

# Multiple Oncogenic Changes (*K-RAS*<sup>V12</sup>, p53 Knockdown, Mutant *EGFRs*, p16 Bypass, Telomerase) Are Not Sufficient to Confer a Full Malignant Phenotype on Human Bronchial Epithelial Cells

Mitsuo Sato,<sup>1</sup> Melville B. Vaughan,<sup>4</sup> Luc Girard,<sup>1</sup> Michael Peyton,<sup>1</sup> Wooschang Lee,<sup>1</sup> David S. Shames,<sup>1</sup> Ruben D. Ramirez,<sup>1,2,6</sup> Noriaki Sunaga,<sup>1</sup> Adi F. Gazdar,<sup>1,3</sup> Jerry W. Shay,<sup>4</sup> and John D. Minna<sup>1,2,5</sup>

<sup>1</sup>Hamon Center for Therapeutic Oncology Research and Departments of <sup>2</sup>Internal Medicine, <sup>3</sup>Pathology, <sup>4</sup>Cell Biology, and <sup>5</sup>Pharmacology, The University of Texas Southwestern Medical Center; and <sup>6</sup>Dallas Veterans Administration Medical Center, Dallas, Texas

## Abstract

We evaluated the contribution of three genetic alterations (p53 knockdown, *K-RAS*<sup>V12</sup>, and mutant *EGFR*) to lung tumorigenesis using human bronchial epithelial cells (HBEC) immortalized with telomerase and Cdk4-mediated p16 bypass. RNA interference p53 knockdown or oncogenic *K-RAS*<sup>V12</sup> resulted in enhanced anchorage-independent growth and increased saturation density of HBECs. The combination of p53 knockdown and *K-RAS*<sup>V12</sup> further enhanced the tumorigenic phenotype with increased growth in soft agar and an invasive phenotype in three-dimensional organotypic cultures but failed to cause HBECs to form tumors in nude mice. Growth of HBECs was highly dependent on epidermal growth factor (EGF) and completely inhibited by EGF receptor (EGFR) tyrosine kinase inhibitors, which induced G<sub>1</sub> arrest. Introduction of EGFR mutations E746-A750 del and L858R progressed HBECs toward malignancy as measured by soft agar growth, including EGF-independent growth, but failed to induce tumor formation. Mutant EGFRs were associated with higher levels of phospho-Akt, phospho-signal transducers and activators of transcription 3 [but not phospho-extracellular signal-regulated kinase (ERK) 1/2], and increased expression of *DUSP6/MKP-3* phosphatase (an inhibitor of phospho-ERK1/2). These results indicate that (a) the HBEC model system is a powerful new approach to assess the contribution of individual and combinations of genetic alterations to lung cancer pathogenesis; (b) a combination of four genetic alterations, including human telomerase reverse transcriptase overexpression, bypass of p16/RB and p53 pathways, and mutant *K-RAS*<sup>V12</sup> or mutant *EGFR*, is still not sufficient for HBECs to completely transform to cancer; and (c) EGFR tyrosine kinase inhibitors inhibit the growth of preneoplastic HBEC cells, suggesting their potential for chemoprevention. (Cancer Res 2006; 66(4): 2116-28)

## Introduction

Human lung cancer develops as a multistep process, usually occurring because of years of smoking-related tobacco exposure

that results in specific proto-oncogene and tumor suppressor gene alterations in lung epithelial cells (1). In fact, the majority of lung cancers have many such changes (1). Identifying the minimal and most crucial set of changes required for lung tumorigenesis and the effect each of these alterations has on the carcinogenic process is vital to develop the best targets for early detection and therapeutic intervention. To address this issue, an *in vitro* model system using human bronchial epithelial cells (HBEC) was recently developed to assess the contribution of specific genetic alterations to lung cancer progression (2, 3). We accomplished this by overexpressing Cdk4 to abrogate the p16/Rb cell cycle checkpoint pathway and ectopic expression of human telomerase reverse transcriptase (hTERT) to bypass replicative senescence, allowing us to develop a series of immortalized HBEC lines without using viral oncoproteins. These HBECs have epithelial morphology, express epithelial markers, are able to differentiate into mature airway cells in organotypic cultures, have minimal genetic changes, and do not exhibit a transformed phenotype (2, 3). We have HBEC lines that are derived from patients with a variety of smoking histories, with and without lung cancer, which also allows us to explore interindividual variation in the tumor formation process.

Two of the genetic alterations that occur almost universally in human lung cancer, inactivation of the p16/pRb pathway and expression of hTERT, were used for establishment of immortalized HBECs and so are already present. The pRb pathway (p16<sup>INK4a</sup>-cyclinD1-Cdk4-pRB pathway) is a key cell cycle regulator at the G<sub>1</sub>-S phase transition. Absence of expression or structural abnormality of Rb protein is seen in >90% of small-cell lung cancers (SCLC) and loss of p16 protein expression by several mechanisms, including methylation or homozygous deletion of p16<sup>INK4a</sup>, is seen in >70% of non-SCLC (NSCLC), both of which result in the inactivation of this pathway (1, 4, 5). Expression of high levels of telomerase is almost universal in lung cancer (1). hTERT is the key determinant of the enzymatic activity of human telomerase and its transcriptional control is a major contributor to the regulation of telomerase activity in many types of human cells (6–10). Because of the central role of the pRb pathway and telomerase expression, we initially evaluated the contribution of ectopically expressing Cdk4 and hTERT on lung cancer development. However, we found that such cells, although immortal and clonable, did not show anchorage-independent growth or an ability to form tumors *in vivo* (2). Other investigators and our group had also immortalized HBECs but these were made using viral oncoproteins, such as human papillomavirus E6/E7 or SV40 large T antigen with or without hTERT (2, 11, 12). These oncoproteins are known to cause

**Requests for reprints:** John D. Minna, Hamon Center for Therapeutic Oncology Research NB8.206, The University of Texas Southwestern Medical Center at Dallas, 6000 Harry Hines Boulevard, Dallas, TX 75390-8593. Phone: 214-648-4900; Fax: 214-648-4940; E-mail: John.Minna@UTSouthwestern.edu.

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malignant transformation through their ability to inactivate Rb and/or p53, as well as provide multiple other functions, which are not characterized. These "other functions" make it difficult to estimate the importance of added genetic or epigenetic changes in HBECs immortalized by viral oncoproteins.

Thus, we designed the current study to determine if the HBECs were genetically tractable and to analyze the effect of additional genetic alterations frequently observed in lung cancer on tumorigenic transformation of HBECs. First, we introduced two well-known genetic alterations seen in lung cancer, one of which is the loss of p53 function, which is observed in 90% of SCLCs and 50% of NSCLCs (1). The other is oncogenic K-RAS, which is frequently seen in NSCLCs (~30%), especially in adenocarcinomas but probably never in SCLCs (1, 13, 14). Furthermore, we introduced a mutant epidermal growth factor (EGF) receptor (EGFR) that has recently been reported in NSCLCs and shown to be correlated with tumor sensitivity to the EGFR tyrosine kinase inhibitors (15, 16). We report here that HBECs immortalized by overexpression of Cdk4 and hTERT and subsequently manipulated to have oncogenic K-RAS, knockdown of p53, or mutant EGFR have acquired part, but not all, of the malignant phenotype by the combination of these genetic alterations. These partially progressed lung epithelial cells show that more changes are needed for the full malignant phenotype. In addition, we have found that these preneoplastic cells are exquisitely sensitive to EGFR inhibition.

## Materials and Methods

**Cells and culture conditions.** The HBEC3 (HBEC3-KT) immortalized normal HBEC line was established by introducing mouse Cdk4 and hTERT into normal HBECs obtained from a 65-year-old woman without cancer (2). NSCLC, NCI-H441, NCI-H358, NCI-H1299, and NCI-H2122 cell lines were obtained from Hamon Center Collection (University of Texas Southwestern Medical Center). HBEC3 was cultured with K-SFM (Life Technologies, Gaithersburg, MD) medium containing 50 µg/mL bovine pituitary extract (Life Technologies) with or without 5 ng/mL EGF (Life Technologies). These cells are resistant to G418 due to the neomycin-resistant gene introduced with the Cdk4 expression vector and to puromycin due to the puromycin-resistant gene introduced with the hTERT expression vector.

