

Telomerase Induces Immortalization of Human Esophageal Keratinocytes Without p16^{INK4a} Inactivation

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Abstract

Normal human somatic cells have a finite life span and undergo replicative senescence after a limited number of cell divisions. Erosion of telomeric DNA has emerged as a key factor in senescence, which is antagonized during cell immortalization and transformation. To clarify the involvement of telomerase in the immortalization of keratinocytes, catalytic subunit of telomerase (hTERT) expression was restored in normal human esophageal epithelial cells (EPC2). EPC2-hTERT cells overcame senescence and were immortalized without p16^{INK4a} genetic or epigenetic alterations. p16^{INK4a} was expressed at moderate levels and remained functional as evidenced by induction with UV treatment and binding to cyclin-dependent kinase 4 and 6. There were no mutations in the p53 gene, and p53 was functionally intact. Importantly, senescence could be activated in the immortalized EPC2-hTERT cells by overexpression of oncogenic H-ras or p16^{INK4a}. Furthermore, the EPC2-hTERT cells yielded basal cell hyperplasia in an innovative organotypic culture system in contrast to a normal epithelium from parental cells. These comprehensive results indicate that the expression of telomerase induces immortalization of normal human esophageal keratinocytes without inactivation of p16^{INK4a}/pRb pathway or abrogation of the p53 pathway.

Introduction

Immortalization is a necessary step toward malignant transformation of normal human somatic cells, which have

intrinsic mechanisms that monitor cell divisions and limit their life span. Progressive shortening of telomeres with each cell division has been found to limit replicative life span of human cells in culture (1–4).

Telomeres are structures at the ends of mammalian chromosomes that provide a protective function, preventing end-to-end chromosomal fusions (5–8). Because conventional DNA polymerase replicates DNA only in the 5′-3′ direction, the so-called DNA end replication problem would occur on lagging strands at the ends of chromosomes. Because no DNA is synthesized beyond the last RNA primer hybridized to the DNA template, removal of the RNA primer generates a persisting gap. Moreover, 100–200 nucleotides of the telomeric DNA are removed from both ends of chromosomes with each round of replication, perhaps due to the action of a 5′-3′ exonuclease (9). When the telomere length reaches a critical threshold, short telomeres are thought to be recognized as damaged DNA and evoke a permanent cell cycle arrest that is termed replicative senescence (10). Cells lacking mechanisms that maintain telomeres ultimately enter a cell death phase termed crisis.

Independent of replicative age, factors such as oncogenic activation, DNA damage, chromatin remodeling, cellular stress, overexpression of cyclin-dependent kinase (Cdk) inhibitors, and deprotection of chromosome ends can also induce a phenotype resembling senescence (11–16). Senescence also has the potential to function in tumor suppression. Consistent with this notion, senescence is alleviated by ablation of either the p16^{INK4a}/pRb or the p14^{ARF}/p53 tumor suppression pathways (17).

Human somatic cells are believed to be immortalized as long as their telomeres are maintained. In fact, most human cancer cells maintain their telomeres through activation of telomerase (18, 19), while some immortalized and cancer cells employ alternative mechanisms to maintain telomeres (20–22). Enforced expression of the catalytic subunit of telomerase (hTERT) can immortalize a variety of normal human somatic cells such as fibroblasts (2), endothelial cells (23), mesothelial and retinal pigment epithelial cells (2), and mammary epithelial cells (24). However, it is controversial whether telomerase alone is sufficient to induce immortalization of human keratinocytes. The factors limiting proliferation of such cells are of particular interest, because squamous cell cancers derived from such cells are among the most common fatal malignancies worldwide and frequently demonstrate p16^{INK4a} inactivation (25–28). Some reports have concluded that

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inactivation of the p16^{INK4a}/pRb pathway is required for immortalization of human keratinocytes in addition to the expression of telomerase (29, 30). By contrast, it has been reported that tissue culture conditions using a fibroblast feeder layer system do not require inactivation of the p16^{INK4a}/pRb pathway in the induction of immortalization of human keratinocytes by ectopic expression of hTERT (31). When hTERT immortalized human oral keratinocytes were examined under the same conditions, it was observed that the ability of hTERT-expressing cells to proliferate was limited by expression of p16^{INK4a} (32).

To address the requirement for immortalization of normal keratinocytes without inactivation of p16^{INK4a}, we retrovirally transduced primary human esophageal cells or keratinocytes (esophageal epithelial cells [EPC2]) with hTERT, immediately and most importantly preceding senescence, and examined the functional roles of the p16^{INK4a}/pRb and p53 pathways in a comprehensive manner not done previously. We observed that EPC2 cells had a limited life span, whereas retroviral introduction of hTERT immortalized EPC2 (EPC2-hTERT) in standard keratinocyte growth conditions (Keratinocyte-sfm; Invitrogen, Carlsbad, CA) in the absence of a fibroblast feeder layer system. EPC2-hTERT cells overcame replicative senescence and have continuously proliferated more than 200 population doublings (PD), 5-fold longer than the life span of parental cells and without evidence of crisis. In addition, p16^{INK4a} is not mutated in coding regions, methylated in the promoter region, or lost due to allelic deletion. In fact, p16^{INK4a} is functionally intact in immortalized EPC2-hTERT cells. Moreover, there was no evidence of alteration in the p53 tumor suppressor gene. However, the senescence program can be activated in these immortalized cells through overexpression of oncogenic H-ras or p16^{INK4a}. This is unexpectedly accompanied by down-regulation of p14^{ARF}. Our results suggest that expression of hTERT is sufficient to immortalize human keratinocytes and does not require inactivation of p16^{INK4a}. Additionally, replicative senescence is suppressed through hTERT expression but can be activated by overexpression of oncogenic H-ras or p16^{INK4a}.

Results

EPC2 Cells Show Progressive Shortening of Telomere Length and Have a Limited Replicative Life Span

EPC2 cells have normal diploid status (33). In these cells, hTERT gene expression was detected in an extremely low level by reverse transcription-PCR (data not shown), and no telomerase activity was found by the telomeric repeat amplification protocol (TRAP) assay (Fig. 1A). Consistent with these observations, the length of telomeres in parental EPC2 cells shortened with passage (Fig. 1B). Three independent experiments demonstrated that EPC2 cells underwent replicative senescence by 40–44 PD, confirmed by senescence-associated β -galactosidase (SABG) staining (Fig. 2, A and B).

