Keratinocyte Growth Conditions Modulate Telomerase Expression, Senescence, and Immortalization by Human Papillomavirus Type 16 E6 and E7 Oncogenes

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ABSTRACT

Keratinocytes undergo a finite number of divisions in culture before senescing. The high-risk human papillomavirus (HPV) E6 and E7 oncoproteins prevent keratinocyte senescence and extend life span by interacting with p53 and pRb, respectively, and also by transcriptionally activating the human telomerase reverse transcriptase (hTERT) gene, which encodes the catalytic subunit of telomerase. We correlated telomerase activity, which was measured by a highly sensitive and quantitative real-time quantitative-PCR-based telomeric repeat amplification protocol assay, with telomere length and the expression of hTERT, p16^{INK4a}, and HPV-16 E6 and E7 in keratinocytes grown under two culture conditions. Primary human foreskin keratinocytes (HFKs) cultured in keratinocyte serum-free medium on plastic senesced at ~13 population doublings (PDs). Senescence was accompanied by a dramatic increase in p16^{INK4a} levels, a marked decrease in telomerase, and only a slight decrease in telomere length. In contrast, HFKs grown in F medium on 3T3 fibroblast feeders maintained elevated telomerase and lower levels of p16^{INK4a} for 60 PDs before senescing ~81 PDs. E7 was shown to act synergistically with E6 to super induce telomerase expression in a feeder environment-dependent manner. Culture of both HFKs and HFK/16E6E7 cells in the feeder environment significantly increased the number of doublings that these cells could undergo without a significant reduction in telomere length. Finally, transfer of either HFKs or HFK/16E6E7 cells from plastic to the feeder fibroblast culture system significantly induced telomerase activity. This induction in telomerase was fully reversible and largely attributable to the medium. Our results suggest that the influence of keratinocyte culture conditions on the expression of telomerase and p16^{INK4a} and on telomere maintenance is responsible, at least partially, for the differences in proliferative capacity, senescence, and HPV-keratinocyte interactions seen in the two culture systems.

INTRODUCTION

Most adult somatic cells have very low or absent telomerase activity. In vitro propagation of primary cells is inevitably accompanied by progressive telomere shortening, which has been identified to be the critical determinant of senescence in human fibroblasts and retinal pigment epithelial cells. Introduction of hTERT,1 the catalytic subunit of telomerase, into these cells restores telomere length and allows the cells to bypass senescence (1, 2). Keratinocytes also undergo a finite number of divisions in culture before they culminate in replicative senescence. Expression of high-risk HPV E6 or E7 oncoproteins significantly extends keratinocyte life span, and expression of both of these proteins completely overcomes the senescent blockade leading to indefinite proliferation and immortalization of keratinocytes [reviewed by Munger and Howley (3)]. Although exogenous expression of hTERT is sufficient to maintain telomere length, it is insufficient to immortalize human keratinocytes by itself, indicating a cell type-specific regulation of the cellular senescence program (4, 5). Several lines of evidence have suggested that p16^{INK4a} is the primary mediator of replicative senescence in keratinocytes. Both inactivation of p16^{INK4a} and expression of telomerase are required to immortalize keratinocytes (4–6). Immortalization of keratinocytes by high-risk HPV E6 and E7 oncoproteins is due partially to their interactions with the cell cycle-regulatory proteins p53 and pRb, respectively (7–11). Abrogation of pRb function by E7 blocks p16^{INK4a}-mediated senescence. E6 reduces p53 levels by targeting it for degradation by the proteasome. In addition, the HPV E6 protein induces telomerase activity through transcriptional activation of the hTERT promoter via p53-independent regulatory mechanisms (12, 13). However, there are indications that E6 is not the sole determinant of telomerase activity because the dramatic rise in hTERT mRNA levels and telomerase activity in late passage HPV-16 E6E7-transduced cervical epithelial cells is not accompanied by an increase in E6 protein expression (14).

Several keratinocyte culture systems have been used to investigate both keratinocyte and papillomavirus biology. These culture systems exhibit significant differences with regard to keratinocyte proliferation, differentiation, and senescence, as well as HPV-keratinocyte interactions. Propagation of keratinocytes was first made possible by growing the cells on mitotically inactivated fibroblast feeders in serum-containing medium (15). This culture system closely resembles the in vivo physiological environment of keratinocytes and is unique in maintaining HPV genomes extrachromosomally (16). Keratinocyte culture was additionally simplified by the development of a serum-free growth medium, which supports proliferation of keratinocytes on plastic substrates in the absence of feeder fibroblasts (17). A third system, organotypic (raft) culture, promotes stratification and full differentiation of keratinocytes. As a result, raft cultures support the full papillomavirus life cycle, including vegetative viral DNA replication and virus production (18).

The choice of culture system can have a significant influence on studies of keratinocyte senescence and immortalization. This has led to a number of discrepancies in the literature. One study suggests that keratinocytes undergo irreversible p16^{INK4a}-related senescence in both the feeder system and the serum-free culture system (19). In contrast, Wright et al. (20) proposed that p16^{INK4a}-initiated senescence could be avoided by growth of keratinocytes in the feeder system. In the presence of feeder fibroblasts, keratinocytes have been shown to grow up to ~50 PDs (21), whereas keratinocytes demonstrate a replicative life span of <20 PDs when cultured in serum-free medium (4, 22). These differences highlight the role of culture conditions in the regulation of proliferation and senescence in keratinocytes.

In this study, we have investigated the effects of culture conditions on cellular senescence and on the immortalization of keratinocytes by high-risk HPV oncoproteins. We also examined the correlation between HPV-16 E6/E7 expression, telomerase activity, and telomere length. Primary HFKs and HPV-16 E6E7-transduced HFKs (HFK/16E6E7 cells) were serially passaged either in K-SFM on plastic (in short, on plastic) or in F medium on 3T3 fibroblast feeders (on feeder fibroblasts) and monitored for expression of HPV-16 E6 and E7, hTERT, p16^{INK4a}, and for telomerase activity and telomere length. Telomerase
activity was accurately measured using a very sensitive and quantitative real-time Q-TRAP assay developed in our lab. Our experiments demonstrated that growth of keratinocytes on feeder fibroblasts reversibly induced telomerase and repressed p16INK4a expression, leading to slower telomere erosion, enhanced proliferative capacity, and an extended life span. This induction of telomerase was mainly attributable to the culture medium rather than the feeder fibroblasts. The HPV-16 E6 protein was capable of inducing telomerase activity in both culture systems, consistent with published studies (12, 22, 23). In contrast, expression of both E6 and E7 led to a superinduction of telomerase activity and maintenance of telomere length, but this synergism between E6 and E7 was seen only in the feeder culture system. Expression of E7 alone had no effect on telomerase expression in either culture system. Finally, HPV-16 E6/E7 expression did not always correlate with hTERT levels, suggesting that additional factors are important for hTERT regulation.

