Knock-in of Oncogenic \textit{Kras} Does Not Transform Mouse Somatic Cells But Triggers a Transcriptional Response that Classifies Human Cancers

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Abstract

\textit{Kras} mutations are present at a high frequency in human cancers. The development of therapies targeting mutated \textit{Kras} requires cellular and animal preclinical models. We exploited adeno-associated virus–mediated homologous recombination to insert the \textit{Kras} G12D allele in the genome of mouse somatic cells. Heterozygous mutant cells displayed a constitutively active Kras protein, marked morphologic changes, increased proliferation and motility but were not transformed. On the contrary, mouse cells in which we overexpressed the corresponding \textit{Kras} cDNA were readily transformed. The levels of Kras activation in knock-in cells were comparable with those present in human cancer cells carrying the corresponding mutation. \textit{Kras}-mutated cells were compared with their wild-type counterparts by gene expression profiling, leading to the definition of a “mutated \textit{Kras}-KI signature” of 345 genes. This signature was capable of classifying mouse and human cancers according to their \textit{Kras} mutational status, with an accuracy similar to or better than published \textit{Ras} signatures. The isogenic cells that we have developed recapitulate the oncogenic activation of \textit{Kras} occurring in cancer and represent new models for studying \textit{Kras}-mediated transformation. Our results have implications for the identification of human tumors in which the oncogenic \textit{Kras} transcriptional response is activated and suggest new strategies to build mouse models of tumor progression. [Cancer Res 2007;67(18):8468–76]

Introduction

The protein encoded by the \textit{Kras} gene is a prototypic small GTPase that acts as a molecular switch, transducing signals initiated at the cell surface from receptor and non–receptor tyrosine kinases to the nucleus.

\textit{Kras} works by cycling between active GTP-bound and inactive GDP-bound conformations (Ras-GTP and Ras-GDP). The competing activities of guanosine nucleotide exchange factors and GTase-activating proteins regulate RAS-GTP levels (1).

According to its GTP status, KRAS activates multiple downstream effectors including the mitogenic Raf/MEK/ERK pathway, and the survival-invasive phosphatidylinositol-3-kinase pathway (reviewed in ref. 2).

Mutations of the \textit{KRAS} gene are among the most common genetic alterations in human cancer occurring in ~50% of colorectal cancers, 75% to 100% of pancreatic cancers, 50% of cholangiocarcinomas, and almost 50% of lung adenocarcinomas (2, 3). Interestingly, activation of the Ras/Raf/Mek/Erk pathway seems also present in cancer lacking \textit{KRAS} mutations (4). This suggests that the mutational profiling of the \textit{Kras} gene might not identify the entire spectrum of tumors in which the Ras pathway is active.

Oncogenic activation of KRAS is often associated with poor prognosis and chemoresistance of the respective malignancies (5, 6). Multiple studies indicate that oncogenic KRAS is required for the proliferation, adhesion, motility, invasion of cancer cells (reviewed in ref. 2). RAS mutations are also associated with a number of human syndromes, such as Costello, Noonan, and cranio-facial-cutaneous disorders (reviewed in ref. 7). In some cases (especially in the Noonan and Costello syndromes), the affected individuals have a higher risk of cancer, suggesting that germ line \textit{Ras} mutations may increase the susceptibility to tumors (7).

Given their importance in human tumorigenesis, a number of cellular and animal models have been developed to study \textit{Kras} oncogenic alleles (8, 9). These included plasmid- and viral-mediated ectopic expression of mutated \textit{Kras} cDNAs in a variety of human and mouse cells of stromal and epithelial origin. Although the latter have been undoubtedly effective in dissecting the oncogenic properties of mutated \textit{Kras} alleles, they often resulted in the overexpression of the corresponding mutated proteins producing misleading results. For example, it has been clearly shown that “dominant transformation by mutated human \textit{Ras} genes requires more than 100 times higher expression than is observed in cancers” (10).

Animal models carrying mutated \textit{Kras} allelic have also been developed. These included elegant genetic experiments in which “latent” \textit{Kras} mutations were inserted in the genome of mouse embryonic stem cells and “activated” in somatic tissues (11, 12). Mouse models of \textit{Kras} mutations have been instrumental in understanding the molecular mechanisms of \textit{Kras}-mediated tumorigenesis. In addition, they have been used to derive mutated \textit{Kras}-specific transcriptional signatures (13). Because \textit{Kras} oncogenic mutations are incompatible with embryonic development, the oncogenic alleles had to be engineered to be activated or expressed only in adult mouse tissues (11, 12).