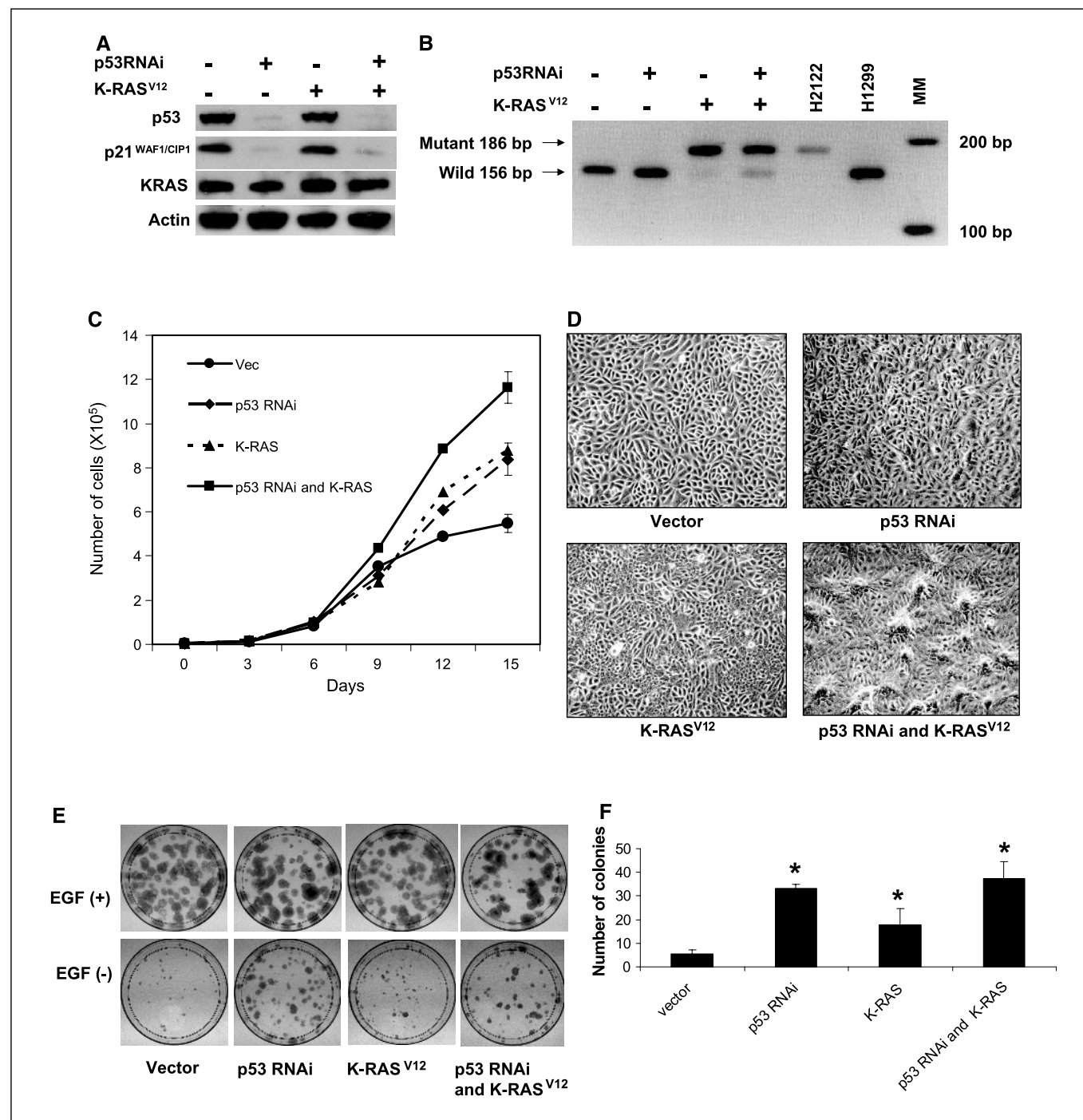
**Viral vector construction and viral transduction.** We used the pSUPER vector (OligoEngine, Seattle, WA) of Brummelkamp et al. (17, 18) as the basis for generating small interfering RNA for stable p53 knockdown. To generate pSUPER.retro-zeocin (pSRZ), *SacII* and *EcoRI* sites were introduced into zeocin-resistant gene fragment amplified from pVgrRXR (a gift from Dr. Preet Chaudhary) and the fragment was cloned into pSUPER.retro using *SacII* and *EcoRI* sites, resulting in the replacement of the puromycin-resistant gene with a zeocin-resistant gene. To generate pSRZ-p53 for p53 knockdown, *EcoRI*- and *HindIII*-digested inserts from pSUPER-p53 (OligoEngine; ref. 18) was cloned into the same sites of pSRZ. pBabe-hyg and an oncogenic K-RAS<sup>V12</sup>, pBabe-hyg-KRAS2-V12, vectors were provided by Dr. Michael White (The University of Texas Southwestern Medical Center, Dallas, TX). To produce viral-containing medium, 293T cells were transiently transfected with viral vector together with pVpack-VSVG and pVpack-GP vectors (Stratagene, La Jolla, CA). Forty-eight hours after the transfection, supernatant of the 293T cells was harvested and passed through a 0.45 µm filter and the viral supernatant was frozen at -80°C. The supernatant was used for infection after adding 4 µg/mL polybrene (Sigma, St. Louis, MO). Forty-eight hours after the infection, drug selection for infected cells was started with 12.5 µg/mL zeocin (Invitrogen, Carlsbad, CA) or 18 µg/mL hygromycin (Clontech, Palo Alto, CA) and continued for 7 to 11 days. HBEC3 cells were infected with four different combinations of the two retroviral vectors: (a) pSRZ and pBabe-hyg (vector control); (b) pSRZ-p53 and pBabe-hyg; (c) pSRZ and pBabe-hyg-KRAS2-V12; and (d) pSRZ-p53 and pBabe-hyg-KRAS2-V12.

To introduce wild and mutant EGFRs into HBEC3 cells, we used the pLenti6/directional TOPO cloning kit. Full-length fragment of wild-type EGFR was amplified from pcDNA3.1-EGFR-wt (a gift from Dr. Joachim Herz, University of Texas Southwestern Medical Center) and cloned into pLenti6/directional TOPO vector according to the instructions of the manufacturer (pLenti-wt-EGFR). The L858R mutation was introduced into pLenti-wt-EGFR by using site-directed mutagenesis kit (Stratagene). The full length of E746-A750 del mutation was amplified from cDNA from HCC827 NSCLC cell line (19) and cloned into pLenti6/directional TOPO vector. Correct sequences were confirmed by sequencing for all vectors. Viral transduction was done following the instructions of the manufacturer. Briefly, the 293FT cells were transiently transfected with viral vector together with viral power (Invitrogen). Forty-eight hours after the transfection, supernatant of the 293FT cells was harvested and passed through a 0.45 µm filter, and frozen at -80°C. The supernatant was used for infection after adding 4 µg/mL polybrene (Sigma). Forty-eight hours after the infection, drug selection for infected cells was started with 5 µg/mL blasticidin (Invitrogen) and continued for 7 days.

**Western blot analysis.** Preparation of total cell lysates and Western blotting were done as described previously (20). Primary antibodies used were mouse monoclonal anti-p53 (Santa Cruz, Santa Cruz, CA), mouse monoclonal anti-p21 (BD Transduction Laboratories, Lexington, KY), mouse monoclonal anti-K-RAS (Santa Cruz), mouse monoclonal anti-EGFR (BD Transduction Laboratories), rabbit polyclonal anti-phospho-EGFR-Tyr<sup>1068</sup> (Y1068), rabbit polyclonal anti-phospho-EGFR-Tyr<sup>845</sup> (Y845), rabbit polyclonal anti-phospho-EGFR-Tyr<sup>992</sup> (Y992), rabbit polyclonal anti-phospho-EGFR-Tyr<sup>1045</sup> (Y1045; Cell Signaling, Beverly, MA), rabbit polyclonal anti-MEK1/2 (Cell Signaling), rabbit polyclonal anti-phospho-MEK1/2 (Cell Signaling), rabbit polyclonal anti-extracellular signal-regulated kinase (ERK) 1 (Cell Signaling), rabbit polyclonal anti-phospho-ERK1 (Cell Signaling), rabbit polyclonal anti-Akt (Cell Signaling), rabbit polyclonal anti-phospho-Akt (Thr<sup>308</sup>), rabbit polyclonal anti-phospho-Akt (Ser<sup>473</sup>), mouse monoclonal anti-phospho-signal transducers and activators of transcription (STAT) 3 (Tyr<sup>705</sup>; Cell Signaling), poly(ADP-ribose) polymerase (PARP; Cell Signaling), and mouse monoclonal antiactin (Sigma) antibodies. Actin protein levels were used as a control for adequacy of equal protein loading. Antirabbit or antimouse antibody (1:2,000 dilution; Amersham, Piscataway, NJ) was used as the second antibody.

**Immunofluorescence staining.** Cells were washed with PHEM [60 mmol/L PIPES, 25 mmol/L HEPES, 10 mmol/L EGTA, and 1 mmol/L MgCl<sub>2</sub> (pH 7.4)] solution, and fixed in 3% paraformaldehyde for 10 minutes at 37°C in PHEM. After additional washes with PBS, the cells were permeabilized with 0.1% Triton in PBS for 10 minutes, blocked with 3% gelatin/3% bovine serum albumin (BSA)/0.2% Tween 20 for 1 hour at 37°C, and incubated with mouse polyclonal anti-p63 antibody (BD Transduction Laboratories) and rhodamine phalloidin (Molecular Probes, Eugene, OR) in gelatin/BSA blocking solution for 16 hours at 4°C. The cells were then incubated with the Alexa Fluor 568 anti-mouse IgG (H + L; Molecular Probes) secondary antibody for 1 hour at 37°C. Finally, cells were stained with 0.5 µg/mL Hoechst 33258 and examined in a fluorescence microscope.

