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Low p16^{INK4a} Expression in Early Passage Human Prostate Basal Epithelial Cells Enables Immortalization by Telomerase Expression Alone

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Abstract

BACKGROUND—There are two principal senescence barriers that must be overcome to successfully immortalize primary human epithelial cells in culture, stress-induced senescence, and replicative senescence. The p16^{INK4a}/retinoblastoma protein (p16/Rb) pathway mediates stress-induced senescence, and is generally upregulated by primary epithelial cells in response to the artificial conditions from tissue culture. Replicative senescence is associated with telomere loss. Following each round of cell division, telomeres progressively shorten. Once telomeres shorten to a critical length, the DNA damage response pathway is activated, and the tumor suppressor p53 pathway triggers replicative senescence. Exogenous expression of telomerase in normal human epithelial cells extends the replicative capacity of cells, and in some cases, immortalizes cells. However reliable immortalization of epithelial cells usually requires telomerase activity coupled with inactivation of the p16/Rb pathway.

METHODS—A lentiviral vector, pLOX-TERT-iresTK (Addgene #12245), containing a CMV promoter upstream of a bicistronic coding cassette that includes *loxP* sites flanking the catalytic subunit of human telomerase gene (*TERT*) and herpes simplex virus type-1 thymidine kinase gene (*HSV1-tk*) was used to transduce normal prostate basal epithelial cells (PrECs) initiated in cell culture from prostate cancer patients undergoing radical prostatectomies.

RESULTS—Transduction of early (i.e., <7) passage PrECs with *TERT* led to successful immortalization. However, attempts to immortalize late (i.e., >7) passage PrECs were unsuccessful. Late passage PrECs, which acquired elevated p16, were unable to overcome the senescence barrier. Immortalized PrECs (TERT-PrECs) retained a normal male karyotype and low p16 expression. Additionally, TERT-PrECs were non-tumorigenic when inoculated into intact male immunodeficient NSG mice.

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CONCLUSIONS—The present studies document that early passage human PrECs have sufficiently low p16 to permit immortalization by *TERT* expression alone. TERT-PrECs developed using this transduction approach provides an appropriate and experimentally facile model for clarifying the molecular mechanism(s) involved in both immortalization of human PrECs, as well as identifying genetic/epigenetic “drivers” for conversion of these immortalized non-tumorigenic cells into fully lethal prostate cancers. Notably, loxP sites flank the exogenous TERT gene in the TERT-PrECs. Cre recombinase can be used to excise TERT, and resolve whether TERT expression is required for these cells to be fully transformed into lethal cancer. Prostate 77: 374–384, 2017.

Keywords

prostate epithelial cells (PrECs); stress-induced senescence; stasis; replicative senescence; telomerase

INTRODUCTION

Normal somatic cells have finite replicative capacity [1,2]. Over time, cells accumulate mutations in their genomic DNA due to incorporation of incorrect DNA bases by polymerases during replication [3] or exposure to environmental mutagens [4]. Limiting the number of cellular divisions serves as a potent anti-cancer mechanism by reducing the number of opportunities for potentially oncogenic mutations in DNA, as well as limiting clonal expansion potential of any transformed cells that may arise. Telomeres are integral to the cellular mechanisms that limit the number of cell divisions. Located at the terminal ends of chromosomes, telomeres are approximately 5–15 kb in length [5] and are composed of a highly conserved, G-rich, repetitive, hexanucleotide sequence (TTAGGG)_n [6,7] in complex with several proteins collectively termed shelterin [8–17]. Due to incomplete lagging strand synthesis during DNA replication, telomeres progressively shorten following each round of cell division [18]. At a critical length, short telomeres activate the DNA damage response (DDR) pathway, and through the tumor suppressor p53 pathway triggers *replicative senescence* [19,20]. To bypass replicative senescence, most cancers activate telomerase, an enzyme that extends telomeres [21]. Normally, telomerase activity is exclusively reserved in stem cells and repressed in somatic cells [22]. Studies in human retinal pigment epithelial cells (RPE-340) and foreskin fibroblasts have shown that exogenous expression of *TERT*, the gene that expresses the catalytic subunit of telomerase, was sufficient to extend the lifespan of these cell types [23]. However, immortalization by *TERT* expression to achieve unlimited replicative capacity in human primary cells has been controversial. Previous attempts to immortalize primary human mammary epithelial cells (HMECs) or human foreskin keratinocytes by *TERT* expression alone were unsuccessful [24].

