Human Lung Epithelial Cells Progressed to Malignancy through Specific Oncogenic Manipulations

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Abstract

We used CDK4/hTERT-immortalized normal human bronchial epithelial cells (HBEC) from several individuals to study lung cancer pathogenesis by introducing combinations of common lung cancer oncogenic changes (p53, KRAS, and MYC) and followed the stepwise transformation of HBECs to full malignancy. This model showed that: (i) the combination of five genetic alterations (CDK4, hTERT, sh-p53, KRASV12, and c-MYC) is sufficient for full tumorigenic conversion of HBECs; (ii) genetically identical clones of transformed HBECs exhibit pronounced differences in tumor growth, histology, and differentiation; (iii) HBECs from different individuals vary in their sensitivity to transformation by these oncogenic manipulations; (iv) high levels of KRASV12 are required for full malignant transformation of HBECs, however, prior loss of p53 function is required to prevent oncogene-induced senescence; (v) overexpression of c-MYC greatly enhances malignancy but only in the context of sh-p53 iKRASV12; (vi) growth of parental HBECs in serum-containing medium induces differentiation, whereas growth of oncogenically manipulated HBECs in serum increases in vivo tumorigenicity, decreases tumor latency, produces more undifferentiated tumors, and induces epithelial-to-mesenchymal transition (EMT); (vii) oncogenic transformation of HBECs leads to increased sensitivity to standard chemotherapy doublets; (viii) an mRNA signature derived by comparing tumorigenic versus nontumorigenic clones was predictive of outcome in patients with lung cancer. Collectively, our findings show that this HBEC model system can be used to study the effect of oncogenic mutations, their expression levels, and serum-derived environmental effects in malignant transformation, while also providing clinically translatable applications such as development of prognostic signatures and drug response phenotypes.

Visual Overview: http://mcr.aacrjournals.org/content/11/6/638/F1.large.jpg.

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Introduction

Human lung cancer develops as a multistep process, usually after prolonged smoke-related tobacco exposure resulting in specific proto-oncogene and tumor suppressor gene alterations in lung epithelial cells (1). Genome-wide analyses have identified multiple genetic and epigenetic alterations in lung tumors (2–5). To translate these findings to the clinic, however, it is essential to identify the best targets for early detection and therapeutic intervention by determining which alterations represent “driver” and which represent “passenger” changes.

Functional tests are a critical step in determining “driver” status and are most commonly conducted by genetically or pharmacologically “correcting” the defect in a lung cancer line and monitoring its effect. Another approach is to introduce putative oncogenic changes into normal lung epithelial cells and ascertain their contribution to malignancy. To undertake the latter, we previously developed an in vitro model of immortalized human bronchial epithelial cells (HBEC) establishing cell lines from more than 30 different individuals (6). HBECs were immortalized by overexpressing Cdk4 and human telomerase reverse transcriptase (hTERT) to emulate 2 of the earliest events and almost
universal events in lung cancer pathogenesis: abrogation of the p16/Rb cell-cycle checkpoint pathway and bypass of replicative senescence. Importantly, this was the first report of immortalizing lung epithelial cells in the absence of viral oncoproteins, as used previously by other groups (7, 8). Viral oncoproteins such as SV40 early region (containing large T and small t antigens) are known to cause malignant transformation through inactivation of retinoblastoma (Rb) and/or p53, as well as inhibit the tumor suppressor gene PP2A phosphatase (9, 10). They also have other less characterized interactions with cellular processes, thereby contributing to cancer pathogenesis in unknown ways. Thus, the critical genetic manipulations that lead to malignant transformation of epithelial cells immortalized with viral oncoproteins are not completely "defined." Moreover, they are likely more oncogenically progressed than nonviral models as shown in mammary epithelial cell systems where full transformation with SV40 early region required 3 manipulations (hTERT, SV40 early region, and RAS; ref. 8) as compared with 6 manipulations (hTERT, dominant-negative p53, CDK4, c-MYC, and cyclin D1) in the absence of SV40 early region (11).

While HBECs used in our study are immortalized, a phenotype they share with cancer cells, they do not display other cancer cell phenotypes such as disruption of the p53 pathway, extensive copy number changes, lack of contact-inhibition, anchorage-independent growth, and the ability to form tumors in immunodecient mice (6, 12). Furthermore, we have shown that immortalized HBECs retain the ability to differentiate into structures found in the normal bronchial epithelium (13, 14). These features make HBECs a physiologically appropriate in vitro model for studying the transformation process of bronchial epithelial cells to lung cancer.

Transformation of lung epithelial cells to full malignancy using defined genetic manipulations has only been described in 2 studies (8, 15). One study using viral oncoproteins-transformed tracheobronchial and small airway epithelial cells with hTERT, SV40 early region, and oncogenic HRAS or KRAS (8). The second study showed fully transformed human small airway epithelial cells (HSAEC) occurred with CDK4, hTERT, mutant p53, mutant KRAS, and either c-MYC, PIK3CA, cyclin D1, or LKB1 manipulations (15). In our bronchial epithelial cell model, we have previously shown the combination of CDK4, hTERT, and p53 knockdown with mutant EGFR, or moderate levels of mutant KRAS\textsuperscript{V12} progressed cells partially but not completely to malignancy, as evidenced by the failure to form tumors in immunodecient mice (12). As lung cancer develops in both the central bronchus and peripheral small airways, development of an in vitro model of malignant transformation in bronchial epithelial cells is essential.

