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13. ABSTRACT *(Maximum 200 Words)*

The RASSF1A locus is epigenetically inactivated in most lung and breast tumors. Expression of RASSF1A is sufficient to revert the growth transformation of human tumor cell lines. We show here that RASSF1A engages the Rb-family cell cycle checkpoint by inhibiting accumulation of cyclin D1 protein. Rare alleles of RASSF1A, isolated from tumor cell lines, encode proteins with alterations that block phosphorylation of RASSF1A on a putative ATM kinase phosphorylation site. These RASSF1A variants cannot inhibit proliferation of tumor cell lines, and suggest that RASSF1A activity is regulated by protein kinase cascades.

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Introduction

Chromosome 3p 21.3 is frequently associated with allele loss and chromosomal deletions in breast and lung cancers[1,2]. RASSF1, which was previously referred to by us as 123F2, is one of nine predicted genes located in a minimal interval of 3p21.3 as defined by analysis of nested deletions found in tumor samples[2]. Two major splice variants of RASSF1, that derive from two different promoter regions, are expressed in normal human mammary epithelial cells [3,4]. The two splice variants differ in the inclusion of an amino terminal Diacyl glycerol (DAG) binding domain. Transcripts for RASSF1 containing the DAG domain, RASSF1A, are not detectable in the majority of breast cancer cell lines that we screened[3,4]. The splice variant lacking the DAG domain, RASSF1C, is transcribed normally in most of the cell lines that we tested[3,4]. The purpose of this research is to determine if RASSF1A is functioning as a tumor suppressor, whose loss contributes to development of cancer. We will investigate the functional consequences of ectopic expression of RASSF1A on the transformed phenotype of cancer derived cell lines with allele loss at chromosome 3p21.3 that lack expression of endogenous RASSF1A. We also propose to characterize the molecular events that mediate function of RASSF1A in cells, including identification of protein partners and molecular targets of RASSF1A. We have shown that expression of RASSF1A but not RASSF1C potently inhibits tumorigenecity of cancer derived cell lines[4]. Our data suggests that RASSF1A engages the RB cell cycle checkpoint by inhibiting accumulation of cyclin D1 protein. We are currently investigating the mechanism of this inhibition.

Body

Task 1. Analysis of the functional consequence of RASSF1A and RASSF1C expression in cancer derived cell lines.

a. Changes in anchorage independent growth

RASSF1A and RASSF1C were ectopically expressed in NCI-H1299, a non small cell lung carcinoma cell line that does not express endogenous RASSF1A. We have shown RASSF1A inhibits anchorage dependent growth of NCI-H1299 cells[4] (attached manuscript in the appendix,Fig 6A in Burbee at al. JNCI 2001 May 2;93(9):691-9.). We will repeat these experiments in breast cancer cell lines, MCF-7 and HTB131, which do not express endogenous RASSF1A[4,5].

b. Occurrence of apoptosis

To determine if RASSF1A expression may be engaging an apoptotic program upon loss of anchorage, transiently transfected H1299 cells were assayed by TUNEL staining following incubation in suspension. H1299 cells expressing RASSF1A were held in suspension for upto 72 hours and did not induce apoptosis (Figure 1). In addition, human mammary epithelial cells and HEK293T cells expressing RASSF1A did not induce apoptosis when assayed by TUNEL staining.

c. Changes in growth rate and cell cycle regulation.

We are unable to establish stable transfected cell lines expressing RASSF1A. H1299 cells transfected with RASSF1A lost expression of ectopic RASSF1A after a few days following selection with drugs and hence no significant differences were observed in the growth curves for the stable RASSF1A cell lines as compared to vector transfected cell lines.

Hence we resorted to single cell based assays using transiently transfected cells to determine effects of RASSF1 expression on regulation of cell cycle and cell growth. HTB131 is a breast cancer derived cell line that does not express endogenous RASSF1A based on RTPCR results obtained previously. To assess the consequences of RASSF1A expression on cell cycle progression, HTB131 cells were assayed for BrdU incorporation following transient transfection with RASSF1A and we found that ectopic expression of RASSF1A resulted in a dramatic inhibition of BrdU incorporation. Expression of RASSF1A and not RASSF1C, in adherent and suspension cultures of H1299 cells, resulted in inhibition of BrdU incorporation (Figure 2a). Expression of RASSF1A in human mammary epithelial cells and a small cell lung cancer derived cell line H1607 also resulted in inhibition of BrdU incorporation. These observations suggest that ectopic expression of RASSF1A can inhibit tumorigenecity through induction of cell cycle arrest.

Fluorescence activated cell sorting (FACS) analysis of propidium iodide stained H1299 cells showed that expresssion of ectopic RASSF1A induced a G1 cell cycle arrest (Figure 2b). To better visualize this result, cells were treated with nocodazole for 12 hours prior to FACS analysis to induce mitotic arrest in those cells capable of progressing though S-phase. The majority of RASSF1A expressing cells remained in G1 relative to untransfected cells, indicating a G1 growth arrest.

Consistent with a role in regulating in G1-S progression, RASSF1A expression dramatically inhibits native cyclin D1 accumulation in human mammary epithelial cells, H1299 and NIH3T3 cells (Figure 3). Expression of RASSF1C and mutants of RASSF1A had only modest effects.

The human papilloma viral protein, E7, can bypass the G1 cell cycle checkpoint imposed by Rb-family of proteins by inhibiting interaction of Rb proteins with E2F transcription factors and other binding proteins. H1299 cells expressing E7 are resistant to RASSF1A-induced cell cycle arrest (Figure 4). Accumulation of cyclin D1 is still blocked in these cells, but is presumably no longer required in the context of loss of Rb-family function. Ectopic expression of cyclin A, which can directly activate Cdk2 to promote G1-S progression, also bypassed RASSF1A induced cell cycle arrest (Figure 4).

d. Formation of tumors in nude mice

We have shown that ectopic expression of RASSF1A in NCI-H1299 cells reverts tumorigenecity in nude mice (attached manuscript in the appendix, Fig 6E in Burbee at al. JNCI 2001 May 2;93(9):691-9)[4]. We will repeat this experiment with MCF7, a breast cancer cell line lacking expression of endogenous RASSF1A.

e. Analysis of functional consequences of mutations identified in RASSF1A found in tumor cell lines.

Characterization of the RASSF1A locus in breast and lung cancers demonstrated that the RASSF1A locus in lung and breast carcinoma demonstrated that the RASSF1A isoform is not expressed in the majority of cell lines and tumors due to methylation of the RASSF1A promoter[4]. A polymorphism in the RASSF1A coding sequence was detected in 12 breast and lung cancer samples that lacked methylation of the RASSF1A promoter region. The polymorphism encodes a substitution of alanine to serine at position 133 of RASSF1A. We also cloned a cDNA from HeLa cells that encodes a substitution of serine131 to phenylalanine. Serine131 has been suggested to be a substrate phosphorylation for ATM kinase[6]. Both RASSF1A (A133S) and RASSF1A (S131F) were severely compromised as compared to RASSF1A with respect to their ability to inhibit BrdU uptake in H1299 cells and human mammary epithelial cells (Table 1).

Mutations in the putative ATM phosphorylation site that interfere with RASSF1 activity also significantly decreased the steady-state phosphorylation of RASSF1C. We are

currently repeating these experiments with RASSF1A and RASSF1A mutants to determine the phosphorylation state of these proteins.

Task 2. To characterize the molecular physiology of RASSF1A.

a. Test interaction of Ras with RASSF1A

Using purified proteins, we find that RASSF1A and RASSF1C selectively bind with GTP bound Ras, which is the active form of Ras (Figure 5a). Anti-ras immunoprecipitation from human mammary epithelial cells brought down a protein of comparable molecular weight to RASSF1A, which was visualized by western blotting using an antibody that we raised against RASSF1 in rabbits (Figure 5b). This interaction was specific to EGF induced cells suggesting that RASSF1A can specifically interact with GTP bound active form of Ras *in vivo*.

b. Characterization of the functional consequence of expression of RASSF1A in NIH3T3 model system

We have taken the approach of using epithelial cells as a model system instead of using fibroblasts since RASSF1A is associated with epithelial cancers of lung and breast origins.

c. Cellular location

RASSF1A and RASSF1C were ectopically expressed in human mammary epithelial cells, H1299 and NIH3T3 cells followed by immunostaining with either anti-tag antibodies or the anti-RASSF1 antibody. Both RASSF1A and RASSF1C appeared to have a microtubular staining pattern and colocalised with anti-tubulin staining. We will attempt to optimize conditions to visualize endogenous RASSF1 proteins using anti-RASSF1 antibody

d. Identifying partners of RASSF1 using yeast two hybrid screens.

DNA binding domain fusion construct of RASSF1A is found to transactivate in the yeast two hybrid system as seen in control experiments and hence will not be suitable for use as bait for a yeast two hybrid screen using a DNA activation domain fusion cDNA library. However the DNA binding domain fusion construct of the DAG domain alone does not transactivate and will be used as bait in a yeast two hybrid screen from a cDNA library.

Key Research Accomplishments

- Ectopic expression of RASSF1A in cancer cell lines, that do not express endogenous RASSF1A, result in loss of anchorage independent growth.
- Loss of anchorage independent growth caused by RASSF1A is not due to induction of apoptosis
- Ectopic expression of RASSF1A results in inhibition of BrdU incorporation in cancer cell lines that do not express endogenous RASSF1A and also in other normal mammalian cell lines, including human mammary epithelial cells.
- RASSF1A induces a G1 cell cycle arrest by invoking the Rb-family checkpoint. This arrest can by bypassed by inactivation of Rb-family proteins using human papilloma viral protein, E7, and also by overexpression of cyclin A, which can directly activate cdk2 and hence allow G1-S progression.
- RASSF1A inhibits accumulation of native cyclin D1 protein to cause a G1 cell cycle arrest.

Reportable Outcomes

Burbee DG, Forgacs E, Zochbauer-Muller S, **Shivakumar L**, Fong K, Gao B, Randle D, Kondo M, Virmani A, Bader S, Sekido Y, Latif F, Milchgrub S, Toyooka S, Gazdar AF, Lerman MI, Zabarovsky E, White M, Minna JD. Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression.
J Natl Cancer Inst. 2001 May 2;93(9):691-9.

Latha Shivakumar, David Burbee, Eva Forgacs, John D. Minna and Michael A. White. Characterization of the role of a putative Ras effector on regulation of cell growth and tumor development. Poster presented at Keystone Symposia 2000 meeting 'Assembly of signaling networks'.

Latha Shivakumar, David Burbee, Eva Forgacs, John D. Minna and Michael A. White. Characterization of the tumor suppressor function of a putative Ras effector. Poster presented at Keystone Symposia 2001 meeting 'Signaling systems-Chemistry, Biology and Pathology'.