**MATERIALS AND METHODS**

**Cell Culture.** Two different keratinocyte culture systems were used in this study. In the feeder system, keratinocytes were cocultivated with mitomycin C-treated 3T3 fibroblasts (either m1, 3T3 or NIH 3T3 as specified in the figure legends) in F medium [3:1 (vol/vol) F-12 Nutrient Mixture (Ham)-DMEM, 5% FBS, 0.4 µg/ml hydrocortisone, 5 µg/ml insulin, 8.4 ng/ml cholera toxin, 10 ng/ml EGF, and 24 µg/ml adenine; Ref. 24]. Fibroblasts were plated at least 30 min and up to 2 days before addition of keratinocytes. Alternatively, keratinocytes were grown directly on plastic tissue culture dishes in keratinocyte-SFM (Invitrocorporation), a chemically defined medium supplemented with 5 ng/ml human recombinant EGF and bovine pituitary extract. All cells were maintained at 37°C in a humidified incubator with 5% CO2 and 95% air, washed twice in PBS (no Ca2+ and Mg2+) and frozen as cell pellets when 80–90% confluent.

Primary HFKs derived from two neonatal foreskins were mixed and cultured one passage on feeder fibroblasts to expand the cells. Then they were divided and subsequently maintained either on plastic or on feeder fibroblasts as described above.

Keratinocytes were also transferred from one culture system to the other as follows. HFKs grown on plastic were trypsinized, pelleted by centrifugation, and then resuspended in F medium and either replated together with trypsinized fibroblasts or onto adherent fibroblasts previously plated in F medium. For HFKs grown on feeder fibroblasts, the fibroblasts were first removed by brief treatment with Versene, and then the HFKs were collected by centrifugation, pelleted by centrifugation, and then resuspended in K-SFM. Alternatively, keratinocytes were grown directly on plastic tissue culture dishes in keratinocyte-SFM (Invitrocorporation), a chemically defined medium supplemented with 5 ng/ml human recombinant EGF and bovine pituitary extract. All cells were maintained at 37°C in a humidified incubator with 5% CO2 and 95% air, washed twice in PBS (no Ca2+ and Mg2+) and frozen as cell pellets when 80–90% confluent.

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**Retroviral Transductions.** At passage 5 for HFKs grown on plastic and passage 4 for HFKs grown on feeder fibroblasts, HFK subcultures were transduced with an amphotrophic LXSN retrovirus expressing HPV-16 E6 and E7 ORFs (25). Transduced HFKs (HFK/16E6E7 cells) subsequently underwent selection by G418 at 100 ng/ml for 2 days and 50 ng/ml for an additional 3 days before the pooled colonies were further serially passaged. Cells were maintained in the same culture system before, during, and after transduction. Control (nontransduced) HFKs were cultured in parallel but were not transduced with an empty LXSN retroviral vector and therefore did not go through G418 selection.

HFKs expressing individual HPV-16 genes were obtained from Tim Veldman and Richard Schlegel (Georgetown University Medical Center) and were used for the experiment presented in Fig. 3. These HFKs had been transduced with an empty LXSN retroviral vector (HFK/LXSN cells) or LXSN retroviral vectors expressing HPV-16 E6 alone, E7 alone, or both E6 and E7 oncoproteins (HFK/16E6, 16E7, or 16E6E7 cells; Ref. 13).

**RNA and Protein Preparation.** RNA samples were obtained to examine the expression of hTERT, p16INK4a, and HPV-16 unspliced E6 and E7 mRNAs. Protein samples were used to detect hTERT and p16INK4a protein and telomerase activity. Both RNA and protein samples were collected at selected passages when HFKs reached 80–90% confluence, unless stated otherwise. Samples of HFKs grown on feeder fibroblasts were harvested after removal of the fibroblasts with Versene. Total RNA was isolated using the RNAqueous Kit (Ambion, Inc., Austin, TX). All RNA samples were initially quantified using absorbance at 260 nm and then treated with DNase I using DNA free (Ambion, Inc.). RNA samples were examined for RNA quality using the Bioanalyzer 2000 (Agilent Technologies), and the RNA concentration was confirmed using the RiboGreen RNA Quantitation Kit (Molecular Probes).

Cell extracts were prepared as follows. HFKs were harvested by trypsinization, washed twice in PBS (no Ca2+ and Mg2+) and frozen as cell pellets at −80°C. The cell pellets were lysed in 1× CHAPS buffer (0.5% 3-(3-cholamidopropyl)-dimethyl-ammonio)-1-propanesulfonate, 10 mM Tris-Cl (pH 7.5), 1 mM MgCl2, 1 mM EDTA, 5 mM 2-mercaptoethanol, 0.1% benzamidine, 10% glycerol, and 10 units/ml RNase inhibitor (Applied Biosystems) for 30 min at 4°C. The lysate was then centrifuged at 12,000 × g for 30 min at 4°C. The protein concentration of the supernatant was determined using the DC Protein Assay Kit (Bio-Rad) in a SpectraMax (Molecular Devices Co.) plate reader.

