

# Immortalization of Human Mammary Epithelial Cells Is Associated with Inactivation of the p14<sup>ARF</sup>-p53 Pathway

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Received 29 July 2003/Returned for modification 21 October 2003/Accepted 2 December 2003

**Inactivation of the ARF-p53 tumor suppressor pathway leads to immortalization of murine fibroblasts. The role of this pathway in immortalization of human epithelial cells is not clear. We analyzed the functionality of the p14<sup>ARF</sup>-p53 pathway in human mammary epithelial cells (MEC) immortalized by human papillomavirus 16 (HPV16) E6, the p53 degradation-defective E6 mutant Y54D, or hTERT. E6-MEC or E6Y54D-MEC maintains high-level expression of p14<sup>ARF</sup>. Late-passage hTERT-immortalized MEC express p53 but down-regulate p14<sup>ARF</sup>. Enforced expression of p14<sup>ARF</sup> induces p53-dependent senescence in hTERT-MEC, while both E6-MEC and E6Y54D-MEC are resistant. We show that E6Y54D inhibits p14<sup>ARF</sup>-induced activation of p53 without inactivation of the p53-dependent DNA damage response. Hence, p53 degradation and inhibition of p14<sup>ARF</sup> signaling to p53 are independent functions of HPV16 E6. Our observations imply that long-term proliferation of MEC requires inactivation of the p14<sup>ARF</sup>-p53 pathway.**

After extensive growth in cell culture, primary cells cease proliferating and develop the phenotype called replicative senescence. Replicative senescence is associated with telomere shortening and involves p53- and p16<sup>ink4a</sup>-dependent mechanisms (reviewed in reference 31). Expression of the catalytic component of telomerase, hTERT, activates telomerase, restores telomeres, and immortalizes some but not all human cells (7, 26, 39, 47).

When placed in culture, primary human mammary epithelial cells (MEC) senesce in two stages. The first is characterized by growth arrest at about 10 population doublings (PDs) and is termed selection or M0 (4, 16). Selection appears to be controlled by the p16<sup>ink4a</sup>-pRb pathway, as cells can be rescued from M0 by expression of human papillomavirus type 16 (HPV16) E7 (16). Some MEC spontaneously lose p16<sup>ink4a</sup> expression (8, 17, 22) and give rise to postselection cells. Postselection MEC can be propagated further to about 40 PD until they reach replicative senescence or M1, which can be overcome by expression of HPV16 E6 (5, 42). Postselection, but not preselection, MEC can be also immortalized by hTERT (26), indicating the critical role of telomerase in overcoming replicative senescence. It has been proposed that HPV16 E6 and E7 induce human epithelial cell immortalization by stimulating hTERT expression and blocking the p16<sup>ink4a</sup>-pRb pathway, respectively (26). MEC grown on feeder layers do not manifest M0 and can be immortalized by hTERT without inactivation of p16<sup>ink4a</sup> (21).

The contribution of p53 inactivation to epithelial cell immortalization is complex. Two dominant-negative (dn) p53 mutations immortalize MEC, while the majority of mutations do not (9, 20). dn p53 together with E7 immortalizes human foreskin keratinocytes (39, 40). This suggests that the role of E6 is to degrade and inactivate p53, while E7 blocks the

p16<sup>ink4a</sup>-Rb pathway. Apart from targeting p53 for degradation, E6 induces telomerase (27) and also has other biological activities affecting cell growth (reviewed in reference 38). Induction of telomerase by E6 is associated with transcriptional activation of the *hTERT* gene (48). Several E6 mutants defective in p53 degradation (8S9A10T, F2V, and Y54H) can activate telomerase and immortalize postselection MEC (18, 26, 30). Cells immortalized by these E6 mutants have an intact DNA damage response to actinomycin D (AcD); therefore, elimination of p53 protein or the DNA damage checkpoint is not required for immortalization (26, 30). p14<sup>ARF</sup> and its murine counterpart p19<sup>ARF</sup> inhibit Mdm2-mediated proteolysis of p53 and thereby increase p53 protein levels (6, 46, 50). Inactivation of p19<sup>ARF</sup> elicits immortalization in murine embryonic fibroblasts (MEF) (24, 41); however, the role of p14<sup>ARF</sup> in immortalization of human cells is not clear. Pre-M1 MEC transduced with hTERT, wild-type E6, or E6 mutant 8S9A10T maintain expression of p14<sup>ARF</sup>, suggesting that elimination of p14<sup>ARF</sup> is not required for immortalization (26).

In this study, we analyzed induction of *hTERT* by E6 and expression levels of *p14<sup>ARF</sup>* and *p16<sup>ink4a</sup>* genes in early and late passages of MEC transduced with hTERT, E6, or E6 mutant Y54D. Like Y54H, this E6 mutant does not induce p53 degradation (30). We found that, in comparison to primary MEC, early passages of E6-MEC, E6Y54D-MEC, and hTERT-MEC did not alter expression of p14<sup>ARF</sup> and p16<sup>ink4a</sup>. In contrast, late passages of MEC immortalized by hTERT down-regulated p14<sup>ARF</sup> and p16<sup>ink4a</sup>, indicating negative selection against cells expressing these genes. MEC immortalized by E6 or E6Y54D, while down-regulating p16<sup>ink4a</sup> to different extents, maintained high levels of p14<sup>ARF</sup> expression, implying that these cells are resistant to p14<sup>ARF</sup> inhibition. Based on these observations, we sought to further explore the role of p14<sup>ARF</sup> in MEC immortalization and the activities of HPV16 E6 in this process.

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## MATERIALS AND METHODS

**Retroviral vectors and infections.** Constructs of HPV16 E6 and various E6 mutants in pLXSN were described before (30); pBabe-puro-hTERT was from R.

Weinberg; pWZL-hygro and pWZL-Hygro-H-Ras(V12) were from S. Lowe; and pBabe-puro-p14<sup>ARF</sup> was from G. Peters. The p14<sup>ARF</sup> cDNA sequence from pBabe-puro-p14<sup>ARF</sup> was recloned as a 0.4-kb *Bam*HI-*Eco*RI fragment into pWZL-hygro. The R248W dn p53 mutant from pBabe-puro-p53R248W was transferred as a *Bam*HI fragment into pLXSN. The LinX-A amphotropic packaging cells were kindly provided by G. Hannon. For production of infectious virus, LinX-A cells were plated in DF1-1 media (4) and were transfected with FuGene6 (Roche). Retrovirus-containing media was harvested 3 days posttransfection of LinX-A cells cultured at 31°C.

