Genetic and Epigenetic Changes in Human Epithelial Cells Immortalized by Telomerase

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Exogenous expression of hTERT, the catalytic component of telomerase, is sufficient for the immortalization of human fibroblasts but insufficient for the immortalization of human foreskin keratinocytes (HFKs) and human mammary epithelial cells (HMECs). These latter cell types can overcome senescence by coexpression of hTERT and human papillomavirus (HPV) E7 or by expression of hTERT and loss of p16^{INK4a} expression, indicating that the retinoblastoma (Rb) pathway, along with a telomere maintenance pathway, plays a role in determining the life span of epithelial cells. In this study, we further characterize hTERT-immortalized HFKs and human adenoid epithelial cells (HAKs) for genotypic and phenotypic alterations that are associated with immortalization. Of five hTERT-immortalized HFK and HAK cell lines examined, four exhibited repression of p16^{INK4a} expression by promoter methylation or specific large-scale deletion of chromosome 9p, the location of p16^{INK4a}. Interestingly, one cell line exhibited complete down-regulation of expression of p14^{ARF}, with only slight down-regulation of expression of p16^{INK4a}. Yet, all of the immortal cells lines exhibited hyperphosphorylated Rb. Cytogenetic analysis revealed clonal chromosome aberrations in three of the five cell lines. All of the cell lines retained a growth block response with the expression of mutant *ras*. When grown on organotypic raft cultures, however, the hTERT-immortalized cells exhibited a maturation delay on terminal differentiation. Our results indicate that immortalization of epithelial cells may require both activation of telomerase and other genetic and/or epigenetic alterations that abrogate normal differentiation. (Am J Pathol 2000; 156:1537–1547)

Telomerase is a ribonucleoprotein that maintains telomere length during cell division.^{1,2} Increased telomerase activity is thought to be a key event in the immortalization of cells and has been detected in many transformed human cell lines, precancerous lesions, and carcinomas.^{3,4} Introduction of the reverse transcriptase component of human telomerase (hTERT) and subsequent telomerase activation has been shown to extend the life span of human fibroblasts and retinal pigment epithelial (RPE) cells.⁵ It has been proposed that telomerase activation is the only step required for the immortalization of cells.^{6,7} Attempts to prolong the life span of epithelial cells such as human foreskin and human mammary epithelium, however, have not been successful, except in conjunction with alterations in the retinoblastoma (Rb) pathway.⁸ Specifically, expression of the cyclin D/cdk inhibitor p16^{INK4a} was down-regulated in the cells when they became immortal. Loss of p16^{INK4a} expression is commonly observed in epithelium-derived cancers,⁹ but its precise role in immortalization and malignant progression is unclear. Expression of p16^{INK4a} steadily increases as cells are passaged in culture, and up-regulation of p16^{INK4a} has been associated with cellular senescence in both fibroblasts and epithelial cells.^{10–15} Yet, fibroblasts can be immortalized by telomerase activation alone without p16^{INK4a} loss, suggesting cell type-specific roles for p16^{INK4a} in senescence. It is possible that p16^{INK4a} is involved in a terminal differentiation program that needs to be overcome for the immortalization of epithelial cells. It has been demonstrated, for example, that restoration of p16^{INK4a} induces myogenic differentiation in rhabdomyosarcoma cells.¹⁶ It has also been shown that p16^{INK4a} is directly involved in regulating $\alpha_v\beta_3$, an integrin that plays a role in extracellular matrix interactions and differentiation.¹⁷ Fibroblasts are considered to be relatively undifferentiated and may not require abrogation of a differentiation associated proliferation block to become immortal, although it has been suggested that p16^{INK4a} is involved in fibroblast-specific differentiation.¹⁸ Another possibility is that p16^{INK4a} is in-

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volved in a stress response to accelerated proliferation. It has been demonstrated, for example, that overexpression of mutant *ras* in fibroblasts causes premature senescence and that this is associated with up-regulation of p16^{INK4a} and p14^{ARF},^{19–21} a gene that shares exons with p16 but utilizes a different promoter and is read in an alternative reading frame.²² It is unknown, however, if natural senescence in culture, particularly in human epithelial cells, is in any way related to the mutant *ras*-mediated stress response observed in fibroblasts. DNA damage has also been hypothesized to play a role in p16^{INK4a} up-regulation, but the mechanism has not been well characterized.^{23,24}