**Real-Time QRT-PCR.** RT reactions were carried out using the cDNA Archive Kit from PE Applied Biosystems. Real-time Q-PCR was done using the Bio-Rad iCycler and either Sybr Green or Taqman chemistries. Table 1 shows the sequence and the location of the primers and Taqman probes used for each assay. The p16INK4a assay was designed to be specific for the major splice variant (variant 1 or v1) of p16INK4a in keratinocytes and does not cross-react with p14ARF (unpublished data). SYBR Green assays were carried out as previously described with an annealing temperature of 60°C, except for the p16INK4a assay, which used 62°C (26). To specifically detect low abundance hTERT mRNA in samples such as those from primary HFKs, we also performed Taqman real-time QRT-PCR assays using Platinum-Quantitative PCR Supermix (Invitrogen) and the primers and probe shown in Table 1. Reaction conditions were as previously reported (27), except that annealing temperature for hTERT was 63°C. Standard curves were created for each run using 10-fold serial dilutions of Topo TA-cloned (Invitrogen) cDNAs of each

### Table 1. Real-time QRT-PCR primers and Taqman probes

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
<th>cDNA clones</th>
</tr>
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<tr>
<td>oCCB-260/oCCB-252</td>
<td>CAAACAAACGCTCGTGTGAT</td>
<td>CTTGTTCCTTTGATGCAG</td>
<td>p3010</td>
</tr>
<tr>
<td>oMDM-170/oMDM-18</td>
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<td>ACCGAAACGCTTAGACTCAC</td>
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<tr>
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<td>CAGGCTTGTCCTCACC</td>
<td>p3644</td>
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<tr>
<td>TS/ACX</td>
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<td>CAGCTGTCCCGGACGGTG</td>
<td>p3575</td>
</tr>
<tr>
<td>oBF-53/oBF-56</td>
<td>AAACGCCCGAATGTTTACGG</td>
<td>TGGCCATCATCAGTCCTG</td>
<td>p3772</td>
</tr>
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* cDNA clone used as template for standard curves.
* Unspliced E6 mRNA.
* This assay was used to generate the data shown in Fig. 1, A and B.
* Primers and Taqman probe (oCB-415: ACCCTTGTCGGAGGTGTCCTTCTGAG) taken from Bieche et al. (27).
* Primers taken from Hou et al. (30).
* Assay specific for splice variant 1.
mRNA (Table 1). The concentration of each standard was determined by PicoGreen dsDNA Quantitation Kit (Molecular Probes). All PCR reactions were run in triplicate. Each sample reaction contained the cDNA from 50 ng of RNA, unless specified otherwise. The measured amount of each specific mRNA was expressed as fg of the respective cDNA clone.

**Western Blot Analysis for p16INK4a and HPV-16 E7.** Equal amount of protein in CHAPS buffer were electrophoresed on 15% Tris-HCl Ready Gel Precast Gels (Bio-Rad) and subsequently transferred to a 0.45-μm nitrocellulose membrane (Bio-Rad). The membrane was blocked in wash solution (0.1% Tween 20 in PBS) containing 5% nonfat dry milk. Mouse anti-p16INK4a monoclonal antibody Ab-4 (NeoMarkers) and mouse anti-HPV-16 E7 monoclonal antibody ED17 (Santa Cruz Biotechnology) were used as the primary antibodies. Protein was visualized using the SuperSignal West Pico (for p16INK4a) or Fempto (for 16E7) Chemiluminescent Substrate (Pierce Biotechnology). Blots were stripped and reprobed with goat polyclonal anti-actin C-11 antibody (Santa Cruz Biotechnology) as a loading control.

**Real-Time Q-TRAP.** Telomerase studies rely on quantitative analyses of telomerase activity. The original TRAP assay uses end point PCR and a gel-based detection system and is only semiquantitative at best (28, 29). The TRAP assay can be made more quantitative through the use of real time Q-PCR (30). Here, we have created a two-step real-time Q-TRAP assay that is efficient, precise, and highly reproducible. The first step of the assay was essentially the same as the conventional protocol (28). In detail, each 40-μl reaction contained 2.0 μg of cDNA from HL-60 326, 1.5 × TRAP reaction buffer [20 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, 10 mM EGTA, and 63 mM KCl], 50 μM of each deoxynucleotide triphosphate, 0.1 μg of TS oligonucleotide substrate (Table 1), and 0.2 μg of T4 gene protein (Amersham Pharmacia Biotech). The reaction mixture was incubated at room temperature for 10 min and then telomerase was inactivated by heating at 95°C for 10 min. The second step of the assay, real-time Q-PCR was used to quantify the number of substrate molecules to which telomeric repeats had been added. Sybr Green real-time Q-PCR assays were carried out essentially as described above for real-time Q-RT-PCR. Each 25-μl reaction contained 300 ng TS and ACX primers (Table 1) and 1.0 μl of the product from the first step of the assay. A standard curve was produced for the real-time Q-TRAP assay using serially diluted 293 cell extracts. All samples were run in triplicate. This assay is linear over at least a 500-fold range (0.008–4 μg of 293 cell protein input).

**Telomere Length.** Cells were harvested for DNA preparation as described above for RNA and protein preparation. Genomic DNA was prepared from cell pellets using the DNA Purification Kit (Promega) according to the manufacturer’s instructions. Telomere length was assessed by Southern analysis of terminal restriction fragments using the TeloTAGGG Telomere Length Assay (Roche). Briefly, ∼1.3 μg of genomic DNA was digested with RsaI and HinfI and resolved by electrophoresis in a 0.8% agarose gel. The DNA fragments were transferred to a nylon membrane and hybridized with a digoxigenin-labeled probe specific for telomeric repeats. Hybridized probe was detected using an alkaline phosphatase-conjugated anti-digoxigenin antibody and chemiluminescence was captured on film.