**Mammary cell immortalization.** Normal human MEC strain 76N, generously supplied by V. Band, and immortal cells derived from it were grown in DF1-1 media (D media) as described earlier (4). 76N MEC were transduced by infection with retrovirus supernatant containing pLXSN-16E6, pLXSN-16E6Y54D, or pBabe-puro-hTERT. MEC were infected for 12 h at 31°C and were transferred to 37°C. Cells were selected with the appropriate antibiotic 48 h postinfection (G418, 75 µg/ml, 14 days; puromycin, 0.5 µg/ml, 6 days; and hygromycin, 50 µg/ml, 3 days). MEC were expanded as two populations. One was always maintained in D media, and the other was propagated for 18 PD in D2 media, which is a defined medium lacking serum and bovine pituitary extract and is selective for immortal MEC (5). The PDs calculated for immortal cells correspond to their outgrowth after antibiotic selection.

**Induction of p53 response by AcD.** Late-passage immortal MEC were plated at 10<sup>6</sup> cells per 10-cm dish 24 h before treatment with AcD (0.5 nM). The cells were harvested for protein and RNA analysis 24 h posttreatment.

**RNA purification.** Cells were grown to 70% confluence, were washed twice with ice-cold phosphate-buffered saline, and were lysed with 4 M guanidinium thiocyanate, and total RNA was extracted by acid phenol-chloroform (10). The integrity of the RNA was verified by 1.2% agarose gel electrophoresis with 7% formaldehyde. Poly(A) RNA was purified from total RNA with an Oligotex mRNA Kit (Qiagen).

**cDNA synthesis and RT-PCR.** Single-stranded cDNA was made from 0.3 µg of poly(A) RNA or 1 µg of total RNA with Superscript II reverse transcriptase (RT) (Gibco) with random primer dN<sub>6</sub>. cDNA was amplified with *Taq* polymerase (Gibco). The following primer pairs were used: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)-G3S (dACCACAGTCCATGCCATCAG) and G3A (dTCCACCACCCTGTTGCTGTA); p14<sup>ARF</sup>-p14S1 (dGTTCTTGCTGACCC TCCGGATT) and p14A2 (dATCAGCACGAGGGCCACAG); p16<sup>ink4a</sup>-p16S1 (dGCCAACGCACCGAATAGTT) and p16A1 (dGGGCAGTTGTGGCCCT GTAG); and hTERT-TERT1784S (dCGGAAGAGTGTCTGGAGCAA) and TERT1928A (dGGATGAAGCGGAGTCTGGA).

**Quantitative RT-PCR.** cDNA was amplified in the real-time PCR cyclor (SmartCycler; Cepheid) by using PCR buffer supplemented with 1.5 mM MgCl<sub>2</sub>, 5% dimethyl sulfoxide, 0.1× SYBR Green (S-7563; Molecular Probes), 1× additive reagent (Cepheid), 0.5 µM primers for p14<sup>ARF</sup> (p14S1 and p16A1) or *GAPDH* (G3S and G3A), and *Taq* polymerase (0.025 U/µl; Gibco). p14<sup>ARF</sup> and *GAPDH* sequences in each cDNA were quantitated by comparison to amplification of external standards of p14<sup>ARF</sup> or *GAPDH* DNA, and the ratio of p14<sup>ARF</sup> to *GAPDH* was calculated. Experiments were repeated twice for each cDNA sample, and the average ratio of p14<sup>ARF</sup>/*GAPDH* was determined.

**Northern blots.** Poly(A) RNA was resolved in 1.5% agarose-7% formaldehyde gel, transferred to GeneScreen (NEN Life Science Products), and hybridized with <sup>32</sup>P-labeled DNA probe at 42°C overnight in solution containing 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50% formamide, 2× Denhardt's solution, 100 µg of salmon sperm DNA/ml, and 1% sodium dodecyl sulfate (SDS). The membrane was washed in 1× SSC with 0.1% SDS at 55°C. Images were analyzed on the PhosphorImager Storm 820 (Molecular Dynamics). p16<sup>ink4a</sup> exon 1β-specific cDNA was generated by PCR with primers p14S1 and p14A2 (dATCAGCACGAGGGCCACAG). The DNA was <sup>32</sup>P labeled in asymmetric PCR with p14A2 primer and [α-<sup>32</sup>P]dATP. The *GAPDH* cDNA probe was generated by PCR with primers G3S and G3A and <sup>32</sup>P-labeled with the Prime-A-Gene Kit (Promega).

**Western blots.** Cells were washed twice with ice-cold phosphate-buffered saline and were lysed on plates by scraping in TSG buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, and 10% glycerol). Protein concentrations were determined with a bicinchoninic acid protein assay (Pierce). Proteins were transferred to Immobilon-P membranes (Millipore) and were processed with the following antibodies: anti-p53 DO-1 (Santa Cruz [1:1,000]); anti-p21 polyclonal (sc-397; Santa Cruz [1:1,000]); anti-p21 monoclonal (OP64; Oncogene Research [1:250]); anti-MDM2 (sc-965; Santa Cruz [1:500]); anti-tubulin (SC-5286; Santa Cruz [1:4,000]); goat-anti-mouse-horseradish peroxidase (Jackson [1:8,000]); goat-anti-rabbit-horseradish peroxidase (Jackson [1:8,000]). p14<sup>ARF</sup> was detected only with Ab3 (2 µg/ml, clone 14P03; NeoMarkers) but not with FL-132 (Santa Cruz), Ab-1 (Oncogene Sciences), Ab2 (clone 14P02; NeoMarkers), or NB200-11

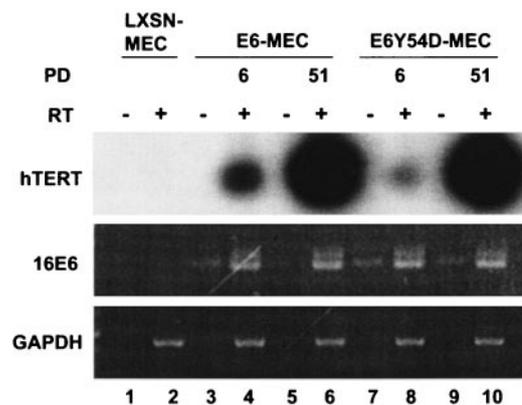


FIG. 1. Induction of hTERT in MEC by HPV16 E6 and E6 mutant Y54D. Poly(A) RNA was extracted from MEC transduced with recombinant retroviruses and was analyzed by RT-PCR for hTERT, E6, or GAPDH. hTERT RT-PCR products were transferred to GeneScreen membranes and were hybridized with a <sup>32</sup>P-labeled hTERT DNA probe generated by asymmetric PCR with primer TERT1928A and [α-<sup>32</sup>P]dATP. The blot is overexposed to demonstrate a lack of detectable hTERT transcripts in primary 76N MEC infected with LXSN retrovirus. "PD" represents PD after selection with G418.

