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Introduction

The evolution of human cells into malignant derivatives is driven by the aberrant function of genes that positively and negatively regulate various aspects of the cancer phenotype, including altered responses to mitogenic and cytostatic signals, resistance to programmed cell death, immortalization, neoangiogenesis, and invasion and metastasis (Hanahan and Weinberg, 2000). The integrity of these gene functions is compromised by substantial genetic and epigenetic alterations observed in most cancer cell genomes. To understand the tumorigenic process, it is imperative to identify and characterize the genes that provide tumor cells with the capabilities requisite for their initiation and progression. However, the identities of those genes that contribute to the tumor phenotype are often concealed by the frequent alterations in genes that play no role in tumorigenesis.

Identifying genes that restrain tumorigenesis (tumor suppressors) has proven especially challenging due to their recessive nature. Further complicating their discovery are the multifaceted mechanisms by which tumor suppressor genes are inactivated including changes in copy number and structure, point mutations, and epigenetic alterations (Balmain et al., 2003). Moreover, the mechanisms by which tumor suppressor genes are inhibited may vary between tumors. With this in mind, a variety of molecular and cytogenetic technologies have been used to establish extensive catalogs of genetic alterations within human cancers (Albertson et al., 2003; Futreal et al., 2004). And while it is generally accepted that highly recurrent aberrations signify changes that are important for tumor development, the causal perturbations underlying tumor genesis are often confounded by the extensive size of alterations and the large number that are incidental to the tumor phenotypes. As such, new strategies to delineate genes with functional relevance to tumor initiation and development are essential to understanding these processes.

One approach to this problem involves the use of *in vitro* models of human cell transformation. In such models, primary cells are transformed into tumorigenic derivatives by the coexpression of cooperating oncogenes (Elenbaas et al., 2001; Hahn et al., 1999; Zhao et al., 2004). These experimental models have been useful in delineating the minimum genetic perturbations required for transformation of various human cell types as well as evaluating the functional cooperation between a gene of interest and a defined genetic context. To date, these models of human cell transformation have incorporated genes already implicated in human tumorigenesis. However, such models also provide a potentially useful platform for the identification of new pathways that contribute to the transformed phenotype.

In this award, we proposed two basic areas of investigation. The first area is the development of methods to investigate the repetoire of the immune system to determine whether auto-antibodies exist that might predict the onset of breast cancer. The second area was the construction and use of shRNA libraries to find genes relevant to breast cancer and hopefully targets that might kill tumor cells. The first area has not progressed much as we have changed how we are approaching the problem of detecting autoantibodies. We have now decided to use phage display as the method of choice and have generated a library of coding fragments of the human genome which we will use to address this problem. Because we are only now getting this system set up, this progress report will focus on the second area of the use of shRNA to explore breast cancer.

We have utilized such an RNAi-based, forward genetic approach to identify genes that suppress oncogenic transformation in a defined human mammary epithelial cell model

Body

RNAi Screen for Suppressors of Epithelial Cell Transformation

Recently, several cell culture models of human cell transformation have been described in which primary human cells are engineered to express combinations of dominantly-acting cellular and viral oncogenes and subsequently measured for anchorage-independent proliferation, an *in vitro* hallmark of transformation (Elenbaas et al., 2001; Hahn et al., 1999; Zhao et al., 2004). We sought to identify short-hairpin RNAs (shRNAs) that cooperate within the context of such a model. As >80% of cancers arise from epithelial tissues (AmericanCancerSociety, 2000), we chose to examine cells derived from human mammary epithelial cells (HMECs) to increase the probability of finding genes with relevance to breast epithelial cancers. The cells utilized for this screen (referred to as TLM-HMECs) were created by sequentially introducing the human telomerase catalytic subunit (hTERT) and the Large T antigen (LT) of SV40 into HMECs (Zhao et al., 2003). In addition, these cells exhibit elevated expression of the endogenous c-*myc* gene resulting from extended culture *in vitro* (Wang et al., 2000). Important to the efficacy of this screen, these cells do not proliferate in the absence of extracellular matrix (<1 colony in 10^4; Figure 1B, left panel) (Zhao et al., 2003). Recent experiments have demonstrated that hyper-activation of the PI(3)K pathway by ectopic expression of a PI(3)K mutant endows these cells with the ability to proliferate in an anchorage-independent manner (Zhao et al., 2003), suggesting that TLM-HMECs are susceptible to transformation by a single genetic event. However, since recent evidence suggests that over-expressed oncogenes can confer different biological effects than oncogenes expressed at endogenous levels (Guerra et al., 2003; Tuveson et al., 2004), we sought to determine whether disruption of endogenous PI(3)K regulation is sufficient to elicit transformation. To this end, an shRNA directed against the PTEN tumor suppressor was introduced into TLM-HMECs by retroviral-mediated gene transfer. PTEN catalyzes the removal of the 3-position phosphate from PtdIns(3,4,5)P_3 and is a well-characterized antagonist of PI(3)K-dependent signals (Vivanco and Sawyers, 2002). PTEN-specific shRNA significantly reduced PTEN expression (Figure 1A). Importantly, reduced PTEN expression conferred robust anchorage-independent proliferation to a level similar to an activated mutant of PI(3)K (myr-p110α) (Figure 1B, right panel), thereby validating that RNAi-mediated loss-of-function of this tumor suppressor is capable of transforming TLM-HMECs.

To identify endogenous suppressors of epithelial cell transformation (SECT) genes (Figure 1C), we infected TLM-HMECs with a retroviral shRNA library we previously constructed in pSM1 (Paddison et al., 2004). This library consists of 28,000 sequence-verified shRNAs targeting ~9,000 genes, with each shRNA linked to a unique 60-nucleotide sequence (DNA "barcode"). These molecular barcodes can be used to monitor relative frequencies of individual shRNAs in complex populations via microarray technology (Hensel et al., 1995). TLM-HMECs infected with a control retrovirus or pSM1-library were assessed for anchorage-independent proliferation (Figure 1C). Only cells infected with the pSM1-library exhibited formation of macroscopic

