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Modeling Tumor Progression by the Sequential Introduction of Genetic Alterations into the Genome of Human Normal Cells

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ABSTRACT: Cancer genomes display a complex blend of genetic lesions affecting oncogenes and tumor suppressor genes. Multiple modeling approaches indicate that 5–15 driver oncogenic events are required to achieve tumor progression in common epithelial cancers. In vitro, a lower number (2–3) of events is typically sufficient to achieve full transformation. We developed cellular models that closely resemble the occurrence of multiple genetic lesions to understand their role in tumor progression. Homologous recombination and transcriptional downregulation were used to recapitulate the co-occurrence of driver mutations targeting oncogenes and inactivation of tumor suppressor genes in human nontransformed epithelial cells. Knockdown of the tumor suppressor genes *PTEN* or *RB1* was combined with mutagenic activation of individual oncogenes (*EGFR*, *KRAS*, *BRAF*, or *PIK3CA*), thus generating a combinatorial model. The simultaneous presence of oncogenic and tumor suppressive events resulted in distinct biochemical properties and anchorage-independent growth abilities. Notably, however, we found that even when up to four individual alterations were concomitantly present they were not sufficient to fully transform the target cells. Our results suggest that the close recapitulation of cancer lesions in not-transformed cells is essential to unveil their oncogenic potential and raise questions concerning the minimal requirements for neoplastic transformation of epithelial cells.
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KEY WORDS: tumor progression; transformation; oncogenes; tumor suppressor

Introduction

Cancer is, in essence, a genetic disease driven by the multistep accumulation of genetic alterations in master genes controlling cell growth and differentiation [Fearon and Vogelstein, 1990]. Cancer-associated mutations are thought to confer a selective growth advantage and, in most instances, are "somatic," as they are present in the tumor but not in the normal tissue from the same patient.

In the past 10 years, the availability of the genome sequence and the development of high-throughput DNA sequencing technologies have allowed the systematic analysis of hundreds of cancer genomes (reviewed in [Stratton, 2011]) and the identification of more than 3000 cancer-associated alleles (http://www.sanger.ac.uk/genetics/CGP/cosmic/). Nevertheless, it is thought that only a subset of the somatic alterations are "drivers," that is, they are causally involved in the neoplastic process and positively selected during tumorigenesis. The remaining genomic variants are thought to be "passengers" and may provide no selective advantage to the tumor, rather they are stochastically retained during repeated rounds of cell division and clonal expansion [Haber and Settleman, 2007]. Indeed, the genomic landscape of a typical colorectal or breast cancer involves a small number of putative driver mutations affecting oncogenes and tumor suppressor genes [Wood et al., 2007].

Intriguingly, not all combinations of cancer mutations are "allowed": some combinations are more frequent than expected just by chance, whereas others are very rare. Mutual exclusivity pattern of mutations often involves genes acting in common signaling pathways, for example *BRAF* V600E mutations typically occur in a mutually exclusive relationship with *RAS* alterations in colorectal cancer, ovarian cancer and melanomas [Davies et al., 2002; Rajagopalan et al., 2002]. On the other hand, cooperative or synergistic interactions are well documented in the case of *HER2* amplification and *PTEN* loss in breast cancers [Yuan and Cantley, 2008]. Altogether, these observations suggest that specific combinations of genetic aberrations are positively or negatively selected during tumor progression.

Although the cancer genome projects have rapidly led to the identifications of hundreds of cancer alleles, their functional annotation has considerably lagged behind. Understanding how molecular alterations cooperate to drive tumor progression is key to provide a molecular framework for developing new therapeutic approaches. Ideally, such functional studies should be performed using model systems closely "recapitulating" the genetic events identified by the genome sequencing efforts.

Additional Supporting Information may be found in the online version of this article.

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Table 1. Genotypes Included in the Combinatorial Genetic "Matrix"

	WT	KI del746–750 ***EGFR***	KI H1047R ***PIK3CA***	KI E545K ***PIK3CA***	KI G13D ***KRAS***	KI V600E ***BRAF***
Nontarget shRNA	+	+	+	+	+	+
shRNA ***PTEN***	+	+	+	+	+	+
shRNA ***RB1***	+	+	+	+	+	+

List of the genetic alterations introduced and combined in the HME-1 immortalized breast epithelial cell line. In bold are listed the genes that have been targeted. The + symbol represents the genotypes that have been generated.
del746–750 EGFR indicates the variant c.2235_2249del; p.Glu746_Ala750del in *EGFR* (GenBank: NM_005228).
H1047R PIK3CA indicates the variant c.3140A>G; p.His1047Arg in *PIK3CA* (GenBank: NM_006218).
E545K *PIK3CA* indicates the variant c.1633G>A; p.Glu545Lys in *PIK3CA* (GenBank: NM_006218).
G13D *KRAS* indicates the variant c.38G>A; p.Gly13Asp in *KRAS* (GenBank: NM_004985.3).
V600E *BRAF* indicates the variant c.1799T>A; p.Val600Glu in *BRAF* (GenBank: NM_004333.4).
The sequence variants reported are described according to the cDNA sequence. Nucleotide position refers to position within coding sequence, where position 1 corresponds to the first position of the start codon.

