

# Immortalization of Human Bronchial Epithelial Cells in the Absence of Viral Oncoproteins

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## ABSTRACT

By expressing two genes (*hTERT* and *Cdk4*), we have developed a method to reproducibly generate continuously replicating human bronchial epithelial cell (HBEC) lines that provide a novel resource to study the molecular pathogenesis of lung cancer and the differentiation of bronchial epithelial cells. Twelve human bronchial epithelial biopsy specimens obtained from persons with and without lung cancer were placed into short-term culture and serially transfected with retroviral constructs containing cyclin-dependent kinase (Cdk) 4 and human telomerase reverse transcriptase (hTERT), resulting in continuously growing cultures. The order of introduction of *Cdk4* and hTERT did not appear to be important; however, transfection of either gene alone did not result in immortalization. Although they could be cloned, the immortalized bronchial cells did not form colonies in soft agar or tumors in nude mice. The immortalized HBECs have epithelial morphology; express epithelial markers cytokeratins 7, 14, 17, and 19, the stem cell marker p63, and high levels of p16<sup>INK4a</sup>; and have an intact p53 checkpoint pathway. Cytogenetic analysis and array comparative genomic hybridization profiling show immortalized HBECs to have duplication of parts of chromosomes 5 and 20. Microarray gene expression profiling demonstrates that the *Cdk4/hTERT*-immortalized bronchial cell lines clustered together and with nonimmortalized bronchial cells, distinct from lung cancer cell lines. We also immortalized several parental cultures with viral oncoproteins human papilloma virus type 16 E6/E7 with and without hTERT, and these cells exhibited loss of the p53 checkpoint and significantly different gene expression profiles compared with *Cdk4/hTERT*-immortalized HBECs. These HBEC lines are a valuable new tool for studying of the pathogenesis of lung cancer.

## INTRODUCTION

*In vitro* studies involving human respiratory epithelium have been hampered by the lack of long-term replicating cultures of bronchial epithelial cells. Attempts to establish such cultures of normal human respiratory epithelium, in most cases, using viral oncoproteins, generated rare variants (*e.g.*, BEAS2B; ref. 1), which become malignant after several passages (2). Recently, the combination of the catalytic subunit of telomerase [human telomerase reverse transcriptase (hTERT)] and genes encoding viral oncoproteins large T and small t

antigen derived from simian virus 40 (SV40) were used in combination to immortalize bronchial epithelial cells (3). We reasoned that SV40 T antigen was needed to overcome the stress induced by growing bronchial epithelial cells in chemically defined medium and in the absence of feeder layers. In the present study, we demonstrate that normal immortal human bronchial epithelial cell (HBEC) lines can be generated without viral oncoproteins by expressing cyclin-dependent kinase (Cdk) 4 and hTERT.

The telomere hypothesis postulates that progressive shortening of the ends of chromosomes (telomeres) in the absence of telomerase or other compensating mechanisms is the mitotic clock that regulates the onset of replicative senescence in normal somatic cells (4–6). The model of mortality stages ( $M_1$  and  $M_2$ ) limiting the replicative capacity of normal somatic cells was first described in fibroblasts (7, 8). Since then, numerous attempts to verify a similar pattern of growth and checkpoint arrest for epithelial cells have been pursued. However, it was found that most epithelial cells propagating in standard culture conditions [*e.g.*, cells cultured in chemically defined medium, without feeder layers, and under normoxia (20%  $O_2$ )] undergo growth arrest early in their life span, well before telomeres become short enough to elicit a telomere-dependent response. This premature growth arrest cannot be explained in terms of telomere shortening. We reported previously that premature growth arrest in epidermal keratinocytes and human mammary epithelial cells when cultivated *in vitro* was associated with up-regulation of the cyclin-dependent kinase (Cdk) inhibitor, p16<sup>INK4a</sup> (9, 10). We found that by overexpressing *Cdk4*, the p16<sup>INK4a</sup>-associated premature growth arrest was prevented, whereas expression of hTERT bypassed telomere-dependent senescence ( $M_1$ ), resulting in immortal cell lines (10, 11). In the current study, we have used this same strategy to generate immortal HBEC lines from small bronchial biopsies obtained during routine patient care. Ectopic *Cdk4* was introduced to eliminate the stress response of the bronchial epithelial cells to the *in vitro* culture conditions, followed by introduction of hTERT to prevent further telomere shortening. We have found that this method reproducibly generates immortalized cells from the bronchial epithelium that have few genetic alterations, do not have a malignant phenotype, and are distinct from immortalized cultures developed by viral oncoproteins. These cultures are important new reagents for understanding the pathogenesis of lung cancer.

## MATERIALS AND METHODS

**Culture of Human Bronchial Epithelial Cells.** Bronchial specimens (~0.5 cm<sup>3</sup>) from areas of the lung histologically not involved with lung cancer were obtained from donors (after obtaining informed consent on institutional review board-approved protocols) undergoing surgical resection for lung and other cancers and transported from the surgical suite to the laboratory in Hanks' balanced salt solution (Gelasco, NY) with 0.4% (v/v) of an antibiotic/antimycotic solution [penicillin (10,000 IU/mL), streptomycin (10,000 mg/

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mL), and amphotericin B (5,000 IU/mL); Gibco, Gaithersburg, MD]. Tissue was dissected and minced into ~4-mm pieces and plated on porcine collagen I (Sigma, St. Louis, MO)-coated tissue culture dishes containing K-SFM media (Invitrogen, Carlsbad, CA). Primary cultures were left undisturbed for 21 days in Modular Incubator Chambers (Billups-Rothenberg, Del Mar, CA) under low oxygen environment (2–3% O<sub>2</sub>) and 5% CO<sub>2</sub> (the low oxygen concentration helped lower p16<sup>INK4a</sup> levels), with changes of media once every 5 days for the first week, and then every other day. When epithelial cell outgrowths were noticeable, we subcultured at a 1:4 ratio with the K-SFM medium, providing a selection against fibroblast and other stromal cell growth (due to the lack of fibroblast growth factors in this medium). Cdk4 was introduced by a retroviral construct (pSRαMSU, G418<sup>+</sup>) into the cultures at population doubling (PD) ~3–20 with G418 selection, followed by introduction of another retroviral construct (pBABE-hTERT, puromycin<sup>+</sup>) at PD ~10–37 as described previously (11). Human papilloma virus (HPV)-16 E6/E7 was introduced by a

retroviral construct (pLXSN, G418<sup>+</sup>) into uninfected cultures at PD 12 with G418 selection. After selection, cells infected with either Cdk4 + hTERT or HPV-16 E6/E7 were serially passaged until they underwent a growth arrest or became immortalized (immortal cell line was defined as >100 PDs). Cells could be switched to normoxic conditions after introduction of Cdk4 without decline in growth rate or life span. However, we kept the culture of HBECs in collagen-coated dishes to facilitate attachment.