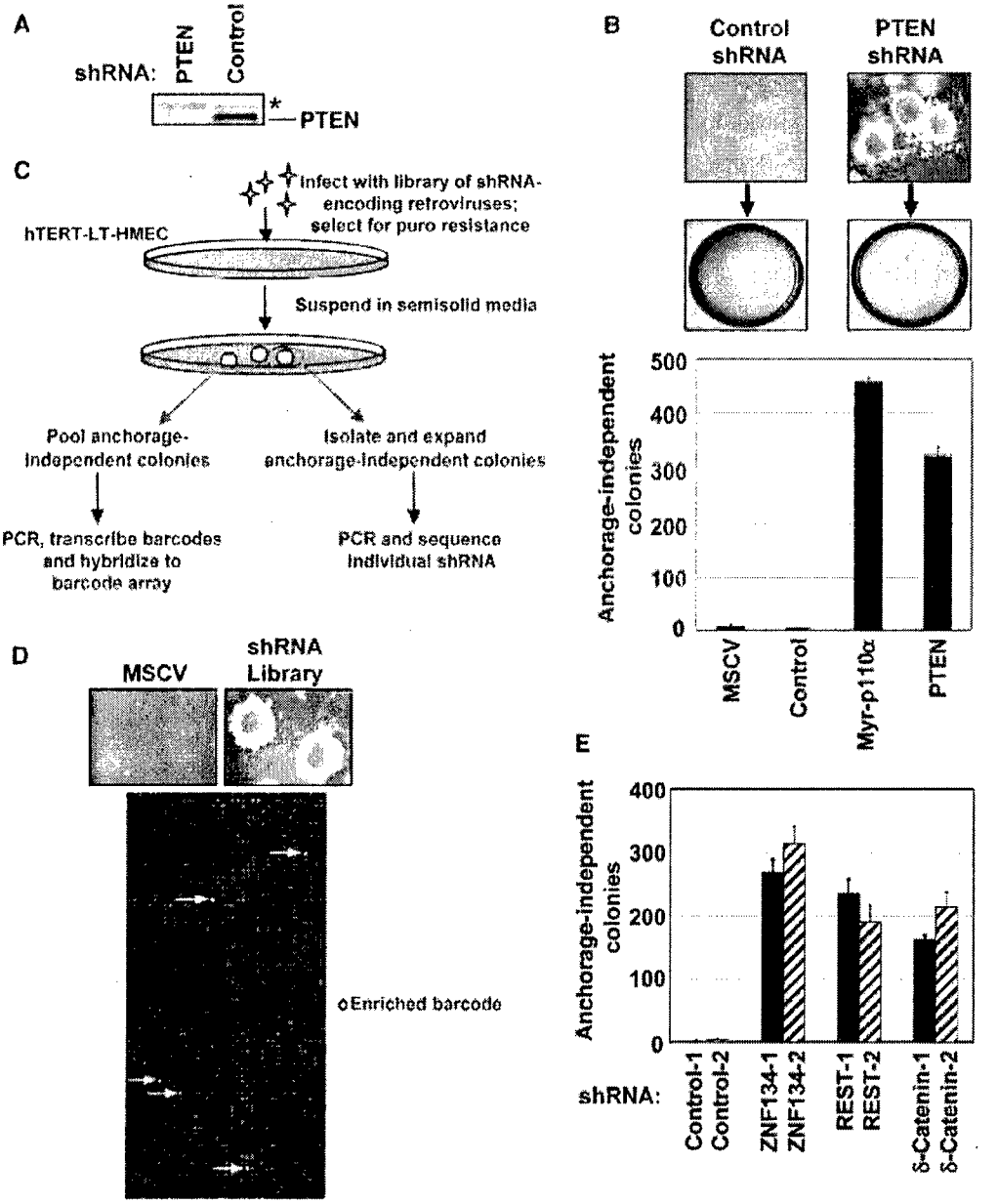


Figure 1. Identification of suppressors of epithelial cell transformation. (A) Cell lysates from TLM-HMECs expressing control- or PTEN-shRNAs were immunoblotted for expression of PTEN. The asterisk denotes a cross-reacting band that serves as a loading control. (B) Cells from (A) were cultured in semisolid media for 3 weeks and photographed at 20x (top panels) or 1x (bottom panels) magnification. Quantitation of macroscopic colony (>0.2mm) formation in semisolid media by TLM-HMECs transduced with empty retrovirus or virus encoding control shRNA, myr-p110α cDNA, or PTEN-shRNA. (C) Outline of the transformation screen. Details provided in Methods. (D) TLM-HMECs transduced with empty or pSM1-shRNA library and cultured in semisolid media for 3 weeks, photographed at 20x (top panels). Bottom panel illustrates a section of a barcode microarray. Barcodes were PCR-amplified from genomic DNA isolated from a pool of 100 anchorage-independent colonies. cRNA was transcribed from a total library preparation (red channel, 635nm) or from PCR-amplified barcode (green channel, 532nm) and hybridized to a barcode microarray. Enriched barcodes (green/yellow) are indicated by arrows. (E) TLM-HMECs expressing shRNAs targeting candidate genes (2 independent shRNAs per gene target) were cultured in semisolid media and quantitated for formation of macroscopic colonies. Experiments were performed in triplicate.

colonies in semisolid media (Figure 1D, top panels), indicating the presence of shRNAs that transform TLM-HMECs. Approximately 100 anchorage-independent clones were pooled and analyzed for the enrichment of barcodes linked to the individual shRNAs (Figure 1D, bottom panel). To support the results from the hybridization studies, we sequenced approximately 200 individual anchorage-independent clones isolated from pSM1-transductants (including 70 colonies used for the barcode microarray hybridization). Sequencing of the proviral shRNAs from these colonies identified twenty-five unique shRNAs. Importantly, these approaches yielded similar results, with 18 of the 25 genes revealed by sequencing of individual clones also identified by barcode microarray analysis. This is the first application of the barcode approach in a mammalian screen, and the high correspondence of identities with the sequenced clones indicates that such an approach harbors promise in more complex experimental designs. In particular, we are hoping we can use this bar coding method to perform synthetic lethal screens.

Most of the shRNAs identified in this screen target genes known or predicted to function in signal transduction or transcriptional regulation (Table 1), consistent with the

Table 1. Suppressors of Epithelial Cell Transformation

Gene	Previously Known Functions	Validated
CDH6*	Type II cadherin; cell-cell adhesion	+
CTNND2*	Stabilization of adherens junctions	+
INPP4B*	PIP2 phosphatase	ND
RASA4*	Ras GAP; calcium-responsive inhibitor of Ras signaling	ND
REST*	Transcriptional repression of neural genes	+
TGFBRII*	TGF-β signaling; cytostatic and apoptotic programs in epithelial tissues	+
VDAC2P*	None	–
ZNF134*	None	+
BCL9	WNT/β-catenin signaling	ND
MAP4K4	TNFα signaling; JNK activation	ND
PKN2	Rho signaling; Akt inhibition	ND
BDKRB2	G protein-coupled receptor	ND
LMO4	Transcriptional regulation; mammary gland development	ND
HAND1	Transcriptional regulation; cardiac morphogenesis	ND
AKT2	PI(3)K effector; survival signaling	ND
STAG3	Meiosis cohesion	ND
DUT	Nucleotide metabolism	ND
RPP30	tRNA processing	ND

This table lists gene targets of unique, sequence-verified shRNAs identified in 200 anchorage-independent colonies isolated from the screen. shRNAs identified in the context of double integrations (seven in total) were disregarded. Ninety percent of isolated anchorage-independent colonies encoded one of eight shRNAs (demarcated by *). For candidate validation, multiple shRNAs directed against independent sequences within a gene target were tested for transformation (ND, not determined).

role of these gene classes in regulating complex cell behaviors. While the majority of these genes have not been directly examined for their relationship to cancer pathogenesis, several are implicated in the regulation of cancer-relevant pathways. Notably, we identified an shRNA targeting CAPRI (*RASA4*), a calcium-sensing Ras GTPase Activating Protein (Ras-GAP) previously shown to inhibit Ras-dependent signaling (Lockyer et al., 2001). This is consistent with the ability of a constitutively-active Ras mutant to transform TLM-HMECs (J.Z. and T.R., unpublished observations). δ-catenin (*CTNND2*) and K-cadherin (*CDH6*) were also isolated (Table 1), suggesting a potential role for adherens junctions in preventing epithelial cell transformation. Strikingly, the type-II receptor for Transforming Growth Factor-β (TGF-β) was also identified, thus implicating the TGF-β tumor suppressor pathway in the control of epithelial cell transformation (see below).

The vast majority (90%) of colonies analyzed in this screen represented shRNAs directed against eight genes (first 8 in Table 1). Therefore, we focused our subsequent investigations on the gene targets of these frequently isolated and potentially more

penetrant shRNAs. In order to validate putative gene targets, we assessed the transforming potential of shRNAs targeting different regions of each candidate. Parental TLM-HMECs were transduced with shRNA-encoding retroviruses and measured for anchorage-independent proliferation. Importantly, for five of six candidate genes tested, two shRNAs transformed TLM cells (Figure 1E, Figure 2B, and data not shown), indicating that the observed phenotype was most likely attributable to reduced function of the intended target and not caused by off-target effects. In contrast, two independent shRNAs directed against the pseudogene VDAC2P did not recapitulate transforming potential of the library-derived VDAC2P-shRNA (data not shown), implying that these shRNAs did not alter VDAC2P expression or that the library-derived shRNA targeted the expression of other genes underlying the transformed phenotype. Consistent with the latter hypothesis, VDAC2P expression was undetectable in TLM-HMECs (data not shown).