So far the role of cancer-associated genetic lesions has been studied mainly by overexpressing the corresponding mutated cDNAs in human cells (under the control of unspecific promoters) or by the ectopic expression of viral oncoproteins [Hahn et al., 1999]. Although these strategies have been remarkably effective, they do not precisely recapitulate the occurrence of the genetic events that accumulate during human cancer development. Accordingly, the definition of the minimal genetic determinants responsible for neoplastic transformation is still incomplete.

We sought to improve some of the limitations of current tumor progression models by using targeted homologous recombination to introduce (knock-in) oncogenic "driver" mutations in immortalized, nontransformed human cells. The approach employs vectors based on Adeno Associated Viruses (AAVs) that facilitate the homologous recombination process [Russell et al., 2002; Zecchin and Di Nicolantonio, 2011].

As recipient cells, we used HME-1, an hTERT-immortalized epithelial cell line of breast origin that can be propagated indefinitely in vitro, but is not tumorigenic. In these cells, we had previously introduced common oncogenic mutations: *EGFR* c.2235_2249del; p.Glu746_Ala750del (hereafter referred as *EGFR* delE746-A750), *KRAS* c.38G>A; p.Gly13Asp (referred as *KRAS* G13D), *BRAF* c.1799T>A; p.Val600Glu (referred as *BRAF* V600E); and *PIK3CA* c.3140A>G; p.His1047Arg and c.1633G>A; p.Glu545Lys (referred, respectively, as *PIK3CA* H1047R and *PIK3CA* E545K) [Di Nicolantonio et al., 2008, 2010]. The knock-in (KI) of any of the above cancer alleles in the genome of HME-1 cells is not sufficient to induce transformation in vitro [Di Nicolantonio et al., 2008; Konishi et al., 2007]. To define which additional molecular events were required for transformation, we evaluated the role of several tumor suppressor genes (such as *TP53*, *PTEN*, and *RB1*) that are frequently inactivated in human cancers (http://www.sanger.ac.uk/genetics/CGP/cosmic/). The knockdown of individual tumor suppressor genes was combined with mutagenic activation of specific oncogenes, thus generating a combinatorial model we will refer to as the "matrix" (Table 1). The "matrix" was then employed to dissect how cancer genes, alone or in combinations, can modulate oncogenic transformation both in vitro and in vivo.

Materials and Methods

Plasmids and Viral Vectors

All experimental procedures for targeting vector construction, AAV production, cell infection, and screening for recombinants have been described elsewhere [Di Nicolantonio et al., 2010]. HME-1 wild-type (WT) cells were engineered to overexpress the KRAS G13D cDNA by infection with a lentiviral vector harboring the mutated *KRAS* allele downstream a constitutive promoter as previously reported [Di Nicolantonio et al., 2010].

The lentivirus production, cell infection, and transduction procedures have been described elsewhere [Vigna and Naldini, 2000].

All prepackaged, high-titer viral particles expressing shRNAs were purchased from Sigma–Aldrich (Milan, Italy). The following shRNAs were employed: TRCN0000040163 or TRCN0000040164 targeting *RB1* and TRCN0000002749 or TRCN0000002746 targeting *PTEN*. Viral transductions were carried out according to the manufacturer's instructions. HME-1 transduced cells were selected for lentiviral integration by Puromycin at 8 μg/ml for at least 10 days. Following selection, HME-1 recombinant cell lines were maintained in Puromycin at 4 μg/ml.

Cells and Cell Culture Reagents

hTERT HME-1 (HME-1), DLD1, and SW48 cell lines were purchased from American Type Culture Collection. RMF/EG fibroblasts were a kind gift of Prof. Robert A. Weinberg (Whitehead Institute for Biomedical Research, Cambridge, MA).

HME-1 were cultured in growth medium containing DMEM/F-12 (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Sigma–Aldrich, Milan, Italy), 20 ng/ml EGF, 10 μg/ml insulin, and 100 μg/ml hydrocortisone. DLD-1 and RMF/EG cells were cultured in DMEM (Invitrogen) with 10% FBS. All cell culture media were supplemented with 50 units/ml penicillin and 50 mg/ml streptomycin. Geneticin (G418) was purchased from Gibco and Puromicin from Sigma–Aldrich.

Protein Analysis

SDS-PAGE Western blotting was performed as previously described [Di Nicolantonio et al., 2008]. The following antibodies were used for Western blotting: anti-total RB1, anti total PTEN, anti-total AKT, anti phospho-AKT S473; anti phospho-MEK1/2 S217/221; anti-total MEK1/2 (Cell Signaling Technology, Danvers, MA); anti P16, anti KRAS, and anti ACTIN (Sigma clone 3B10–2F2) and anti-VINCULIN (Millipore, Billerica, MA).