**RNA extraction and reverse transcription-PCR/RFLP analysis.** We modified previously reported reverse transcription-PCR (RT-PCR)/RFLP method designed to distinguish mutated from wild-type *K-RAS* alleles (21). Total RNA was extracted using RNeasy mini kit (Qiagen, Valencia, CA). Four micrograms of total RNA were reverse transcribed with Superscript II First-Strand Synthesis using oligo-dTMP primer system (Invitrogen). PCR amplification was carried out with 3,704 K-RAS<sub>NTN</sub> sense (GACTGAATATAAAGTTGTGGTAGTTGACCT) and 3,672 K-RAS-RT-R antisense (5'-TCC-TCTTGACCTGCTGTGTCG-3) primers, creating *Bst*NI restriction patterns that distinguished mutated from wild-type *K-RAS* alleles. PCR reactions were done in a 25 µL reaction mixture containing 1.5 mmol/L MgCl<sub>2</sub>, 187.5 µmol/L of each deoxynucleotide triphosphate, 10 pmol of each primer, and 1.25 units of HotStar Taq DNA Polymerase (Qiagen). Cycling conditions were one incubation of 15 minutes at 95°C, followed by 35 cycles of a 20-second denaturation at 94°C, 60-second annealing at 58°C, and 90-second extension at 72°C, and a final elongation at 72°C for 7 minutes. PCR products were cut with *Bst*NI, electrophoresized on 1% agarose gel with



**Figure 1.** Characterization of p53 knocked down and K-RAS<sup>V12</sup>-expressing HBEC3 cells. **A**, Western blots showing the suppression of p53, p21<sup>WAF1/CIP1</sup>, and K-RAS in p53RNAi-, mutant K-RAS<sup>V12</sup>-, and p53RNAi and mutant K-RAS<sup>V12</sup>-expressing HBEC3 cells. p53 and p21<sup>WAF1/CIP1</sup> are clearly knocked down in p53RNAi-expressing cells, whereas the expression levels of K-RAS in K-RAS<sup>V12</sup>-transfected and p53RNAi and K-RAS<sup>V12</sup>-transfected HBEC3 cells approximate that of vector alone-transfected HBEC3 cells. **B**, RFLP analysis of K-RAS cDNA showing mutant K-RAS<sup>V12</sup> transcripts are predominantly expressed in mutant K-RAS<sup>V12</sup>-expressing and p53RNAi and mutant K-RAS<sup>V12</sup>-expressing HBEC3 cells. H2122 and H1299 are used as positive controls for mutant K-RAS<sup>V12</sup> and wild-type K-RAS, respectively. Brummelkamp et al. (18) reported a new vector system, named pSUPER, which generated small interfering RNAs in mammalian cells to functionally inactivate p53. Subsequently, they developed a retroviral version of pSUPER, named pSUPER.ret (pRS), to obtain stable knockdowns and showed stable and specific knockdown of oncogenic K-RAS<sup>V12</sup> (17). To see the long-term effect of p53 inactivation, we used the pSUPER.ret system for p53 knockdown. Because a puromycin-resistant gene in pRS was already integrated in HBEC3 in the process of introducing Cdk4, we developed pRS-zeocin vector (pSRZ) by replacing the puromycin-resistant gene in pRS vector with a zeocin-resistant gene. Subsequently, the published p53 target small interfering RNA sequence was cloned into pSRZ (17, 18), yielding pSRZ-p53 vector. **C**, increased saturation density in p53 RNAi and mutant K-RAS<sup>V12</sup>-expressing HBECs. HBEC3 cells (2,000) were cultured in triplicate 12-well plates and counted every 3 days. ●, vector-expressing HBEC3 cell; ◆, p53RNAi-expressing HBEC3 cells; ▲, mutant K-RAS<sup>V12</sup>-expressing HBEC3 cells; ■, p53RNAi and mutant K-RAS<sup>V12</sup>-expressing HBEC3 cells. **D**, cells were grown as described in (C) and pictures were taken on day 12. **E**, liquid colony formation assay for vector-, p53RNAi-, mutant K-RAS<sup>V12</sup>-, and p53RNAi and mutant K-RAS<sup>V12</sup>-expressing HBEC3 cells in the presence or absence of EGF (5 ng/mL). A total of 200 cells were plated per dish and cultured for 2 weeks before staining with methylene blue. **F**, quantitation of the number of colonies in the absence of EGF. Columns, mean of three independent experiments; bars, SD. \*,  $P < 0.01$ , one-way ANOVA with Bonferroni's posttest.



**Table 1.** Tumorigenicity assay in nude mice for HBEC3 cells

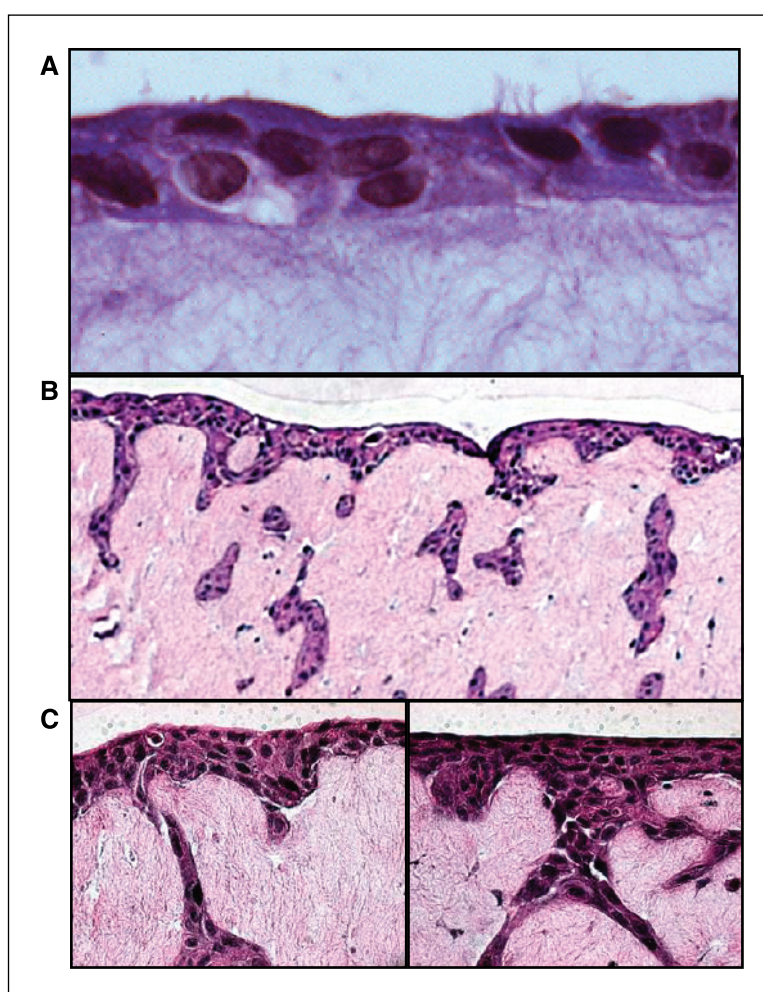
Cell line	No. nude mice injected	No. cells per mouse	Observation period (d)	Percentage of tumors
NCI-H358	10	$5 \times 10^6$	90	100
NCI-H441	5	$5 \times 10^6$	61	100
NCI-H1299	40	$5 \times 10^6$	17-38	100
HBEC3				
Nontreated	5	$1 \times 10^7$	90	0
K-RAS <sup>V12</sup>	5	$5 \times 10^6$	90	0
p53RNAi	5	$5 \times 10^6$	90	0
p53RNAi and K-RAS <sup>V12</sup>	5	$5 \times 10^6$	90	0
p53RNAi and K-RAS <sup>V12</sup>	5	$2.5 \times 10^6$	90	0
With Matrigel				
p53 RNAi and EGFR-L858R	5	$2.5 \times 10^6$	90	0

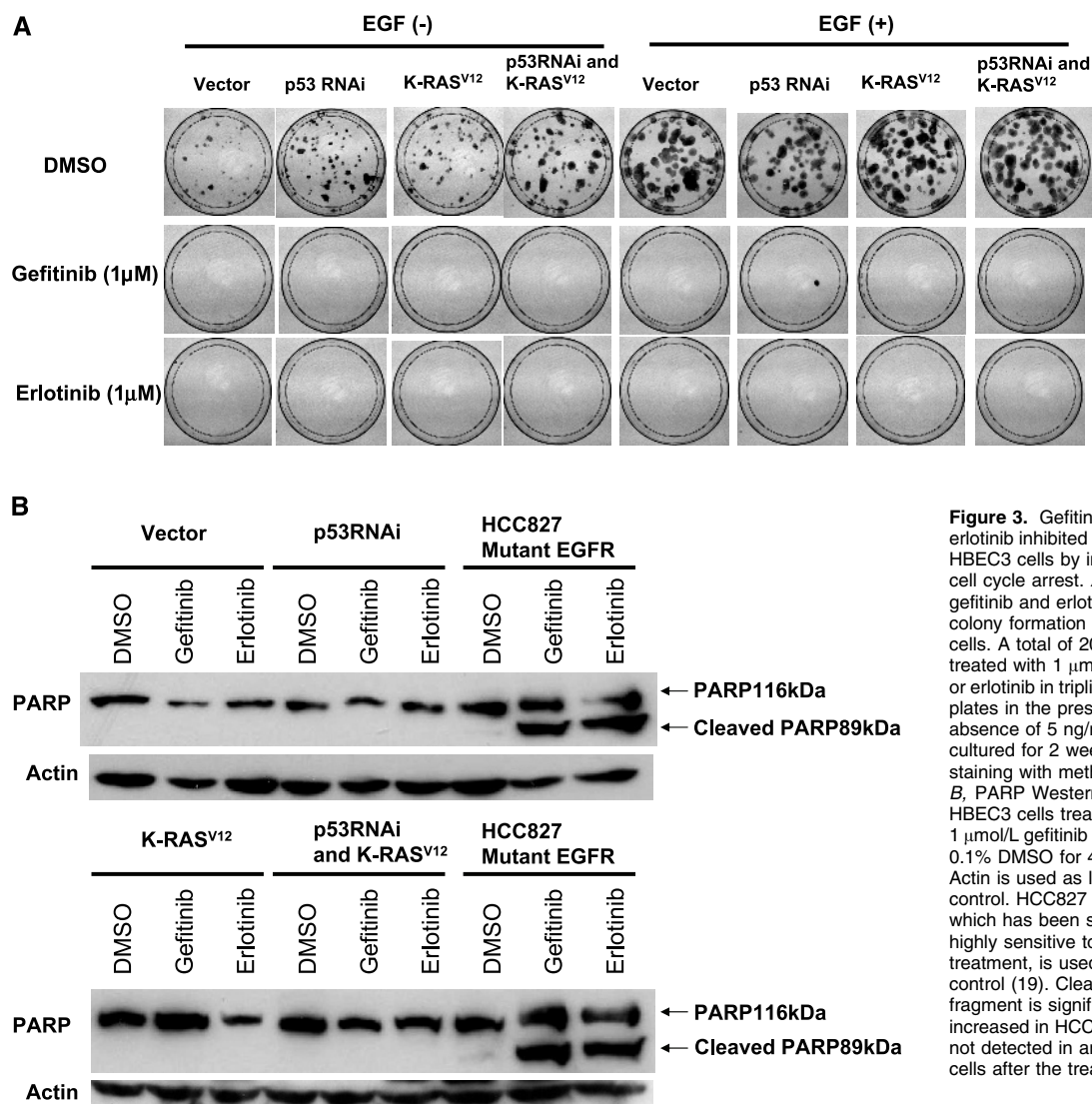
ethidium bromide, and visualized by UV. NCI-H2122 cell line containing a endogenous mutant K-RAS at codon 12 was used as a control for K-RAS<sup>V12</sup> allele, and NCI-H1299 cell was used as a control for the wild-type K-RAS allele.