**RESULTS**

**Senescence Is Initiated by Different Events in Keratinocytes Cultured under Different Conditions.** Both cell culture conditions and viral oncopathies have a profound impact on the replicative capacity of keratinocytes. We were first interested in whether there are differences in p16INK4a and hTERT expression and telomerase activity in keratinocytes cultured in different systems. HFKs pooled from two neonatal foreskins were expanded one passage on feeder fibroblasts and then divided and serially cultured either on plastic in K-SFM or on feeder fibroblasts in F medium. At each passage, portions of the cells were harvested for RNA, DNA, and protein analyses. HFKs grown on plastic ceased dividing at passage 8 (p8) or ∼13 PDs. In contrast, HFKs grown on feeder fibroblasts maintained a high growth rate up to p26 (∼60 PDs), then started to grow slowly and finally stopped dividing at ∼p57 (81 PD). hTERT mRNA and telomerase activity could be detected in very early passage HFKs (Fig. 1, A and B). However, both hTERT mRNA and telomerase activity dropped quickly to an undetectable level in senescent HFKs grown on plastic (Fig. 1A). Senescence was accompanied by only a slight decrease in telomere length (Fig. 1C, compare Lanes 1 and 6 to Lane 2). In contrast, levels of hTERT mRNA and telomerase activity persisted until nearly 60 PDs in HFKs grown on feeder fibroblasts and then decreased with the onset of senescence (Fig. 1B). There was no significant reduction in telomere length between PD 15 and PD 43, indicating that telomerase levels in early to intermediate passage HFKs grown on feeder fibroblasts are sufficient to maintain telomere length (Fig. 1C, compare Lanes 3 and 4). The drop in telomerase activity after PD 60 correlated with significant telomere erosion immediately preceding senescence (Fig. 1C, Lane 5). Analyses of hTERT mRNA and telomerase activity in feeder fibroblasts showed extremely low levels, ruling out the possibility that the hTERT mRNA and telomerase activity seen in HFKs grown on feeder fibroblasts was attributable to incomplete removal of the fibroblasts before harvest (data not shown). Overall, telomerase activity correlated well with hTERT mRNA, suggesting that hTERT is the limiting subunit of telomerase (Fig. 1, A and B). This correlation was also seen in all other studies described below.

p16INK4a has been found to be tightly linked to senescence of keratinocytes cultured in serum-free media (4, 5). We therefore assayed p16INK4a mRNA levels in our HFK RNA samples using a real-time QRT-PCR assay that detects the major splice variant (v1) of p16INK4a mRNA and not p14ARF mRNA (unpublished results). As expected, senescence of HFKs grown on plastic was accompanied by a dramatic increase in p16INK4a mRNA levels (Fig. 1D). This increase in the level of p16INK4a was confirmed at the protein level using Western blotting (Fig. 1D, inset). A gradual increase in p16INK4a expression was also seen in early passage HFKs grown on feeder fibroblasts, but the levels of p16INK4a remained considerably below those seen in the senescent HFKs grown on plastic. In addition, p16INK4a mRNA levels stabilized by 40 PDs and increased only slightly when these cells eventually senesced after 60 PDs. This suggests that p16INK4a is not directly involved in triggering senescence in HFKs grown on feeder fibroblasts.

**Keratinocyte Culture Conditions Significantly Influence the Induction of Telomerase by HPV-16 E6 and E7 Oncoproteins.** There is strong evidence that immortalization of keratinocytes requires both inactivation of the Rb/p16INK4a pathway and activation of telomerase (4, 5). This can be accomplished by the high-risk HPV E7 and E6 oncoproteins, respectively. We demonstrated above that expression of p16INK4a and telomerase are significantly different in HFKs grown on plastic compared with HFKs grown on feeder fibroblasts. We next wanted to see if culture conditions affect the induction of telomerase and maintenance of telomeres by the HPV-16 E6 oncoprotein.

HFKs grown either on plastic or on feeder fibroblasts were transduced at p5 (8 PDs) or p4 (7 PDs), respectively, by a retroviral vector expressing the HPV-16 E6 and E7 ORFs. Pooled G418-resistant colonies (subsequently referred to as HFK/16E6E7 cells) were continuously cultured under the same conditions as before transduction. HFK/16E6E7 cells grew vigorously under both culture conditions and demonstrated no significant differences in growth characteristics between early and late PDs. Both HPV-16 E6/E7 mRNA and E7 protein were monitored at selected passages. We were unable to detect E6 protein using standard Western blotting techniques. The E6 and E7 ORFs are present in the same mRNA when expressed from the retroviral vector, and as expected, their expression was closely correlated over time (Fig. 1E). Moreover, a significant increase in E6/E7 mRNA and E7 protein levels was observed between early and late PDs in HFK/16E6E7 cells grown both on plastic and feeder fibro-
Fig. 1. Effects of keratinocyte culture conditions on hTERT, p16 INK4a, and HPV-16 E6/E7 expression and telomerase activity. HFKs and HFK/16E6E7 cells were derived and serially passaged either in K-SFM on plastic or in F medium on m13T3 feeder fibroblasts as described in “Materials and Methods.” RNA, DNA, and protein samples were collected at selected PDs as indicated. The same RNA samples were analyzed in A, B, D, and E. hTERT, p16 INK4a, and HPV-16 E6 and E7 mRNAs were assayed using real-time QRT-PCR. Values are reported as fg of the respective plasmid control (Table 1)/50 ng total RNA unless otherwise specified. Telomerase activity was analyzed by real-time Q-TRAP as described in “Materials and Methods.” Telomerase activity values are relative and expressed as ng of a 293 cell control extract with equivalent activity. The scales for both hTERT mRNA and telomerase activity are different in A and B. A, hTERT mRNA and telomerase activity in HFKs and HFK/16E6E7 cells grown on plastic. HFKs stopped dividing at 13 PDs (labeled senescence). Culture of HFK/16E6E7 cells was terminated at 74 PDs. The arrow labeled transduction indicates the point at which HFKs were transduced with the HPV-16 E6/E7 retroviral vector to generate HFK/16E6E7 cells. Separate curves for HFKs (left bottom set) and HFK/16E6E7 cells are labeled accordingly. B, hTERT mRNA and telomerase activity in HFKs and HFK/16E6E7 cells grown on feeder fibroblasts. HFKs started to grow slowly at 110 PDs and senesced at 81 PDs. Culture of HFK/16E6E7 cells was terminated at 121 PDs. The graph is labeled as in A. Separate curves for HFKs (bottom set) and HFK/16E6E7 (top set) cells are labeled accordingly. C, telomere length analysis of HFKs and HFK/16E6E7 cells grown on plastic and on feeder fibroblasts. The blots show terminal restriction fragments resolved by electrophoresis and visualized with a telomere-specific probe as described in “Materials and Methods.” DNA samples were obtained from the cells described in A and B. The presence or absence of HPV-16 E6/E7, growth conditions (S, K-SFM on plastic; F, medium on feeder fibroblasts), and PDs at time of harvest are indicated at the top of each lane. The size (in kb) and location of the molecular weight markers are shown at the left of each panel. D, p16 INK4a mRNA expression in nontransduced HFKs grown on plastic and on feeder fibroblasts. Each real-time QRT-PCR reaction included the cDNA from 5 ng of total RNA. Curves are labeled by real time QRT-PCR assay (E6 or E7) and culture environment [plastic (top set) or feeder fibroblasts (bottom set)]. Inset shows Western blot analysis of HPV-16 E7 and actin protein in HFK/16E6E7 cells cultured on either plastic or feeder fibroblasts for the specified number of PDs.
bombs. Surprisingly, E6/E7 mRNA levels were dramatically higher (~5–10-fold) in HFK/16E6E7 cells grown on plastic compared with those grown on feeder fibroblasts. A comparable difference in E7 protein levels was also seen but only when extracts were further diluted (Fig. 1E inset and data not shown). These results suggest that HFKs expressing higher levels of HPV-16 E6 and E7 proteins have a selective growth advantage and, furthermore, that higher E6 and/or E7 levels are required for long-term growth on plastic. Interestingly, HFK/16E6E7 cells grown on plastic demonstrated a biphasic E6/E7 mRNA curve with a local minimum at 50 PDs. However, no concomitant changes of cell growth, morphology, or crisis were observed at this point.