PD was calculated as follows: outgrowths were trypsinized, resuspended as a single cell suspension, and counted (Coulter Cell and Particle Counter, Z1 Single Threshold Model; Beckman Coulter, Hialeah, FL). After counting the cells, the initial primary cultures were reseeded and considered to be at PD = 0. Unattached cells were collected and counted the next day to determine the number of remaining attached cells on the primary culture. Because the attachment efficiency was >98%, the number of unattached cells was disregarded in calculating subsequent PDs. Gained PD was calculated at each

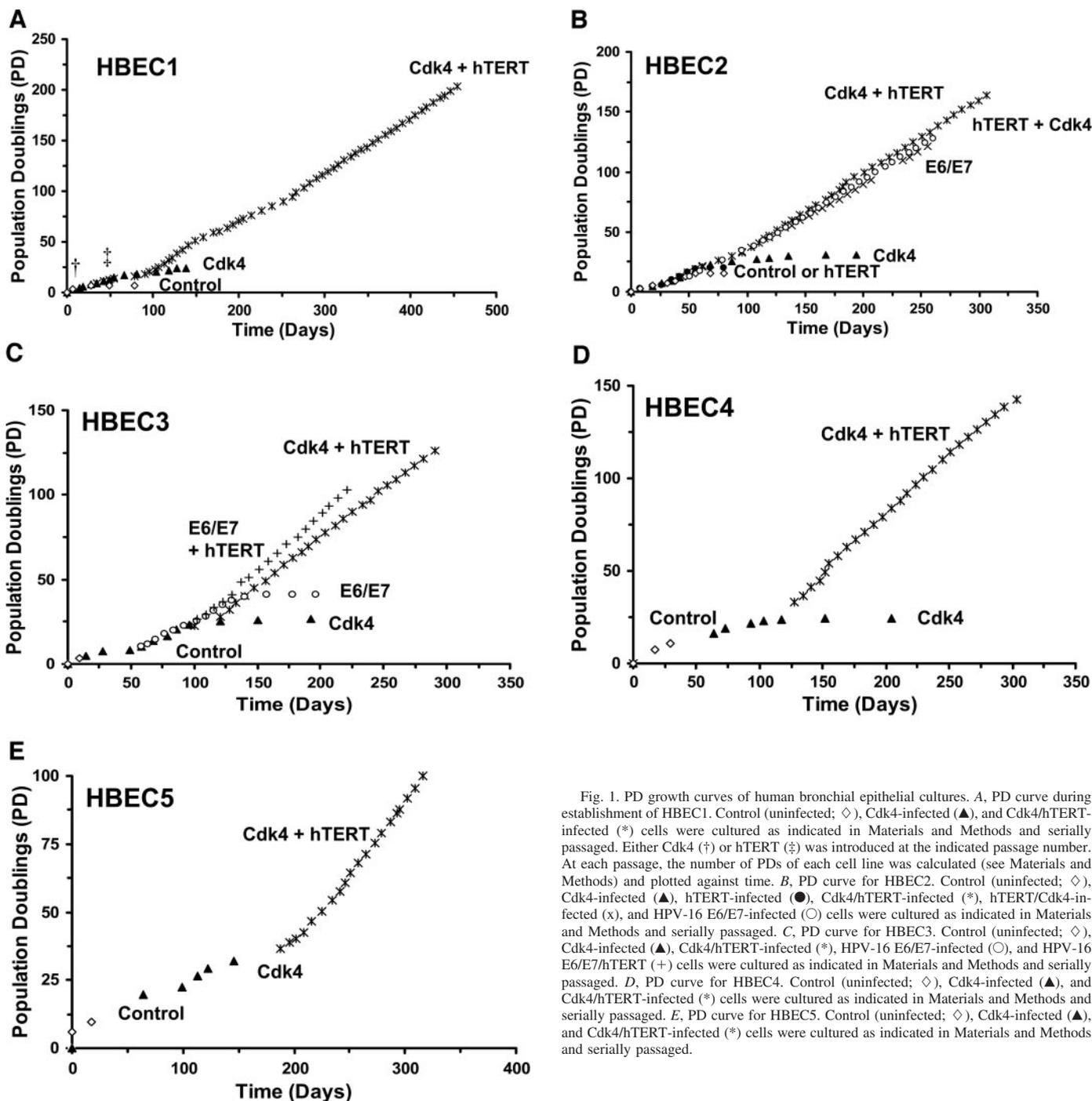


Fig. 1. PD growth curves of human bronchial epithelial cultures. A, PD curve during establishment of HBEC1. Control (uninfected;  $\diamond$ ), Cdk4-infected ( $\blacktriangle$ ), and Cdk4/hTERT-infected ( $*$ ) cells were cultured as indicated in Materials and Methods and serially passaged. Either Cdk4 ( $\dagger$ ) or hTERT ( $\ddagger$ ) was introduced at the indicated passage number. At each passage, the number of PDs of each cell line was calculated (see Materials and Methods) and plotted against time. B, PD curve for HBEC2. Control (uninfected;  $\diamond$ ), Cdk4-infected ( $\blacktriangle$ ), hTERT-infected ( $\bullet$ ), Cdk4/hTERT-infected ( $*$ ), hTERT/Cdk4-infected ( $\times$ ), and HPV-16 E6/E7-infected ( $\circ$ ) cells were cultured as indicated in Materials and Methods and serially passaged. C, PD curve for HBEC3. Control (uninfected;  $\diamond$ ), Cdk4-infected ( $\blacktriangle$ ), Cdk4/hTERT-infected ( $*$ ), HPV-16 E6/E7-infected ( $\circ$ ), and HPV-16 E6/E7/hTERT ( $+$ ) cells were cultured as indicated in Materials and Methods and serially passaged. D, PD curve for HBEC4. Control (uninfected;  $\diamond$ ), Cdk4-infected ( $\blacktriangle$ ), and Cdk4/hTERT-infected ( $*$ ) cells were cultured as indicated in Materials and Methods and serially passaged. E, PD curve for HBEC5. Control (uninfected;  $\diamond$ ), Cdk4-infected ( $\blacktriangle$ ), and Cdk4/hTERT-infected ( $*$ ) cells were cultured as indicated in Materials and Methods and serially passaged.

passage by using the following equation:  $n = \log(N_H/N_I)/\log 2$ , where  $n$  is the number of PDs,  $N_H$  is the number of cells harvested at the end of the growth period, and  $N_I$  is the number of cells inoculated. Gained PDs were added at each passage to compute the cumulative PD depicted in the growth curve figures (Fig. 1A–E).

**Retroviral Vector Construction and Infection.** Retroviral parent vector pSR $\alpha$ MSU (G418<sup>+</sup>) expressing mouse Cdk4 was developed from the generous gift of Charles J. Sherr (St. Jude Children's Research Hospital, Memphis, TN). This is the same construct used previously (11). HBECs were infected with medium containing Cdk4wt helper-free viral supernatant produced from pSR $\alpha$ MSU (G418<sup>+</sup>) expressing the Cdk4 vector in the presence of 4  $\mu$ g/mL Polybrene (Sigma) for 10 to 12 hours. Cells were allowed to recover for 72 hours followed by drug selection with 30  $\mu$ g/mL G418 (Calbiochem, San Diego, CA) for 10 days. Bronchial cells were then infected with a second retroviral construct, pBABE (puromycin<sup>+</sup>) expressing hTERT. This is the same construct used previously (10, 11). Bronchial cells were infected with conditioned media containing hTERT helper-free viral supernatant [produced by pBABE (puromycin<sup>+</sup>) in the presence of 4  $\mu$ g/mL Polybrene followed by selection with 250 ng/mL puromycin (Sigma) for 3 to 4 days, initiated 72 hours later. Retroviral parent vector pLXSN was obtained from A. D. Miller (Seattle, WA). The HPV-16 E6/E7 genes were cloned into a vector under transcriptional regulation of the Moloney murine leukemia virus promoter-enhancer sequences [obtained from D. A. Galloway (Seattle, WA)]. These retroviral vectors also contain the gene conferring neomycin resistance under transcriptional regulation of the SV40 promoter. Plasmid DNA was transfected into the ecotropic packaging cell line PE501 by calcium phosphate precipitation and selected with G418 (1  $\mu$ g/mL). Derived viral supernatant was used to infect the amphotrophic packaging cell line PA317 (12) to generate clones containing unrearranged proviral copies of pLXSN parental vector and pLXSN vector containing HPV-16 E6/E7. PA317 clones were selected on G418 (1  $\mu$ g/mL). Medium containing released viruses produced from confluent dishes was filtered (pore size, 0.45  $\mu$ m) and used to infect parental HBEC2 and HBEC3 cells in the presence of 4  $\mu$ g/mL Polybrene (Sigma) for 10 to 12 hours. Bronchial cells were allowed to recover for 72 hours followed by drug selection with 30  $\mu$ g/mL G418 (Calbiochem) for 10 days.