Whether telomerase activation and subsequent immortalization can predispose cells to malignant transformation is an issue that remains controversial. Earlier studies have concluded that immortalization of human fibroblasts and RPE cells by hTERT was not associated with cancer-specific alterations.^{6,7} For example, the hTERT-immortalized cells still maintained the ability to hypophosphorylate Rb under conditions that cause cell cycle blocks, such as DNA damage or confluency. Telomerase activation was also suggested to confer karyotypic stability. If this is true, an argument could be made that constitutive telomerase activation is protective against the development of cancer because it prevents the genetic instability associated with telomere loss. Telomerase activation and subsequent immortalization of cells, however, clearly would provide a selective growth advantage for the development of malignancy. Telomerase activation occurs in up to 97% of *in vivo* malignancies and the vast majority of immortal cell lines,^{3,4} and it has been demonstrated that expression of hTERT can predispose human embryonic kidney cells to tumorigenic transformation by coexpression of SV40 large T antigen and mutant *H-ras*.²⁵ Nevertheless, the ability to immortalize cells by hTERT could provide the potential to generate relatively normal immortal cells for research endeavors that have been limited by a finite supply of primary cells and tissue. Our observation, however, that immortalization of HFKs and HMECs by hTERT requires abrogation of the Rb pathway raises the question of whether the immortal cells are actually "normal."

In this study, we have analyzed hTERT-immortalized HFKs and human adenoid epithelial cells (HAKs) to characterize events associated with immortalization and to determine whether the cells exhibit phenotypic alterations that make them abnormal. Our results indicate that both epigenetic and genetic changes occur on immortalization by hTERT and that immortalization is associated with an aberrant delay in the epithelial program of terminal differentiation.

Materials and Methods

Cell Culture and Organotypic Rafts

Human adenoid epithelial cells (HAKs) and human foreskin keratinocytes (HFKs) were isolated as previously described.²⁶ Cells were grown in keratinocyte serum-free

media (K-SFM; Gibco-BRL, Gaithersburg, MD) in 50 μ g/ml G418 after retroviral infection. Clones were picked by cylinder isolation of colonies as previously described and split into 1:4 or 1:8, using trypsin/EDTA. Cells in crisis or senescence were fed three to four times weekly, split if necessary, and maintained until there was no visible sign of proliferation (usually 2 months). All experiments described were performed within 100 population doublings (pd) of crisis. The protocol for organotypic rafts has been described.^{26–28} Briefly, dermal equivalents are generated with collagen and normal human fibroblasts. Epithelial cells are seeded on top of the dermal equivalents and grown submerged for 7 days in Rheinwald Green media. The "rafts" are then raised to the air-liquid interface, using a stainless steel grid on organ culture plates, and fed from beneath daily. After 12 days, the rafts were frozen in OCT or fixed in methacarn for immunohistochemical analyses.

Northern Blot

Total RNA was isolated from subconfluent cells with a kit (Qiagen). Twenty micrograms was run on a 1.2% gel and transferred to Hybond N membrane. For detection of p14^{ARF} expression, exon 1 of the INK4 locus was amplified and labeled by random priming with [³²P]CTP. Blots were hybridized and washed as described.²⁹ The 36B4 probe was generated by polymerase chain reaction (PCR), labeled by random priming as described,⁸ and hybridized to the same stripped blot as above.

Ras Infection, TGF- β , β -Galactosidase Staining, and 5-Azadeoxycytidine Treatment

The pCTV3-H-Ras61L construct was made by cloning the H-Ras61L cDNA from the pAX142 vector into pCTV3, and viral supernatants were generated in Phoenix amphotropic packaging lines as described.^{30–32} Exponentially growing cells were infected overnight in 4 μ g/ml polybrene. Infected cells were washed in regular media and refed. Cells were passed on the following day, and selective medium containing 8 μ g/ml hygromycin (Boehringer-Mannheim) was added on the following day. After 5–10 days, cells were fixed and stained for β -galactosidase expression and collected for protein and RNA. β -Galactosidase staining of cell cultures was performed as described.³³ To test TGF- β sensitivity, subconfluent newly passaged cells were treated with various concentrations of porcine TGF- β (0, 0.1, 1.0, 10.0, and 100.0 μ mol/L) (R&D Systems, Minneapolis, MN) for 6 days, after which cells were collected for protein.

