

# 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* as 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-ras*<sup>V12</sup>, 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

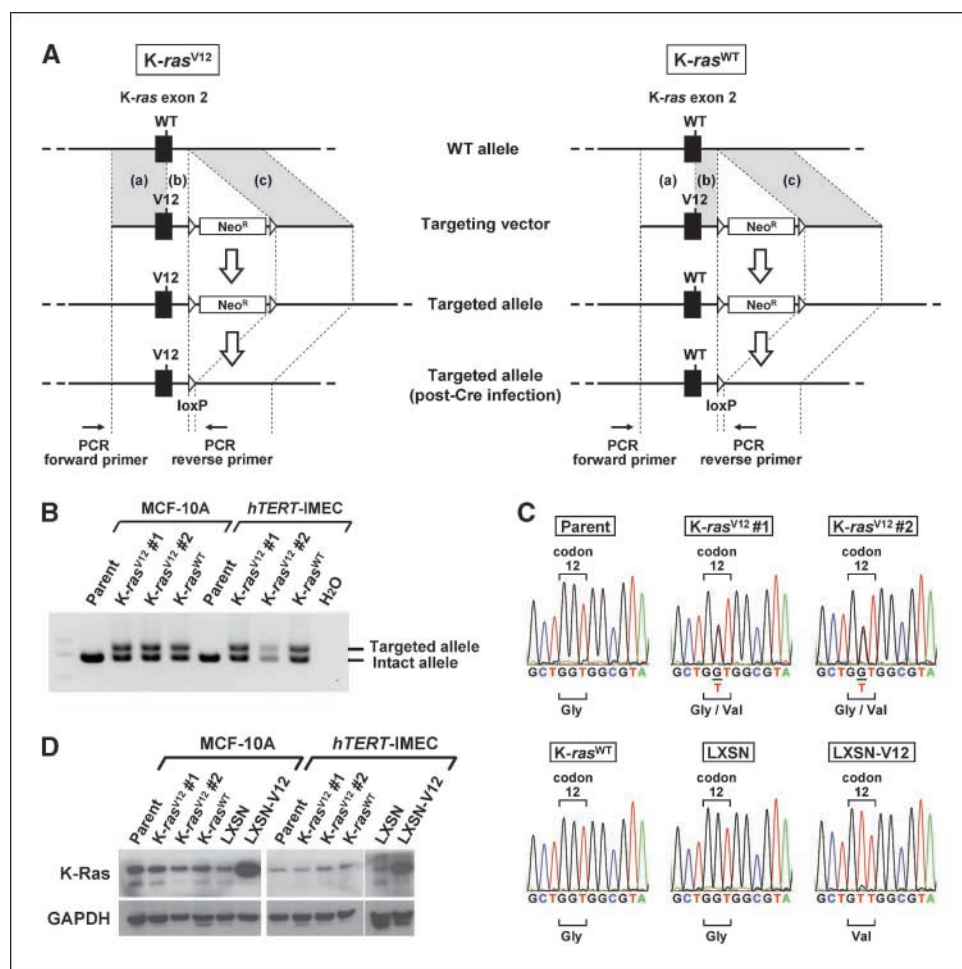
**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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**Figure 1.** Targeted knock-in of oncogenic K-ras<sup>V12</sup>. **A**, scheme for targeted knock-in of the K-ras<sup>V12</sup> oncogene.

The endogenous K-ras gene locus surrounding exon 2, targeting vector containing V12 mutation, two types of targeted alleles, and targeted alleles post-Cre infection are described. If crossover arises within regions (a) and (c) during homologous recombination, resulting knock-in clones carry K-ras<sup>V12</sup> alleles (left column). However, crossover that occurs within regions (b) results in the generation of K-ras<sup>WT</sup> alleles (right column). Filled boxes, open boxes, and open triangles, K-ras exon 2, neomycin resistance gene cassettes, and loxP sites, respectively. Primers indicated at the bottom were used for the PCR amplification in **B**. **B**, DNA gel electrophoresis showing PCR products using gDNA from K-ras knock-in clones (post-Cre infection) and their parental counterparts. Primers used for PCR are denoted in **A**, and their sequences are provided in Supplementary Table S1. **C**, chromatographs indicating the K-ras mRNA sequences at codons 11 to 14 in MCF-10A derivatives. Nucleotide sequences and amino acids at codon 12 are indicated below the chromatographs. **D**, immunoblotting for K-Ras protein in K-ras knock-in and constitutively expressing cells.