**Telomeric Repeat Amplification Protocol.** After harvesting of cells from culture, 10<sup>5</sup> cells were aliquoted into a DNase-free, RNase-free 1.5-mL microfuge tube. This aliquot of cells was pelleted by centrifugation in a tabletop centrifuge (5415D; Eppendorf) at 6,000 rpm for 6 minutes at room temperature. The supernatant was aspirated and discarded, and the remaining cell pellet was snap frozen in liquid nitrogen and stored at –80°C until analysis by

telomeric repeat amplification protocol (TRAP) assay (13–15). One thousand cells were used in a nonradioactive polymerase chain reaction (PCR)-based assay using the TRAPeze telomerase detection kit (Intergen, Purchase, NY). The reaction mixture was prepared using the TRAP buffer, deoxynucleoside triphosphate mix, and TRAP-primer mix (containing an anchored CX primer and the Internal Standard <sup>32</sup>P primers) from Intergen's kit. A modified TS primer (5'-Cy5-AATCCGTCGAGCAGAGTT; Integrated DNA Technologies, Inc.), 20  $\mu$ g of ultrapure bovine serum albumin (Ambion, Austin, TX), and 2 units of Taq DNA polymerase were added to this mixture, and PCR cycles were performed as recommended (Intergen). After PCR, 20  $\mu$ L of the TRAP reaction mixtures were separated on 10% nondenaturing acrylamide gels, and the gels were fixed in 0.5 mol/L NaCl, 50% EtOH, and 40 mmol/L sodium acetate (pH 4.2) for 30 minutes and exposed, without drying, to phosphor screens overnight and visualized on a PhosphorImager using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

**Ultraviolet Irradiation.** Ultraviolet (UV) irradiation was performed in a homemade box built with four UV-B bulbs (Philips Lighting). Applied UV-B dose was measured by radiometer (IL-1400A radiometer/photometer; International Light, Newburyport, MA). Infected and uninfected control cells were plated in duplicate at 5 × 10<sup>5</sup> cells per 100-mm culture dish containing growth medium and incubated overnight. After two rinses with 1 × PBS, liquid was aspirated, and the cells were irradiated at different doses: 50, 100, 200, and 400 J/m<sup>2</sup> UV-B. Growth medium was added to the cells immediately after irradiation, and cells were incubated for 24 hours. Untreated dishes from each cell line served as a control. After incubation, cells were harvested for protein extracts.

**Immunoblotting.** Protein extracts prepared from logarithmically growing cells were lysed in 2% SDS in 50 mmol/L Tris-HCl, and total protein concentration was determined using the BCA assay (Pierce, Rockford, IL). Fifty micrograms of each sample were electrophoresed on a 12% and 15% SDS polyacrylamide gel and transferred to nitrocellulose (Amersham, Arlington Heights, IL), the nitrocellulose was blocked in 5% nonfat dry milk and incubated in primary antibody [p16<sup>INK4a</sup> (clone G175-1239; 2 mg/mL; PharMingen, San Diego, CA), p53 [Ab-6; 1 mg/mL; Oncogene Research Products, Boston, MA), p14<sup>ARF</sup> (Ab-1; 1 mg/mL; Oncogene Research Products); and actin (AC-40; 1:3,000; Sigma)] for 1 hour at room temperature. The membranes were washed with 1 × PBS/0.1% Tween 20 and incubated with horseradish peroxidase-conjugated sheep antimouse IgG (Amersham) at a dilution of 1:3,000 when p16<sup>INK4a</sup> or actin was the primary antibody and a dilution of 1:5,000 when p53 and p14<sup>ARF</sup> were the primary antibodies, and specific

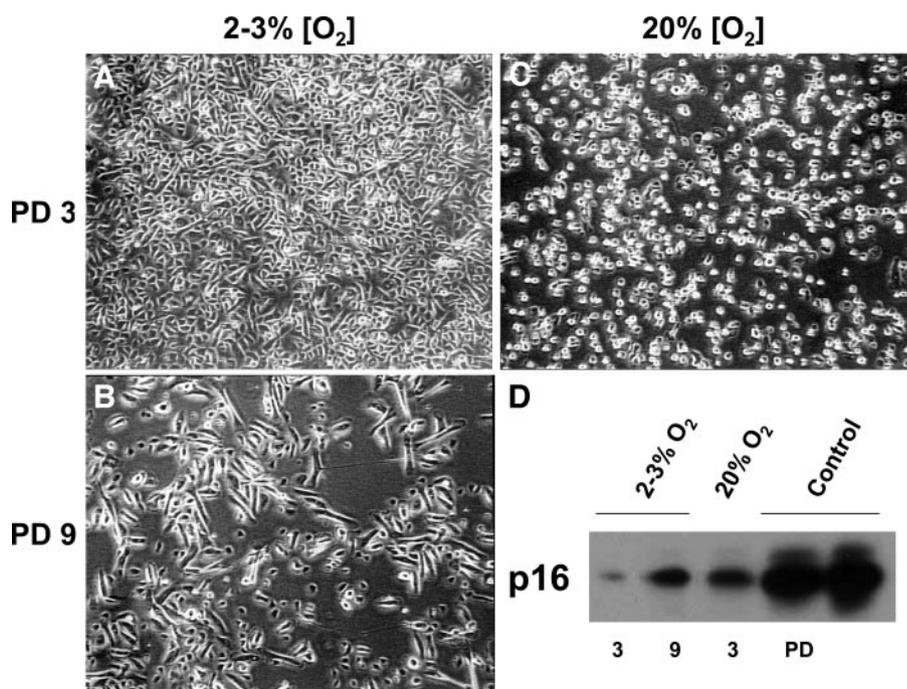


Fig. 2. Premature growth arrest induced by normoxia is associated with up-regulation of p16<sup>INK4a</sup> expression. A, photomicrograph of bronchial cells at PD 3, cultured under low oxygen conditions (2–3% O<sub>2</sub>). B, photomicrograph of bronchial cells at PD 9, cultured under low oxygen conditions. C, photomicrograph of bronchial cells at PD 3 cultured under normoxia conditions (20% O<sub>2</sub>). D, Western blot of p16<sup>INK4a</sup> expression under the three different conditions described in A–C. Control refers to p16<sup>INK4a</sup> expression of HeLa.

