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Proliferation of adult human bronchial epithelial cells without a telomere maintenance mechanism for over 200 population doublings

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Abstract

To date, there is no direct evidence of telomerase activity in adult lung epithelial cells, but typical culture conditions only support cell proliferation for 30 to 40 population doublings (PD), a point at which telomeres remain relatively long. Here we report that in *in vitro* low stress culture conditions consisting of a fibroblast feeder layer, rho-associated coiled coil protein kinase inhibitor (ROCKi), and low oxygen (2%), normal human bronchial epithelial basal progenitor cells (HBECs) divide for over 200 PD without engaging a telomere maintenance mechanism (almost four times the “Hayflick limit”). HBECs exhibit critically short telomeres at 200 PD and the population of cells start to undergo replicative senescence. Subcloning these late passage cells to clonal density, to mimic lung injury *in vivo*, selects for rare subsets of HBECs that activate low levels of telomerase activity to maintain short telomeres. CRISPR/Cas9 knockout of human telomerase reverse transcriptase (hTERT) or treatment with the telomerase-mediated telomere targeting agent 6-thio-2'-deoxyguanosine (6-thio-dG) abrogates colony growth in these late passage cultures (>200PD) but not in early passage cultures (<200PD). To our knowledge, this is the first study to report such long-term growth of HBECs without a telomere maintenance mechanism. This report also provides direct evidence of telomerase activation in HBECs near senescence when telomeres are critically short. This novel cell culture system provides an experimental model to understand how telomerase is regulated in normal adult tissues.

Keywords

ROCK inhibitor; conditional reprogramming; low oxygen; senescence; telomerase

Introduction

Telomerase activity is high in embryonic stem cells and during select phases of fetal development (1). However, telomerase is down-regulated upon differentiation to somatic

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Author Contributions

Conception and design: JRP, JM, ET, JWS; Performing experiments: JRP, JM, ET, SS, IM; Analysis and interpretation: JRP, JM, ET, SS, IM, JWS; Drafting the manuscript for intellectual content: JRP, JWS

cells (2). Initially, telomerase activity following birth was only associated with advanced human tumors and cancer cell lines while most somatic tissues tested were telomerase negative (3). Further investigations detected telomerase activity in a subset of fast proliferating normal human cells, including hematopoietic tissues (4–7), skin (8, 9), hair follicles (10), and intestinal mucosa (11). Epithelial cell turnover in the lung occurs at a slower pace in the absence of damage. For example, ciliated tracheal cells have a half-life of six months and ciliated bronchial cells have a half-life of seventeen months in mouse lung (12).

Telomerase is a conserved ribonucleoprotein enzyme complex (13) that uses an RNA template (*TERC*) to reverse transcribe and add TTAGGG_n DNA sequences at mammalian chromosome ends during DNA replication (14, 15). Shelterin, a 6 protein complex, associates or binds to telomeric repeats and protects the ends from being recognized as DNA breaks (16). The important role of telomerase in regeneration of adult tissue is underscored by the manifestation of genetic diseases in individuals with telomere spectrum disorders, ranging from bone marrow failure to idiopathic pulmonary fibrosis (IPF) (17, 18). In fact, IPF is the most prevalent pathology associated with mutations in telomerase and a significant percentage of IPF patients have mutations in *TERT*, *TERC*, or other genes associated with telomere stability (19–22); thus short telomeres are a risk factor for IPF (20). An emerging concept is that IPF results from chronic epithelial damage and defective regeneration, but the cause of damage and the molecular mechanism of impaired regeneration are unknown (23). Critically short telomeres have been shown to impair regeneration of adult stem cells in both humans and mice, resulting in age-related pathologies (24).

Hayflick and Moorhead demonstrated that fetal lung fibroblasts grown in standard cell culture conditions with fetal bovine serum in 21% atmospheric oxygen levels had a limited replicative lifespan *in vitro* of about 50 to 60 population doublings (PD), which became known as the Hayflick limit (25, 26). Since then others have demonstrated that oxygen sensitivity is one of the extrinsic factors limiting the replicative lifespan of both human (27) and murine fibroblasts (28). Shortened telomeres correlated with and appeared to be a hallmark of human fibroblasts cultured *in vitro* for extended passages, but whether shortened telomeres had a causative role in replicative senescence was unknown (29). While a few cell types can be immortalized by just the ectopic introduction of *TERT* (catalytically active and rate limiting component of telomerase) (30), most human cell lines (under typical culture conditions) cannot be immortalized by exogenous *TERT* expression alone (31, 32). Thus, it remains to be determined if the Hayflick limit as originally described for fetal human lung fibroblasts is due to critically shortened telomeres or cell culture shock-induced premature senescence (33).