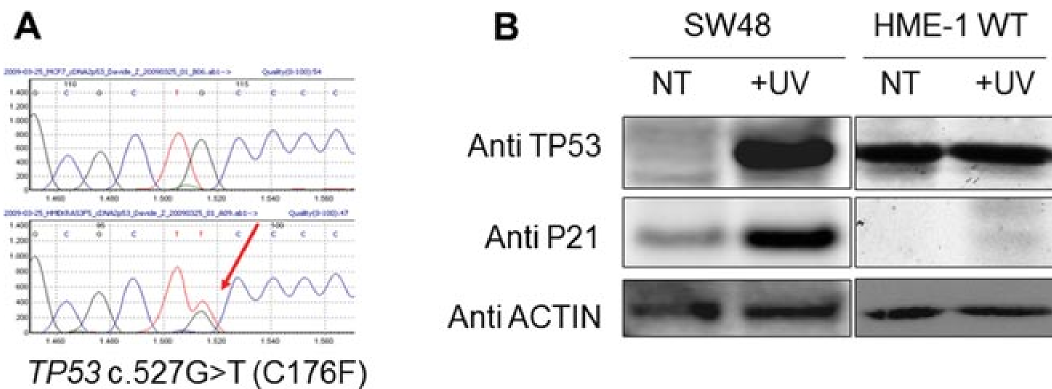

Figure 1. Analysis of TP53 status in HME-1 cells. **A:** The coding regions of the *EGFR*, *PIK3CA*, *KRAS*, *BRAF*, *TP53*, *PTEN*, and *RB1* genes were analyzed by conventional Sanger sequencing approach. The nonsynonymous c.527G>T mutation (C176F) was detected in exon 5 of the *TP53* gene **B:** Functional analysis of the TP53 checkpoint to genotoxic stress. The SW48 (TP53 proficient) and the HME-1 (harboring the c.527G>T; C176F mutation) cell lines were treated with UV. Expression of TP53 or P21 was evaluated by immunoblotting with the indicated antibodies

Soft Agar Anchorage-Independent Growth Assay

To assess anchorage-independent growth, triplicate samples of 3000 cells from each HME-1 derived cell line, or triplicate samples of 1000 DLD1, were mixed 10:1 with 5% agarose in their specific growth medium, for a final concentration of 0.5% agarose. The cell mixture was plated on the top of a solidified layer of 1% agarose growth medium in 24-well plates. Cells were fed every 2–3 days with 100 μl of growth medium. Cells were stained with 0.02% iodonitrotetrazolium chloride (Sigma–Aldrich) and photographed after 16 days. Quantification of the area occupied by colonies in each well was performed by the Metamorph Offline software (Meta Imaging Series 6.1).

Xenograft Models

All animal procedures were approved by the Ethical Commission of the University of Turin and by the Italian Ministry of Health. A colony of immunocompromised NOD/SCID mice was maintained in-house under aseptic sterile conditions. Mice were administered autoclaved food and water ad libitum. Mice manipulation and operations were performed under sterile conditions, and animals received antibiotics in the drinking water up to 1 week after all surgical procedures.

Three-weeks-old female NOD/SCID mice weighing 8–12 grams were anesthetized by isoflurane gas, the mammary epithelium was removed from both the number 4 inguinal mammary glands, whereas 2.5×10^5 unirradiated RMF/EG fibroblasts and 2.5×10^5 irradiated (4 Gy) fibroblasts were injected into the cleared fat pads 24 hr after irradiation. Two weeks after, 4×10^5 human breast epithelial HME-1 or MDA MB 231 cells were injected into the humanized site. To this aim, epithelial cells were resuspended in a 1:1 solution of medium and matrigel to a concentration of 4×10^5 cells/30 μl before the injection.

For xenografted tissues analysis, mammary glands were carefully excised, fixed in 10% formalin overnight and included in paraffin. For each gland, 2-μm to 3-μm serially cut sections were obtained from a formalin-fixed and paraffin-embedded block. The sections were stained with hematoxylin and eosin mixture, as described elsewhere (http://ccm.ucdavis.edu/bcancercd/52/prcl_HandE.html).

An expert human pathologist was asked to examine sections of the stained mouse mammary glands to detect the presence of tumors.

Statistics

Statistically different numbers of soft agar colonies ($P < 0.05$) were assessed by the Newman–Keuls Multiple Comparison Test for each experiment performed. Each experiment was performed in duplicate on at least two independent clones for each genotype.

Results

Construction of Recombinant Human Cells Harboring Combinations of Cancer Mutations