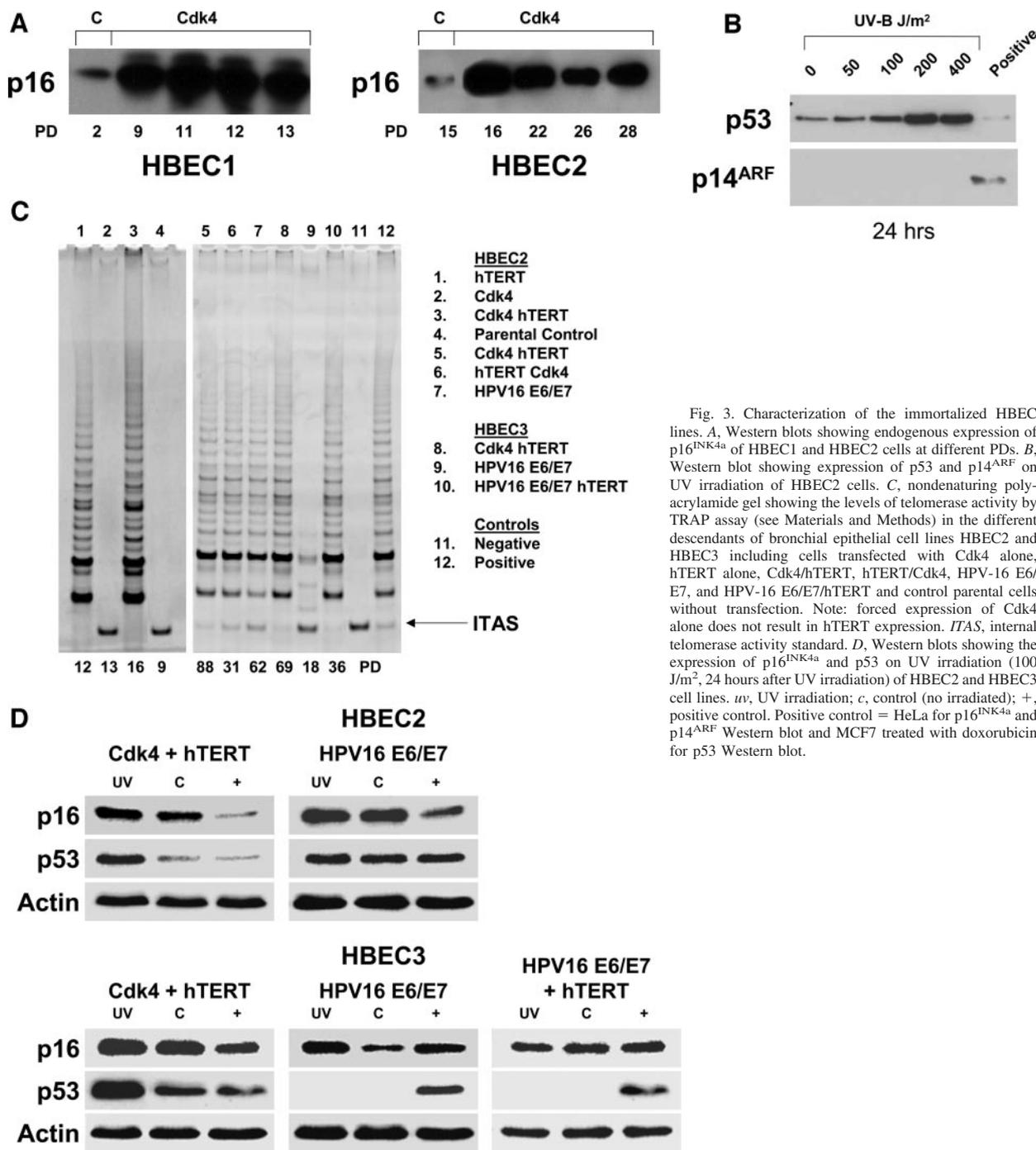


Fig. 3. Characterization of the immortalized HBEC lines. *A*, Western blots showing endogenous expression of p16<sup>INK4a</sup> of HBEC1 and HBEC2 cells at different PDs. *B*, Western blot showing expression of p53 and p14<sup>ARF</sup> on UV irradiation of HBEC2 cells. *C*, nondenaturing polyacrylamide gel showing the levels of telomerase activity by TRAP assay (see Materials and Methods) in the different descendants of bronchial epithelial cell lines HBEC2 and HBEC3 including cells transfected with Cdk4 alone, hTERT alone, Cdk4/hTERT, hTERT/Cdk4, HPV-16 E6/E7, and HPV-16 E6/E7/hTERT and control parental cells without transfection. Note: forced expression of Cdk4 alone does not result in hTERT expression. *ITAS*, internal telomerase activity standard. *D*, Western blots showing the expression of p16<sup>INK4a</sup> and p53 on UV irradiation (100 J/m<sup>2</sup>, 24 hours after UV irradiation) of HBEC2 and HBEC3 cell lines. *uv*, UV irradiation; *c*, control (no irradiated); *+*, positive control. Positive control = HeLa for p16<sup>INK4a</sup> and p14<sup>ARF</sup> Western blot and MCF7 treated with doxorubicin for p53 Western blot.

protein bands were detected using a chemiluminescent substrate (SuperSignal Substrate; Pierce).

**Karyotyping.** Cell lines were incubated with Colcemid (Invitrogen) for 2 hours and harvested with trypsin. Cells were then treated with hypotonic KCl solution (0.075 mol/L) for 30 minutes at 37°C, fixed and washed three times with methanol/acetic acid (3:1), resuspended, and dropped onto slides. GTG-banded chromosome preparations were made using standard methods. At least 20 cells were analyzed from each culture, and chromosome images were captured and analyzed.

**DNA Extraction.** Genomic DNA was isolated from frozen cell pellets by homogenization, SDS/proteinase K (Life Technologies, Inc., Rockville, MD) digestion, phenol-chloroform extraction, and EtOH precipitation (16).

**Expression and Comparative Genomic Hybridization Microarrays.** RNA fluorescent labeling reaction and hybridization were performed with 5 µg of RNA using the Affymetrix GeneChips HG-U133-Plus2 according to the

manufacturer's instructions.<sup>10</sup> The array consists of 54,675 probe sets (29,180 unique genes). All array data are available on-line in a Minimum Information About a Microarray Experiment (MIAME) compliant database.<sup>11</sup> Analysis was performed with Affymetrix MicroArray Suite 5.0 and with an in-house Visual Basic software MATRIX 1.25.<sup>12</sup> This program imports multiple CHP files (saved as text files) from MicroArray Suite 5.0 into an Excel spreadsheet and performs various analyses including normalization, grouping and comparison of arrays using log ratios, scatter plots, color display, and hierarchical clustering. Comparative genomic hybridization (CGH) microarrays or array CGH (aCGH) microarrays were performed as described previously (17, 18) using 10

<sup>10</sup> <http://www.affymetrix.com>.

<sup>11</sup> [http://microarray.swmed.edu/smcdbs/warray\\_db.html](http://microarray.swmed.edu/smcdbs/warray_db.html).

<sup>12</sup> Girard, L. and Minna, J. D. Matrix, a microarray analysis tool in EXCEL, manuscript in preparation.