While telomerase activity has not been previously reported in adult human lung tissue, it may be transiently expressed in basal progenitor cells during injury repair. Recently, we demonstrated that less stressful conditions for long-term expansion of primary human bronchial epithelial basal progenitor cells (HBECS) *in vitro* include co-culturing with an irradiated fibroblast feeder layer, rho-associated coiled coil kinase inhibitor (ROCKi), and 2% oxygen (ROCKi conditions) (34). Differentiated lung epithelial cells are exposed to 21%

atmospheric oxygen *in vivo*, but basal lung stem cells residing near the basement membrane are exposed to much lower oxygen levels. Therefore, low oxygen culture conditions reduce the oxidative stress and DNA damage that occur in standard cell culture conditions that is not representative of *in vivo* conditions (35). Low oxygen tension has also been shown to control proliferation and maintain the undifferentiated state of a variety of stem cells (36). For these reasons, the improved ROCKi conditions were modified from conditional reprogramming conditions as originally described (37). In the present studies, we observed that HBECs in ROCKi conditions exhibit an extended lifespan compared to HBECs in standard culture conditions without engaging a telomere maintenance mechanism, and the population of cells senesce when telomeres reached a critically short telomere length but a rare stem-like cell can engage a telomere maintenance mechanism, such as reactivating low levels of telomerase.

Materials and Methods

Experimental Design

The objectives of this study were to test if HBECs in reduced stress culture conditions, with an irradiated fibroblast feeder layer and a ROCK inhibitor in low oxygen (ROCKi conditions), would exhibit an extended lifespan compared to HBECs serially passaged in parallel in standard culture conditions. HBECs in ROCKi conditions were serially passaged long-term to test whether HBECs would senesce when a critically short telomere length was reached, or engage a telomere maintenance mechanism. In the beginning, HBECs were grown at high density (10,000 cells/cm²) in ROCKi conditions until reduced growth rate and senescent cells were observed (PD 214). Then, HBECs were grown at clonal density in ROCKi conditions to select for cells with increased growth capacity. Overtime, growth rate, telomere length, chromosomal stability, and senescent and stem cell markers were measured. HBECs were also tested overtime for engagement of alternative lengthening of telomeres (ALT) or telomerase activity as telomere maintenance mechanisms.

3T3 J2 cell culture

The 3T3 (J2 strain) Swiss mouse fibroblast cell line was purchased from Tissue Culture Shared Resource (TCSR), Lombardi Comprehensive Cancer Center, Georgetown University and tested negative for mycoplasma. The cell line does not produce murine viruses and was irradiated at 30 Gray (Gy) with gamma radiation to provide an irradiated fibroblast feeder layer.

HBECs co-cultured with irradiated 3T3 J2 feeder cells

Primary HBECs were obtained through a material transfer agreement from The CF Center Tissue Procurement and Cell Culture Core at The University of North Carolina, Chapel Hill, Marisco Lung Institute which were procured under the University of North Carolina Office of Research Ethics Biomedical Institutional Review Board. The HBECs in this study were harvested from the tracheobronchial airways of a 21-year-old male who died from head trauma and tested negative for mycoplasma. Primary HBECs were co-cultured with irradiated 3T3 J2 feeder cells with ROCK inhibitor and 2% O₂ (ROCKi conditions) as described previously (34). Briefly, freshly irradiated (30 Gy) 3T3 J2 cells and primary

HBECs were seeded in a 1:1 ratio (10,000 cells/cm² each) on uncoated dishes in Bronchial Epithelial Growth Medium (BEGM) (Lonza, Walkersville, MD, USA) supplemented with 5% FBS and 10 μ M ROCK inhibitor Y-27632 (Enzo Lifesciences, Farmingdale, NY, USA). Co-cultures were maintained in trigas chambers with 2% O₂, 7% CO₂, and 91% N₂ at 37°C. Population doublings (PD) were calculated as follows: co-cultures were washed with 0.02% EDTA in PBS for 5 min at 37°C to remove fibroblasts followed by a brief wash with PBS before trypsinization. HBECs were single cell suspended and counted with a TC20 automated cell counter (BioRad, Hercules, CA, USA). The number of PDs gained at each passage was calculated with the following equation: $n = \log(N_H/N_I)/\log_2$, where N is the number of PDs, N_H is the number of cells harvested at the end of the growth period, and N_I is the initial number of cells seeded. Gained PDs were cumulatively added at each passage and plotted versus time in the growth curve figures.