Transduction of low passage HFKs with the 16E6E7-expressing retrovirus immediately induced hTERT mRNA and telomerase activity (Fig. 1, A and B), consistent with published studies (12, 13). Paradoxically, both hTERT mRNA and telomerase activity were dramatically higher in HFK/16E6E7 cells grown on feeder fibroblasts than in similar cells grown on plastic. As discussed above, this is opposite to the pattern of E6 and E7 expression in HFK/16E6E7 cells grown on feeder fibroblasts and on plastic (Fig. 1E). In other words, the higher E6 and E7 expression levels in HFK/16E6E7 cells grown on plastic did not result in a correspondingly stronger induction of telomerase. These data suggest a synergistic activation of hTERT mRNA and telomerase activity by E6 and/or E7 and the feeder culture environment. In HFK/16E6E7 cells grown on plastic, hTERT mRNA levels and telomerase activity remained low until ~50 PDs, although E6/E7 mRNA and E7 protein levels increased significantly during this period (compare Fig. 1, A and E). In contrast, hTERT mRNA, telomerase activity, and E6/E7 mRNA levels rose sharply in parallel after 50 PDs. This increase in telomerase activity at late PDs was not seen when HFK/16E6E7 cells were grown on feeder fibroblasts (Fig. 1B).

Telomere length assays were carried out to determine whether induction of telomerase by HPV oncogenes is sufficient to prevent telomere erosion. Surprisingly, telomere length decreased progressively in HFK/16E6E7 cells grown on plastic, indicating that even the high telomere levels in late passage HFK/16E6E7 cells grown on plastic are insufficient to maintain or elongate telomeres (Fig. 1C, Lanes 7–9). HFK/16E6E7 cells cultured on feeder fibroblasts for 15 PDs had longer telomeres than HFK/16E6E7 cells cultured on plastic for the same number of PDs (Fig. 1C, compare Lanes 7 and 10). In addition, the cells grown on feeder fibroblasts maintained telomere length for at least 56 PDs (Fig. 1C, Lane 11). At very late passages, these cells show severe erosion of a subpopulation of telomeres, suggesting that a subpopulation of the cells have lost telomerase activity (Fig. 1C, Lane 12). These data demonstrate that superinduction of telomerase by HPV-16 E6 and E7 oncoproteins and the feeder culture environment leads to significantly better telomere maintenance.

Previous studies have shown that inactivation of pRb by E7 induces p16INK4a expression through accumulation of active E2F1 (31). We also saw an induction of p16INK4a mRNA and protein in HFK/16E6E7 cells grown in either culture system (data not shown). Levels of p16INK4a mRNA and protein increased progressively with passage number and essentially paralleled E6/E7 mRNA. This increase was more prominent in HFK/16E6E7 cells grown on plastic.

**Telomerase and p16INK4a Expression in Keratinocytes Are Significantly Modulated by Growth of Keratinocytes on Feeder Fibroblasts.**

There are multiple possible explanations for the higher telomerase levels in HFKs and HFK/16E6E7 cells grown on feeder fibroblasts compared with those grown on plastic. One possibility is the selective outgrowth of a telomerase-positive subpopulation such as stem cells when keratinocytes are grown on feeder fibroblasts or the loss of such a population when keratinocytes are grown on plastic. Alternatively, hTERT expression could be directly induced or repressed by different culture conditions. We tested the second hypothesis by transferring HFKs and HFK/16E6E7 cells from one culture system to the other.

In the first experiment, p5 HFKs, which had previously been grown on plastic and frozen, were revived and cultured again on plastic. After one passage, the HFKs were divided and then serially passaged both on plastic and on feeder fibroblasts. Telomerase activity was monitored at each passage (Fig. 2A). As expected, telomerase activity was low and decreased progressively with passage in HFKs grown on plastic. In contrast, telomerase activity was induced to a remarkably higher level (14-fold) in p7 HFKs that had been transferred onto feeder fibroblasts. In addition, significantly higher levels of telomerase activity persisted in subsequent passages of HFKs grown on feeder fibroblasts although the initial high level was not maintained. To determine whether induction of telomerase activity by the feeder environment is reversible, we took the p7 and p9 HFKs growing on feeder fibroblasts and replated them on plastic. In both cases, telomerase activity decreased at the next passage to the same levels seen in HFKs continuously cultured on plastic. These results indicate that culture of keratinocytes on feeder fibroblasts induces telomerase activity and that this induction is fully reversible.

RNA samples from the same HFKs were also assayed for p16INK4a expression (Fig. 2C). As seen previously, p16INK4a mRNA levels rose steadily with passage in HFKs grown on plastic. Unlike telomerase activity, however, it took several passages to reach maximum p16INK4a mRNA levels. Transferring p6 HFKs grown on plastic onto feeder fibroblasts reduced or blocked this rise in p16INK4a expression. Moreover, p16INK4a mRNA continued to increase when HFKs grown on feeder fibroblasts were transferred to and cultured on plastic. Thus, growth of HFKs on feeder fibroblasts not only induces telomerase expression but also represses p16INK4a expression.