To overcome the limitations of the abovementioned models, we have exploited the use of somatic homologous recombination to build mouse tumor progression models closely recapitulating the
genetic alterations found in human tumors. Our approach is based on the distinct properties of adeno-associated viruses (AAV) promoting homologous recombination in mammalian somatic cells. This strategy allows the introduction of point mutations or deletions in a given genomic locus, thus allowing its expression under physiologic conditions similar to the situation found in human disease.

As a test case, we used AAV-mediated homologous recombination to modify the genome of mouse somatic cells by introducing Kras G12D point mutation. We present evidence that this strategy successfully generates mutant Kras mouse cell models that can be exploited to evaluate the biological and the oncogenic properties of the corresponding mutations. Furthermore, we show that the isogenic wild-type and mutant cells can be used to identify a mutated Kras signature that classifies human tumors on the basis of their KRAS genetic status.

Materials and Methods

Cell culture and reagents. MLP29 have been previously described (14) and cultured following standard procedures. Geneticin (G418) was purchased from Life Technologies. Antibodies used for immunoblotting included anti-AKT, anti-phospho-AKT S473, anti–mitogen-activated protein kinase (MAPK), and anti–phospho-MAPK (Cell Signaling Technology); the anti-actin antibody was purchased from Sigma.

Construction of the targeting vector and identification of recombinant clones. Details on the experimental strategies used to generate the Kras knock-in cells are available in the Supplementary Methods.

RNA extraction and reverse transcription-PCR analysis. To verify the expression of the mutated alleles in the targeted MLP29 clones, total RNA was extracted using RNeasy Mini/Midi Kit (Qiagen). Retrotranscription was carried out with Moloney murine leukemia virus reverse-transcriptase RNase H minus (Promega). Expression analysis of the mutated mKras allele was done by PCR with the following primers: FW, 5′-gcctgctgaaaatgactgag-3′; RV, 5′-tgctgaggtctcaatgaacg-3′. For expression profiling, the RNA extraction was done using Trizol plus RNA purification kit (Invitrogen Life Technology). RNA qualitative and quantitative assessment was done using the Bioanalyzer 2100 (Agilent Technologies).

RAS activation assay. Details on the experimental strategies used to assess the amount of RAS-GTP in cellular lysates are available in the Supplementary Methods.

Sequence analysis. cDNA was prepared from MLP WT and MLP KI ras G12D cells as described above. Reverse transcription-PCR was carried out with the following primers (FW, 5′-GCCTGCTGAAAATGACTGAG-3′; RV, 5′-TGCTGAGGTCTCAATGAACG-3′). Purified PCR products were sequenced using the reverse primer 5′-TCCAGAGACAGGTTTCTCCA-3′. Sequencing and mutational analysis was carried out as previously described (16).

Morphologic analysis. Representative pictures of MLP29 clones were taken with ImageReady software (Adobe) using a microscope (DMIL; Leica) and a 20 × 0.30 objective (Leica) equipped with a digital camera (DFC320; Leica).

Cell viability, wound healing, motility, and anchorage independence assays. Details on the experimental strategies used to assess the biological properties of the targeted cells are available in the Supplementary Methods.

Construction of the lentiviral vector expressing mKRAS WT and G12D. Full-length cDNAs coding for mKras WT and mKras G12D were amplified from cDNA extracted from MLP29 KI G12D and then cloned in pPRRsin.PPT.CMV.MCS MM.WPRE. The lentivirus production, cell infection, and transduction procedures have been described elsewhere (15).
Results

Construction of the AAV Kras KI vector. AAV is a human, replication-defective parovirus. The wild-type genome possesses two open reading frames, termed rep and cap, flanked by two inverted terminal repeats. We built a recombinant AAV, in which both of the open reading frames were deleted and replaced with exogenous mouse Kras sequences. The inverted terminal repeats, necessary for the packaging of the vector, were the only elements maintained from the wild-type virus. The homologous recombination cassette cloned within the inverted terminal repeats consisted of two 1 kb sequences ("homology arms"), one of which contained the mutated Kras exon. The selectable marker neomycin transferase gene was introduced between the homology arms. The Neo cassette was flanked by two LoxP sites to allow Cre recombination-mediated excision of the Neo cassette from the targeted cells’ genome (Fig. 1A). The regions of homology of the Kras locus were amplified from genomic DNA obtained from the target mouse somatic cells (MLP29 cells; see below). The Kras G12D mutation was introduced in the exon containing homology arm by standard site-directed mutagenesis (Fig. 1A). Details on the construction of infective AAV carrying the Kras homology arms are reported in Materials and Methods.