Endogenous TGF-β Signaling Suppresses Cellular Transformation

As negative regulators of oncogenic transformation, genes isolated in this screen may represent candidate tumor suppressor genes or impinge upon pathways critical to the genesis of cancer. Notably, this approach identified the Transforming Growth Factor-β Receptor II (TGF-βRII) as a regulator of transformation (Table 1). Observations in mouse models and in human tumors indicate that the TGF-βRII (as well as several components of TGF-β signaling) is a potent tumor suppressor in numerous tissues including the mammary and colonic epithelia (Derynck et al., 2001; Siegel and Massague, 2003). The TGF-β pathway is a potent inhibitor of epithelial cell proliferation, but has not been previously implicated in regulating oncogenic transformation *in vitro*. To verify the role of TGF-βRII in suppression of transformation, two retroviral-encoded shRNAs targeting independent sequences within TGF-βRII were introduced into TLM-HMECs. These shRNAs reduced TGF-βRII expression levels and impaired phosphorylation of SMAD2, a substrate and transducer of endogenous TGF-β receptor signaling (Figure 2A). Importantly, TGF-βRII-targeted shRNAs also elicited robust anchorage-independent proliferation in TLM cells (Figure 2B), thus validating the identification of TGF-βRII in our screen. Reduced TGF-β signaling did not alter proliferation on an adhesive cell culture surface (Figure 2C), suggesting that loss of extracellular matrix (ECM) interactions may induce TGF-β signaling or alter the threshold of endogenous TGF-β signaling necessary to elicit a cytostatic response.

To further examine the role of endogenous TGF-β signaling in restraining cell transformation, we inhibited TGF-β signal transduction by alternative mechanisms and assessed the consequences on anchorage-independent proliferation. TLM-HMECs were transduced with retroviruses encoding a previously characterized dominant-negative mutant of TGF-βRII or SMAD7, a negative regulator of TGF-β receptor signaling (Siegel and Massague, 2003). Expression of either cDNA conferred growth in semisolid media (Figure 2D), indicating that the transforming capacity of TGF-βRII shRNAs is not an RNAi-specific phenomenon. Conversely, ectopic activation of TGF-β signaling by a constitutively active mutant of TGF-βRI (T204D) was able to restrain anchorage-independent proliferation elicited by PTEN knockdown (Figure 2E), suggesting a genetic interaction between PI(3)K and TGF-β signaling in the context of cell transformation.

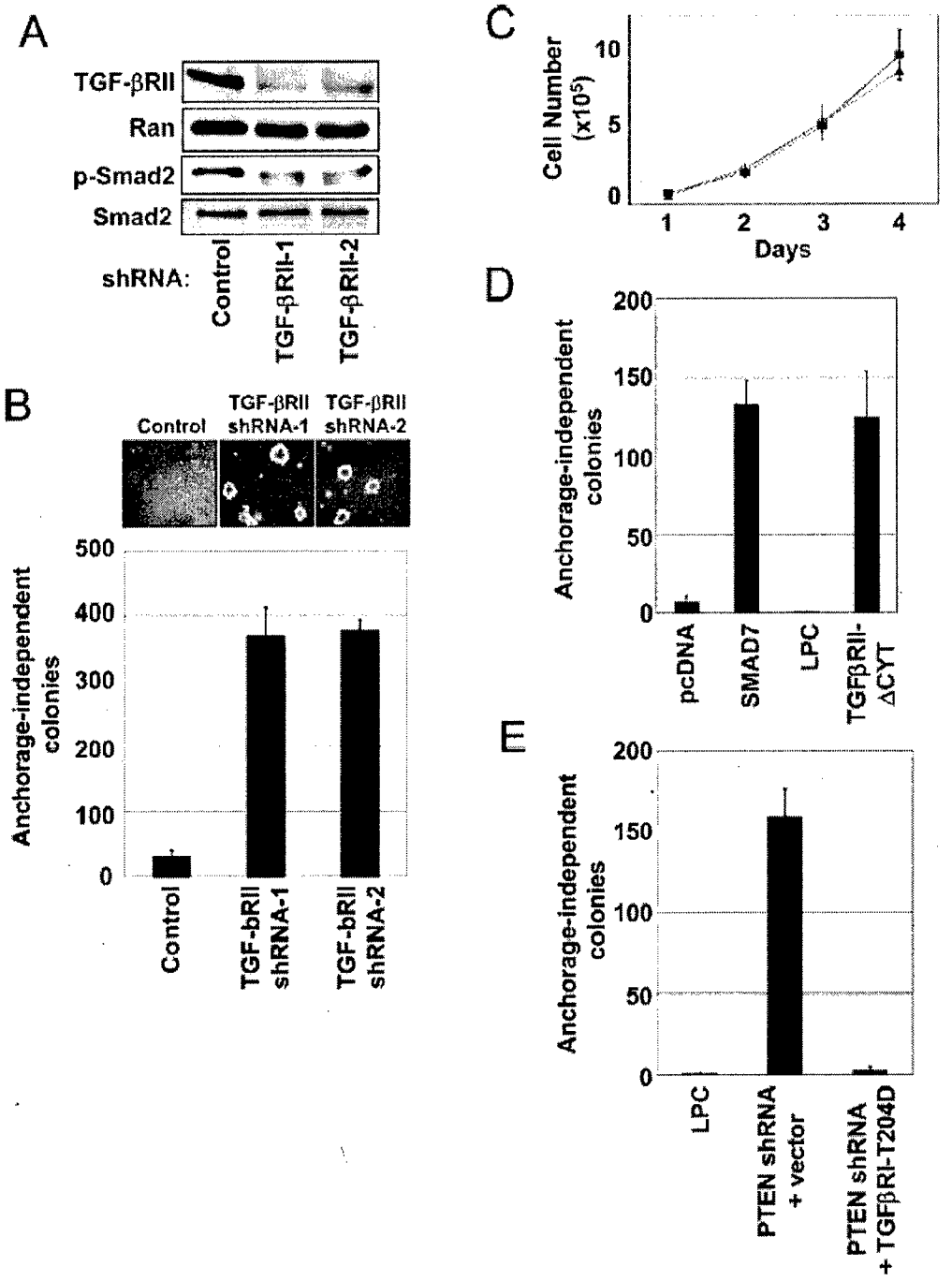


Figure 2. TGF-β signaling suppresses epithelial cell transformation. (A) Cell lysates from TLM-HMECs expressing control- or TGF-βRII-shRNAs were immunoblotted for expression of TGF-βRII, Ran (loading control), serine-465/467- phosphorylated SMAD2, or total SMAD2. (B) Cells from (A) were cultured in semisolid media for 3 weeks, photographed at 20x, and quantitated for formation of microscopic colonies. (C) Growth curves of cells expressing control (_), or two independent TGF-βRII-shRNAs (_ or _) grown in monolayer culture. (D) TLM-HMECs were stably transfected with empty plasmid (pcDNA3) or plasmid expressing SMAD7 or transduced with empty retrovirus (LPC) or retrovirus encoding a dominant-negative mutant of TGF-βRII (TGFβRII-ΔCYT). Resulting polyclonal cell lines were assayed for anchorage-independent proliferation. (E) TLM-HMECs were transduced with control (LPC) or PTEN-shRNA encoding retrovirus. PTEN-shRNA expressing cells were subsequently infected with empty retrovirus or virus expressing a constitutively active mutant of TGFβRI (TGFβRI-T204D). Each derivative cell line was assessed for proliferation in semisolid media.

