).

(v) Incubate the mixtures at 30 °C for 30 min, to allow telomerase-mediated extension of the TS primer.

(vi) Amplify by PCR in a thermocycler: 94 °C for 30 s denaturation, 59 °C for 30 s annealing and 72 °C for 1 min extension, for 30–33 cycles.

PROTOCOL

- (vii) Add 5 μl of loading dye to each sample. Load 25 μl of each sample on a 12.5% non-denaturing PAGE gel in $0.5\times$ TBE. Run the gel for ~ 3.5 h at 200 V for a 19 cm vertical gel or until the xylene runs 70–75% of the gel length. Run the gel for 1.5 h at 400 V for a 10–12 cm gel.
- (viii) Stain the gel with SYBR Green at a dilution of 1:10,000 in $0.5\times$ TBE for 40 min (protect from light).
- (ix) Visualize the gel with a 254 or 302 nm UV transilluminator that has an SYBR Green filter.

? TROUBLESHOOTING

- (x) To obtain quantitative values of telomerase activity, analyze the TRAP assay using a CCD imaging system and image analysis program. For this, quantify the amount of telomerase product generated (TPG), which corresponds to the number of TS primers (600 molecules) extended by telomerase by at least four telomeric repeats during a 30 min incubation at 30 °C. To estimate TPG, measure the total signal intensity of the bands corresponding to all TRAP product ladder bands from all samples, including the non-heat-treated control (x) and heat-treated sample extracts (x_0), CHAPS lysis buffer only control (r_0) and TSR8 quantification control (r). In addition, measure the signal from the internal control in non-heat-treated samples (c) and TSR8 quantification control (c_R). The assay generates a linear quantitative curve for the three densities examined per cell type, that is, 1,000, 10,000 and 20,000 cells. Based on regression analysis, the number of TPG generated is determined per 1,000 cells using the following formula: $\text{TPG (units)} = (x - x_0)/c/(r - r_0)/c_R \times 100$.

(E) Telomere length analysis ● TIMING 3 d

- (i) Extract genomic DNA from cells using a genomic DNA extraction kit (Promega) as per the manufacturer's instructions. Genomic DNA can also be extracted using lab-specific standard methods.
- (ii) Use the TeloTAGGG Telomere Length Assay kit (Roche) to determine telomere length, as described in the following steps. First digest 250 ng of DNA using the restriction enzymes *HinfI* and *RsaI*.
- (iii) Separate the digested DNA by electrophoresis on a 0.8% agarose gel in $1\times$ Tris-acetate-EDTA buffer at 5 V cm^{-1} until the bromophenol blue dye is approximately 10 cm from the wells (2–4 h depending on gel length).
- (iv) Southern transfer the digested DNA by the capillary transfer method. For this, submerge the gel in 0.25 M HCl for 5–10 min (until the bromophenol blue turns yellow), rinse the gel two times with dH_2O , denature for 2×15 min in 0.5 M NaOH and 1.5 M NaCl, rinse the gel two times with dH_2O and finally neutralize the gel for 2×15 min in 0.5 M Tris-HCl and 3 M NaCl, pH 7.5.
- (v) Transfer the digested DNA from the gel to the nylon membrane via capillary or vacuum transfer at RT using $20\times$ SSC as a transfer buffer for 16 h.
- (vi) Fix the transferred DNA onto a wet blotting membrane by UV crosslinking (120 mJ). Rinse the membrane with $2\times$ SSC.
▲ CRITICAL STEP If the hybridization will not be performed immediately, air-dry the membrane and store at 4 °C.
- (vii) Pre-hybridize the membrane in pre-warmed digoxigenin (DIG) easy hybridization solution (from the kit) for 30–60 min at 42 °C with gentle agitation. Remove the pre-hybridization solution and hybridize the membrane with telomere-specific probe diluted in DIG easy hybridization solution for 3 h at 42 °C with gentle agitation.
- (viii) Wash off the hybridization solution (2×5 min with stringent wash buffer I at RT; 2×20 min with stringent wash buffer II at 50 °C). Incubate the membrane in blocking solution for 30 min at RT with gentle agitation. Incubate with anti-DIG-alkaline phosphatase working solution (from the kit, 75 mU ml^{-1} , 1:10,000) for 30 min at RT with gentle agitation.
- (ix) Wash off the antibody solution (2×15 min with washing buffer at RT). Then add CDP-Star chemiluminescence substrate (from the kit) for 2–5 min at RT to detect the anti-DIG-alkaline phosphatase-labeled DNA. Finally, expose the membrane to chemiluminescent film for 5–20 min at RT.
- (x) Finally, determine the mean terminal restriction fragment length as $\Sigma(\text{OD}_i)/\Sigma(\text{OD}_i/L_i)$, where OD_i is the densitometer output and L_i is the length of the DNA position at i . The amount of telomeric DNA is estimated by integrating the volume of each band using ImageQuant.