(Novus). Processed blots were developed with SuperSignal West Dura extended-duration substrate (Pierce). Images were detected and quantitated with the LAS-1000+ luminescent image analyzer (Fuji).

## RESULTS

**Induction of hTERT by HPV16 E6 in MEC.** HPV16 E6 and immortalization-competent E6 mutants induce telomerase in MEC (18, 27). Activation of telomerase by E6 correlates with increased transcription of *hTERT* (35, 48). However, the temporal expression of *hTERT* by E6 mutants defective in p53 degradation was not tested. Figure 1 shows RT-PCR of hTERT and E6 in early and late passages of MEC transduced with E6 or the E6 mutant Y54D. In primary 76N MEC infected with the control LXSN retrovirus, *hTERT* transcripts were below detection under these RT-PCR conditions (Fig. 1, lane 2). In MEC transduced with E6 or E6Y54D, induction of the *hTERT* gene was detected by PD 6 (Fig. 1, lanes 4 and 8). There was a further dramatic increase in the amount of hTERT transcription in late-passage cells (Fig. 1, lanes 6 and 10). Of note, levels of E6 RNA did not change (compare lanes 4, 6, 8, and 10). This suggests that, apart from E6, other secondary events modulate the level of *hTERT* in MEC. Similar findings were recently reported in primary cervical epithelial cells transduced with E6/E7 oncogenes (2) and in human foreskin keratinocytes transfected with the HPV16 genome (45). Based on these observations, we investigated whether MEC expressing E6 or constitutive *hTERT* exhibits other adaptations in key pathways during passage beyond senescence checkpoints.

**Expression of p53, p14<sup>ARF</sup>, and p16<sup>ink4a</sup> in early and late passages of MEC transduced with hTERT, E6, or E6 mutant Y54D.** Pre-M1 MEC transduced with *hTERT*, E6 or E6 mutant 8S9A10T, which does not degrade p53, have high levels of expression of p14<sup>ARF</sup> while down-regulating p16<sup>ink4a</sup> (26). However, later passages of immortal MEC were not tested in this report. We analyzed levels of p53, p14<sup>ARF</sup>, and p16<sup>ink4a</sup> in

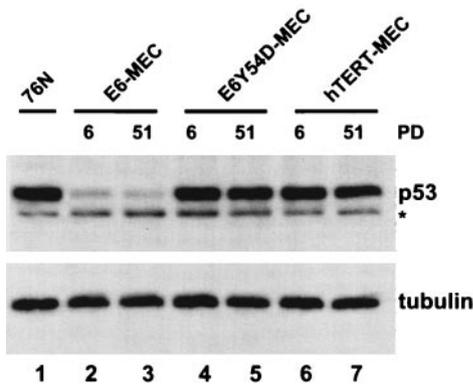


FIG. 2. Levels of p53 protein in early and late passages of MEC transduced with wild-type HPV16 E6, E6 mutant Y54D, or *hTERT*. Total cell lysate equal to 20  $\mu$ g of protein was loaded per lane and was analyzed by Western blotting. p53 protein was detected with DO-1 antibody, and tubulin was used as loading control. PD, PD after retrovirus infection and antibiotic selection; \*, a nonspecific band.

early (PD 6) and late (PD 51) passages of MEC infected with retroviruses expressing wild-type E6, E6 mutant Y54D, or *hTERT*. To avoid clonal variations, we tested pooled populations of cells. As expected, MEC expressing E6 had low levels of p53 protein (Fig. 2, lanes 2 and 3), while both E6Y54D-MEC (lanes 4 and 5) and *hTERT*-MEC (lanes 6 and 7) had levels of p53 similar to those of primary 76N cells. This confirms that E6-mediated p53 degradation is not required for MEC immortalization (26, 30). In each cell population, the level of p53 protein remained consistent at early or late passage, suggesting that there is no negative selection against expression of p53 during propagation of these MEC.

We examined the expression of the *p14<sup>ARF</sup>* and *p16<sup>ink4a</sup>* genes by RT-PCR (Fig. 3). The primary 76N strains used in this study are post-M0 MEC and express detectable *p16<sup>ink4a</sup>* mRNA. A uniform population of preselection 76N cells does not exist for comparison to determine whether *p16<sup>ink4a</sup>* was down-regulated during their selection. Immortal MEC are selected by culture in D2 media, which lacks serum and bovine pituitary extract (5). Cell growth in serum-free media may select a subpopulation of cells. Therefore, in addition to the MEC selected in D2, we tested late-passage cells that were propagated in parallel cultures in complete D media. Comparison of 76N MEC (Fig. 3, lane 1) or cells infected with pLXSN empty vector (lane 2) to early-passage cells revealed a slight increase in the levels of *p14<sup>ARF</sup>* transcript, with *p16<sup>ink4a</sup>* levels only marginally decreased in E6Y54D-MEC and *hTERT*-MEC (lanes 3, 4, and 5). A different picture emerged from analysis of late-passage cells (lanes 6 to 11). Both E6-MEC and *hTERT*-MEC down-regulated *p16<sup>ink4a</sup>* (lanes 9 and 11), while E6Y54D-MEC exhibited elevated levels of *p16<sup>ink4a</sup>* (lane 10). Interestingly, E6-MEC (lane 9) and E6Y54D-MEC (lane 10) both maintained high levels of *p14<sup>ARF</sup>*, while *hTERT*-MEC (lane 11) down-regulated *p14<sup>ARF</sup>*. This pattern of *p16<sup>ink4a</sup>* and *p14<sup>ARF</sup>* expression was not dependent on D2 media, as cells always grown in D media (lanes 6, 7, and 8) had similar levels of expression (compare *p14<sup>ARF</sup>* in lanes 6 and 9; lanes 7 and 10; and lanes 8 and 11).

Another report for which Northern blotting was used did not

detect down-regulation of *p14<sup>ARF</sup>* in early-passage (pre-M1) MEC transduced with *hTERT*, E6, or the p53 degradation-defective E6 mutant 8S9A10T (26). Therefore, we tested levels of *p14<sup>ARF</sup>* expression in MEC by Northern blotting (Fig. 4A) and quantitative real-time RT-PCR (Fig. 4B). Both methods confirmed that expression of *p14<sup>ARF</sup>* was down-regulated in late-passage *hTERT*-MEC while being maintained in E6-MEC and E6Y54D-MEC (Fig. 4A, compare lanes 3 to 5, and B). Real-time RT-PCR revealed a 15-fold reduction of *p14<sup>ARF</sup>* transcript from early to late passage in *hTERT*-MEC. We also confirmed that levels of *p14<sup>ARF</sup>* protein in 76N and early- and late-passage *hTERT*-immortalized MEC (Fig. 5B, lanes 3, 4, and 7) were barely detectable with one of five commercially available antibodies and with use of enhanced high-sensitivity chemiluminescence, indicating that levels of endogenous proteins are very low. *p16<sup>ink4a</sup>* protein was not detected by immunoblot in lysates from these cells (data not shown).