This observation is interesting in light of evidence from several systems demonstrating that TGF-β and PI(3)K signals are integrated at multiple levels to regulate survival and proliferation (Conery et al., 2004; Remy et al., 2004; Seoane et al., 2004). Further investigation is required to determine the functional nodes through which these two pathways interact during HMEC transformation.

Inactivation of REST in Human Tumors

Consistent with a role in suppressing oncogenic transformation, four of the SECT genes we identified in the screen or initial experiments are either established tumor suppressors (TGF-βRII and PTEN) or regulators of cancer-relevant signaling machinery including the Ras proto-oncogene (*RASA4*) and cadherin complexes (*CTNND2*). Therefore, it is probable that other genes identified in our screen also represent candidate tumor suppressors and may be found altered in tumors. A hallmark of tumor suppressor loci is their high frequency of loss of heterozygosity (LOH) in tumors. Consequently, we examined whether our candidate genes reside in genetic loci targeted for such chromosomal aberrations in human tumors. Array-based comparative genomic hybridization (aCGH) has evolved into a high-throughput method for cataloguing such copy number aberrations (CNAs) with high resolution. To this end, aCGH has been successfully utilized to characterize genomic alterations in the context of pancreatic adenocarcinoma(Aguirre et al., 2004), and more recently used to define CNAs in a large collection of human colon tumors and cell lines (Martin/DePinho, manuscript in preparation). We wish to apply this information to breast tumor arrays but the CAN data was not available at the time of this study. As described (Aguirre et al., 2004), overlapping CNAs from individual colon tumor samples and tumor-derived cell lines were used to define minimal common regions (MCRs) of loss or gain. These discrete MCRs were further prioritized based on parameters of confidence and significance including: (1) recurrence in multiple independent samples, (2) high Log_2 ratio of change (e.g. depth of deletion), (3) focal nature (e.g. MCR $\leq$ 2.0Mb), and (4) MCR encompassing no more than 5 annotated genes (Aguirre et al., 2004). From this analysis, nine high-confidence MCRs of recurrent focal deletions were identified within the colon cancer genome, in sum representing only 34 annotated genes (Figure 3A). Consistent with its role in the pathogenesis of human cancers (Ruas and Peters, 1998), the $p16^{INK4A}$ tumor suppressor was present within one of these focal deletions (Figure 3A).

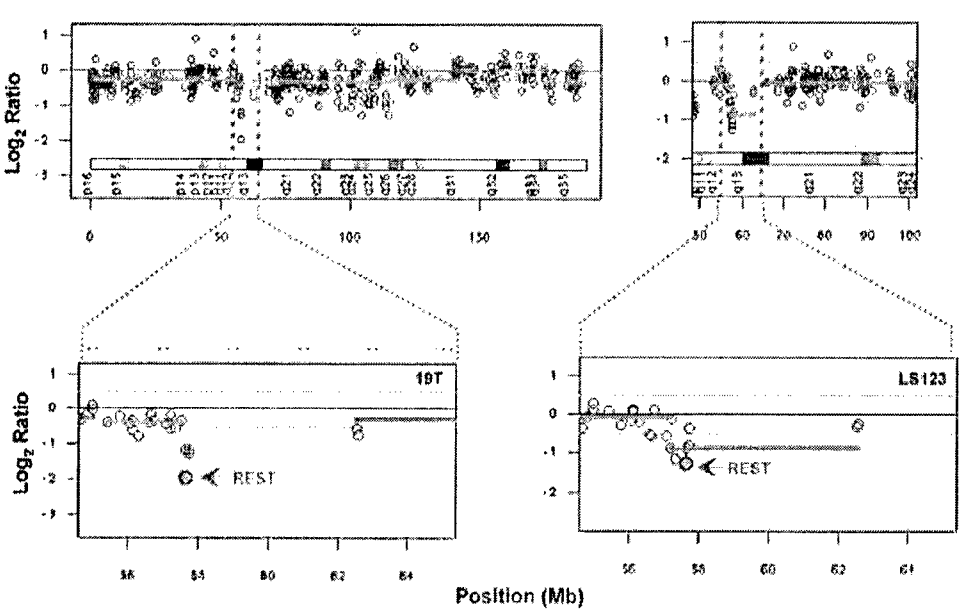
Remarkably, without *a priori* knowledge of the candidate genes listed in Table 1, this genomic approach identified high-confidence MCRs that included two highly penetrant candidates from our RNAi screen: *TGFBR2* and *REST* (Figure 3A). RE1-Silencing Transcription Factor (REST)/ Neuron Restrictive Silencing Factor (NRSF) is a transcriptional regulator best characterized for its role in repressing neuronal genes including neurotrophins and cell-adhesion molecules in non-neuronal tissues (Chong et al., 1995; Schoenherr and Anderson, 1995). Intriguingly, a variety of human tumors including those arising in breast, ovary, and lung activate expression of neuron-specific genes. In some instances, the inappropriate expression of these neural genes elicits an autoimmune response that culminates in neurological disorders, collectively known as paraneoplastic neurologic degenerations (PND; see discussion) (Albert and Darnell, 2004). Such aberrant neural gene expression suggests that these cancers harbor defects in regulators of neuronal programs. Consequently, we investigated a potential role for

A

High Confidence Loci in Colon Cancer

Chromosome	Minimum Common Region (Mb)	Peak Probe Value (log_2ratio)	# of Genes	Candidate Tumor Suppressor
3	1.66	-2.98	4	TGFBR2
4	0.22	-1.22	5	REST
8	0.41	-1.31	4	
9	0.55	-1.22	4	CDKN2A ($p16^{INK4A}$)
10	0.84	-1.24	2	
11	0.08	-4.12	2	
14	0.61	-1.57	5	
17	0.23	-3.4	5	
21	0.15	-1.36	3	

B



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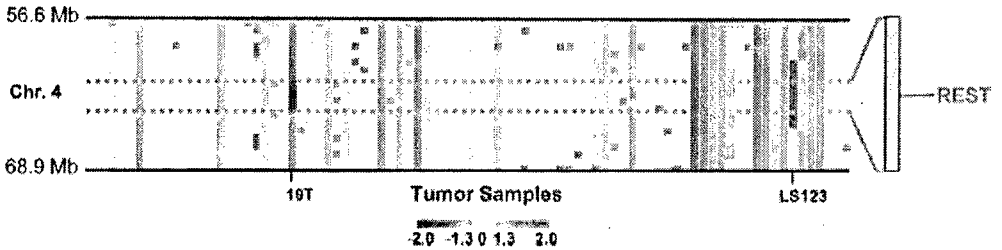

Figure 3. Deletion of REST in human tumors. (A) High-confidence recurrent CNAs in colon cancer. Recurring focal CNAs in colon adenocarcinoma and tumor cell derivatives were identified and prioritized as previously described(Aguirre et al., 2004) with modifications. Each of the identified minimum common regions (MCR) consists of <2.0Mb and ≤ 5 annotated genes. Peak probe values represent the minimum absolute log_2-ratio detected within each MCR. Also shown are the chromosomal location, the number of NCBI annotated genes residing within the MCR, and candidate tumor suppressor genes within each MCR. SECT genes are highlighted in blue. (B) aCGH profiles of chromosome 4 in a primary tumor (19T) and a colon cancer cell line (LS123) showing deletion of a discrete region, with MCR defined by vertical red lines. Raw data and segmentation analysis are represented by blue circles and red horizontal lines, respectively. (C) Segmentation data of aCGH profiles from each of 42 primary colon tumors and 38 colon tumor-derived cell lines. Chromosomal gain or loss within a section of chromosome 4 is represented by a color gradient (red=gain, blue=loss). Gray boxes represent uninformative probe hybridizations.