Preparing 3T3 conditioned media

3T3 J2 irradiated (30 Gy) fibroblasts were seeded in 15 cm fibronectin (Sigma-Aldrich, St. Louis, MO, USA) coated dishes at 10,000 cells/cm² with 30 mL of BEGM. Media was conditioned for 72 h before sterile filtering and freezing aliquots at -80°C.

Terminal Restriction Fragment (TRF) assay (telomere length)

DNA was isolated from 1–2×10⁶ cell pellets using the manufacturer's instructions (Qiagen, Valencia, CA, USA). Three to 5 μ g DNA was digested with 2 different restriction enzymes (HinfI and RsaI) (New England Bio, Ipswich, MA, USA) and incubated at 37°C overnight. Digested DNA was separated on a 0.85% agarose gel overnight at 53 V for 21 h. The telomere signals were detected by Southern blot analysis as previously indicated (38). In brief, after DNA was transferred from gel to a positive-charged nylon membrane, the transferred DNA was fixed by UV crosslinking. The cross-linked membrane was then hybridized with the hypersensitive DIG-labeled telomere probe overnight at 42 °C. After hybridization, the membrane was washed with buffer 1 (2X SSC, 0.1% SDS) at room temperature for 15 min and then washed twice with buffer 2 (0.5X SSC, 0.1% SDS) at 55 °C for 15 min. Next, the membrane was washed with DIG wash buffer (1X maleic acid buffer with 0.3% Tween-20) for 5 min. Then the membrane was incubated with 1X DIG blocking solution for 30 min at room temperature. After blocking, the membrane was incubated with anti-DIG antibody (Roche, Basel, Switzerland) in 1X blocking solution (1 to 10,000 dilution) for 30 min at room temperature. The membrane was then washed with DIG buffer (Roche) two times at room temperature for 15 min. After washing, telomere signals were detected by incubating with CDP-Star (Roche) for 5 min.

Telomere Shortest Length Assay (TeSLA)

The TeSLA assay was performed as described in detail previously to measure the average and the shortest telomere lengths (39).

Collection of chromosome spreads

HBECs were treated with 5 μ M colchicine (Sigma-Aldrich) and incubated for 12 hours at 37°C to enrich the fraction of mitotic cells. Successively, cells were collected and re-

suspended in 75 mM KCl hypotonic solution (Sigma-Aldrich) for 20 min at 37°C, followed by fixation in Carnoy solution (3:1 v/v Methanol/Acetic Acid) (Fisher Scientific, Hampton, NH, USA). Finally, cells were dropped onto slides and air dried.

Ploidy analysis

Ploidy was evaluated performing chromosome count. Cells treated with colchicine and fixed as reported in the previous section, were stained and mounted 4',6-diamidino-2-phenylindole (DAPI)/Vectashield (Vector Laboratories, Burlingame, CA, USA). Metaphase pictures were captured with Axiovert 200M Fluorescent Microscope (Carl Zeiss, Oberkochen, Germany) and images (100 metaphases for each sample) analyzed with ImageJ v1.52a.

Telomeric qualitative fluorescent in situ hybridization (FISH)

HBEC cells were fixed and seeded as described in the previous section (Collection of chromosome spreads). After 48 h, slides were rinsed in 1X PBS for 15 min and fixed in formaldehyde 4% (Sigma-Aldrich) in PBS for 2 min. Successively, slides were rinsed in 1X PBS and incubated in acidificate pepsine solution for 10 min at 37°C, then washed in 1X PBS. The slides were fixed again in formaldehyde 4% in PBS for 2 min, washed in 1X PBS two times and dehydrated in 70, 90 and 100% ethanol (Fisher Scientific) for 5 min each and air dried. The slides were denatured for 3 min at 80°C with 20 µL of hybridization mixture contained 70% deionized formamide, 1M Tris pH 7.2, 8.56% buffer MgCl₂, 5% maleic blocking reagent and 25 µg/ml Cy3-conjugated PNA Tel-C (CCCTAA)₃ probe (PNA Bio Inc., Thousand Oaks, CA, USA) and incubated overnight at 4°C in a humid chamber. Slides were washed two times for 15 min in wash solution containing 70% formamide, 10mM Tris pH 7.2, 0.1% BSA and washed three times for 5 min in a solution containing 0.1 M Tris pH 7.5, 0.15 M NaCl and 0.08 % Tween-20. The slides were dehydrated by ethanol series, air-dried and counterstained with Vectashield/DAPI (Vector Laboratories, Burlingame, CA). Images were acquired at 100X magnification using an Axiovert 200M Fluorescent Microscope (Carl Zeiss, Oberkochen, Germany).