In the present study, we aimed to conrm the genetic tractability of HBECS and fully transform cells to malignancy using oncogenic manipulations commonly found in lung tumors. Loss of p53 function and oncogenic KRAS are 2 well-known genetic alterations in lung cancer occurring in approximately 50% and 30% of non–small cell lung carcinoma (NSCLC), respectively (16, 17). Aberrant expression of c-MYC, through amplification or overexpression, is found in approximately 20% of NSCLCs (1). While it is known that protein levels of oncogenic RAS can inuence its oncogenic ability (18), expression of oncogenic KRAS\textsuperscript{V12} can also lead to premature senescence of normal human epithelial cells (19). The prevalence of KRAS alterations in NSCLC indicates however that malignant transformation requires the cell to adapt to this oncogenic stress, perhaps assisted through preceding oncogenic transformations (20). Here, we present one of the rst reports of full malignant transformation of lung epithelial cells with dened genetic manipulations. Furthermore, we characterize the effect of oncogenic stress and environmental effects such as growth factors upon tumorigenic transformation in HBECS, illustrate divergent clonal heterogeneity, and determine the capability of this in vitro model for developing and testing lung cancer therapeutics.

Materials and Methods

Cells and culture conditions

HBEC3 (HBEC3KT), HBEC4 (HBEC4KT), and HBEC17 (HBEC17KT) immortalized normal HBEC lines were established by introducing mouse Cdk4 and hTERT into normal HBECs (6). HBECs were cultured with keratinocyte serum-free medium (KSF; Life Technologies Inc.) media containing 50 µg/mL of bovine pituitary extract (BPE; Life Technologies Inc.) and 5 ng/mL of EGF (Life Technologies Inc.). Partially transformed HBECs (soft agar clones) were also cultured with RPMI-1640 (Life Technologies Inc.) media supplemented with 10% FBS (R10). Lung cancer cell lines were established by our laboratory and maintained in RPMI-1640 (Life Technologies Inc.) with 5% FBS (21, 22). All cell lines were DNA-fingerprinted (PowerPlex 1.2 Kit; Promega) and Mycoplasma-free (e-Myc Kit; Boca Scientific).

Viral vector construction and viral transduction

Stable p53 knockdown and moderate expression of KRAS\textsuperscript{V12} was achieved as described previously (12). Expression of high KRAS\textsuperscript{V12} levels used a lentiviral vector, pLenti6-KRAS\textsuperscript{V12}, as described previously (23). Lentiviral vectors expressing KRAS\textsuperscript{V12}, KRAS\textsuperscript{C12}, and KRAS\textsuperscript{V12} were constructed from pLenti6-KRAS\textsuperscript{V12} as described previously (24). A c-MYC-expressing retroviral vector (designated pMSCV-MYC) was constructed by ligating a BamHI/SalI-digested c-MYC insert from pCTV3K (ref. 25; a gift from Dr. J. Michael Ruppert, University of Alabama, Tuscaloosa, AL) into BglII/XhoI-digested pMSCV-hyg (Clontech). Lentivirus and retrovirus-containing medium were produced as described previously (12). Transduced cells were selected with zeocin (12.5 µg/mL), blasticidin (2 µg/mL), or hygromycin (20 µg/mL) for 7 to 14 days. The presence of mutant KRAS\textsuperscript{V12} in stable cell lines was conrmed using a reverse transcription PCR (RT-PCR)/RFLP assay, as described previously (12).
Immunoblotting and southern blotting

Preparation of total cell lysates and Western blotting were carried out as described previously (26). Primary antibodies are listed in Supplementary Table S1 and were detected with horseradish peroxidase (HRP)–conjugated anti-rabbit or anti-mouse secondary antibodies (1:2,000 dilution; Thermo Fisher Scientific). Actin or HSP90 protein levels were used as loading controls. Southern blotting for HBEC clones derived from large soft agar colonies was carried out as described previously (27) using Phototope-Star Detection Kit (New England Biolabs). DNA was probed with the blasticidin-resistance gene amplified from pLent6-KRASV12 vector using 5′-ATGGCCAAGCCTTTGTCT-CAAG-3′ and 5′-TTAGCCCTCCACACATAACC-3′ primers.

Biochemical and in vitro transformation assays

Senescent cells were stained with Senescence β-Galactosidase Staining Kit (Cell Signaling) and blue-stained senescent cells were counted under a microscope (×20 total magnification). Percentage of positively stained cells was averaged across 6 fields. Cell-cycle analysis was conducted on subconfluent populations of cells harvested 48 hours after seeding, unless otherwise stated, and cell-cycle analysis was conducted as described previously (28). Cell proliferation assays were conducted by seeding 5,000 cells in 6-well plates and cultured for 2 weeks before staining colonies (12); 200 to 600 viable cells were seeded in triplicate in 100-mm plates and cultured for 2 weeks before seeding colonies with methylene blue. Acute KRASV12 toxicity assays were conducted by transducing cells with KRASV12 or LacZ lentivirus and selecting for 3 days with blasticidin before anchorage-dependent colony formation assays. Anchorage-independent (soft agar) growth assays were conducted as previously described (12) seeding 1,000 viable cells in 12-well plates. MTS assays to measure drug response to cisplatin, or 2,000/140 nmol/L (gemcitabine/cisplatin), 1,000/298 nmol/L (paclitaxel/carboplatin), 1,000/3,501 nmol/L (pemetrexed/cisplatin) were conducted as previously described (29). Cells were treated for 96 hours with 4-fold dilutions from a maximum dose of 1,000/3,501 nmol/L (paclitaxel/carboplatin), 1,000/298 μmol/L (pemetrexed-cisplatin), or 2,000/140 μmol/L (gemcitabine/cisplatin). Each experiment was carried out in quadruplicate with 8 replicates per experiment. Fitting of data to dose–response inhibition curves, calculation of ED50 values, and comparisons based on one-way ANOVA with Dunnett posttest were conducted using GraphPad Prism version 5.00 for Windows (GraphPad Software).