**Enforced expression of *p14<sup>ARF</sup>* in *hTERT*-MEC induces p53-dependent senescence.** Enforced expression of *p19<sup>ARF</sup>* in MEF or *p14<sup>ARF</sup>* in human tumor cell lines induces p53-dependent growth arrest (24, 46). Diploid fibroblasts expressing exogenous *p14<sup>ARF</sup>* develop the enlarged flat-cell morphology and stain positive for senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal), similar to that seen in cells in replicative senescence (13, 46, 49). It appears that *p14<sup>ARF</sup>*-induced growth arrest requires intact p53-p21<sup>cip1</sup> signaling, because *p53<sup>-/-</sup>* as well as *p21<sup>cip1</sup>* diploid human fibroblasts are resistant to *p14<sup>ARF</sup>* inhibition (49). Accordingly, E6 rescues human diploid fibroblasts from *p14<sup>ARF</sup>* inhibition (13, 46). However, expression of *hTERT* in diploid fibroblasts does not rescue these cells from *p14<sup>ARF</sup>*, indicating that *p14<sup>ARF</sup>*-induced senescence is a pathway independent from telomerase (49).

Our RT-PCR data suggest that E6- and E6Y54D-immortal-

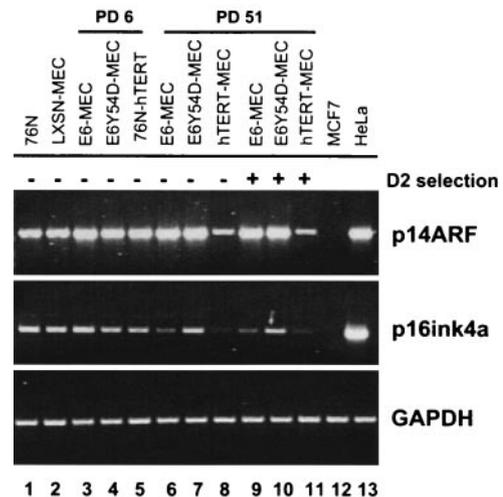


FIG. 3. RT-PCR of *p14<sup>ARF</sup>* and *p16<sup>ink4a</sup>* in early and late passages of immortal MEC. Cells transduced with specified retroviruses were grown in D or D2 media and were harvested at different PDs. RNA was analyzed by RT-PCR: *p14<sup>ARF</sup>*, 30 cycles, primers p14S1 and p16A1; *p16<sup>ink4a</sup>*, 34 cycles, primers p16S1 and p16A1; and *GAPDH*, 20 cycles, primers G3S and G3A. MCF7 cells do not express *p14<sup>ARF</sup>* and *p16<sup>ink4a</sup>* due to deletion of the *INK4a* locus. HeLa cells are from an HPV18-positive cervical cancer cell line and express *p14<sup>ARF</sup>* and *p16<sup>ink4a</sup>*.

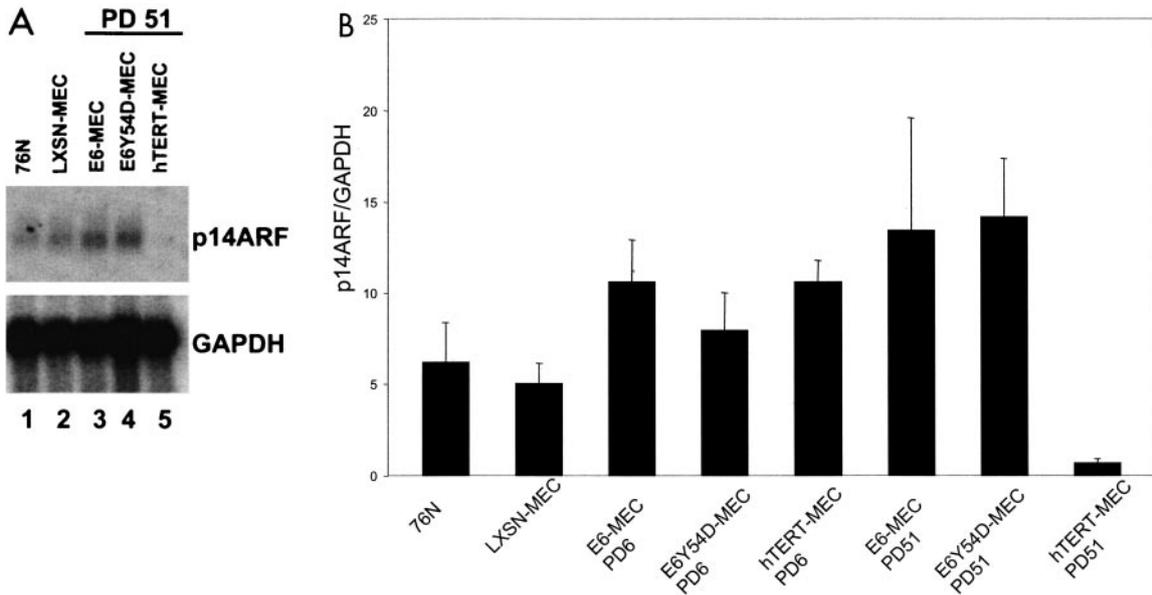


FIG. 4. Expression of p14<sup>ARF</sup> is down-regulated in late passages of MEC immortalized by hTERT but is maintained in MEC immortalized by E6 or E6 mutant Y54D. (A) Northern blot. Poly(A) RNA from specified cells was hybridized with <sup>32</sup>P-labeled probes specific for p14<sup>ARF</sup> or GAPDH. (B) Quantitative RT-PCR. cDNA was amplified in the real-time PCR cyclor with SYBR Green detection of PCR products. p14<sup>ARF</sup> and GAPDH sequences in each cDNA were quantitated with external standards, and the ratio of p14<sup>ARF</sup> to GAPDH was calculated. Experiments were repeated twice for each cDNA sample, and the average p14<sup>ARF</sup>/GAPDH ratio is shown.

ized MEC can tolerate high levels of p14<sup>ARF</sup>, while hTERT-immortalized cells cannot. To test this, we expressed exogenous p14<sup>ARF</sup> in immortal MEC by means of retrovirus-mediated gene transfer. Cells were infected with pWZL-hygro-p14<sup>ARF</sup> or pWZL-hygro control retrovirus. After selection with hygromycin, cells were harvested at specified days for cell counts and protein or RNA studies. Figure 5A shows RT-PCR results for MEC infected with the p14<sup>ARF</sup> retrovirus. Increased p14<sup>ARF</sup> RNA levels are seen after hygromycin selection in hTERT and E6Y54D-MEC after infection with the p14<sup>ARF</sup> virus. Lysates from all cells show increased p14<sup>ARF</sup> protein

analyzed by Western blotting with the Ab3 antibody after infection with pWZL-hygro-p14<sup>ARF</sup> retrovirus but not with the control pWZL-hygro virus (Fig. 5B, lanes 10 to 17).

