Knock-in of Mutant K-ras in Nontumorigenic Human Epithelial Cells as a New Model for Studying K-ras–Mediated Transformation

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Abstract
The oncogenic function of mutant ras in mammalian cells has been extensively investigated using multiple human and animal models. These systems include overexpression of exogenous mutant ras transgenes, conditionally expressed knock-in mouse models, and somatic cell knockout of mutant and wild-type ras genes in human cancer cell lines. However, phenotypic discrepancies between knock-in mice and transgenic mutant ras overexpression prompted us to evaluate the consequences of targeted knock-in of an oncogenic K-ras mutation in the nontumorigenic human breast epithelial cell line MCF-10A and hTERT-immortalized human mammary epithelial cells. Our results show several significant differences between mutant K-ras knock-in cells versus their transgene counterparts, including limited phosphorylation of the downstream molecules extracellular signal-regulated kinase and AKT, minor proliferative capacity in the absence of an exogenous growth factor, and the inability to form colonies in semisolid medium. Analysis of 16 cancer cell lines carrying mutant K-ras genes indicated that 50% of cancer cells harbor nonoverexpressed heterozygous K-ras mutations similar to the expression seen in our knock-in cell lines. Thus, this system serves as a new model for elucidating the oncogenic contribution of mutant K-ras expressed in a large fraction of human cancer cells. [Cancer Res 2007;67(18):8460–7]

Introduction
The K-ras oncogene is frequently mutated in a variety of human neoplasms, including adenocarcinomas of the pancreas (90%), colon (45%), and lung (35%; ref. 1). Missense mutations preferentially occur at codons 12, 13, and 61, which encode constitutively hyperactive forms of Ras (2). Given the importance of Ras to carcinogenesis, its physiologic and pathologic functions in normal and neoplastic cells have been extensively investigated. In addition to a variety of conventional transgenic approaches, several lines of knock-in mice carrying mutant K-ras oncogenes at the endogenous locus have been generated recently (3–5). These animals develop neoplasms in several organs with or without cooperative genetic alterations (3–10). In addition, murine embryo fibroblasts derived from these animals show oncogenic properties that differ from murine fibroblasts constitutively expressing the ras gene. Unlike exogenous ras expression, targeted knock-in of a mutant K-ras gene did not elicit signs of senescence but did confer limited features of transformation, such as enhanced growth rate, reduced dependency on serum, and loss of contact inhibition (4, 5).

In human cells, studies have been mostly limited to exogenous ras overexpression particularly with mutant H-ras (11–19). In addition, somatic cell knockout of K-ras has been accomplished in several human cancer cell lines with varying results (20, 21). Specific RNA interference (RNAi)–mediated knockdown of mutant K-ras has also been shown to affect soft agar colony formation and tumorigenicity in nude mice using a human pancreatic cell line model (22). However, we sought to observe the effects of endogenous mutant K-ras expression in a human nontumorigenic system to gain insight into its contribution to the early stages of neoplastic transformation. Here, we created targeted knock-in of a mutant K-ras gene, K-rasV12, in multiple lines of nontransformed human breast epithelial cells immortalized spontaneously (MCF-10A) or by hTERT introduction [hTERT-immortalized mammary epithelial cells (hTERT-IMEC)]. Our results show that the endogenous expression of a mutant K-ras gene causes significantly distinct phenotypes compared with exogenous overexpression, including minimal activation of downstream signaling pathways, continued reliance on exogenous epidermal growth factor (EGF), and the inability to form colonies in semisolid medium. Thus, these knock-in cell lines provide new research tools for elucidating the contribution of mutant K-ras toward cellular transformation.