Cells were treated with 5-azadeoxycytine (Sigma) at 1.5×10^{-6} mol/L at subconfluency for 7 days before collection for Western or Northern analyses. Untreated subconfluent cells were collected at the same passage.

Western and Immunohistochemical Analyses

Western analyses were performed as described, using monoclonal antibodies for p16^{INK4} (Pharmingen, San Di-

ego, CA), Rb (Pharmingen), p27^{KIP} (Transduction Labs), cyclin D (Pharmingen), cdk4 (Transduction Labs), or p53 (Oncogene Research). Forty micrograms of protein lysate for Rb analysis and 20 μ g of lysate for analysis of other proteins were run on polyacrylamide gels of the appropriate concentration and blotted onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA). Detection was performed using a Renaissance chemiluminescence kit (NEN, Boston, MA).

Standard immunocytochemistry and hematoxylin and eosin (H&E) staining techniques were used on methacarn-fixed or OCT frozen and acetone-fixed sections as described.³⁴ Antibodies were AE2 (Bioscience) for K1 analysis and clone A9 (a gift from Tom Carey, University of Michigan) for α 6 β 4 analysis.

Tumorigenicity Studies

Cells that were 30–50 pd postcrisis were injected into 4- to 6-week-old athymic nude female mice (Simonson) at 3×10^6 cells/site, one site per animal, two animals per cell line, subcutaneously into the dorsal anterior quadrant. Mice were observed for more than 3 months for tumorigenic growth at the site of injection.

Comparative Genome Hybridization Analysis

Comparative genome hybridization (CGH) was performed as described, with modifications.^{35–37} Reference DNA was isolated from precrisis hTERT HFK cells, and test DNA was isolated from hTERT postcrisis cells. Test DNA was labeled with biotin-14-dATP (Gibco BRL) and reference DNA with digoxigenin-11-dUTP (Boehringer-Mannheim), using the standard protocol provided with the Boehringer-Mannheim nick translation kit. Normal male metaphase spreads were pretreated and hybridized for 3–5 days at 37°C as described.^{35–37} Biotinylated DNA sequences were detected using avidin-fluorescein isothiocyanate (FITC) (Vector Labs). Signals were amplified once. Digoxigenin-labeled DNA sequences were visualized using rhodamine-conjugated sheep anti-digoxigenin Fab fragments (Boehringer-Mannheim). The preparations were then counterstained with 4,6-diamidino-2-phenylindole-dihydrochloride (DAPI) (50 ng/ml) for 10 minutes at room temperature and mounted in Vectashield (Vector Labs). Image acquisition and processing were performed using an epifluorescence microscope (Nikon Microphot-SA) equipped with a 100-W mercury lamp and a standard CCD camera interfaced to a DELL PC. Gray-level images were recorded separately for each fluorochrome, using specifically aligned filter sets for DAPI, FITC, and rhodamine. Digital images were processed with Cytovision software developed by Applied Imaging. After correction of centromere placement and determination of the chromosome axis, individual FITC/rhodamine profiles were calculated for each chromosome. Mean ratio profiles were then determined from 9–25 metaphase spreads. Chromosomes were identified by inspection of inverted digital DAPI images.

Bisulfite Genomic Sequencing

After digestion with *Eco*RI, 1.5 μ g of genomic DNA was bisulfite modified as previously described, with minor modifications.³⁸ PCR amplification of the p16^{INK4a} promoter was performed with a sense primer (5' GTA GGT GGG GAG GAG TTT AGT T) binding –355 to –334 relative to the translation start site [43], and one of two antisense primers (5' TCT AAT AAC CAA CCA ACC CCT CC or 5' CTA CCT AAT TCC AAT TCC CCT ACA) binding at positions –95 to –73 or +209 to +233.³⁹ Reaction conditions were 34 cycles at 95°C for 30 s, 60°C for 15 s, 72°C for 75 s, and 34 cycles at 95°C for 30 s, 64°C for 15 s, and 72°C for 75 s. PCR products were cloned, and at least 10 individual epigenotypes per sample were determined by automated sequencing. Analysis of non-CpG cytosines indicated the efficiency of bisulfite conversion at 99%.