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 at a density of  $1 \times 10^5$  cells/T25 cm<sup>2</sup> tissue culture flask at day 0. Cell numbers were counted with a Z1 Cell and Particle Counter (Beckman Coulter) and medium was changed from day 1 until day 19 every 3 days. Due to the high proliferative index of retrovirally infected LXSN-V12 cells, these cells were counted every day up to day 7 or 8. At least two independent experiments were carried out for both MCF-10A and hTERT-IMEC derivatives.

**Targeted knock-in of the K-ras<sup>V12</sup> oncogene.** Targeted knock-in of oncogenic K-ras<sup>V12</sup> was conducted with an adeno-associated viral vector as described (26, 27). The targeting construct containing a neomycin resistance gene regulated by a SV40 early promoter was constructed by PCR, using genomic DNA (gDNA) from the colon cancer cell line SW480 as template for the homology arms. The targeting vector was transduced into cells and antibiotic selection was done with 120  $\mu$ g/mL (MCF-10A derivatives) or 100  $\mu$ g/mL (hTERT-IMEC derivatives) of G418 (Life Technologies) in multiple 96-well plates. Neomycin-resistant colonies were expanded, replicated and pooled and PCR-based screening was done to identify cells that had undergone homologous integration of the targeting vectors followed by further PCR screening of individual colonies from positive pools. Targeted cells were infected 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.

**Retrovirus.** pLXSN carrying oncogenic K-ras<sup>V12</sup> was a generous gift from Dr. Hiroyasu Esumi (National Cancer Center Research Institute East, Chiba, Japan; ref. 28). Retroviral vector was produced using AmphiPack-293 cells (BD Clontech) according to the manufacturer's instructions and used for cell infection with 8  $\mu$ g/mL of polybrene (Sigma-Aldrich). Antibiotic selection was started the next day and continued at least for 1 week.

**gDNA and RNA extraction, cDNA synthesis, PCR, and direct sequencing.** gDNA and total RNA were prepared from cells using QIAamp DNA Blood kit and RNeasy kit (Qiagen), respectively. cDNA was synthesized with First-Strand cDNA Synthesis kit (Amersham Biosciences). PCR amplification was done using GeneAmp 9700 (Applied Biosystems) and Platinum Taq (Invitrogen). PCR primers to amplify cDNA were designed so that forward and reverse primers were located at distinct exons. Automated direct sequencing of PCR products for the K-ras and p53 genes was carried out by the Johns Hopkins DNA Analysis Facility. Primer sequences for PCR and direct sequencing are available on request.

**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 Invitrolon 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-p44/p42 mitogen-activated protein kinase (MAPK) rabbit antibody (Cell Signaling Technology), anti-phospho-p44/p42 MAPK (Thr<sup>202</sup>/Tyr<sup>204</sup>) mouse antibody (Cell Signaling Technology), anti-AKT rabbit antibody (Cell Signaling Technology), anti-phospho-AKT (Ser<sup>473</sup>) rabbit antibody (Cell Signaling Technology), anti-p53 (DO-1) mouse antibody (Santa Cruz Biotechnology), anti-p21<sup>WAF1</sup> (Ab-1) mouse antibody (EA10; Oncogene),

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,  $3 \times 10^4$  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 3 weeks of incubation, the colonies were stained with iodinitrotetrazolium 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  $\mu$ L suspension of  $5 \times 10^6$  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 MyiQ (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-ras*<sup>V12</sup> allele as well as an endogenous WT allele, whereas the third clone retained WT *K-ras* in both targeted and nontargeted alleles (*K-ras*<sup>WT</sup> clone; Fig. 1A). We presume that *K-ras*<sup>WT</sup> 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-ras*<sup>WT</sup> clones serve as the perfect control because the presence or absence of one base substitution at codon 12 is the only difference between *K-ras*<sup>V12</sup> and *K-ras*<sup>WT</sup> 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-ras*<sup>V12</sup> 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-ras*<sup>V12</sup> 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-ras*<sup>V12</sup> 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).

**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  $\gamma$ -irradiation (Supplementary Fig. S2B). These results confirmed that *p53* function remains intact in all cell lines.