Ectopic hTERT Expression Induces Immortalization of EPC2 Cells

We transduced hTERT in parallel with an empty vector in EPC2 cells at 42 PD, just before senescence, to determine if

hTERT expression alone is sufficient to induce immortalization. Telomerase activity in hTERT-transduced cells was confirmed by the TRAP assay (Fig. 1A). As expected, telomeres were elongated in hTERT-expressing cells (Fig. 1B). Whereas cells transduced with the empty vector underwent senescence at 44 PD, similar to parental cells, hTERT-expressing cells did not undergo senescence and have continuously grown more than 200 PD (Fig. 2, A and B). These cells have never demonstrated a slow growth phase (30). These results were confirmed in five additional independent transduction experiments at different PD (data not shown). We next checked the karyotype of EPC2-hTERT cells and parental EPC2 cells. EPC2-hTERT cells, which overcame senescence, exhibited normal karyotype, consistent with that found in parental cells (data not shown).

Senescent and Immortalized EPC2 Cells Express Normal p16^{INK4a}

To explore the molecular basis underlying senescence of EPC2 cells, we examined the state of the p16^{INK4a}/pRb and p14^{ARF}/p53 pathways. p16^{INK4a} was strongly up-regulated in senescent EPC2 cells (Fig. 3A). Interestingly, cyclin D1 and cyclin D2 were also up-regulated in senescent cells. However, pRb expression was reduced and the protein was hypophosphorylated (Fig. 3A). Whereas p53 and p21^{WAF1/CIP1} expression was unaffected, p14^{ARF} expression decreased with serial passages and was strongly down-regulated during senescence (Fig. 3B). These findings are consistent with a p16^{INK4a}-mediated senescence.

Because inactivation of p16^{INK4a} has been reported to play a pivotal role in immortalization of human keratinocytes, we determined if p16^{INK4a} inactivation occurred in the immortalized hTERT-expressing cells. The expression of p16^{INK4a} protein remained at moderately high levels in early and intermediate PD of immortalized hTERT-expressing EPC2 cells (Fig. 3A). In late PD of immortalized cells, p16^{INK4a} expression decreased but remained higher than in early passage parental cells (Fig. 3A). We asked if this down-regulation of p16^{INK4a} found in late PD was caused by a genetic or epigenetic alteration such as deletion, mutation, or promoter hypermethylation that is frequently observed in human cancers, especially esophageal squamous cell carcinoma. DNA sequencing revealed no mutations in the coding sequence, and a PCR-based assay showed no loss of heterozygosity (LOH) adjacent to or within the p16^{INK4a} gene by a PCR-LOH assay (Table 1). We determined the methylation status of the p16^{INK4a} promoter in early and late PD immortalized cells by methylation-specific PCR (MSP). Only unmethylated DNA was found at both time points (Fig. 4A).

p16^{INK4a} is a specific inhibitor of the cyclin D1/Cdk4 or cyclin D1/Cdk6 complexes, and tumor-associated mutations regularly disrupt p16^{INK4a} binding to Cdks (34). To determine whether p16^{INK4a} expressed in immortalized cells was functional, we asked whether the protein was bound to Cdk4 and Cdk6. Cell lysates from immortalized cells were immunoprecipitated with antibodies against either Cdk4 or Cdk6 followed by immunoblotting for p16^{INK4a}. p16^{INK4a} was readily detected in such immunoprecipitates as were other Cdk inhibitors, such as p21^{WAF1/CIP1} and p27^{Kip1} (Fig. 4B). The binding of p16^{INK4a} to Cdk4 or Cdk6 was observed even in late PD of immortalized

cells (data not shown). Of note, Cdk4 and Cdk6 levels remained constant in parental or immortalized cells (Fig. 4C). We then asked whether the p16^{INK4a} promoter in immortalized cells remained responsive to stimuli known to induce p16^{INK4a} expression as in skin keratinocytes (35, 36). UV irradiation induced expression of p16^{INK4a} in EPC2-hTERT cells, demonstrating that the p16^{INK4a} promoter remains responsive in these cells (Fig. 4D). Taken together, these results indicate that the p16^{INK4a} gene and protein remained intact during immortalization of EPC2 cells induced by hTERT.

Enforced Expression of p16^{INK4a} Induces Senescence in Immortalized EPC2 Cells

An adenovirus vector expressing p16^{INK4a} was infected in late PD of EPC2-hTERT cells (Fig. 5A). Consistent with a previous report (13), the p16^{INK4a}-expressing vector shifted pRb to a hypophosphorylated status, inhibited DNA synthesis (data not shown), and induced morphological changes of flattening and spreading in 100% of cells (data not shown), whereas a control *lacZ*-expressing vector did not affect morphology and cell growth. These senescent changes were reinforced by the presence of SABG staining (Fig. 5B). Therefore, the p16^{INK4a}/pRb pathway is functional in immortalized EPC2-hTERT cells.

Immortalized EPC2-hTERT Cells Have No Changes in the p14^{ARF}/p53 Pathway

Although it has been reported that the p53 pathway is less involved in senescence of human keratinocytes than in fibroblasts and not necessarily inactivated during immortalization

of human keratinocytes (29, 30), expression of dominant negative p53 extends the life span of human keratinocytes (21). Therefore, we asked whether abrogation of this pathway is involved in immortalization of hTERT-expressing EPC2 cells. Western blot analysis showed equivalent p53 levels in parental EPC2 and immortalized EPC2-hTERT cells (Fig. 3B). There were no appreciable changes in the expression of p14^{ARF} and p21^{WAF1/CIP1} proteins during immortalization (Fig. 3B). In addition, there were no mutations found in the p53 gene by direct DNA sequencing (Table 1). We also checked the function of p53 in immortalized EPC2-hTERT cells (176 PD). Hydroxyurea and UV irradiation are known to induce DNA damage and activate p53. As shown in Fig. 6, p53 induction was observed after either UV irradiation or hydroxyurea treatment, resulting in up-regulation of p21^{WAF1/CIP1} (Fig. 6). Thus, immortalized hTERT-expressing EPC2 cells maintain an intact p53 pathway.