Knock-in of the Kras G12D mutation in mouse liver progenitor cells. As a recipient cellular model to test our knock-in approach, we selected mouse liver progenitor cells (MLP29). MLP29 are somatic mouse cells previously derived from embryonic liver (14). MLP29 display a number of features making them appealing for genetic and biological manipulation. They can be propagated indefinitely in vitro but are not oncogenic. Furthermore, they can be used to assess cellular phenotypes including growth factor–dependent proliferation, motility, and “invasive growth” (14). Their transcriptional profile has been previously ascertained, highlighting genes involved in cancer progression (17).

Kras is mutated at a very high frequency in cholangiocarcinomas which originate from liver biliary ducts (3). MLP29 cells are bipotent progenitors expressing markers of both hepatocytic and bile duct lineage, which makes them an appealing model to study KRAS-mediated carcinogenesis (14). We also verified that the Kras sequence is wild-type in MLP29 cells, confirming that they are a suitable model for genetic manipulation of the corresponding genomic locus.

MLP29 cells were infected with the recombinant AAV-Kras-G12D vector described above and selected for 21 days in the presence of neomycin until single resistant colonies appeared. Clones in which homologous recombination had occurred were identified by PCR analyses on the corresponding genomic DNA as described in Fig. 1B. Targeting frequencies were 1:300. Correct targeting of the Kras point mutation into the corresponding genomic locus was verified by sequencing at the DNA level (data not shown) and at the mRNA level (Fig. 1C). PCR-positive clones displayed a heterozygous G > A nucleotide change corresponding to a glycine to glutamic acid modification at codon 12 of the Kras coding sequence (Fig. 1C).

Two independently identified clones (denominated KI-1 and KI-2) carrying the Kras G12D mutation were amplified and subjected to further analysis. Interestingly, one of the clones (referred to as NEO+) that scored positively at the PCR screening, did not display the G > A nucleotide change. This indicates that the homologous recombination had involved the NEO cassette but only a portion of the 5’ homology arm (Supplementary Fig. S1). We reasoned that clone NEO+ differs from clones KI-1 and KI-2 only for the Kras mutation, and would therefore represent the ideal isogenic control for the mutated clones. The NEO+ clone was therefore amplified and used throughout the study.

To exclude the possibility that the Neo resistance cassette might affect the expression of the mutated allele purified recombinant TAT-CRE protein was used to excise the Neo cassette using the flanking loxP sites (Supplementary Fig. S2A). Allele-specific quantitative PCR was then used to assess the expression of the mutant G12D allele (Supplementary Fig. S2B). These experiments showed that the expression of the Neo cassette did not negatively affect the expression of the mutated allele. The “cre-out” cells were included as controls in some of the biochemical and biological experiments (see below).

Kras knock-in cells display distinct biochemical and biological features. The Kras protein cycles between the active GTP-bound and the inactive GDP-bound conformations. The G12D oncogenic mutation is thought to lock Kras into the GTP-bound
conformation by reducing its constitutive GTPase activity, and thus, constitutively activating Ras signaling. To assess whether the knock-in of the \textit{Kras} G12D mutation would activate the Kras protein in MLP29 cells, we did a pull-down assay using the recombinant CRIB domain of BRAF, which is known to bind Ras only in its GTP-bound conformation. Figure 2A shows that both mutant clones (KI-1 and KI-2) display constitutively active Kras, whereas in the matched wild-type cells and in the perfectly isogenic NEO+ clone, Kras is inactive.

Next, we compared the levels of Kras activation in the KI clones with those present in naturally occurring colorectal cancer cells carrying the corresponding mutations. The levels of Kras activation in mouse KI cells were comparable with those present in human cancer cells (Fig. 2A). Interestingly, when we ectopically expressed (by lentiviral-mediated transduction) a mutated \textit{Kras} G12D cDNA, the targeted cells displayed levels of GTP-RAS higher than those present in the knock-in or in the cancer cells (Fig. 2A). Overall, these data show that the knock-in system directly phenocopies the oncogenic activation of K\textit{RAS} present in human tumors.