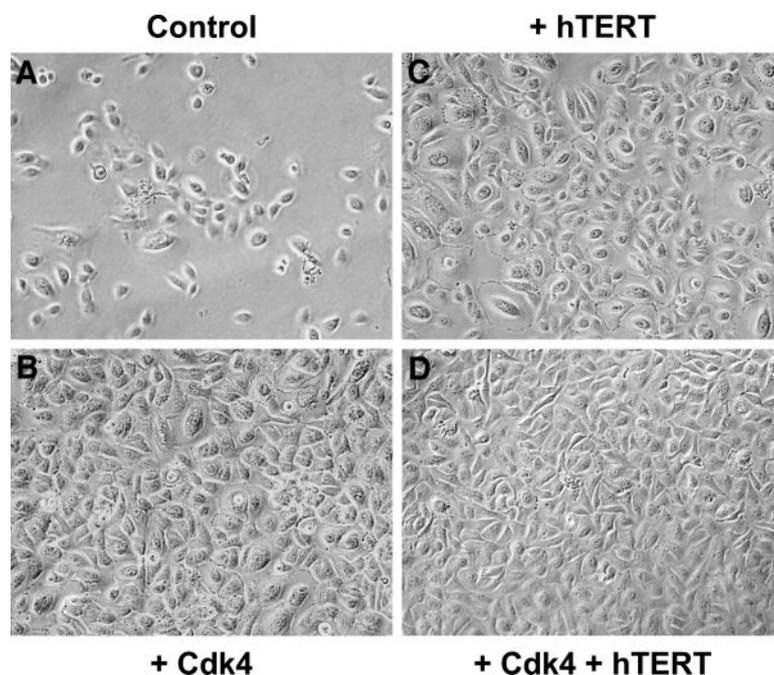


Fig. 4. Immortalization of HBECs requires both Cdk4 and hTERT. *A*, photomicrograph of control (uninfected) bronchial cells at PD 7. *B*, photomicrograph of human bronchial cells at PD 24–32 infected with retrovirus containing Cdk4 (selected with 30  $\mu\text{g}/\text{mL}$  G418 for 10 days). *C*, photomicrograph of human bronchial cells at PD 8 infected with retrovirus containing hTERT (selected with 250 ng/mL puromycin for 4 days). *D*, photomicrograph of human bronchial cells at PD 77 infected with retroviruses containing Cdk4 and hTERT.

$\mu\text{g}$  of genomic DNA. Analysis of aCGH data in Fig. 5 was done using Caryoscope software.<sup>13</sup> Analysis of aCGH data provided in the supplemental figures was done using MATRIX, which sorts array data according to chromosome location [based on GoldenPath hg16;<sup>14</sup> it calculates a 5-point moving average to reduce local variations and then generates a color display with red for DNA gain and green for DNA loss].

## RESULTS

**Immortal Human Bronchial Epithelial Cell Lines.** Bronchial biopsies were obtained from donors undergoing surgical resection for lung cancer and other cancers from areas of the lung histologically not involved with cancer. A commercially available source of proximal neonatal bronchial epithelial cells was obtained from Clonetics. Immortalized cell lines were generated by serial introduction of retroviral expression vectors for Cdk4 followed by hTERT. Control HBECs growth-arrested at PDs 9–16, whereas the life span of cells infected with Cdk4 was extended to PD 24–32 (Fig. 1). On coinfection with hTERT, cells have exceeded 100 PDs and continue growing (Fig. 1). For comparison of the Cdk4/hTERT-immortalized HBECs with viral oncoprotein-immortalized HBECs, we introduced expression vectors for HPV-16 E6 and E7 into the same parental cells. In one case, E6 and E7 alone resulted in immortalization (with spontaneous expression of telomerase), whereas all others required E6, E7, and the hTERT expression vector. Using these methods, we have immortalized 12 primary bronchial epithelial biopsy cultures, indicating the generality of this method for the reproducible production of extended life span bronchial epithelial cell cultures.

**Premature Growth Arrest Associated with Normoxic Culture Conditions and Increased Expression of p16<sup>INK4a</sup>.** Culturing bronchial epithelial cells under normoxic (20%) conditions was associated with increased expression of p16<sup>INK4a</sup> compared with cultures in low oxygen levels (2–3% O<sub>2</sub>; Fig. 2). Thus, we initially cultured parental bronchial cells in low oxygen conditions, under which they only experience normoxic conditions transiently during subculture and medium changes. However, even under these culture conditions, an

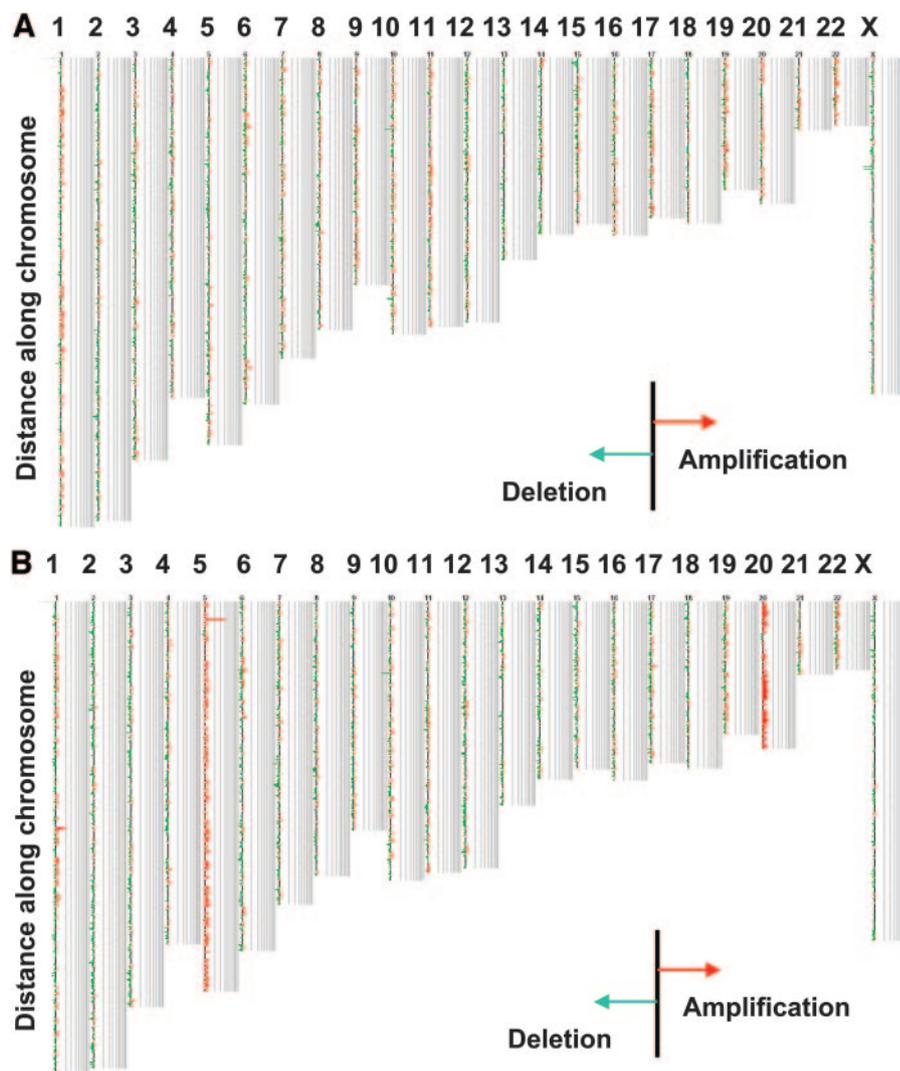
increase in p16<sup>INK4a</sup> expression was detected at PD 10–16, resulting in growth arrest (Fig. 2). This may be due to additional stresses such as chemically defined medium condition or growth in the absence of feeder layers. We reasoned that to further increase life span, a method of bypassing p16<sup>INK4a</sup>-induced growth arrest was needed. Thus, Cdk4 was exogenously overexpressed to bypass the p16<sup>INK4a</sup>-induced premature growth arrest, as reported previously for other epithelial cell types (11). Overexpression of Cdk4 extended the life span of HBECs (~PD 24–32; Fig. 1*A–E*) and was associated with up-regulation of p16<sup>INK4a</sup> to very high levels (Fig. 3*A*), but not with up-regulation of p53 or p14<sup>ARF</sup> (Fig. 3*B*). This result suggests that pathways implicated in the up-regulation of p16<sup>INK4a</sup> during premature growth arrest may involve only the INK4a arm of the INK4a/Arf gene locus. In addition, as reported previously (11), elongation of life span of HBECs on introduction of Cdk4 did not reactivate or up-regulate endogenous telomerase in 12 separate culture (Fig. 3*C*), and thus Cdk4-expressing cells senesced (Figs. 1*A–E* and 4). Thus, we introduced hTERT into Cdk4-expressing cells, which resulted in the introduction of unlimited proliferative capacity (Figs. 1*A–E*, 3*B*, and 4). In addition, after the combination of Cdk4 and hTERT was expressed in the HBECs, they could be switched to normoxic conditions without any sign of change in growth rate or life span.

