

hTERT Extends the Life of Human Fibroblasts without Compromising Type I Interferon Signaling

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Abstract

Primary cells are often used to study viral replication and host-virus interactions as their antiviral pathways have not been altered or inactivated; however, their use is restricted by their short lifespan. Conventional methods to extend the life of primary cultures typically utilize viral oncogenes. Many of these oncogenes, however, perturb or inactivate cellular antiviral pathways, including the interferon (IFN) response. It has been previously shown that expression of the telomerase reverse transcriptase (*TERT*) gene extends the life of certain cell types. The effect that *TERT* expression has on the innate antiviral response to RNA- and DNA-containing viruses has not been examined. In the current study, we introduced the human *TERT* (*hTERT*) gene into a primary human embryonic lung (HEL-299) cell strain, which is known to respond to the type I IFN, IFN- β . We show that the resulting HEL-TERT cell line is capable of replicating beyond 100 population doublings without exhibiting signs of senescence. Treatment with IFN- β resulted in the upregulation of four model IFN stimulated genes (ISGs) in HEL-299 and HEL-TERT cells. Both cell lines supported the replication of herpes simplex virus type 1 (HSV-1) and vesicular stomatitis virus (VSV) and impaired the replication of both viruses upon IFN- β pretreatment. Introduction of the viral oncoprotein, simian virus 40 (SV40) large T-antigen, which is frequently used to immortalize cells, largely negated this effect. Taken together, our data indicate that expression of hTERT does not alter type I IFN signaling and/or the growth of two viruses, making this cell line a useful reagent for studying viral replication and virus-cell interactions.

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Introduction

In performing studies that examine cellular immune responses to viral infections, it is often necessary to work with primary cells, as the efficacy of intrinsic and innate immune pathways are frequently diminished in immortalized cells [1–4]. One disadvantage of using primary cells is their limited proliferative capacity in cell culture, which is due in part, to the progressive shortening of telomeres [5].

Telomeres are repetitive nucleoprotein structures that serve to cap the ends of chromosomes, facilitating their replication, and prevent their ends from appearing as DNA breaks [6]. Telomeres are maintained by a complex known as telomerase, whose essential core consists of the catalytic subunit telomerase reverse transcriptase (*TERT*) and the telomerase RNA template component (*TERC*) [7,8]. Along with a number of other factors, *TERT* is loaded onto the 3' overhang of existing telomeric DNA and utilizes *TERC* as a template to add repeats of a guanine-rich sequence, 5'TTAGGG3', in all vertebrates; concordantly, DNA primase and DNA polymerase are recruited to the new telomeric repeats, subsequently synthesizing the complementary 5' strand [6]. In the absence of active telomerase, erosion of the telomeres occurs with each successive round of replication, resulting in the loss of telomeric (~100 bps) sequence [9,10]. Once telomeres are

reduced from their normal 15 kb length to ~4 kb, DNA damage sensors trigger p53- and pRb-dependent mechanisms that result in cellular senescence, inducing a G₁ cell cycle arrest [11].

Replicative senescence is thought to be a mechanism of cellular lifespan regulation, preventing diseases such as cancer, and is intrinsic to the health of an organism [12–15]. However, for technical reasons it can be desirable to extend the proliferative capacity and prevent the senescence of a primary cell culture or strain. One way to avoid or reverse replicative senescence is transformation with viral oncogenes, such as the simian virus 40 (SV40) large T antigen (TAg) or the human papillomavirus (HPV) E6 and E7 proteins [16–18]. In both cases, these viral proteins reverse senescence through the inactivation of p53 and/or pRb. While this allows cells to resume progression through the cell cycle and replicate, these cells still undergo telomeric erosion and ultimately undergo a phenomenon termed crisis [16], where massive cell death occurs due to gross genomic rearrangements and instability in the absence of telomeres. While the estimated 1 in 10⁷ cells (for human cells) that survive crisis exit immortalized [19,20], this transformation results in the dysregulation of several cellular pathways, including the antiviral type I interferon (IFN) response [1].

The IFN response is an innate antiviral pathway that, upon detection of viral molecular patterns, results in the production and release of the cytokine and type 1 IFN, IFN- β [21–23]. IFN- β binds to its cognate receptor in both an autocrine and paracrine manner, activating a signal transduction cascade that ultimately upregulates numerous interferon-stimulated genes (ISGs), which function to limit viral replication. The IFN response serves as a major restriction point for many viruses as evidenced by the increased pathogenesis of these viruses in animal models in which either the type I IFN receptor, IFNAR, or a key signaling molecule, STAT1, are deleted [24–30]. One example is herpes simplex virus type-1 (HSV-1), a large double-stranded DNA-containing virus that is estimated to infect 70–90% of adults [31]. Notably, HSV-1 encodes for viral proteins that inactivate or delay this IFN response [32,33]. Studies examining how HSV-1 counteracts the IFN response are often performed in primary cultures or cell strains, such as human embryonic lung (HEL) cells, because these cells possess a robust IFN response and phenotypes that are apparent in HEL cells are often greatly diminished in transformed lines [1,34]. A potential drawback with using HEL cells is their rapid progression into senescence.

As part of their differentiation program, human cells cease expressing hTERT, while continuing to produce other essential telomerase subunits such as TERC [35]. It has been shown by a number of labs that the lifespan of fibroblasts is efficiently extended by the reintroduction of hTERT into these cells [36,37]. Exogenous expression of hTERT presumably allows terminally differentiated fibroblasts to resume the extension of their telomeres, delaying or avoiding the production of signals that trigger replicative senescence and in turn prevents the chromosomal damage encountered by replication through crisis [38]. Unlike transformation with viral oncogenes, fibroblasts that exogenously express hTERT do not, for the most part, exhibit an oncogenic phenotype [39]. Notably, the effect that life-extension by exogenous expression of hTERT on innate antiviral pathways, and in particular the IFN response, has not been examined.

Here we report the creation of a life-extended HEL cell line via transduction of a human diploid primary-like cell strain, HEL-299, with a retrovirus encoding *hTERT*. HEL-299s were chosen as a parental cell line since they are both capable of supporting high levels of HSV-1 and VSV replication and retain a strong innate immune restriction of viral replication [40,41]. Our results show that the derivative cell line, HEL-TERT, unlike the parental cells, replicated to at least 100 population doublings, exhibited telomerase activity, and failed to undergo either replicative senescence or crisis. Morphologically, HEL-TERT cells appeared indistinguishable from HEL-299 cells. HEL-TERTs responded to IFN- β by upregulating representative ISGs and supported the replication of HSV-1 and VSV to similar levels as HEL-299 cells. Additionally, the introduction of the SV40 large TAg counteracted the IFN- β -directed restriction of HSV-1 and VSV replication. In summary, our data indicate that hTERT extends the replicative potential of human fibroblasts while not perturbing the type 1 IFN response, making these cells a valuable tool in virological and virus-cell interaction studies.