**In vitro and in vivo cell growth assays.** To determine growth curves, cells were cultured in triplicate wells in 12-well plates and counted every 3 days. Liquid colony formation assays were done as previously described (22). Briefly, 200 viable cells were plated in triplicate 100 mm plates and were cultured in K-SFM medium supplemented with 50 µg/mL bovine

pituitary extract with or without 5 ng/mL EGF. To measure the effect of gefitinib or erlotinib, 1 µmol/L of each drug was added to the medium and the medium was replaced every 3 days. Surviving colonies were counted 14 days later after staining with methylene blue. For soft agar growth assays, 1,000 viable cells were suspended and plated in 0.37% Sea Kem agar (FMC, Philadelphia, PA) in K-SFM medium supplemented with 20% of fetal bovine serum and 50 µg/mL bovine pituitary extract with or without 5 ng/mL EGF in triplicate 12-well plates, and were layered over a 0.50% agar base in the same medium as the one used for suspending the cells. To measure

**Figure 2.** Effect of p53 knock down and mutant K-RAS<sup>V12</sup> on three-dimensional organotypic culture of HBEC3 cells. A, stained paraffin cross-sections of organotypic cultures of HBEC3 cells showed that they formed a confluent layer of cells on the upper surface of the culture with the presence of cilia-like structures. Low-magnification (B) and high-magnification (C) p53RNAi and mutant K-RAS<sup>V12</sup>-expressing HBEC3 cells showed a histologic change similar to metaplasia and dysplasia and they invaded into the fibroblast and collagen underlayer.





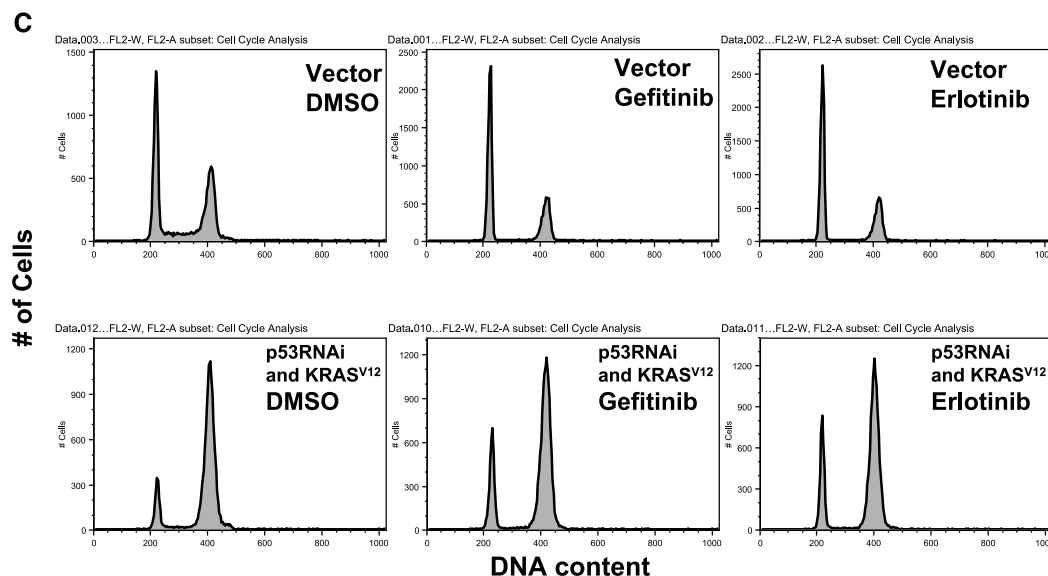
**Figure 3.** Gefitinib and erlotinib inhibited the growth of HBEC3 cells by inducing G<sub>1</sub> cell cycle arrest. **A**, effect of gefitinib and erlotinib on liquid colony formation of HBEC3 cells. A total of 200 cells were treated with 1 μmol/L gefitinib or erlotinib in triplicate 100 mm plates in the presence or the absence of 5 ng/mL EGF and cultured for 2 weeks before staining with methylene blue. **B**, PARP Western blot for HBEC3 cells treated with 1 μmol/L gefitinib or erlotinib or 0.1% DMSO for 48 hours. Actin is used as loading control. HCC827 cell line, which has been shown to be highly sensitive to gefitinib treatment, is used as positive control (19). Cleaved 89 kDa fragment is significantly increased in HCC827 cells but not detected in any of HBEC3 cells after the treatment.

the effect of gefitinib or erlotinib, 1 μmol/L of each drug was drug added to agar base layer. The number of microscopically visible colonies (>50 cells) was counted 4 weeks later. *In vivo* tumorigenicity was evaluated by injection of cells in nude mice. Male BALB/c nude (*nu/nu*) 3- to 6-week-old mice (Charles River Laboratories, Wilmington, DE) were irradiated on day 0 of the experiment in groups of five animals by a 5-minute exposure to 350 cGy from a cesium source. The next day, each mouse was given an injection s.c. on its flank  $0.25 \times 10^7$  to  $1 \times 10^7$  viable HBEC3 cells in 0.2 mL PBS containing different combinations of ectopically introduced genes. Coinjection of Matrigel (BD Bioscience, San Jose, CA) was also tested for HBEC3 cells expressing p53 RNA interference (RNAi) and K-RAS<sup>V12</sup>. Mice were monitored every 2 to 3 days for tumor size. All animal care was in accord with institutional guidelines and approved Institutional Animal Care and Research Advisory Committee protocols. The NSCLC, NCI-H358, NCI-H441, and NCI-H1299 cell lines ( $5 \times 10^6$  cells) were used as positive controls.

**Three-dimensional organotypic culture assay.** Cultures were established as previously described for skin equivalents (23) except that airway fibroblasts were used in place of skin cells. Briefly, type I collagen and IMR90 fibroblasts were mixed and were allowed to polymerize. The collagen gels were released and incubated for a period of 4 to 10 days to allow the

fibroblasts to contract the gels, creating a "submucosa." Cloning rings were then placed atop the gels and HBEC cells were plated into the rings at a concentration of  $2 \times 10^5/\text{cm}^2$ . After allowing the cells to attach for 4 hours, the rings were removed and organotypic cultures submerged for 4 days in keratinocyte feeder layer medium containing ascorbic acid, then emerged to the air-liquid interface for up to 28 days in culture, after which time the cultures were harvested, fixed, and prepared for histology. Organotypic cultures were immersed in 10% neutral buffered formalin overnight at 4°C followed by dehydration, paraffin embedding, and thin sectioning; 5 and 10 μm sections were then rehydrated and stained with H&E to view overall morphology (<http://www.protocol-online.org/prot/Histology/Staining/>). Stained slides were then viewed using an Axioscop-2 or Axioplan-2E microscope (Carl Zeiss, Thornwood, NY; [www.zeiss.com](http://www.zeiss.com)) and photographed with Hamamatsu ORCA monochrome charge-coupled device camera (Hamamatsu, Bridgewater, NJ; [www.hamamatsu.com](http://www.hamamatsu.com)).

**Cell cycle analysis.** Cells were harvested 48 hours after the treatment of 1 μmol/L gefitinib, erlotinib, or 0.1% DMSO, fixed with 70% ethanol, treated with 5 mg/mL RNase A (Roche Molecular Biochemicals), stained with 50 μg/mL propidium iodide, and analyzed by flow cytometry for DNA synthesis and cell cycle status [FACSCalibur instrument, (Becton Dickinson) with FlowJo software].