**Effects of Expression in Human Bronchial Epithelial Cells of Cdk4/hTERT and HPV-16 E6/E7 on p16<sup>INK4a</sup> and p53 Expression.** To determine the effect of viral oncoproteins and Cdk4/hTERT on checkpoint mechanisms, we evaluated the expression of p16<sup>INK4a</sup> and p53 after UV irradiation (Fig. 3*D*). In both HBEC2 and HBEC3 cell lines immortalized with Cdk4/hTERT, we detected a significant increase in the levels of p16<sup>INK4a</sup> expression before UV irradiation compared with control HeLa cells; there was no further increase in the expression of p16<sup>INK4a</sup> on UV irradiation. In addition, in both HBEC2 and HBEC3 immortalized cell lines, we detected low levels of p53 expression before UV irradiation compared with control MCF7 cells treated with doxorubicin, and there was a significant increase in p53 expression in the HBECs on UV irradiation. This result suggests that in the Cdk4/hTERT-immortalized cells, the p53 checkpoint pathway remains intact, despite the block of the p16<sup>INK4a</sup> checkpoint pathway by ectopic Cdk4.

<sup>13</sup> <http://dahlia.stanford.edu:8080/caryoscope/index.html>.

<sup>14</sup> <http://genome.ucsc.edu>.

Fig. 5. Representative aCGH microarray analysis of parental and immortal HBECs. aCGH microarray analysis of parental HBECs before immortalization (A) and immortalized HBEC1 Cdk4/hTERT cells (B). The array used to generate the aCGH data contains 45,000 cDNA elements, and results with the HBEC (test) DNA are given in red, whereas the sex-matched control DNA results are given in green. This figure was generated using the program Cytoscope. Each human chromosome is depicted by a vertical line. Each line perpendicular to the chromosome line represents the genomic DNA copy number of the gene represented by an individual cDNA that has been precisely positioned along the chromosome according to the current human genome sequence information. Increases in test DNA copy number (amplifications) are represented by red lines, whereas decreases in test DNA copy number (deletions) are represented by green lines. The distance of the amplification or deletions is given in logarithmic units. For reference, the changes along chromosomes 5 and 20 represent duplications; also note a small regional amplification of 5p in HBEC1, which we know is not telomerase. See the supplemental figures for detailed analysis for other HBEC cultures including HPV E6/E7-immortalized cells.



In HBECs immortalized with HPV-16 E6/E7 (and strongly expressing telomerase either spontaneously or after introduction of a hTERT expression vector), there was a moderate increase of p16<sup>INK4a</sup> in cells before UV irradiation (compared with control), and there was no further increase on UV irradiation. In HBEC3 E6/E7 cells (in which telomerase expression is extremely weak and comes from an endogenous source), expression of p16<sup>INK4a</sup> was low before UV irradiation compared with control, and there was a significant increase on UV irradiation. By contrast, in HBEC3 E6/E7/hTERT cells (in which telomerase is strongly positive after introduction of an exogenous expression vector), there was no increase of p16<sup>INK4a</sup> on UV irradiation, mimicking the results detected in HBEC2 E6/E7 (strongly positive telomerase expression). This result suggests that expression of telomerase (and thus, maintenance of telomeric ends) may be involved in p16<sup>INK4a</sup>-dependent stress signaling mechanisms. In HBEC2 E6/E7 cells (in which telomerase is strongly expressed from an endogenous source), we detected that expression of p53 remained at the same level compared with control with or without UV irradiation (Fig. 3D). In HBEC3 E6/E7 (weakly positive telomerase expression) and HBEC3 E6/E7/hTERT (strongly positive telomerase expression) cell lines, expression of p53 was not detected before or after UV irradiation. These results suggest HPV-16 E6/E7 abrogates p53 regulation independent of the level of telomerase expression.

**Gene Expression Profile, Array Comparative Genomic Hybridization Profile, and Karyotypic Analysis.** We next examined aCGH profiles of five HBEC lines including parental, Cdk4/hTERT-immortalized, and HPV-16 E6/E7-immortalized cell lines (Fig. 5A and B; supplemental figures 1–8). We identified duplication of chromosome 5 and 20 regions in all immortalized cell lines (Cdk4/hTERT and HPV-16 E6/E7), but not in the parental preimmortalized cultures. In addition to duplication of chromosomes 5 and 20, HBEC2 HPV-16 E6/E7 cell line showed regional macrodeletions in chromosomes 6p and 20q (supplemental figures 1–8). In contrast to HBEC1–4, the HBEC5 Cdk4/hTERT (whose parental bronchial epithelial culture, NHBE, was obtained from Clonetics) showed multiple regional amplifications and deletions in several chromosomes (supplemental figures 1–8). BEAS2B (T antigen-immortalized HBEC line) also showed multiple regional microamplifications and amplifications on chromosomes 5, 8q, 9, 16q, 17q, and 20q and multiple regional microdeletions and deletions on chromosomes 8p, 16p, and X. None of these karyotypic results appears to be related to smoking history.

Characterization of gene expression array data derived from Affymetrix U133-Plus2 chips showed that both Cdk4/hTERT-immortalized and HPV-16 E6/E7-immortalized cells had gene expression signatures that clustered together and were distinct from those of lung cancer cell lines (Fig. 6). However, within the HBEC cluster, different

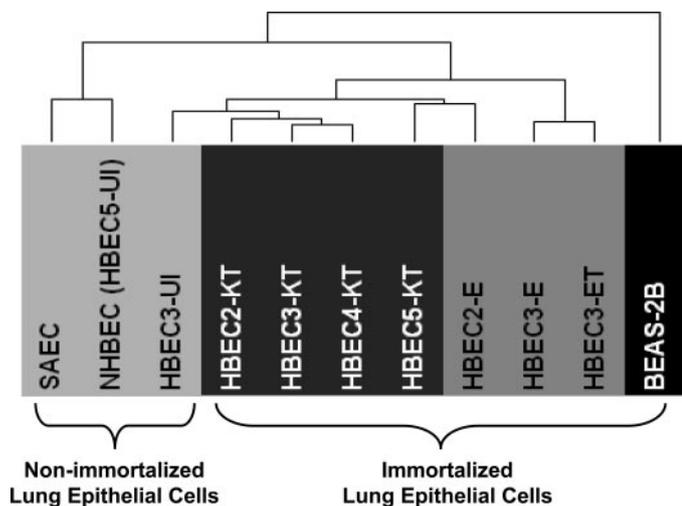


Fig. 6. Cluster analysis of parental and immortalized bronchial epithelial cells. Using Affymetrix U133-Plus2 expression profile data from the various HBEC cultures, we performed unsupervised hierarchical clustering of Cdk4/hTERT-immortalized bronchial epithelial cell lines (HBEC2,3,4,5KT for Cdk4/hTERT), HPV-16 E6/E7-immortalized bronchial epithelial cell lines (HBEC2-E, immortalized and spontaneously expressed high levels of endogenous telomerase; HBEC3-E, preimmortal cells with an extended life span but without endogenous telomerase expression; HBEC3-ET, immortalized cells have addition of exogenous hTERT expression vector), parental bronchial epithelial cells [HBEC3-UI, uninfected with expression vectors; NHBC HBEC5-UI (Clonetics), uninfected normal HBECs used to generate HBEC5 immortal cells], small airway epithelial (SAEC) cells (Clonetics), and BEAS2B, the SV40 T antigen-immortalized bronchial epithelial cell line. Note that the nonimmortalized cultures cluster together, whereas the immortalized lines cluster together, with those immortalized by Cdk4/hTERT being distinct from the HPV-16 E6/E7-immortalized lines.