In addition to replicative senescence, cells can also undergo *stress-induced senescence (stasis)*, independent of telomere length [25] and without activation of the DDR pathway [26]. Cell senescence in response to stress is mediated by the p16^{INK4a}/retinoblastoma protein (p16/Rb) pathway and can occur before telomere lengths are critically short. The tumor suppressor protein, p16, binds and inhibits cyclin-dependent kinases 4/6 (CDK4/6), which are positive regulators of cell cycle progression. In the absence of CDK4/6 activity, Rb is not phosphorylated. As a result, hypophosphorylated Rb is able to bind to the

transcription factor E2F1, inhibiting the transcription of E2F1 target genes for cell cycle progression, thereby arresting cells in G1 [27]. Studies in HMECs have shown that cells in stasis have elevated p16 levels, and no extensive genomic instability or telomere attrition [26]. While the exact mechanisms of stasis have yet to be satisfactorily characterized, the well known phenomenon of “culture shock” offers some insight on possible sources that induce stasis [28]. Cells explanted from organisms and cultured under artificial conditions are exposed to several stressful triggers, including abnormal levels of nutrients, growth factors, and oxygen [29]. Consequently, the artificial conditions of tissue culturing activate the p16/Rb pathway in cells, resulting in cellular stasis. Interestingly, modifying the formulation of culture media including serum content, lipid content, and the addition of the anti-stress mediator oxytocin, can forestall stasis in HMECs and increase the number of population doublings before cell senescence ensues [26]. Likewise, culturing cells under low oxygen tension (i.e., in situ normoxia) conditions [30], or on top of irradiated fibroblast “feeder” cell layers [31,32] can also prolong cell culture lifespan. Studies with HMECs demonstrated a 10 to 25-fold increase in p16 expression in cells following 15–25 population doublings in standard in vitro culture conditions. However, a subset of HMECs achieve additional long-term replicative capacity (approximately 80 population doublings) due to repression of p16 via methylation of the p16 gene [33].

Viral strategies suppressing the activity of both p53 and the p16/Rb pathway have been successfully implemented to transform primary human cells. Common examples include the introduction of human papilloma virus (HPV) E6 and E7, which exert their inhibitory effects on p53 and Rb, respectively [24]. Similarly, adenovirus E1A inactivates Rb proteins and E1B inhibits p53 [34]. Simian virus 40 (SV40) large T antigen inhibits both p53 and Rb [35]. While these viral transformation strategies extend the number of population doublings, immortalization also requires the activation of telomerase. Without a means of telomere length extension or maintenance, proliferation-associated telomere attrition eventually leads to destabilized telomeres and lethal levels of chromosomal instability.

Previous attempts to immortalize human epithelial cells by exogenous *TERT* expression were thus only achievable when coupled with inactivation of the p16/Rb pathway [24]. This strategy allowed cells to bypass both senescence barriers, (i) stasis and (ii) replicative senescence, as well as telomere crisis. Ectopic expression of c-Myc in HMECs (HMEC-spiral K) [36] and primary human prostate epithelial cells (PrECs) [37] was sufficient to immortalize these epithelial cells, presumably because c-Myc has been shown to simultaneously activate telomerase while suppressing the Rb/ p16^{INK4a} checkpoint [37]. Interestingly, HMECs exposed to a highly stressful serum-free culturing condition that rapidly induced p16 expression were refractory to c-Myc induction of telomerase, whereas cells not exposed to this stress-inducing culturing condition could be immortalized by c-Myc [38]. Previously, Herbert and colleagues reported that *TERT* expression was sufficient to immortalize HMECs when cells were cultured on feeder layers. Notably, HMECs cultured on plastic culture dishes had significant p16 induction, whereas HMECs cultured on feeder cells maintained relatively low p16 levels by comparison [39]. These findings suggested that immortalization of human epithelial cells by *TERT* expression alone is achievable, as long as p16 levels are sufficiently low in the proliferative pool of cells.