As a preliminary step, we evaluated the status of the tumor suppressor genes *TP53*, *PTEN*, and *RB1* in the recipient cell line HME-1 and its derivatives carrying individual oncogenic mutations [Di Nicolantonio et al., 2008, 2010]. Sequence analyses showed that *PTEN* and *RB1* genes were WT. The nonsynonymous change c.527G>T p.Cys176Phe (referred as *TP53* C176F) was detected in exon 5 of *TP53* (Fig. 1A). This variant is one of the most frequent *TP53* somatic mutations and has been detected in multiple tumor types including breast cancers (http://www-p53.iarc.fr/; http://www.sanger.ac.uk/genetics/CGP/cosmic/). At the functional level, the C176F variant has been shown to impair the TP53-mediated checkpoint in response to genotoxic stress [Dearth et al., 2007; Shi et al., 2002]. We experimentally assessed the functional status of TP53 by treating HME-1 cells with UV radiation, a commonly used genotoxic stimulus [Latonen and Laiho, 2005]. The protein levels of TP53 and its main downstream effector cyclin-dependent kinase inhibitor P21/WAF were not markedly increased following UV treatment in HME-1, differently from what was observed in the TP53 proficient SW48 cell line (Fig. 1B). These results indicate that HME-1 parental cells, as well as their isogenic KI derivatives, carry a functionally inactive TP53. As these cells are unable to grow in anchorage-independent conditions [Di Nicolantonio et al., 2008], the concomitant presence of well-established oncogenic events such *EGFR*, *KRAS*, *PIK3CA*, or *BRAF* mutant alleles together with loss of TP53 function are not sufficient to confer a transformed phenotype to HME-1 cells.

Having confirmed that *PTEN* and *RB1* are WT in HME-1, we proceeded to knock down their expression by means of short hairpin RNAs (shRNAs). In each case, we identified at least two independent

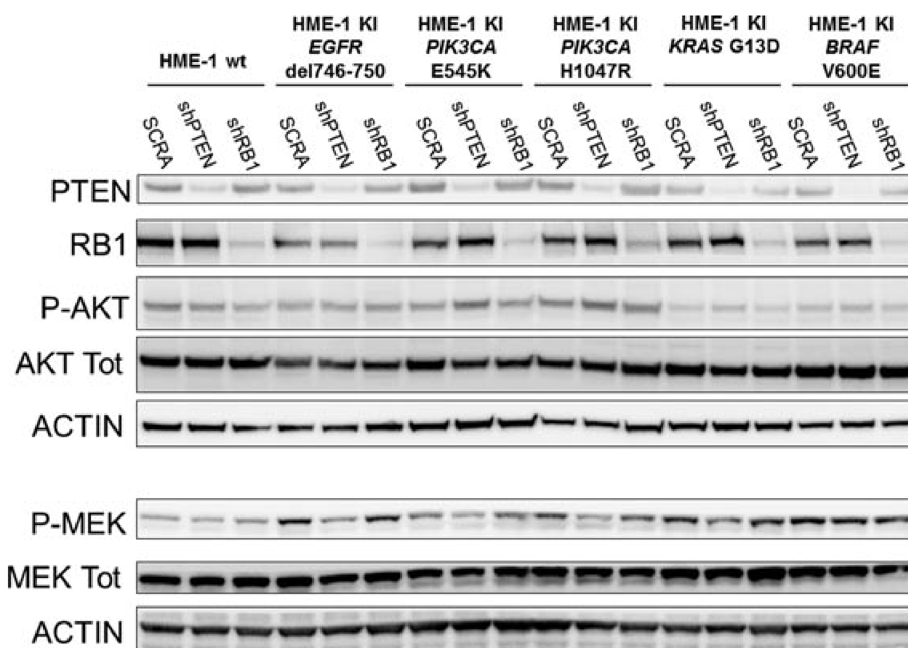

Figure 2. Biochemical analysis of HME-1 cells carrying the indicated genetic alterations. The effective silencing of *PTEN* or *RB1* was tested by Western blot analysis of the corresponding proteins in WT, *EGFR* (del746–750), *PIK3CA* (E545K and H1047R), *KRAS* (G13D), or BRAF (V600E) KI infected with Scramble (SCRA) nontarget, *PTEN*, or *RB1*-targeting shRNAs. Activation of AKT was measured using anti-phospho-AKT antibody, whereas MEK activation was detected by anti-phospho-MEK1/2 antibody. Antibodies against AKT, MEK1/2, and anti-ACTIN were used as controls.

shRNAs that efficiently reduced the expression of the corresponding protein (Supp. Fig. S1).

To exclude variability, at least two independent KI clones were targeted for each genotype present in the entire suite of HME-1 cells. The effective knockdown of the corresponding transcripts was confirmed by Western blot (Fig. 2, upper panels). A nontargeting shRNA (hereafter referred as "scramble") was used as a control in all experiments. In summary, we generated a combinatorial genetic matrix that systematically couples *EGFR* (*delE746-A750*), *KRAS* (*G13D*), *BRAF* (*V600E*), and *PIK3CA* (*H1047R* and *E545K*) activating mutations with *PTEN* and *RB1* inactivation (Table 1).

Biochemical Properties Induced by Specific Combinations of Cancer Mutations

We have previously reported that knock-in of a single cancer allele in HME-1 cells results in the activation of the corresponding proteins and triggers specific signaling pathways . We investigated how the combined activation of individual oncogenes and the inactivation of *RB1* or *PTEN* tumor suppressors modulate the downstream signaling in the mutant cells of the "matrix." We focused on the two main oncogenic routes affected by the *EGFR*, *KRAS*, *BRAF*, *PIK3CA* oncogenic mutations, in particular, the MEK-ERK and the PI3K-AKT axes.