In vivo tumorigenicity assays and histologic analysis

In vivo tumorigenicity was evaluated by injection of cells in female 5- to 6-week-old nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice. Each mouse was given a subcutaneous injection on its flank of 3 to 5 × 10⁶ viable cells in 0.2 mL of PBS. Mice were monitored every 2 to 3 days for tumor formation for up to 6 months. All animal care was in accord with institutional guidelines and approved Institutional Animal Care and Use Committee (IACUC) protocols. Formalin-fixed, paraffin-embedded (FFPE) xenograft tumor tissue was sectioned and stained with hematoxylin and eosin (H&E), Alcian Blue–PAS, PAS, and mucicarmine for histologic analysis. Immunohistochemical staining for p63 (BioCare; clone BC4A4), Napsin A, CK5, and CK7 was carried out commercially by ProPath. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining was conducted using DeadEnd Fluorometric TUNEL System (Promega).

Statistical testing and microarray analysis

For comparison of colony formation and senescent cells between different genetically manipulated cell strains, we used a two-tailed Student t test and P < 0.05 was considered significant. mRNA microarray profiling was conducted with Illumina HumanHT-12 v4 Expression BeadChips (Illumina Inc.) following the manufacturer’s guidelines and analyzed with in-house Visual Basic software MATRIX V1.483. Functional analysis of differentially expressed genes was conducted using Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Inc.). The data discussed in this publication have been made available in the National Center for Biotechnology Information’s Gene Expression Omnibus (GEO) public repository (http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE40828. The predictive ability of the soft agar gene signature was tested using 2 independent mRNA microarray lung tumor datasets; 209 primary lung adenocarcinomas and squamous cell carcinomas (SCC; SPORE dataset) GSE41271, and 442 primary lung adenocarcinomas [National Cancer Institute (NCI) Director’s Challenge Consortium dataset; ref. 30]. A detailed description of these 2 datasets and the prediction analysis is given in the Supplementary Methods.

Results

Oncogenic transformation of HBECs correlates with level of exogenous KRASV12 expression

NSCLC cell lines harboring a mutation in KRAS express wide-ranging endogenous levels of the protein (Fig. 1A). Thus, to create HBECs that express comparable levels of oncogenic KRAS protein, we used 2 different expression vectors: a retroviral vector, which resulted in modest levels of expression, and a lentiviral vector that resulted in high levels of expression (Fig. 1A). Compared with HBEC3 expressing moderate levels (retroviral-mediated) of KRASV12 protein, HBEC3 expressing high levels of KRASV12 (lentiviral-mediated) exhibited a significant increase in soft agar colonies in the background of both wild-type p53 (HBEC3) and p53 knockdown (HBEC3Δp53; Fig. 1B). To confirm the contribution of the level of KRASV12 expression toward HBEC transformation, we examined 7 clonal populations of HBEC3Δp53.KRASV12 (sh-p53 and lentiviral-delivered KRASV12; Fig. 1C). Clones with high levels of KRAS expression had increased anchorage-dependent (liquid) colony-forming ability (Fig. 1D and Supplementary Fig. S1A).
High levels of mutant KRASV12 induce senescence in HBECs, in a p53-dependent manner

Lentiviral-mediated introduction of high levels of KRASV12 caused a large subset of HBEC3 cells to display significant morphologic changes, including flattened, enlarged shape, and a vacuole-rich cytoplasm, suggestive of oncogene-induced senescence. This effect was not observed following exogenous expression of moderate levels of KRASV12 (Supplementary Fig. S1B). Senescence-associated β-galactosidase (SA-β-gal) staining confirmed the morphologic changes corresponded with senescence (Supplementary Fig. S1C). Significantly less RAS-induced senescence was observed in HBEC3 with stable p53 knockdown (Fig. 1E and Supplementary Fig. S1C). An acute, senescence-associated p53-mediated KRASV12 toxicity was shown in anchorage-dependent colony formation assays seeded 4 days after transduction with KRASV12 lentivirus. Significant toxicity was observed in HBECs with wild-type p53 (HBEC3 and another HBEC line, HBEC4) following transduction with KRASV12 lentivirus compared with control LacZ lentivirus but not in HBECs with p53 knockdown (Fig. 1F and Supplementary Fig. S1D and S1E), suggesting induced senescence was p53-dependent. p53-mediated mitogenic or oncogenic KRAS stress was also observed with other codon 12 KRAS mutants (KRASV12 and D12; Supplementary Fig. S1F and S1G), whereas overexpression of wild-type KRAS in HBEC3 did not induce a senescence phenotype (Supplementary Fig. S1F and S1G). Immunoblotting for cleaved caspase-3 confirmed KRASV12-induced toxicity was bypassed with p53 knockdown. A, immunoblot for KRAS protein expression in HBEC3 cells infected with KRASV12 using either a moderately expressing retroviral (pBabe-hyg-KRASV12) or high-expressing lentiviral (pL6-KRASV12) vector. Actin was used as loading control. B, anchorage-independent (soft agar) colony formation in HBEC3 or LacZ lentivirus. Harvested 7 days after infection with p53 or p53 knockdown. G, immunoblot of HBEC3 or p53, KRASV12 and KRAS wild-type. KRAS was used as loading control. C, immunoblot of HBEC3 with high (lentiviral) or moderate (retroviral) levels of KRASV12 in the background of both wild-type p53 and p53 knockdown (sh-p53; t test). C, immunoblot of HBEC3 or p53, KRASV12 and KRAS wild-type expressing retroviral (pBabe-hyg-KRASV12) or high-expressing lentiviral (pL6-KRASV12) vector. Actin was used as loading control. D, anchorage-dependent colony formation ability of HBEC3 or p53, KRASV12 and KRAS wild-type in the absence (Control LacZ) or presence (sh-p53) of p53 knockdown. E, quantification of SA-β-gal staining found KRASV12-induced senescence in HBEC3 cells was significantly lower in cells with p53 knockdown compared with p53 wild-type (t test). F, anchorage-dependent colony formation assay to compare acute KRASV12-induced toxicity in HBEC3 and HBEC4 with wild-type p53 or p53 knockdown. G, immunoblot of HBEC3 cell lysates harvested 7 days after infection with KRASV12 or LacZ lentivirus. ** P < 0.01; *** P < 0.001. Full-length blots are presented in Supplementary Fig. S8.
These results show that oncogenic KRAS mediates a potent cellular stress response in CDK4/htERT–immortalized HBECs, whereby the cells resist oncogenic transformation by engaging cellular senescence. Loss of p53 function however impedes this cellular senescence response, indicating that in these cells, p53 signaling is a primary mediator of RAS-induced senescence. Furthermore, as high levels of oncogenic KRAS expression are required for malignant transformation, it shows that loss of p53 function is a critical co-oncogenic step in the malignant transformation of the large majority of HBECs.