Materials and Methods

Cells and Viruses

HEL-299 cells from the American Type Culture Collection (CCL-137), HEL telomerase life-extended (HEL-TERT), and HEL-TERT SV40 large TAg transformed (HEL-TERT-T) cells (the latter two of which were created as part of this work, as

detailed below) were maintained in Minimum Essential Medium Eagle Alpha Modification (α MEM) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 10 U/mL penicillin, and 10 U/mL streptomycin. In addition, HEL-TERT cells were kept under drug selection using hygromycin-B (Sigma) at 50 μ g/mL while HEL-TERT-T cells were maintained under selection with hygromycin-B at 50 μ g/mL and phleomycin at 10 μ g/mL. HeLa, GP2-293, Vero, and L7 (Vero cells that contain the *ICP0* gene [42]) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5% FBS, 2 mM L-glutamine, 10 U/mL penicillin, and 10 U/mL streptomycin.

HEL-299 (passage 4) cells were transduced with the retroviral vector, pMX-hTERT-hygro vector. pMX-hTERT-hygro was created by subcloning the *hTERT* (catalytic subunit of human telomerase) and hygromycin resistance genes from the vector, pBABE-hygro-hTERT [43] (Addgene plasmid 1773), into the retroviral vector, pMX-GFP [44]. A control vector, pMX-dTERT-hygro, was created by excising a BamHII fragment, which removes the N-terminal 849 residues of hTERT (Uniprot: O14746) [45] (including the TERC-interaction and most of the reverse-transcriptase domains), from pMX-hTERT-hygro. Retroviral stocks were generated using the Pantropic Retroviral Expression System (Clontech) as recommended by the manufacturer. HEL-299 cells were transduced with filtered retroviral stocks and two days later placed under selection with hygromycin B at 100 μ g/mL, which was lowered to 50 μ g/mL 7 days later for subsequent culturing. HEL-TERT SV40 large TAg-expressing cells were created by transduction with the vector, pLVX-LgT-zeo. pLVX-LgT-zeo was created by subcloning the CMV promoter, SV40 TAg ORF, SV40 early promoter, and zeomycin resistance genes from pBABE-zeo largeTcDNA [46] (Addgene plasmid 1779) into the lentiviral vector, pLVX-AcGFP-N1 (Clontech), replacing the region containing the CMV promoter, AcGFP ORF, phosphoglycerate kinase promoter, and puromycin resistance genes. Lentiviral stocks were prepared essentially as described above for pMX-hTERT-hygro with the inclusion of the lentiviral packaging vector, psPAX2 (Addgene plasmid 12260) during lentiviral stock preparation. HEL-TERT cells were transduced with filtered lentiviral stocks and two days later placed under selection with phleomycin (Invivogen) at 20 μ g/mL for 42 days, which was lowered to 10 μ g/mL for long term culturing.

KOS was the wild type strain of HSV-1 used in our viral experiments [47]. 7134 is an ICP0-null mutant HSV-1 strain in which the *E. coli lacZ* gene has replaced the *ICP0* open reading frame [48]. KOS and 7134 were grown on Vero cells and titered on Vero or L7 cells, respectively [49,50]. The vesicular stomatitis virus recombinant, VSV-eGFP, contains the enhanced green fluorescent protein gene between the G and L genes [51] and was a gift from Dr. Asit Pattnaik. VSV-eGFP stocks were grown and titered on Vero cells. Sendai virus (SeV, Cantrell strain) was purchased from Charles River Laboratories.

β -galactosidase Staining

To detect senescence, HEL-299, and moderate and high passage HEL-TERT cells were plated at 1×10^5 cells per well in 12 well plates and grown to confluence. The cells were fixed in 3.7% formaldehyde, washed twice with $1 \times$ phosphate buffered saline (PBS), and stained for β -galactosidase activity as previously described [52]. Cells were viewed with a Nikon Eclipse TE2000-U microscope and photographed with a digital camera (Canon).

Life-Extension Characterization

Low passage HEL-299 and HEL-TERT cells were plated in 60 mm dishes at $1-2 \times 10^3$ cells per dish. Prior to reaching confluence,

the cells were trypsinized, counted with a hemocytometer, and replated at the above-mentioned amount. This was repeated until cells reached senescence and died. Using cell counts and days in culture, the population doublings were determined for each cell line.

Telomeric Repeat Amplification Protocol (TRAP) Assay

TRAP assays were performed essentially as described [53]. 2×10^5 HEL-299, HEL-TERT, and HeLa cells were collected, pelleted, and frozen at -80°C . The cell pellets were resuspended in 200 μL of CHAPS lysis buffer (0.5% CHAPS, 10 mM Tris-HCl pH 7.5, 1 mM MgCl_2 , 1 mM EGTA, 3.5% 2-mercaptoethanol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 $\mu\text{g}/\text{mL}$ aprotinin, and 1 $\mu\text{g}/\text{mL}$ leupeptin) and incubated on ice for 30 minutes before cell pellets were collected by centrifugation. Telomeric repeats were amplified in a solution of 10 ng of cell extract, $1 \times$ Taq buffer (NEB), 0.2 mM dNTPs, 0.04 $\mu\text{g}/\mu\text{L}$ of T4 Gene 32 Protein (NEB), and 2 U of the Taq polymerase (NEB) containing 0.5 ng/ μL of the primers: TS (5'-AATCCGTCGAG-CAGAGTT-3') and ACX (5'-GCGCGG(CTTACC)₃CTAACC-3') by polymerase chain reaction (PCR) in an MJ Mini Personal Thermal Cycler (Bio-Rad). Final PCR products were gel electrophoresed on 20% polyacrylamide gel, visualized with ethidium bromide staining, and photographed with a VisiDoc-It Imaging System (UVP).

Quantitative Reverse Transcriptase Real Time PCR

HEL-299, HEL-TERT, and HEL-TERT-T cells were plated at 1×10^5 cells per well. Twenty-four hours post-plating, cells were mock treated or treated with human IFN- β at 1000 U/mL (AbD Serotec). At 9 h post treatment, cells were washed twice with PBS and harvested in Trizol (Invitrogen) to isolate total RNA. RNA was converted into cDNA using iScript cDNA synthesis kit (Bio-Rad) according to manufacturers recommendations. For each sample, real time PCR was performed using FastStart SYBR green master (Rox) (Roche) in a StepOnePlus Real-Time PCR System (Applied Biosystems). Transcripts were amplified using the following primer sets: *hTBP* (5'-TGCACAGGAGCCAAGAGT-GAA-3' and 5'-CACATCACAGCTCCCCACCA-3'), *ISG15* (5'-GGTGGACAAATGCCAGCAAC-3' and 5'-ATGCTGGTG-GAGGCCCTTAG-3'), *IFIT1* (5'-TAGCCAACATGTCCTCA-CAGAC-3' and 5'-GTGCCTTGAGCAAAGCCCTAT-3'), *IFIT2* (5'-ACGCATTTGAGGTCATCAGGGTG-3' and 5'-CCAGTTCGAGGTTATTTGGATTTGGTT-3') [54], and *Mx1* (5'-AGAAGGAGCTGGAAGAAG-3' and 5'-CTGGAGCAT-GAAGAAGT-3') [55]. All transcript levels were normalized to *hTBP*.