Additional Genetic Alterations Are Required for Transformation

Because immortalization is a critical step toward malignant transformation, we asked whether immortalized hTERT-expressing cells were prone to transformation or acquired other features of transformed cells. First, we examined the phenotype of immortalized EPC2-hTERT cells in organotypic culture. As shown in Fig. 7, EPC2-hTERT cells exhibit complete stratified epithelia with evidence of basal cell hyperplasia. However, none of the cells are dysplastic or invade through the basement membrane, characteristics of malignant transformation.

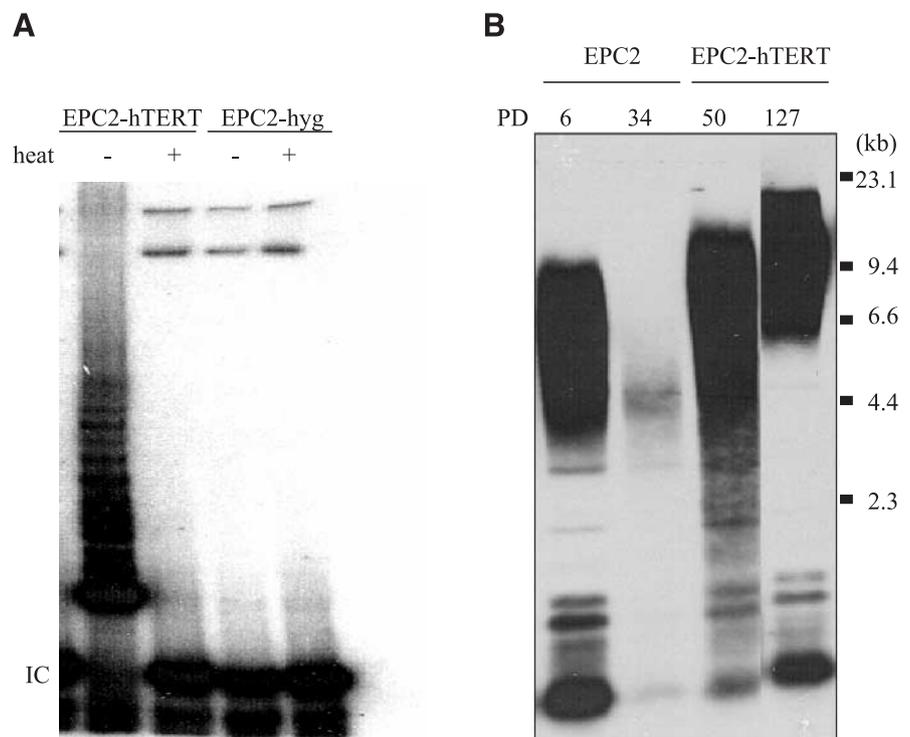


FIGURE 1. EPC2-hTERT cells have increased telomerase activity and telomere length compared with parental EPC2 cells. **A.** Cell extracts from EPC2-hTERT and EPC2-hyg cells were assayed for telomerase activity by the TRAP assay. Samples of equal mass were heat inactivated as a negative control. Strong telomerase activity was demonstrated by a 6-bp ladder in EPC2-hTERT extracts but was undetectable in extracts of EPC2-hyg cells infected with a control virus. *IC* indicates amplification of a 36-bp internal control. **B.** Telomere length was measured by Southern blot using telomeric repeats as a probe. Telomere shortenings occurred after serial passage of parental EPC2 cells. Ectopic expression of hTERT increased telomere length.

Because immortalized EPC2-hTERT cells demonstrated basal cell hyperplasia but not malignant transformation in organotypic culture, we transduced mutant *H-ras* into immortalized EPC2-hTERT cells to determine if transformation could be induced. Expression of oncogenic *H-ras* induced up-regulation of p16^{INK4a}, hypophosphorylation of pRb (Fig. 8A), and inhibition of DNA synthesis (Fig. 8B), and 100% of cells had a large and flat morphology consistent with senescence (data not shown), the latter of which was confirmed by SABG staining (Fig. 8C). Consistent with

findings in senescent parental EPC2 cells, p14^{ARF} expression was down-regulated in *H-ras*-induced senescence in immortalized EPC2-hTERT cells and accompanied by increased cyclin D1 expression (Fig. 8A). However, p21^{WAF1/CIP1} was mildly increased (Fig. 8A). Expression of oncogenic *H-ras* can transform a variety of immortalized cells but activates senescence in cells expressing functional p16^{INK4a} and p53 (12). Our immortalized EPC2-hTERT cells were resistant to transformation and remained susceptible to senescence by expression of oncogenic *H-ras*, presumably due to the