Materials and Methods
Cell culture and growth assays. The nontransformed human breast epithelial cell line MCF-10A (23) and its derivatives were grown in DMEM/F12 (1:1) without phenol red supplemented with 2% charcoal dextran–treated fetal bovine serum (FBS; Hyclone), 20 ng/mL EGF, 10 μg/mL insulin, 0.5 μg/mL hydrocortisone, and 0.1 μg/mL cholera toxin (hereafter denoted as “supplemented DMEM/F12”). All supplements were purchased from Sigma-Aldrich unless otherwise noted. hTERT-IMEC and hTERT-IMEC no. 2 were generous gifts and established by Drs. Myles Brown (Harvard Medical School, Boston, MA) and Jerry W. Shay (The University of Texas, Dallas, TX), respectively (24, 25), and these cells and their derivatives were cultured in MEMB supplemented with Bullet kit (10 ng/mL human EGF, 5 μg/mL insulin, 0.5 μg/mL hydrocortisone, gentamicin sulfate, amphotericin B, and bovine pituitary extract; Cambrex). Cancer cell lines used in this study were cultured in DMEM supplemented with 5% FBS except for colon cancer cell lines for which McCoy’s 5A medium was used.

For functional analysis of p53, subconfluent cell cultures were γ-irradiated at 0.7 Gy/min using a Gammacell 40 Exactor (MDS Nordion), and cell lysates were harvested 36 h after irradiation and subjected to Western blotting as described below. To evaluate the phosphorylation status of

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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extracellular signal-regulated kinase (ERK) and AKT, a monolayer of cultured breast epithelial cells was washed with HBSS (Invitrogen) thrice and maintained in medium with or without EGF for 24 h before the harvest of cell lysates.

For cell growth in EGF-free conditions, exponentially growing cells were washed with HBSS thrice and seeded in relevant medium without EGF. One day later, cells were harvested by trypsinization and counted with a Z1 Cell and Particle Counter (Beckman Coulter). PX12 was conducted with an adenovirus encoding Cre recombinase to remove the selection cassette followed by single-cell dilution and screening by PCR for successful Cre recombination. Primer sequences for PCR are shown in Supplementary Table S1.

Immunoblotting and F-actin staining. Whole-cell protein extracts prepared in Laemmli sample buffer were resolved by SDS-PAGE using NuPAGE gels (Invitrogen), transferred to Invitrogen polyvinylidene difluoride membranes (Invitrogen), and probed with primary and horseradish peroxidase–conjugated secondary antibodies. The primary antibodies used in this study are anti-c-K-Ras (Ab-1) mouse antibody (OP24; Calbiochem), anti-p21WAF1 (Ab-1) mouse antibody (EA10; Oncogene), anti-p53 (DO-1) mouse antibody (Santa Cruz Biotechnology), anti-p27KIP1 (Ab-1) mouse antibody (Cell Signaling Technology), and anti-AKT rabbit antibody (Cell Signaling Technology). Peroxidase–conjugated secondary antibodies were used to detect the primary antibodies. Antigen was detected by enhanced chemiluminescence (ECL) on X-ray film.
and anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mouse antibody (6C5; Abcam). Densitometric analysis was done with Quantity One version 4.6.1 (Bio-Rad). For F-actin staining, monolayer cultures of MCF-10A and hTERT-IMEC derivatives were processed with Alexa Fluor 488 phalloidin (Invitrogen) as per manufacturer’s instruction.

**Colony formation assay in semisolid medium and acinar morphogenesis assay.** For colony formation assays, 3 × 10⁴ exponentially growing cells were cast in 3 mL of top layer medium composed of supplemented DMEM/F12 and 0.4% UltraPure agarose (Invitrogen) and poured on top of a 2 mL bottom layer containing 0.6% agarose in six-well tissue culture plates. Supplemented DMEM/F12 was added to the wells once a week. After 2 mL bottom layer containing 0.6% agarose in six-well tissue culture plates. Supplemented DMEM/F12 was added to the wells once a week. After 2 weeks of incubation, the colonies were stained with indo1 nitrotetrazolium chloride (Sigma-Aldrich) and photographed the next day. Two independent experiments were done in triplicate. Morphogenesis assays were carried out as described previously (29), except that EGF was supplemented to the medium at a final concentration of 20 ng/mL for MCF-10A and 10 ng/mL for hTERT-IMEC. Photographs were taken under phase-contrast microscopy after 2 weeks of incubation.