Increased phosphorylation of AKT was observed when silencing of *PTEN* was combined with *PIK3CA* mutational activation (KI H1047R or E545K), whereas the same effect was not triggered by *PTEN* knock down in the wt cells (Fig. 2). This suggests that negative feedback loops may occur in HME-1 not-transformed cells, buffering the aberrant activation of AKT signaling pathways at least in particular genetic contexts.

A general decrease in the activation of AKT was observed in all genotypes harboring an activating mutation in *KRAS* or *BRAF* (Fig. 2). Moreover, silencing of *PTEN* decreased the levels of phospho-MEK in different KI genetic backgrounds (Fig. 2), suggesting that a negative cross-talk between PI3K and MAPK pathways may take place when one of the two signaling cascades are genetically activated in HME-1 cells.

On the other hand, reduced expression of *RB1* had minimal or no effect on the MEK or PI3K signaling pathways, consistently with previous reports [El-Naggar et al., 2009]. To confirm that *RB1* silencing had resulted in functional inactivation of this tumor suppressor gene, we evaluated if our cellular models showed defects in cell cycle. Indeed, we found that shRNA-mediated suppression of *RB1* affected the cell cycle distribution toward the G2 phases in cells analysed 24 hr after synchronization (data not shown). We also verified that the cell cycle effector P16 was expressed and that its levels were not affected by the silencing of *RB1* (Supp. Fig. S2).

In summary, the combined activation of oncogenic and tumor suppression events resulted in distinct patterns of intracellular signaling pathways.

In Vitro Systematic Evaluation of the Oncogenic Properties of the Mutant Cell Lines

We then focused on evaluating the tumorigenic properties of the HME-1 cell matrix. Multiple studies indicate that the in vitro assay, which more closely assesses the tumorigenic potential of cancer cells is their ability to grow in anchorage-independent fashion in soft agar [Freedman and Shin, 1974].

We previously reported that *EGFR*, *KRAS*, and *PIK3CA* mutant HME-1 cells are virtually unable to grow in soft agar, whereas *BRAF* V600E knock-in cells form a negligible number of colonies [Di Nicolantonio et al., 2008]. Considering that HME-1 are also *TP53* defective, this indicates that the presence of a constitutively active oncogene and the inactivation of a single tumor suppressor gene are not able to sustain *per se* the transformed phenotype.