**Figure 3** Continued. C, FACS profiles of vector (control) or p53 RNAi and mutant K-RAS<sup>V12</sup>-expressing HBEC3 cells treated with 1  $\mu$ mol/L of gefitinib or erlotinib or 0.1% DMSO for 48 hours. Cells were harvested after the treatment, stained with propidium iodide, and analyzed by using the flow cytometer. X axis, DNA content; Y axis, cell number. The fractions of cells in G<sub>1</sub> phase are significantly increased in both vector and the combined HBEC3 p53 knock down and mutant K-RAS cells, with the reduction of S-phase cells after the treatment of gefitinib or erlotinib. Compared with vector cells treated with DMSO, the combined HBEC3 p53 knockdown and mutant K-RAS cells treated with DMSO show a significant increase in G<sub>2</sub>-M phase cells.

**Microarray analysis.** RNAs were labeled and hybridized to Affymetrix HG-U133-Plus2 GeneChips according to the protocol of the manufacturer (<http://www.affymetrix.com>). This array contains 54,675 genes (29,180 unique genes). Microarray analysis was done using Affymetrix MicroArray Suite 5.0 and in-house Visual Basic software MATRIX 1.26.

**Real-time RT-PCR for DUSP6/MKP-3.** The expression of *DUSP6/MKP-3* was analyzed by quantitative real-time RT-PCR. Primers were designed to ensure a single 107 bp amplicon using the standard Taqman assay-on-demand PCR protocol with a 10-minute hot start. Products were resolved on 2% agarose (Sigma). A probe sequence was designed using Primer-Express software (Applied Biosystems). The probe was labeled with TAMRA (quencher) and FAM (reporter) and synthesized by Integrated DNA Technologies. To establish the efficiency of this assay, we used a 5-fold serial dilution of cDNA over six concentrations. These samples were run on the Gene Amp 7700 Sequence Detection System (Applied Biosystems) in triplicate. The resultant curve had a slope of  $-3.396$  and  $R^2$  coefficient of  $0.9939$ . For quantitative analysis of *DUSP6/MKP-3*, we used *GAPDH* (Applied Biosystems assay-on-demand) as an internal reference gene to normalize input cDNA. Quantitative real-time RT-PCR was done in a reaction volume of 25  $\mu$ L, including 1  $\mu$ L cDNA. We used the comparative  $C_t$  method to compute relative expression values.

**Statistical analyses.** For comparison of saturation density and colony formation between the different genetically manipulated cell strains, we used one-way ANOVA with Bonferroni's *post hoc* test correction and for comparisons of the effect of EGFR on growth the Mann-Whitney *U* test.

## Results

**RNAi-mediated p53 knockdown and K-RAS<sup>V12</sup> introduction in HBEC3s.** We used retroviral vector-mediated RNAi technology to generate HBEC3 clones stably knocked down for p53. HBEC3-expressing Cdk4 and hTERT cells were infected with pSRZ-p53 (see Materials and Methods and Fig. 1 caption), selected with zeocin, and tested for p53 and p21<sup>WAF1</sup> protein expression. Western blot analysis showed clear suppression of p53 and p21<sup>WAF1</sup>

(Fig. 1A). Next, we introduced mutant K-RAS<sup>V12</sup> into pSRZ-expressing and pSRZ-p53-expressing HBEC3 cells using pBabe-hyg-KRAS2-V12 retroviral vector followed by hygromycin selection. Western blot analysis showed that the expression levels of K-RAS in K-RAS<sup>V12</sup>-transfected and p53RNAi and K-RAS<sup>V12</sup>-transfected HBEC3 cells approximated that of vector alone-transfected HBEC3 cells (Fig. 1A). Because antibodies that recognize only wild or mutant K-RAS are not available, we did RT-PCR/RFLP analysis to distinguish between K-RAS<sup>V12</sup> and wild-type K-RAS mRNA expression. The analysis revealed that mutant K-RAS<sup>V12</sup> transcripts were the predominant form expressed in the K-RAS<sup>V12</sup>-transfected HBEC3 alone or with p53 RNAi cells (Fig. 1B), indicating that most of the K-RAS protein expressed in HBEC3 cells infected with pBabe-hyg-KRAS2-V12 was the mutant form. We also did immunocytochemistry of p63 (a stem cell marker) and found that mutant K-RAS and/or p53 knockdown did not alter the p63 expression levels of HBEC3 cells (data not shown).

**p53 knockdown and K-RAS<sup>V12</sup> introduction into HBEC3s increase saturation density.** We assessed the effect of p53 knockdown and expression of mutant K-RAS<sup>V12</sup> on cell growth and found no significant difference in growth rate in the exponential growth phase between p53RNAi-expressing, K-RAS<sup>V12</sup>-expressing, p53RNAi and K-RAS<sup>V12</sup>-expressing, and vector-expressing HBEC3 cells. However, p53RNAi-expressing ( $P < 0.01$ ), K-RAS<sup>V12</sup>-expressing ( $P < 0.01$ ), and p53RNAi and K-RAS<sup>V12</sup>-expressing ( $P < 0.001$ ) HBEC3 cells achieved significantly higher final saturation densities in confluent cultures compared with vector-transfected control (in all cases here and below using one-way ANOVA with Bonferroni's *post hoc* test; Fig. 1C and D). Also, the final density of the combined p53RNAi and K-RAS<sup>V12</sup>-expressing HBEC3 cells was significantly higher than that of HBEC3 cells with either p53 knockdown ( $P < 0.01$ ) or mutant K-RAS<sup>V12</sup> ( $P < 0.01$ ) alone (Fig. 1C and D). We conclude from these studies that introduction of these



**Table 2.** Soft agar colony formation assay for HBEC3 cells treated with gefitinib or erlotinib

	HBEC3			
	Vector	p53RNAi	K-RASV12	p53RNAi and K-RASV12
EGF(+)				
DMSO	1.3 ± 0.47	11 ± 1.6	5.7 ± 1.2	23 ± 2.1
Gefitinib (1 μmol/L)	0	0	0	0
Erlotinib (1 μmol/L)	0	0	0	0
EGF(-)				
DMSO	0	0	0	1.3 ± 0.94
Gefitinib (1 μmol/L)	0	0	0	0
Erlotinib (1 μmol/L)	0	0	0	0

NOTE: Number of colonies after 14 days are shown as average ± SD. HBEC3 cells (1,000) were plated in agar and treated with 1 μmol/L gefitinib or erlotinib in triplicate 12-well plates for up to 2 weeks in the presence or the absence of EGF and colonies (50-100 cells) were counted.

genetic changes produced part of the malignant phenotype, increased saturation density.

**p53 knockdown and K-RAS<sup>V12</sup> introduction permits anchorage-independent growth and partial bypass of EGF dependence.** HBEC3 cells are able to form colonies in liquid medium but not in soft agar (i.e., they do not display anchorage-independent growth). We then tested the p53 and K-RAS<sup>V12</sup>-manipulated variants to see if they had acquired this ability. In addition, because HBEC3 cells express robust levels of EGFR (Fig. 4A) and EGF is in the K-SFM synthetic medium, we tested the dependence of colony formation in liquid and semisolid medium on EGF. Supplementation of EGF dramatically enhanced liquid colony formation in all HBEC3 cells (Fig. 1E). In liquid colony formation in the presence of EGF, no significant difference in the number of colonies was seen between p53RNAi-expressing, K-RAS<sup>V12</sup>-expressing, p53RNAi and K-RAS<sup>V12</sup>-expressing, and vector-expressing HBEC3 cells. In contrast, there were significant differences in the number of colonies in the absence of EGF between these four strains (Fig. 1F). In the absence of EGF, p53RNAi-expressing (5.9 fold,  $P < 0.001$ ) and p53RNAi and K-RAS<sup>V12</sup>-expressing (6.6-fold,  $P < 0.001$ ) HBEC3 cells formed a markedly increased number of colonies compared with vector control, whereas K-RAS<sup>V12</sup>-expressing HBEC3 cells formed significantly increased (3.2-fold,  $P < 0.001$ ) number of colonies

compared with vector control (in all cases here and below using one-way ANOVA with Bonferroni's *post hoc* test; Fig. 1F). In the presence of EGF, p53RNAi-expressing, K-RAS<sup>V12</sup>-expressing, and p53RNAi and K-RAS<sup>V12</sup>-expressing HBEC3 cells formed a significantly increased number of soft agar colonies compared with vector control, 7.3-fold ( $P < 0.001$ ), 6.7-fold ( $P < 0.001$ ), and 16.5-fold ( $P < 0.001$ ), respectively, whereas in the absence of EGF, p53 RNAi and p53RNAi and K-RAS<sup>V12</sup>-expressing HBEC3 cells formed very few colonies (Fig. 4C). We conclude from these studies that introduction of these genetic changes led to anchorage-independent growth and both oncogenic K-RAS or p53 knockdown led to partial bypass of dependence on EGF. However, the cells still remain dependent on EGF signaling to express this anchorage-independent growth although an unexpected finding was the ability of p53 knockdown to partially alleviate this EGF dependence.