Western Blot

HEL-299, HEL-TERT, and HEL-TERT-T cells were plated at 1.5×10^5 of cells per well in a 12-well plate. 24 h later, cells were either mock treated or treated with IFN- β at 1000 U/mL for 16 hours before being washed with PBS and then lysed into Red Loading Buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.01% phenol red, 42 mM DTT) plus with protease inhibitors (1 $\mu\text{g}/\text{mL}$ aprotinin, 1 $\mu\text{g}/\text{mL}$ leupeptin, 1 mM phenylmethylsulfonyl fluoride). Samples were resolved on a 4–12% Bis-Tris gradient polyacrylamide gel, transferred to nitrocellulose, blocked with 5% BSA in Tris-buffered saline with 0.1% Tween-20 (TBS-T) for 1 h at room temperature. Membranes were probed with an antibody against IFIT1 (PA3-848, Thermo Scientific) diluted in 5% BSA/TBS-T overnight at 4°C . Membranes were washed three times with TBS-T, probed with HRP-conjugated goat-anti-rabbit IgG diluted in 5% BSA/TBS-T for 1

h at room temperature, washed three times with TBS-T, developed with chemiluminescent substrate (Femto ECL, Pierce Laboratories), and detected using an Image Station 4000R (Kodak) and Carestream Molecular Imaging software. The membranes were then striped and probed with β -actin ((I-19)-R, Santa Cruz Biotechnology) as previously described [40]. Images were assembled using Adobe Photoshop and Adobe Illustrator (Adobe Systems).

Plaque Reduction Assays

Plaque assays for KOS and 7134 on HEL-299, HEL-TERT, and HEL-TERT-T cells ($-/+$ IFN- β) were carried out as previously described [40]. Images of viral plaques were captured by scanning the immunohistochemically stained plates with a flatbed scanner (Canon).

HSV-1 Viral Yield Assays

To examine HSV-1 productive infection, HEL-299, HEL-TERT, and HEL-TERT-T cells were plated at 1×10^5 cells per well in 12 well plates. One day post-plating, cells were mock-treated or treated with 1000 U/mL of human IFN- β . Sixteen hours post-treatment, cells were infected with either KOS or 7134 at 5 plaque forming units (PFU)/cell, washed with PBS ($-/+$ IFN- β) after 1 hour to remove unabsorbed virus, and placed back in growth medium ($-/+$ IFN- β). At 24 hours post-infection, cells were harvested and frozen at -80°C . Virally infected samples were thawed and sonicated, and standard plaque assays were performed on either Vero cells (for KOS) or L7 cells (for 7134) to determine viral titers.

VSV Viral Yield Assays

To measure VSV replication, HEL-299, HEL-TERT, and HEL-TERT-T cells were plated, mock-treated or treated with IFN, and infected as for the HSV-1 yield assays except that cells were infected with VSV-eGFP at 0.1 PFU/cell. At 24 hours post-infection, cells were harvested and frozen at -80°C . Virally infected samples were thawed and sonicated, and standard plaque assays were performed on Vero cells to determine viral titers.

Antiviral Cytokine-Production Assay

To assess the ability of various cell lines to produce antiviral cytokines, HEL-299, HEL-TERT, or HEL-TERT-T cells were plated at 1×10^5 in 12-well plates. The next day, the cells were either mock infected with serum-free α MEM or infected with SeV at 100 hemagglutination units (HAU) per 10^6 cells in serum-free medium for 1 hour, after which the virus was removed from the cells and fresh α MEM containing 10% FCS was added to the cells. Twenty-four hours post infection, SeV-infected HEL cells were irradiated with ultraviolet light to inactivate the virus. To test for the production of antiviral cytokines secreted from these cells, duplicate Vero cell monolayers (2×10^5 cells per well in 12-well plates) were exposed to HEL supernatants. In addition, one set of Vero cells were treated with either fresh α MEM or α MEM containing IFN- β at 1, 10, 100, or 1000 U/mL as positive controls. Six hours later, untreated and treated Vero cells were infected with VSV-eGFP at ~ 200 PFU/well. At 1 hour post infection, the Vero cells were overlaid with α MEM containing 10% FCS and 1% methylcellulose. Twenty-four hours post-VSV infection, the methylcellulose was removed, Vero cells were washed with PBS and fixed with 3.7% formaldehyde, and fluorescent plaques were counted.

Results

HEL-TERT Cells Exhibit an Expanded Proliferative Capacity

HEL-299 cells are a primary strain that have been used to study viral replication and the type 1 IFN response [40,56,57]. This cell strain, nonetheless, can only be passaged in culture a limited number of times before undergoing senescence [58]. We wanted to determine whether ectopic expression of hTERT in HEL-299 cells would allow for a longer period of culturing. HEL-299s were transduced with a retrovirus encoding both hTERT and hygromycin resistance. The resulting antibiotic resistant mass population, hereafter called HEL-TERT, were then used in subsequent experiments. To first examine whether hTERT conferred an extended ability to replicate, HEL-299 and HEL-TERT cells were maintained in culture for an extended period of time, comparing the number of population doublings to days in culture. As expected, HEL-299s proliferated just under 60 days in culture and underwent a total of 23.5 population doublings (from two experiments); at which point the cells ceased to divide and underwent widespread cell death two weeks later (Figure 1). It should be noted that in a couple of instances, HEL-299 cells were able to undergo approximately 35 population doublings (data not shown). In contrast, the HEL-TERT cells were maintained in culture for 185 days and went through 114 population doublings (Figure 1), at which point the experiment was terminated. Transduction of HEL-299 cells with a retroviral vector that either expresses the green fluorescent protein or contains a deletion in *hTERT* failed to extend the life span of the HEL-299s (data not shown). These results demonstrate that expression of hTERT significantly extends the life span of HEL-299 cells.

HEL-TERT Cells Contain Active Telomerase

To establish that transduced hTERT resulted in telomerase activity in HEL-TERT cells, we performed TRAP assays. In this assay, telomerase activity is monitored by examining the laddering or amplification of 6 base-pair 5'TTAGGG3' telomeric repeats [59]. HeLa cells, which express hTERT [60], exhibited the characteristic 6 bp laddering, while the non-immortalized HEL-299 failed to do so (Figure 2). Unlike the parental cell line, the HEL-TERT cells showed a clear laddering effect, indicating that exogenous hTERT is active and capable of extending telomeres. As a control, we determined that our samples did not contain a PCR inhibitor by amplifying the cellular promyelocytic leukemia (*PML*) gene (data not shown). Thus, HEL-TERT cells contain active telomerase, suggesting that the extended proliferative capacity of this cell line can be attributed to the maintenance of telomeres.