**Xenograft growth assay.** Female athymic nude mice, obtained at 4 to 6 weeks of age (Harlan), were injected s.c. in the flanks with 100 μL suspension of 5 × 10⁴ cells. A 1:1 mixture of HBSS and Matrigel (BD Biosciences) were used to suspend the cells for each cell line. Injection sites were examined for tumors weekly for 10 weeks. The NIH Guide for the Care and Use of Laboratory Animals were followed in all experiments.

**Quantitative real-time reverse transcription-PCR analysis for K-ras.** Real-time PCR was done with MyQ (Bio-Rad) using cDNA as templates. Reactions were done in triplicate and repeated twice. A standard curve was generated for each session using serially diluted samples, and gene expression in each sample was determined in reference to the standard curve. K-ras gene expression in each sample was normalized to GAPDH. Oligonucleotide primers used for real-time PCR are shown in Supplementary Table S1.

### Results

**Targeted knock-in of a mutant K-ras gene.** We conducted targeted knock-in of a heterozygous K-ras mutation by replacing an endogenous wild-type (WT) K-ras allele with an activating mutant allele in MCF-10A and hTERT-IMEC cell lines. We used these human breast epithelial cell lines because they are well characterized, being among the most commonly used cell types in studies of Ras function (11, 12, 14, 15), and we have shown previously that MCF-10A cells are amenable to gene targeting (30).

Moreover, immortalization of breast epithelial cells is readily achievable by hTERT introduction affording us the ability to work with other cell lines in addition to MCF-10A (24, 25). The targeting vector was constructed so that it would replace glycine with valine at codon 12 (V12), which is one of the most common amino acid substitutions of K-Ras found in human cancers (31). Targeted knock-in is achieved via homologous recombination of the transduced targeting vector with the K-ras gene locus. For both MCF-10A and hTERT-IMEC cell lines, we established three independently derived K-ras–targeted clones. For each parental cell line, two out of three targeted clones contained a single K-rasV¹² allele as well as an endogenous WT allele, whereas the third clone retained WT K-ras in both targeted and nontargeted alleles (K-rasWT clone; Fig. 1A). We presume that K-rasWT clones were generated by crossover of the 5’ homology arm of the targeting vector with the endogenous K-ras gene at a position between codon 12 and the proximal end of the 5’ arm, leading to retention of WT sequence. K-rasWT clones serve as the perfect control because the presence or absence of one base substitution at codon 12 is the only difference between K-rasV¹² and K-rasWT clones. By transiently introducing Cre recombinase, all the exogenous sequences originating from the targeting vector were removed except for a 96 bp sequence surrounding a loxP site in intron 2. Correct targeting was shown by PCR amplification of gDNA derived from K-ras knock-in clones after Cre-loxP recombination (Fig. 1B). For comparative controls, WT and mutant K-rasV¹² cDNA retroviral vectors were stably transduced into MCF-10A and hTERT-IMEC cells. Table 1 describes the designation for each knock-in clone and retrovirally (LXSN) infected cells derived from either MCF-10A or hTERT-IMEC.

Reverse transcription-PCR followed by direct sequencing (RT-PCR-DS) showed equivalent amounts of mutant and WT K-ras gene expression in the K-rasV¹² clones, indicating efficient transcription of the targeted allele (Fig. 1C for MCF-10A clones and Supplementary Fig. S1 for hTERT-IMEC clones). As expected, a high level of gene expression was found in cells retrovirally infected with exogenous K-rasV¹² compared with the endogenous WT K-ras gene. Using Western blot analysis, the amount of K-Ras protein in knock-in clones was equivalent to that of control cells, in stark contrast to the large amount of protein seen with virally transduced K-Ras (Fig. 1D).