Conclusions:

Reintroduction of RASSF1A expression in tumor cells that do not express endogenous RASSF1A, results in dramatic inhibition of tumorigenecity. Our data suggests that the predominant mechanism by which RASSF1A exerts growth control is through inhibition of accumulation of cyclin D1 protein and by engaging the Rb checkpoint in the G1 phase of the cell cycle. We have identified rare polymorphisms of RASSFf1 with mutations around the ATM phosphorylation consensus sequence. The resulting proteins have lost activity, and show reduced levels of phosphorylation in cells. It is possible that individuals carrying these polymorphisms may have increased risk for development of some types of neoplastic disease.

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Table 1: Consequences of RASSF1 Expression on BRDU Incorporation and Cyclin D1 Expression.*

	BRDU Positive Cells	Cyclin D1 Positive Cells
RASSF1A:	20 +/- 8%	9+/- 2%
RASSF1C:	67 +/- 8%	44 +/- 4%
1A(S131F):	65 +/- 11%	62 +/- 2%
1A(S131E):	65 +/- 1%	61 +/- 1%
1A(A133S):	55 +/- 5%	49 +/- 5%

* Values are normalized to vector control (set at 100%) Error is SEM from at least three independent experiments.

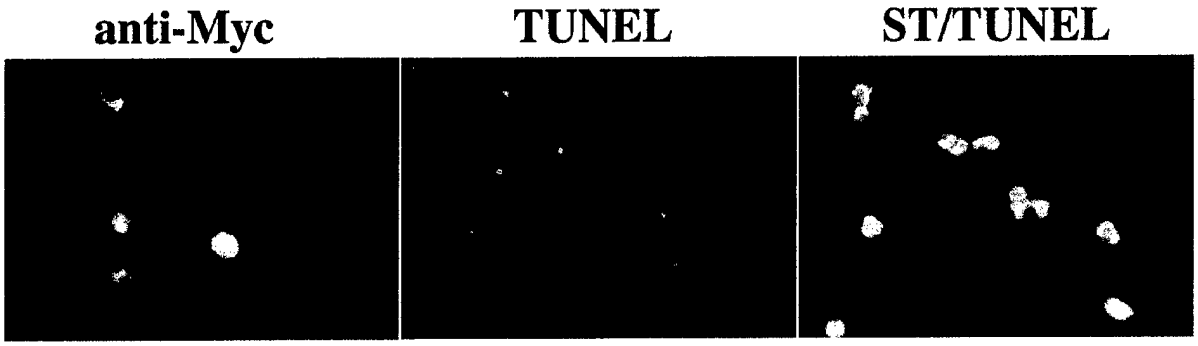

Figure 1. RASSFA expression does not induce apoptosis in suspension cultures

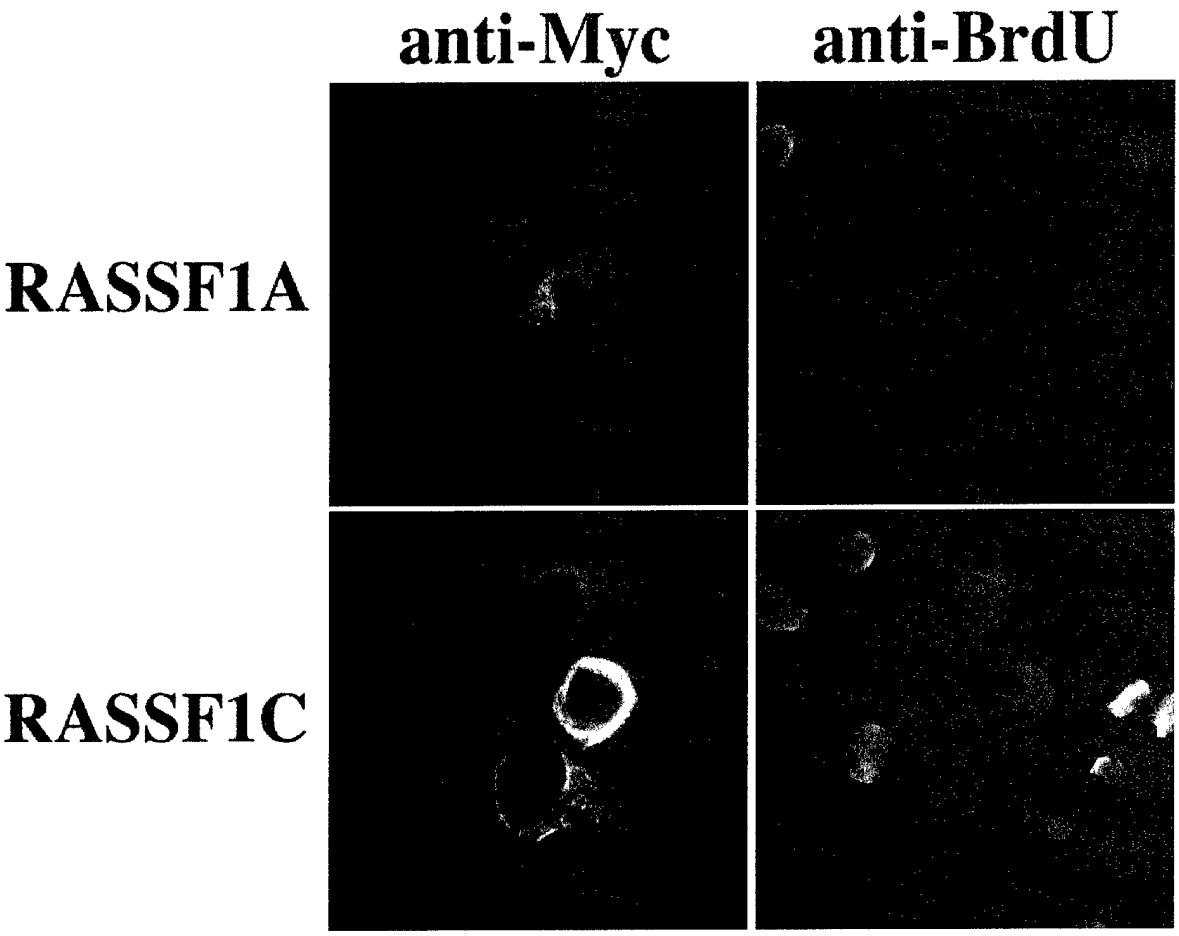

Figure 2a. Expression of RASSSF1A inhibits cell proliferation

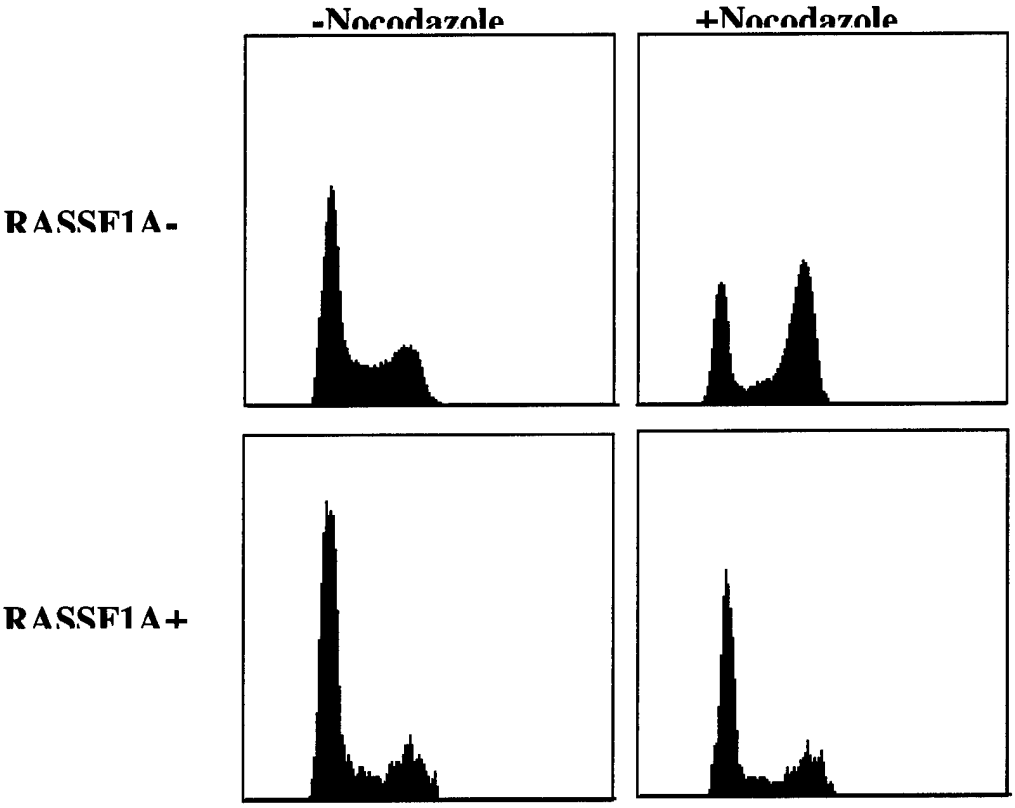

Figure 2b. RASSF1A induces G1 cell cycle

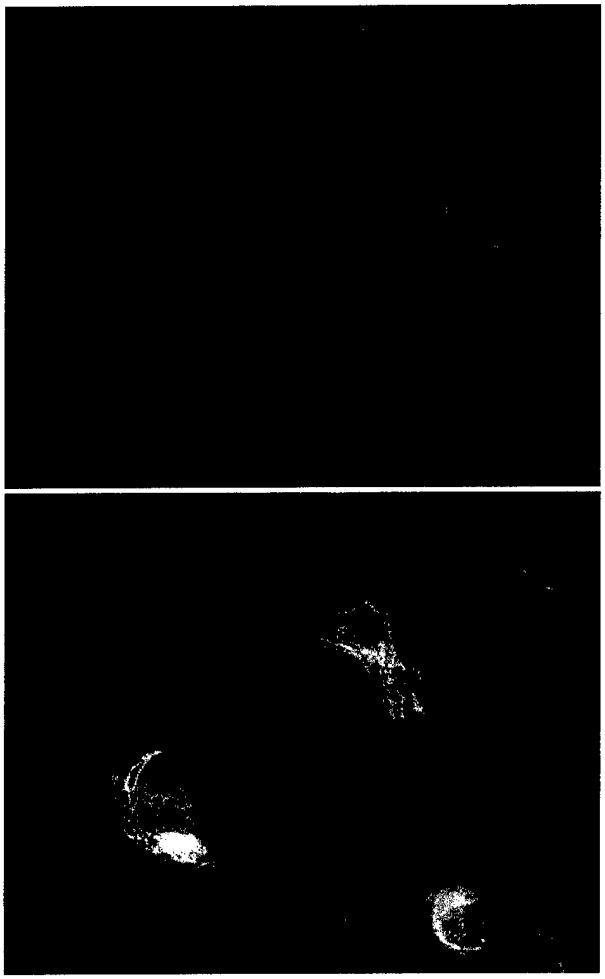

Figure 3. RASSF1A prevents accumulation of cyclin D1

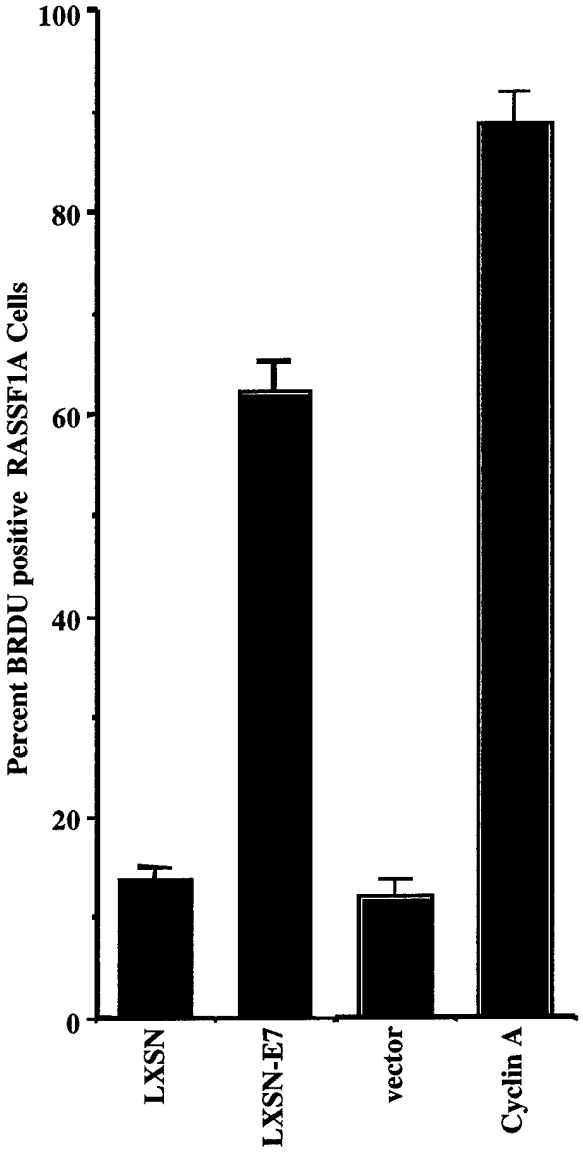

Figure 4. Bypass of the Rb checkpoint can rescue RASSF1A induced G1 arrest

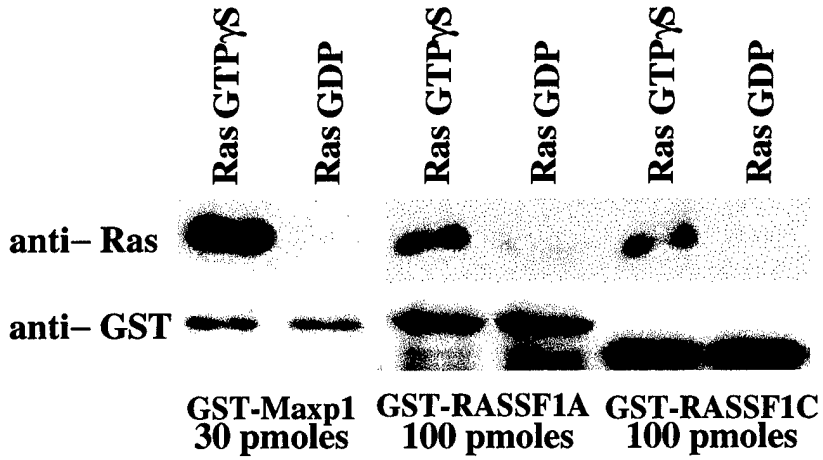

Figure 5a. RASSF1A specifically interacts with GTP-Ras

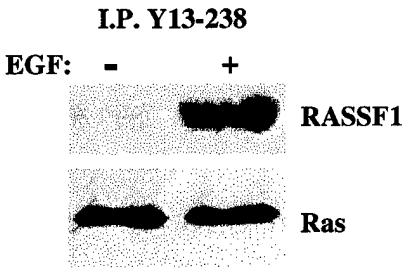

Figure 5b. RASSF1A coimmunoprecipitates with Ras in an EGF dependent manner

Figure Legends:

Figure 1. RASSF1A expression does not induce apoptosis in suspension cultures. H1299 cells were transiently transfected with myc-tagged RASSF1A. After 48 hours of incubation in suspension cultures, cells were spun onto glass coverslips, fixed, stained with anti-Myc to detect RASSF1A expression and TUNEL labeled to detect fragmented DNA. Cells were treated with 1uM staurosporine for 4 hours in suspension as a positive control for TUNEL labeling (ST/TUNEL)

Figure 2. RASSF1A expression inhibits cell proliferation. 24 hours after transfection with the indicated constructs, H1299 cells were incubated for an additional 24 hours in the presence of BrdU. RASSF1A expression was detected as in A. BrdU incorporation was detected with anti-BrdU. Quantitation by microscopic observation of three independent experiments is shown in table 1.

Figure 3. RASSF1A induces G1 cell cycle arrest. H1299 cells transiently transfected with RASSF1A were incubated for 12 hours in 10uM nocodazole, or left untreated. Cells were then trypsinized, fixed, and stained with anti-myc and propidium iodide. Two-color FACS sorting was used to determine the population of RASSF1A expressing cells with 2N versus 4N DNA content.

Figure 4. RASSF1A prevents accumulation of cyclin D1. H1299 cells were transfected with RASSF1A or RASSF1C and stained with anti-cyclin D1 and anti-Myc antibodies. Overlays are shown using rhodamine red X-conjugated anti-rabbit IgG antibodies to detect rabbit anti-cyclinD, and FITC-conjugated anti-mouse IgG to detect the 9E10 anti-myc antibody. Quantitation by microscopic observation of at least three independent experiments is shown in table1.

Figure 5. Bypass of the Rb checkpoint rescues the RASSF1A induced G1 arrest. H1299 cell lines from pooled populations infected with LXSN or LXSN-E7 replication defective retroviruses were transiently transfected with RASSF1A and stained for expression and BRDU incorporation. H1299 cells were transiently transfected with RASSF1A together with cyclin A, and assayed for expression of RASSF1A, cyclin A and BRDU incorporation. The percentage of cell expressing RASSF1A and incorporating BRDU was quantitated by microscopic observation. Bars represent standard error from the mean of values obtained from three independent experiments.