In the normal human prostate, the proliferative pool of epithelial cells is organized in stem cell units composed of basally located adult stem cells. It is critical to note that these stem cells do not depend on androgen receptor (AR) signaling to self-renew or to give rise to either neuroendocrine or transit amplifying (TA) cell progeny [40]. Cellular turnover in these progeny is driven by a transcriptionally dependent cascade initiated by an indirect, extracellular-activated, reciprocal paracrine interaction between the stroma and epithelia [40]. Factors secreted by prostate epithelium stimulate the supporting glandular stromal cells to express AR protein. Androgen supplied via circulation binds to the AR within prostate stromal cells and initiates AR-dependent transcription of specific target genes within prostate stromal cells, resulting in their production and secretion of a series of peptide growth factors known as “andromedins” which include IGF-1, EGF, FGF7, FGF10 [40]. These stromally derived paracrine andromedins diffuse across the basement membrane into the epithelial compartment where they bind to their respective cognate receptors and initiate cell signaling cascades. Under the appropriate context, these signaling cascades result in the AR-negative prostate TA cells undergoing several rounds of proliferation before differentiating into intermediate cells (ICs) that co-express both basal and luminal epithelial markers [40]. The ICs subsequently differentiate into proliferatively quiescent, AR-positive secretory-luminal epithelial cells [40]. Ligand induced AR signaling in the AR-positive secretory-luminal epithelial cells stimulates secretory functions, but inhibits cell cycle entrance [40–42].

Prostatic TA cells have a remarkably low rate of proliferation. Less than 0.2% of these cells proliferate per day [43]. This translates into a turnover time of 500 days in the normal prostate [43]. Thus, it is rather unexpected that with such a low rate of proliferation, p16 protein levels are low to undetectable in the epithelial cells of the prostate [44], suggesting that the low rate of proliferation cannot be attributed to senescence. When normal human prostatic tissue is dissociated and placed in low calcium, serum-free defined media, with appropriate added growth factors (insulin, epidermal growth factor, bovine pituitary extract to supply FGFs, transferrin, hydrocortisone, triiodothyronine, and ethanolamine), normal basal stem and TA PrECs grow out, and while not immortalized, they can be serially passaged 8–10 times before becoming proliferatively quiescent [45–47]. In the present report, we document that early passage basal PrECs have sufficiently low p16 levels to permit immortalization by *TERT* expression alone.

MATERIALS AND METHODS

Cell Culture

Normal prostate tissue was isolated from non-cancer containing tissue locations within radical prostatectomy specimens obtained from localized prostate cancer patients according to NA_00001575 protocol approved by the Johns Hopkins Institutional Review Board, as previously reported [48]. Single cell suspensions were obtained by digesting the prostate tissue overnight at 37°C in collagenase solution containing 0.28% collagenase I from Sigma-Aldrich, 1% DNase I from Sigma-Aldrich, 10% fetal calf serum (FCS) and 1× concentration of Gibco® Antibiotic-Antimycotic solution, in RPMI 1640. After incubation, cells were centrifuged at 250g and the supernatant was removed from the cell pellet. To prevent

homotypic interactions between epithelial cells, the cell pellet was re-suspended in a reducing buffer containing 1 mM 1,4-Dithiothreitol (DTT) in phosphate buffered saline (PBS) for 30min at 37°C to disrupt disulfide bridge formation between E-cadherin proteins. Following treatment, the cells were centrifuged at 250g until a pellet was formed, and the supernatant was subsequently removed. Cells were re-suspended in PBS containing 0.25% trypsin and 1 mM ethylene-diaminetetraacetic acid (EDTA) for 30min at 37°C. To inactivate trypsin, FCS was added to a final concentration of 10%. Cells were washed twice by centrifuging (250g) the suspension to a pellet, removing the supernatant, and resuspending the cells in PBS. To remove cell aggregates, the cell suspension was passed through a cell strainer (BD Falcon). Based on the percentage of viability as determined by trypan blue exclusion, 20,000 viable PRECs per cm² were inoculated into tissue culture flasks containing Keratinocyte Serum-Free Media (KSFM) supplemented with insulin, epidermal growth factor, bovine pituitary extract to supply FGFs, transferrin, hydrocortisone, triiodothyronine, and ethanolamine obtained from Life Technologies (i.e., K-SFM complete medium, Invitrogen). PRECs were passaged when the cultures reached 80% confluence.