(F) Karyotyping of cell lines ● TIMING 4 h

- (i) Cell lines should be fed with fresh medium the day before the procedure.
▲ CRITICAL STEP To achieve successful karyotyping, it is important that the cells are actively dividing. Therefore, culture wells no more than 50% confluent should be used for this procedure.
- (ii) The following day, add KaryoMAX colcemid solution directly to the plate to get a final concentration of 0.02 g ml^{-1} .
! CAUTION Colcemid is toxic; wear protective gear. Incubate for 2 h at 37 °C.
- (iii) Remove the culture medium containing colcemid and add 0.5 ml of Tryple X. After 1 min, discard Tryple X and add 1 ml of culture medium. Triturate the cells with a fine-tipped plastic pipette and transfer to a 15 ml tube.
- (iv) Centrifuge cells at $200g$ for 5 min. Discard the supernatant and resuspend in 2 ml of 0.075 M KCl hypotonic solution by vortexing for a few seconds. Incubate in a water bath for 30 min at 37 °C.
- (v) Add 0.5 ml of freshly prepared methanol/acetic acid (3:1) fixative accompanied by continuous swirling. Centrifuge cells at $200g$ for 4 min at RT.

- (vi) Discard the supernatant and repeat Step v three more times.
 - **PAUSE POINT** Store at 4 °C if cells are not to be analyzed right away.
- (vii) With pre-wetted glass Pasteur pipette carefully transfer a drop of the cell suspension onto a humidified surface of a cold slide held at a 45° angle.
- (viii) Air-dry the slide and stain with Giemsa stain. Wash with water, air-dry and clear cells by passing through two changes of xylene. Coverslip on permount.

(G) Cell cycle analysis ● TIMING 4 d

- (i) Select wells at 50% confluency. Feed them with fresh culture medium the night before the assay.
- (ii) On the day of the assay, treat one well with γ -irradiation (40 Gy) and then maintain the cells for an additional 72 h. Treat another well with 5 mM hydroxyurea for 72 h.
- (iii) On the third day, treat the cells with a 6 h pulse of BrdU (10 $\mu\text{g ml}^{-1}$).
- (iv) Wash off the BrdU and dissociate to single-cell suspension using Tryple X (described above).
 - ▲ **CRITICAL STEP** If cells appear to be clumping or if the suspension becomes overtly viscous, treat with DNase I for 10 min at 37 °C to avoid mechanical shearing during trituration and centrifugation.
- (v) Centrifuge at 300g for 5 min and discard the supernatant. Wash once with HBSS and fix the cells by adding 0.7 ml of cold 200 proof ethanol solution to a 0.3 ml suspension of cells in 2 ml Eppendorf tubes.
 - **PAUSE POINT** At this point, cells may be stored in 70% ethanol at -20 °C for several weeks before staining and flow cytometric analysis.
- (vi) Centrifuge cells (300g, 5 min) and wash once with HBSS. Treat the cells with 2 N HCl for 20 min.
- (vii) Neutralize the HCl by adding 1 ml of 2 M sodium borate. After 5 min, centrifuge cells (300g, 5 min) and wash with HBSS twice.
- (viii) Label with 100 μl of rat anti-BrdU antibody (1:400 in HBSS) overnight at 4 °C.
- (ix) Wash cells with HBSS and label with FITC-conjugated goat anti-rat IgG (1:200 in HBSS) for 2 h at RT.
- (x) Wash with HBSS twice and counter-stain with 1 ml of propidium iodide (40 $\mu\text{g ml}^{-1}$ in PBS).
 - ▲ **CRITICAL STEP** Propidium iodide can also bind to double-stranded RNA. If necessary, add 50 μl of RNase A solution (10 $\mu\text{g ml}^{-1}$) and incubate for 3 h at 4 °C before carrying out the assay.
- (xi) Analyze the cells for DNA content on a Coulter Elite flow cytometer, or other available flow cytometer or cell sorter with cytometric capability. Determine the percentage of cells in G1, S and G2/M phase using an appropriate algorithm^{32,33} or commercial program (FlowJO).