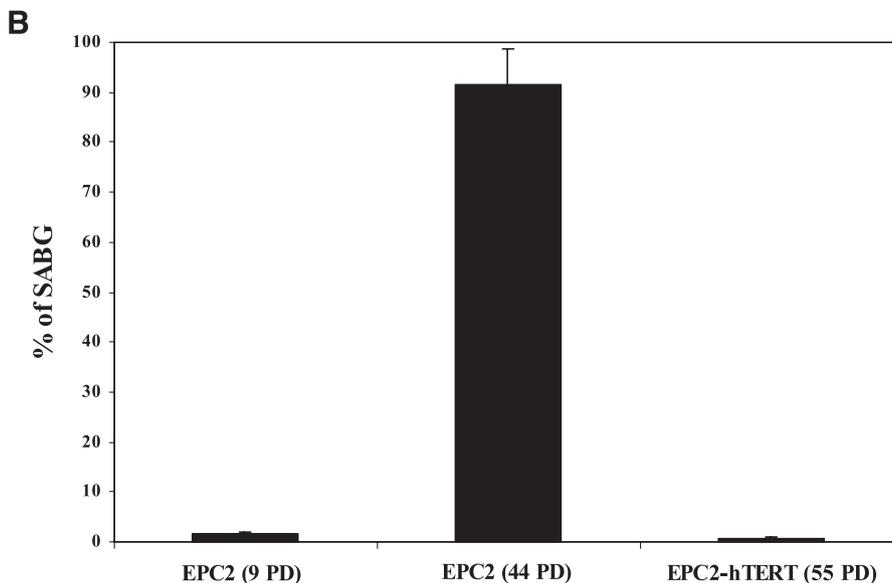
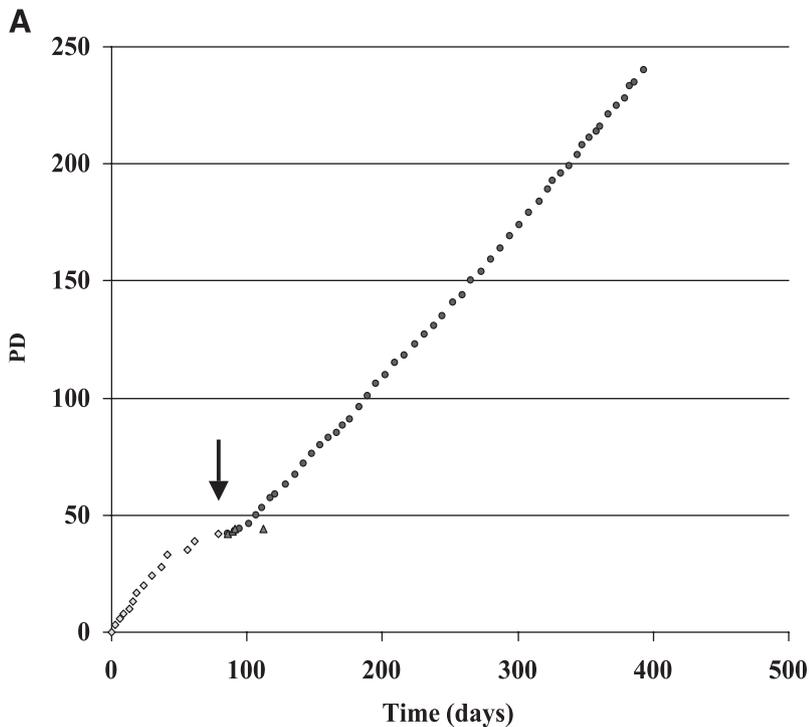


FIGURE 2. Ectopic expression of hTERT induces immortalization of EPC2 cells. **A.** The downward arrow indicates the time of transduction. While EPC2-hyg cells underwent senescence at a similar PD to parental cells, EPC2-hTERT cells overcame senescence and continuously grew, without a slow growth phase. ◇, EPC2; ●, EPC2-hTERT; ▲, EPC2-hyg. **B.** Senescence is evaluated by SABG staining in EPC2 cells (9 and 44 PD) and EPC2-hTERT cells (55 PD) and depicted in a quantitative fashion. Bars, SD.

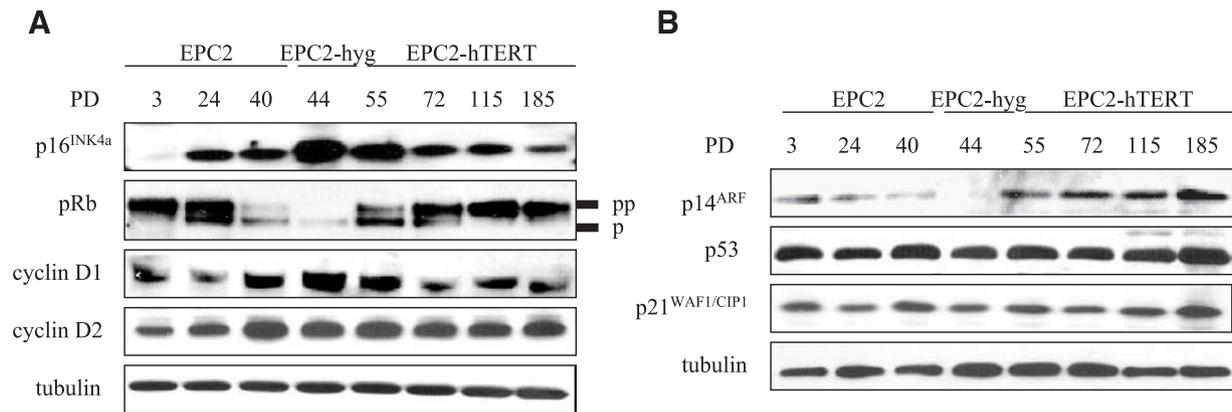


FIGURE 3. Expression and functional analysis of the p16^{INK4a}/pRb pathway and the p14^{ARF}/p53/p21^{WAF1/CIP1} pathway in EPC2, EPC2-hyg, and EPC2-hTERT cells. Western blot for (A) p16^{INK4a}, pRb phosphorylation status, cyclin D1, and cyclin D2 and for (B) p14^{ARF}, p53, and p21^{WAF1/CIP1} in serial passages of parental EPC2, EPC2-hyg, and EPC2-hTERT cells. Hyperphosphorylation (pp) and hypophosphorylation (p) forms of pRb are indicated. Tubulin served as a protein loading control. The esophageal cancer cell line TE11, which has a mutated p53 gene, was used as a positive control for the detection of p53 protein (data not shown).

expression of functional p16^{INK4a}. Supporting the observed resistance of immortalized EPC2-hTERT cells to transformation by oncogenic H-*ras*, we did not find up-regulation of *c-myc* by Western blot analysis (data not shown). *c-myc* can cooperate with oncogenic *ras* to induce transformation and has been reported to be up-regulated in cells immortalized by hTERT (37). These results verify that the p16^{INK4a}/pRb response pathway is intact in immortalized cells.