Figure 6a. RASSF1A specifically interacts with GTP-Ras. Purified recombinant GST-Maxp1(aa 229-455), GST-RASSF1A, or GST-RASSF1C was mixed with 100 pmoles of purified recombinant human Ras loaded with GTPγS or GDP in Ras binding buffer. GST proteins were immobilized on glutathione agarose beads washed and separated on SDS PAGE. Levels of immobilized Ras proteins are shown in the top panel. Immobilized GST proteins are shown below. Results shown are representative of multiple experiments.

Figure 6b. RASSF1A coimmunoprecipitates with Ras in an EGF-dependent manner. Confluent serum-starved cultures of HME cells were stimulated or not with 10 ng/ml EGF for 10 minutes. Cells were harvested, and endogenous Hras and Kras protein was immunoprecipitated with Y13-238. The precipitates were analyzed by SDS-PAGE for the presence of Ras using a pan-Ras monoclonal antibody (bottom panel) and RASSF1 using RASSF1 polyclonal antibodies (top panel).

Epigenetic Inactivation of RASSF1A in Lung and Breast Cancers and Malignant Phenotype Suppression

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Abbreviations: DAG, diacylglycerol; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NHBE, normal human bronchial epithelial; NSCLC, non-small cell lung cancer; SAE, small airway epithelial; SCLC, small cell lung cancer.

Background: The recently identified RASSF1 gene locus is located in a 120 kb homozygous deletion region of chromosome 3p21.3 that frequently undergoes allele loss in lung and breast cancers suggesting that it may contain one or more tumor suppressor genes. We explored the hypothesis that RASSF1 encoded a tumor suppressor gene for lung and breast cancer. **Methods**: We assessed expression of two RASSF1 gene products, RASSF1A and RASSF1C, and the methylation status of their respective promoters in 27 non-small-cell lung cancer (NSCLC) cell lines and 107 resected NSCLCs, 47 small-cell lung cancer (SCLC) cell lines, 22 breast cancer cell lines, 39 resected breast cancers, 104 non-malignant lung samples, and breast and lung epithelial cultures. We also transfected a lung cancer cell line that lacks RASSF1A expression with a vector containing RASSF1A cDNA to determine whether exogenous expression of RASSF1A would affect *in vitro* growth and *in vivo* tumorigenicity. All statistical tests were two-sided. **Results**: RASSF1A mRNA was detected in non-malignant epithelial cultures, but not in 100% of the SCLC, 60% of the NSCLC, or 57% of the breast cancer lines. By contrast, RASSF1C was expressed in all non-malignant cell cultures and nearly all cancer cell lines. RASSF1A expression was induced in lung cancer lines by 5-aza-2' deoxycytidine treatment. RASSF1A CpG promoter hypermethylation was detect in 100% of SCLC, 63% of NSCLC, and 64% of breast cancer lines, 30% of primary NSCLCs, 49% of primary breast tumors, but in none of the non-malignant lung tissues. Furthermore, promoter hypermethylation in patients with resected NSCLCs was associated with impaired survival ($P = .046$). Exogenous expression of RASSF1A, but not RASSF1C, decreased *in vitro* colony formation and *in vivo* tumorigenicity. **Conclusion**: RASSF1A is a potential tumor suppressor gene that undergoes epigenetic inactivation in lung and breast cancers through hypermethylation of its promoter region.

Allelic loss of human chromosome 3p is an early and frequent event in the development of lung and breast cancers (*1-4*). Identification of a series of nested 3p21.3 homozygous deletions in small-cell lung cancers (SCLCs) directed an intensive effort to positionally clone tumor suppressor genes from a 630 kb region, which was recently narrowed to a 120 kb subregion by identification of a breast cancer homozygous deletion (*5-7*). Sequencing of the entire 630 kb region identified at least 25 genes, several of which may encode tumor suppressor genes for lung cancer (*6*). Nine genes are located in or border the breast cancer-defined subregion. One of these genes, which spans 7.6 kb of genomic DNA, has a predicted Ras association domain and homology to the Ras effector Nore1 (Fig. 1); it has therefore been termed RASSF1 (*8,9*).