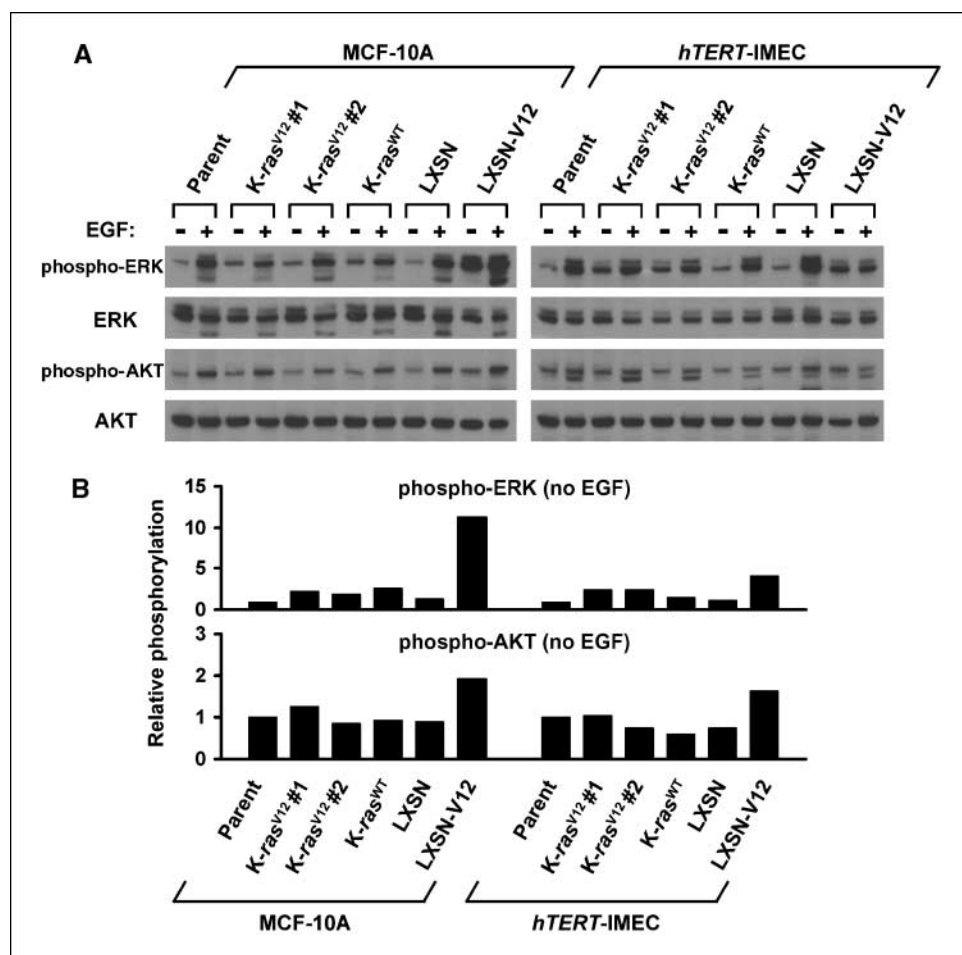
**Ras phosphorylation of downstream effectors in K-ras knock-in clones.** Ras is a critical mediator for several signal transduction pathways, including the MAPK and phosphatidylinositol 3-kinase (PI3K) pathways (2). It is well established that ectopic introduction of mutant *ras* can lead to the constitutive activation

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

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

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

**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.



of these pathways via hyperphosphorylation of key effector molecules. To address the biochemical consequences of targeted K-ras<sup>V12</sup> 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<sub>1</sub> 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<sup>V12</sup> 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<sup>V12</sup> 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<sup>V12</sup> 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<sup>V12</sup> 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<sup>V12</sup> 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<sup>V12</sup> clones, several biological variables were evaluated. Morphologically, K-ras<sup>V12</sup> clones seemed identical to their control counterparts. Using phase-contrast microscopy and F-actin staining of monolayer cultures, K-ras<sup>V12</sup>