The combination of p53 knockdown and KRASV12 in HBECs significantly increases in vitro transformation, which is further augmented with c-MYC overexpression.

The single introduction of p53 knockdown, mutant KRASV12 or c-MYC overexpression resulted in quantitatively modest but significant increases in soft agar colony number (Fig. 2A–C). The combination of p53 knockdown and KRASV12 (HBEC3p53,KRASV12) resulted in a significant increase in transformation not observed in other dual combinations (p53 knockdown+c-MYC (HBEC3p53,MYC) or KRASV12+c-MYC (HBEC3KRASV12,MYC)), whereas introduction of all 3 manipulations (HBEC3p53,KRASV12,MYC) resulted in the most transformed phenotype (Fig. 2B). Higher expression of c-MYC was achieved if overexpressed in HBEC3p53 (p53 knockdown), HBEC3KRASV12 (KRASV12), or HBEC3p53,KRASV12 (p53 knockdown and KRASV12) cells (Fig. 2A), suggesting partial transformation of immortalized HBEC3 with either p53 knockdown or addition of mutant KRASV12 is required for the cells to tolerate high levels of c-MYC.

Combination of sh-p53+KRASV12 or sh-p53+KRASV12+c-MYC oncogenically transforms HBEC3 in vivo

To test the tumor-forming ability of HBEC3p53,KRAS and HBEC3p53,KRAS,MYC, cells grown in defined KSFM media were injected subcutaneously into NOD/SCID mice. We had previously found HBEC3p53 with moderate levels of oncogenic KRASV12 (using a retroviral expression vector) failed to form tumors when injected into immunodeficient mice (12). In contrast, transforming with higher levels of KRASV12 resulted in tumor growth in 5 of 24 (21%) injections (Table 1). Despite the significant increase in in vitro anchorage-independent growth observed in HBEC3p53,KRAS,MYC compared with HBEC3p53,KRAS (Fig. 2B), HBEC3p53,KRAS,MYC was only slightly more tumorigenic in vivo with tumors in 3 of 10 (30%) injections (Table 1). Histopathologic analysis showed that the oncogenically manipulated HBEC populations formed SCCs, adenocarcinomas, adenosquamous carcinomas, and poorly differentiated carcinomas with typical morphologic features of each histology (Table 1 and Fig. 3). Squamous and adenocarcinoma differentiation was confirmed by p63 and mucicarmine/Alician Blue-PAS positivity, respectively, and pathology was verified by an independent lung cancer pathology expert. Adenocarcinomas were found to be strongly cytokeratin 5–positive, whereas negative for Napsin A and the squamous cell marker, cytokeratin 7 (Supplementary Fig. S2A). In all cases, adenocarcinoma differentiation was TITF1(NKX2-1)–negative (data not shown), most likely a reflection of the HBECs being derived from central airway cells. The fact that glandular cells are negative for both TITF-1 and Napsin A is not unexpected as the vast majority of adenocarcinomas (and glandular component in adenosquamous carcinomas) in whole tissue sections are either positive for both or negative for both. The development of different tumor histologies from the same HBEC-manipulated population suggests either clonal selection or the cells respond to differentiation signals in vivo.

Exploration of interindividual differences in HBEC transformation

To compare interindividual differences in malignant transformation using the same combination of oncogenic changes, we tested HBEC17, derived from a different individual than HBEC3. We observed a similar transformed phenotype in vitro (Supplementary Fig. S2B) however, in contrast to HBEC3, HBEC17 was significantly more resistant to full in vivo transformation (Table 1). The difference in tumor formation rate between HBECs derived from different donors suggests the existence of interindividual differences in susceptibility to specific oncogene-induced malignant transformation.
Growth in serum-containing medium enhances full tumorigenic transformation of HBECs

In addition to viral oncoproteins, many previous studies that succeeded in transforming normal cells to malignancy also used serum-containing medium instead of defined serum-free medium (31–33). We have previously shown that CDK4/hTERT–immortalized HBECs with no additional oncogenic manipulations require EGF, a supplement present in KSFM medium, for cell growth, but oncogenic transformation with p53 knockdown and KRASV12 allows the cells to become EGF-independent (12). In the present study, we show while HBEC3 cannot tolerate growth in RPMI-1640 supplemented with serum (FBS), media commonly used for growth of cancer cell lines, HBEC3p53, KRAS,