The RASSF1 gene encodes several major transcripts that are produced by alternative promoter selection and alternative mRNA splicing: RASSF1A encodes a 39 kDa predicted peptide that contains a Ras association domain and a predicted N-terminal diacylglycerol (DAG) binding domain (Figure 1), and RASSF1C encodes a 32 kDa predicted peptide that contains a Ras association domain (*6,10*). We characterized RASSF1A and RASSF1C as potential tumor suppressor genes in lung and breast cancer. Our previous study failed to identify point mutations at the RASSF1 locus (*6*). However, as will be seen, we found that expression of the RASSF1A transcript was lost in many lung and breast cancers. Because loss of gene expression can be caused by tumor acquired aberrant methylation, we assessed the methylation status of the RASSF1A promoter region in these tumors (*11*). In addition, we tested the ability of RASSF1A to suppress the malignant phenotype. Previously Dammann et al. (*12*) showed that the RASSF1A promoter is hypermethylated in lung cancer cells and that exogenous expression of RASSF1A suppresses tumorigenesis in nude mice. We have confirmed and extended those findings by analyzing the expression and methylation status of both RASSF1A and RASSF1C genes in lung and breast cancers.

SUBJECTS AND METHODS

Patient population

Resected lung tumor samples and clinical data were collected from patients after obtaining appropriate Institutional Review Board approval and patients' written informed consent. Lung and breast tumor cell lines generated by us have been described (*13-15*). Primary tumor samples and corresponding non-involved lung tissues were obtained from 107 NSCLC patients who had received curative resection surgery at the Prince Charles Hospital, Brisbane, Australia between June 1990 and March 1993 and for whom clinical and survival data of 5 or more years were available (*16,17*). Among the 107 patients there were: 76 males, 31 females, aged 28-81 (mean age 61 years at diagnosis); 61 stage I, 21 stage II, 24 stage IIIA, and 1 stage IIIB disease (*18*); 45 adenocarcinomas, 43 squamous cell carcinomas, 11 adenosquamous carcinomas, 4 large cell carcinomas, 3 atypical carcinoids, and 1 typical carcinoid; 98 patients were smokers with mean exposure of 31 pack-years, and 9 were never smokers or nonsmokers.

We also obtained 39 primary breast tumors from patients aged 31-84 years undergoing breast cancer treatment in the UT Southwestern Hospital system. There were: 3 stage I; 15 stage IIA; 2 stasge IIB; 8 stage IIIA; 5 stage IIB; and 6 stage IV; 30 were infiltrating ductal carcinomas; 4 were invasive lobular carcinomas; 1 was a lobular carcinoma in situ; 2 were ductal carcinomas in situ; and 2 were breast adenocarcinomas at metastatic sites. Clinical information was obtained by retrospective review of clinical records. DNA was prepared from the tumors by standard methods (*19*).

Cell lines and cell cultures

cDNAs and genomic DNAs were obtained from the following cell lines, most of which have been deposited in the American Type Culture Collection. These lines represented the spectrum of lung cancer histologies. These cell lines include (all Hxxxx lines have the prefix NCI-) SCLCs: H69, H82, H128, H146, H182, H187, H196, H209, H249, H289, H290, H345, H378, H524, H526, H592, H735, H738, H740, H748, H774, H841, H847, H862, H889, H1092, H1105, H1184, H1304, H1339, H1450, H1607, H1618, H1672, H1688, H1963, H2028, H2029, H2081, H2108, H2171, H2195, H2227, HCC970; and NSCLCs: H23, H28, H125, H157, H226, H358, H720, H727, H838, H920, H1155, H1299, H1437, H1466, H1573, H1648, H1770, H1792, H1819, H1838, H1993, H2009, H2052, H2077, H2087, H2347, H2452, H2882, H2887, HCC44, HCC78, HCC95, HCC193, HCC515, HCC827, and HCC1171. Normal human bronchial epithelia (NHBE) and small airway epithelial (SAE) cell cultures were obtained from Clonetics and grown and harvested as directed by the vendor.

Expression analysis of RASSF1 isoforms

The identification of the RASSF1 gene (initially called 123F2) and its major isoforms RASSF1C and RASSF1A were reported as part of the overall characterization of the genes in the larger 630 kb 3p21.3 homozygous deletion region (*6*) (Fig. 1A-C).

Sequence information from exons 1A and 3 was used to design the forward primer, PKCDF, 5'-GGCGTCGTGCGCAAAGGCC-3', and the reverse primer, R182, 5'-GGGTGGCTTCTTGCTGGAGGG-3' (Fig. 1C). This primer pair was used in reverse transcript (RT)-PCR screens of lung, heart, and pancreatic tissue-specific cDNA libraries (Clontech). The

RASSF1A cDNA sequence was identical to that of the RASSF1C cDNA from the second exon to the carboxyl terminus, but the two cDNA's had different 5' exons (RASSF1A, GenBank # AF102770: exons 1A and 1C; RASSF1C, Genbank # AF040703: exon 1 (Fig. 1). We also isolated tissue specific isoforms from heart (RASSF1D, GenBank # AF102771) and pancreas (RASSF1E, GenBank #AF102772) cDNA libraries.

Primers derived from exon-intron junctions (available on-line at the Journal website as supplementary data) were used for genomic DNA single strand conformation polymorphism mutation analysis of the coding regions of RASSF1A and RASSF1C on a panel of NSCLC, SCLC, and breast cancer cell line DNAs, and aberrantly migrating fragments were sequenced as described (*16,20*).

RNA analysis

Isoform-specific RT-PCR assays were used for analysis of RASSF1A and RASSF1C expression. Primers for RASSF1C were Nox3, 5'-CTGCAGCCAAGAGGACTCGG-3'. and R182; and for RASSF1A either PKCDF or NF, 5'-TGCAAGTTCACCTGCCAC-3', and R182 (Fig. 1C). Total RNA was isolated from previously described lung and breast cancer cell lines grown in RPMI-1640 supplemented with 5% fetal bovine serum (complete medium) (*13-15*) by Trizol extraction (Gibco-BRL). A total of four μg of total RNA was reverse transcribed using Gibco-BRL Superscript First Strand cDNA kit . All cDNA preparations were tested for the ability to amplify a non-transcribed genomic sequence immediately upstream of the first exon of the RASSF1A transcript. Any cDNAs that produced a product from this sequence were discarded because they were contaminated with genomic DNA .

Methylation analysis

The methylation status of the presumed RASSF1A and RASSF1C promoter regions was determined by methylation-specific PCR. Genomic DNAs from lung cancer cell lines not expressing RASSF1A (NCI lines H1299, H1184, H1304, H841, H2108, H128), or expressing RASSF1A (H1792, H2009), were modified by sodium bisulfite treatment. Bisulfite treatment converts cytosine bases to uracil, but has no effect on methylcytosine. PCR amplification followed by sequencing of the PCR fragments allows determination of which specific CpG dinucleotides in the promoter region are modified by methylation (*21-23*). PCR primers (sequences available on line) were designed to amplify genomic sequences in the presumed promoter regions of RASSF1A (cosmid Luca12 GenBank # AC002481 nucleotides 17,730 to 18,370) and RASSF1C (GenBank # AC002481 nucleotides 21,022 to 21,152 and 21,194 to 21,332). The resulting PCR fragments were sequenced by automated fluorescence-based DNA sequenced to determine the methylation status.

The data on CpG methylation in RASSF1A non-expressing lung cancer cell lines (data available on line) were used to design methylation-specific PCR (*22*) primers for the RASSF1A 5' promoter region: the primers to detect the methylated form were 5'-GGGTTTTGCGAGAGCGCG-3' (forward) and 5'-GCTAACAAACGCGAACCG-3' (reverse); and the primers to detect the unmethylated form were 5'-GGTTTTGTGAGAGTGTGTTTAG-3' (forward) and 5'-CACTAACAAACACAAACCAAAC-3' (reverse). Each primer set generates a 169 base pair product. Methylation-specific PCR cycling conditions consisted of one incubation of 15 min, 95 °C, followed by 40 cycles of 30 sec denaturation at 94 °C, 50 sec at annealing temperature (64 °C for methylation-specific, and 59 °C for unmethylated-specific primers), 30 sec extension at 72 °C, and a final extension at 72 °C for ten min. PCR products were analyzed

in 2% agarose gels. Lymphocyte DNA, methylated in vitro by CpG (Sss I) methylase (New England Biolabs) following the manufacturer's directions, was used as a positive control. A water blank was used as a negative control.

Azacytidine treatment

We also assessed the expression of RASSF1A after exposure to 5-aza-2'-deoxycytidine, a drug that inhibits DNA methylation. We exposed the RASSF1A non-expressing NSCLC line NCI-H157 to 0.5 μM 5-aza-2'-deoxycytidine for 48 hours, after which we isolated total RNA, and performed RT-PCR for RASSF1A, RASSF1C, and GAPDH. RT-PCR of GAPDH transcripts was performed using forward primer GAPDH-C (5'-CATGACAACTTTGGTATCGTG-3'), and reverse primer GAPDH-D (5'-GTGTCGCTGTTGAAGTCAGA-3'). RT-PCR products were separated by agarose gel electrophoresis and visualized after staining with ethidium bromide.

Antibody preparation

The carboxyl terminal portion of RASSF1A was fused to GST and expressed in E. coli. Lysates were purified and injected into three New Zealand White rabbits. Rabbits were bled at three-week intervals after injection. After the third bleeding, rabbits were bled out.

Transfection studies

RASSF1A and RASSF1C cDNAs were each cloned into pcDNA3.1+ (Invitrogen), re-sequenced to confirm that the cDNAs were in the correct orientation and reading frame,

transcribed, and translated *in vitro* with commercial kits (CloneTech). The expression vector containing RASSF1A produced a 42 kda protein and that containing RASSF1C produced a 32 kda protein on SDS-polyacrylamide gels, close to their respective predicted molecular weights of 39 and 32 kda (data not shown).

The RASSF1A expression vector was transfected into NSCLC NCI-H1299 cells expressing RASSF1C but not RASSF1A, using Lipofectamine plus (Gibco-BRL) according to manufacturer's recommendations. In cases of transient transfection, cells were collected 48 hours after thansfection. Because the expression vector contains a neomycin resistance gene, clones expressing RASSF1A were selected in complete medium supplemented with G418 (800 μg/mL). Stable clones were maintained in complete medium supplemented with G418 (600 μg/mL). We confirmed that the clones were expressing the transfected RASSF1A gene by isolating total RNA from individual clones and performing RT-PCR as described above. At the same time we also transfected NCI-H1299 cells with the vector containing no inserts and isolated stable vector clones.