### Table 1. Designation of K-ras knock-in and constitutively expressing cells created in this study

<table>
<thead>
<tr>
<th>Designation</th>
<th>Description</th>
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<tr>
<td>Parent</td>
<td>Parental cell lines</td>
</tr>
<tr>
<td>K-rasV¹² #1, #2</td>
<td>Clones #1 and #2 with targeted knock-in of K-ras G12V mutation (knock-in type (a)–(c) shown in Fig. 1A)</td>
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<tr>
<td>K-rasWT</td>
<td>Clones undergoing targeted knockin of WT K-ras gene (knock-in type (b)–(c) shown in Fig. 1A)</td>
</tr>
<tr>
<td>LXSN</td>
<td>Bulk cells infected with empty LXSN retroviral vector</td>
</tr>
<tr>
<td>LXSN-V¹²</td>
<td>Bulk cells infected with LXSN retroviral vector expressing a G12V mutant K-ras gene</td>
</tr>
</tbody>
</table>

NOTE: The nomenclature in this table is applied to both MCF-10A and hTERT-IMEC derivatives.

**Assessment of p53 signaling in K-ras knock-in clones.** It has been reported that immortalized cell lines can acquire p53 mutations in vitro resulting in genotypes advantageous for cell growth (24). The abrogation of this pathway could potentially affect the ability to address the oncogenic potential of mutant K-ras. Thus, all knock-in clones were subsequently assessed for their p53 status using two different methods. Genomic sequencing analysis of the entire coding region of p53 in MCF-10A, hTERT-IMEC, and all knock-in clones derived from these cell lines revealed only WT sequence, although some single nucleotide polymorphisms were identified (Supplementary Fig. S2A). The p53 pathway was also functionally examined by assessing increases in p53 and p21 protein by Western blot after cells were exposed to 8 Gy γ-irradiation (Supplementary Fig. S2B). These results confirmed that p53 function remains intact in all cell lines.

**Ras phosphorylation of downstream effectors in K-ras knock-in clones.** Ras is a critical mediator for several signal transduction pathways, including the MAPK and phosphatidylinositol 3-kinase (PI3K) pathways (2). It is well established that ectopic introduction of mutant ras can lead to the constitutive activation...
of these pathways via hyperphosphorylation of key effector molecules. To address the biochemical consequences of targeted K-ras\textsuperscript{V12} knock-in, the MAPK and PI3K pathways were examined by assessing ERK and AKT phosphorylation, respectively. MCF-10A and hTERT-IMEC require EGF for continuous growth in culture, and removal of this cytokine induces a G\textsubscript{1} arrest. This facilitates the ability to assess the effects of mutant K-Ras on the phosphorylation of downstream molecules in two ways. First, activation of the EGF receptor can also result in MAPK and PI3K signaling serving as an internal positive control for each cell line (2). Second, in the absence of EGF, ERK and AKT are minimally phosphorylated. Therefore, even minute amounts of phosphorylation of these molecules by mutant K-Ras can be detected.

Thus, whole-cell lysates were prepared from cells cultured with or without EGF and then examined by Western blot using phospho-specific anti-ERK and anti-AKT antibodies. In the absence of EGF, phosphorylation of ERK was minimal in MCF-10A-K-ras\textsuperscript{V12} clones, similar to levels seen in control cells. In contrast, there was marked ERK phosphorylation seen in MCF-10A-LXSN-V12 cells (Fig. 2A and B). Appreciable increase of AKT phosphorylation was not seen in MCF-10A-K-ras\textsuperscript{V12} clones in the absence of EGF, although there was a slight increase observed in MCF-10A-LXSN-V12 cells compared with parental and control cells. The series of hTERT-IMEC cells exhibited similar results except for a slight up-regulation of ERK phosphorylation in K-ras\textsuperscript{V12} clones compared with parental and control cells in the absence of EGF. LXSN-V12 cells derived from hTERT-IMEC displayed increased ERK phosphorylation in the absence of EGF, although the magnitude of this response was not as great as in the MCF-10A-LXSN-V12 cells. In addition, a small increase in phospho-AKT from basal levels was seen in LXSN-V12 cells similar to the MCF-10A system.