We then did biochemical analysis to assess activation of the Kras pathway in WT and knock-in cells. To this end, we measured activation of MAPK and AKT using anti–phospho-specific antibodies (Supplementary Fig. S3). The results indicate that knock-in of the \textit{G12D} allele does not affect or even reduce the activation of the RAS downstream signaling pathways. These data are in accordance with previous work by Guerra et al. and Tuveson et al., which also reported that knock-in of mutated \textit{Kras} in mouse cells does not increase (but can actually reduce) the levels of MAPK activation (12, 18).

Next, we assessed whether MLP29 cells expressing \textit{Kras} mutant alleles might display distinct biological properties. Oncogenic \textit{K\textit{RAS}} mutations are thought to affect a number of cellular features including cell morphology, proliferation, and motility. MLP29 \textit{Kras} knock-in clones growing in \textit{vitro} could be readily distinguished from the wild-type or the NEO+ isogenic counterparts based on their morphology (Fig. 2B; Supplementary Fig. S4). Upon reaching confluence, the knock-in clones grew in a disorganized manner, whereas the wild-type cells formed a tight and organized monolayer (Fig. 2B; Supplementary Fig. S4). The proliferative capability of \textit{Kras} mutant cells was slightly but significantly increased (Fig. 2C). Mutant cells were more motile than their wild-type counterparts as measured in a transwell motility assay (Fig. S1B). Additionally, the knock-in cells displayed an increased wound-healing potential (Fig. 3B).

These results showed that the presence of a mutant \textit{Kras} alleles confers distinct biological properties to MLP29 cells including morphologic changes, increased motility, and proliferation. Importantly, in all the biochemical and biological assays that we did, the two mutated clones and the wild-type and the NEO+ cells showed homogeneous behavior. This indicates that clonal variability does not significantly affect the properties of the different cell lines.

**Knock-in of the \textit{Kras} G12D Mutation does not transform mouse liver progenitor cells.** A number of studies suggest that \textit{K\textit{RAS}} mutations can lead to the transformation of mammalian cells. Most of the experiments supporting this notion have been done by transfection-mediated ectopic expression of mutated \textit{K\textit{RAS}} cDNAs under the control of viral promoters. A previous report indicated that transformation by mutated human \textit{RAS} genes requires more than “100 times higher expression than is observed in cancers” (10). To directly compare the knock-in and the transfection methodology, we engineered MLP29 cells that expressed a G12D \textit{Kras}–mutated cDNA under the control of a cytomegalovirus promoter. The results were unequivocal; whereas MLP29 cells carrying the \textit{G12D} heterozygous mutation in the \textit{K\textit{RAS}} genomic locus were not capable of growing in soft agar, the corresponding cells expressing the \textit{G12D} cDNA readily formed colonies in the same conditions (Fig. 4). In these experiments, the ectopic expression of wild-type \textit{Kras} was sufficient to trigger transformation in MLP29 cells. This is probably due to the high transduction efficiency of lentiviral vectors. Our results indicate that the expression of a heterozygous oncogenic \textit{Kras} allele under its own promoter is not sufficient to transform mouse liver epithelial (MLP29) cells. The somatic knock-in approach closely recapitulates the genetic status of human tumors (such as cholangiocarcinomas) carrying mutated \textit{K\textit{RAS}}.

**Definition of a gene expression signature for \textit{Kras} KI.** To identify genes whose expression is affected by mutated \textit{Kras}, total RNA was extracted from the KI-1 and KI-2 clones, and from two controls, the NEO+ clone and wild-type cells. Transcriptome profiling was carried out in parallel on two different DNA microarray platforms, Affymetrix and Illumina, for systematic cross-validation of the results. The data have been deposited in the NCBI’s Gene Expression Omnibus and are accessible through the GEO Series accession number GSE8711. Preliminary clustering analysis allowed clear segregation of the two independently derived KI clones from the two control samples, confirming that clonal variation does not significantly affect our cellular model systems (data not shown). After filtering for detection, genes with
significant differential expressions between KI clones and controls were identified using significance analysis for microarray (19). Comparison of the results obtained in the two microarray platforms yielded a panel of 345 genes, the expressions of which were concordantly up-regulated or down-regulated by mutated \textit{Kras} (Supplementary Table S1; Supplementary Fig. S5), hereafter referred to as the "\textit{Kras-KI Signature}.”