Karyotypic Analysis

Metaphase spreads of HAK and HFK cell lines between 10 and 50 pd postcrisis were prepared on glass coverslips by standard cytogenetic methods, and 10–40 G-banded cells were analyzed for karyotypic abnormalities.

Mutational Analysis

The sequences of the coding regions and splice junctions of exons 1 and 2 of p16^{INK4a} were determined by automated sequencing after amplification as described, with minor modifications.⁴⁰ Primer sequences were as follows: exon 1: sense 5' GAA GAA AGA GGA GGG GCT G, antisense 5' GCG CTA CCT GAT TCC AAT TC; exon 2: sense 5' GGA AAT TGG AAA CTG GAA GC, antisense TCT GAG CTT TGGAAG CTC T.

Results

Loss of p16^{INK4a} or p14^{ARF} Expression

Earlier studies indicated that loss of p16^{INK4a} expression or abrogation of the Rb pathway by HPV E7 was necessary for immortalization of HFKs by hTERT.⁸ In this study we extend our observations on the mechanisms of epithelial cell immortalization by hTERT to human adenoid epithelium (HAK). As was the case with HFKs, retroviral mediated expression of hTERT in HAK cells was insufficient for immortalization. Telomerase-positive hTERT-expressing clones senesced or went through a severe crisis before becoming immortal. All of the hTERT HFK and HAK immortal cell lines were shown to have high telomerase activity and to maintain telomere lengths with passaging in culture (data not shown). As has been described previously,⁸ hTERT HFK clones (cl 22 and 398) that went through crisis and became immortal exhibited loss of p16^{INK4a} expression as compared to normal HFKs (Figure 1a, lanes 1–3). Of three hTERT HAK clones that emerged from crisis, two (cl 7 and cl 12) exhibited loss of p16^{INK4a} expression (Figure 1a, lanes 5 and 6). One

clone (cl 41), however, did not show complete loss of p16^{INK4a}, but instead exhibited loss of expression of p14^{ARF} as assayed by Northern analysis (Figure 1b, lane 8). Western analysis failed to detect ARF in any of the lines (data not shown), presumably because of low ARF protein levels in keratinocytes in general.⁴¹ Interestingly, the immortalized cell lines did not exhibit any consistent alterations in p21, p53, p27^{KIP}, or cdk4 expression (Figure 1a). However, there was slightly lower expression of cyclin D as compared to normal HFK or HAK, particularly in those cell lines that had complete loss of p16^{INK4a} expression, and a slightly higher expression of Mdm2 in all of the postcrisis cells. All of the immortal cells, including the ARF-negative cell line cl 41, also exhibited more hyperphosphorylated Rb as compared to normal precrisis cells when grown in subconfluent conditions, indicating a probable defect in the Rb pathway (Figure 1a).

Cytogenetic Alterations in Cell Lines

Examination of the hTERT-immortalized cells by cytogenetic analyses within 50 population doublings (pd) of crisis revealed clonal chromosome aberrations in some of the cell lines but not in others (Table 1). Two cell lines, TERT HFK cl 398 and TERT HAK cl 41, showed no evidence of cytogenetic abnormalities and had normal diploid chromosome numbers. One cell line, hTERT HAK cl 12, contained an extra chromosome 5, whereas another, hTERT HAK cl 7, contained translocations of chromosomes 2 and 7 and several nonclonal aberrations. Interestingly, the cell line hTERT HFK cl 22 exhibited an isochromosome 9q as the only cytogenetic abnormality visible by G-banding. Loss of heterozygosity (LOH) analyses using PCR primers that amplified polymorphic sequences on 9p, the location of p16^{INK4a}, revealed that this cell line had sustained a deletion of one 9p arm (data not shown). CGH showed that this, along with amplification of chromosome 9q, was the only apparent chromosome aberration in this cell line (Figure 2). Thus at least one p16^{INK4a} allele in hTERT HFK cl 22 was lost by specific deletion of the 9p arm.