To further confirm the minimal effect of mutant K-ras knock-in, an additional independently derived parental hTERT-IMEC, designated as hTERT-IMEC no.2, was used for K-ras gene targeting. A K-ras\textsuperscript{V12} knock-in clone was established, and correct targeting and proper expression of the targeted allele were verified by the same PCR and RT-PCR-DS assays as described above (Supplementary Fig. S3A and B). Absence of p53 mutations in this clone was also confirmed (data not shown). Western blot analysis of ERK and AKT phosphorylation in this clone showed results identical to the original hTERT-IMEC K-ras\textsuperscript{V12} cells (Supplementary Fig. S3C and D), indicating that our results were reproducible across multiple cell lines. Overall, these data from multiple pairs of isogenic clones show that in the absence of EGF, a single mutant K-ras allele stimulates minimal or nondetectable signaling through the MAPK and PI3K pathways as measured by ERK and AKT phosphorylation.

**Oncogenic properties of K-ras knock-in clones.** To assess the oncogenic properties of targeted K-ras\textsuperscript{V12} clones, several biological variables were evaluated. Morphologically, K-ras\textsuperscript{V12} clones seemed identical to their control counterparts. Using phase-contrast microscopy and F-actin staining of monolayer cultures, K-ras\textsuperscript{V12} clones derived from hTERT-IMEC displayed increased ERK phosphorylation in the absence of EGF, although the magnitude of this response was not as great as in the MCF-10A-LXSN-V12 cells. In addition, a small increase in phospho-AKT from basal levels was seen in LXSN-V12 cells similar to the MCF-10A system.

### Figure 2.

ERK and AKT phosphorylation in K-ras knock-in clones and constitutive K-ras-expressing cells. **A**, phosphorylation of ERK and AKT in the presence or absence of EGF. Cell lysates were obtained from K-ras knock-in and constitutively expressing cells maintained with or without EGF for 24 h. **B**, densitometric analysis of ERK and AKT phosphorylation in the derivatives of MCF-10A and hTERT-IMEC in the absence of EGF. Data from Western blotting in **A** are analyzed. Phosphorylation of ERK and AKT is normalized to total ERK and AKT protein levels, respectively, and shown relative to the data in each parental cell line.
and control cells exhibited a “cobble stone” appearance typical of epithelial cells. However, LXSN-V12 cells showed an obvious and altered morphology appearing as highly refractile cells with multiple protrusions (MCF-10A) or spindle-shaped cells (hTERT-IMEC; Fig. 3A). Proliferation assays were then done in the absence of EGF to determine the effect of K-rasV12 on growth factor independence, often a hallmark of a transformed phenotype. For MCF-10A derivatives, LXSN-V12 cells showed dramatic proliferation compared with K-rasV12 clones, which exhibited only slight growth within 19 days in culture (Fig. 3B). Control cell lines remained arrested as described previously (30). For hTERT-IMEC cells, no appreciable proliferation was observed in any derivatives including K-rasV12 clones up to 19 days after cell plating with the notable exception of LXSN-V12 cells, which rapidly proliferated in the absence of EGF similar to the MCF-10A-LXSN-V12 cells.