Lentiviral Vector and Packaging

The lentiviral vector, pLOX-TERT-iresTK (Addgene plasmid # 12245), contains a CMV promoter upstream of a bicistronic coding cassette that includes the *TERT* gene and herpes simplex virus type-1 thymidine kinase (HSV1-tk) gene [49]. Integration of the provirus, which contains the coding cassette, is flanked by *loxP* sequences. As previously described [50], second generation packaging plasmids were used for viral packaging. The CMVDR8.9 plasmid, which contains the viral *gag*, *pol*, and *rev* genes, and the envelop plasmid pMD.G (Addgene plasmid # 12259) were co-transfected with pLOX-TERT-iresTK at a 4:8:1 ratio (pLOX-TERT-iresTK:CMVDR8.9:pMD.G) into HEK293T cells using Lipofectamine® 2000 Transfection Reagent diluted in Gibco™ Opti-MEM™ I Reduced Serum Media. Cells were incubated for 24 hr at 37°C. The media was removed and replaced with Opti-MEM™. Cells were incubated for an additional 24 hr, and virus was harvested and concentrated by ultracentrifugation for 2 hr at 100,000g.

Transduction of PRECs

Concentrated virus was added to culture media containing 8 mg/ml of polybrene (Sigma–Aldrich) and incubated for 24 hr at 37°C. The media containing virus and polybrene were removed and replaced with media lacking virus and polybrene. PRECs treated with virus were passaged when PRECs were 80% confluent.

Senescence-Associated β -Galactosidase Staining

Senescence was determined using a previously described β -galactosidase staining approach [51,52]. PRECs were cultured on Nunc™ Lab-Tek™ chamber slides for 2 days. Cells were washed twice with PBS, fixed in 3% formaldehyde for 3–5 min, and washed in PBS following fixation. Fixed cells were incubated at 37°C and treated with β -galactosidase stain solution containing 1 mg/ml 5-bromo-4-chloro-3-indolyl β -galactoside (X-Gal), 2mM citric acid/sodium phosphate adjusted to pH 6.0, 250 μ M potassium ferrocyanide, 250 μ M potassium ferricyanide, 7.5 μ M NaCl, and 100 μ M MgCl₂. Cells with blue cytoplasmic and nuclear staining were considered positive for senescence-associated β -galactosidase activity.

Western Blotting

Approximately 10^5 cells were treated with lysis buffer containing (20mM Tris-HCl pH 7.8, 140 mM NaCl, 1mM EDTA, 0.5% sodium deoxycholate, 0.5% Nonidet P-40) supplemented with PhosSTOP phosphatase inhibitor tablet and protease inhibitor tablet (Roche), and 1mM DTT as previously described [53]. Suspended cells were incubated on ice for 30 min. Cleared lysate was collected by centrifuging the lysed cell suspension at 10,000g for 10min at 4°C, followed by collection of the supernatant. Protein lysates from 100,000 cells were run on SDS–PAGE and transferred to PVDF membranes. Membranes were blocked in Tris-Buffered Saline and 0.1% Tween 20 (TBST) containing 5% milk for 1 hr. Proteins of interest were probed with primary antibodies diluted in blocking buffer. Anti-p16 (sc-467, Santa Cruz), anti-p27 (554069, BD Transduction Labs), β -catenin (05–511, Millipore), and secondary antibodies conjugated with horseradish peroxidase (HRP) purchased from Amersham Biosciences were used for Western blotting. Membranes were treated with Amersham ECL Western blotting detection reagent according to the manufacturer's instructions and were subsequently exposed to film.

Cytogenetic Analysis and Mycoplasma Testing

The cytogenetic analysis of the *TERT* immortalized PrECs (ID#: 11220) was performed by the Cytogenetics Core Facility, Johns Hopkins Cancer Center. All lines were mycoplasma negative using the MycoSensor PCR Assay kit (Agilent Technologies).