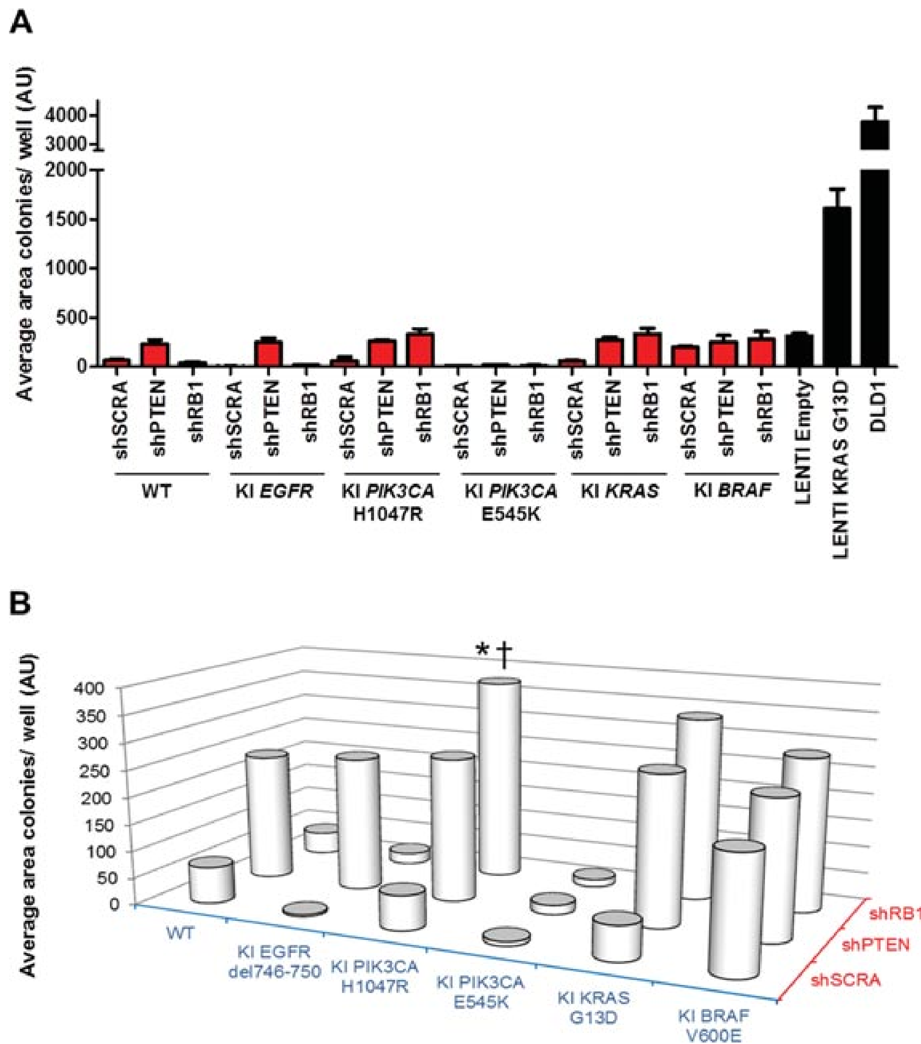

Figure 3. Transformed potential of HME-1 cells carrying single and multiple genetic alterations. **A:** Anchorage-independent growth was evaluated on HME-1 cells WT, *EGFR* (del746–750), *PIK3CA* (H1047R, and E545K), *KRAS* (G13D), or *BRAF* (V600E) KI (Knock-in) infected with Scramble (shSCRA), *PTEN*, or *RB1*-targeting shRNAs (sh*PTEN* or sh*RB1*). HME-1 cells infected with lentiviral control vector (LENTI Empty) or with lentiviral vector expressing the G13D KRAS cDNA (LENTI KRAS G13D) were also included. DLD1 cancer cells were used as positive control. The area occupied by colonies was analyzed with the MethaMorph software. Columns indicate the mean area of one field calculated in one representative experiment out from three. Error bars are standard deviations. AU = arbitrary units. **B:** The same data shown in (**A**) for HME-1 "matrix" models were plotted on a 3D histogram. Knocked-in cancer alleles are listed on the *x*-axis, and shRNAs used to transduce KI clones are listed on the *z*-axis. * $P < 0.05$ compared with hTERT HME-1 WT infected with the same shRNA (Newman–Keuls multiple comparison test). † $P < 0.05$ compared with respective KI genotype + SCRAMBLE shRNA (Newman–Keuls multiple comparison test).

We therefore evaluated whether loss of *PTEN* or *RB1* in combination with the other oncogenic events may be sufficient to drive neoplastic transformation in HME-1 cells by profiling the entire cell "matrix" with anchorage-independent growth assays. HME-1 ectopically expressing *KRAS* G13D allele was employed as a control of the experiments, together with the fully transformed cancer cell line DLD1. The lentiviral-infected cells expressed significantly higher levels of KRAS compared to both WT and *KRAS* KI HME-1 cells (Supp. Fig. S3).

We found that the ectopic expression of mutant KRAS in the parental cells was at least 10 times more powerful in conferring anchorage-independent growth capability then any of the combinations we tested (Fig. 3A).

We next carefully compared the "growth" capabilities within the matrix using statistical analysis (Fig. 3B). We found that combinations involving the BRAF V600E or the *PIK3CA* E545K mutations promoted, respectively, the highest and lowest number of colonies in soft agar. *PTEN* inactivation was more effective than *RB1* suppression in conferring anchorage-independent growth properties across all tested genotypes. Finally, the silencing of a given tumor suppressor gene exerted a different effect depending on the preexisting knock-in genetic background. This phenomenon can be observed comparing the H1047R *PIK3CA* knock-in clones silenced for the expression of *RB1* with the other knock-in cells in which *RB1* was inactivated. Indeed, the concurrent presence of *PIK3CA* H1047R and the inactivation of *RB1* produced the strongest cooperative effect among all combinations tested, as HME-1 harboring both alterations formed a statistically relevant higher number of colonies with respect to the cells in which the same alterations were introduced as single hits (Fig. 3B). These results were confirmed in at least two independent knock-in clones for each genotype, showing that the individual phenotypes observed