Prolonged Culture of HEL-TERTs does not Result in Senescence

As fibroblasts reach senescence, they exhibit characteristic changes in cellular morphology, such as an increase in area, due to dysregulation of cytoskeleton elements [5,61]. Additionally, senescent cells can be detected by their upregulation of a lysosomal β -galactosidase [62]. When we compared low (6 population doublings) and high (20 population doublings) passage HEL-299 cells, we noted that many of the higher passage cells exhibited a clear enlargement of the cytoplasm, with a change from their typical narrow, drawn-out morphology to one that was shortened and/or broader (Figure 3A). When we compared the HEL-TERT cells to low passage HEL-299 cells, we were able to

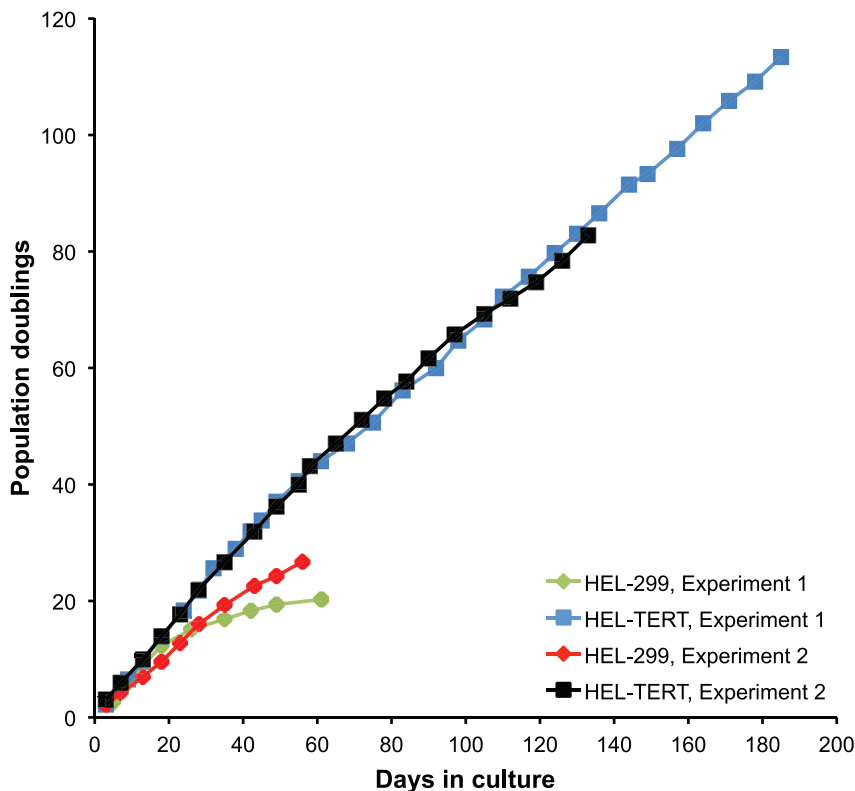


Figure 1. HEL-TERT cells are life-extended compared to HEL-299 cells. HEL-TERT and HEL-299 cells were plated as duplicate cultures in 60 mm dishes at 1×10^5 and 2×10^5 cells per plate, respectively. For each passaging, cells were counted and re-plated. Population doublings were determined by using cell counts and days in culture. doi:10.1371/journal.pone.0058233.g001

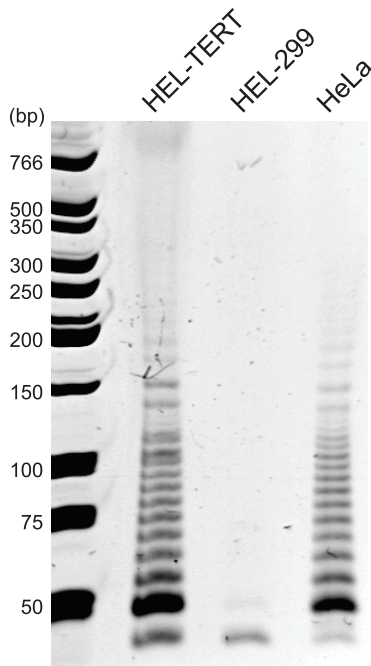


Figure 2. Telomerase activity is detectable in HEL-TERT and HeLa cells but not HEL-299 cells. HEL-299 (3 population doublings), HEL-TERT (3 population doublings), and HeLa cells were examined for telomerase activity using the TRAP assay. HeLa cells were used as a positive control for telomerase activity. The numbers at the left side of the figure are DNA size markers (bp: base pair). doi:10.1371/journal.pone.0058233.g002

detect little if any morphological changes either shortly after transduction with hTERT or at 100 population doublings later (Figure 3A). As part of these studies, we also examined another HEL-TERT derivative cell line that expresses SV40 large Tag (hereafter named HEL-TERT-T). SV40 large Tag is known to alter the IFN-response [63]. From this experiment, HEL-TERT-T cells appeared to have an altered cellular morphology, with the cells decreasing in length and broadening in width. When we examined all the cell types for senescence-associated β -galactosidase activity, β -galactosidase activity was clearly detected in older HEL-299 cells, whereas we failed to detect β -galactosidase activity in either low passage HEL-299 or low or high passage HEL-TERT, or high passage HEL-TERT-T cells (Figure 3B). These results indicate that not only do HEL-TERT cells retain their ability to proliferate but also fail to exhibit signs of senescence.

Treatment of HEL-TERT Cells with Human IFN- β Induces Strong ISG Expression

Because the IFN response has been reported to be altered in immortalized cells [2–4], we decided to examine the effect that exogenous hTERT had on ISG levels. HEL-299, HEL-TERT, and HEL-TERT-T cells were stimulated with IFN- β for 9 h and the transcript levels of four prototypic ISGs (*ISG15*, *IFIT1*, *IFIT2*, and *Mx1*) were monitored by qRT-PCR. Both HEL-299 and HEL-TERT cells showed robust upregulation in the transcript levels of all four genes after the addition of IFN- β (Figure 4). Three of the ISGs induced to similar levels between the two cell lines while *ISG15* was induced to slightly higher levels in the HEL-TERTs. On the other hand, the overall upregulation of these genes upon IFN- β treatment was greatly diminished in HEL-TERT-T cells. When we examined IFIT1 protein levels, we found

that, as expected [64], unstimulated HEL-299 and HEL-TERT cells contained little to no detectable IFIT1; however, IFIT1 was readily detected 9 hours after IFN- β treatment (Figure 5). Notably, IFN-treated HEL-299 and HEL-TERT cells showed comparable levels of IFIT1 protein. HEL-TERT-T cells, on the other hand, showed persistent production of IFIT1 and a greatly reduced difference between the unstimulated and IFN-treated states (as compared to that found in the other two cell lines), in agreement with a previous report [65]. Thus, the ectopic expression of hTERT in HEL-299 cells via retroviral transduction does not largely affect the ability of HEL cells to induce the expression of these four ISGs by IFN- β nor does it lead to a dysregulation of ISG protein production (i.e., IFIT1) as does expression of TAG.