In Vivo Tumorigenicity Assay

Using a Johns Hopkins Animal Care and Use Committee approved protocol, intact adult male immunodeficient NSG mice (lacking mature T cells, B cells, and natural killer cells) [54] were inoculated subcutaneously in the flank with the 2×10^6 viable TERT-PrECs in 200 μ l of Matrigel as described previously [53].

RESULTS

Human Prostate Basal Epithelial Cells Grown in Culture Gradually Accumulate p16 Protein Prior to Senescence

Early (i.e., <5) passage basal PrECs grow exponentially with a doubling time of 41 hr and overall, have very low levels of p16 protein expression, Figure 2A. Consistent with previous reports [55,56], normal human PrECs used in this study accrued features characteristic of senescence following 7–11 passages and cease to grow altogether. Pre-senescent, log phase, proliferating PrECs at passage 3 (eight population doublings) displayed typical epithelial polygonal cell morphology, Figure 1A. In contrast, the majority of the PrECs at passage 9 (20 population doublings) exhibited declines in proliferation rate and possessed the distinct enlarged and flattened cell morphology indicative of cellular senescence, Figure 1B. Previously, senescent cells have been reported to have elevated levels of lysosomal β -galactosidase protein [57], and therefore, stain positive for β -galactosidase activity under slightly acidic conditions (pH 6) [52]. Although lysosomal β -galactosidase exhibits maximal activity at a pH closer to 4.0 [58], senescent cells with significantly increased lysosomal content will preferentially stain positive with the chromogen X-gal, while employing the

suboptimal, slightly acidic, pH 6 [59]. Using this assay, we observed that approximately 5% of the early passage PrECs were positive for β -galactosidase activity; in contrast, more than 30% of late stage PrECs (equivalent to 20 population doublings) stained positively for senescence-associated β -galactosidase, Figure 1A versus B. Associated with this increase in cellular senescence was a concomitant increase in p16 protein expression with increased passage number, Figure 2A.

When p16 Expression Is Low, Exogenous *TERT* Expression Is Sufficient to Immortalize PrECs

Normal human somatic cells lack telomerase [60]. This includes prostate tissues [61] and PrECs derived from normal prostate [37]. Previous attempts by others to immortalize telomerase-negative human epithelial cells (HMECs and keratinocytes) by introducing *TERT* have been unsuccessful without abrogation of p16 [24]. We hypothesized that introducing *TERT* at an early passage, before PrECs had significant p16 induction, would permit immortalization. At passage 8, PrECs exhibit a significant increase in p16 levels compared to earlier passages, Figure 2A. Therefore, PrECs at passage 6 were transduced with a lentiviral expression construct containing the *TERT* gene. Taking advantage of the limited replicative capacity of PrECs in culture, cells that successfully integrated *TERT* following transduction were expected to have extended proliferative capacity, and could therefore be selected. In our studies, untreated PrECs ceased to proliferate beyond passage 8, which corresponds to approximately 20 population doublings. However, early passage PrECs virally transduced with *TERT* immortalized and continued to proliferate well beyond 60 population doublings, Figure 2B. In contrast, PrECs at passage 8 with high p16 expression were not immortalized following viral transduction with *TERT*, suggesting that *TERT* expression is unable to reverse growth suppression and senescence in PrECs when p16 expression is elevated.

Stress Induced p16 Expression in *TERT*-PrECs Is Suppressed

TERT immortalized PrECs have significantly lower p16 expression compared to passage 9 matched PrECs, Figure 2C. Additionally, as described above, elevated p16 expression appears to prevent *TERT* immortalization of PrECs. These two observations raise the issue of whether *TERT* driven immortalization suppresses p16 expression. To determine if p16 expression is indeed suppressed, *TERT*-PrECs were cultured under conditions expected to elicit a stress response and induce elevated p16 expression [27]. Growth factor deprivation causes normal human PrECs to exit cell cycle progression and induces apoptosis [62]. Typically coinciding with p16/Rb-induced senescence is the accumulation of p27^{Kip1} (p27). In cell cycle progression, p27 blocks the transition from G1 to S phase by inhibiting the activity of the cyclin E kinase/cyclin-dependent kinase 2 complex (cyclin E/cdk2) [63]. While p16 and p27 can have both cytoplasmic and nuclear localization, their role in cell cycle regulation is restricted to the nuclear compartment of the cell [64]. In response to growth factor inhibition, cell cycle exit is accompanied with nuclear localization of p16 [65] and p27 [66,67], which was observed in PrECs cultured in the absence of growth factors, Figure 2D. However, *TERT*-PrECs did not have an increase in nuclear localization of p16 or p27 in response to growth factor withdrawal, Figure 2D, suggesting that p16/Rb response was suppressed in *TERT*-PrECs.