Table 1. In vivo tumorigenicity of manipulated HBECs

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Mediuma</th>
<th>Tumor formation rateb</th>
<th>Latency, dc</th>
<th>Histology</th>
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<tr>
<td>HBEC3 p53, KRAS</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Population</td>
<td>KSFM</td>
<td>5/24 (21%)</td>
<td>152</td>
<td>SCC (2) Adenosquamous (2) Poorly differentiated carcinoma (1)</td>
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<tr>
<td>Population</td>
<td>R10</td>
<td>7/17 (41%)</td>
<td>128</td>
<td>Large cell/giant cell carcinoma (3) Adenocarcinoma (2) SCC (1) Adenosquamous (1)</td>
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<tr>
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<td>—</td>
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<td>—</td>
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<td>—</td>
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<tr>
<td>Clone 9</td>
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<td>Population</td>
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<td>3/10 (30%)</td>
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<td>Adenosquamous (2) SCC (1)</td>
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<td>Population</td>
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<td>Clone 1</td>
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<td>Clone 7</td>
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<td>0/9 (0%)</td>
<td>—</td>
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</tr>
<tr>
<td>Population</td>
<td>R10</td>
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<td>Population</td>
<td>KSFM</td>
<td>0/10 (0%)</td>
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<tr>
<td>Population</td>
<td>R10</td>
<td>3/8 (38%)</td>
<td>n.d.</td>
<td>SCC (2) Sarcomatoid carcinoma features (1)</td>
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</table>

— medium supplemented with 10% FBS.
— number of subcutaneous tumors/number of injections (%).
— median time (days) for subcutaneous xenografts to reach 250 mm³; n.d., not determined.

Growth in serum-containing medium enhances full tumorigenic transformation of HBECs

In addition to viral oncoproteins, many previous studies that succeeded in transforming normal cells to malignancy also used serum-containing medium instead of defined serum-free medium (31–33). We have previously shown that CDK4/hTERT–immortalized HBECs with no additional oncogenic manipulations require EGF, a supplement present in KSFM medium, for cell growth, but oncogenic transformation with p53 knockdown and KRASV12 allows the cells to become EGF-independent (12). In the present study, we show while HBEC3 cannot tolerate growth in RPMI-1640 supplemented with serum (FBS), media commonly used for growth of cancer cell lines, HBEC3p53, KRAS,
is adaptable and will proliferate (Supplementary Fig. S3A). Serum-supplemented media has been shown to induce differentiation of epithelial cells in culture (34). Therefore, to further delineate the differences between HBEC3 compared with HBEC3p53,KRAS and HBEC3p53,KRAS,MYC following growth in serum-supplemented media, cells grown in either serum-free KSFM or serum-supplemented (5% FBS) KSFM for 96 hours were then analyzed for expression of a panel of lung differentiation and cancer stem cell (CSC) markers by quantitative RT-PCR (qRT-PCR). All 3 cell lines expressed high levels of basal markers (KRT5 and KRT14), low levels of central airway epithelial cell markers (MUC1 and TUBB4), with undetectable expression of peripheral airway markers (CC10 and SPC) in line with cells derived from bronchial epithelial cells (Supplementary Fig. S3B). Aldehyde dehydrogenase (ALDH) activity has been shown to be a marker of CSCs in the lung (28). We therefore measured the expression of 2 ALDH isozymes, ALDH1A1 and ALDH1A3, to find all the 3 cell lines that expressed ALDH1A3 with much lower expression of...
ALDH1A1. Growth in serum-containing medium resulted in all 3 cell lines exhibiting significant decrease in the expression of basal markers (KRT5 and KRT14), most notably in HBEC3 (Supplementary Fig. S3C). Expression of central airway markers, particularly MUC1, increased more than 10-fold in HBEC3 when cells were grown in serum-supplemented medium, whereas HBEC3p53,KRAS and HBEC3p53,KRAS,cMYC showed little if any increase in these markers. Expression of CSC markers ALDH1A1 and ALDH1A3 increased in HBEC3 and HBEC3p53,KRAS but not in HBEC3p53,KRAS,cMYC. Morphologically, growth in serum-supplemented medium resulted in HBEC3 cells developing a flattened morphology representative of a differentiated state (Supplementary Fig. S3D), whereas this was largely absent in HBEC3p53,KRAS cells and completely absent in HBEC3p53,KRAS,cMYC. Furthermore, HBEC3p53,KRAS,cMYC and to a lesser extent HBEC3p53,KRAS developed an elongated mesenchymal morphology after short-term growth in serum (Supplementary Fig. S3D). Together, these data show that serum-supplemented medium induces differentiation in parental HBEC3 cells; however, oncogenic transformation enables the cells to resist serum-induced differentiation and instead undergo epithelial-to-mesenchymal transition (EMT).

We reasoned that partially transformed HBECs that have adapted to growth in serum-supplemented RPMI-1640 may differ in tumorigenicity compared with cells grown in KSFM. Therefore, to compare the effect of genetic and environmental manipulations, HBEC3p53,KRAS and HBEC3p53,KRAS,cMYC were grown in either defined KSFM (serum-free) medium or RPMI medium supplemented with 10% FBS (R10) for at least 3 weeks, then injected subcutaneously in NOD/SCID mice (Table 1). Growth in serum-containing medium markedly increased in vivo tumorigenicity, decreased tumor latency, and tumors in general were more undifferentiated (e.g., large cell and giant cell carcinomas), particularly in HBEC3p53,KRAS,cMYC (Table 1 and Fig. 3). This effect is similar to what is observed in patients where poorly differentiated lung tumors are generally associated with aggressive tumor growth. To determine if the rate of apoptosis differed between poor and well-differentiated xenograft tumors, FFPE sections were analyzed with a TUNEL assay. There was no significant difference in the rate of apoptosis in relation to differentiation although a moderate well-differentiated SCC and giant cell carcinoma showed the greatest amount of TUNEL staining (Supplementary Fig. S3E). Overall, the increase in tumorigenicity of oncogenically progressed HBECs after growth in serum shows the influential role of exogenous serum-derived factors in the malignant progression of lung cancer.