HSV-1 and VSV Replicate to Comparable Levels, +/- IFN- β , in HEL-299 and HEL-TERT Cells

As another measure to assess whether the IFN response is active and functional in HEL-TERT cells, we examined the replication of three viruses in the presence of IFN- β . For these studies, we chose HSV-1, which is largely resistant to type I IFNs, as well as both an ICP0-null mutant of HSV-1 and VSV, as these latter two viruses are sensitive to type I IFNs [66–68]. Initially, we examined the ability of wildtype (WT) and ICP0-null HSV-1 to form plaques on untreated and IFN- β -treated HEL-299, HEL-TERT, and HEL-TERT-T cells. Both viruses had visually comparable plaque sizes on both HEL-299 and HEL-TERT cell types (Figure 6), even on higher passage HEL-TERT cells (data not shown). Plaques appeared to be slightly smaller on HEL-TERT-T cells, which is most likely due a decrease in the size of the cells that occurred upon transduction of TAG (Figure 3A). When the cells were pretreated with IFN- β , there was a large decrease in plaque size for both WT HSV-1 and the ICP0-null mutant on HEL-299 and HEL-TERT cells, while plaque size on the HEL-TERT-T cells remained largely the same. The ability of WT virus to form plaques was similar on the three cell lines in untreated cells (Figure 7A), though the ICP0-null virus showed slight increases of 2-fold and 4.5-fold on HEL-TERT and HEL-TERT-T cells, respectively. Upon the addition of IFN- β , the plaquing efficiencies of the WT and ICP0-null viruses were decreased \sim 10 fold and \sim 50-100-fold, respectively, on both HEL-299 and HEL-TERT cells (Figure 7C). In contrast to the introduction of hTERT into HEL-299 cells, expression of large TAG greatly diminished the ability of IFN- β to restrict the plaquing of either WT or the ICP0-null virus (3-fold for either) (Figure 7C), resulting in a nearly 80-fold increase in the plating efficiency of the ICP0-null virus on IFN-treated HEL-TERT-T cells as compared to IFN-treated HEL-299 cells (Figure 7B). To further examine the effect of hTERT on HSV-1 replication, we also performed viral yield assays in the three cell types. (Figure 7D). WT HSV-1 replicated to comparable levels in all three cell lines, with IFN-pretreatment producing a slight reduction in yields from both HEL-299 and HEL-TERT but not from HEL-TERT-T cells. Like WT HSV-1, the ICP0-null mutant replicated nearly as well among the three cell types in untreated cells; however, IFN-pretreatment resulted in a 100-fold decrease of viral yields in HEL-299 and HEL-TERT cells while producing little to no effect in HEL-TERT-T cells.

To monitor reductions in VSV production, we again performed viral yield assays in the presence and absence of IFN- β . Just as for HSV-1, VSV grew equally well among the three cell lines in untreated cells. In IFN- β -treated cells, however, VSV growth in both HEL-299 and HEL-TERT cells was reduced by $>10^6$ -fold while it was reduced by only 200-fold in pretreated HEL-TERT-T cells (Figure 8). Thus, HEL-TERT cells are similar to HEL-299s in their ability to support the replication of two genetically distinct

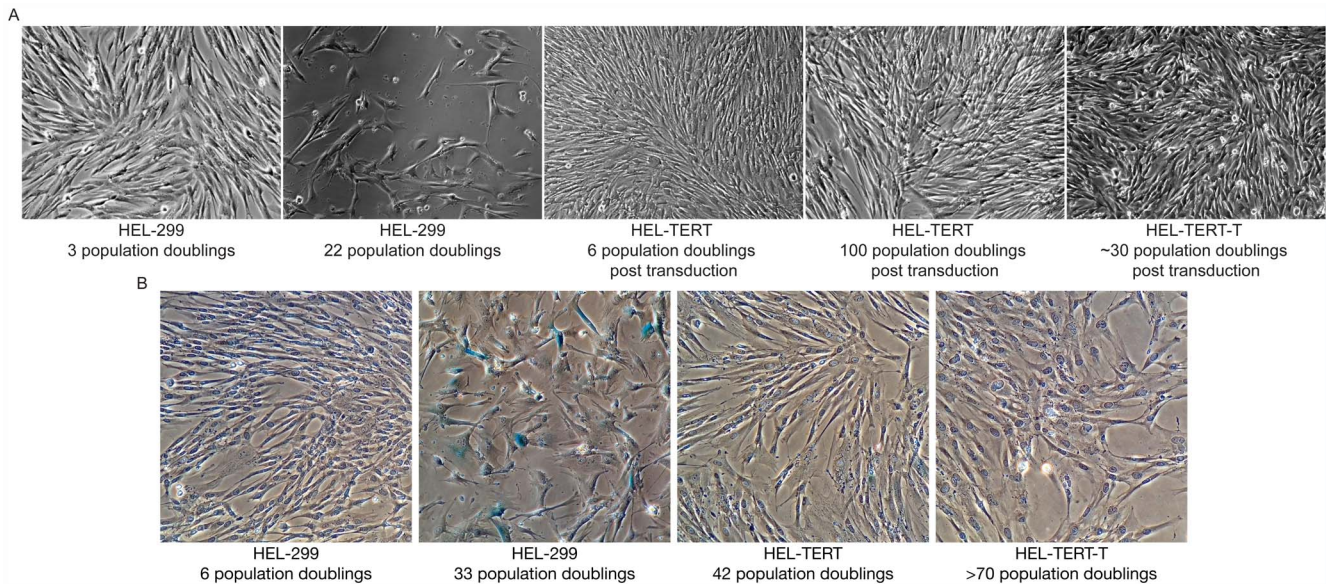


Figure 3. HEL-299, HEL-TERT, and HEL-TERT-T cell morphology and senescence. **A.** Transduction of HEL-299 cells with hTERT does not alter morphology. Light microscopy of live HEL-299 cells at 3 (left panel) and 22 population doublings (left middle panel), HEL-TERT cells after 6 (middle panel) and 100 (right middle panel), and HEL-TERT-T (far right panel) after approximately 30 population doublings. **B.** HEL-TERT cells fail to exhibit at least one sign of senescence. HEL-299 cells at 6 (left panel) and 33 (left middle panel), HEL-TERT cells after 42 (right middle panel), and HEL-TERT-T cells at approximately 70 (right panel) population doublings were stained for β -galactosidase activity. doi:10.1371/journal.pone.0058233.g003

viruses, and they retain an IFN response that is as functional as the parental cell line. Overall, ectopically expressed hTERT does not appear to adversely affect viral replication or the type I IFN response in a human lung fibroblast cell strain.

Effect of hTERT on Antiviral Cytokine Production

In addition to determining its effect on ISG upregulation and on the efficacy of an IFN-induced antiviral state, we decided to assess whether ectopic hTERT expression altered the ability of cells to produce IFN and other antiviral cytokines in response to infection.