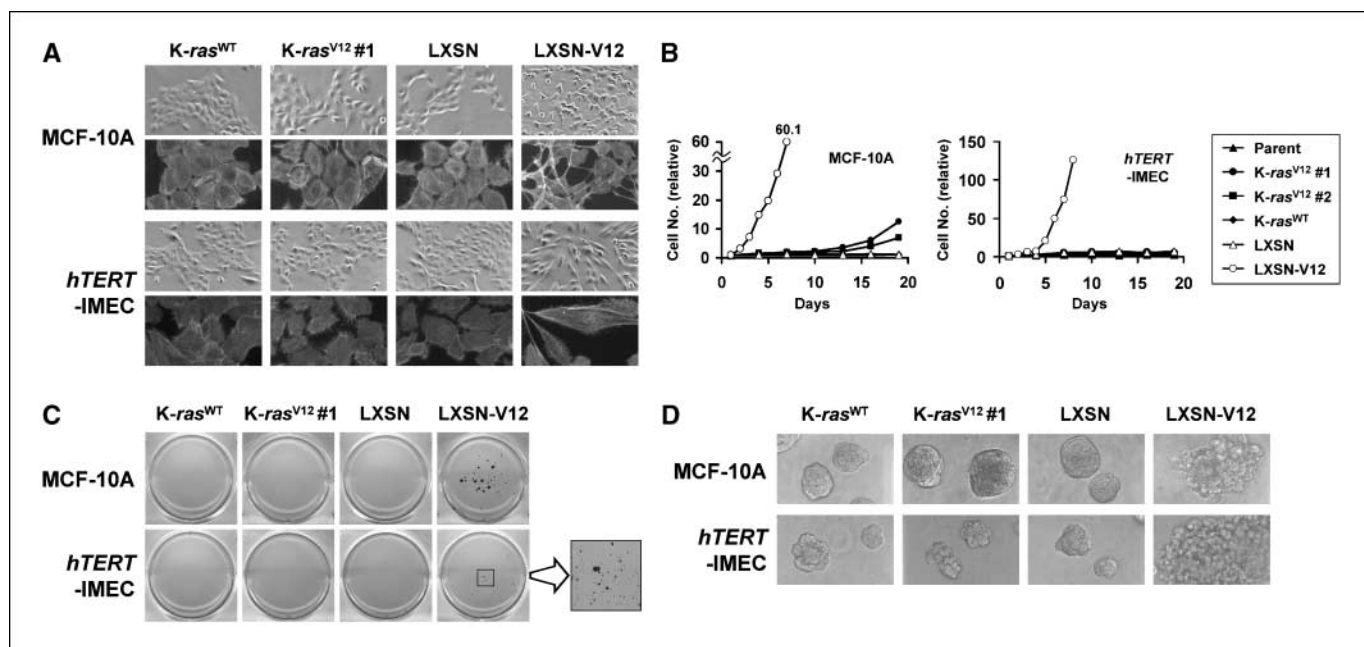
and control cells exhibited a "cobble stone" appearance typical of epithelial cells. However, LXSN-V12 cells showed an obvious and altered morphology appearing as highly refractile cells with multiple protrusions (MCF-10A) or spindle-shaped cells (*hTERT*-IMEC; Fig. 3A). Proliferation assays were then done in the absence of EGF to determine the effect of *K-ras*<sup>V12</sup> on growth factor independence, often a hallmark of a transformed phenotype. For MCF-10A derivatives, LXSN-V12 cells showed dramatic proliferation compared with *K-ras*<sup>V12</sup> clones, which exhibited only slight growth after 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-ras*<sup>V12</sup> 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-ras*<sup>V12</sup> 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-ras*<sup>V12</sup> clones did not exhibit any morphologic alterations compared with their control counterparts.

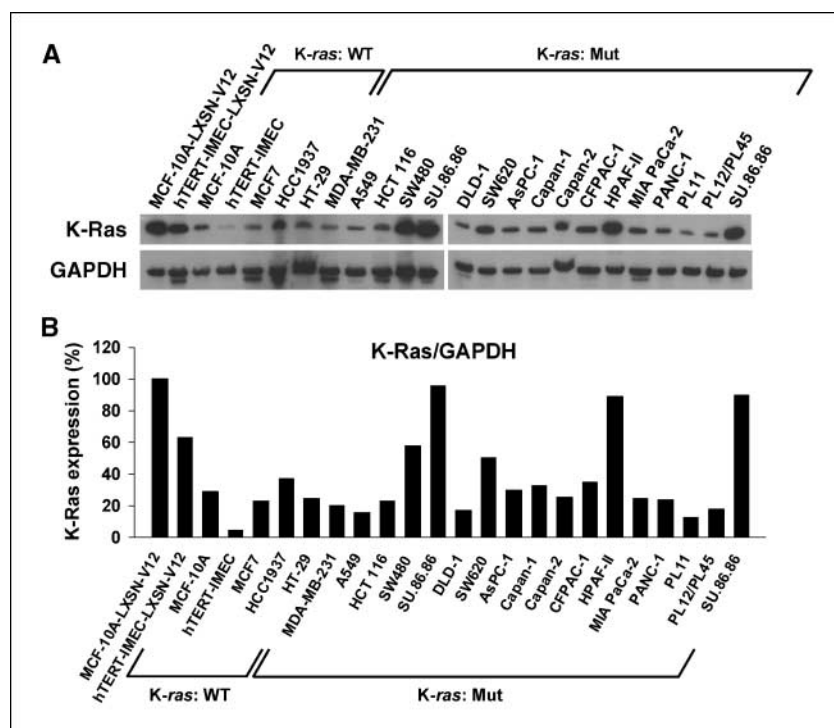
To assess the *in vivo* tumorigenicity of *K-ras*<sup>V12</sup> clones, xenograft assays in athymic nude mice were done. After 10 weeks postinoculation, no tumors developed in any derivatives of either MCF-10A or *hTERT*-IMEC, including LXSN-V12 cells (data not shown). HCT 116 colon cancer cells inoculated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