Methylation of the p16^{INK4a} Promoter

Loss of p16^{INK4a} expression has been shown to result from deletion, point mutation, and methylation of CpG islands in the promoter.⁴² Sequencing analyses of p16^{INK4a} exons 1 and 2 revealed no point mutations or small deletions in the cell lines that had lost p16^{INK4a} expression (data not shown). The methylation state of the p16^{INK4a} promoter was examined in these cell lines by treatment with the demethylating agent 5-azadeoxycyti-

Figure 1. A: Western analyses for expression of cell cycle proteins in hTERT immortalized keratinocytes. All cells were subconfluent when collected. **Lane 1:** Normal precrisis human foreskin keratinocytes (HFK) expressing hTERT; **lanes 2 and 3:** two postcrisis hTERT expressing HFK; **lane 4:** normal precrisis human adenoid keratinocytes (HAK); **lanes 5–7:** three postcrisis hTERT expressing HAK. **B:** Northern analyses for the expression of p14^{ARF} in immortalized keratinocytes. All cells are the same as in **A**, with the addition of HPV16 E7 expressing foreskin keratinocytes in **lane 4**. The probe 36B4 is a control for RNA loading.

Table 1. Karyotype Analyses of hTERT Cell Lines

Cells	Karyotype
HFK (normal)	46, XY
hTERT HFK CI 22	47, XY, iso (9)(q)
hTERT HFK CI 398	46, XY
HAK (normal)	46, XX
hTERT HAK CI 7	major: 47, XX, add (2)(q37), add (7)(q22), +add (7)(q32) minor: 48, XX, add (2)(q37), add (7)(q22), +add (7)(q32), +20 46, XX, add (2)(q37), add (7)(q22)
hTERT HAK CI 12	47, XX, +5
hTERT HAK CI 41	46, XX

dine. After 7 days of treatment in 5-azadeoxycytidine, re-expression of p16^{INK4a} protein was observed in all of the cell lines that initially had lower or no expression, indicating that the p16^{INK4a} promoter was down-regulated by methylation in the untreated cell lines (Figure 3). There was slight up-regulation of p14^{ARF} expression on 5-azadeoxycytidine treatment in hTERT HAK ci 41, suggesting that at least one allele of the p14^{ARF} promoter in this cell line might also be down-regulated by methylation (data not shown). Interestingly, treatment with azadeoxy-

cytidine and concomitant up-regulation of p16^{INK4a} or p14^{ARF} caused a senescent-like phenotype, as measured by β -galactosidase staining and morphological alteration, in all of the cell lines tested (data not shown). However, 5-azadeoxycytidine treatment also caused a senescent-like phenotype in normal HFK- and HPV E7-expressing cells without P16^{INK4a} or p14^{ARF} up-regulation, indicating that 5-azadeoxycytidine treatment may nonspecifically affect the expression of other genes involved in the senescent phenotype.

We also examined the methylation state of the p16^{INK4a} promoter in the different cell lines by bisulfite methylation analysis.³⁸ This method utilizes bisulfite-induced modification of genomic DNA, under conditions in which cytosine is converted to uracil, but 5-methylcytosine remains nonreactive. When clones derived from PCR-amplified bisulfite-treated DNA are sequenced, only cytosines that were methylated in the original sample present as cytosines, whereas all other cytosines are converted to thymine. All of the cell lines that showed down-regulation of p16^{INK4a} exhibited methylation of specific CpG sites in the p16^{INK4a} promoter, whereas DNA from precrisis cells did not exhibit extensive methylation (Figure 4). Interestingly, there was some methylation in hTERT HAK ci 41, which reflects the lower but not completely absent expression of p16^{INK4a} in the cell line. The CpG sites that were found to be methylated in postcrisis cells have been

Figure 2. Comparative genome hybridization (CGH) analysis of hTERT HFK ci 22. Precrisis hTERT HFK ci 22 DNA was labeled with Rhodamine and postcrisis hTERT HFK ci 22 with FITC, and CGH was performed as described in Materials and Methods. Shifts into the red represent deletions. Shifts into the green represent amplifications. The only aberrations observed in hTERT HFK ci 22 were a deletion of 9p and amplification of 9q.