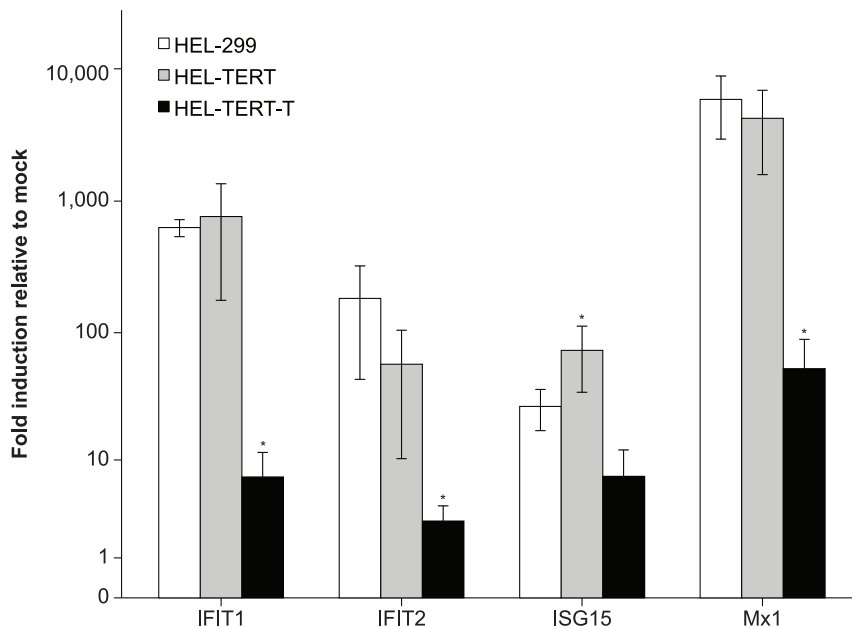


Figure 4. HEL-TERT but not HEL-TERT-T cells show ISG induction at levels similar to HEL-299 cells after IFN stimulation. HEL-299 and HEL-TERT cells were treated or mock treated with 1000 U/mL of human IFN- β . At 9 hours post treatment, total RNA was isolated from cells and reversed transcribed into cDNA for qRT-PCR analysis to monitor *IFIT1*, *IFIT2*, *ISG15*, and *Mx1* transcript levels. Data represents the means of 6 samples; error bars represent the standard errors of the means. * $p < 0.05$, one-way ANOVA, Bonferroni's multiple comparison post-test, compared to HEL-299 levels. doi:10.1371/journal.pone.0058233.g004

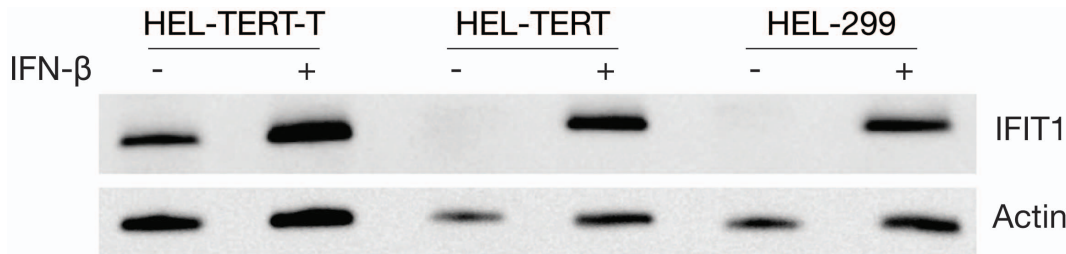


Figure 5. IFIT1 protein production is induced to similar levels by IFN- β in HEL-299 and HEL-TERT cells. HEL-299, HEL-TERT, and HEL-TERT-T cells were mock treated or treated with 1000 U/mL of IFN- β and harvested 9 hours later. Cell lysates were analyzed for IFIT1 or β -actin protein production by western blot.

doi:10.1371/journal.pone.0058233.g005

We used infection by SeV, which is known to be a strong inducer of IFNs and other antiviral cytokines in human cells [69]. For this assay, media from uninfected and infected HEL cells are placed onto naïve Vero cells, which respond to but cannot produce IFN, and restrictions on VSV plaquing are monitored [70,71]. Media from mock-infected HEL cells had no effect on the ability of VSV to plaque on Vero cells while IFN- β pretreatment, at the highest level tested (1000 U/mL), was able to reduce the number of plaques formed by approximately 35-fold (Table 1). When we tested the ability of media from SeV-infected HEL cells, we found that media from all three cell types were capable of lowering the number of plaques that formed by 5–7 fold. These reductions were similar to the antiviral activity of 100 U/mL of IFN- β . Although we are unable to distinguish between IFN- β or among the IFN- α subtypes with this assay, the protective effect produced by media from HEL-299 and HEL-TERT cells was identical and suggests that hTERT does not affect the activation of IFN-production in response to viral infection.

Discussion

Due to their unperturbed DNA damage, senescence, and antiviral pathways, primary cells are considered biologically relevant cells when studying how these cellular processes affect viral replication. However, their limited ability to proliferate makes their use in examining these pathways technically challenging. For example, the establishment of a cell line depleted for a particular cellular protein is generally difficult to generate

because of the rapid and inevitable onset of senescence. Thus, in studying cell-virus interactions, there is a need for life-extended cell lines that retain many of the characteristics of a primary cell (e.g., antiviral responses) while allowing for the analysis of specific cellular genes or proteins (e.g., depletion, gene knockout). It is possible to immortalize primary cells with viral and cellular oncogenes, but immortalization can result in alterations of cellular processes and inhibit antiviral pathways, affecting the replication of wild-type and mutant viruses [72–75]. Another approach is to use the *TERT* gene, which has been reported to extend the life of human fibroblasts [36], and avoids many of the problems associated with cellular or viral oncogene immortalization or transformation [39]. Prior to this study, the effect of hTERT expression on the IFN response had not, to the best of our knowledge, been examined.

The traditional approach used to immortalize primary cells has been the introduction of cellular or viral oncogenes. The most commonly used of these include E1A and E1B from adenovirus [76], E6 and E7 from human papillomavirus [17], and large TAg from SV40 [20,77]. In general, these proteins bypass senescence by the inactivation of one or both of the tumor suppressor proteins, p53 and pRb [78–80]. Unfortunately, in addition to perturbing the cell cycle, many of these viral proteins also serve to antagonize or inactivate antiviral pathways in order to promote viral replication. E1A, E6, E7, and large TAg are capable of disrupting the activity of, among others, cellular histone deacetylases [81] and CBP/p300 [82–84] resulting in widespread transcriptional and epigenetic changes [85–89]. In the case of