REST in human tumor suppression. Deletions of varying size encompassing the *REST* locus on chromosome 4 were detected in a significant proportion of tumors, with evidence of genetic loss in 14 of 42 primary tumors and in 13 of 38 cell lines (Figure 3C), suggesting that chromosomal deletions targeting the *REST* locus are a frequent event in colon cancer. Importantly, microdeletions encompassing the *REST* gene were detected in a primary tumor specimen (CRC_19T) and a colon cancer cell line (LS123), thus defining a minimal common region. This MCR (Figure 3B, top panels) encompasses only five known genes, with the *REST* gene residing at the peak amplitude of each of these focal deletions (Figure 3B, bottom panels). Collectively, the unbiased identification of *REST* as a target of recurrent microdeletions as well as frequent larger deletions strongly suggest that *REST* is targeted for inactivation during colon cancer pathogenesis.

REST is widely expressed throughout non-neuronal tissues including the colonic epithelium (Supplemental Figure 1) (Chong et al., 1995; Schoenherr and Anderson, 1995). The above results suggest that loss of REST expression may confer a selective advantage during the evolution of tumor cells. This hypothesis predicts that cells with defective REST function may be sensitive to reconstitution of REST. We examined this prediction by ectopically expressing REST in colon cancer cells that have lost (SW1417) or retain (SW620) endogenous REST expression (Figure 4A). Exogenous REST expression elicited a mild decrease in the proliferation of SW620 colon cancer cells (Figure 4B). In contrast, ectopic REST expression significantly reduced colony formation in SW1417 cells (>50-fold; Figure 4B), indicating that these cells are highly dependent on the absence of REST for their proliferation *in vitro*. Coupled with the function of REST in suppressing epithelial cell transformation (Figure 1E), these data strongly support a role for REST in tumor suppression.

In order to establish a more causal relationship between disruption of REST function and tumorigenesis, we analyzed primary colon tumors and colon tumor cell lines for the presence of mutations within the *REST* coding region. We sequenced exons 2-4 of the *REST* gene in a total of 86 colon cancers (48 tumors and 38 cell lines). We identified a single-nucleotide deletion within *REST* exon 4 of cells derived from a colorectal adenocarcinoma (DLD-1)(Figure 4A). This frameshift mutation results in a premature termination in the center of the coding sequence, yielding a protein with a predicted size of approximately 85kDa. This frameshift mutant, referred to as REST-FS, was detected by N-terminal REST antibodies in lysates from DLD-1 cells but not that of other colon cancer cell lines (Figure 4B, top panel). To confirm this, the frameshift mutation was engineered into a Flag-tagged REST cDNA and stably introduced into cells. Expression of an 85kDa protein was detected using REST- (Figure 4B, bottom panel) and FLAG-specific antibodies (Figure 4C, top panel).

Tumor cells harboring this frameshift mutation also encoded and expressed a wild-type REST allele (data not shown). Unfortunately, the primary tumor from which these cells were derived is not available, preventing analysis to determine whether the frameshift mutation occurred *in vivo*. However, we hypothesized if expression of REST-FS was important to the genesis of this cancer, the mutant allele should exhibit dominant-negative activity. REST-FS lacks the C-terminal repressor domain that interacts with CoREST, a corepressor molecule that mediates transcriptional repression and silencing by REST(Andres et al., 1999). To determine if REST-FS displays properties of a dominant negative protein, we transduced the HMEC derivative (TLM cells) used in our

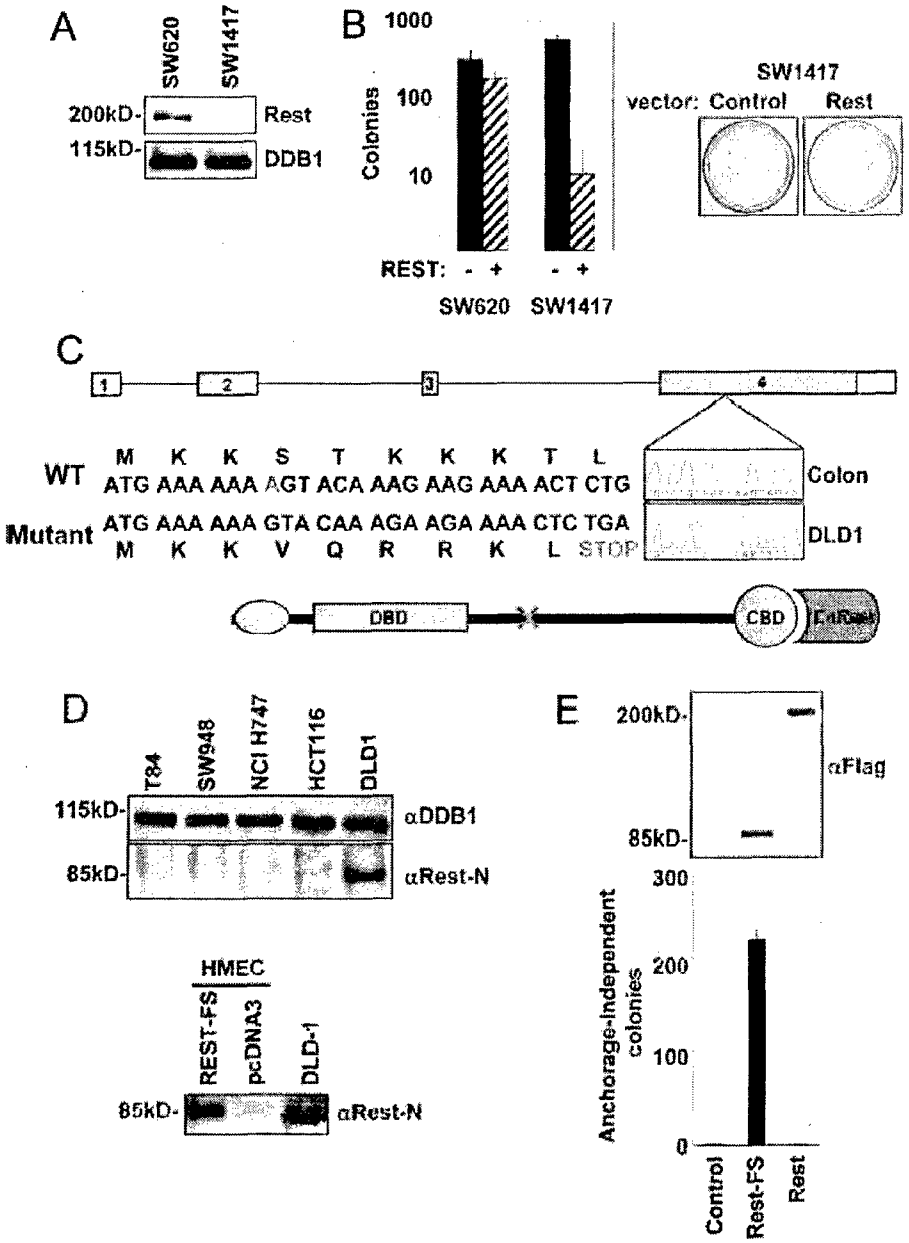

Figure 4. Aberrant REST function in colon cancer. (A) REST expression was analyzed in cell lysates from SW620 and SW1417 colon cancer cell lines antibodies recognizing the C-terminus of REST. (B) SW620 and SW1417 cells were transduced with control- or REST-expressing retrovirus, seeded at 5,000 cells per dish, and cultured for 2 weeks under puromycin selection. (C) Single nucleotide deletion within exon 4 of the *REST* gene (top schematic) was observed in DLD-1 cells by sequence analysis. The premature termination (indicated by red x, bottom schematic) results in a protein retaining the DNA-binding domain (DBD) and N-terminal SIN3-binding domain, but lacking the repressor domain responsible for interaction with CoRest (CBD). (D) The presence of truncated REST was analyzed by immunoblotting with antibodies recognizing the N-terminus of REST (top panel) or DDB1 (loading control, middle panel) against cell lystates from colon cancer cell lines. Protein lysates derived from HMECs transfected with empty vector (pcDNA3) or vector encoding the frameshift REST mutant (REST-FS) were probed with antibodies raised against the N-terminus of REST (bottom panel). (E) TLM-HMECs transduced with control retrovirus or retroviruses encoding flag-REST or flag-REST-FS were analyzed for exogenous cDNA expression using flag-specific antibodies (top panel) or assayed for anchorage-independent proliferation (bottom panel).