Discussion

Because somatic cells do not express telomerase, telomeres progressively shorten with each cell division. Telomeric attrition causes disruption of the telomere structure, which normally protects the ends of chromosomes. Critically short telomeres are then thought to trigger the process of replicative senescence (1–3). Consistent with this notion, telomeres have been found to be short in a variety of senescent human cells (3, 38, 39). Parental EPC2 cells approaching senescence have short telomeres and no detectable telomerase activity. Although the mechanisms through which short telomeres are recognized remain to be fully elucidated, cell cycle checkpoint proteins such as p16^{INK4a}, pRb, and p53 have been reported to be involved in cell cycle arrest during senescence (17, 40, 41). Up-regulation of p16^{INK4a} and hypophosphorylation of pRb were observed in EPC2 cells, without accumulation of p53. These results suggest that the p16^{INK4a}/pRb pathway fosters senescence in human keratinocytes. Furthermore, we found up-regulation of cyclin D1 and cyclin D2 in senescent cells. It is conceivable that cyclin D1 and cyclin D2 in this context bind to and inactivate Cdk2 (42–44). Thus, there may be a potential contribution of high cyclin D1 and cyclin D2 levels in promoting senescence in this pathway for esophageal keratinocytes, although, generally, cyclin D1 and cyclin D2 will promote proliferation or extend the replicative life span of cells in other contexts.

In addition, a senescence phenotype can also be induced by aberrant expression of oncogenes, damaged DNA, chromatin

remodeling, cellular stress, overexpression of Cdk inhibitors, or deprotection of chromosome ends (11–16, 45). In this study, we observed a senescence phenotype in immortalized EPC2-hTERT cells with enforced expression of either p16^{INK4a} or oncogenic H-*ras*. Interestingly, senescence induced by oncogenic H-*ras* also revealed up-regulation of p16^{INK4a} and hypophosphorylation of pRb, without accumulation of p53. This result supports a previous report showing that p16^{INK4a}-deficient human fibroblasts are resistant to oncogenic *ras*-induced senescence (46). p21^{WAF1/CIP1} was mildly up-regulated during activation of senescence by oncogenic H-*ras*. Interestingly, p21^{WAF1/CIP1}-associated senescence results in a different cellular morphology than morphological changes associated with p16^{INK4a}/pRb-induced senescence (47). By contrast, we observed morphological differences between cells associated with replicative senescence compared with oncogenic H-*ras*-activated senescence (data not shown). Our present results suggest that both telomere-dependent and telomere-independent senescence is preferentially regulated through the p16^{INK4a}/pRb pathway in human keratinocytes.

We also observed down-regulation of p14^{ARF} in telomere-dependent replicative senescence in parental EPC2 cells and telomere-independent oncogenic H-*ras*-activated senescence in immortalized EPC2-hTERT cells. Accordingly, it has been previously reported that senescent epidermal keratinocytes express little p14^{ARF} (48). It is conceivable that hypophosphorylated pRb results in decreased E2F activity and therefore

Table 1. Genetic and Epigenetic Analysis

	EPC2, EPC2-hyg	EPC2-hTERT
p16 ^{INK4a} mutations	ND	N (80 PD, 185 PD)
p16 ^{INK4a} LOH	ND	N (106 PD, 185 PD)
p16 ^{INK4a} methylation	N	N (185 PD)
p53 mutations	N	N (80 PD)

N, not found; ND, not determined.

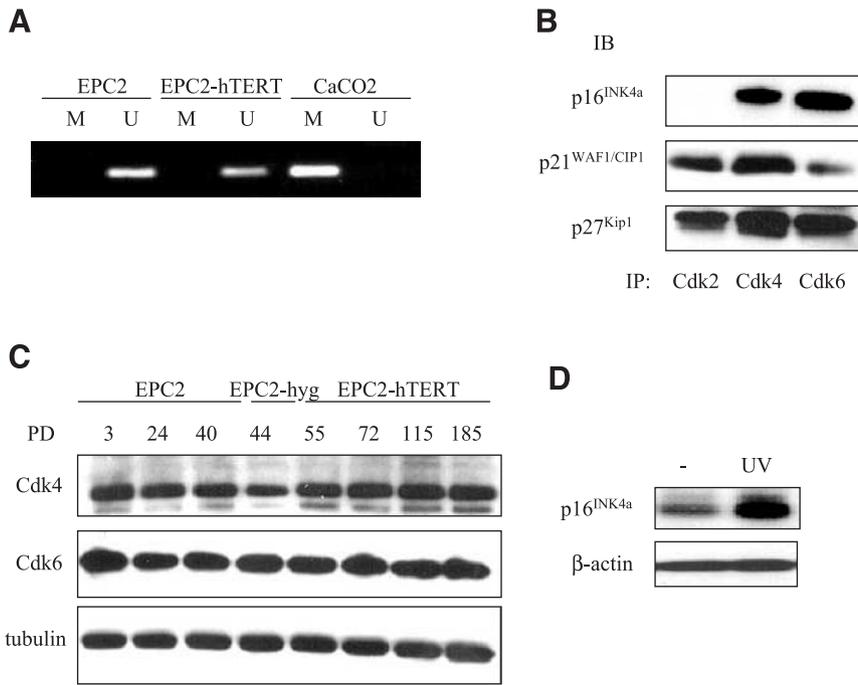


FIGURE 4. p16^{INK4a} promoter and functional assays in immortalized EPC2-hTERT cells. **A.** MSP (unmethylated [U] or methylated [M]) of the p16^{INK4a} gene was evaluated on 2% agarose gel stained with ethidium bromide. The p16^{INK4a} promoter was not methylated in EPC2-hTERT cells. CaCO2 colon cancer cells served as a positive control for methylation. **B.** Immunoprecipitations (IP) of EPC2-hTERT at 68 PD with Cdk2, Cdk4, or Cdk6 antibody were immunoblotted (IB) and probed with p16^{INK4a}, p21^{WAF1/CIP1}, or p27^{Kip1} antibody. p16^{INK4a} expressed in immortalized EPC2-hTERT cells maintained the ability to bind Cdk4 or Cdk6. **C.** Western blot for Cdk4 and Cdk6 remained expressed in serial passages. Tubulin served as a protein loading control. **D.** EPC2-hTERT cells (176 PD) were treated with 60 J/m² of UV. Expression of p16^{INK4a} was up-regulated after UV treatment in immortalized EPC2-hTERT cells. β-actin served as a protein loading control.