ImmunoFISH staining

HBEC cells were seeded at 1X10⁵ density on slides previously sterilized with ethanol 100%. After 48 h, slides were rinsed in 1X PBS for 5 minutes and then fixed in 4% formaldehyde in PBS at 4°C for 20 min. Slides were permeabilized with 0.1% Triton™ X-100 (Sigma-Aldrich) in PBS at room temperature for 10 min and blocked with 10% bovine serum albumin in PBS at 37°C for 30 min. Cells were then incubated with the mouse primary anti-promyelocytic leukaemia (PML) antibody (PG-M3; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:100 in PBS for 3 h at room temperature. After washing with 0.05% Triton™ X-100 in PBS for 5 min, cells were incubated with a secondary goat anti-mouse IgG conjugated with Alexa 488 (Invitrogen, Carlsbad, CA, USA) diluted 1:300 in PBS for 1 h at room temperature. After immunostaining, telomeric FISH was performed as described in "Telomeric Qualitative FISH" section. Pictures were taken at 100X magnification with multiple Z-stack (5–10) at 0.4 µm intervals using an Axiovert 200M Fluorescent Microscope (Carl Zeiss). Images were analyzed with ImageJ v1.52a.

C-circle assay

The C-circle assay was performed as described previously (40). Briefly, Hinf1/Rsa1/Alu1-digested genomic DNAs were incubated with phi29 polymerase (New England Biolabs, Ipswich, MA, USA) and reaction buffer (0.75 U of phi29 polymerase, 0.1 mg/ml bovine serum albumin, 0.2 mM deoxynucleoside triphosphate mix, and 1× phi29 buffer) at 30°C for 12 h and then at 65°C for 20 min. Samples were loaded into slot blots and then hybridized with ³²P-labeled C-probe (CCCTAA)₃ under native conditions to measure the amplified C-circle level.

Droplet Digital TRAP assay (telomerase activity)

Quantitation of telomerase enzyme activity was performed as described previously (41).

CRISPR/Cas9

A single guide RNA (sgRNA) targeting exon 1 of wild-type hTERT, sequence GCGGGGAGCGCGCGGCATCGCGG, was inserted into the pSpCas9(BB)-2A-GFP (PX458) plasmid expressing Cas9 and GFP. PX458 was a gift from Feng Zhang (42) (Addgene plasmid #48138). ROCKi HBECs (PD 14 or PD 324) were seeded at 3×10³ cells per 8 cm² well of a 6-well plate 24 hours before transfection with Transfex (ATCC) and 2 µg of PX458 plasmid with sgRNA insert. GFP⁺ HBE cells (highest expressing 5%) were sorted by fluorescence-activated cell sorting (FACS) to 96-well plates 48 hours post-transfection; each receiving well of a 96-well plate contained BEGM +5% FBS +10 µM Y-27632 +1×10³ irradiated 3T3 J2 fibroblasts.

6-thio-2'-deoxyguanosine treatment

For treatment at high density, HBECs were seeded in triplicate at 30,000 cells per well of a 6-well plate with 8×10⁴ irradiated 3T3s, in BEGM with 5% FBS and 10 µM ROCK inhibitor and incubated in a 5% CO₂ incubator at 37°C for 7 days. Cells were treated with 3 µM 6-thio-deoxyguanosine or DMSO/water (1:1) control 24 h after seeding and then every 3 days. On the final day, cells were counted and reseeded for further treatment. For treatment at clonal density, HBECs were seeded in triplicate at 100 cells per well of a 6-well plate with 8×10⁴ irradiated 3T3s, in BEGM with 5% FBS and 10 µM ROCK inhibitor and incubated in a 5% CO₂ incubator at 37°C for 10 to 14 days. Cells were treated with 3 µM 6-thio-deoxyguanosine (Metkinen Oy, Kuopio, Finland) or DMSO/water (1:1) control 24 h after seeding and then every 3 days. On the final day, media was aspirated and cells fixed/stained with 6% glutaraldehyde/0.5% crystal violet (Sigma-Aldrich) for 1 h at room temperature with rocking. Dishes were rinsed with warm H₂O until clear and the resulting clones in each condition were imaged and counted.