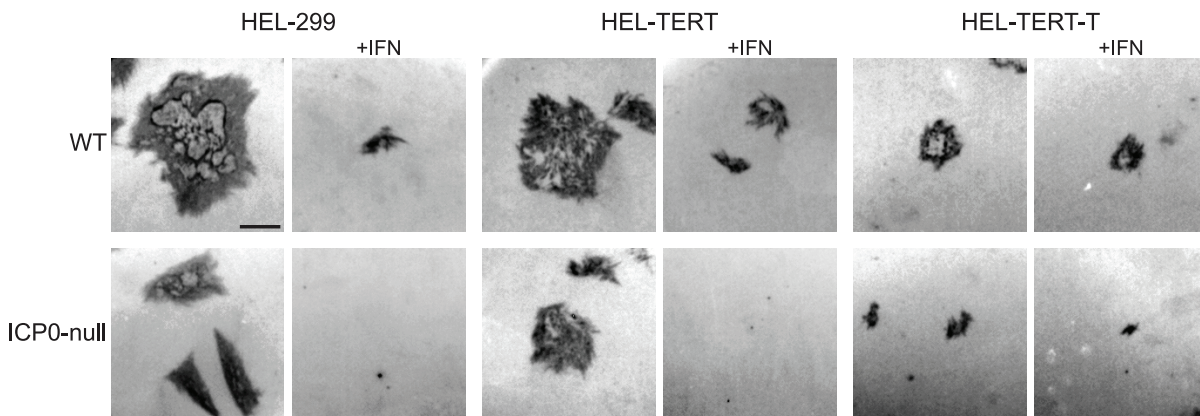


Figure 6. HSV-1 shows similar plaque size and morphology on HEL-299 and HEL-TERT cells. HEL-299, HEL-TERT, and HEL-TERT-T cells were mock or pretreated with IFN- β for 16 h and then infected with WT HSV-1 or an ICP0-null mutant, and plaques for both viruses were visualized by immunohistochemistry three days post-infection. Bar = 1 mm.

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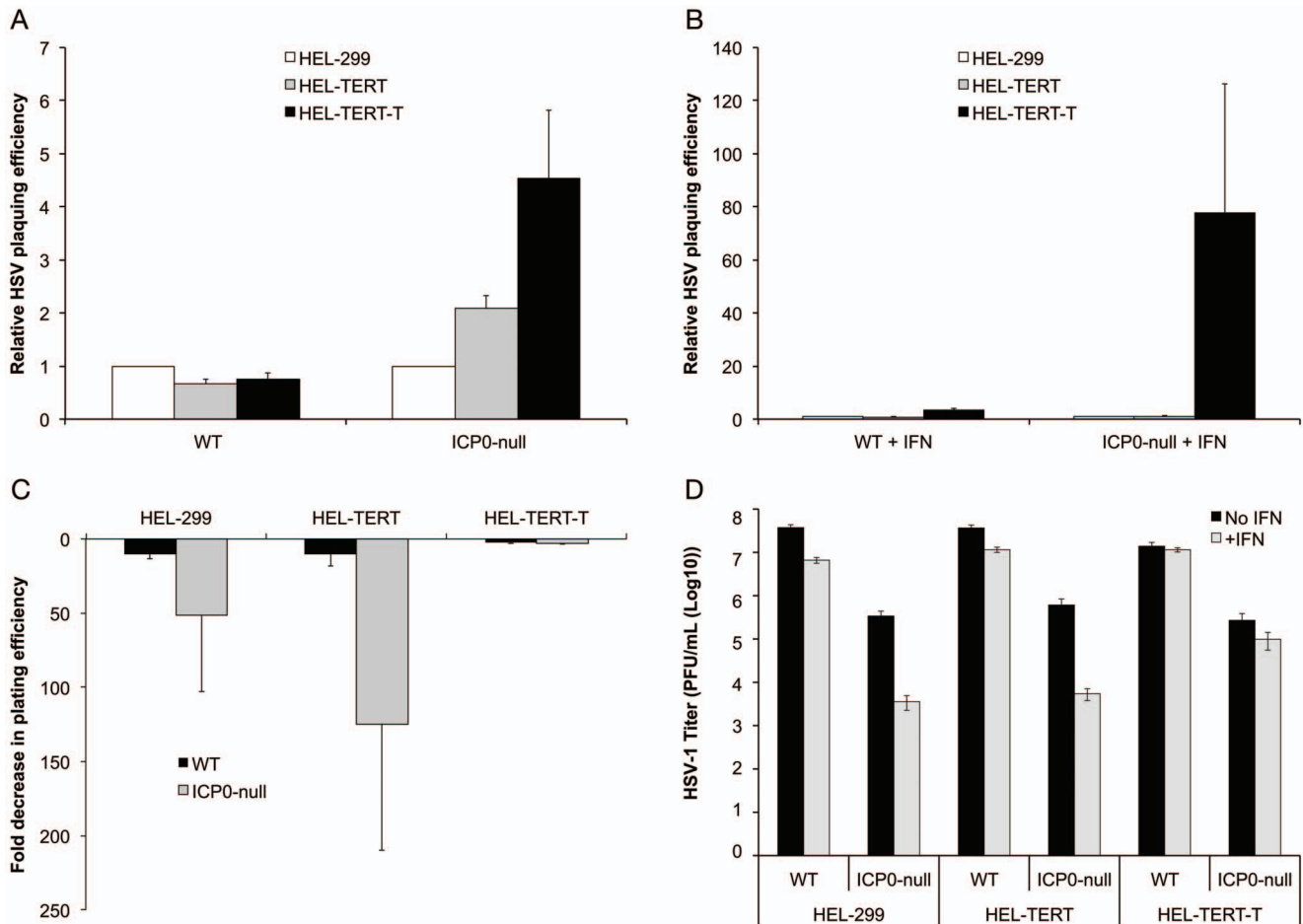


Figure 7. Replication of HSV-1 is diminished by IFN- β in HEL-299 and HEL-TERT but not HEL-TERT-T cells. **A and B.** HEL-299, HEL-TERT, and HEL-TERT-T cells were mock (A) or pre-treated with IFN- β (1000 U/mL) (B) and were infected 16 h post treatment with 10-fold serially diluted stocks of WT HSV-1 or an ICP0-null mutant. Plaques were visualized by immunohistochemistry 3 days post-infection. An average of three experiments is shown. Data is presented as the ratio of plaques formed on the indicated cell line to that on HEL-299 cells. **C.** Data generated for A and B, but presented as a ratio of the number of plaques formed on mock-treated cells compared to that on IFN-treated cells. **D.** HEL-299, HEL-TERT, and HEL-TERT-T cells were mock or pre-treated with IFN- β (1000 U/mL) and were infected (16 h post treatment) with either WT HSV-1 or the ICP0-null mutant at an MOI of 5 PFU/cell. Samples were harvested 24 h post-infection. Viral titers were determined by plaque assays. An average of three experiments is shown. In all cases, error bars represent the standard errors of the means. doi:10.1371/journal.pone.0058233.g007

E1A, this interaction prevents the major type I IFN transcription factor, STAT1, from binding to CBP/p300 and upregulating ISGs [90]. Likewise, E6 is capable of preventing activation of the IFN response by blocking the transcriptional activity of IRF3 [91]. E1B proteins inhibit apoptosis and are capable of inactivating the cellular DNA damage response [92–94]. Large TAg, on the other hand, has recently been reported to activate STAT1 and induce ISGs upregulation in the absence of IFN-exposure [65]. In agreement with this, we saw persistent production of IFIT1 protein in TAg-transduced cells. However, the expression of large TAg, independent of SV40 infection, has also been shown to decrease the phosphorylation of the cellular translation factor eIF2 α by an IFN effector, double-stranded RNA protein kinase (PKR) [95]. This decrease in phosphorylation increases the translation capability of viral mRNAs. Similarly, our results show that while large TAg may lead to high levels of ISG protein production, it functionally inactivates the IFN response. Furthermore, immortalization by mechanisms not involving viral oncogenes may inactivate antiviral pathways, as observed with the loss

of induction of ISGs in immortalized cells derived from Li-Fraumeni patients [1].