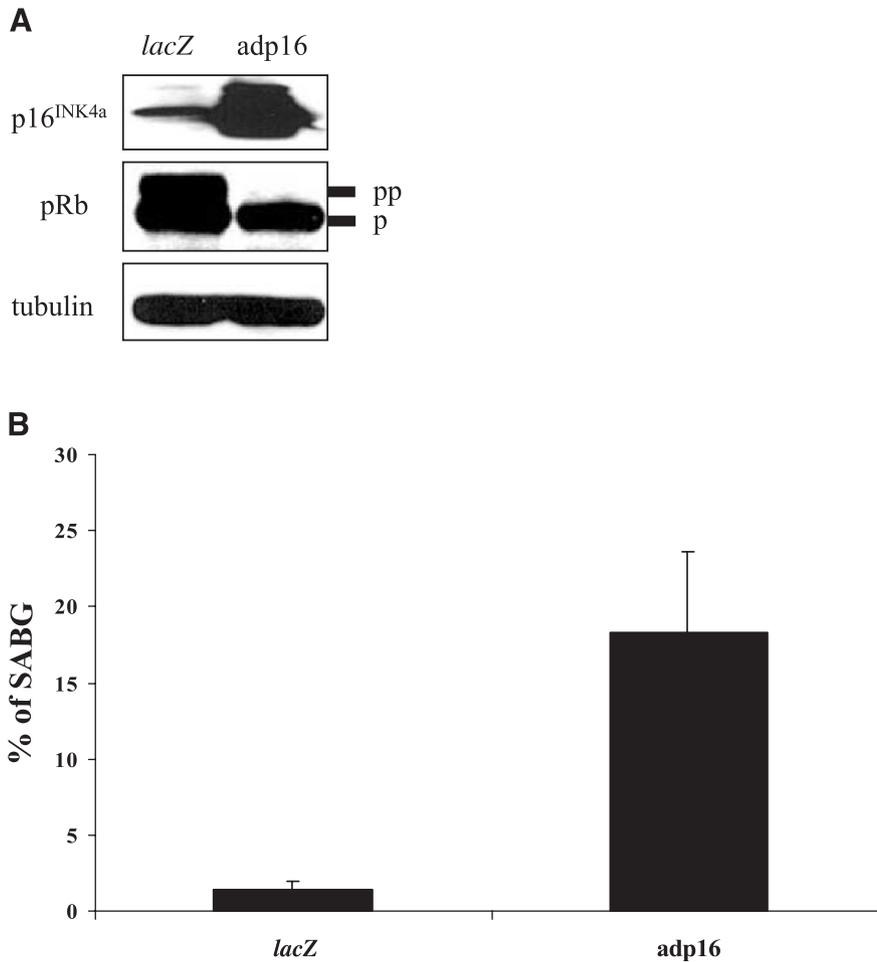


FIGURE 5. Adenoviral overexpression of p16^{INK4a} in immortalized EPC2-hTERT cells. **A.** Cell lysates were prepared from EPC2-hTERT cells at 215 PD infected with 100 multiplicities of infections of adenovirus vector expressing either p16^{INK4a} (*adp16*) or *lacZ* 72 h after infection. Enforced expression of p16^{INK4a} induced hypophosphorylation of pRb in EPC2-hTERT cells. Hyperphosphorylation (pp) and hypophosphorylation (p) forms of pRb are indicated. Tubulin was served as a protein loading control. **B.** Enforced expression of p16^{INK4a} induced senescence in immortalized EPC2-hTERT cells. Senescence cells were evaluated 2 weeks after adenovirus infection by positive SABG staining. Bars, SD.

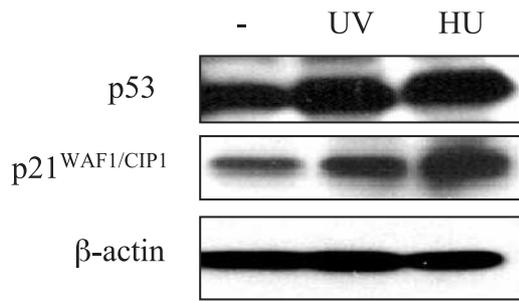


FIGURE 6. DNA damage activates the p53 pathway. UV and hydroxyurea (HU) treatment induced stabilization of p53 in EPC2-hTERT cells (176 PD), resulting in induction of p21^{WAF1/CIP1}. β-actin served as a protein loading control.

decreased p14^{ARF} transcription (49). One apparent consequence of down-regulation of p14^{ARF} during senescence is that p53 is not induced, although p53 could still be induced and targeted for subsequent degradation by MDM2.

Several lines of evidence reveal that the reconstitution of telomerase into certain human somatic cells prevents telomeric attrition and senescence, resulting in immortalization (2, 19, 23). By contrast, ectopic expression of hTERT in human keratinocytes was insufficient in some studies to induce immortalization, suggesting that they have telomere-independent mechanisms that limit their life span (29, 30, 32). In this study, we have demonstrated that immortalization of human esophageal keratinocytes can be mediated by ectopic expression of hTERT immediately preceding senescence. The immortalized cells have intact p16^{INK4a}/pRb and p53 pathways, suggesting that ectopic expression of telomerase is sufficient to induce their immortalization and that inactivation of the p16^{INK4a}/pRb pathway is not necessary. Thus, our data indicate that keratinocytes do not possess a telomere-independent clock that activates p16^{INK4a}-mediated replicative senescence. Differences in biological properties between esophageal and other keratinocytes, such as those originating in the epidermis, oral cavity, cornea, and conjunctiva (29, 30, 32), may include donor age and latent diseases. These and other factors may account in part for the observed discrepancies in keratinocyte response and tissue culture conditions, which reportedly abolished the requirement of the p16^{INK4a} inactivation for immortalization of epidermal keratinocytes and mammary epithelial cells (24, 31). Another possibility may relate to the timing of hTERT overexpression in human oral keratinocytes that

transduced hTERT in early PD as represented in previous studies, whereas we transduced hTERT just before replicative senescence. Our study is the first to provide a thorough evaluation of the integrity of the p16^{INK4a} pathway during hTERT-induced immortalization, which had been lacking in previous studies with oral or epidermal keratinocytes.