β-galactosidase staining

A senescence β-galactosidase staining kit (Cell Signaling, Danvers, MA, USA) was used to perform staining for β-galactosidase activity at pH 6, a biomarker of senescent human cells (43).

Immunofluorescence staining

HBECs spanning low to high PD were cultured on Nunc™ Lab-Tek II™ 4-well chamber slides™ (ThermoFisher, Waltham, MA, USA) and fixed with 4% paraformaldehyde before incubating with blocking buffer (1X PBS, 0.3% Triton X-100, 5% normal goat serum) (Jackson ImmunoResearch, West Grove, PA, USA). Blocked cells were incubated with primary antibodies diluted in blocking buffer against keratin 5 (Ker5) (Abcam, Cambridge, UK) and NP63 (Biolegend, San Diego, CA, USA) overnight at 4°C. The wells were washed three times with 1X PBS, incubated with species specific Alexa Fluor conjugated secondary antibody (Thermo Fisher) for 1 hour at room temperature, washed three times with 1X PBS, and counterstained with Vectashield/DAPI (Vector Laboratories). Images were acquired at 20X magnification using an Axiovert 200M Fluorescent Microscope (Carl Zeiss).

Data analysis.

A student's paired *t* test with a two-tailed distribution was used to analyze significance. P-values less than 0.05 were considered significant. The graphs and statistics were generated with Microsoft Excel 2010 software.

Results

ROCKi conditions extend the lifespan of human bronchial epithelial basal progenitor cells and telomeres are critically short at about 200 population doublings.

ROCKi conditions supported the *in vitro* culture of primary human bronchial epithelial basal progenitor cells (HBECs) beyond 200 population doublings (Figure 1A, blue line). When HBECs were switched from less stressful ROCKi conditions back to standard growth media and 2% oxygen around PD 70 (red line) or PD 200 (green line), cells only proliferated for a few passages before undergoing cell culture stress-induced premature senescence. This indicates that normal cell cycle checkpoints are intact and are activated in more stressful culture conditions. In addition, we have previously reported (34) that growth of HBECs in the presence of ROCKi and feeder layer in 21% oxygen results in senescence of HBECs at PD 80, underscoring the necessity of 2% oxygen along with ROCKi and feeder layer for the long-term life-extension exhibited by HBECs in ROCKi conditions. Measurement of telomere length over time (Figure 1B) shows gradual shortening of telomeres up to PD 214. Mouse 3T3 J2 lethally irradiated feeder fibroblasts are telomerase positive with long telomeres and were depleted for telomere length and telomerase assays by culturing HBECs for two passages in filtered media conditioned by the feeder layer. To get an accurate measurement of the shortest telomeres which closely correlate with cellular senescence (44, 45), the telomere shortest length assay (TeSLA) was employed. TeSLA quantifies the length of all the shortest telomeres that are below detection using other telomere measurement methods (39). TeSLA showed a gradual shortening of telomere length up to PD 214, at which point the average telomere length was ~1 kb with about 86% of telomeres below 1.6 kb (Figure 1C). In standard growth conditions, primary HBECs only proliferate for 30 to 40 PD (34, 46), at which point telomeres are still well above an average of 4 kb. Therefore, the growth arrest of primary HBECs in standard growth conditions is likely due to stress-induced premature senescence rather than critically short telomeres.

Human bronchial epithelial basal cells engage a telomere maintenance mechanism upon reaching critically short telomere lengths.