Figure 4. c-MYC overexpression or growth in serum-containing media induces EMT in HBEC3p53,KRAS. A, phase contrast photomicrographs showing the morphologic effect observed in HBEC3p53,KRAS (left) following overexpression of c-MYC (middle) or switching from defined serum-free media to serum-containing media (right; × 20 magnification). B, immunoblot for EMT markers in oncogenically manipulated HBEC3 and HBEC17 grown in either KSFM (serum-free) or serum-containing (R10) media. The presence (+) or absence (−) of a band is shown. C, EMT-related genes altered 4-fold or greater in pairwise analysis of HBEC3p53,KRAS and HBEC3p53,KRAS,cMYC comparing cells grown in serum or defined medium (KSFM). Values log2 transformed. *, P < 0.05; ***, P < 0.01. Full-length blots are presented in Supplementary Fig. S8.
HBEC3 (represented vertically) were clustered using centered Pearson tumorigenic clones; green, nontumorigenic clones) and probes expressed (SAM, FDR) comparing HBEC3 “(1)” and “(2)” spanning a 3-week interval. E, a supervised analysis ability to form large soft agar colonies (representative soft agar colonies (arrowhead). These large colonies were isolated, expanded, pictures of 2 clones; figures of 2 clones; images of 2 clones; and genome-wide mRNA expression profiling of HBEC3 soft agar clones identifies a clinically applicable signature of prognosis. A, uncloned, parental populations of HBEC3 soft agar clones (clone 1, 5, and 11) of each manipulation (sh-p53, KRASV12, and c-MYC) nor dual combination of sh-p53 or KRASV12 with c-MYC (Fig. 2C). Seven HBEC3p53,KRASV12,c-MYC clones were isolated from these large colonies and repeat soft agar assays confirmed that the large colony phenotype was maintained (Fig. 5A). Southern blotting showed the clones represent independent transformation events as indicated by discrete digestion patterns (Supplementary Fig. S4A) and immunoblotting confirmed HBEC3p53,KRASV12,c-MYC clones maintained their exogenously introduced oncogenic manipulations (Figs. 1C and 5B, respectively; summarized in Supplementary Table S2). While immortalized but nontransformed HBECs preferentially grow in serum-free conditions, they require serum for anchorage-independent growth (Supplementary Fig. S4B). Remarkably, following isolation from soft agar (in KSFM+20% FBS) 9 of 11 clones were serum growth factor–dependent as they

Malignant transformation of HBECs is enhanced by epithelial-to-mesenchymal transition, induced by either c-MYC or growth in serum

In HBEC3p53,KRASV12 cells, overexpression of c-MYC (in defined KSFM medium) or growth in serum-containing RPMI-1640 medium led to increased oncogenic transformation, as shown by soft agar colony formation and in vivo tumor growth. Both of these manipulations also led to HBEC3p53,KRASV12 cells exhibiting a more mesenchymal-like morphology (Fig. 4A). Loss of E-cadherin (epithelial marker) and gain of vimentin and N-cadherin (mesenchymal markers) confirmed an EMT (Fig. 4B). c-MYC- or serum-induced EMT was also seen in HBEC17 cells, derived from another individual, with p53 and KRASV12 manipulations (HBEC17p53,KRASV12, Fig. 4B). Whole-genome mRNA profiling of HBEC3 and HBEC3p53,KRASV12 cells grown in KSFM or R10 confirmed a significant over-representation of EMT-related genes (P = 1.04 × 10⁻¹⁶ and P = 1.38 × 10⁻⁰⁹ for HBEC3p53,KRASV12 and HBEC3p53,KRASV12,MYC, respectively; Fig. 4C), with upregulation of EMT-promoting genes following growth in serum-containing media.

Isogenic soft agar clones of oncogenically manipulated HBEC3 represent independent genetic events with distinct in vivo growth, tumor histology, and differentiation

The genetic combinations of shp53+KRASV12 or shp53+KRASV12,c-MYC in HBEC3 led to the formation of a small subset (0.5%–1.5% of all soft agar colonies) of very large, macroscopically visible (>1 mm) colonies when grown in soft agar, which was not observed with any single manipulation (shp53, KRASV12, or c-MYC) nor dual combination of sh-p53 or KRASV12 with c-MYC (Fig. 2C). Seven HBEC3p53,KRASV12,c-MYC clones and 4 HBEC3p53,KRASV12,MYC clones were isolated from these large colonies and repeat soft agar assays confirmed that the large colony phenotype was maintained (Fig. 5A). Southern blotting showed the clones represent independent transformation events as indicated by discrete digestion patterns (Supplementary Fig. S4A) and immunoblotting confirmed HBEC3p53,KRASV12,c-MYC and HBEC3p53,KRASV12,MYC clones maintained their exogenously introduced oncogenic manipulations (Figs. 1C and 5B, respectively; summarized in Supplementary Table S2). While immortalized but nontransformed HBECs preferentially grow in serum-free conditions, they require serum for anchorage-independent growth (Supplementary Fig. S4B).Remarkably, following isolation from soft agar (in KSFM+20% FBS) 9 of 11 clones were serum growth factor–dependent as they
Oncogenic transformation of HBECs increases sensitivity to standard lung cancer chemotherapies