**Expression levels of mutant K-Ras protein in cancer cell lines.** *K-ras*<sup>V12</sup> knock-in clones clearly show distinct phenotypes compared with overexpression of exogenous mutant *K-ras*. Consequently, the physiologic relevance of *K-ras*<sup>V12</sup> 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



**Figure 3.** Distinct biological phenotypes between *K-ras* knock-in clones and *K-ras* constitutively expressing cells. **A**, representative photomicrographs of *K-ras* knock-in and constitutively expressing cells obtained by phase-contrast microscopy (top,  $\times 200$  magnification) and F-actin staining (bottom,  $\times 600$  magnification). **B**, growth rate of *K-ras* knock-in and constitutively expressing cells in EGF-free medium. Cell numbers at each time point are expressed relative to those in day 1. Note that the relative cell numbers of parent, *K-ras*<sup>WT</sup>, and LXSN (left) and parent, *K-ras*<sup>V12</sup> #1, *K-ras*<sup>V12</sup> #2, *K-ras*<sup>WT</sup>, and LXSN (right) in each time point are near 1.0 and overlapping. **C**, representative colony formation of MCF-10A and *hTERT*-IMEC cells and their *K-ras* derivatives in semisolid medium. Photographs of *K-ras*<sup>WT</sup>, *K-ras*<sup>V12</sup> #1, control LXSN, and constitutively overexpressing LXSN-V12 cells (left). Colonies of *hTERT*-IMEC-LXSN-V12 are better visualized under higher magnification (right). **D**, three-dimensional culture of MCF-10A and *hTERT*-IMEC cells and their *K-ras* derivatives grown in Matrigel. Photographs of *K-ras*<sup>WT</sup>, *K-ras*<sup>V12</sup> #1, control LXSN, and constitutively overexpressing LXSN-V12 cells. Magnification,  $\times 400$ .

**Figure 4.** K-Ras expression in human cancer cells. **A**, Western blotting of K-Ras in 19 cancer cell lines and breast epithelial cells. A protein sample for SU.86.86 was applied to both gels to allow for comparison between the two blots. **B**, densitometric analysis of Western blotting. Data are normalized to GAPDH protein and shown relative to the protein level in MCF-10A cells retrovirally expressing K-ras.



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. S4B). This pattern of gene expression is identical to the *K-ras*<sup>V12</sup> 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-ras*<sup>V12</sup> 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),<sup>3</sup> 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

<sup>3</sup> <http://www.sanger.ac.uk/perl/genetics/CGP/cosmic?action=bycancer&ln=KRAS&sn=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, 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-ras*<sup>V12</sup> versus *K-ras*<sup>WT</sup> 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 p19<sup>ARF</sup> 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 2.** Details of the 16 human cancer cell lines harboring *K-ras* mutations categorized by *K-ras* expression and allelic status

<i>K-ras</i> allelic status	Cell line	Cancer type	Predominant allele	Codon	Mutation	
					Nucleotide	Amino acid
<i>K-ras</i> nonoverexpressed						
Heterozygous (equivalent)	Capan-2	Pancreas	—	12	GTT	Valine
	PL11	Pancreas	—	12	GTT	Valine
	HCT 116	Colon	—	13	GAC	Aspartic acid
	DLD-1	Colon	—	13	GAC	Aspartic acid
	MDA-MB-231	Breast	—	13	GAC	Aspartic acid
Heterozygous (imbalanced)	CFPAC-1	Pancreas	Mutant	12	GTT	Valine
	Panc-1	Pancreas	Mutant	12	GAT	Aspartic acid
	PL12/PL45	Pancreas	Mutant	12	GAT	Aspartic acid
Homozygous	Capan-1	Pancreas	Mutant	12	GTT	Valine
	AsPC-1	Pancreas	Mutant	12	GAT	Aspartic acid
	MIA PaCa-2	Pancreas	Mutant	12	TGT	Cysteine
	A549	Lung	Mutant	12	AGT	Serine
<i>K-ras</i> overexpressed						
Heterozygous (imbalanced)	HPAF-II	Pancreas	Mutant	12	GAT	Aspartic acid
	SU.86.86	Pancreas	Mutant	12	GAT	Aspartic acid
Homozygous	SW480	Colon	Mutant	12	GTT	Valine
	SW620	Colon	Mutant	12	GTT	Valine

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.

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