At PD 214, the growth rate of ROCKi HBECs slowed down and the entire population of cells appeared to be undergoing telomere-induced replicative senescence as demonstrated by increasing numbers of large and β -galactosidase positive cells at pH 6, a biomarker of senescent human cells (43) (Figure 2A,B). Prior to terminating the experiment, HBECs were seeded at clonal density in ROCKi conditions to partially phenocopy a lung injury to determine if there were any rare cells with increased growth capacity within the remaining cell population. Interestingly, we observed that about 15% of the cells seeded were able to grow into robust colonies that could be serially passaged (Figure 2C). Telomere length of ROCKi HBEC colonies measured by TRF (Figure 3A) and TeSLA (Figure 3B), showed a gradual increase in telomere length between PD 240 and PD 335 that then plateaued and was maintained at an average of 1.7 kb for an additional 60 PDs, indicating HBECs activated a telomere maintenance mechanism. The change in telomere length over time measured by TRF and TeSLA was supported by telomere fluorescent *in situ* hybridization (FISH) on metaphase spreads of ROCKi HBECs from low (PD 23), mid (PD 230), and high PD (PD 390) (Figure 3C). Prior to subculturing HBECs at clonal density at PD 214, there was no evidence for subsets of cells with longer telomeres as determined by TeSLA analyses (Figure 1C) and telomere *in situ* hybridization (data not shown).

Recently, a rare stem cell in the distal lung has been identified that co-expresses keratin 5 (Ker5) and Np63 (a p63 splice variant) and is essential for lung regeneration (47, 48). Characterization of ROCKi HBECs pre- and post- clonal expansion showed positive immunofluorescent staining for both of the lung progenitor cell markers at low (PD 28), mid (PD 236), and high PD (PD 429) (Figure 4). Ker5 is a cytosolic structural protein which localizes to the cytoplasm throughout passages, as expected (Figure 4A-C). In low PD HBECs pre-cloning, the transcription factor Np63 primarily localized to the cytoplasm (Figure 4D) while at mid PD post-cloning, a subset of ROCKi HBECs exhibited Np63 localization in the nucleus (Figure 4E). By late PD, HBECs serially cloned for ~200 PD exhibited complete localization of Np63 in the nucleus (Figure 4F). P63, a member of the p53 family, is a transcription factor with multiple isoforms; the N isoforms are antagonistic of p53. In addition, p63 is essential for epithelial stem cell proliferation (49) and its nuclear transport has been shown to enhance proliferation and abrogate differentiation in squamous cell carcinoma (50). Nuclear localization of Np63 indicates that not only is it expressed by the HBECs, but its cellular localization could facilitate changes in gene expression.

To investigate the telomere maintenance mechanism employed by HBECs in ROCKi conditions after clonal expansion, we first tested for the telomerase-independent mechanism of alternative lengthening of telomeres (ALT). The C-rich circular DNA characteristic of cells employing ALT was not detected in low (PD 65), mid (PD 214), and high PD (PD 411) ROCKi HBECs (Figures 5A). Also, ALT associated promyelocytic leukaemia (PML) bodies, a hallmark of most ALT cells (51), did not co-localize with telomeres (Figures 5B,S1A,B). Finally, the absence of ALT was further supported by the lack of long and heterogeneous telomeres (Figures 3A,B) (51). To rule out that the observed replicative lifespan extension was due to chromosomal end-fusions, we performed Bal 31 digestion,

which is a highly specific single/double-stranded exonuclease that digests telomere ends, which would be protected from digestion if end-to-end fusions occurred (Figure 5C). Bal31 digestion resulted in complete degradation of telomere DNA, indicating there were no chromosome end-fusions “protecting” telomeres. Additionally, telomere fusions were not observed in late passage HBECs using pancentromere-telomere FISH (data not shown) and chromosome counts in ROCKi HBECs (Figure 5D).

Low levels of telomerase are activated in human bronchial epithelial basal cells with critically short telomeres.

We next tested if telomerase activation could be detected after PD 214. Droplet digital PCR quantitation of telomerase activity using the telomere repeat amplification protocol (ddTRAP) for HBECs at PD 314 indicated low, but above background levels, of telomerase activity compared to cells at PD 32 and 235 (Figure 6A), suggesting telomerase may be transiently expressed in a subset of cells. To investigate whether the low levels of telomerase activity were functional, we transfected PD 317 and PD 437 HBECs with PX458 CRISPR/Cas9 GFP⁺ plasmid expressing guide RNA to knockout the *TERT* gene. CRISPR transfection resulted in the growth of less than 1% of cells sorted by FACs to four 96-well plates compared to untransfected cells with about 38% colony growth (Figure 6B). In an independent experiment, PD 14 (long telomeres) and PD 324 (short telomeres) HBECs were also transfected with PX458 CRISPR/Cas9 expressing guide RNA to knockout *TERT*. CRISPR transfection resulted in about 80% reduced growth of colonies in PD 324 HBECs but not PD 14 HBECs (Figure S2). If late passage cells did not have telomerase we would have expected similar colony formation to early passage HBECs. To further test the role of telomerase in late PD ROCKi HBECs, we treated HBECs with the telomerase mediated telomere targeting agent 6-thio-2'-deoxyguanosine (6-thio-dG), which has been shown to induce rapid telomere dysfunction and cell death in telomerase positive cells, but not telomerase negative cells (52). Treatment of PD 253 and 263 HBECs with 6-thio-dG for 3 passages resulted in greatly reduced colony size and number (Figure 6C and D).