TERT-PrECs Have a Normal Karyotype and Are Not Tumorigenic In Vivo

Chromosome analysis of 957E/*TERT*, another *TERT* immortalized PrEC line, revealed several genomic alterations [68]. The 957E/*TERT* cell line was established from benign prostate epithelial cells collected from a patient with familial prostate cancer, and was successfully immortalized by transducing early passage cells with a retrovirus expressing *TERT* [68]. It is uncertain if the observed chromosomal alterations were present prior to *TERT* introduction. Based on cytogenetic analysis, the TERT-PrECs generated in this study had a normal male karyotype with no gross chromosomal abnormalities, Figure 3. Critically short telomeres can induce significant genomic alterations, such as aneuploidy, non-reciprocal translocations, and copy number alterations [69–71]. Therefore, telomere-driven genomic alterations, detectable by standard karyotyping analyses, do not appear to contribute to the immortalization of the TERT-PrECs in our study. Importantly, immortalized TERT-PrECs are not tumorigenic when xenografted into intact male immunodeficient NSG mice, even in animals that were followed for more than 1 year post-inoculation.

DISCUSSION

The seminal studies by Bodnar and colleagues demonstrated that exogenous expression of telomerase in three types of normal human cells (foreskin fibroblasts, retinal pigmented epithelial cells, and endothelial cells) acquired an “extended lifespan” [23,72]; thus establishing telomere-associated replicative senescence as a barrier to proliferative capacity in normal human cells. Less than a year later, studies by Kiyono and colleagues uncovered a more complex narrative in the immortalization of normal human cells. The latter study revealed that, while human foreskin fibroblasts achieved immortalization by telomerase activity alone, immortalization of human foreskin keratinocytes and HMECs required inactivation of the p16/Rb pathway in addition to activation of telomerase [24]; thus establishing the importance of the p16 dependent, stress-induced senescence as an additional barrier to increased proliferative capacity. Several follow-up studies in numerous epithelial tissue types, especially in HMECs, PrECs, and skin keratinocytes, have since confirmed replicative senescence and stress-induced senescence as the two primary barriers to achieving limitless replicative potential in human cells [37,38,51,73–75]. Overall, the introduction of *TERT* in epithelial cells provides an extended, although still limited, lifespan; while inactivation of the p16/Rb pathway in combination with *TERT* expression immortalized these cells.

Previous studies in keratinocytes and HMECs have demonstrated that co-culture with fibroblast feeder layers suppresses stress-induced senescence; thus permitting immortalization by *TERT* before p16 levels significantly increase [32,39]. Our studies in PrECs confirm that immortalization is achievable with *TERT* expression alone, provided that p16 levels are low and all cells have not yet senesced. Previous attempts to immortalize PrECs using retroviral-mediated *TERT* introduction reported to be unsuccessful, despite having detectable telomerase activity [76]. Potentially, p16 levels may have been significantly elevated prior to telomerase introduction; therefore, preventing immortalization. As stated above, despite viral transduction of *TERT*, we observed that once

the population of PrECs was committed to senescence, they were unable to achieve unlimited proliferation.