Because hTERT expression has been detected even in squamous cell carcinoma *in situ* (50), telomerase reactivation might take place as an early step in the development of esophageal squamous cell cancer. This is supported by findings that 80–100% of esophageal cancers express telomerase (51, 52). Extended cell replication facilitated by telomerase might increase the risk of other genetic or epigenetic alterations, leading to malignant transformation. In fact, EPC2-hTERT cells (late PD) demonstrated basal cell hyperplasia in organotypic culture but were not yet transformed. Thus, hTERT expression appears to be important for immortalization but insufficient for malignant transformation (53, 54), consistent with the failure of oncogenic *H-ras* to transform immortalized EPC2-hTERT cells. Whether hTERT expression acts exclusively through telomere elongation in EPC2 cells or mediates other functions remains to be determined (55, 56).

In summary, replicative senescence in human esophageal keratinocytes depends on telomere length and is induced by the p16^{INK4a}/pRb pathway. Ectopic hTERT expression is sufficient to induce immortalization of human esophageal keratinocytes without inactivation of p16^{INK4a} or abrogation of p53, which is underscored by the robustness of our data and not done in a comprehensive fashion previously. Importantly, the senescence program can be imposed in immortalized human esophageal keratinocytes by overexpression of p16^{INK4a} or oncogenic *H-ras*, indicating that the senescence program remains intact.

Materials and Methods

Cell Culture, Retroviral Vectors, and Retroviral Transduction

Normal human esophageal keratinocytes (EPC2) were isolated and cultured in Keratinocyte-sfm (Invitrogen) (33). Organotypic culture was performed as previously described (33). The pBabe hygromycin-resistant retrovirus vector expressing hTERT was obtained from R. Weinberg (Whitehead Institute, Cambridge, MA). The pBabe puromycin-resistant retrovirus vector expressing *H-ras* V12 was obtained from S. Lowe (Cold Spring Harbor Laboratory, Cold Spring Harbor,

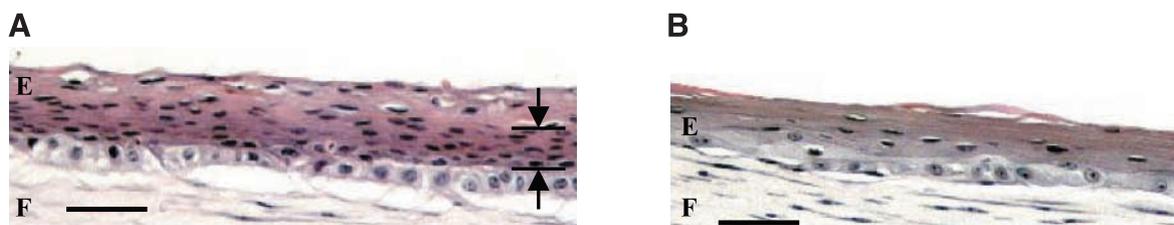


FIGURE 7. H&E staining of organotypic culture. Organotypic culture demonstrates basal cell hyperplasia (arrows) in EPC2-hTERT cells (A) versus a normal phenotype in parental EPC2 cells (B). Squamous epithelium (E) and fibroblasts (F) in collagen matrix are indicated. (Scale bar corresponds to 50 μm.)

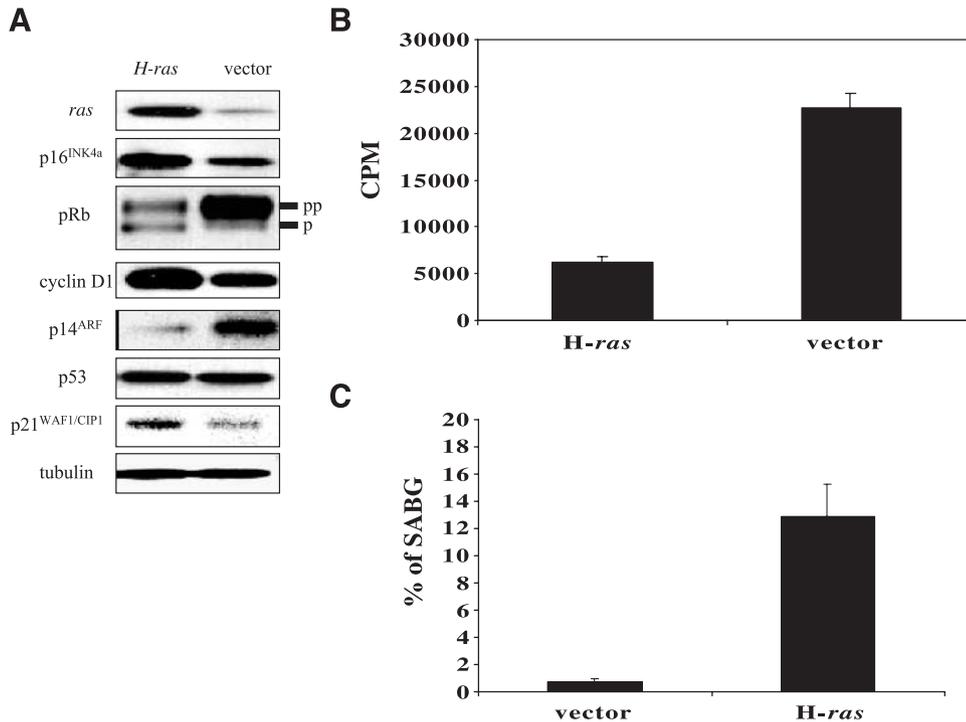


FIGURE 8. Retroviral over-expression of H-ras activates a senescence phenotype in immortalized EPC2-hTERT cells. **A.** Expression of the *ras*, p16^{INK4a}, pRb, cyclin D1, p14^{ARF}, p53, and p21^{WAF1/CIP1}. Cell lysates were prepared from EPC2-hTERT at 215 PD infected with a retrovirus vector expressing oncogenic H-ras or empty vector on day 13 after infection. Hyperphosphorylation (pp) and hypophosphorylation (p) forms of pRb are indicated. Tubulin served as a protein loading control. **B.** Thirteen days after infection, cells were incubated with the growth media containing [³H]thymidine for 24 h. [³H]Thymidine incorporation assay indicated inhibition of DNA synthesis in EPC2-hTERT cells by expression of H-ras. Bars, SD. **C.** Transduction of H-ras induced senescence in immortalized EPC2-hTERT cells. Senescent cells were confirmed by positive SABG staining on day 13 after adenoviral infection. Bars, SD.