Discussion

The results of both the CRISPR and 6-thio-dG experiments demonstrate low density cloning of ROCKi HBECs after PD 214 selects for cells that can engage low levels of telomerase activity analogous to injury repair in the lung, but only at higher passage number when telomeres are critically short. We previously reported the human *TERT* gene is very close to a telomere end on chromosome 5p and that *TERT* can be transcriptionally activated when telomeres are short using a mechanism termed telomere looping or telomere position effects over long distances that may explain the activation of telomerase in these cells at late PD (53). Other adult epithelial tissues traditionally accepted to be telomerase silent may share a similar mechanism for upregulating telomerase activity at old age when telomeres reach a critically short length. Importantly, when combined with oncogenic mutations, activation of telomerase at old age (with short telomeres) in epithelial tissues may support cancer progression.

In summary, we have developed methods for maintaining normal human lung basal progenitor cells in culture long-term by mimicking *in vivo* conditions and reducing cell culture stress through co-culture with an irradiated fibroblast feeder layer and supplementation with ROCK inhibitor in low (2%) oxygen (ROCKi conditions). These modified conditional reprogramming conditions allowed for proliferation of human lung basal progenitor cells well past the 30 to 40 population doublings previously reported *in vitro* (46, 54), a point at which HBECs still possess relatively long telomeres. We suggest that senescence of cultured cells results from both extrinsic and intrinsic factors. One set of senescence triggers is the extrinsic culture environment that can be initiated by a multitude of disparate stress factors, while the second set of senescence triggers is intrinsic and depends on the machinery that monitors telomere length (33). In addition, as recently reviewed (55), the senescence research field would benefit from clearly defining more specific descriptors of the term cellular senescence.

The ROCKi conditions in this report supported an *in vitro* cell culture model of lung progenitor cell aging that was not previously available. Combined with cloning, analogous to lung injury *in vivo*, ROCKi conditions allowed for the observation that rare lung progenitor cells activate low levels of telomerase when telomeres are critically short. Cloning also selected for HBECs co-expressing Ker5 and Np63, markers of rare stem cells in the distal lung essential for lung regeneration (47, 48). This novel cell culture system provides an experimental model to understand how telomerase is regulated in normal adult tissues. While previous studies have associated telomerase mutations or short telomeres with pulmonary fibrosis and impairment of alveolar stem cell function, this study is the first to report direct evidence of telomerase activity in human lung progenitor cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations

6-thio-dG	6-thio-2'deoxyguanosine
ALT	alternative lengthening of telomeres
BEGM	bronchial epithelial growth medium

ddTRAP	droplet digital telomere repeat amplification protocol
FACS	fluorescence-activated cell sorting
FISH	fluorescent <i>in situ</i> hybridization
Gy	Gray
HBECs	human bronchial epithelial basal progenitor cells
hTERT	human telomerase reverse transcriptase
IPF	idiopathic pulmonary fibrosis
IRJ2	irradiated J2 fibroblasts
Ker5	keratin 5
PD	population doublings
PML	promyelocytic leukaemia
ROCKi	rho-associated coiled coil kinase inhibitor
sgRNA	single guide RNA
TeSLA	telomere shortest length assay
TRF	telomere restriction fragment

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Figure 1. Primary HBECs in co-culture with irradiated fibroblasts in the presence of ROCK inhibitor and 2% oxygen (ROCKi conditions) exhibit extended lifespan beyond 200 population doublings (PD), about four times the Hayflick limit.