We then assessed how many genes known to be transcriptionally regulated by oncogenic Ras were regulated in our mutated \textit{Kras} isogenic model, and used the hypergeometric distribution test to assess statistical significance. As a source of \textit{RAS}-responsive genes, we used two gene lists: one obtained by Bild et al. from human primary cells transiently transduced with adenoviral \textit{RAS} expression vectors (the "HMEC-\textit{HRAS} signature"; ref. 20), and one by Sweet-Cordero et al. from a model of somatic Ras mutation-driven mouse lung carcinogenesis (the "mouse lung signature"; ref. 13). For the analysis, the two lists were mapped onto our mouse Affymetrix platform, obtaining a total of 1,091 probe sets, 921 for the mouse lung signature and 201 for the HMEC-\textit{HRAS} signature.

We found that a significant fraction of the published Ras-responsive genes were also regulated in MLP29 knock-in cells (\(P < 10^{-5}\) for both published lists when a fold change threshold of 2 is chosen).

As a second approach, we directly compared the three lists of signature genes, all mapped on our mouse Affymetrix platform. We found that 52 of the \textit{Kras-KI} signature genes (15.1%) were also present in the mouse lung signature, with an observed/expected ratio of 2.13, and a hypergeometric \(P < 10^{-7}\). The overlap between the \textit{Kras-KI} and the HMEC-\textit{HRAS} signature was slightly worse in terms of coverage percentage, but significant (15 genes, O/E ratio = 2.98, \(P < 10^{-7}\)). However, comparison between the two published signatures revealed an even worse overlap (18 genes, O/E ratio = 2.31, \(P < 10^{-3}\)). Overall, 67 genes of the \textit{Kras-KI} signature are represented in published \textit{RAS} signatures, showing that knock-in of \textit{Kras} in somatic cells leads to the modulation of Kras-initiated pathways ultimately driving a specific transcriptional response.

The \textit{Kras-KI} signature classifies mouse and human lung cancers. \textit{KRAS} mutations are present at a high frequency in non–small cell lung cancers, in which they are thought to play a central role in tumor progression and have been associated with poor response to therapy and reduced survival (21). As discussed in the Introduction, sequencing of the \textit{KRAS} gene might not identify the entire spectrum of tumors in which the \textit{KRAS} pathway is oncogenically deregulated.

To verify whether the \textit{Kras-KI} signature might be able to reflect and predict the mutational status of \textit{KRAS} and the oncogenic activation of the corresponding pathway, we analyzed published microarray data sets of experimentally induced mouse and human lung cancers.

We initially focused on microarray data generated in the previously mentioned model of Kras-driven mouse lung carcinogenesis (13). The data set is composed of 50 samples, of which 31 were from lung cancers with mutated \textit{Kras} and 19 were from normal lungs with wild-type \textit{Kras}. We mapped 162 genes from the \textit{KRAS-KI} signature onto 173 probe sets of this data set (Supplementary Table S2), and assessed their ability to discriminate WT and mutated \textit{Kras} samples using the signal-to-noise ratio (SNR) metric (22). The average SNR for the signature was 0.81. Such values could not be reached by random gene lists of the same size of the signature, as highlighted in a Monte Carlo simulation (\(P < 0.00005\); Supplementary Fig. S6). Strikingly, the hierarchical clustering based on the expression of signature genes divided the 50 samples into two clearly distinct clusters containing either only WT or mutated \textit{Kras} samples. A marked concordance was observed in the behavior of the signature genes between MLP29 cells and the lung samples, with the \textit{Kras-KI}
clones coclustering with the mouse lung cancer samples, and the WT and NEO+ clones coclustering with the normal lung samples (Fig. 5). These results show that the Kras-KI signature is capable of discriminating mouse lung cancers based on their Kras mutational status.

To assess the class prediction ability of the signature in human cancer, we mapped the Kras-KI signature on microarray data generated by Bhattacharjee and colleagues (23) including 94 human lung adenocarcinoma samples for which they annotated the mutational status of codons 12 and 13 of KRAS (see Supplementary Table S2). Also in this case, we observed a significant enrichment for genes with high SNR ($P < 0.0005$; Supplementary Fig. S6). Notably, the Kras-KI signature was found to have a less significant SNR when used to discriminate normal lung tissue from lung cancers of various types ($P = 0.0056$; Supplementary Fig. S6), indicating that it specifically discriminates KRAS mutational status rather than neoplastic progression. We then used the nearest mean classifier approach (24) to classify each lung adenocarcinoma based on the expression of the signature genes in all the other samples. Briefly, average expression was calculated for each gene in the KRAS-mutated and WT group, excluding the sample undergoing classification (leave-one-out; see Supplementary Methods).