Another feature of transformed cells is their ability to form colonies in semisolid medium. Previous studies have reported colony formation in soft agar for both MCF-10A and hTERT-immortalized breast epithelial cells on exogenous overexpression of H-ras (11, 12, 19). We therefore did a similar analysis using our knock-in clones. In semisolid medium, K-rasV12 clones as well as control cells did not form colonies, whereas LXSN-V12 cells caused sporadic colonies in both MCF-10A and hTERT-IMEC systems (Fig. 3C). Human breast epithelial cells have also been shown to form acinar structures when grown in three-dimensional cultures with Matrigel, and oncogenic changes can lead to aberrant overgrowth of these structures (32). MCF-10A and hTERT-IMEC cells and their derivatives were subsequently cultured under these conditions to assess any changes in acinar morphology (Fig. 3D). For both MCF-10A and hTERT-IMEC cells, LXSN-V12 showed striking structural changes, including lack of acinar formation and aggressive cellular proliferation with cluster formation. In contrast, K-rasV12 clones did not exhibit any morphologic alterations compared with their control counterparts.

To assess the in vivo tumorigenicity of K-rasV12 clones, xenograft assays in athymic nude mice were done. After 10 weeks postinoculation, no tumors developed in any derivatives of either MCF-10A or hTERT-IMEC, including LXSN-V12 cells (data not shown). HCT 116 colon cancer cells inoculated in parallel served as controls and rapidly formed tumors within 2 weeks. Collectively, these experiments revealed marked phenotypic differences between endogenously expressed hemizygous K-rasV12 alleles versus constitutive overexpression of this mutant gene.

Expression levels of mutant K-Ras protein in cancer cell lines. K-rasV12 knock-in clones clearly show distinct phenotypes compared with overexpression of exogenous mutant K-ras. Consequently, the physiologic relevance of K-rasV12 knock-in clones as related to expression levels in human cancer cells was investigated. Using a combination of quantitative real-time RT-PCR and Western blotting, we examined mRNA and protein levels of K-ras in 19 human cancer cell lines (16 with K-ras mutations and 3 without K-ras mutations) and compared them with parental MCF-10A and hTERT-IMEC cells, along with their retroviral LXSN-V12 derivatives. Quantitative real-time RT-PCR showed a markedly higher level of K-ras mRNA from LXSN-V12 cells compared with the endogenous expression found in noncancerous or cancerous cell lines (Supplementary Fig. S4A). At the protein level, the majority of cancer cells expressed similar amounts of K-ras protein compared with the parental MCF-10A cell line, although the amount in hTERT-IMEC was significantly lower (Fig. 4A and B). However, four cancer cell lines (SW480, SU.86.86, SW620, and HPAF-II) exhibited protein levels comparable with the levels found only of LXSN-V12 cells, which rapidly proliferated in the absence of EGF similar to the MCF-10A-LXSN-V12 cells.

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in LXSN-V12 cells, despite having amounts of mRNA that were approximately equal to cell lines not overexpressing K-ras. One potential explanation for the observed discrepancy between mRNA and protein levels in these cell lines may be perturbed posttranscriptional/translational regulation.

We then queried for allelic imbalance of mutant versus WT alleles in the 16 cancer cell lines that harbor mutant K-ras. By sequencing the mutated regions of K-ras reported previously in these cell lines (31) using gDNA as template and RT-PCR-DS analysis, the allelic ratios of mutant to WT K-ras were assessed for both DNA and mRNA. K-ras mutational sequencing using gDNA as template showed mutant to WT allelic ratios that were identical to RT-PCR-DS, suggesting that gene expression was directly correlated with relative gene dosage (data not shown). From this analysis, it was found that 5 of 16 cancer cell lines express WT and mutant K-ras alleles equally, as shown by the equivalent peak intensities of mutant versus WT nucleotides with RT-PCR-DS (Table 2; Supplementary Fig. S4). This pattern of gene expression is identical to the K-rasV12 knock-in clones (Fig. 1C; Supplementary Fig. S1). It should be noted that these five cell lines did not show overexpression of K-Ras protein by Western blot similar to K-rasV12 knock-in clones (Figs. 1D and 4A and B). Moreover, three additional cell lines that also do not overexpress K-Ras protein, (CFPAC-1, Panc-1, and PL12/PL45) again displayed heterozygous expression of mutant and WT K-ras, although in these cell lines, there was slightly more expression of the mutant allele. In contrast, only 2 of 16 cell lines examined showed a gene/allelic expression pattern similar to that of virally transduced LXSN-V12 cells. These cell lines (SW480 and SW620) exhibited marked overexpression of mutant K-Ras protein based on the absence of WT sequence by RT-PCR-DS. These data collectively indicate that K-ras gene expression in roughly half of cancer cells that harbor a mutant K-ras gene is more closely recapitulated by targeted knock-in of an oncogenic K-ras, rather than overexpression of a transgene.