Figure 3. Western analysis showing re-expression of p16^{INK4a} on 5-azadeoxycytidine treatment. Cells are the same as in Figure 1. They were collected as subconfluent untreated (–) or after being treated for 7 days (+) with 5-azadeoxycytidine.

Figure 4. Methylation-specific sequencing analysis of p16^{INK4a} promoter. The numbers on the x-axis represent CpG sites in the p16^{INK4a} promoter as previously described. Site 10 is a polymorphism found in the population that was present in the HFK lines only. **A:** Precrisis hTERT HFK; **B:** postcrisis hTERT HFK clones 22 and 398; **C:** precrisis HAK; **D:** postcrisis hTERT HAK clones 7, 12, and 41.

shown previously to be important in the down-regulation of p16^{INK4a} expression.⁴³ Thus, in most cases, loss of expression p16^{INK4a} in the cell lines was epigenetic, being caused by methylation, except for the case of hTERT HFK cl 22, where one allele was lost by deletion (see above).

Retention of Growth Control in Cells That Have Lost p16^{INK4a} or p14^{ARF}

It has been hypothesized that loss of p16^{INK4a} and/or p14^{ARF} expression may allow cells to bypass senescence induced by overexpression of mutant *ras* oncogene.¹⁹ Retroviruses expressing *H-ras* with a mutation at codon 61 were used to infect normal HFKs or the different hTERT-immortalized HFK or HAK cell lines. After selection in hygromycin, normal HFKs with mutant *H-ras* exhibited a growth block that had characteristics of senescent HFKs, including staining with β -galactosidase and increased cytoplasm-to-nuclear ratios as compared to vector alone (Figure 5). Interestingly, hTERT-immortalized cell lines that had lost p16^{INK4a} expression or p14^{ARF} expression also exhibited a significant growth block, β -galactosidase staining, and alterations of morphology with mutant *H-ras* (Figure 5), indicating that loss of

p16^{INK4a} or p14^{ARF} alone was insufficient for abrogation of the ras-induced growth block. The cells also retained the ability to be growth arrested after TGF- β treatment and confluency (data not shown).

Because telomerase activation and p16^{INK4a} loss occur frequently in the development of cancer, we wanted to determine whether the immortal cell lines were tumorigenic. All of the different cell lines were found to be negative for tumorigenicity in athymic nude mice 3 months after injection, although hTERT HFK cl 22 gave rise to a benign epithelial cyst in one of two injected sites (data not shown).

Differentiation of p16^{INK4a}- or p14^{ARF}-Negative Cell Lines on Organotypic Raft Cultures

One advantage of using keratinocytes in this study is that they can be induced to undergo complex terminal differentiation when grown on organotypic raft cultures.^{26,27} This makes it possible to determine how hTERT immortalization and loss of p16^{INK4a} or p14^{ARF} expression affect the process of differentiation. When grown on organotypic raft cultures, normal early passage HFKs form a proliferating basal layer of dividing cells, on top of which is the spinous suprabasal layer, followed by the cornified

Figure 5. Effect of mutant H-ras on cells. Cells were infected with either vector or mutant 61L H-ras-expressing retrovirus and assayed for β -galactosidase activity as described in Materials and Methods.

layer (Figure 6a). The postcrisis hTERT-immortalized HFKs that had lost p16^{INK4a} expression exhibited relatively normal terminal differentiation when examined by H&E (Figure 6b). However, there were alterations in the expression of differentiation-associated proteins in the immortal cells. For example, in normal squamous epithelium and in HFK organotypic rafts, cytokeratin K1 is not expressed in the basal layer but is expressed in all of the suprabasal and cornified layers (Figure 6c). The p16^{INK4a}-negative hTERT-immortalized HFK clones, however, displayed delayed expression of K1, with several initial suprabasal layers of cells that were K1 negative (Figure 6d).