Figure 5. Expression of genes of the KRAS-KI signature in normal and neoplastic mouse lung and in Kras knock-in and control clones. The 173 probe sets corresponding to the RAS-KI signature (rows) were used to cluster samples from mouse lung together with MLP29 clones. The color codes for the samples (bottom).
For comparison, we adopted the same classification procedure using genes from the two previously mentioned Ras signatures obtained in the mouse lung model (mouse lung signature; ref. 13) and in HMECs (HMEC-HRAS signature; ref. 20), respectively. The results of this classification are presented in Table 1, and show that all three signatures have a very similar performance. These data suggest that, albeit nontransforming, knock-in of mutated Kras in mouse cells generates a transcriptional signature, the capability of which in predicting the presence of Kras mutations in human lung cancer is comparable to those previously published.

Discussion

Ommics technologies and the availability of the human genome sequence have allowed unprecedented progress in the identification of genes mutated in human cancers, leading to the detection of hundreds of cancer-associated alleles (16, 25, 26). Compared with the genomic discovery stage, the functional validation phase of the corresponding alleles is now lagging behind. For example, hundreds of point mutations affecting kinase genes have been recently described (16, 25, 26), but their biochemical and biological properties are largely unknown.

Mammalian cell lines and mouse strains have been widely used as model systems to functionally characterize cancer alleles carrying point mutations or deletions. In the first case, the cDNA corresponding to the mutated alleles are ectopically expressed by means of plasmid transfection or retroviral infection in human or mouse cell lines upon selection with markers. The derivative cells are then used to assess the biochemical and biological properties of the mutated cDNA with a variety of standardized assays. Classic examples of this approach are the focus-forming assay, which measures loss of contact inhibition, and the soft agar growth assay, which evaluates the anchorage-independent potential of neoplastic cells.

These studies have yielded remarkable results but are typically hampered by at least three issues. First, the expression is achieved by transient or stable transfection of cDNAs often resulting in the overexpression of the target allele at levels that do not accurately recapitulate what happens in human cancers. Second, the expression of the mutated cDNA is mainly achieved under the control of non–endogenous viral constitutively active promoters such as SV40, cytomegalovirus, and long terminal repeats (27). As a result, the mutated alleles cannot be appropriately modulated in the target cells. Finally, functional experiments have often been done using cells of stromal origin such as embryonal or 3T3 mouse fibroblasts. Stromal cells are not the primary targets of oncogenic transformation in the large majority of solid cancers.

Another approach has been to use homologous recombination to introduce the point mutations in a locus-specific fashion into the genome of mouse embryonic stem cells. This strategy works very efficiently and allows the generation of mouse strains carrying specific cancer alleles. Very often, however, mutated dominant oncogenes (such as Kras) cannot be transmitted through the mouse germ line (28) because the resulting embryos are not viable. Complex genetic strategies (such as the use of tissue-specific CRE-Lox systems, or latent alleles) have been devised to allow the expression of the mutated alleles only in adult mouse somatic tissues (12, 18). These have been used for a variety of experimental approaches including the demonstration that oncogenic Kras alleles can immortalize primary fibroblasts (12, 18).

To overcome these limitations, we exploited a novel approach to introduce cancer alleles in the genome of mouse epithelial somatic cells. Similar to most mammalian somatic cells, most adult mouse cells display a low frequency of homologous recombination that has thus far limited their use for genetic and functional analysis. To overcome this problem, we used a shuttle vector based on the backbone of AAVs. The latter has been found to increase the rate of targeted homologous recombination in human and mouse cells (29, 30).

As a model system to test our strategy, we chose the Kras G12D mutation, which is commonly found in human tumors such as colorectal, lung and pancreatic cancers. Mutated Kras proteins are, at least in theory, good therapeutic targets because they act as central switches in cellular processes leading to oncogenesis (2). Despite many efforts, the development of inhibitors directly targeting Kras has thus far failed, apparently due to difficulties in targeting the intrinsic RAS GTPase activity or the lack of specificity of farnesyltransferase inhibitors (31). The development of cellular and animal models that more closely recapitulate human diseases is a prerequisite to identifying molecules inhibiting the Kras signaling pathway rather than Kras itself.