subgroups were formed by nonimmortalized, Cdk4/hTERT-immortalized, and HPV-16 E6/E7-immortalized bronchial epithelial cells (Fig. 6). Gene expression profiles of the Cdk4/hTERT-immortalized and parental cells were more similar than either was to HPV preimmortalized cells (Fig. 6; see supplemental figure 9 for gene list). These results suggest that Cdk4/hTERT immortalization involved different changes in gene expression than HPV immortalization and that the gene expression profile of Cdk4/hTERT-immortalized cells is closer to that of the starting parental bronchial epithelial cells.

**Malignant Phenotype.** Both Cdk4/hTERT- and HPV-16 E6/E7-immortalized HBEC lines were tested for malignant transformation characteristics by both *in vitro* and *in vivo* tumorigenicity assays. None of the cell lines formed colonies in soft agar after 21 days compared with non—small-cell lung cancer cell line NCI-H1299 and thus were unable to grow in an anchorage-independent manner (data not shown). Likewise, Cdk4/hTERT- or HPV-16 E6/E7-immortalized HBEC lines were not able to form tumors in nude mice (5–10 × 10<sup>6</sup> cells injected per mouse) after 3 months of observation compared with control non—small-cell lung cancer line H1299, which showed tumor formation by week 2 (data not shown).

**Human Bronchial Epithelial Cells Express Stem Cell Marker p63.** The HBECs were immunostained for p63, a p53 homolog essential for regenerative proliferation in epithelial development. All five HBEC lines expressed the stem-like cell marker p63 (Fig. 7), suggesting the immortalized cell lines had characteristics of pulmonary stem cells. In work to be reported elsewhere,<sup>15</sup> we also know the Cdk4/hTERT-immortalized HBECs have the ability to differentiate in organotypic cultures to form cilia.

<sup>15</sup> Vaughan, M. B., Ramirez, R., Sato, M., Sheridan, S., Milchgrub, S., Dimajo, J. M., Minna, J. D., and Shay, J. W. Differentiation of immortalized human bronchial epithelial cells in organotypic 3D cultures, manuscript in preparation.

## DISCUSSION

In the present study, we have reproducibly immortalized HBECs from routine cultures of bronchial biopsy specimens using Cdk4 and hTERT expression vectors without introducing viral oncoproteins. By contrast, past cultures were derived using viral oncoproteins, such as the SV40 or adenovirus-12 SV40 hybrid virus (1), plasmids containing SV40 large T antigen gene (3), the whole genome of HPV-16 or -18, or a plasmid encoding the E6 and E7 genes of HPV-16 (19–22). By overexpressing Cdk4 to overcome the p16<sup>INK4a</sup>-mediated stress response elicited when culturing human epithelial cells in chemically defined medium and in the absence of feeder layers (10, 11), in combination with hTERT to overcome replicative senescence, we have established that primary HBECs can be immortalized with high frequency. This avoids the use of viral oncoproteins that cause many effects including genomic instability and malignant transformation (3, 2, 22). In fact, our comparison studies using HPV-16 E6 and E7 viral oncoproteins resulted in bronchial epithelial cells with more genetic changes and different gene expression signatures compared with those immortalized with Cdk4 and hTERT.

We observed that culture of HBECs in chemically defined medium, without feeder layers, and under ambient (21%) O<sub>2</sub> conditions was associated with up-regulation of p16<sup>INK4a</sup>, leading to premature growth arrest of the cells (PD ~2–3, passage 1; Fig. 2). The role of the ambient (21%) and low oxygen (2–5%) on the ability to immortalize fetal and adult cells with hTERT has been assessed previously (23), as well as the consequences of culturing epithelial cells under inadequate conditions (9, 10). We reasoned that culturing primary bronchial epithelial cells in collagen-coated dishes and low oxygen conditions (2–5%) would help to keep p16<sup>INK4a</sup> expression, whereas overexpressing Cdk4 would bypass cell cycle checkpoints, thus allowing further extension of life span. Consistently, ectopic Cdk4-overexpressing cells showed a 2- to 3-fold prolonged life span compared with controls.

After extension of life span by Cdk4, direct immortalization was achieved by introduction of hTERT. To determine whether the cells became transformed on immortalization, we assessed their phenotype for malignant transformation and compared their gene expression profiles by microarray analysis. None of the immortalized bronchial cell lines formed colonies in soft agar or tumors in nude mice. Gene expression profiles showed immortalized bronchial cell lines clustered together with each other and with nonimmortalized bronchial cells, but the immortalized bronchial cell lines were distinct from lung cancer cell lines and viral oncoprotein-immortalized bronchial epithelial cells. Thus, immortalization of HBECs with Cdk4 and hTERT provides a method to generate cellular reagents, which are closer to their normal counterparts, thus making them new useful cellular reagents for studying the molecular pathogenesis of lung cancer. These studies include identification of molecular markers that precede disease, which are useful in early detection, and identification of novel therapeutic targets. Furthermore, comparisons of Cdk4/hTERT-immortalized cell lines and their control counterparts by aCGH revealed that these immortal cell lines were remarkably genomically stable, except for the duplication of chromosome 5 and 20. In contrast, the HPV-16 E6/E7-immortalized cells showed, in addition to duplication of chromosomes 5q and 20q, regional macrodeletions in chromosomes 6p and 20q. The duplication of chromosomes 5 and 20 in the immortal cell lines is intriguing. Low copy number gains of chromosomes 5 and 20 previously have been reported to be associated with cellular immortalization by viral oncoproteins (24–28). In addition, two human mammary epithelial hTERT-immortalized cell lines have been found to be trisomic for chromosome 20 (29). These results suggest that gain of one or more genes on these chromosomes might contribute to cellular immortalization. However, another explanation

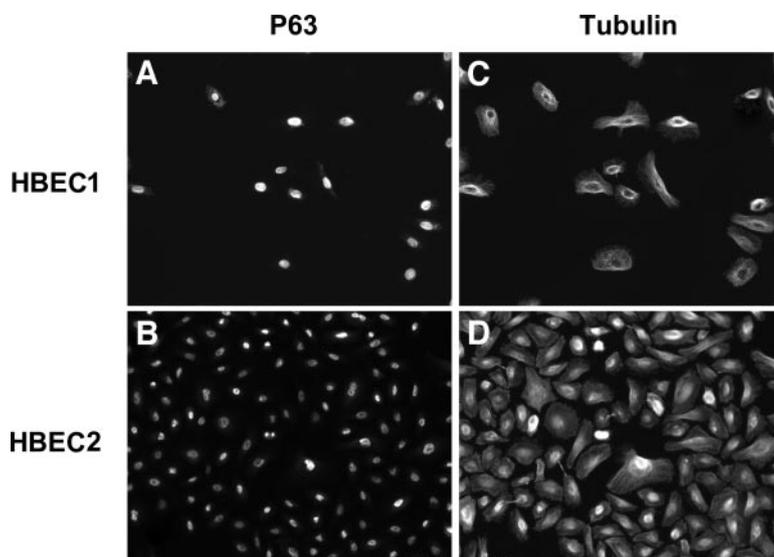


Fig. 7. Immortalized bronchial epithelial cell lines express epithelial cell cytokeratins and stem cell marker p63. Immunostain of the same slide of HBEC1 cells with anti-p63 (A) and anti-tubulin (C). Immunostain of HBEC2 cells with anti-p63 (B) and anti-tubulin (D).

could be that chromosomal duplications are the result of mitotic nondisjunction due to integration of the proviral cDNA into the cell's genome after retroviral transfection. Additional studies are required to determine whether *in vitro* manipulation also contributed to the chromosomal duplications.