**Discussion**

The importance of K-ras to human carcinogenesis is exemplified by its mutational frequency in several human cancers (1). However, understanding the contribution of mutant K-ras toward human carcinogenesis remains incomplete. Although numerous studies have shown that forced overexpression of mutant ras can lead to properties characteristic of transformation in human cells, our data and reports by others suggest that this pattern of gene expression may only apply to a subset of human malignancies (33). In the current study, we used gene targeting to knock in an oncogenic mutation within the K-ras gene in nontransformed human breast epithelial cells. Our system provides a new model to study the cellular function of mutant K-ras expressed from its native promoter that recapitulates the heterozygous gene expression patterns present in a large fraction of human cancer cells.

K-ras mutations have been found in ~5% of human breast cancers (Sanger COSMIC database version 28), and a recent study reported a mutational frequency of 12.5% in human breast cancer cell lines (34). This low rate of K-ras mutation suggests that mutation of this gene may not be a significant factor in the initiation of most breast cancers. Given the low frequency of mutation, one could hypothesize that K-ras mutations found in primary breast cancers are “passenger” mutations and not involved with breast carcinogenesis at all. However, the fact that all breast cancer K-ras mutations to date have been found exclusively at the codon 12 and 13 hotspots (31, 34) suggests that these mutations are functionally significant in a small subset of breast cancers.

It is possible, on the other hand, that the significance of K-ras mutations toward human carcinogenesis varies depending on cell and tissue type. Given the higher K-ras mutational frequencies

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3 [http://www.sanger.ac.uk/perl/genetics/CGP/cosmic?action=bycancer&ln=KRAS&sm=breast]
found in other neoplasms, such as pancreas, colon, and lung cancers, mutation of K-ras may have a larger contribution toward the carcinogenic process in these organs compared with breast epithelial cells; therefore, our results may not be globally applicable to other tissue types. In principle, this question could be addressed using nontumorigenic hTERT-immortalized epithelial cell lines derived from these organs. Although several groups have established such cell lines (35–37), to our knowledge, none are commercially available, which in some cases can restrict access to these reagents. In addition, we have historically found that some human cell lines, including MCF-7, MDA-MB-231, and HCC1500, are either refractory to or have an extremely low rate of homologous recombination depending on the gene being targeted, although we have not yet tried newer generation vectors in these cell lines. Thus, continued improvements in somatic cell gene targeting may enable us to evaluate the oncogenic properties of mutant K-ras in other nontumorigenic epithelial cell types that acquire K-ras mutations at high frequency during cellular transformation.

Given that the expression of an endogenous mutant K-ras allele yields a minimal phenotype in breast epithelial cells, it is tempting to speculate that previous studies using overexpression of mutant K-ras yielded results that may have underestimated the true number of genetic "hits" needed to achieve a fully transformed state. In support of this hypothesis, others have shown that a critical amount of oncogenic ras gene expression that far exceeds levels found in human cells is required for transformation in vitro (14, 13), (33). Because transformation relies on the accumulation of multiple genetic or epigenetic events in oncogenes and tumor suppressor genes (38), it is reasonable to postulate that additional genetic alterations, including amplification/overexpression of mutant K-ras itself, may cooperate with heterozygous K-ras mutations to promote the carcinogenic process (39). Our system offers an opportunity to elucidate such genetic cooperation by conducting targeted knock-in of additional mutations within oncogenes and tumor suppressor genes in K-rasV12 versus K-rasWT cell lines.