We next wanted to know if the very high telomerase levels seen in HFK/16E6E7 cells grown on feeder fibroblasts are also a direct consequence of the culture environment (Fig. 1B). We therefore repeated the culture changing experiment outlined above for HFKs, only this time, we used the HFK/16E6E7 cells (Fig. 2B). As before, growth on feeder fibroblasts induced telomerase activity and this induction was fully reversible. However, telomerase activity was induced to dramatically higher levels in HFK/16E6E7 cells compared with nontransduced HFKs. HPV-16 E6/E7 mRNA levels were assayed to rule out the possibility that the induction of telomerase activity in these cells is attributable to an increase in E6 expression. An inverse relationship between telomerase activity and E6/E7 expression was found in HFK/16E6E7 cells grown on plastic and on feeder fibroblasts, indicating that this is not the case (data not shown). These results lend additional support to the conclusion that HPV-16 E6 and/or E7 proteins and factors in the feeder culture system synergistically induce telomerase activity.

**Superinduction of Telomerase Activity in Keratinocytes Grown on Feeder Fibroblasts Requires both the HPV-16 E6 and E7 Proteins.**

It is well documented that the HPV-16 E6 protein mediates telomerase activation by transcriptional induction of hTERT (13, 23, 32). However, only one study demonstrated a role for E7 in this process and that study used keratinocytes cultured on feeder fibroblasts (23). Our studies described above demonstrated that a retroviral vector expressing both the E6 and E7 proteins superinduced telomerase in keratinocytes cultured in the feeder system. To determine whether the E7 protein is involved in this superinduction, HFKs transduced with an empty LXSN retroviral vector (HFK/LXSN) or
analyses are as described for performed with HFK/16E6E7 cells at the indicated passage numbers. Annotations and A same as in Fig. 1. The same symbols are used in real-time Q-TRAP as described in samples were collected at the indicated passages, and telomerase activity was analyzed by Transitions from one culture system to the other are indicated by grown on feeder fibroblasts were replated back on plastic for one or more passages. At p6, a portion of these HFKs was replated on m 1 3T3 feeder fibroblasts in F medium and serially passaged in the feeder system until p12. At p7 and p9, some of the HFKs grown on feeder fibroblasts were replated back on plastic for one or more passages. Transitions from one culture system to the other are indicated by dashed arrows. Protein samples were collected at the indicated passages, and telomerase activity was analyzed by real-time Q-TRAP as described in “Materials and Methods.” B, similar experiments were performed with HFK/16E6E7 cells at the indicated passage numbers. Annotations and analyses are as described for A. C, p16INKnca expression is depressed in HFKs grown on feeders fibroblasts. RNA samples from the same passages of HFKs as in A were collected and examined for p16INKnca mRNA expression using real-time QRT-PCR. Units are the same as in Fig. 1. The same symbols are used in A and C.

LXSN retroviral vectors expressing HPV-16 E6 alone, E7 alone, or both E6 and E7 oncoproteins (HFK/16E6, 16E7, or 16E6E7) were analyzed (13). The LXSN retroviral vector expressing 16E6E7 is different from the 16E6E7 retroviral vector used for the experiments presented in Figs. 1 and 2 and makes an E6 pre-mRNA that is spliced much more efficiently (data not shown). These transduced HFKs were initially established on plastic and then were serially passaged either on plastic or on feeder fibroblasts. Telomerase activity was assessed at each passage and is shown in Fig. 3. When the keratinocytes were cultured on plastic (Fig. 3, left panel), telomerase activity in HFK/16E7 cells was as low as that in p9 HFK/LXSN controls. In contrast, both HFK/16E6 and HFK/16E6E7 cells exhibited significantly elevated (>10-fold) but equivalent telomerase levels. However, when these same keratinocytes were cultured on feeder fibroblasts (Fig. 3, right panel), HFK/16E6E7 cells exhibited dramatically higher telomerase levels than HFK/16E6 cells. Again, E7 had no effect on telomerase activity when it was expressed alone. Unspliced E6 mRNA (encoding full-length functional E6 protein) and E7 mRNA were also quantified to determine whether these results were because of differences in E6 or E7 expression (data not shown). E7 mRNA levels varied <2-fold in HFK/16E7 and HFK/16E6E7 cells in the two culture systems. However, Western blot analysis indicated that E7 protein levels were significantly lower in HFK/16E6E7 cells compared with HFK/16E7 cells (data not shown). Unspliced E6 mRNA was ~20-fold lower in HFK/16E6E7 cells compared with HFK/16E6 cells when both cell lines were grown on feeder fibroblasts. Lower levels of US E6 mRNA presumably result in lower levels of full-length E6 protein. These results are consistent with our previous conclusion that dramatically less E6 is required for induction of telomerase activity when E7 and the feeder environment are present. To summarize these results, when keratinocytes are grown on plastic, E7 has no effect on telomerase activity, either by itself or in combination with E6. In contrast, the E7 protein is responsible for the superinduction of telomerase in E6-expressing keratinocytes grown on feeder fibroblasts. Taken together, these experiments indicate that both the HPV-16 E6 and E7 proteins and factors in the feeder culture environment synergistically induce telomerase expression in keratinocytes.

**Induction of Telomerase Activity in Keratinocytes Grown in the Feeder System Is Due Predominantly to the Culture Medium.** Multiple factors in the feeder culture system may be involved in the induction of telomerase activity in keratinocytes. Possible factors include direct cell-cell contact, cell contact with the ECM, and/or soluble factor(s) that are components of the culture medium or are secreted from the feeder fibroblasts. As a first step in the identification of the responsible factor(s), HFK/16E6E7 cells that had originally been grown on plastic in K-SFM were replated onto different culture substrates, including feeder fibroblasts, plastic, and plastic either

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**Fig. 2. Growth of keratinocytes on feeder fibroblasts reversibly induces telomerase activity and represses p16INKnca expression.** A, telomerase activity was induced in keratinocytes grown on feeders. p5 HFKs were serially passaged on plastic in K-SFM until p12. At p6, a portion of these HFKs was replated on m 1 3T3 feeder fibroblasts in F medium and serially passaged in the feeder system until p12. At p7 and p9, some of the HFKs grown on feeder fibroblasts were replated back on plastic for one or more passages. Transitions from one culture system to the other are indicated by dashed arrows. Protein samples were collected at the indicated passages, and telomerase activity was assessed by real-time Q-TRAP as described in “Materials and Methods.” B, similar experiments were performed with HFK/16E6E7 cells at the indicated passage numbers. Annotations and analyses are as described for A. C, p16INKnca expression is depressed in HFKs grown on feeders fibroblasts. RNA samples from the same passages of HFKs as in A were collected and examined for p16INKnca mRNA expression using real-time QRT-PCR. Units are the same as in Fig. 1. The same symbols are used in A and C.