Tumorigenicity Testing

The *in vitro* growth characteristics of NSCLC NCI-H1299 clones that expressed RASSF1A were tested for anchorage-dependent and anchorage-independent soft agar growth. NCI-H1299 cells were grown to 80 to 90% confluence in complete medium. Cells were collected and counted. 5×10^5 cells were transfected with 1 μg of purified plasmid DNA using lipofectamine reagent (Gibco-BRL). Samples were performed in triplicates or higher replicates. After 48 hours of growth in plates non-selective medium, cells were trypsinized and diluted usually ten to twenty-five fold into complete medium containing 800 μg/ml G418, and plated into fresh 100

mm dishes. Medium was changed twice weekly. After 14 days of growth, medium was removed, plates washed with PBS, and colonies then stained with 1% methylene blue in 50% (v/v) ethanol.

For the anchorage-independent soft agar growth assays, 10^3 RASSF1A-expressing clones were resuspended in soft agar. The basement layer consisted of 0.50% agar in complete medium and the cells were suspended in the top layer, which consisted of 0.33% Noble agar (Sigma) in complete medium supplemented with 600 μg/mL G418), and colonies counted after 21 days. For retrovirally infected cells, anchorage independent growth assays were performed as follows: 5,000 or 10,000 viable selected cells from each infection were plated in 0.3% soft agar over a 0.5% agar base in Dulbecco's Modified Eagles Medium (GIBCO BRL) with 10% heat-inactivated fetal calf serum. Colonies were counted after 21 days.

The RASSF1A and RASSF1C cDNAs were also placed in the retroviral vector pBABEpuro, and re-sequenced to confirm that the genes were in the correct sequence and orinetation (*24*). Virus was prepared in the 293 cell based Phoenix packaging cell line as described from cells infected with either vector alone or with constructs containing the RASSF1A or RASSF1C cDNAs (*24*). Culture supernatants were collected (37 °C, 10 min, 1500 rpm) and used to infect NSCLC NCI-H1299 cells as described (*24*). Because the viral vector contains a puromycin resistance gene, the infected cells were selected with 1 μg/mL puromycin for 7 days. Cells surviving the puromycin selection and thus containing the transgene were pooled, total cell extracts made and western blot analysis performed as described to verify protein expression of the transfected genes (*25*).

We also tested the ability of RASSF1A-infected cells to grow *in vivo* in nude mice. Male BALB/c nude (nu/nu) 3-6 week old mice (Charles River Laboratories) were irradiated on day

zero of the experiment in groups of five animals by exposure to 350 cGy for five minutes from a cesium source. The next day, each mouse was injected subcutaneously on its flank with 0.2 ml of sterile phosphate-buffered saline containing 10^7 viable parental, vector control, or RASSF1A retroviral infected NSCLC NCI-H1299 tumor cells. Mice were monitored every 2-3 days for tumor size, and once tumors reached >1500 mm^3, mice were euthanized. All animal care was in accord with institutional guidelines.

Statistical analysis

Statistical analysis was performed using χ^2 and Fisher's exact test for differences between groups. Overall survival curves were calculated using the Kaplan-Meier method and survival curves were compared by the logrank statistic (*26*). All analyses including univariate, multivariate and Cox analyses were performed using SPSS Windows Version 9.0.1. All statistical test were two-sided.

RESULTS

Characterization of RASSF1 gene

RASSF1A is encoded by exons 1A, 1C, and 2-5. RASSF1C is encoded by exons 1 through 5 (Fig. 1). The start sites for RASSF1A and RASSF1C are ~2 kb apart and have two independent CpG island-containing putative promoter regions. The two transcripts encode unique proteins that contain distinct domains. RASSF1A contains a DAG binding domain, encoded by exons 1A and 1C, and a Ras association domain, encoded by exons 2 through 5. RASSF1C lacks the DAG binding domain but has the Ras association domain. The Ras association domain is >50% identical and >70% similar to the carboxyl terminal 225 residues of mouse Nore1 (*9*). The Ras association domain, consisting of a core of 90 amino acids, is flanked on the amino terminal side by a region

homologous with one found in Nore1 and *C. elegans* orthologue T24F1.3 proteins. Immediately adjacent to the DAG domain of RASSF1A is a peptide sequence PxxP, which consists of the minimal homology required for an SH3-binding domain. RASSF1A contains a central linker that contains a number of prolines, as well as acidic and hydroxyl-bearing residues. These regions, called PEST sequences, are associated with proteins that are rapidly turned over by ubiquitination-dependent pathways. For RASSF1C, the amino-terminal region unique to this isoform is enriched for these residues. Within the PEST sequences common to both RASSF1A and RASSF1C is found a serine residue that is phosphorylated in vitro by DNA-dependent, ATR, and ATM kinases (*27*).

To determine if the genes were mutated in lung and breast cancers, we performed extensive mutational analysis using single strand conformation polymorphism assays on genomic DNA tested for RASSF1A alterations. An additional 77 lung cancer cell line samples had been tested previously and no RASSF1C alterations were found (not shown) (*6*) Using the RASSF1A sequence as a reference, we found several polymorphisms including: codon 21 (AAG to CAG) Lys to Gln; codon 28 (CGT to CGA) no amino acid change; codon 49 (GGC to GGT) no amino acid change; codon 53 (CGC to TGC) Arg to Cys; codon 129 (GAC to GAG) Asp to Glu; codon 133 (GCT to TCT) Ala to Ser; and codon 330 (TAT to TGT) Tyr to Cys.

Expression of RASSF1A and RASSF1C in lung and breast cancer cell lines

RASSF1 is located within a region of frequently affected by allele loss in tumor growth; thus we investigated whether RASSF1A and RASSF1C are expressed in lung and breast cancer cell lines. We used isoform-specific RT-PCR to distinguish the expression of RASSF1A and RASSF1C in lung and breast tumor cell lines and normal lung and breast epithelial cultures (Figure 2). RASSF1A was expressed in both normal lung epithelial cultures (normal human bronchial epithelial and small

airway epithelial cultures), and a normal breast epithelial culture (Figure 2, C), but was not expressed in 32/32 (100%) SCLC lines, 17/26 (65%) NSCLC cell lines and 15/25 (60%) breast cancer cell lines. A sampling of this data is shown in Fig. 2. By contrast, RASSF1C was expressed in nearly all of the lung and breast cancer cell lines, with a few exceptions in lung and breast cancer lines with known homozygous deletions that include the RASSF1 locus. In uncultured lung adenocarcinomas, RASSF1A was expressed in only two of five cases, while RASSF1C was expressed in all cases (Fig. 2, C).

During RT-PCR analysis for RASSF1A we frequently noted two closely spaced bands in RASSF1A expressing tumors and normal human bronchial epithelial cultures (Fig. 2). We sequenced these RT-PCR products and found that the larger band corresponded to RASSF1A while the smaller product represents a different transcript, RASSF1F (GenBank Accession #AF286217). This transcript skips exon 1C to produce an mRNA encoding a predicted truncated peptide of 92 amino acids ending within the DAG binding domain (Fig. 1, D). The biologic function, if any, of RASSF1F is unknown.

Methylation Status of the RASSF1A promoter region

Aberrant promoter methylation in tumors can lead to the loss of gene expression, as has been demonstrated for several tumor suppressor genes in human cancers (*11*). To assess whether the loss of RASSF1A expression was the result of promoter hypermethylation, we determined the CpG methylation status in the 5' region of RASSF1A (from –800 to +600 bp of the predicted RASSF1A transcript start site) by sequencing sodium bisulfite-modified DNA from eight lung cancer cell lines. Six of six lung cancer cell lines not expressing RASSF1A exhibited methylation of almost all CpG dinucleotide sites in the putative promoter region (data available on line). The two lung cancer cell

lines that did express RASSF1A were either not methylated at these CpG sites or showed limited methylation. In contrast, no methylation was found in CpG sites in the presumed RASSF1C promoter region of these eight cell lines. To confirm that promoter hypermethylation may contribute to the lack of expression of RASSF1A in the lung cancer cell lines, we assessed the expression of RASSF1A after exposure to 5-aza-2'-deoxycytidine, a drug that inhibits DNA methylation. We exposed the RASSF1A non-expressing NSCLC line NCI-H157 to 0.5 μM 5-aza-2'-deoxycytidine and found re-expression of RASSF1A by this cell line but little or no change in the expression of the housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase), or in the expression of RASSF1C after this treatment (Fig. 3).

Methylation-specific PCR analysis of the promoter region of RASSF1A in lung and breast cancers

To determine the methylation-status of the promoter region of RASSF1A in primary lung and breast cancers, we used methylation-specific PCR analysis. Genomic DNA from a large number of primary resected NSCLCs, paired lung tissues resected from the same patients but not involved with the cancer, primary resected breast cancers, and a large panel of lung and breast cancer cell lines were subsequently treated with sodium bisulfite and tested for the presence of methylated and unmethylated CpG dinucleotides in the promoter region of RASSF1A. Shown in Figure 4, are the results from the representative primary resected lung and breast tumor samples, paired uninvolved lung tissues, and SCLC tumor lines. All of the primary resected NSCLC and non-tumor paired samples contained unmethylated promoter sequences, which was expected because these tumor resection specimens were not microdissected and thus contaminated with stromal cells. However, 32 of 107 (30%) primary NSCLCs, 100% of the SCLC lines, and 19 of 39 (49%) primary breast

cancers exhibited the methylated RASSF1A allele (Fig. 4 and Table 1). By contrast, no methylated alleles were detected in 104 paired resected non-malignant lung tissues (Fig. 4 and Table 1).

In the panel of lung and breast cell lines we found a high frequency of methylated RASSF1A alleles (Table 1). Because the lung and breast tumor cell lines represent essentially clonal populations of tumor cells without contaminating normal cells, we tabulated the frequency of detecting the methylated and unmethylated RASSF1A alleles (Table 2). While the lung and breast cancer lines often derive from clinically more aggressive lesions than the average population of tumors (*13-15*), our previous studies have shown that tumor cell lines continue to retain the genetic alterations found in the uncultured tumor specimens from which they were derived (*14,15*). The presence of only the methylated allele is consistent with either the methylation of both parental alleles or the retention of the methylated allele and loss of the unmethylated 3p allele. All of the SCLC cell lines showed only the methylated allele or lacked RASSF1A entirely due to a homozygous deletion, consistent with the nearly universal 3p21.3 allele loss in SCLC (*1,14,28*). Of the NSCLCs cell lines, 13 out of 27 (48%, Table 2.) had only the methylated RASSF1A allele and 10 of 27 (37%) only the unmethylated allele, consistent with a lower rate of 3p21.3 allele loss in this tumor type (*1*). Likewise, 10 of 22 samples (45%, Table 2.) of breast cancers cell lines had only the methylated allele and 7 of 22 (32%) had only the unmethylated allele, again consistent with the rate of 3p21.3 allele loss found in breast cancer (*15*). As expected, two tumor lines previously shown to have homozygous deletions involving the 3p21.3 region were negative for the presence of either the methylated or unmethylated allele (Table 2) (*6,7*).