A) Growth curves of primary HBECs serially passaged with ROCK inhibitor (ROCKi) and irradiated J2 fibroblasts (IRJ2) in 2% oxygen (blue line) or switched to BEGM in 2% oxygen without ROCK inhibitor starting at PDs 70 and 200 (red and green lines). B) TRF and C) TeSLA measurements show HBECs cultured long-term in ROCKi conditions exhibit progressively shorter telomere length up to PD 214.

Figure 2. Assessment of morphology and senescence in ROCKi HBECs over time.

A) Comparison of phase contrast images of PD 32 (left) and PD 159 (right) ROCKi HBECs show increases in subsets of enlarged cells after PD 100. B) A subset of PD 161 ROCKi HBECs show pH-dependent β -galactosidase staining (blue). C) PD 250 HBECs were seeded at 666 cells/10cm² dish in triplicate in ROCKi conditions for 7 days prior to fixation/Crystal violet staining. The average number of colonies formed compared to cells seeded was about 15%; Avg: average $\pm$ standard deviation

Figure 3. Cloning of primary ROCK1 HBECs beyond 200 PD selects for a subpopulation of cells with a telomere maintenance mechanism.

A) TRF and B) TeSLA measurements show lengthening of telomeres between PD 240 to 335 which then plateaued and was maintained at about 1.7 kb until PD 391. C) Telomere FISH on metaphase spreads at low (PD 23), mid (PD 230), and high PD (PD 390) support measurement of extremely short telomeres (signal free ends) at mid PD and slightly lengthened telomeres at late PD. TelC: C-rich telomere probe

Figure 4. ROCKi HBECs immunostain for both of the lung progenitor cell markers keratin 5 (Ker5) and NP63.

(A-C) Ker5 is a cytosolic structural protein which localizes to the cytoplasm throughout passages, as expected. (D-F) NP63 is a transcription factor that primarily localizes to the cytoplasm at low PD (PD 28) while at mid PD (PD 236) a subset of ROCKi HBECs exhibit NP63 localization to the nucleus; NP63 localization is localized to the nucleus in most cells at late PD (PD 429).

Figure 5. ROCKi HBECs do not exhibit alternative lengthening of telomeres (ALT) as a telomere maintenance mechanism but maintain chromosome stability.

A) The C-rich circular DNA characteristic of cells using ALT was probed for by Southern blot in low (PD 65), mid (PD 214), and high PD (PD 411) HBECs compared to telomerase positive (TEL+) 293FT cells and ALT positive (ALT+) Saos2 cells. (-): water only control

B) PD 420 HBEC nuclei in interphase were co-immunostained for PML bodies (green) and telomeres (red) with C-rich probe (TelC) and counterstained with DAPI. No ALT-associated PML bodies were visualized.

C) TRF analysis of PD 65 and PD 353 ROCKi HBECs without (0hr) or with (1.5hr, 3hr) Bal31 pre-digestion show no telomeric DNA in lanes that were

protected from Bal31 digestion by end fusions. D) Chromosome counts of 100 metaphase spreads per PD demonstrate a diploid karyotype with the vast majority being 46 chromosomes at low (PD 28) and mid PD (PD 235), while late PD (PD 392) HBECs after cloning exhibit increased ploidy to 47 chromosomes in 43% of the cell population.

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Figure 6. Late Passage (PD 314) ROCKi HBECs exhibit low levels of telomerase and targeting telomerase by CRISPR TERT knockout or treatment with a telomerase mediated toxic compound inhibits clonal growth.

A) Droplet digital PCR for TRAP (ddTRAP) activity of PD 32, 235, and 314 ROCKi HBECs; TRAP positive control cell line (Pos CTL) is 293FT. B) Transfection of PD 317 ROCKi HBECs with CRISPR/Cas9 PX458 plasmid to knockout hTERT results in the growth of 1 colony out of 384 (0.3%) cells sorted by FACs to four 96-well plates compared to untransfected cells with 38.5% colony growth. PD 417 ROCKi HBECs transfected with CRISPR/Cas9 hTERT plasmid also resulted in the growth of 1 colony out of 384 (0.3%) compared to untransfected cells with 37.5% colony growth. Black inset bars = $\pm$ standard deviation C) Treatment of ROCKi HBECs with 3 μ M 6-thio-dG for 3 passages at clonal density inhibits colony formation compared to control as demonstrated by reduced colony size and number observed in PD 263 colonies after crystal violet staining. Black inset bars = $\pm$ standard deviation; *P<0.05