**p53 knockdown and expression of mutant K-RAS<sup>V12</sup> does not give a full malignant phenotype.** In tumorigenicity assays, none of HBEC3 derivatives formed s.c. tumors in nude mice. Because Matrigel (BD Bioscience) accelerates tumor growth when coinjected with cells in athymic mice (24), we injected HBEC3 cells expressing p53 RNAi and K-RAS<sup>V12</sup> together with Matrigel. However, even with Matrigel, the HBEC3 cells expressing p53 RNAi and

**Table 3.** Effect of gefitinib and erlotinib on cell cycle progression in HBEC3 cells

	Nontreated (DMSO)			Gefitinib/erlotinib		
	G <sub>1</sub>	S	G <sub>2</sub> -M	G <sub>1</sub>	S	G <sub>2</sub> -M
Vector	45.4 ± 0.2	27.5 ± 6.0	28.7 ± 3.9	76.6 ± 5.6*	2.6 ± 0.5 <sup>†</sup>	28.0 ± 2.0
p53RNAi	41.4 ± 2.9	27.6 ± 3.7	31.0 ± 4.5	69.5 ± 0.2*	7.4 ± 2.8*	27.0 ± 3.4
K-RAS <sup>V12</sup>	49.5 ± 6.3	31.7 ± 10.6	17.8 ± 12.2	81.4 ± 4.6*	7.7 ± 2.4	16.6 ± 2.8
p53RNAi and K-RAS <sup>V12</sup>	12.9 ± 3.2	11.8 ± 6.2	73.7 ± 3.8	25.6 ± 4.8*	5.3 ± 1.2	70.8 ± 3.7

NOTE: Data are percentages (mean ± SD). Averaged values of three independent experiments are shown. Watson Pragmatic algorithm was used to calculate each cell cycle distribution. Because the algorithm contains approximations, the total of each distribution is not exactly 100%.

\* $P < 0.01$  in comparison with the respective control.

<sup>†</sup> $P < 0.05$  in comparison with the respective control.

K-RAS<sup>V12</sup> did not form tumors. In contrast, tests of  $5 \times 10^6$  NSCLC, NCI-H358, NCI-H441, and NCI-H1299 cells reproductively formed progressively growing nude mouse xenograft tumors in the 17- to 90-day observation period (Table 1). We conclude from these studies that even with these gain-of-function and loss-of-function manipulations, a full malignant phenotype is not achieved (*in vivo* tumor formation).

**Oncogenic manipulation leads to an invasive phenotype in a three-dimensional organotypic culture assay.** To evaluate the effect of oncogenic manipulation in HBEC3s on their ability to differentiate and to invade, we did three-dimensional organotypic culture. HBEC3 cells only expressing hTERT and Cdk4 cells formed a confluent layer of cells on the upper surface of a fibroblast and collagen gel under layer and developed both ciliated (Fig. 2A) and mucous-producing cell types. In stark contrast, the cells expressing hTERT, Cdk4, K-RAS<sup>V12</sup>, and p53 RNAi showed histologic change similar to metaplasia/dysplasia and they invaded into the fibroblast and collagen gel similar to cancer cells invading into the submucosal layer (Fig. 2B and C). We conclude from these studies that p53 knockdown and K-RAS<sup>V12</sup> are additive in malignant transformation leading to the development of anchorage-independent growth and the ability to invade in a three-dimensional culture system.

**Gefitinib and erlotinib inhibit proliferation and colony formation of HBEC3 cells by inducing G<sub>1</sub> cell cycle arrest.** The dependency of HBEC3 cell on EGF signaling prompted us to investigate the effect of tyrosine kinase inhibitors, gefitinib and erlotinib, on cell proliferation in mass culture and colony formation of these cells. Both gefitinib and erlotinib at 1  $\mu$ mol/L completely inhibited the mass culture proliferation of all HBEC3 cells both in the presence and the absence of EGF (data not shown). Gefitinib or erlotinib at 1  $\mu$ mol/L also completely inhibited both anchorage-dependent and anchorage-independent colony formation in all HBEC3 cells (Fig. 3A; Table 2). Thus, although oncogenic manipulation partially relieved EGF dependence, EGF tyrosine kinase inhibitors remain potent inhibitors of HBEC growth. To investigate the mechanisms of this growth inhibition by tyrosine kinase inhibitors, we did apoptosis and cell cycle analyses. Western blot for PARP cleavage, an indicator of caspase-mediated apoptosis, showed that cleaved 89 kDa fragment was not detected in any of HBEC3 cells treated with tyrosine kinase inhibitors but was significantly increased in HCC827 EGFR mutant cells after the treatment with tyrosine kinase inhibitors for 48 hours (Fig. 3B). Cell cycle analysis also did not show sub-G<sub>1</sub> DNA fractions indicative of apoptosis in HBEC3 cells treated with tyrosine kinase inhibitors. Instead, the cell cycle analysis showed increase in the fraction of cells in G<sub>1</sub> phase in all the HBEC3 cells treated with either of the drugs, with reduction of S-phase cells (Fig. 3C; Table 3). These results suggest that growth inhibition for HBEC3 cells by tyrosine kinase inhibitors is mainly caused by G<sub>1</sub> cell cycle arrest and not apoptosis. Comparing the results of fluorescence-activated cell sorting (FACS) analysis for the control cells treated with DMSO, we found that the combined HBEC3 p53 knockdown and mutant K-RAS cells showed a significantly increase in G<sub>2</sub>-M phase cells compared with either manipulation alone, suggesting dramatic cell cycle deregulation results from this oncogenic combination (Fig. 3C; Table 3).

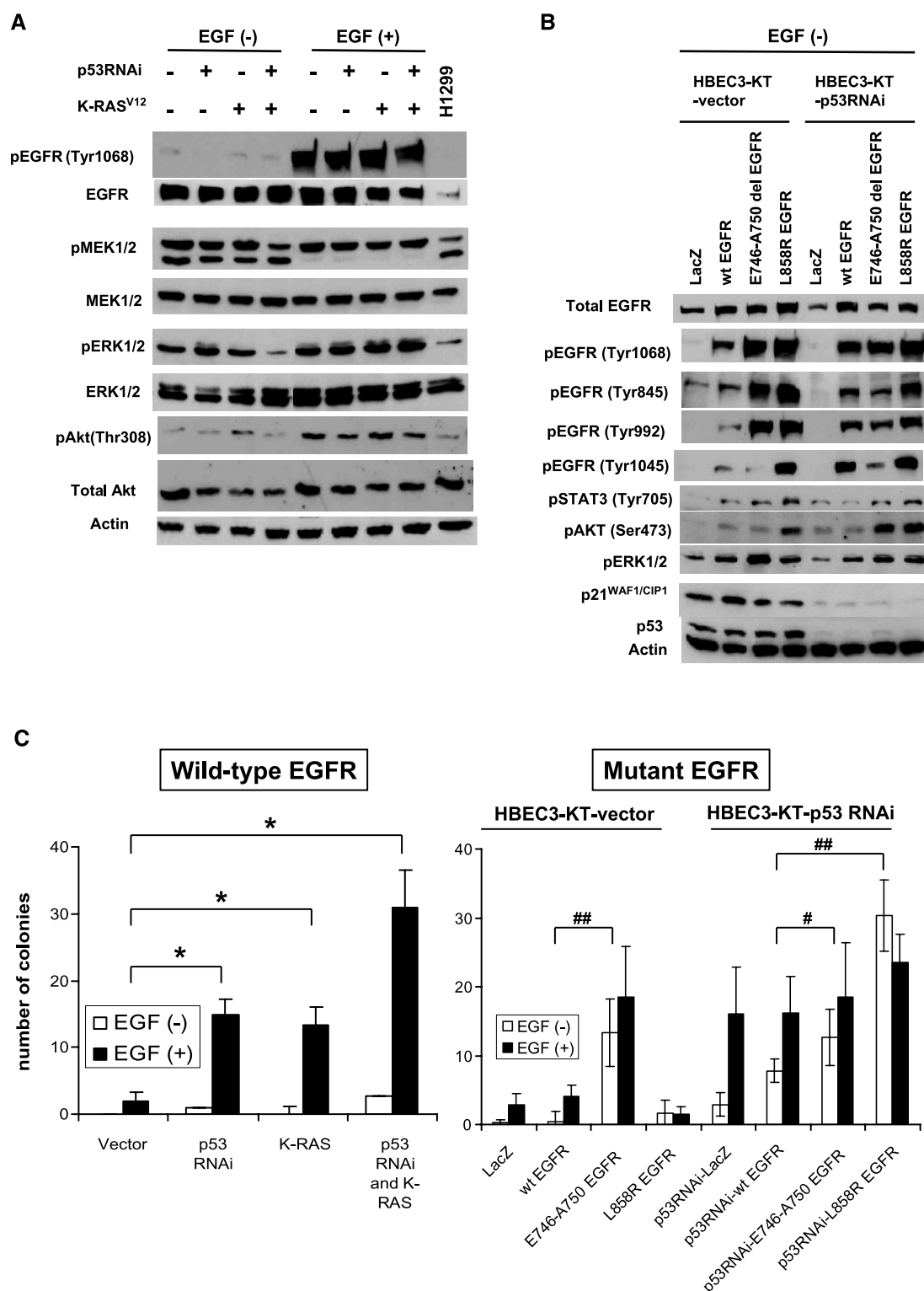
**Effect of K-RAS<sup>V12</sup> and EGF supplementation on expression of phospho-EGFR, phospho-MEK, phospho-ERK, and phospho-Akt.** We measured the expression of phosphorylated and total EGFR, mitogen-activated protein kinases (MAPK), and Akt

proteins in the HBEC3 cells in the presence and absence of EGF. Addition of EGF resulted in massive induction of phospho-EGFR, a slight induction of phospho-ERK, and a modest induction of phospho-Akt<sup>Thr308</sup> in all HBEC3 cells (Fig. 4A). In the absence of EGF, two immunoreactive phospho-MEK1/2 bands were detected whereas the faster migrating band was not detected in the presence of EGF, representing a shift to the hyper phosphorylated form (Fig. 4A). Surprisingly, introduction of K-RAS<sup>V12</sup> did not show a significant effect on phosphorylation of MAPKs in the presence or absence of EGF but led to a slight increased phospho-Akt<sup>Thr308</sup> in the absence of EGF (Fig. 4A). Also, surprisingly, both phospho-MEK1/2 and phospho-ERK were down-regulated in p53RNAi and K-RAS<sup>V12</sup>-expressing HBEC3 cells compared with the other three cell lines in the absence of EGF (Fig. 4A).