For a subset of 61 lung and breast tumor cell lines, we performed both expression and methylation analysis and found a significant association ($P < 0.0001$, Fisher's Exact Test) between the presence of methylated RASSF1A alleles and loss of RASSF1A expression. In 12 tumors

RASSF1A was expressed in the absent of a methylated allele, in 44 tumors RASSF1A was not expressed in the presence of a methylated allele, in 4 samples RASSF1A was not expressed in the absence of methylated allele (presumably by some other inactivating mechanism), while the one exception (a breast cancer) of RASSF1A expression in the presence of a methylated allele, an unmethylated allele was also present. These data show the critical association of RASSF1A methylation with RASSF1A expression.

We next assessed whether there was any association between RASSF1A promoter methylation and clinical findings in the primary NSCLC patients. We found no statistically significant association between RASSF1A methylation and age, sex, tumor–node–metastasis pathologic stage (*18*), or tumor histology in 107 resected NSCLCs (data not shown). In addition, we found no statistically significant association between RASSF1A methylation and age, TNM pathological stage, tumor histology, estrogen or progesterone receptor status, or HER2/Neu expression in 39 primary resected breast cancers (data not shown).

Lung cancer survival among patients differed by the methylation status of RASSF1A ($P = .046$) (Fig. 5). Also by univariate analysis, in this group of 107 NSCLC patients treated with an attempt at curative surgical resection, tumor (T1,T2, T3) and nodal stages (N1 and N2) as well as reported weight loss were significant univariate predictors of adverse survival outcome. Neither smoking history (yes/no or pack years with 40 pack year cutoff) or treatment differences (all patients had surgical resection of lobectomy or pneumonectomy, and only five had prior radiotherapy or chemotherapy) accounted for the adverse survival. Because a multivariate analysis is of limited use with a small sample size, we performed a Cox proportional hazards regression analysis using RASSF1A methylation and the main univariate factors (tumor and nodal stage and weight loss). RASSF1A methylation was not found to be an independent prognostic factor of survival. However,

this result could be due to small numbers because even N stage (a known prognostic factor) was also no longer an independent factor in the analysis. Currently, we are studying a much larger cohort of NSCLC patients to determine whether RASSF1A methylation is an independent factor.

Effect of Exogenous Expression of RASSF1A on Tumor Cell Phenotype

To assess the effect of ectopic expression of RASSF1A on a lung-tumor derived cell line devoid of endogenous RASSF1A expression, we first cloned RASSF1A cDNA into the plasmid pcDNA3.1+, an expression vector that contains the gene for neomycin resistance, and transfected NCI-H1299 cells. After selection in G418 for 14-21 days, we determined colony formation of NCI-H1299 cells in both anchorage-dependent and anchorage-independent assays. Expression of RASSF1A in NCI-H1299 cells resulted in a 40-60 % decrease in the colony formation in liquid medium on plastic, and ~ 90% decrease in soft agar colony formation compared with cells transfected with the pcDNA3.1 vector alone (Fig. 6, B). Wild-type p53 control transfections gave ~80% and 95% reduction in the same assays in NCI-H1299 cells, which have an intragenic p53 homozygous deletion (*29*). Several clones of NCI-H1299 cells transfected with RASSF1A were isolated in selective G418 medium and found to express RASSF1A by RT-PCR (Fig. 6, C). In each of these clones, soft agar colony formation was reduced by approximately 90% compared with the vector-transfected control clones (Fig. 6, D), but otherwise the clones grew well *in vitro*.

We infected NCI-H1299 cells, which lack endogenous expression of RASSF1A, with retroviral expression vectors containing RASSF1A or RASSF1C. We then tested the ability of the cells to grow in an anchorage-independent manner. Cells expressing RASSF1A had a marked reduction in the ability to form soft agar colonies compared with cells infected with vector or a retrovirus

containing RASSF1C (Fig. 6, A). Vector controls had 3,200 colonies (100%) for 10,000 cells plated, 19% of control for the RASSF1A isoform and 108% of control for the RASSF1C isoform. Otherwise these RASSF1A and RASSF1C infected cells grew well *in vitro* and showed no signs of toxicity or apoptosis (data not shown).

Finally, we tested the ability of the retroviral infected NCI-H1299 cells to form tumors in nude mice. Cells infected with the retroviral vector (parental cells) formed tumors rapidly (Figure 6, E). By contrast, cells infected with RASSF1A retroviral vector and expressing the RASSF1A protein had much lower tumorigenicity *in vivo* (Fig. 6, E).

DISCUSSION

We have found strong evidence that RASSF1A functions as a tumor suppressor gene that undergoes epigenetic inactivation in cancers by methylation of the CpG islands in the promoter region. Whereas normal lung and breast epithelial cells expressed both RASSF1A and RASSF1C, many lung and breast cancers failed to express RASSF1A but did express RASSF1C. Exposure of a NSCLC line to the de-methylation agent 5-aza-2'deoxycytidine restored expression of RASSF1A. These tumor cell lines and uncultured primary lung and breast tumors frequently acquired RASSF1A 5' CpG island hypermethylation not found in tissues not involved with cancer. Finally, forced expression of RASSF1A by transfection using several different vectors suppressed anchorage-independent growth and tumor formation in nude mice. Although there was a ~40% reduction in colony formation in liquid media after transfection, lung cancer clonal isolates stably expressing exogenously introduced RASSF1A, showed no toxicity or morphology changes *in vitro*. Independently Dammann et al. have recently reported similar results implicating RASSF1A as a 3p21.3 tumor suppressor gene inactivated by methylation of the CpG promoter region (*12*). Also,

we find that loss of expression of RASSF1A in a sample of resected NSCLCs is associated with decreased survival.

There is mounting evidence that tumor suppressor genes can be inactivated by tumor-acquired methylation of their promoter regions; indeed, this method of tumor suppressor gene inactivation may be more common than amino acid sequence-altering mutations (*11*). We found only the methylated RASSF1A allele in 45 to 100% of tumor cell lines, depending on the tumor type, which was consistent with either methylation of both parental alleles or loss of the unmethylated allele. Because the 3p21.3 region, where RASSF1 is located, undergoes allele loss in a variety of human tumors including those of the head and neck, kidney, and cervix (*30*), it will be important to extend the RASSF1A studies to these types of cancers. Although the methylation studies were prompted when we failed to find any tumor-acquired amino acid sequence altering mutations in either RASSF1A or RASSF1C (*6*), in our studies we did find several polymorphisms in the RASSF1A coding region, several of which altered the amino acid sequence. Studies are in progress to determine whether any of these polymorphisms have functional consequences.

Analysis of the protein sequence of the RASSF1 isoforms revealed several domains that may aid in identification of specific cellular functions. The presence of a Ras association domain in both RASSF1 isoforms suggests that these proteins may function as effectors of Ras signaling (or signaling of a Ras-like molecule) in normal cells. If so, the observation that RASSF1A can function as a tumor suppressor gene implies that RASSF1 acts in opposition to Ras effector pathways that stimulate proliferation. RAS mutations rarely occur in SCLC or in breast cancer and only are found in ~30% of NSCLCs (usually in adenocarcinomas) (*2*). Thus, the observation of RASSF1A methylation with the associated loss of expression in many tumors without RAS mutations suggests that inactivation of RASSF1A expression may be a tumorigenesis mechanism distinct from the

production of RAS mutations leading to activation of RAS signallling in tumors. However, it is important to note that although many proteins have been identified that contain Ras association domains motifs by database analysis (*8*), the majority of these proteins have not been validated as *bona fide* Ras interactors. Therefore, studies are presently under way to assess the role of RASSF1 in Ras-dependent growth control. Kim et al. found that both RASSF1A and RASSF1C also possess a putative ATM (ataxia telangiectasia mutated) kinase phosphorylation site in their common exon (Figure 1), based on *in vitro* phosphorylation studies (*27*). Dammann et al. isolated RASSF1 transcripts using a yeast two hybrid assay with the DNA repair protein XPA (xeroderma pigmentosum A) as bait (*12*). These findings suggest that RASSF1 products may participate in the DNA damage response or in DNA damage-induced regulation of other cellular signaling events. As for signalling involving cell proliferation, the presence of a putative DAG binding domain in RASSF1A but not in RASSF1C suggests studies to test the role of tumor promoters interacting with the RASSF1A isoform in a novel light: tumor promoters act on proteins with DAG binding domains by facilitating their movement to the cell membrane, thus allowing them to interact with the cell's signaling components. Thus, tumor promoters such as phorbol esters would be expected to move RASSF1A to the membrane, where it would be expected to act in its normal function as a growth suppressor until RASSF1A expression is lost.

Our previous work has shown that 3p21.3 allele loss occurs early in lung cancer pathogenesis (*1,3,4,28*). Other studies have shown that promoter methylation of other tumor suppressor genes (e.g., for $p16^{Ink4A}$) can be detected in preneoplastic lung tissues or histologically non-involved lung tissue (*31*). RASSF1A promoter methylation may also represent a potentially important marker for the development of invasive lung and breast cancer. Because many smokers have genetic alterations in their respiratory epithelium as a result of damage by tobacco carcinogens (*3,4,28,32*), the

discovery of a marker such as RASSF1A promoter methylation may be of great use for both early detection and for prognosis in monitoring chemoprevention efforts. Furthermore, RASSF1A may represent another potential target for pharmacological re-expression as a novel mode for cancer treatment.

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NOTES

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FIGURE LEGENDS

Figure 1. Map of the RASSF1 locus, transcripts, and protein domains. (A) The exon-intron structure of the RASSF1 locus with the location of the CpG islands in the predicted promoter regions (the

location of which are shown by double-headed arrows) of RASSF1A and RASSF1C. RASSF1A transcription is predicted to come from the most centromeric promoter region located within a CpG island. RASSF1F also commences at this promoter, but is missing exon 1C. Transcription of RASSF1C is predicted to begin in the most telomeric promoter region, which is ~2 kb from that of RASSF1A. The RASSF1C transcript commences with exon 1. A fourth RASSF1 transcript, RASSF1B (GenBank #AF132677), reported by Dammann et al. (*12*) probably commences within an intermediate promoter region, and also encodes its own unique 5' exon (not shown). Also not shown are RASSF1D (expressed in heart tissue) and RASSF1E (expressed in pancreatic tissue), tissue-specific forms with minor alterations from RASSF1A. Blocks represent exons; lines represent introns. (B) Schematic of the RASSF1A transcript and predicted protein sequence domains. The location of the various primers (PKCDF, NF, R182, and R292) used for isoform-specific RT-PCR analyses are indicated. The figure indicates the site of exons as tick marks. In the schematic below, the potential src homology 3 (SH3) binding region, putative diacylglycerol (DAG) binding domain, PEST sequence, Ras association domain, and ATM (ataxia telangiectasia mutated) phosphorylation site are labelled as colored boxes (*27*). (C) Schematic of the RASSF1C transcript and predicted protein sequence domains. The location of the various primers (NOX3, R182, and R292) used for isoform-specific RT-PCR analyses are indicated. The figure indicates the site of exons as tick marks above the peptide schematic; and domain sequences are labelled as in part B. (D) Schematic of the RASSF1F transcript and predicted protein sequence domains. The figure is labelled as before.