To determine if oncogenic transformation of HBECs altered their sensitivity to standard lung cancer chemotherapies, we tested 3 platinum-based doublets (paclitaxel-cisplatin, gemcitabine-cisplatin, and pemetrexed-cisplatin) currently in use for NSCLC treatment. Oncogenic manipulation of HBEC3 with sh-p53 and KRASV12 resulted in a significant increase in sensitivity to gemcitabine-cisplatin and pemetrexed-cisplatin doublet therapy in vitro (approximately 6- and 10-fold, respectively) but not paclitaxel-cisplatin (Supplementary Table S3 and Supplementary Fig. S5; one-way ANOVA; \( P < 0.001 \)). Overall, the soft agar clones of HBEC3p53\(^{fl}\)KRAS\(^{fl}\) showed sensitivities comparable with the parental HBEC3p53\(^{fl}\)KRAS\(^{fl}\) cell line with clone 5 and 7 showing intermediate sensitivity. Thus, these tumorigenically progressed HBECs could provide a cell context appropriate, isogenic model system for identifying genetic differences regulating sensitivity and resistance to platinum doublet chemotherapy used in the treatment of NSCLCs.

mRNA profiles of tumorigenic versus nontumorigenic HBEC3p53\(^{fl}\)KRAS\(^{fl}\) soft agar clones predict prognosis and histology in clinical lung tumor specimens

The identification of tumorigenic and nontumorigenic clones of HBEC3p53\(^{fl}\)KRAS\(^{fl}\) that share the same genetic manipulations (sh-p53 and KRASV12\(^{fl}\)) and biologic selection (large, soft agar colonies) presents a unique cell model to characterize spontaneous transformation events that progress HBECs to full malignancy. Biochemical assays suggest the expression level of KRASV12 is a major contributor toward full transformation (Figs. 1C and D and 5C), whereas other tumorigenic events such as dysregulation of cell cycle did not differ between clones (Supplementary Fig. S6; summarized in Supplementary Table S2). To analyze molecular differences between tumorigenic versus nontumorigenic clones, the clones were profiled with whole-genome mRNA expression microarrays. The mRNA expression profile of each clone remained stable in culture as shown by comparing mRNAs collected at a 3-week interval in unsupervised clustering (Fig. 5D). Tumorigenic and nontumorigenic clones separated into 2 distinct clusters suggesting the clones exhibit a strong expression profile associated with their tumorigenic phenotype, and supervised analysis comparing tumorigenic with nontumorigenic clones found 171 unique genes (203 probes) that were differentially expressed [Significance Analysis of Microarrays (SAM), False Discovery Rate (FDR) = 5%; Supplementary Table S4; Fig. 5E].

We tested the ability of the expression patterns of the 171 genes to predict overall survival and disease-free survival in 2 independent lung tumor cohorts; the SPORE dataset of resected early-stage NSCLCs (adeno- and squamous carcinomas; \( n = 209 \)) and a second dataset of primary lung adenocarcinoma samples (\( n = 442 \); NCI Director’s Challenge Consortium dataset). Prediction models were built using supervised principal component analysis and datasets were interchanged as training and testing datasets to test for robustness. Irrespective of dataset, the soft agar clone tumorigenic versus nontumorigenic signature was able to identify patients with significantly worse overall (Fig. 5F) and recurrence-free (Supplementary Fig. S7) survival. The successful application of a gene signature derived from an isogenic in vitro model of HBEC transformation in predicting outcome in clinical lung tumor samples indicates the power of such a system and provides a preclinical model for testing the functional importance of the genes in the signature.

Discussion

In this study, we sought to characterize the stepwise progression of lung cancer pathogenesis by introducing defined genetic manipulations commonly found in lung cancer into an in vitro HBEC model system (Fig. 6). We found the expression level of mutant KRAS is a critical transformative factor in HBECs, however, inactivation of p53 signaling is required to evade the tumor-suppressive barrier of RAS-induced senescence. We also show that EMT is an important oncogenic step, where c-MYC or growth in serum-containing medium both spontaneously induced an EMT and led to increased tumorigenicity.

In HBECs derived from multiple individuals, we show 5 genetic changes (hTERT and Cdk4 to immortalize the cells, followed by p53 knockdown, mutant KRASV12\(^{fl}\), and c-MYC overexpression) together with serum-induced EMT are able to transform cells to a fully malignant state. Tumor xenografts of transformed HBECs were typical of lung cancer but varied in histology, suggesting histologically distinct lung cancers from the central bronchus may originate from a multipotent stem-like cell. Interestingly, clonal analysis of sh-p53+KRASV12\(^{fl}\) transformed HBECs found the isogenic cells exhibited distinct phenotypes in terms of in vivo tumorigenicity, xenograft histology, and drug response. The mRNA profile that distinguished tumorigenic from nontumorigenic clones was also able to identify a subset of primary lung tumor patients with significantly poorer survival.
tolerate high levels of oncogenic KRAS, a driver of malignant transformation. Transformation studies in HMECs, which undergo spontaneous methylation-mediated p16 silencing, also report that high levels, but not low levels, of oncogenic RAS engage in senescence machinery (36, 37). However, in HMECs, HRAS-induced senescence is mediated through TGF-β signaling in a p53-independent manner. Thus, the mechanism of RAS-induced senescence differs between in vitro epithelial cell models, most likely a consequence of immortalization methods or the cell of origin.