initial screen with wild-type or mutant REST and assessed anchorage-independent proliferation. Cells expressing REST-FS, but not wild-type REST, exhibited robust colony formation (Figure 4C, bottom panel). The ability of REST-FS to phenocopy the transforming activity of REST shRNAs strongly suggests that this mutant interferes with the functions of endogenous REST that restrain epithelial cell transformation. Taken together, these data provide strong support for the hypothesis that REST is a tumor suppressor in human cells.

REST Suppresses PI(3)K Signaling

The implication that REST is involved in regulating the transformed state of epithelial cells led us to determine which molecular circuits might be affected by loss of REST function. Activation of PI(3)K-dependent signaling by a variety of mechanisms has been shown to confer transformation in HMECs (Zhao et al., 2003), indicating that this pathway provides a potent stimulus to the transformed phenotype. Furthermore, deregulation of PI(3)K signaling occurs in a wide spectrum of human cancers (Vivanco and Sawyers, 2002). As such, we examined the impact of disrupting REST function (Figure 5A) on the activation of the PI(3)K pathway. TLM-HMECs expressing control- or REST-shRNA were deprived of growth factors, restimulated with EGF for various times, and subsequently analyzed for activating phosphorylation of Akt (serine-473), an essential effector of PI(3)K signaling. Stimulation of Akt phosphorylation by EGF was enhanced in cells expressing REST-shRNA throughout the timecourse (Figure 5B). Consistent with increased signaling downstream of Akt, phosphorylation of ribosomal S6 protein and translational inhibitor 4E-BP1 was also upregulated in cells expressing REST shRNA (Figure 5C). In agreement, recent experiments have demonstrated that ectopic expression of eIF-4E (the target of 4E-BP1) confers transformation to mammary epithelial cells and promotes tumor formation *in vivo* (Avdulov et al., 2004; Wendel et al., 2004). These results indicate that impaired REST function confers an increase in both the intensity and duration of PI(3)K-dependent signaling. In order to investigate whether PI(3)K signaling is required for REST-shRNA induced transformation, we utilized a dominant-negative mutant of the PI(3)K regulatory subunit, p85. This mutant (referred to as Δp85) has previously been shown to abolish H-Ras^{V12}-induced and SV40 st-induced cell transformation (Rodriguez-Viciana et al., 1997; Zhao et al., 2003). TLM-HMECs expressing control-, PTEN-, or REST-shRNAs were transduced with a control or Δp85-encoding retrovirus. Expression of Δp85 (Figure 5D, top panel) did not alter growth of cells on an adhesive cell culture surface (data not shown). Consistent with the role of PTEN as an antagonist of PI(3)K signaling, Δp85 abrogated anchorage-independent growth of PTEN-shRNA cells (Figure 5D, bottom panel). Similarly, REST-shRNA induced transformation was inhibited in the presence of Δp85 (Figure 5D, middle and bottom panels), indicating that PI(3)K signaling is required for transformation conferred by loss of REST. Coupled with the aberrant activation of PI(3)K-dependent signals in response to reduced REST expression, these results suggest that inhibition of PI(3)K signaling might be a mechanism through which REST suppresses transformation and tumorigenesis.

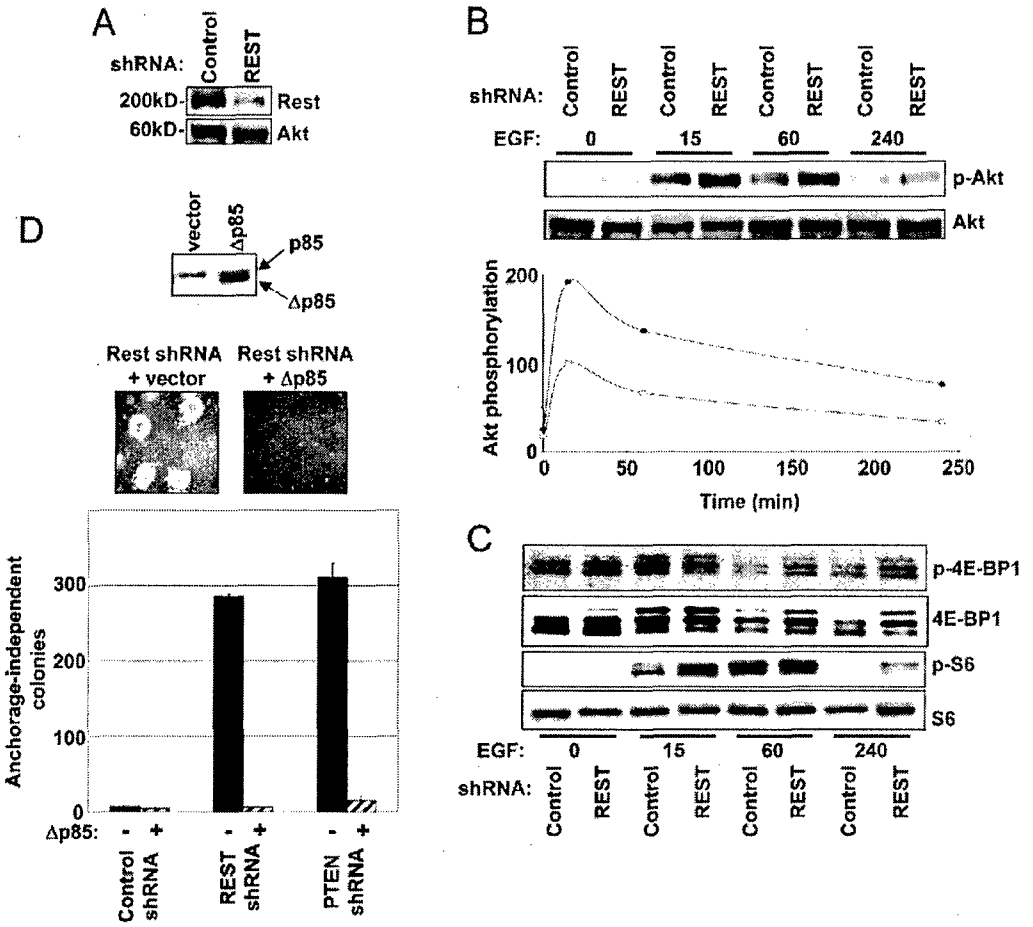

Figure 5. Loss of REST function stimulates PI(3)K signaling and requires it for HMEC transformation. (A) Cell lysates from TLM-HMECs expressing control- or REST-shRNAs were immunoblotted for expression of Akt (loading control, bottom panel) or REST (C-terminal antibody, top panel). (B) TLM-HMECs expressing control or REST-shRNA were starved in basal media without growth factors for 20 hours and restimulated with 10ng/mL EGF for indicated times. Cell lysates were immunoblotted with antibodies recognizing p-Akt (ser473) or total Akt. Akt phosphorylation was measured as a ratio of p-Akt/total Akt and represented as a percentage of phosphorylated Akt at 15 minutes of EGF stimulation in control-shRNA cells (control-shRNA _, REST-shRNA_). (C) Cell lysates from (A) were probed with antibodies recognizing p-S70 of 4E-BP1, total 4E-BP1, p-S235 and p-S236 of S6, and total S6 as indicated. (D) REST loss requires PI3K signaling for transformation. TLM-HMECs expressing control, REST, or PTEN-shRNA were transduced with empty or Δp85-expressing retrovirus. Immunoblot analysis of endogenous p85 and exogenous Δp85 as indicated (top panel). Cells were analyzed for anchorage-independent proliferation (middle and bottom panels).