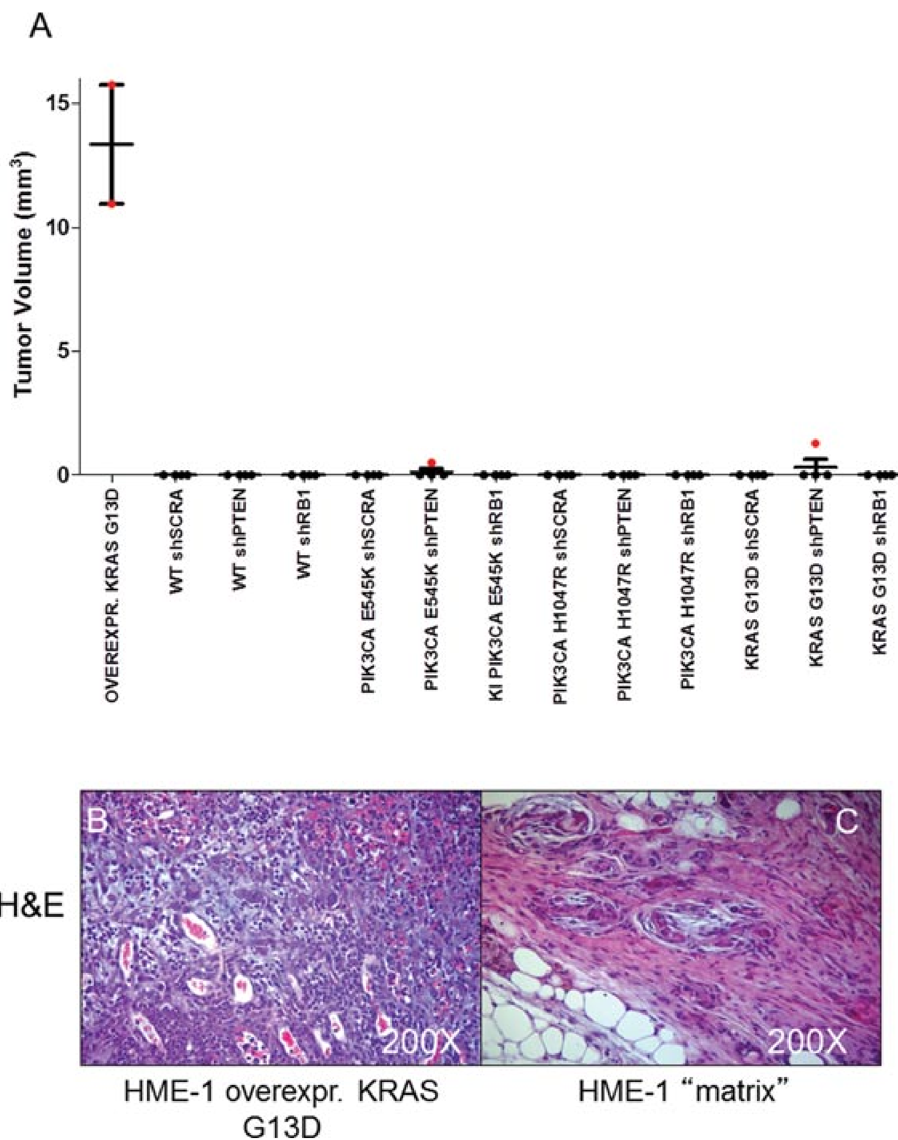

Figure 4. **A**: Tumor formation in NOD SCID humanized mammary glands transplanted with HME-1 cells ectopically expressing G13D KRAS mutant cDNA (OVEREXPR. KRAS G13D) or with HME-1 cells of the "matrix" (WT, *PIK3CA* E545K, *PIK3CA* H1047R, or *KRAS* G13D KI cells infected with Scramble –shSCRA-, *PTEN*, or *RB1*-targeting shRNAs). Dots represent individual mammary glands transplanted with HME-1 of the indicated genotype. Red dots indicate samples in which hallmarks of tumor were detected by histological analysis of the transplanted glands. Tumor volumes were measured from longitudinal paraffin sections of the glands stained with Hematoxylin/eosin. Representative sections of mammary glands injected with HME-1 cells ectopically expressing KRAS G13D cDNA (**B**) or with HME-1 cells of the "matrix" (**C**) stained with Hematoxylin/eosin.

within the HME-1 "matrix" were not due to clonal variability (Supp. Fig. S4).

In Vivo Evaluation of the Oncogenic Properties of the Mutant Cell Lines by Orthotopic Transplantation in Mouse Models

Multiple studies indicate that the microenvironment can actively contribute to tumor initiation, progression, and metastasis (reviewed in [Hu and Polyak, 2008]). We therefore set out to evaluate how a suitable orthotopic environment, such as the mammary tissue, may influence the growth of the "matrix."

We focused on the combinatorial genotypes of the "matrix" that have been found in human breast tumors, such as *PTEN* or *RB1* inactivation and *PIK3CA* (H1047R and E545K) or *KRAS* (G13D) oncogenic activation (http://www.sanger.ac.uk/genetics/CGP/cosmic/). Considering the low oncogenic potential of the "matrix" in vitro, we exploited an orthotopic transplantation method, which was reported to efficiently support the engraftment of normal human mammary epithelial organoids in the mammary fat pad of NOD SCID mice [Kuperwasser et al., 2004]. Cleared and humanized inguinal mammary glands of NOD SCID mice were injected with 4×10^5 HME-1 cells of each of the genotypes. HME-1 cells ectopically expressing mutant KRAS and the breast tumor cell line MDA-MB-231 were used as positive controls.

Mice were sacrificed 10 weeks later to assess the engraftment and the growth of human epithelial cells. Although the matrix cells models were generally unable to form tumors, KRAS G13D overexpressing HME-1 as well as MDA-MB-231 cells readily generated palpable

tumors 6 weeks after injection (Fig. 4A and data not shown). Histological analysis confirmed that the "matrix" cells were unable not only to form tumors but also to promote the early phases of breast tumor progression, such as formation of displastic glands (Fig. 4B and C).

Discussion

The cancer genome projects elucidated the molecular landscapes of human tumors. The next logical step is to understand how the cancer-specific genetic alterations cooperate to drive tumor progression. This knowledge is required to provide a molecular framework for developing new therapeutic approaches.

In this work, we sought to evaluate how individual molecular events frequently found in human tumors cooperate to trigger the transformation of human cells. Targeted homologous recombination was used to introduce (knock-in) oncogenic "driver" mutations in immortalized-but nontransformed-human cells.

Subsequently, shRNA-mediated silencing was used to knockdown individual tumor suppressor genes thus generating a combinatorial model we refer to as the "matrix." The "matrix" was then employed to dissect how cancer genes, alone or in combinations, can modulate oncogenic transformation both in vitro and in vivo.