Figure 2. RASSF1A and RASSF1C mRNA expression detected by isoform-specific reverse transcriptase polymerase chain reaction (RT-PCR) in a sampling of lung cancer cell lines (A), breast cancer lines (B), and resected lung tumors and normal human lung and breast epithelial cultures (C) .

All RT-PCR products were separated on agarose gels and identified by staining with ethidium bromide. Arrows indicate location of transcripts (A) Lung cancer lines tested in lanes: 1 = H157; 2 = H358; 3 = H727; 4 = H740; 5 = H748; 6 = H838; 7 = H1184; 8 = H1299; 9 = H1304; 10 = H1437; 11 = H1450; 12 = H1770; 13 = H1792; 14 = H1963; 15 = H1993; 16 = H2009; 17 = H2077; 18 = H2108; 19 = HHCC44; 20 = HCC78. (B) Breast cancer lines tested in lanes: 1 = HCC38; 2 = HCC1187; 3 = HTB19; 4 = HTB20; 5 = HTB22; 6 = HTB23; 7 = HTB24; 8 = HTB25; 9 = HTB26; 10 = HTB27; 11 = HTB121; 12 = HTB129; 13 = HTB130; 14 = HTB131; 15 = HTB132; 16 = HTB133; 17 = HCC1395; 18 = HCC1428; 19 = HCC1569; 20 = HCC1806; 21 = HCC2157. (C) Resected lung adenocarcinoma samples (ADC 1 through 5) and cultures of normal small airway epithelial cells (SAEC), normal human bronchial epithelial (NHBE), and normal human breast epithelial (NHBRE) cultures.

Figure 3. Expression of RASSF1A after treatment of lung cancer cells with 5-aza-2'-deoxycytidine. NCI-H157, a non-small-cell lung carcinoma (NSCLC) cell line that expresses RASSF1C but not RASSF1A, was grown in the presence (+ lanes) and absence (- lanes) of 0.5 μM 5-aza-2'-deoxycytidine (5Aza-CdR) for 48 hours. Total RNA was isolated, cDNA prepared, and isoform-specific reverse transcriptase polymerase chain reaction performed for RASSF1A, RASSF1C, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control.

Figure 4. Methylation-specific polymerase chain reaction (PCR) for the detection of methylated RASSF1A 5' CpG sequences in primary resected non-small-cell lung carcinomas (NSCLC) and their accompanying normal lung (upper panel), small-cell lung carcinoma (SCLC) cell lines (middle panel), and primary breast cancers (lower panel). For resected NSCLCs, U = results with primers

specific for unmethylated sequences; M = results with primers specific for methylated sequences. P = results with peripheral blood lymphocyte DNA, which is unmethylated or *in vitro* methylated (IVMD); and H20 = negative controls with water blanks. For SCLCs, all of the samples show RASSF1A methylation (shown) and all are negative for the unmethylated allele (not shown). Lane 20 is negative control. For the breast cancers tumors 1-8 are positive for RASSF1A methylation while tumor 9 was negative. Representative samples are shown. PCR products were separated on agarose gels and bands were detected after staining with ethidium bromide.

Figure 5. Kaplan-Meier survival curve for 107 patients with resected non-small-cell lung carcinoma (NSCLC) based on RASSF1A methylation status (N = 32 methylated, 75 not methylated). For RASSF1A unmethylated patients the number of cases = 75, censored = 39, events = 36, mean overall survival 52 months (95% CI 44, 59), median overall survival 49 months (95% CI 44, 59); for RASSF1A methylated patients, number of cases = 32, censored 9, events = 23, mean overall survival 37 months (95% CI 27, 46), median overall survival 28 months (95% CI 9, 47). The log rank test test statistic for equality of survival distributions for RASSF1A methylation was 3.97 with df 1, P = 0.0463. The patients at risk for each group at 3 time points were: RASSF1A unmethylated 12 months (n = 63), 36 months (n = 34), and 60 months (n = 16); for RASSF1A methylated 12 months (n = 24), 36 months (n = 13), and 60 months (n = 5).

Figure 6. Effect of RASSF1A on the *in vitro* growth of the non-small-cell lung carcinoma (NSCLC) cell line NCI-H1299. Inhibition of NSLC H1299 lung cancer cell anchorage independent growth by RASSF1A. (A) NCI-H1299 cells were infected with the pBABEpuro retrovirus expression vectors containing vector control, RASSF1A, or RASSF1C. Infected cells

(10,000 per plate) were suspended in 0.33% agar and the suspension was layered over a 0.5% agar base. Colonies greater than 0.2 mm in diameter were counted after 21 days. All transfections were verified to express an increased amount over background of RASSF1 protein(s) with a rabbit anti-RASSF1-GST fusion antibody shown in the lower right panel of (A). C = positive control generated by transient transfection of RASSF1A; V = infection with the retroviral vector control (note run over from positive control); 1A = infection with the retroviral vector containing RASSF1A; 1C = infection with the retroviral vector containing RASSF1C. (B) Anchorage-dependent and anchorage-independent colony formation after transfection of NCI-H1299 cells with empty vector or pcDNA3.1+ expression vectors containing wild-type p53 or RASSF1A. For analysis of anchorage-dependent growth, NCI-H1299 cells were transfected with pcDNA-derived expression vectors and diluted after two days of non-selective growth into 100 mm dishes with selective medium. Transfected cells were plated in liquid medium (anchorage-dependent) or soft agar (anchorage-independent) containing 800 μg/mL of G418. Colonies were stained with methylene blue in anchorage-dependent experiments after 14 days. Results represent the average 8-12 experiments in liquid medium and three soft agar experiments. Standard deviations shown or are <2%. Solid bars = anchorage-dependent growth (CI 95 is 0-36 for p53; 52-60 for RASSF1A); open bars = anchorage-independent growth (CI 95 0-6 for p53; 0-39 for RASSF1A). (C) Expression of exogenously introduced RASSF1A by Northern blot in stable clonal transfectants of NCI-H1299 transfected with the pcDNA-derived expression vectors, that were used for soft agar colony formation assays. The vector control (vector) and four separate clones with various levels of RASSF1A mRNA expression are shown. These clones are several of the same clones used in the anchorage independent growth assay shown in figure D. Ethidium bromide staining of the ribosomal RNA is shown as a loading

control. The clones were also verified to express the RASSF1A isoform by RT-PCR using isoform specific primers (data not shown). (D) Soft agar colony formation in NCI-H1299 clones stably expressing RASSF1A. Two vector controls and three RASSF1A expressing clones were plated in 0.33% soft agar with colonies counted 21 days later. The mean and standard deviation are shown. One vector control produced slightly fewer clones than the other. (CI 95 is 0-4 for F1A.4; 2-16 for F1A.5; 3-14 for F1A.19). (E) Effect of RASSF1A on the *in vivo* growth of the non-small-cell lung carcinoma (NSCLC) cell line NCI-H1299. Suppression of H1299 xenograft tumorigenicity after retroviral transfection with pBABEpuro RASSF1A expression vector. H1299 cells were infected, selected in 1 μg/mL puromycin as described in the methods and verified to express RASSF1A. 10^7 viable tumor cells were injected into the flanks of each of 5 Balb/c (nu/nu) nude previously irradiated mice. Tumor size was monitored over time and size is shown in cubic millimeters. The H1299 parent cells represent averages of tests in >20 mice. Mice that were injected with RASSF1A-infected NCI-H1299 cells grew no measureable tumors.

Table 1. Frequency of methylation-specific polymerase chain reaction assay for the detection of RASSF1A CpG island methylated alleles in lung and breast cancers.

DNA Sample source*	# Tested	# Methylation allele (+) (%)
Primary resected NSCLCs	107	32 (30%)
Corresponding non-malignant lung	104	0 (0%)
NSCLC lines	27	17 (63%)
SCLC lines	47	47 (100%)
Primary resected breast cancers	39	19 (49%)
Breast cancer lines	22	14 (64%)

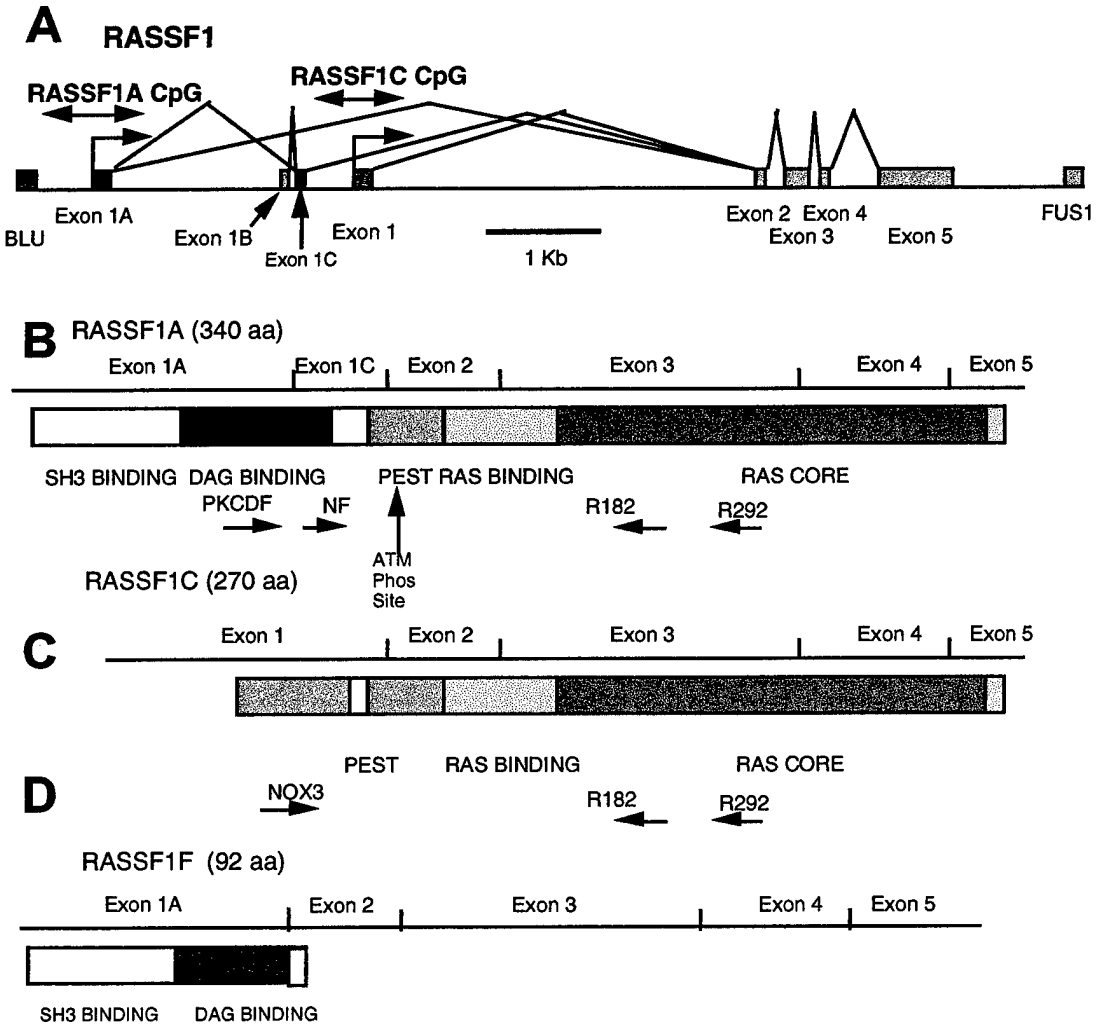
* NSCLC = non-small-cell lung carcinoma; SCLC = small-cell lung carcinoma

Table 2. Presence of methylated and unmethylated RASSF1A alleles in 97 lung and breast cancer cell lines.