AAV-mediated knock-in of the Kras G12D mutation was attempted in mouse liver progenitor cells (MLP29), a somatic immortal epithelial cell line previously established in our laboratory that is not transformed and does not form tumors in immunocompromised mice.

Clones displaying correct targeting of the Kras genomic locus were identified at a frequency of 1:300. Knock-in cells displayed

Table 1. Classification of human lung cancer samples by the Kras-KI, mouse lung, and HMEC signatures

<table>
<thead>
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<th>KRAS mutational status</th>
<th>Wild-type</th>
<th>Mutated</th>
<th>Total</th>
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<td>Wild-type</td>
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<td>5</td>
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<tr>
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<td>29</td>
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<td>Total</td>
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<td>HMEC signature class prediction</td>
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<td>Error (%)</td>
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NOTE: Nearest-mean classification with leave-one-out was used to classify 94 adenocarcinoma samples according to the Kras-KI, mouse lung, and HMEC signatures expression. The results of the classification are compared with the results of Kras sequencing at positions 12 and 13.

5 http://www.sanger.ac.uk/genetics/CGP/cosmic/
constitutively active Kras protein, marked morphologic changes and increased proliferation and motility. Interestingly, these cells were not transformed as measured by the soft agar assay. Importantly, targeted cells did not show oncogene-induced senescence and were not transformed in contrast with results previously reported for mouse cells overexpressing a mutated HRAS cDNA (32). We confirmed that in our cell models, overexpression of mutated Kras CDNA led to full transformation, indicating that the levels of Kras activation are critical for this phenotype. Amplification and/or overexpression of mutated Kras is very uncommon in human cancers, suggesting that the levels of Kras activation relevant for tumor progression are those achieved by the presence of point mutations in a heterozygous state. In line with this, the levels of Kras activation (as measured by a GTP load assay) in the knock-in cells were highly comparable with those found in naturally occurring tumor cells carrying the corresponding heterozygous mutations.

Our results are the first to show the direct introduction of an oncogenic mutation (such as the Kras G12D allele) in the genome of mouse epithelial somatic cells. Notably, the biochemical and transforming properties of our knock-in cell models are remarkably similar to those obtained from cells derived from mice carrying analogous Kras alleles (12, 18). We conclude that the isogenic cells that we have developed recapitulate the oncogenic activation of Kras occurring in human tumors. To further characterize and validate our cellular models, we did transcriptional profiling of the wild-type and knock-in cells using two independent microarray platforms that resulted in a distinct Kras KI signature of 345 genes. The signature was then tested for its ability to classify mouse and human cancers in which the mutational status of Kras was also known. The signature unequivocally distinguished mouse lung samples carrying the G12D mutation from the corresponding normal tissues. When applied to a large cohort of human non-small cell lung tumors for which the transcriptional profile was available, the signature was able to correctly predict the wild-type status of the Kras locus in most cases. Interestingly, a large fraction of cases displaying a wild-type Kras were instead defined as “mutant” by the signature. We therefore used two additional Ras signatures, obtained in mouse and human models alongside with that obtained in the mouse KI cells to compare their performance, and the results were very similar.

There are two possible interpretations for these results, (a) the signatures might have misclassified the wild-type samples due to poor specificity or (b) the signatures detected the functional status of Kras hyperactivation, possibly deriving from mutations at Kras codons other than 12 and 13 or involving activation of signaling molecules acting in the same pathway. The latter hypothesis is consistent with the notion that molecules acting upstream or downstream of Ras could also be deregulated in cancer (2). However, further investigations are required to address this issue.

In conclusion, we have developed a new cellular model closely recapitulating the Kras genetic lesions occurring in human cancers. Our findings support the concept that Kras mutations, in the absence of overexpression, promote distinct biological effects and drive a specific transcriptional response, but are not sufficient to trigger full transformation in epithelial cells.

Given the knock-in efficiency that we observed, our work points to new experimental strategies to build tumor progression models by “in vivo delivering” of oncogenic mutations using AAV-mediated homologous recombination in mouse somatic cells. Considering the recent successes and the unique phenotypes seen with mosaic conditional knockout mouse models of cancer (33, 34), we also envision that orthotopic transplantation of genetically manipulated somatic murine cells would provide a physiologically relevant model of human carcinogenesis.

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