(H) Phenotypic analysis ● TIMING 1–2 weeks

- (i) Differentiate cells from the hSC11V-TERT line by plating on poly-L-ornithine- and laminin-coated plates (100 ng cm^{-2} from a 1 $\mu\text{g ml}^{-1}$ stock), in the presence of culture medium (DMEM/F12/N2/T3/hydrocortisone, as noted) supplemented with 10% FBS and 20 ng ml^{-1} each of GDNF and BDNF. Maintain the cultures for a week with 2/3 fresh medium change every 3 d.
- (ii) Fix the cells with 4% paraformaldehyde for 5 min.
- (iii) Permeabilize the cells with PBS containing 0.1% saponin and 1% NGS for 15 min at RT.
- (iv) Incubate the cells overnight at 4 °C with primary antibody (nestin (1:200), β III tubulin (1:600), neurogenin2 (1:100), MAP-2 (1:100), Islet1 (1:5), ChAT (1:200), NMDA-NR1 (1:800) or others) diluted in PBS containing 0.05% saponin and 0.5% NGS. Incubate cells with fluorescent dye-labeled secondary antibodies at RT for 1 h at dilutions ranging from 1:200 to 1:400.

? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible cause	Possible solution
7	Low viable cell number count	Too much elapsed time between tissue acquisition, dissociation and definitive culture or storage Insufficient mincing of tissue into small size, resulting in suboptimal enzyme access Insufficient dissociation due to inactive enzyme	Chop tissue into small pieces at the location of tissue acquisition; transport the tissue on ice Make sure to chop into small pieces, optimally < 1 mm^3 Make sure to prepare fresh enzyme mixture and reconstitute DNase I just before dissociation



TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible cause	Possible solution
15	No colonies seen after puromycin selection	Viral titer too low Inactive virus Puromycin concentration too high and toxic for the cells	Titrate before use on 3T3 fibroblast line. If viral supernatant is used, partially purify before use Store properly at -80°C and do not use viral aliquots stored at 4°C Re-estimate minimum puromycin dose needed to kill uninfected cells, using smaller dose increments
17D(ix)	No product ladder seen Product band seen in all lanes including primer/dimer/PCR contamination control	Telomerase activity not initiated Primer-dimer PCR artifacts	Telomerase is very heat sensitive; make sure extraction and TRAP reaction are carried out at $<25^{\circ}\text{C}$ The band pattern of primer-dimer is distinguishable from authentic telomerase activity. The band spacing of primer-dimer is usually an unevenly spaced 6 bp ladder. In addition, bands 3–6 are weaker in intensity
17H(iv)	Differentiation induces more than one cell type after selection	Mixing of two clones during puromycin selection of transduced cell lines	Plate cells at a lower density so that they grow as colonies. Re-select a few morphologically different colonies with cloning rings

ANTICIPATED RESULTS

Our prototypic example, the hSC11V-TERT line, has been maintained over several years of continuous passage, long ago surpassing 120 measured PDs. Without losing its karyotypic stability or obedience of cell cycle checkpoints, it has retained high-level telomerase activity as assessed by TRAP, and extended telomere length compared to its control lines. The line gives rise predominantly to neurons of ventral phenotype, with persistent generation of additional progenitors; upon transplantation with prolonged survivals, we have noted no evidence of overt tumors or anaplastic degradation. By this benchmark, successful overexpression of hTERT in human neural progenitor cells may be expected to result in the generation of homogeneous lines that have the capacity to (a) divide for sustained periods without anaplastic or neoplastic transformation, (b) remain true to their lineage and derivative phenotypes, (c) retain their responses to appropriate differentiation cues and (d) generate fully functional, terminally differentiated progeny, including appropriately post-mitotic neuronal progeny.

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