NY). Each vector and empty vectors were transfected into phoenix retrovirus producer cells (33). Retroviral supernatants were collected 48 and 72 h posttransfection. Parental EPC2 cells were prepared for retroviral transduction at 42 PD, just before senescence. After transduction with either pBabe-hygro-hTERT (EPC2-hTERT) or pBabe-hygro (EPC2-hyg), cells were incubated with 10 μ g/ml of hygromycin B (Roche, Basel, Switzerland). Clones were pooled and expanded. For H-ras infection, immortalized EPC2-hTERT cells at 215 PD were used and incubated with 0.5 μ g/ml of puromycin.

Adenoviral Vectors and Infection

Recombinant adenoviral vectors expressing p16^{INK4a} and *lacZ* were generated as previously described (57). We prepared EPC2-hTERT cells at 215 PD. Cells (3×10^5) were plated on 6-cm dishes 24 h before infection. Infection was performed by adding 100 multiplicities of infections of adenovirus.

Determination of Replicative Life Span

Parental EPC2, EPC2-hTERT, and EPC2-hyg cells were routinely maintained in subconfluent conditions. The number of PD was determined using the following formula: PD = $\log(N/N_0) / \log 2$, where *N* is the number of collected cells and *N*₀ is the number of seeded cells.

[³H]Thymidine Incorporation Assay

We prepared 5×10^4 H-ras-transduced cells and empty vector-transduced cells per well into 12-well plates 24 h before the assay. Growth media containing 1 μ Ci/ml of methyl-

[³H]thymidine were added to the cells and incubated for 24 h. The cells were lysed with 0.5-N NaOH, and high molecular weight nucleic acid was precipitated with cold 10% TCA. Precipitated cells were collected by filtration through Whatman GF/C filters (Clifton, NJ). Dried filters were placed into Ready Value Liquid Scintillation Cocktail, and the radioactivity was counted with LS6500 Multi-Purpose Scintillation Counter. The assay was done in a triplicate fashion.

Senescence-Associated β -Galactosidase Activity

Senescent cells were identified by SABG staining using the Senescence β -Galactosidase Staining Kit (Cell Signaling, Beverly, MA) according to the manufacturer's protocol. Stained cells were scored by counting five high-power fields under light microscopy.

Telomerase Activity and Telomere Length Assays

Telomerase activity was determined by the TRAP assay using TRAPEZE Telomerase Detection Kit (Intergen Co., Purchase, NY). Telomere length was measured by Southern blot of *Hinf*I- and *Rsa*I-digested genomic DNA from parental and derivative cells using the Telomere Length Assay Kit (PharMingen, San Diego, CA).

DNA Damage Treatment

UV irradiation was performed using a Stratallinker 1800 UV Crosslinker (Stratagene, La Jolla, CA) with 60 J/m², and protein was extracted after 24 h. Hydroxyurea treatment was done at 20 mM for 48 h.

Western Blot Analysis

Ten micrograms of protein from whole-cell extracts were separated by SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Billerica, MA). The membranes were incubated with antihuman p16^{INK4a} (PharMingen), G3-245 against pRb (PharMingen), DCS-6 against cyclin D1 (PharMingen), G132-43 against cyclin D2 (PharMingen), H-22 against Cdk4 (Santa Cruz, Santa Cruz, CA), C-21 against Cdk6 (Santa Cruz), 14P02 against p14^{ARF} (Oncogene Science, Boston, MA), DO-1 against p53 (Oncogene Science), EA10 against p21^{WAF1/CIP1} (Oncogene Science), Kip1/p27 (Transduction Laboratories, Lexington, KY), AC-74 against β -actin (Sigma Chemical Co., St. Louis, MO), and DMA1A + DM1B against tubulin (Neo Markers, Union City, CA). Staining was detected by using ECL Plus (Amersham, Piscataway, NJ).

Immunoprecipitation

Cell lysates were prepared by lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, protease inhibitors cocktail [complete, Mini, Roche]) and incubated with anti-Cdk2 (M2; Santa Cruz), Cdk4 (H-22, Santa Cruz), or Cdk6 (C-21, Santa Cruz) antibody. Bound proteins were collected on Protein G Agarose (Invitrogen). Pellets were washed three times with wash buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl) and resuspended in electrophoresis sample buffer.

LOH Analysis

Genomic DNA was extracted from EPC2 and EPC2-hTERT cells using the PUREGENE DNA Isolation Kit (Gentra Systems, Minneapolis, MN). Three microsatellite markers (D9S126, D9S1748, and D9S162) were used to detect LOH in the *CdkN2/INK4a* locus. PCR mixtures contained 25-ng genomic DNA, 10 pmol each of forward primer labeled with [³²P]ATP and reverse primer, 1.5-mM MgCl₂, 200- μ M deoxy-nucleotide triphosphates, and 0.5-U Taq DNA polymerase. After denaturation at 94°C for 5 min, PCR was performed with 35 cycles at 94°C for 15 s, 65°C for 15 s, and 72°C for 30 s, with a final extension at 72°C for 7 min. After heat denaturation at 95°C for 5 min, PCR products were loaded on 6% denaturing polyacrylamide gels. Gels were dried and exposed to X-ray film for evaluation of LOH.

Methylation-Specific PCR

The methylation status of the p16^{INK4a} gene promoter was examined by MSP. Bisulfite modification of genomic DNA and MSP were performed as published previously (58). Primer sets used for MSP were 5'-CAGAGGGTGGGGCGGACCGC-3' and 5'-CGGGCCGCGGCCGTGG-3' for unmodified DNA, 5'-TTATTAGAGGGTGGGGCGGATCGC-3' and 5'-GACCCC-GAACCGCACCCTAA-3' for methylated DNA, and 5'-TTATTAGAGGGTGGGGTGGATTGT-3' and 5'-CAACCC-CAAACCAACCATAA-3' for unmethylated DNA (58). DNA extract from CaCO₂ was served as a positive control of methylated promoter (59).

DNA Mutational Analysis

We amplified exons 3–9 of the p53 gene and exons 1–3 of the p16^{INK4a} gene from genomic DNA and sequenced the PCR products for detection of any mutations in coding sequences.

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