Our approach in this study was to extend the life of human fibroblasts with hTERT. Cells transformed with hTERT arrest in response to serum starvation, maintain anchorage dependence, double at a rate similar to untransformed cells, and do not exhibit genomic instability [39]. While it has been reported that expression of hTERT can alter the expression of a limited number of genes, none of these have an apparent role in antiviral pathways [96]. We found that hTERT expression does not interfere with the upregulation of four representative ISGs (*ISG15*, *IFIT1*, *IFIT2*, and *Mx1*), does not lead to aberrant ISG protein production, nor does its expression affect the replication of two genetically distinct viruses, HSV-1 and VSV. This is in agreement with previous work demonstrating that exogenous expression of hTERT in fibroblasts does not affect the replication of human cytomegalovirus [97,98] nor does it affect the upregulation of the IFN-induced senescence mediator, IFI16, upon IFN-stimulation [99]. Furthermore, unlike SV40 large TAg, exogenous hTERT

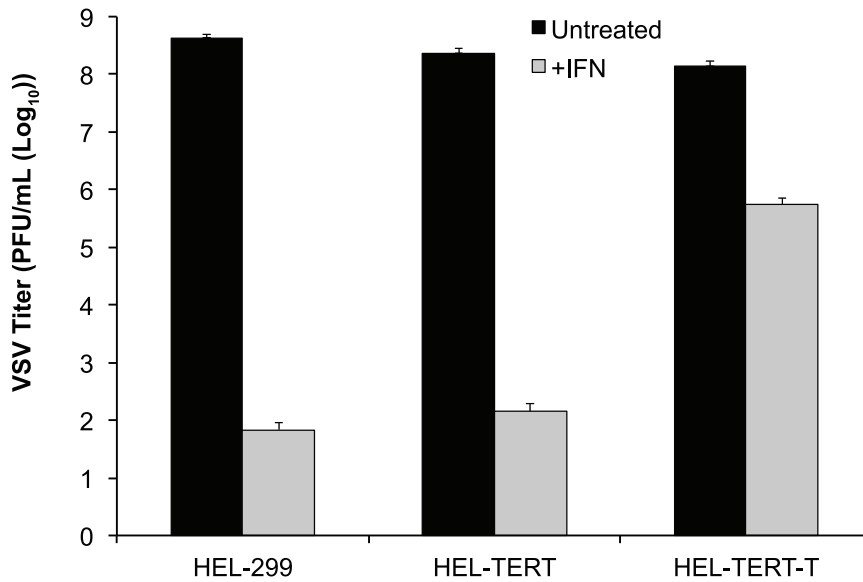


Figure 8. Replication of VSV is diminished by IFN- β in HEL-299 and HEL-TERT but not HEL-TERT-T cells. HEL-299, HEL-TERT, and HEL-TERT-T cells were mock or pre-treated with IFN- β (1000 U/mL) and were infected (16 h post treatment) with VSV-eGFP at an MOI of 0.1 PFU/cell. Samples were harvested 24 h post-infection. Viral titers were determined by plaque assays. An average of three experiments is shown. In all cases, error bars represent the standard errors of the means.
doi:10.1371/journal.pone.0058233.g008

Table 1. Ectopic hTERT expression does not affect the ability of HEL cells to produce antiviral cytokines.

	Treatment	Number of plaques on Vero cells
HEL-299	Mock	224.0 \pm 19.4
	SeV	40.6 \pm 4.44
HEL-TERT	Mock	234.8 \pm 19.3
	SeV	35.0 \pm 6.54
HEL-TERT-T	Mock	219.6 \pm 14.7
	Sev	28.6 \pm 7.60
IFN- β	Untreated	215.0 \pm 5.0
	1 U/mL	227.5 \pm 3.5
	10 U/mL	200.5 \pm 9.5
	100 U/mL	27.5 \pm 0.50
	1000 U/mL	6.00 \pm 2.0

HEL-299, HEL-TERT, and HEL-TERT-T cells were mock- or SeV-infected at 100 HAU/10⁶ cell. At 24 hpi, the media were transferred to naïve Vero cell monolayers. 6 h later, the Vero cells were infected with ~200 PFU of VSV-eGFP per well. At 24 hpi with VSV-eGFP, the Vero cells were fixed and the number of fluorescent plaques counted. Data shown represents the average of two experiments performed in duplicate (\pm standard errors of the means).
doi:10.1371/journal.pone.0058233.t001

did not impair the ability of IFN to restrict the replication of VSV or an ICP0-null mutant of HSV-1, both viruses being quite sensitive to the effects of IFN- β , nor did it hinder the ability of

HEL cells to produce antiviral cytokines in response to viral infection. While we did observe slight differences in the levels of induction for the four ISGs between HEL-299 and HEL-TERT cells, these differences failed to translate into an appreciable effect on the ability of IFN- β to suppress replication of VSV or the ICP0-null HSV-1 mutant. hTERT overexpression has been reported to enhance the formation of apoptotic markers during HSV-1 infection in HeLa cells, which express the human papillomavirus E6 and E7 oncoproteins, and sensitizes them to apoptosis [100]. Our results, however, suggest that exogenous expression of hTERT in a primary cell strain has little impact on viral replication.

In conclusion, HEL-TERTs are permissive for HSV-1 and VSV growth, have a robust antiviral response, and a significantly enhanced lifespan. They recapitulate the phenotype of an HSV-1 ICP0-mutant, which is known to be complemented by the loss of proteins involved in the DNA damage response [101], antiviral pathways [102], or overexpression of certain cyclins [103], suggesting that these pathways are unperturbed. Because the phenotypes of certain HSV-1 mutants are only apparent in primary cells, we believe the HEL-TERT cell line to be an ideal choice due to their longevity and robust antiviral response. Additionally, they will allow for the establishment of derivative cell lines that are depleted or overexpress targets of interest, facilitating a better understanding of cellular pathways (including the IFN response) and the viruses that alter these pathways.

Author Contributions

Conceived and designed the experiments: MCS ETG MPL DJD. Performed the experiments: MCS ETG MPL. Analyzed the data: MCS ETG MPL DJD. Contributed reagents/materials/analysis tools: MCS ETG. Wrote the paper: MCD ETG DJD.

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