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**Fig. 3. E7 acts synergistically with E6 to induce telomerase in keratinocytes only in the feeder culture system.** Control HFKs transduced with an empty LXSN retroviral vector (HFK/LXSN) and HFKs transduced with HPV-16 E6, E7, and E6E7-expressing retroviral vectors (HFK/16E6, HFK/16E7, or HFK/16E6E7 cells, respectively) were serially passaged either in K-SFM on plastic (left panel) or in F medium on NIH 3T3 feeder fibroblasts (right panel). The same cells were used in both panels and were originally cultured on plastic. Protein samples were collected at each passage and assayed for telomerase activity. Telomerase activity is shown on the same scale in both panels.
preconditioned with 3T3 fibroblasts or coated with collagen I, collagen IV, fibronectin, laminin, or poly-d-lysine. For each substrate, HFK/16E6E7 cells were maintained in either K-SFM, F medium, or 3T3 feeder-conditioned F medium for 3.5–5 days before measurement of telomerase activity. F medium induced telomerase activity in HFK/16E6E7 cells grown on plastic to levels close to those present in HFK/16E6E7 cells grown on feeder fibroblasts (Fig. 4A). Maximum induction required ~3 days in culture (Fig. 4B). E medium, another culture medium widely used in keratinocytes culture, also induced telomerase activity to an equivalent degree (data not shown). These data indicate that it is the medium rather than the fibroblasts that is predominantly responsible for induction of telomerase. However, conditioned F medium consistently gave a slightly greater induction of telomerase activity when cells were grown on plastic (Fig. 4A), suggesting that fibroblasts may secrete a soluble factor(s) that also contributes to telomerase induction. None of the other culture substrates listed above contributed to telomerase induction when cells were grown in F medium or conditioned F medium (data not shown).

Taken together, these data indicate that the culture medium is the predominant factor responsible for the induction of telomerase in keratinocytes grown in the feeder system and that the fibroblasts play only a minor role.

The major components of F medium that are not found in K-SFM are serum and cholera toxin. To identify the factor(s) in F medium responsible for telomerase induction, HFK/16E6E7 cells were cultured on plastic for 3 days in either K-SFM or F medium modified by the addition or absence of serum, cholera toxin, or EGF (Fig. 4C). Equivalent low levels of telomerase activity were seen in HFK/16E6E7 cells grown in K-SFM and F medium lacking cholera toxin. In addition, F medium lacking either serum or EGF was considerably less effective than complete F medium at inducing telomerase. However, cholera toxin and serum each failed to induce telomerase activity when added separately or together to K-SFM (Fig. 4C and data not shown). This indicates that cholera toxin and serum are each necessary but not sufficient for F medium mediated telomerase induction in HFK/16E6E7 cells.

**DISCUSSION**

Keratinocytes exhibit remarkable differences in replicative life span when they are cultured in different systems. Previous studies have highlighted the role of p16INK4a in early senescence of keratinocytes grown on plastic (20). Here, we demonstrate for the first time that telomerase activity can be reversibly induced by growth of keratinocytes on feeder fibroblasts. Induction and maintenance of telomerase activity significantly slowed telomere shortening and therefore likely contributes to the extended life span of primary keratinocytes in the feeder culture system. Furthermore, we show that alterations in keratinocyte biology due to culture environment lead to dramatic differences in HPV-keratinocyte interactions. Specifically, synergistic induction of telomerase activity to very high levels by the HPV-16 E6 and E7 oncoproteins was seen only in keratinocytes in the feeder culture system. The p16INK4a/pRb pathway by culture substrate. HFK/16E6E7 cells grown on plastic for 3 days in culture (Fig. 4A), and data not shown). This indicates that cholera toxin and serum are each necessary but not sufficient for F medium mediated telomerase induction in HFK/16E6E7 cells.

**DISCUSSION**

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Our data are consistent with recent studies that early senescence in keratinocytes grown in SFM on plastic is due to culture shock and is mediated through p16INK4a (20). Early attempts to immortalize keratinocytes by expression of hTERT failed when the keratinocytes were grown in K-SFM on plastic (4). Immortalization also required abrogation of the p16INK4a/pRb pathway by expression of the HPV-16 E7 protein, mutations in the CDKN2A/INK4A locus, or down-regulation of p16INK4a (expression (4, 5, 20). Growth of keratinocytes on feeder fibroblasts leads to down-regulation of p16INK4a expression and hTERT expression is sufficient to immortalize these keratinocytes
of subtle differences in cell culture protocols. In our experiments, primary HFKs grown on plastic senesced after 13 PDs (Fig. 1). Senescence was accompanied by both a dramatic increase in \( p_{16^{INK4a}} \) mRNA and protein and a loss of hTERT mRNA and telomerase activity. This senescence likely represents the cell culture stress-induced growth arrest previously described (20) because the telomeres were only slightly shortened and therefore are unlikely to have triggered replicative senescence (mortality stage 1 or M1). Interestingly, rapid loss of telomerase has been shown to induce apoptosis independent of telomere length (34–36). Thus, in our experiments, loss of telomerase may also have contributed to the cessation of growth of HFKs cultured on plastic. We also found that cell culture stress-induced senescence could be overcome by either expression of HPV-16 E6 and E7 proteins or by growth of the keratinocytes on feeder fibroblasts, consistent with the studies mentioned above. Growth of HFKs on feeder fibroblasts stabilized \( p_{16^{INK4a}} \) expression at intermediate levels and permitted the cells to grow rapidly for 60 PDs before they entered a slow growth phase followed by senescence. Surprisingly, significant hTERT mRNA and telomerase activity were present in these cells throughout the rapid growth phase and actually exceeded levels of hTERT mRNA and telomerase activity present in early to intermediate passage HFK/16E6E7 cells grown on plastic. These levels of telomerase were sufficient to maintain telomere length during the rapid growth phase and likely contribute to the extended life span of HFKs grown on feeder fibroblasts (Fig. 1C). The slow growth phase of these cells was accompanied by a dramatic drop in telomerase activity and significant telomere erosion immediately preceding senescence (Fig. 1, B and C). This was not accompanied by a significant increase in \( p_{16^{INK4a}} \) mRNA or protein (Fig. 1D). These data suggest that senescence was not mediated by \( p_{16^{INK4a}} \) and implicate a telomerase-dependent mechanism, indicating that this is M1 or true replicative senescence. This conclusion is supported by studies showing that HFKs expressing exogenous hTERT can undergo at least 160 PDs when grown on feeder fibroblasts (20). Taken together, our data support claims that senescence in keratinocytes grown on plastic represents a tissue-culture artifact and that the \( Rb/p_{16^{INK4a}} \) checkpoint may represent a stress-induced consequence of inadequate culture environment rather than a telomere-independent second mechanism of cellular senescence (20, 37). The feeder culture system simply represents a more favorable and supportive environment for keratinocyte proliferation.