Proliferation of hTERT-MEC was strongly inhibited by p14<sup>ARF</sup> (Fig. 6). By day 6 postinfection (about three PDs), these cells developed the enlarged flat-cell phenotype with vacuoles characteristic of senescent MEC. By day 12 postinfection, most of the cells had this appearance. In contrast to induced senescence in human fibroblasts (13, 49), we did not detect increased SA-β-gal staining in immortal MEC infected with p14<sup>ARF</sup> or activated H-RasV12 (data not shown). Lack of

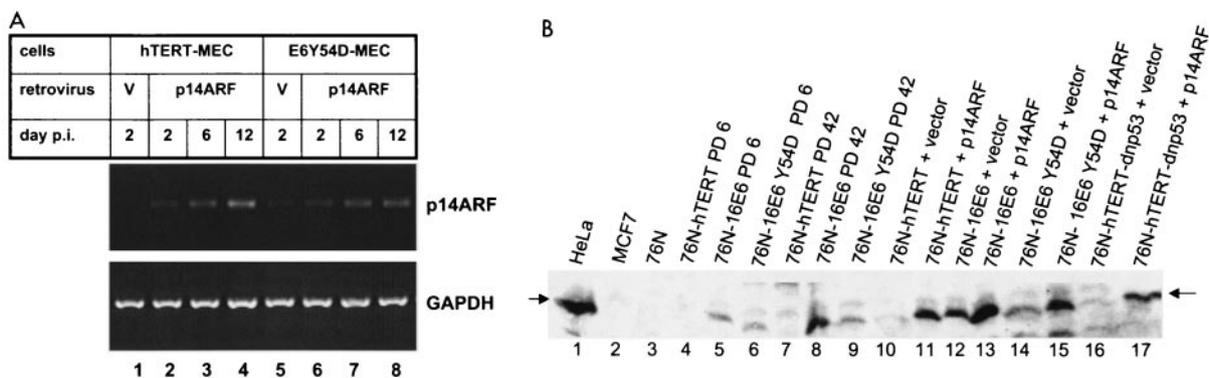


FIG. 5. Detection of p14<sup>ARF</sup> RNA and protein in MEC infected with p14<sup>ARF</sup> retrovirus. (A) RT-PCR detection of p14<sup>ARF</sup>. Cells were infected with control pWZL-hygro or pWZL-hygro-p14<sup>ARF</sup> retrovirus. The day 2 time point is immediately before hygromycin selection was started, p14<sup>ARF</sup> sequences were amplified in 30 cycles of RT-PCR with primers p14S1 and p14A2. (B) Western blot of p14<sup>ARF</sup>. Total cell lysate equal to 50 μg of protein was loaded per lane and was analyzed by Western blot. p14<sup>ARF</sup> protein was detected with the Neomarkers antibody. Lanes 1 and 2 are HeLa (positive) and MCF7 (negative) controls. Early-passage primary MEC strain 76N (lane 3) and hTERT immortal MEC at early (lane 4) and late (lane 7) passage do not express detectable p14<sup>ARF</sup> protein, which was detectable in wild-type HPV 16 E6 (lanes 5 and 8) and mutant Y54D (lanes 6 and 9) at both early and late passages. Lanes 10 to 17 used lysates from these cells after infection with control (pWZL-hygro) or pWZL-hygro-p14<sup>ARF</sup> retrovirus.

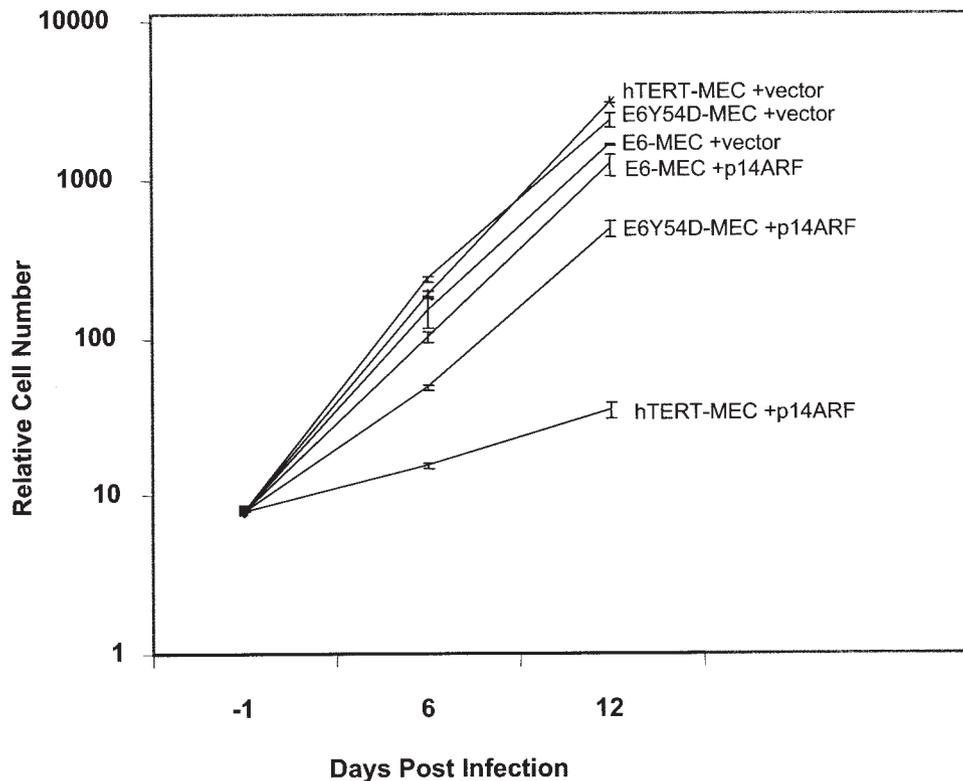


FIG. 6. Growth of immortal MEC infected with p14<sup>ARF</sup> retrovirus. Immortal MEC (PD75) were infected with pWZL-hygro-p14<sup>ARF</sup> retrovirus or pWZL-hygro empty vector. After selection with hygromycin, cells were harvested and counted at the specified days postinfection. Day -1 is when cells were plated for infection. Cells were infected on the next day, designated as day 0. The growth curve is the average of two independent experiments. Note that this is a semilogarithmic graph.