Our data show microenvironmental signals such as those provided by growth media can strongly influence the transformation of HBECS. Switching immortalized, but nontransformed HBECS from defined, serum-free culture medium to serum-containing medium induced inhibition of cell growth with induction of central airway differentiation markers. When immortalized HBECS with additional oncogenic manipulations are switched to serum-containing medium, however, the cells are able to bypass serum-induced differentiation and instead become mesenchymal and more tumorigenic, with a greater frequency of undifferentiated tumors. Overexpression of c-MYC in HBECS also induced an EMT and increased tumorigenicity. c-MYC has been shown to induce EMT in TERT-immortalized HMECs (38). In our study, c-MYC-induced EMT only in the presence of p53 and KRAS alterations and not with c-MYC overexpression alone (data not shown). While c-MYC overexpression or growth in serum-containing media both caused HBECS to undergo EMT and increase tumorigenicity, the presence of both manipulations had a synergistic effect resulting in full malignant transformation in HBECS from 2 individuals. This suggests their tumor-promoting effects signal through mutually exclusive pathways.

In the present study, we found interindividual differences in HBEC transformation suggesting HBECs derived from different donors can vary in their susceptibility to specific oncogene-induced malignant transformation. In terms of in vitro anchorage-independent transformation, neither cell line showed anchorage-independent growth following immortalization (CDK4 and hTERT), yet the combination of 5 genes (Cdk4, bTERT, sh-p53, KRASV12, and cMYC) resulted in a colony-forming efficiency of approximately 60% in HBEC3 compared with less than 15% in HBEC17. These differences could potentially stem from multiple factors including germline polymorphisms [such as single-nucleotide polymorphisms (SNP)], somatically acquired mutations derived from either the patient (such as age or environmental exposures) or laboratory practices (such as time in culture), or epigenetic mechanisms all of which may predispose the cells to oncogenic transformation. The patients from whom HBEC3 and HBEC17 were established, differ in respect to known risk factors for lung cancer such as age and smoking history, and it is likely they also differ in respect to unknown germline and/or somatic alterations. Thus, a comprehensive survey of genomic alterations present in HBECs before genetic manipulation (such as by whole-genome sequencing) would provide better indication of the level of existing premalignant

Figure 6. Model of malignant transformation of in vitro HBECs following stepwise introduction of common lung cancer mutations. The experimental data presented in this article identify the following steps: step 1, CDK4 and hTERT immortalized, HBECs are nontransformed and lack of anchorage-independent growth in soft agar; step 2, in vitro transformation as defined by anchorage-independent growth in soft agar is achieved with the single manipulation of loss of p53, moderate KRASV12 expression or both, whereas expression of high levels of KRASV12 expression leads to in vitro transformation with significant cellular senescence; step 3, partial in vivo transformation with subcutaneous tumor growth in immunocompromised mice in 30% to 80% of injections is observed with the combination of p53 loss and high KRASV12; step 4, an EMT occurs following overexpression of cMYC or growth in serum-containing media; step 5, combination of cMYC overexpression and growth in serum-containing media results in complete oncogenic transformation of HBECs with tumor growth in vivo observed in more than 80% of injections in immunocompromised mice. Clonal selection of partially transformed HBECs identifies tumorigenic and nontumorigenic clones.
susceptibilities, but these experiments are beyond the scope of the current study.

The lung can be divided into central and peripheral compartments (39). SCCs usually arise from the central compartment, whereas adenocarcinomas may arise from either compartment, illustrating the importance of studying oncogenic transformation in both central and peripheral lung epithelial cell models. Many HBEC-derived tumors in our study were p63-positive with squamous differentiation, which likely reflects a stem/basal cell origin. A fewer number of tumors showed distinct dual squamous and adenocarcinoma differentiation or adenocarcinoma differentiation alone. A study using SV40-immortalized tracheobronchial and small airway epithelial cells found both cell types could be fully transformed with oncogenic RAS (8). Another study using nonviral oncoproteins immortalized HSAECs with CDK4, hTERT, and a dominant-negative form of p53 (p53R175H) and transformed the cells using low levels of KRASV12, plus c-MYC, PIK3CAH1047R, cyclin D1, or LKB1H194A (15). In HBECS, we have shown p53 mutation is not required for immortalization (6), and moreover, it increases oncogenic transformation (6, 12). This disparity may signify intrinsic differences between centrally and peripherally derived immortalized epithelial cell models. Taken together, however, our study of defined oncogenic transformation of HBECS [both in the present study and previously (12)] and the study in HSAECs by Sasai and colleagues (15) largely correspond with respect oncogenic transformation of bronchial epithelial cells. We previously showed transformation of HBECS with low levels of KRASV12 (with CDK4, hTERT, and p53 knockdown) resulted in a modest increase in anchorage-independent growth but no tumor formation in vivo (12). Sasai and colleagues reported that transformation of HSAECs with low levels of KRASV12 (with CDK4, hTERT, and p53CT) failed to yield anchorage-independent or in vivo growth. The authors were able to fully transform HSAECs using low levels of KRASV12 only with additional genetic alterations.

In conclusion, using the HBECS system as a progression model of lung cancer, we were able to study early transformative events in bronchial epithelial cells and the mechanisms used to overcome tumor-suppressive barriers. We show HBECS can be transformed to full malignancy by introducing defined genetic manipulations to produce histologically similar lung tumors in xenografts, indicating our in vitro HBEC model retains characteristics of the tissue of origin. Furthermore, we show HBECS can model preneoplastic changes and spontaneous transformation events such as oncogene-induced senescence and EMT and have clinically translatable applications as shown in isogenic clones exhibiting distinct drug response and tumorigenic phenotypes. Thus, genetically defined in vitro models such as HBECS will be an invaluable tool to assess the contribution of specific genes toward lung cancer pathogenesis, pertinent to recent whole-genome sequencing efforts, and to screen for novel therapeutic compounds directed at oncogenically acquired, tumor-specific vulnerabilities.

Disclosure of Potential Conflicts of Interest
J.D. Minna has ownership interest (including patents) in NCI and University of Texas Southwestern Medical Center. No potential conflicts of interest were disclosed by the other authors.

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