The analyses of heterozygous K-ras mutations using conditional knock-in mice have already shown biological and biochemical phenotypes that differ from conventional transgenic approaches (4, 5). However, previous studies have also shown discrepancies between human and murine cells on mutant ras gene introduction. For example, inactivation of a tumor suppressor such as p53, p16, or p19ARF in primary murine cells with subsequent ectopic expression of ras confers a fully transformed phenotype (40–44); however, transformation of human cells requires additional genetic events (13–18). Moreover, quantitative differences in Ras downstream signaling pathways have been described between human versus murine cells (45, 46). Thus, the creation of human cell lines with targeted K-ras alleles serves as a more accurate counterpart for comparison with human cells ectopically expressing mutant K-ras.

As mentioned previously, multiple K-ras knockout studies have been done in which either the WT or mutant K-ras allele was disrupted in three different human cancer cell lines (20, 21, 47). Specific inactivation of mutant K-ras by RNAi in the human pancreatic cancer cell line Capan-1 has also been done (22). Although there are discrepancies between these studies, they collectively showed that disruption of mutant K-ras can affect the tumorigenicity of human cancer cells. However, these somatic knockout and knockdown cancer cell lines cannot address the biological consequences of K-ras mutations at the early stages of carcinogenesis when mutations of ras genes are thought to occur (48). In addition, the cancer cell lines used in the K-ras knockout studies display an unusually high rate of mutation known as microsatellite instability (MIN), a genetically unstable phenotype caused by mutations or silencing of nucleotide mismatch repair genes (49). Therefore, it is possible that these cancer cell lines may harbor or acquire additional genetic alterations in culture that could affect analysis of Ras signaling (38). Indeed, we have shown recently the discovery of genetic heterogeneity between single-cell clones after targeted knock-in of the hMLH1 gene in the MIN

<table>
<thead>
<tr>
<th>Table 2. Details of the 16 human cancer cell lines harboring K-ras mutations categorized by K-ras expression and allelic status</th>
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<tbody>
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<td>K-ras allelic status</td>
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<tr>
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<td>MDA-MB-231</td>
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<td>Heterozygous (imbalanced)</td>
</tr>
<tr>
<td>Panc-1</td>
</tr>
<tr>
<td>PL12/PL45</td>
</tr>
<tr>
<td>Homozygous</td>
</tr>
<tr>
<td>AsPC-1</td>
</tr>
<tr>
<td>A549</td>
</tr>
<tr>
<td>K-ras overexpressed</td>
</tr>
<tr>
<td>SU86.86</td>
</tr>
<tr>
<td>SW480</td>
</tr>
<tr>
<td>SW620</td>
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cancer cell line HCT 116 (50). This property of MIN may also explain the differing degrees of tumorigenicity seen in studies using the same MIN DLD-1 colon cancer K-ras knockout clones (20, 47). Thus, the experimental data derived from these genetically targeted MIN cancer cell lines need to be interpreted with caution.

To circumvent these issues, the current study has used noncancerous human epithelial cells immortalized spontaneously (MCF-10A) or by hTERT transduction. These cells are genetically stable with defined genetic/epigenetic alterations in a limited number of genes (23, 24) and amenable to gene targeting as shown in this study and our previous work (30). We have therefore created a new model for the future study of K-ras–mediated carcinogenesis, which will ultimately lead to novel insights into the pathogenesis of human cancers and serve as the underpinnings for the development of new targeted therapies.

References


Knock-in of Mutant K-ras in Nontumorigenic Human Epithelial Cells as a New Model for Studying K-ras-Mediated Transformation

Hiroyuki Konishi, Bedri Karakas, Abde M. Abukhdeir, et al.