SA- $\beta$ -Gal staining in senescent human epithelial cells was also noted in other studies (V. Band, personal communication). To test whether p14<sup>ARF</sup>-induced growth arrest in hTERT-MEC is dependent on functional p53, we introduced the dn p53 mutant R248W into hTERT-MEC by retrovirus-mediated gene transfer. The p53R248W inactivates p53-mediated transactivation and extends life span but does not immortalize 76N MEC (9). When infected with p14<sup>ARF</sup> retrovirus, the hTERT-p53R248W-MEC (T53-MEC) cells displayed no growth arrest and no phenotypic change in comparison to infection with empty vector (data not shown). This proves that p14<sup>ARF</sup>-induced growth arrest in hTERT-MEC is p53 dependent.

We also tested the effect of exogenous p14<sup>ARF</sup> on growth of MEC immortalized by E6 or E6Y54D. Based on high-level expression of endogenous p14<sup>ARF</sup> in late passages of these cells, we predicted that both E6-MEC and E6Y54D-MEC would be resistant to exogenous p14<sup>ARF</sup>. As expected, growth of E6-MEC was not inhibited by p14<sup>ARF</sup> (Fig. 6) and there was no difference in phenotype. Interestingly, proliferation of E6Y54D-MEC was transiently inhibited by p14<sup>ARF</sup>. By day 6 postinfection with p14<sup>ARF</sup>, the E6Y54D-MEC were a mixed population of small cells and large flat cells mostly without vacuoles. By day 12 postinfection the population was composed mostly of small cells with a doubling rate identical to that of wild-type E6. Therefore, enforced expression of p14<sup>ARF</sup> in E6Y54D-MEC induces transient growth arrest followed by rapid outgrowth of cells resistant to p14<sup>ARF</sup> despite the presence of normal levels of wild-type p53.

**Activation of p53 by p14<sup>ARF</sup> is inhibited in MEC immortalized by E6 mutant Y54D.** p14<sup>ARF</sup>-induced growth arrest is associated with the p53 stabilization and accumulation of the products of p53-responsive genes such as *MDM2* and *p21<sup>cip1</sup>* (23, 46). The fact that E6Y54D-MEC rapidly overcome p14<sup>ARF</sup>-induced growth inhibition suggests that E6Y54D can inactivate p14<sup>ARF</sup>-induced activation of p53.

Figure 7A shows levels of p53, p21<sup>cip1</sup>, and MDM2 proteins in cells infected with p14<sup>ARF</sup> or control retrovirus. Figure 7B, C, and D depict normalized levels of proteins in comparison to cells infected with pWZL-hygro virus. In hTERT-MEC there was a 3.5-fold increase in p53 protein at day 2 postinfection (Fig. 7A, compare lanes 6 and 7, and B), before the cells' morphology changed. This level of p53 was maintained through day 12 postinfection (Fig. 7B). Accumulation of MDM2 (Fig. 7A, compare lanes 6 and 7, and D) was detected at day 2 postinfection and increased sevenfold by day 6, when the cells developed the large flat-cell senescent phenotype. Accumulation of p21<sup>cip1</sup> (Fig. 7C) was also detected at day 2 postinfection (compare lanes 6 and 7 in Fig. 7A). Interestingly, at day 6 postinfection, we reproducibly detected accumulation of smaller peptides cross-reacting with the p21<sup>cip1</sup> antibody (Fig. 7A, lane 8). The relative abundance of these faster-migrating p21<sup>cip1</sup>-specific peptides increased further by day 12 postinfection (Fig. 7A, lane 9). These proteins were also detected by a second p21<sup>cip1</sup> monoclonal antibody (data not shown), implying that they are p21<sup>cip1</sup> encoded. A p21<sup>cip1</sup> antiserum showed only a single band at the predicted position (data not shown). Total