Culture conditions and HPV oncogenes could regulate hTERT expression and telomerase activity in keratinocytes in several ways. Telomerase activity is present in the proliferating basal cell compartment of a normal squamous epithelium \textit{in vivo} and repressed as keratinocytes differentiate (38–40). The observation that telomerase activity is maintained in keratinocytes grown on feeder fibroblasts may indicate that the feeder culture environment better mimics the environment present in the basal layer of the epidermis. We have preliminary data suggesting that the differences in telomerase activity between the two culture systems are not attributable to differences in the differentiation state of the keratinocytes (unpublished results). However, HPV-16-immortalized keratinocytes are resistant to calcium and serum-induced terminal differentiation, so this may play a role in the induction of telomerase by E6 and E7 (41). Our investigations identified three components of F medium that are required for the full induction of telomerase. Cholera toxin was essential, whereas serum and EGF played contributory roles (Fig. 4C). However, none of these components were sufficient to induce telomerase when added to K-SFM. Cholera toxin stimulates the cyclic AMP pathway through the stimulatory guanine nucleotide binding protein (Gs) component of adenylate cyclase (42). Cholera toxin and EGF are known to stimulate the proliferation of human epidermal keratinocytes in culture, as well as to prevent the induction of differentiation by serum (21, 43, 44). EGF is present in both K-SFM and F medium and is therefore not sufficient to mediate telomerase induction by itself. Another factor that could play a role in the induction of telomerase in the feeder culture system is cell-cell contact between keratinocytes because keratinocytes grow in clusters in F medium and are dispersed in K-SFM. However, addition of calcium to K-SFM caused keratinocytes to cluster but only slightly affected telomerase activity (unpublished data). We also considered the potential role of the feeder fibroblasts in telomerase induction. Bi-directional interactions between feeder fibroblasts and keratinocytes via cell-cell and cell-ECM contact and paracrine signals promote the growth and proliferation of epithelial cells (45–47). For example, undifferentiated human embryonic stem cells can be maintained in a feeder fibroblast-free culture environment consisting of an appropriate ECM and fibroblast-conditioned media (48). In this culture environment, ES cells maintain telomerase activity. However, these investigators did not determine whether ECM and conditioned medium are essential for telomerase activity. In our studies, we found only a minor role for paracrine factors secreted by feeder fibroblasts and no positive effects of different extracellular matrices on telomerase activity (Fig. 4A and unpublished data).

Most studies on the activation of telomerase by high-risk HPVs have attributed this activation exclusively to the E6 protein (12, 13, 22, 32). It is important to point out that these studies all cultured keratinocytes in SFM on plastic. We also found no effect of E7 on telomerase expression when keratinocytes were cultured in K-SFM on plastic (Fig. 3, left panel). This was true for E7 alone or in combination with E6. In contrast, we demonstrated a very strong synergistic activation of telomerase by the coexpression of E6 and E7 in keratinocytes grown on feeder fibroblasts in F medium (Figs. 1B and 3, right panel). Synergistic activation of telomerase activity appears to be at the transcriptional level because hTERT mRNA was similarly induced (Fig. 1, A and B). This effect was particularly striking because the levels of E6/E7 mRNA and E7 protein were much lower in HFK/16E6E7 cells grown on feeder fibroblasts compared with those cultured on plastic (Fig. 1E). We presume that levels of the E6 protein were similarly reduced. Our data suggests that the HPV-16 E7 protein sensitizes keratinocytes grown on feeder fibroblasts to E6-mediated transactivation of the hTERT promoter. One other telomerase study used a similar feeder fibroblast keratinocyte culture system and also observed that the E7 protein augments activation of the hTERT promoter by E6 (23). Synergistic activation of telomerase by E6 and E7 in keratinocytes cultured on feeder fibroblasts is biologically relevant because only these cultures maintained telomere length for >50 PDs. Other investigators have also reported that expression of HPV-16 E6 and E7 in HFKs grown on plastic is insufficient to maintain telomere length in all cells in the culture (4, 22). The synergistic activation of telomerase may be partially responsible for the cooperative immortalization of keratinocytes by E6 and E7 (49–51). In addition, it is likely that the feeder culture system more closely mimics the \textit{in vivo} environment and that this synergism also occurs \textit{in vivo}.

We observed a significant increase in E6/E7 mRNA and E7 protein between early and late passage HFK/16E6E7 cells grown in both culture systems (Fig. 1E). In addition, much higher levels of E6/E7 mRNA were seen when the cells were serially cultured on plastic. We can hypothesize that these changes in expression represent a selection for E6 and/or E7 functions. Both E6 and E7 are translated from the same bicistronic mRNA expressed from the retroviral vector. Although expression of full-length functional E6 protein can be affected by alternative splicing in the E6 region, we found no evidence for differential expression of E6 and E7 through changes in alternative...
ACKNOWLEDGMENTS

We thank Dick Schlegel and Alison McBride for many helpful discussions and suggestions, Emiko Soeda for HKFs, Tim Veldman for E6- and E7-transduced HKFs, Paul Lambert and Fang Yuan for cell culture protocols, Alison McBride for p16\(^{INK4a}\) antibody, and Dick Schlegel, Alison McBride, and Astrid Baege for critical reading of the manuscript.

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Keratinocyte Growth Conditions Modulate Telomerase Expression, Senescence, and Immortalization by Human Papillomavirus Type 16 E6 and E7 Oncogenes

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