Early studies by Geron Corporation observed that one clone of a *TERT* immortalized retinal pigmented epithelial cell line had undetectable levels of p16 expression after approximately 100 population doublings, while another *TERT* immortalized clone that had undergone a similar number of population doublings had p16 levels comparable to the late passage parental line. The authors attributed this phenomenon to clonal variation, perhaps in components of the p16/Rb pathway downstream of p16 [77]. HMECs in culture have been reported to bypass stress-induced senescence by methylation of the p16 promoter; thus suppressing p16 expression and resulting in hyper-phosphorylation of Rb [78]. Previous reports of telomerase-immortalized human keratinocytes [79] and HMECs [24,80] were accompanied by spontaneous methylation of the p16 promoter. Similarly, the *TERT*-PrECs in our studies may also have repressed p16 expression; however, the methylation status of the p16 promoter is unknown. While the mechanism of p16 suppression observed here has yet to be characterized, we propose two potential explanations permitting *TERT* immortalization of PrECs, Figure 4. In both models, prior to *TERT* introduction, early passage cells are able to proliferate. In Model A, following a limited number of cell passages, a subset of cells activates the p16/Rb pathway in response to the stresses of tissue culturing (e.g., abnormal levels of nutrients, growth factors, and oxygen [29]) and undergo stress-induced senescence (stasis). Additional passaging of the remaining proliferating cells eventually succumbs to replicative senescence due to activation of the p53 pathway in response to critically shortened telomeres, Figure 4, Model A. In contrast, introduction of *TERT* to early passage PrECs prior to stress-induced senescence immortalizes the subset of cells that would have otherwise undergone replicative senescence. In the second scenario, the introduction of *TERT* expression in pre-senescent cells prevents or bypasses activation of the p16/Rb and p53 pathways; thus bypassing both replicative and stress-induced senescence, Figure 4, Model B. The literature seems to support the plausibility of the former explanation (Model A), rather than the latter (Model B).

CONCLUSION

Transduction with the lentiviral *TERT* expression vector developed by Salmon et al. [49] provides a robust method for the routine generation of immortalized human PrECs, which maintain a normal karyotype and are non-tumorigenic. Immortalized *TERT*-PrECs generated with this approach provides an appropriate and experimentally facile model for clarifying the molecular mechanism(s) involved in immortalization of human prostatic epithelial cells. Furthermore, since *TERT*-PrECs are non-tumorigenic, this cell model can also be used to study the genetic/epigenetic changes required to produce lethal cancers when inoculated in intact male immunodeficient NSG mice. Such studies would help identify the “drivers” for conversion of these immortalized non-tumorigenic cells into fully lethal prostate cancers. A notable advantage of these *TERT*-PrECs is that the virally transduced *TERT* gene is excisable from these cells. The coding cassette, which includes *TERT*, is engineered with flanking *loxP* sequences that are susceptible to Cre recombinase. The ability to excise *TERT* in this cell model allows for the opportunity to study p16/Rb, p53, and telomeres when exogenous telomerase activity is removed from these cells.

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Fig. 1.

A: Phase contrast image of PrECs grown in culture after three passages and stained for β -galactosidase expression. **B:** Phase contrast image of PrECs after nine passages and stained for β -galactosidase activity. Examples of cells staining positive for β -galactosidase activity are indicated with small arrows.

Fig. 2.

A: Western blots of p16 protein levels from cell lysates collected from PrECs at different passages. Each lane was loaded with 10^5 cells. **B:** Number of population doublings of untreated PrECs and PrECs transduced at passage 6 with a *TERT* lentiviral expression vector (TERT-PrECs). **C:** Western blot of p16 protein levels from cell lysates collected from PrECs and TERT-PrECs. Each lane was loaded with 10^5 cells. **D:** Western blot of p16 and p27 protein levels from cytoplasmic and nuclear extracts. Beta-catenin, which is predominately expressed in the cytoplasm, was used as a loading and cell compartment specificity control. Cells were treated with and without growth factors as indicated.

Fig. 3.
Representative normal 46X,Y male karyotype of *TERT* immortalized PrECs this study.

Fig. 4.

Two proposed models permitting *TERT* immortalization of PrECs. In Model A, a subset of PrECs activates the p16/RB pathway in response to tissue culturing. The remaining proliferating cells eventually undergo replicative senescence due to activation of the p53 pathway in response to critical telomere shortening. Introduction of *TERT* to early passage PrECs prior to stasis immortalizes the subset of cells that would have otherwise undergone replicative senescence. In Model B, all cells senesce by activating either the p16/Rb pathway

or the p53 pathway. Introduction of *TERT* to early passage PrECs allows cells to bypass both stasis and replicative senescence.

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