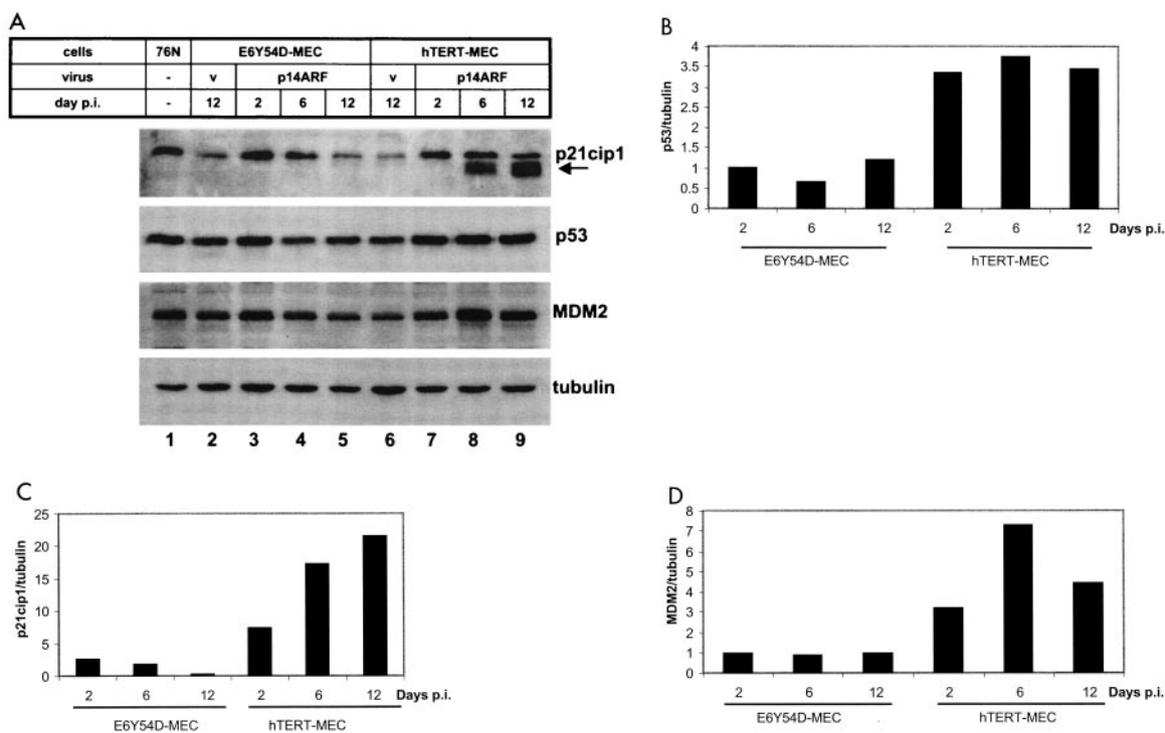


FIG. 7. Accumulation of p53, p21<sup>cip1</sup>, and MDM2 proteins in p14<sup>ARF</sup>-infected cells. (A) Cells were infected with p14<sup>ARF</sup> retrovirus or empty vector as described in the Fig. 6 legend, and total cell lysates corresponding to 20  $\mu$ g of protein were analyzed by Western blot. The arrow indicates smaller p21<sup>cip1</sup>-specific peptides detected with OP64 antibody (Oncogene Research). Virus "V," empty vector pWZL-hygro. (B to D) Levels of p53, p21<sup>cip1</sup>, and MDM2 were quantitated with the LAS-1000+ luminescent image analyzer (Fuji) and were normalized to tubulin. Protein levels in cells infected with the empty vector were set at 1.

levels of p21<sup>cip1</sup>-specific peptides in hTERT-MEC increased more than 20-fold by day 12 postinfection (Fig. 7C). In E6-MEC and T53-MEC infected with p14<sup>ARF</sup> retrovirus, there was no accumulation of p21<sup>cip1</sup> protein (data not shown).

In contrast to hTERT-MEC, stabilization of p53 or accumulation of MDM2 was not detected in E6Y54D-MEC infected with p14<sup>ARF</sup> (Fig. 7A, lanes 3 to 5, and B and D). There was a 2.7-fold increase in levels of p21<sup>cip1</sup> in E6Y54D-MEC by day 2 postinfection (Fig. 7C). The abundance of p21<sup>cip1</sup> gradually returned to basal noninduced levels by day 12 postinfection (Fig. 7A, compare lanes 2 to 5, and C).

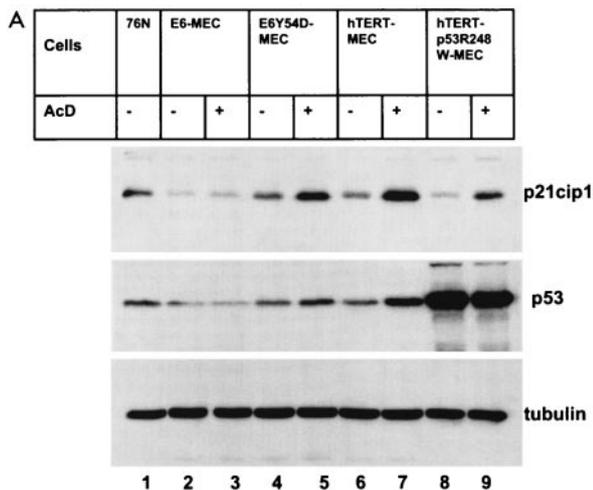
**AcD-induced DNA damage response in immortal MEC.** Analysis of levels of p53, p21<sup>cip1</sup>, and MDM2 proteins in cells infected with p14<sup>ARF</sup> retrovirus implies that activation of p53 is blocked in E6Y54D-MEC. p53 is activated in response to diverse stress signals (reviewed in reference 3). MEC immortalized by the E6 mutant 8S9A10T, F2V, or Y54H, which does not degrade p53, have an intact DNA damage response to AcD (26, 30). Therefore, we tested activation of p53 by AcD in immortal MEC containing E6, E6Y54D, hTERT, or hTERT + p53R248W (Fig. 8). As expected, there was no accumulation of p53 or p21<sup>cip1</sup> in E6-MEC (compare lanes 2 and 3). Both E6Y54D-MEC (lanes 4 and 5) and hTERT-MEC (lanes 6 and 7) accumulated p53 and p21<sup>cip1</sup> proteins, indicating activation of p53 and induction of p21<sup>cip1</sup>. However, E6Y54D-MEC accumulated less p53 and p21<sup>cip1</sup> than did hTERT-MEC, indicating that activation of p53 by AcD may be partially compromised by E6Y54D. In contrast to the response induced by p14<sup>ARF</sup>, there was no accumulation of small p21<sup>cip1</sup>-specific

peptides in hTERT-MEC treated with AcD. Evaluation of p53 stabilization in T53-MEC was not possible due to the large amounts of p53 protein in cells not treated with AcD. Interestingly, a small degree of p21<sup>cip1</sup> induction was still present in T53-MEC expressing dn p53 mutant R248W (compare lanes 8 and 9), indicating residual p53 function or p53-independent activation of p21<sup>cip1</sup> by AcD.

## DISCUSSION

In this study we address the role of the p14<sup>ARF</sup>-p53 and p16<sup>ink4a</sup>-pRb pathways in MEC immortalization. It has been proposed that replicative senescence is dependent only on telomere shortening, while the p16<sup>ink4a</sup>-dependent mechanism of replicative senescence is an artifact created by inappropriate growth conditions (36). However, even when MEC are grown on feeder layers, p16<sup>ink4a</sup> protein levels are down-regulated in late passages of MEC (21). Collectively, our data and other studies (26, 39) indicate negative selection against cells expressing p16<sup>ink4a</sup> during immortalization and/or propagation of MEC and keratinocytes—either grown on feeder layers or not. Activation of the p16<sup>ink4a</sup>-pRb pathway represses telomerase activity in cancer cell lines (11, 12). The inverse correlation between expression of p16<sup>ink4a</sup> and hTERT in MEC raises the question whether down-regulation of p16<sup>ink4a</sup> can account for increased levels of hTERT in late passage MEC immortalized by 16E6 or E6-Y54D.

Studies on the role of ARF in cell immortalization are complicated by the partial overlapping of ARF and p16<sup>ink4a</sup> genes.



Mutations or deletions in the *INK4a* locus frequently inactivate both genes, and disruption of *ARF* might be due to bystander effect of selection against  $p16^{\text{ink}4a}$ . In murine cells, inactivation of *p19<sup>ARF</sup>* leads to immortalization of MEF and pre-B cells (24, 33). Rare selective inactivation of *p14<sup>ARF</sup>* with retention of *p16<sup>ink4a</sup>* has been reported in human tumors (19, 34, 44), melanoma cell lines (29), a melanoma-neural system tumor syndrome family (37), and a postcrisis clone of human adenoid epithelial cells immortalized by hTERT (15). These data suggest a role for  $p14^{\text{ARF}}$  in human cancer and cell transformation, independent from  $p16^{\text{ink}4a}$ . The observation that E6-MEC with inactivated p53 function maintain expression of *p14<sup>ARF</sup>* while down-regulating *p16<sup>ink4a</sup>* indicates that inactivation of *p14<sup>ARF</sup>* in hTERT-MEC is not a consequence of selection against  $p16^{\text{ink}4a}$  and implies an independent role for  $p14^{\text{ARF}}$  in MEC immortalization.