Normal adenoid epithelial cells also terminally differentiate on organotypic raft cultures with a basal layer of dividing cells and somewhat less complex stratification than that of HFK (Figure 6, e and f). As with the hTERT-immortalized HFKs, hTERT-immortalized HAKs exhibited changes in the expression of differentiation-associated proteins. For example, in normal HAKs the integrin $\alpha 6 \beta 4$ is expressed only in the basal layer (Figure 6g). hTERT-immortalized HAKs that lost p16^{INK4a} or p14^{ARF} expression, however, exhibited $\alpha 6 \beta 4$ expression in all layers (Figure 6h). Thus two different hTERT-immortalized keratinocyte subtypes that lost p16^{INK4a} or p14^{ARF} expression

showed delayed maturation when induced to terminally differentiate. The possibility exists that hTERT expression alone, rather than loss of p16^{INK4a} or p14^{ARF} expression, caused the altered phenotype. Analysis of normal precrisis HFKs and HAKs that expressed hTERT alone was limited because of poor growth in the raft cultures, presumably because they were near the end of their proliferative life span. However, as with normal cells, expression of $\alpha 6\beta 4$ was limited to the basal layer (data not shown).

Discussion

In this study we have shown that human epithelial cells immortalized by hTERT down-regulate p16^{INK4a} expression by genetic and/or epigenetic alterations, and that other changes such as loss of expression of p14^{ARF} and chromosome aberrations are present in the cells, although they are not extensive. Our data indicate that hTERT-immortalized epithelial cells that have lost p16^{INK4a} or p14^{ARF} expression still retain certain growth controls, but they exhibit alterations in the expression of terminal differentiation markers when induced to differentiate on organotypic raft cultures. These results suggest that epithelial cells, along with telomerase activation, may require the abrogation of a terminal differentiation program to become immortal.

It remains unclear why p16^{INK4a} is up-regulated in keratinocytes as they approach senescence. Senescence in epithelial cells has generally not been associated with the expression of markers of terminal differentiation, although there is evidence in certain systems that exogenous expression of p16^{INK4a} plays a role in differentiation.^{16,17,44–46} It has also been shown that deletion or altered regulation of p16^{INK4a} occurs concomitantly with the loss of differentiation associated with tumor progression in mouse skin.⁴⁷ Exogenous expression of p16^{INK4a} by itself does not induce expression of markers of terminal differentiation, and differentiation can occur without up-regulation of p16^{INK4a}.^{12,44} It is possible that up-regulation of p16^{INK4a} in epithelial cells is insufficient to induce the complete terminal differentiation program in monolayer culture, but that it is sufficient to induce a component of the pathway that causes senescence. Although epidermal differentiation was not specifically addressed in p16^{INK4a}/ARF mouse knockout studies, the mice were reported to develop normally,⁴⁸ suggesting that p16^{INK4a} and p14^{ARF} are expendable for proper differentiation. Experiments to replace p16^{INK4a} and/or p14^{ARF} in the epithelial cell lines that have lost expression of these genes will be useful in determining whether p16^{INK4a} or p14^{ARF} expression is directly involved in the differentiation program. Certainly, one possibility is that p16^{INK4a} and p14^{ARF} are simply effector proteins that are activated as part of a terminal differentiation or senescence program. It will be important, therefore, to deter-

mine what upstream factors cause p16^{INK4a} and p14^{ARF} up-regulation, as these may be the factors that trigger senescence.