**Tyrosine kinase domain mutant EGFRs enhanced anchorage-independent growth of HBEC3 cells.** EGFR with mutations in the tyrosine kinase domain have been discovered in lung cancers predominantly arising in never smokers (25). These mutant EGFRs are suspected as having oncogenic properties. To determine if mutant EGFRs commonly found in lung cancer (E746-A750, L858R) have oncogenic ability, we evaluated the tumorigenicity of wild-type and mutant EGFR transfected HBEC3s by soft agar colony formation assays. In the absence of EGF, E746-A750 del expressing HBEC3 cells formed significantly increased number of colonies compared with wild type-expressing cells in both p53 wild-type ( $P < 0.001$  by Mann-Whitney *U* test) and p53 knocked down cells ( $P < 0.01$  by Mann-Whitney *U* test), whereas L858R mutant-expressing HBEC3 cells increased the number of colonies only in p53 knocked down cells ( $P < 0.001$  by Mann-Whitney *U* test; Fig. 4C). In contrast to p53 RNAi and mutant K-RAS-expressing HBEC3s that formed very few number of colonies ( $2.7 \pm 2.0$  of 1,000) in the absence of EGF, the E746-A750 del mutant transfectants formed substantial number of colonies even in the absence of EGF (Fig. 4C), suggesting that E746-A750 del mutant reduced the EGF dependence of HBEC3s in terms of anchorage-independent growth. Of interest, the L858R mutant only showed this independence when p53 was removed by knockdown (Fig. 4C). Again, in these p53 knocked down HBEC3s carrying a control vector (used for EGFR introduction), the cells remained dependent on EGF for soft agar growth. These results indicate that both types of mutant EGFRs possess oncogenic properties compared with wild-type EGFR. They also provide functional differences between the deletion and missense EGFR mutants, including differences in p53 interaction. We also did nude mice injection assays for p53 knocked down HBEC3s carrying L858R mutant, which formed the most number of colonies in the absence of EGF (Fig. 4C). However, they did not form tumor in nude mice (Table 1).

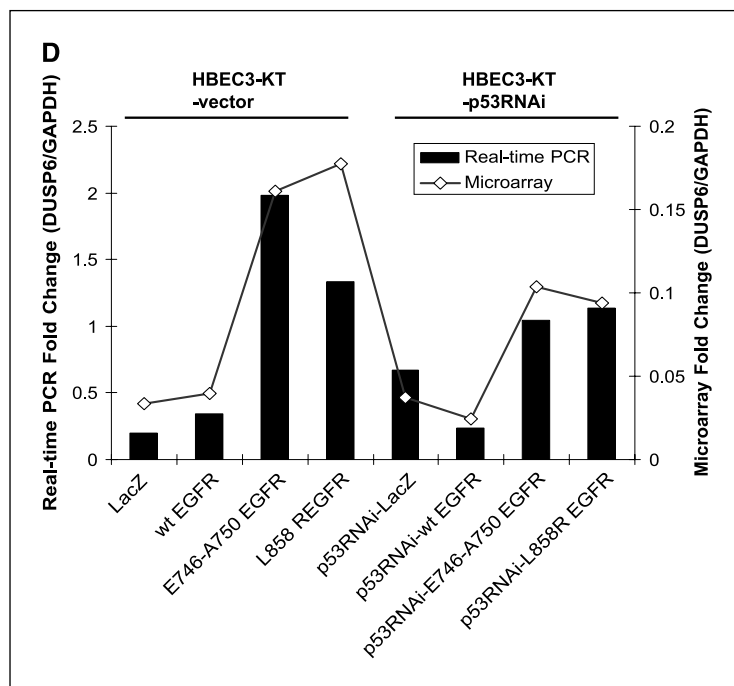
**Introduction of both wild-type and mutant EGFR into HBEC3s resulted in constitutive activation of EGFR.** Phosphorylation level of EGFR was evaluated by Western blotting with four (Y845, Y992, Y1045, and Y1068) phosphorylation-specific antibodies. To reduce the background for Western blotting, cells were first starved in the medium without bovine pituitary extract and EGF for 24 hours before harvest. In the absence of exogenous EGF, we found EGFR mutants and wild-type EGFR to exhibit induced levels of phosphorylated EGFR, suggesting the existence of autocrine ligands stimulating EGFR (Fig. 4B). In p53 wild-type cells, wild-type EGFR showed phosphorylation of Y845, Y992, and Y1068 to a lesser extent than when mutant EGFRs were present (Fig. 4B). In contrast, in p53 knocked down cells, such a difference





**Figure 4.** Transduction analysis for oncogenically manipulated HBEC3 cells. **A**, effect of K-RAS<sup>V12</sup> and EGF supplementation on expression of phospho-EGFR, phospho-MEK, phospho-ERK, and phospho-Akt. HBEC3 cells were grown in the presence or absence of 5 ng/mL EGF and were immunoblotted to detect phospho-EGFR (*pEGFR*), EGFR, phospho-MEK1/2 (*pMEK1/2*), MEK1/2, phospho-ERK (*pERK*), ERK, phospho-Akt (*pAkt*; Thr<sup>308</sup>), and Akt. Actin was used as loading control. **B**, effect of wild-type and mutant EGFR introduction on expression of phospho-EGFRs, phospho-STAT3, phospho-ERK, and phospho-Akt in HBEC3 cells. Wild type- or mutant EGFR-introduced HBEC3 cells were grown in the absence of EGF and were immunoblotted to detect phospho-EGFRs (Y1068, Y1045, Y992, and Y845), EGFR, phospho-STAT3, phospho-Akt (Y473), and phospho-ERK. Actin was used as loading control. **C**, soft agar colony formation assay for oncogenically manipulated HBEC3 cells. A total 1,000 of each HBEC3 cell strains were plated in agar and 4 weeks later microscopically visible colonies were counted. Columns, mean of three independent experiments; bars, SD. \*,  $P < 0.01$ , one-way ANOVA with Bonferroni's posttest; #,  $P < 0.01$ , Mann-Whitney test; ##,  $P < 0.001$ , Mann-Whitney test.

**Figure 4** Continued. D, microarray analysis and real-time PCR validation for *DUSP6/MKP-3* gene. Columns, fold changes of the mRNA levels of *DUSP6/MKP-3* gene relative to those of *GAPDH* gene in HBEC3 cells transfected with wild-type or mutant EGFR.



was not seen (Fig. 4B). This result suggests that the activation of wild-type EGFR might be suppressed by p53. Interestingly, the Y1045 site was highly phosphorylated in L858R transfectants but not in E746-A750 del transfectant (Fig. 4B), indicating that Y1045 is unique in distinguishing between the two types of EGFR mutations (Fig. 4B).

**AKT and STAT3 were phosphorylated at higher level in mutant EGFRs than wild-type EGFR.** The EGFR L858R mutant showed increased level of phosphorylated Akt and STAT3 in both p53 wild-type and p53 knocked down cells, whereas the EGFR E746-A750 del mutant showed increased level of phosphorylated Akt only in p53 knocked down cells and slightly increased level of phosphorylated STAT3 in both p53 wild-type and p53 knocked down cells (Fig. 4B). By contrast, no significant difference in phosphorylated ERK was seen between wild-type and mutant EGFRs (Fig. 4B). These results suggest that mutant EGFRs selectively transduces signals through Akt and STAT3, which is consistent with previously reported data (26).

***DUSP6/MKP-3* gene was up-regulated in mutant EGFR transfected HBEC3 cells.** Microarray analysis for HBEC3 cells transfected with wild-type or mutant EGFRs shows that mRNA of *DUSP6/MKP-3*, whose protein is a dual-specificity phosphatase that dephosphorylate the active form of ERK (27, 28), is significantly up-regulated in mutant EGFR transfectants compared with wild-type and vector-transfected cells (Fig. 4D). Real-time PCR analysis for *DUSP6/MKP-3* also showed this result (Fig. 4D).

## Discussion

We have taken HBECs immortalized using overexpression of Cdk4 (to circumvent p16-mediated cell culture growth arrest) and hTERT (to prevent telomere erosion) and genetically manipulated them by stably knocking down p53, expressing oncogenic K-RAS<sup>V12</sup> and mutant EGFR, alone or in combination. The results show that these additional genetic changes, commonly found in human lung cancer,

progress the HBEC3 cells part, but not all, of the way toward malignancy. The human cells exhibit higher saturation density, anchorage-independent growth, invade in an organotypic culture assay but do not form tumors in mouse xenografts. Although, in general, the cells remain dependent on EGF, p53 knockdown and mutant EGFR reduce this EGF dependence. In addition, their growth and ability to form colonies in liquid and semisolid medium is dramatically reduced by EGFR-directed tyrosine kinase inhibitors. These studies indicate that more than four genetic alterations are required for the full cancer transformation of HBECs.