In summary, we have established a general and practical approach to generate normal immortalized HBECs from clinically accessible specimens. These cultures provide models that can be genetically manipulated in a stepwise fashion to progress the cells toward malignancy that may be useful for studying the early stages of preneoplasia and progression, differentiation, and morphogenesis and for comparisons with lung cancer for the identification of new genetic and epigenetic differences between tumor and normal cells. These results suggest it should be possible to generate immortalized cultures representing several or all of the different steps in lung cancer pathogenesis. The ability to genetically manipulate these cells will allow us to systematically test the importance for the malignant phenotype of the many different changes found in lung cancer. Finally, HBECs with defined genetic changes may be useful for screening and testing new chemopreventive and therapeutic agents that would have specificity against proteins encoded by these changes.

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## REFERENCES

- Reddel RR, Ke Y, Gerwin BI, et al. Transformation of human bronchial epithelial cells by infection with SV40 or adenovirus-12 SV40 hybrid virus, or transfection via strontium phosphate coprecipitation with a plasmid containing SV40 early region genes. *Cancer Res* 1988;48:1904–9.
- Reddel RR, Salghetti SE, Willey JC, et al. Development of tumorigenicity in simian virus 40-immortalized human bronchial epithelial cell lines. *Cancer Res* 1993;53:985–91.
- Lundberg AS, Randell SH, Stewart SA, et al. Immortalization and transformation of primary human airway epithelial cells by gene transfer. *Oncogene* 2002;21:4577–86.
- Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. *Nature (Lond)* 1990;345:458–60.
- Harley CB. Telomere loss: mitotic clock or genetic time bomb? *Mutat Res* 1991;256:271–82.
- Harley CB, Vaziri H, Counter CM, Allsopp RC. The telomere hypothesis of cellular aging. *Exp Gerontol* 1992;27:375–82.
- Shay JW, Werbin H, Wright WE. Telomere shortening may contribute to aging and cancer: a perspective. *Mol Cell Differ* 1994;2:1–18.
- Wright WE, Pereira-Smith OM, Shay JW. Reversible cellular senescence: implications for immortalization of normal human diploid fibroblasts. *Mol Cell Biol* 1989;9:3088–92.
- Herbert BS, Wright WE, Shay JW. p16<sup>INK4a</sup> inactivation is not required to immortalize human mammary epithelial cells. *Oncogene* 2002;21:7897–900.
- Ramirez RD, Morales CP, Herbert BS, et al. Putative telomere-independent mechanisms of replicative aging reflect inadequate growth conditions. *Genes Dev* 2001;15:398–403.
- Ramirez RD, Herbert BS, Vaughan MB, et al. Bypass of telomere-dependent replicative senescence (M1) upon overexpression of Cdk4 in normal human epithelial cells. *Oncogene* 2003;22:433–44.
- Miller AD, Rosman GJ. Improved retroviral vectors for gene transfer and expression. *Biotechniques* 1989;7:980–6, 989.
- Kim NW, Piatyszek MA, Prowse KR, et al. Specific association of human telomerase activity with immortal cells and cancer. *Science (Wash DC)* 1994;266:2011–5.
- Piatyszek MA, Kim NW, et al. Detection of telomerase activity in human cells and tumors by a telomeric repeat amplification protocol (TRAP). *Methods Cell Sci* 1995;17:1–15.
- Wright WE, Shay JW, Piatyszek MA. Modifications of a telomeric repeat amplification protocol (TRAP) result in increased reliability, linearity and sensitivity. *Nucleic Acids Res* 1995;23:3794–5.
- Herrmann BG, Frischauf AM. Isolation of genomic DNA. *Methods Enzymol* 1987;152:180–3.
- Pollack JR, Perou CM, Alizadeh AA, et al. Genome-wide analysis of DNA copy-number changes using cDNA microarrays. *Nat Genet* 1999;23:41–6.
- Pollack JR, Sorlie T, Perou CM, et al. Microarray analysis reveals a major direct role of DNA copy number alteration in the transcriptional program of human breast tumors. *Proc Natl Acad Sci USA* 2002;99:12963–8.
- Coursen JD, Bennett WP, Gollahon L, Shay JW, Harris CC. Genomic instability and telomerase activity in human bronchial epithelial cells during immortalization by human papillomavirus-16 E6 and E7 genes. *Exp Cell Res* 1997;235:245–53.
- Viallet J, Liu C, Emond J, Tsao MS. Characterization of human bronchial epithelial cells immortalized by the E6 and E7 genes of human papillomavirus type 16. *Exp Cell Res* 1994;212:36–41.
- Willey JC, Broussoud A, Sleemi A, et al. Immortalization of normal human bronchial epithelial cells by human papillomaviruses 16 or 18. *Cancer Res* 1991;51:5370–7.
- Zhang L, Liu T, Liu H, Gu C. HPV16 E6E7 fragments transform immortalized human bronchial epithelial cells into neoplastic cells [in Chinese]. *Zhonghua Bing Li Xue Za Zhi* 2000;29:350–3.
- Forsyth NR, Evans AP, Shay JW, Wright WE. Developmental differences in the immortalization of lung fibroblasts by telomerase. *Aging Cell* 2003;2:235–43.
- Cuthill S, Agarwal P, Sarkar S, Savelieva E, Reznikoff CA. Dominant genetic alterations in immortalization: role for 20q gain. *Genes Chromosomes Cancer* 1999;26:304–11.
- Hukku B, Rhim JS. Role of chromosome 5 in immortalization and tumorigenesis of human keratinocytes. *Cancer Genet Cytogenet* 1993;68:22–31.
- Jin Y, Zhang H, Tsao SW, et al. Cytogenetic and molecular genetic characterization of immortalized human ovarian surface epithelial cell lines: consistent loss of chromosome 13 and amplification of chromosome 20. *Gynecol Oncol* 2004;92:183–91.
- Savelieva E, Belair CD, Newton MA, et al. 20q gain associates with immortalization: 20q13.2 amplification correlates with genome instability in human papillomavirus 16 E7 transformed human uroepithelial cells. *Oncogene* 1997;14:551–60.
- Tsao SW, Wong N, Wang X, et al. Nonrandom chromosomal imbalances in human ovarian surface epithelial cells immortalized by HPV16–E6E7 viral oncogenes. *Cancer Genet Cytogenet* 2001;130:141–9.
- Troester MA, Hoadley KA, Sorlie T, et al. Cell-type-specific responses to chemotherapeutics in breast cancer. *Cancer Res* 2004;64:4218–26.

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## Immortalization of Human Bronchial Epithelial Cells in the Absence of Viral Oncoproteins

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