In addition to this description of using siRNAs to identify potential tumor suppressors, we have generated an entire new library of shRNAs which we call the second generation library in a novel vector pSM2 (Silva et al., 2005). This new generation of shRNA libraries (shRNA^{mir}) takes into consideration our advancing understanding of microRNA biogenesis. As has previously proven successful in plants and in animals, second-generation shRNA^{mirs} are modeled after endogenous microRNAs, specifically being harbored within the backbone of the primary mir-30 microRNA. This natural configuration proved to be up to 12 times more efficient in the production of the mature synthetic miRNAs than simple hairpin designs. Additionally, we have

biochemically characterized processing of these synthetic microRNAs, allowing us to predict the mature small RNA product(s) that will be generated from each vector. This has allowed selection of target sequences that maximize efficiency by directing preferential incorporation of the correct strand into RISC. Using these criteria, we have produced and sequence-verified more than 140,000 shRNAs covering a substantial fraction of the predicted genes in the mouse and human genomes. We have assayed a selected subset of shRNAs from the library for their ability to knock-down the expression of targeted genes by quantitative RT-PCR. We have also tested this set in a phenotypic assay and compared the performance of the first- and second-generation library designs. Overall, the shRNA^{mir} libraries that we have constructed provide a convenient, flexible and effective tool for studying gene function in human cells. We feel this new library will be much more thourough in identifying genes relevant to breast cancer in the future.

In addition to the generation of this new library, we have been working on the generation of vectors that work well in single copy to knock down gene expression. The reason single copy knockdown is so important is that it is essential if one wants to use bar codes to screen for genes that drop out of a population. In order for a particulat shRNA retrovirus to disappear from a library of infected cells because it is toxic, it is necessary that the majority of the individual viral insertions express enough shRNA to produce a phenotype. In our version one library we selected for hairpins that functioned, so rare high expressors will come through the screen. However, usually these version one vectors do not work well at single copy on the average. To get around this we designed a new series of Lenti virus vectors, the PRIME vectors, that express the shRNA-Mir30 from a strong polII promoter, CMV. We found that the inclusion of GFP in between the CMV and mir30 construct in the same transcript made the shRNA much more potent (Stegmeier et al., 2005). This allowed us to observe significant knockdown at single copy.

Key research accomplishments

1. Generation of a shRNA version 1 library.
2. Use of this library in a human mammary epithelial transformation model to identify new tumor suppressor genes.
3. Identification of several genes including PTEN, TGF-beta Receptor II and the transcriptional repressor REST among 20 others that can suppress transformation of mammary cells.
4. Generation of version 2 library in a micro-RNA based vector pSM2 that contains over 140,000 shRNAs to human and mouse genomes.
5. Generation of the PRIME vector series of Lentiviruses that give very good single copy knockdown of gene expression. This is essential for synthetic lethality bar code screens.

With respect to the Statement of Work, we have accomplished the goals of year 1 and part of year 2 shown below.

Statement of Work.
Year 1.

Task 1 (Months 1-12)
In the first year we anticipate beginning to work out the conditions for using the bar coding method to follow retroviruses containing hairpins as mixtures in complex libraries. We now have a library of 22,000 hairpins covering about 8,000 genes. We will be performing exploratory screens and optimizations to test the quality of the barcoding method. We must have this method working well to carryout the synthetic lethal screens.

We accomplished this goal in two ways. The first is we performed a bar code screen for potential tumor suppressors and identified several genes described above. Secondly, we have improved our vectors to allow single copy knockdown as described in Stegmeier et al. 2005. This is absolutely essential for the bar coding experiments we have proposed to kill cancer cells.

Task 2 (Months 1-24 and possibly longer, an ongoing effort)
We will continue to expand the library during this period to encompass more genes. This will be done in collaboration with Dr. Greg Hannon.

We have accomplished this goal by the generation of a second generation library in the mir30 context as described in Silva et al., 2005. This covers 140,00 human and mouse shRNAs.

Task 3 (Months 6-24)
We also will begin the process of analyzing the human genome for coding sequences to set up the bio-informatics analysis to generate a list of sequences we wish to express to look for auto-antibodies. We should begin synthesizing oligo nucleotides to cover human genes.

We have performed the analysis and designed oligonucleotides to cover the human genome. We have also cloned those oligos and are ready to begin devising ways to express them. We have decided against expressing the oligos on microarrays but in stead plan to express them using phage display and immunoprecipitate the peptides recognized by autoantibodies. These purified epitopes will be recovered by PCR and deconvoluted by microarray hybridization. Thus, we willl NOT be purchasing a microarrayer as originally proposed. Instead we will use the funds for the microarrayer to purchase microarrays to deconvolute out immunoprecipitations.

Year 2.

Task 4 (Months 13-24)
In this period we plan to begin to carryout screen to look for genes which when knocked down by shRNA will interfere with the growth of cells containing defined mutations that lead to breast cancer. We will start with known tumor suppressors such as loss of p53 and Rb. We will use the barcoding methods. We may also screen for genes that sensitize cells to killing by gamma IR.

We have not yet begun this aim but hope to soon.

Task 5 (Months 18-36)
We will begin to synthesize shRNA clones corresponding to the mouse genome.

We have gone a long way toward accomplishing this aim, having about 60,000 sequence verified shRNAs in our version 2 library already.

Task 6 (Months 12-24) We will expand the library of short coding regions for the autoantibody project and work out conditions to express these protein fragments in bacteria in a high through-put fashion.

We are ready to begin this task but have only just cloned the oligos which we had synthesized on microarrays.

Reportable outcomes

1. Westbrook, T.F., Martin, E.S., Schlabach, M.R., Leng, Y., Liang, A.C., Feng, B., Zhao, J.J., Roberts, T.M., Mandel, G., Hannon, G., DePinho, R., Chin, L., and S.J. Elledge (2005) A Genetic Screen for Candidate Tumor Suppressors Identifies REST. *Cell* 121:837-48.
2. Silva, J.M., Li, M.Z., Chang, K., Ge, W., Golding, M.C., Rickles, R.J., Siolas, D., Hu, G., Paddison, P.J., Schlabach, M.R., Sheth, N., Bradshaw, J., Burchard, J., Kulkarni, A., Cavet, G., Sachidanandam, R., McCombie, W.R., Cleary, M.A., Elledge, S.J., Hannon, G.J. (2005) Second-generation shRNA libraries covering the mouse and human genomes. *Nat. Genet.* 37:1281-8.
3. Stegmeier, F., Hu, G., Rickles, R.J., Hannon, G.J. and Elledge, S.J. (2005) A lentiviral microRNA-based system for single-copy polymerase II-regulated RNA interference in mammalian cells. *Proc. Natl. Acad. Sci. USA.* 102:13212-7.