Several studies have reported that introduction of oncogenes, such as K-RAS or HRAS and c-myc, result in malignant transformation of HBECs (29–31). However, until the present study, there has not been immortalized cell lines with wild-type p53 function. (Prior studies were done with viral oncoprotein immortalized cells abrogating p53 function; refs. 11, 12, 29, 30, 32.) In the present study, we have shown that >90% inhibition of p53 protein in immortalized HBECs enhances the clonal and soft agar colony formation and results in partial loss of contact inhibition, indicating that loss of p53 function contributes importantly to the malignant progression of HBECs. In addition, the combination of p53 knockdown and oncogenic K-RAS<sup>V12</sup> enhanced these changes further, suggesting these two genetic alterations have additive effects on tumorigenicity. Taken together, these results show that this model system provides a powerful new approach to assess the contribution of individual genetic alterations in HBECs in the malignant process.

RAS was first identified as an oncogene by virtue of its ability to overcome cell-to-cell contact inhibition of proliferation and this ability has been well documented in many types of cells (33, 34). In the present study, not only oncogenic K-RAS<sup>V12</sup> but also p53 knockdown resulted in partial loss of contact inhibition and the combination of them enhanced this ability. Recently, Meerson et al. (35) reported results consistent with this finding. They showed that p53 knockdown in WI38 human embryonic lung fibroblasts

reduced density-dependent inhibition of growth by abolishing G<sub>1</sub> phase arrest. Although density-dependent inhibition of growth is a complex phenomenon and its precise mechanism is not well understood, this phenomenon is thought to be indicative of tumorigenic potential. Thus, their results and ours suggest that p53 may function as a tumor suppressor even when the cells are not under stresses, such as genotoxic damage and irradiation. Recently, other studies also reported that p53 function is involved in regulating cell motility and adhesion (36, 37).

It is unclear how the mutant K-RAS transfected HBECs preferentially express the mutant compared with wild-type *K-RAS* allele. These cells express mutant K-RAS mRNA predominantly without changing the total K-RAS protein levels, suggesting that wild-type K-RAS expression is suppressed at the transcriptional level in these cells. The mechanism of transcriptional regulation of K-RAS has not been fully elucidated and we are unable to explain the mechanism of this observation. However, the hypothesis that oncogenic RAS inhibits the transcription of wild-type RAS is compatible with our observations. Because the tumor suppressor function of wild-type K-RAS has been shown in mice, one possibility is that mutant RAS exerts its oncogenic ability in part by suppressing the expression of wild-type RAS. This hypothetical function of oncogenic RAS seems very attractive in terms of better understanding the mechanism of oncogenic RAS, and thus it will be of interest to further investigate these findings.

Previous studies have found high levels of EGFR expression in both immortalized and nonimmortalized HBECs (38). EGF supplementation also results in a slight increase of growth rate at normal cell density in HBECs (38). Several studies have shown the existence of an EGFR autocrine loop involving EGF, transforming growth factor- $\alpha$ , and amphiregulin in HBECs (39, 40). In addition, tobacco smoke induces proliferation of primary HBEC through an EGFR autocrine loop mediated by tumor necrosis factor- $\alpha$ -converting enzyme and amphiregulin, suggesting tobacco smoke induction of the EGFR autocrine loop in lung cancer pathogenesis (41). In the present study, we found that HBEC3 cells expressed high level of EGFR that was stimulated to phospho-EGFR with EGF whereas their colony-forming ability in both liquid and soft agar was highly dependent on EGF supplementation. Signal transduction studies in HBEC3 cells suggest that this may in part be due to the up-regulation of the Akt pathway. With p53 knockdown and K-RAS oncogenic manipulation, the EGF dependence was partially relieved, suggesting the potential for autocrine growth factor production. Thus, previous studies and our results suggest that EGF autocrine loop may play an important role in cell proliferation and tumorigenic progression of HBECs.

Gefitinib (Iressa) and erlotinib (Tarceva) are orally available tyrosine kinase inhibitors that target EGFR (42–45). Gefitinib has been approved as a third-line therapy for NSCLC patients. Erlotinib has been shown to be active and well tolerated in patients with NSCLC, providing survival benefit (46). Although these drugs are being developed as anticancer drugs, recent studies have shown that gefitinib inhibits cell proliferation in immortalized normal or precancerous breast cells, supporting its role as a chemopreventive agent (47). Because we found robust expression of EGFR in HBEC3 cells and their high dependency of growth on EGF, we considered the possibility that tyrosine kinase inhibitors are also effective in oncogenically manipulated HBEC3 cells. We observed that 1  $\mu$ mol/L gefitinib or erlotinib dramatically inhibited both anchorage-dependent and anchorage-independent cell growth of HBEC3 cells. Apoptosis and cell cycle analyses showed that this inhibition

was caused mainly by not apoptosis but G<sub>1</sub> cell cycle arrest, which is consistent with previous papers reporting that gefitinib and erlotinib induce G<sub>1</sub> cell cycle arrest in several types of cells (48–52). These results provide part of a preclinical rationale for the development of these drugs for the prevention of human lung cancer. It is important to point out that the concentrations used in the present studies are actually achieved in patients with current standard drug practices (53, 54). In addition, interestingly, we found that G<sub>2</sub>-M fraction significantly increase with the combination of p53 knock down and mutant K-RAS cells compared with either oncogenic manipulation alone. We speculate that in the presence of intact p53 function, cell cycle progression induced by mutant K-RAS in HBECs is suppressed by the ability of p53 to induce G<sub>1</sub> arrest, whereas in the absence of p53, mutant K-RAS exerts its ability to progress the cell cycle from G<sub>1</sub> to S phases, resulting in significantly increased G<sub>2</sub>-M phase fraction. Consistent with this hypothesis, one paper showed that ectopic expression of mutant N-RAS impaired the G<sub>1</sub> and G<sub>2</sub> cell cycle arrest only in p53-defective cells (55). In addition, it will be interesting to see whether similar types of cell cycle deregulation are also found when mutant EGFR is combined with loss of p53 function in these cells.

Introduction of tyrosine kinase domain EGFR mutants enhanced anchorage-independent growth of HBEC3s, providing evidence of their oncogenic properties. In addition, signal transduction analysis showed that they stimulated Akt and STAT3 but not Erk1/Erk2 signals, consistent with previously reported results (26). Our discovery of DUSP6/MKP-3 mRNA up-regulation in mutant EGFR transfectants provides an explanation for this. Because DUSP6/MKP-3 protein is a dual-specificity phosphatase that dephosphorylates the active form of ERK (27, 28), it is possible that Erk1/Erk2 phosphorylation in EGFR mutant cells is down-regulated by DUSP6/MKP-3. In addition, the findings that Akt and STAT3 were not highly phosphorylated in HBECs that showed robust level of phosphorylated EGFR suggests that a negative feedback regulatory pathway may be activated for Akt and STAT3 as well, and that the mutant EGFRs bypass this regulation.

Although p53 knockdown, K-RAS<sup>V12</sup>, and mutant EGFR progress HBEC3 cells toward malignancy, the manipulated cells are not fully malignant. What additional genetic alterations are required for full malignant transformation in HBEC3 cells? For this question, there may be a clue from recent work of Hahn et al., who showed that defined genetic alterations, including the early region of the SV40 genome, the *hTERT* gene, and an oncogenic allele of H-ras, resulted in malignant transformation in human embryonic epithelial and fibroblast cells (56). By precisely analyzing the early region of SV40, they have shown that small T antigen, which is transcribed from the early region together with large T antigen, may play an important role in carcinogenesis. Small T antigen has been shown to bind and to target phosphatase 2A (PP2A), which regulates the RAS/MAPK cascade. Our unpublished studies have shown no mutation but frequent loss of PP2A expression in lung cancer, raising the possibility that PP2A is involved in lung carcinogenesis. Thus, a next step would be to inactivate PP2A in HBEC3 cells in addition to p53 knockdown and K-RAS<sup>V12</sup>. It will also be of interest to introduce other genetic alterations observed in lung cancer, such as MYC family overexpression, FHIT inactivation, RASSF1A inactivation, and PTEN inactivation. Although we used a previously reported target sequence for p53 knockdown, which has also been used in several papers (17, 18, 57) and has no other BLAST hits, we are unable to completely exclude the possibility that off-target effects might affect our phenotypic analysis.



In conclusion, we have shown that using the immortalized HBEC model, p53 knockdown, K-RAS<sup>WT</sup>, and mutant EGFR in the presence of p16 bypass and human telomerase contribute to lung cancer tumorigenesis, but additional genetic alterations are required for full malignant transformation of HBECs. In addition, we note that p53 knockdown relaxes the dependence on EGF in the presence of both wild-type and mutated EGFR. However, these oncogenically manipulated HBEC cells remain highly dependent on EGFR signaling for expression of key portion of the malignant phenotype. This dependence along with activated EGFR in bronchial preneo-

plasia suggests the use of EGFR inhibition by tyrosine kinase inhibitors as chemoprevention agents for lung cancer.

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## Multiple Oncogenic Changes (*K-RAS*<sup>V12</sup>, p53 Knockdown, Mutant *EGFRs*, p16 Bypass, Telomerase) Are Not Sufficient to Confer a Full Malignant Phenotype on Human Bronchial Epithelial Cells

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