We found that even when multiple cancer-associated events were concomitantly present the levels of transformation were not comparable to that of naturally occurring cancer cells.

On the other hand, HME-1 cells, similarly to other not-transformed epithelial human cells previously described [Campbell et al., 2007; Kim et al., 2011], were fully transformed by the ectopic expression of mutated *KRAS*.

Interestingly, these results are different from what was previously reported for other mammary immortalized epithelial cells overexpressing mutant *KRAS*, in which the acquisition of transformed features in vitro did not translate into in vivo tumorigenic properties (Konishi et al., 2007). It is likely that the differences in the model system or the mouse transplantation techniques can account for this discrepancy.

Considering that HME-1 cells are susceptible to transformation and that the strategy we employed was suited to assess tumor properties both in vitro and in vivo, the lack of tumorigenicity observed for the genetic "matrix" models is unlikely due to the particular cell recipient employed.

These results clearly indicate that studies involving overexpression of oncogenes offer a simplistic view of the transformation process and that the precise recapitulation of specific cancer lesions in not-transformed cellular models is a fundamental prerequisite to understand their oncogenic potential.

It is remarkable that the cell models we employed retained a not-transformed phenotype both in vitro and in vivo even when up to four individual alterations (the pre-existing hTERT ectopic expression, *TP53* mutation and two more newly introduced) were present at the same time.

These data somehow contrast with those generated in genetically engineered mouse models, where two hits are often sufficient to induce complete transformation. This is the case of *KRAS* mutational activation combined with *TP53* mutation [Jackson et al., 2005] that is capable of driving lung tumorigenesis in mice, or the combined mutational activation of *BRAF* and knockout of *PTEN*, that is sufficient to induce melanomas [Dankort et al., 2009]. It is possible that the incomplete (shRNA mediated) downregulation of the tumor suppressors could explain at least in some instances this discrepancy. Furthermore, the molecular mechanisms and the genetic determinants underlying transformation in murine tissues can be different from those occurring in human cancers [Rangarajan and Weinberg, 2003]. Importantly, the timeframe involved in the development of tumorigenesis in genetically modified mice generally allows for additional genomic evolution, while this unlikely happens in our cell culture system.

Indeed, our data are in agreement with the observations that multiple oncogenic mutations can co-occur in human lesions with no malignant potential, such as benign human epidermal tumors [Hafner et al., 2010]. Our results are also consistent with the finding that an average of 80 mutations are present in a human breast tumor. [Wood et al., 2007].

At the biochemical level, the combination of specific genetic aberrations elicited a distinct pattern of activation of the AKT and MEK signaling pathways. Nevertheless, our results also suggest that the activation of feedback loops may occur in HME-1 not transformed cells following the introduction of specific genetic alterations. In this regard, although loss of *PTEN* was previously reported to correlate with the activation of AKT in tumor cell lines [Dahia et al., 1999; Wu et al., 1998], *PTEN* knockdown in HME-1 was not able to increase by itself the phosphorylation of AKT.

Moreover, a negative cross-talk between PI3K and MAPK pathways was observed in few genotypes of the HME-1 "matrix" when one of the two cascades was genetically activated. Interactions between MAPK and PI3K pathways have been described, and several studies demonstrated that blockade of one pathway leads to the activation of the other by relieving negative feedback loops acting upstream [Carracedo et al., 2008; Chandarlapaty et al., 2011; Mirzoeva et al., 2009; Normanno et al., 2006; Serra et al., 2011]. We hypothesize that these negative regulatory mechanisms may still be active and eventually enforced in HME-1 mutated cells, allowing the cross-talk between MEK and AKT pathways.

Along this line, we speculate that the biological and the biochemical effects of the genetic alterations introduced may be "mitigated" by the activation of several feedback loops still effective in this not-transformed cellular recipient.

It is therefore possible that additional mutations targeting key regulatory pathways are essential to destabilize the not-transformed cellular system and to drive the tumorigenic process.

Analysis of the genetic "matrix" also highlighted differences in the anchorage-independent growth abilities among individual genotypes. Surprisingly, cells harboring the *PIK3CA* E545K mutation showed a reduced colony formation capability compared to *PIK3CA* H1047R mutants. The finding that the two mutations within this cancer gene have different biochemical characteristics has been previously reported [Zhao and Vogt, 2008]. Indeed, the *PIK3CA* gain of function triggered by the E545K variant requires interaction with RAS, whereas the H1047R kinase domain mutation depends on the interaction with p85 [Zhao and Vogt, 2010]. We believe that the cell models and the type of analysis we presented may help in elucidating why distinct signaling properties of individual mutations result in different tumor-associated phenotypes.

In conclusion, we find that concomitant deregulation of up to four cancer genes does not lead to overt transformation in human nontransformed cells. This likely reflects the complexity and multiplicity of genetic events that drive tumor progression and is presently being unraveled by the sequencing of cancer genomes. The sequential introduction of newly discovered cancer alleles in cell models such as the one described here may lead to greater understanding of the molecular mechanisms that promote and sustain the transformed phenotype.

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