Pre-M1 MEC transduced with *hTERT*, E6 or E6 mutant 8S9A10T maintain high-level *p14<sup>ARF</sup>* mRNA (26), suggesting that elimination of  $p14^{\text{ARF}}$  is not required for MEC proliferation. However, advanced passages of those cells were not tested. In addition, 76N MEC immortalized by Bmi-1 do not down-regulate  $p14^{\text{ARF}}$  protein, as shown by Western blot analysis (14). In our experiments, the level of endogenous  $p14^{\text{ARF}}$  protein in primary and hTERT immortal 76N MEC was below the detection limit of Western blotting with several commercially available polyclonal and monoclonal antibodies. MEC immortalized by HPV 16 E6, E6Y54D, and *hTERT* plus dn p53 had low but detectable  $p14^{\text{ARF}}$  protein, consistent with the RT-PCR data. In this study down-regulation of *p14<sup>ARF</sup>* RNA is shown in two independent populations of late-passage hTERT-MEC. Analysis of the *p14<sup>ARF</sup>* expression in other epithelial cells (and other MEC strains) immortalized by *hTERT* will clarify if inactivation of *p14<sup>ARF</sup>* is a common event in such cells.

Enforced expression of  $p14^{\text{ARF}}$  induces p53-dependent growth arrest and senescence in hTERT-MEC. This is accompanied by accumulation of p53,  $p21^{\text{cip}1}$ , and MDM2, which is in agreement with  $p14^{\text{ARF}}$ -induced senescence in fibroblasts (23, 46, 49). Interestingly, in addition to full-size  $p21^{\text{cip}1}$ , we found accumulation of smaller  $p21^{\text{cip}1}$ -specific peptides. Such peptides are not detected in hTERT-MEC treated by AcD. The

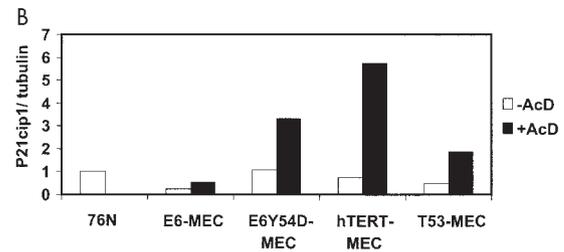


FIG. 8. Accumulation of p53 and  $p21^{\text{cip}1}$  proteins in AcD-treated cells. (A) Late-passage immortal MEC were treated with AcD, and total cell lysates (20  $\mu\text{g}$ ) were analyzed by Western blot as described in the Fig. 7 legend. (B) Levels of  $p21^{\text{cip}1}$  were quantitated and normalized to tubulin as described in the Fig. 7 legend.  $p21^{\text{cip}1}$  levels in 76N cells were set at 1.

truncated  $p21^{\text{cip}1}$  peptides represent amino-terminal fragments of  $p21^{\text{cip}1}$ , since the carboxy-terminal  $p21^{\text{cip}1}$  polyclonal anti-serum detects only one full-size  $p21^{\text{cip}1}$  in the  $p14^{\text{ARF}}$ -infected hTERT-MEC (data not shown). The N terminus of  $p21^{\text{cip}1}$  has cdk inhibitory activity, while the C terminus binds and inhibits PCNA (32). Other studies have not shown the induction of  $p21^{\text{cip}1}$  fragments in cells expressing exogenous  $p14^{\text{ARF}}$  but used different cell strains and antibodies (23, 46, 49). The origin and significance of the  $p21^{\text{cip}1}$ -derived peptides are a subject for further investigation.

Previous studies showed that E6 mutants 8S9A10T, F2V, and Y54H do not degrade p53 yet could immortalize MEC (26, 30), identical to the phenotype that we show here for E6Y54D. Interestingly, these mutant E6 proteins all retained ability to bind the ubiquitin ligase E6AP, which is necessary for E6-mediated p53 degradation. Pre-M1 MEC expressing these mutants, as well as hTERT-MEC, express normal levels of p53 and  $p14^{\text{ARF}}$  and preserve the p53-dependent DNA damage checkpoint. However, here we show that, while cells immortalized by E6Y54D express p53,  $p14^{\text{ARF}}$  signaling to p53 is inactivated and  $p14^{\text{ARF}}$ -induced senescence is blocked. Nonetheless, cells harboring E6Y54D remain susceptible to p53 activation by DNA damage.  $p14^{\text{ARF}}$  is not strictly required for p53-dependent DNA damage response but may modulate it (25). The mechanism for how E6Y54D restricts  $p14^{\text{ARF}}$ -induced activation of p53 requires further investigation. Different stress signals activate p53 through multiple pathways (3). In tumor cells, the p53-dependent response to hypoxia-like stress may be inactivated while response to AcD is intact (1). The present model is that ARF stabilizes p53 by targeting MDM2 to the nucleolus and blocking p53 ubiquitination by MDM2 (reviewed in reference 43). E6Y54D may block p53 activation by interaction with the p53 protein or by inhibiting another protein(s) required for p53 activation. Interestingly, a recent study shows that immortalization-competent E6 mutants defective in p53 degradation, including E6Y54H, bind and degrade the p53 transcriptional coactivator hADA3 (28). We are currently examining the relationship between E6 binding to cellular targets and inhibition of the  $p14^{\text{ARF}}$ -p53 pathway.

The mechanism of E6 stimulation of *hTERT* expression is of active interest. We show a dramatic increase in expression of *hTERT* in late-passage MEC immortalized by wild-type E6 or E6Y54D. This was not accompanied by an increase in the level of E6. Similar findings were reported in human keratinocytes

transduced with HPV16 E6/E7 (2). Our observations in MEC suggest initial E6-dependent induction of low levels of *hTERT* followed by E6-independent induction of high levels of *hTERT* expression during propagation of cells. Alternatively, different cells transduced with E6 initially express variable levels of *hTERT*, and only those with high levels of expression become immortal and are selected for during propagation.

#### ACKNOWLEDGMENTS

We thank Scott Lowe, Robert Weinberg, Gordon Peters for providing constructs, Greg Hannon for retroviral packaging cells, Vimla Band for 76N cells and stimulating discussion, Joanna Parish, Ting Yu and Caroline Lai for assistance with computer graphics and review of the manuscript, and other members of the lab for useful comments. Judith Alamares assisted with the real-time PCR.

This work was supported by NIH grant R01 CA73558.

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