Conclusions

Suppressors of Epithelial Cell Transformation

In the study described above, we applied an shRNA-based genetic approach to identify genes suppressing oncogenic transformation of human mammary epithelial cells. Within the context of this model, we have identified two previously established tumor suppressor genes (*PTEN* and *TGFBR2*) that actively restrain HMEC transformation. In addition, we have identified several genes that impinge upon pathways implicated in cancer pathogenesis. For example, we isolated the calcium-sensing Ras-GAP, CAPRI (*RASA4*), a previously described negative regulator of the Ras proto-oncogene (Lockyer et al., 2001). Consistent with a role for regulation of Ras signaling in our experimental system, activated Ras can transform TLM-HMECs. Likewise, identification of δ-catenin (*CTNND2*), a member of the p120 catenin family that regulates cadherin stability and trafficking (Reynolds and Roczniak-Ferguson, 2004), implicates a role for adherens junctions in constraining transformation of HMEC. Notably, disruption of adherens junction components has been shown to alter several growth-regulatory pathways (eg. β-catenin, PI(3)K) and has been linked to cancer progression in a variety of tissues (Cavallaro and Christofori, 2004; Vasioukhin et al., 2001).

The novelty of this genetic approach is in the unbiased identification of new and unanticipated tumor suppressor functions. In this regard, our studies provide significant evidence for the identification of a novel tumor suppressor, the transcriptional repressor REST/NRSF (see below).

Involvement of REST/NRSF in Human Cancer

Transcription factors often coordinately control complex programs of gene expression during development, and as such, are logical candidates underlying the aberrant activation of developmental programs in cancer. Here, we present several lines of evidence that REST may play a role in tumor suppression in humans. First, reduced

REST function (mediated by RNAi or expression of dominant-negative REST) promotes transformation of human epithelial cells. Conversely, reconstitution of REST expression elicits a dramatic proliferation defect in colon cancer cells that have lost endogenous REST function. Strikingly, an independent aCGH-based search for genomic loci characterized by recurrent microdeletions identified the REST locus as a high-confidence target in colorectal cancer. This high-confidence list includes two previously established tumor suppressors, $p16^{INK4A}$ and TGFβRII (Derynck et al., 2001; Ruas and Peters, 1998; Siegel and Massague, 2003), the latter of which was also identified as a SECT gene. In addition, larger deletions encompassing the REST gene were frequent in colon tumors and tumor cell lines. Furthermore, we isolated a frameshift mutation of the REST coding region in colorectal tumor cells that displays properties of a dominant-negative, transforming human epithelial cells to a level similar to REST shRNAs. Finally, we show that impaired REST function enhances the intensity and duration of PI(3)K signaling, a pathway that is aberrantly activated in many if not all human cancers(Vivanco and Sawyers, 2002). Importantly, PI(3)K activity was required for cellular transformation conferred by reduced REST function, indicating that suppression of PI(3)K signaling may be an important mechanism underlying the ability of REST to restrain the transformed state. The mechanism by which REST inhibits this oncogenic pathway is not yet clear. REST regulates a complex transcriptional program, and as such, may impinge on PI(3)K signaling through multiple networks. However, it should be noted that BDNF and other neurotrophins are among the transcriptional targets repressed by REST. BDNF activates the TrkB receptor and has recently been shown to suppress anoikis via PI(3)K-dependent pathways (Douma et al., 2004), providing a plausible mechanism for activation of PI(3)K signaling in the absence of REST. Taken together, these data provide compelling support for a role for REST in human tumorigenesis and further validate the genetic approach we have undertaken.

Deregulation of neuronal programs has been implicated in cancer through the phenomena of paraneoplastic neurological degenerations (PNDs) (Albert and Darnell, 2004). In these diverse neurological disorders, tumors originating in non-neuronal tissues activate expression of neural peptides that elicit an immune response to the tumor as well as the host nervous system. Although the expression of some of these neural antigens is common or universal in some cancers (breast, ovarian, small-cell lung cancers) (Albert and Darnell, 2004), the occurrence of PNDs in cancer patients is rare, suggesting that other factors play a role in the autoimmune response. Nonetheless, aberrant activation of neuronal gene expression raises the possibility that regulators of neurogenesis are malfunctioning in these tumors. To investigate this possibility, we searched for previously identified PND antigens among known REST targets and discovered RE1-binding sites in several PND antigen encoding genes. Furthermore, promoters of two of these PND antigens (synaptotagmin and glutamic acid decarboxylase) were recently shown to be directly bound by REST, and moreover, transcription of synaptotagmin was induced upon expression of a dominant-negative REST mutant similar to the one we identified in tumors (Ballas and Mandel, in press). This supports the hypothesis that defects in the REST pathway are tied to PND pathology and tumorigenesis. In further support of a role in tumorigenesis, REST expression was recently shown to be absent in a subset of SCLC cells (Neumann et al., 2004). Additionally, microarray profiling also demonstrates down-regulation of REST expression in prostate and small cell lung

cancers, two malignancies that often display distinct neuroendocrine phenotypes (Dhanasekaran et al., 2001; Garber et al., 2001; Rhodes et al., 2004). These findings suggest that REST may play a broader role in human malignancies in addition to colorectal cancer. In particular, many breast cancers show neural gene expression signatures which could be due to disruption of the REST pathway.

Genetic Screening for Tumor Suppressor Pathways

While further studies will be needed to determine the extent to which REST, or other genes we have identified, are involved in human cancer, our results point to the utility of this approach for identifying recessive cancer relevant genes. Although many of the genes isolated via this strategy may not be frequent targets of mutation in tumors, they may nonetheless reveal novel pathways relevant to tumorigenesis. While we have identified several suppressors of cellular transformation, it is clear that this screen was not saturated, and consequently, there remains significant potential for this method in the future discovery of SECT genes. Underscoring this, the shRNA library used in our screen was designed to target only ~9,000 genes, representing less than one-third of the annotated genes in the human genome. Furthermore, only one shRNA corresponding to any given SECT gene was isolated in this screen. For several of these genes, multiple corresponding hairpins were present within the pSM1 library. This suggests that many SECT candidates were not identified, because this library lacked a sufficiently penetrant shRNA to elicit the transformed phenotype. This is not surprising since the pSM1 library was constructed before many of the parameters affecting siRNA efficiency had emerged. For instance, the stability of siRNA ends has been shown to bias the incorporation of sense/anti-sense strands into the RISC complex (Khvorova et al., 2003; Schwarz et al., 2003). Such siRNA design "rules" improve the potency of target gene suppression and will undoubtedly be incorporated into future generations of mammalian shRNA libraries, thus providing more potent tools in identifying SECT genes.

SECT candidates identified in this screen were selected for their transforming capacity in cooperation with the genetic milieu of TLM-HMECs (ectopic expression of hTERT, SV40 LT, and elevated endogenous c-myc expression). However, distinct classes of SECT genes are likely to be revealed in models incorporating alternative combinations of genetic perturbations. Additional SECT candidates may also be discovered in transformation models of alternative cell types, reflecting the different signaling requirements in cells derived from various human tissues (Hamad et al., 2002; Rangarajan et al., 2004), or by interrogating different cancer relevant phenotypes such as invasion, migration or angiogenesis. Indeed, as *in vitro* models of human cell transformation are engineered to more accurately reflect the molecular changes and heterotypic cellular complexity found in human cancers, we anticipate that this general strategy will enable a much more complete understanding of the pathways and processes that cancer cells usurp during tumorigenesis.

New technological advances

Importantly, the new shRNA libraries (Silva et al, 2005) and new PRIME vectors we have developed (Stegmeier et al., 2005) should make more thorough screens possible in the future. In addition, we are hoping these new methods will allow the loss of function bar code screening for genes whose inhibition kills cancer cells to become a reality.

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Appendices

None.