The loss of p16^{INK4a} or p14^{ARF} expression on immortalization with hTERT appears to be fairly specific, although other genetic alterations were observed. One cell line, hTERT HFK cl 398, exhibited only loss of p16^{INK4a} expression by promoter-specific methylation, presumably of both p16^{INK4a} alleles, and no other apparent cytogenetic alterations. The cell line hTERT HFK cl 22 exhibited only aberrations of one chromosome 9 and methylation of the promoter of the other p16^{INK4a} allele. Two cell lines, hTERT HAK cl 7 and 12, showed alterations of chromosomes 2 and 7 or chromosome 5, respectively. The role of these alterations, if any, in the immortalization process is unknown, and other undetected alterations may exist in the cells. The observation that one cell line, hTERT HAK cl 41, exhibited loss of p14^{ARF} instead of complete loss of p16^{INK4a} raises the possibility that loss of p14^{ARF} is an alternative pathway to immortalization by hTERT. p14^{ARF} has been shown to increase steady-state levels of p53 by binding Mdm2 in mouse fibroblasts,^{49–53} and loss of p14^{ARF} or p53 is commonly observed with immortalization of mouse cells.⁵⁴ Loss of p53 also occurs frequently during immortalization of human keratinocytes,⁵⁵ but not necessarily in the presence of high telomerase activity.⁸ We cannot rule out the possibility that loss of p14^{ARF} expression was the result of the same genetic aberration that led to the obvious reduction of p16^{INK4a} levels in the cell line. hTERT HAK cl 41 behaved similarly to those cell lines that had lost p16^{INK4a} expression, including increased hyperphosphorylated Rb during subconfluent growth, retention of growth controls, and delayed maturation on the raft cultures. These results suggest that hTERT HAK cl 41 may contain an unidentified aberration in the Rb pathway. We did not observe significant reduction of p53 or p21 in the cell line that had lost p14^{ARF} expression, suggesting that loss of p14^{ARF} may have different consequences in human keratinocytes. Alternatively, p14^{ARF} loss may only have noticeable effects on levels of p53 and p21 in experimental conditions, such as differentiation, that were not analyzed in this study. The availability of the p14^{ARF}-negative cell line may be useful in further characterizing the role of p14^{ARF} in keratinocyte senescence.

Our finding that keratinocytes that have lost p16^{INK4a} or p14^{ARF} expression still exhibit fairly normal growth controls is of significant interest, particularly the observation that loss of p16^{INK4a} or p14^{ARF} by itself does not preclude a growth block mediated by overexpression of mutant *ras*. It has been hypothesized from mouse knockout studies that one of the main functions of p16^{INK4a} is to prevent excessive proliferation upon stimulation by constitutively active *ras*.¹⁹ Our data suggest that the response to mutant *ras* is more complex than simple p16^{INK4a} up-regulation. Perhaps both p16^{INK4a} loss and

Figure 6. Differentiation of HFK, HAK, hTERT immortalized HFK, and hTERT immortalized HAK on organotypic raft cultures. **a:** H&E staining of normal HFK. **b:** H&E staining of postcrisis hTERT HFK cl 22. **c:** Keratin 1 immunostaining of normal HFK. **d:** Keratin 1 immunostaining of postcrisis hTERT HFK cl 22. **e:** H&E staining of normal HAK. **f:** H&E staining of hTERT HAK cl 7. **g:** $\alpha 6\beta 4$ immunostaining of normal HAK. **h:** $\alpha 6\beta 4$ immunostaining of hTERT HAK cl 7.

p14^{ARF} loss or both p16^{INK4a} loss and p53 loss must occur to bypass *ras*-mediated senescence. Another alternative is that the response to mutant *ras* in human keratinocytes is different from that observed in mouse fibroblasts.

Whether telomerase activation and subsequent immortalization might eventually lead to malignancy remains a point of controversy. The hTERT-immortalized keratinocytes were nontumorigenic and still fairly normal with regard to growth control within the first 100 population doublings after crisis, although one cell line did have the ability to form a benign growth in nude mice. Previous studies using HPV-immortalized HFKs have shown that the formation of cysts often precedes tumorigenic conversion.⁵⁶ However, telomerase activation and p16^{INK4a} or p14^{ARF} loss by themselves do not appear to be sufficient for full malignant transformation. Our results indicate that chromosome aberrations can and do occur in the presence of telomerase activity, but, like the results of others,^{6,7,57} they do not appear to be extensive. Whether maintenance of genetic stability by telomerase activation is actually protective against the development of cancer is a question that has to be studied in more detail.

In conclusion, our results indicate that telomerase activation can allow a growth advantage to epithelial cells so that they sustain stable epigenetic or genetic alterations in p16^{INK4a}, p14^{ARF}, or other genes to become immortal. The immortal cells retain an apparently normal response to mutant *ras* and other factors but exhibit aberrant differentiation. Clearly, our results demonstrate that it is important to address issues such as these if hTERT-immortalized cells are to be used in therapy or for studies of normal cell function.

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