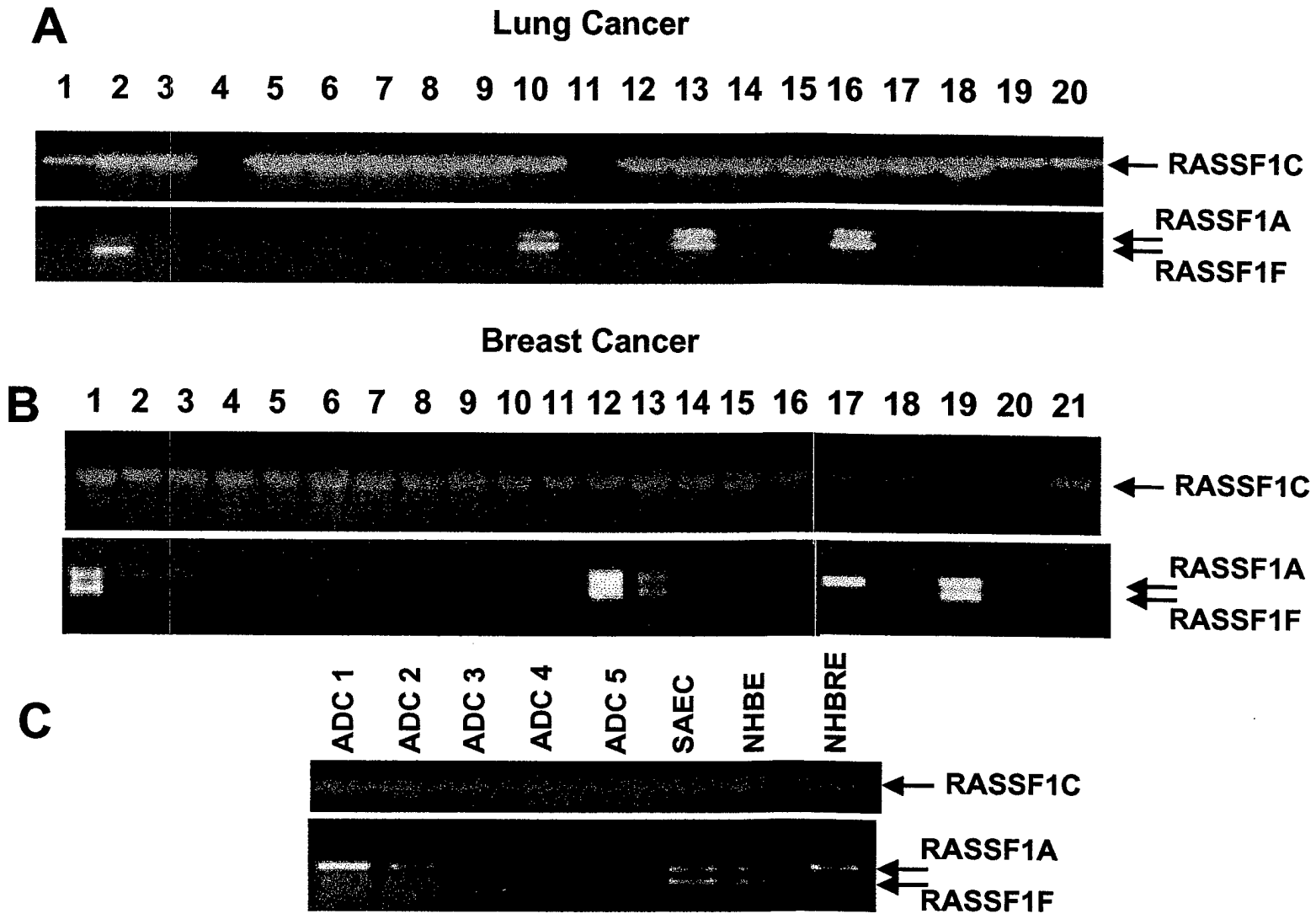
RASSF1A CpG Genotype		SCLC*	NSCLC	Breast	Total
Methylated allele	Unmethylated allele				
+	+	0	4	4	8
+	-	47	13	10	70
-	+	0	10	7	17
-	-	1	0	1	2†
Totals		48	27	22	97

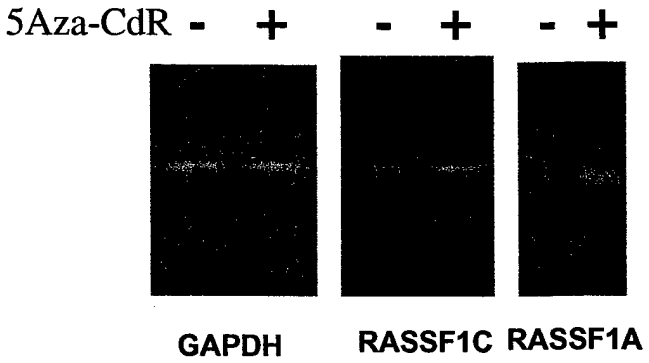
* SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer.

† The two tumor cell lines with methylation-specific polymerase chain reaction genotypes lacking both methylated and unmethylated alleles (SCLC line NCI-H740 and breast cancer line HCC1500) were known to have homozygous deletions including the RASSF1 locus in chromosome region 3p21.3.


A
RASSF1
RASSF1A CpG
RASSF1C CpG
BLU
Exon 1A
Exon 1B
Exon 1C
Exon 1
1 Kb
Exon 2
Exon 4
Exon 3
Exon 5
FUS1
B
RASSF1A (340 aa)
Exon 1A
Exon 1C
Exon 2
Exon 3
Exon 4
Exon 5
SH3 BINDING
DAG BINDING
PKCDF
NF
PEST RAS BINDING
ATM
Phos
Site
RAS CORE
R182
R292
RASSF1C (270 aa)
C
Exon 1
Exon 2
Exon 3
Exon 4
Exon 5
PEST
RAS BINDING
RAS CORE
D
NOX3
R182
R292
RASSF1F (92 aa)
Exon 1A
Exon 2
Exon 3
Exon 4
Exon 5
SH3 BINDING
DAG BINDING

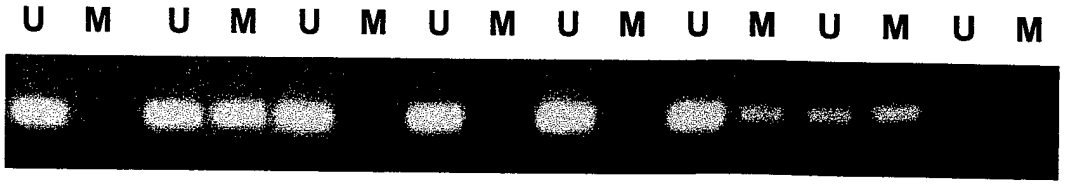
Burbee Fig. 1


A
Lung Cancer
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
RASSF1C
RASSF1A
RASSF1F
Breast Cancer
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
RASSF1C
RASSF1A
RASSF1F
C
ADC 1
ADC 2
ADC 3
ADC 4
ADC 5
SAEC
NHBE
NHBRE
RASSF1C
RASSF1A
RASSF1F



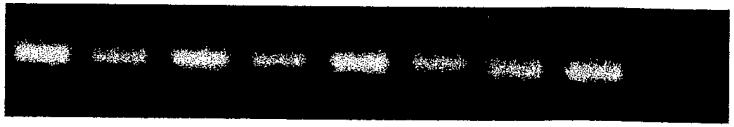
Burbee Fig. 3

Primary Resected NSCLCs

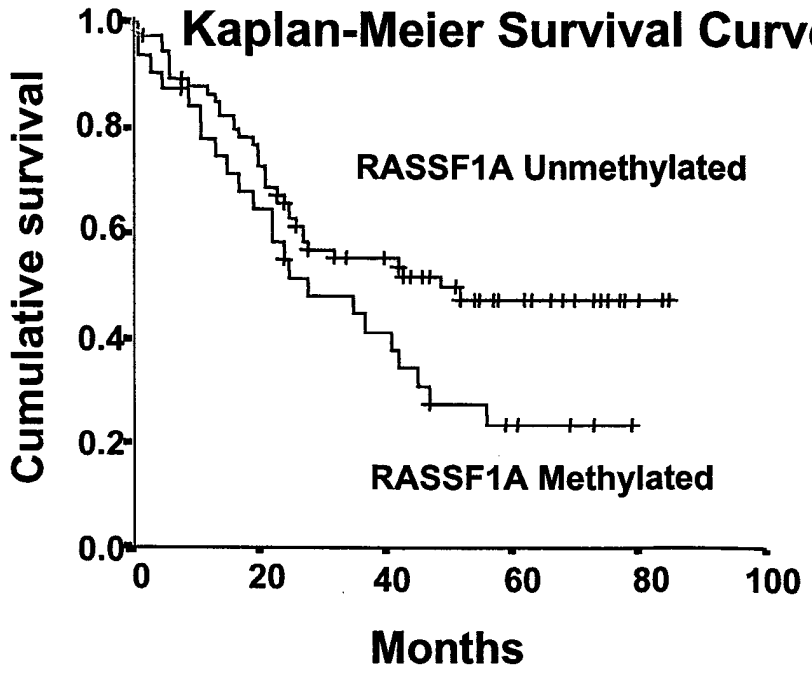


SCLCs

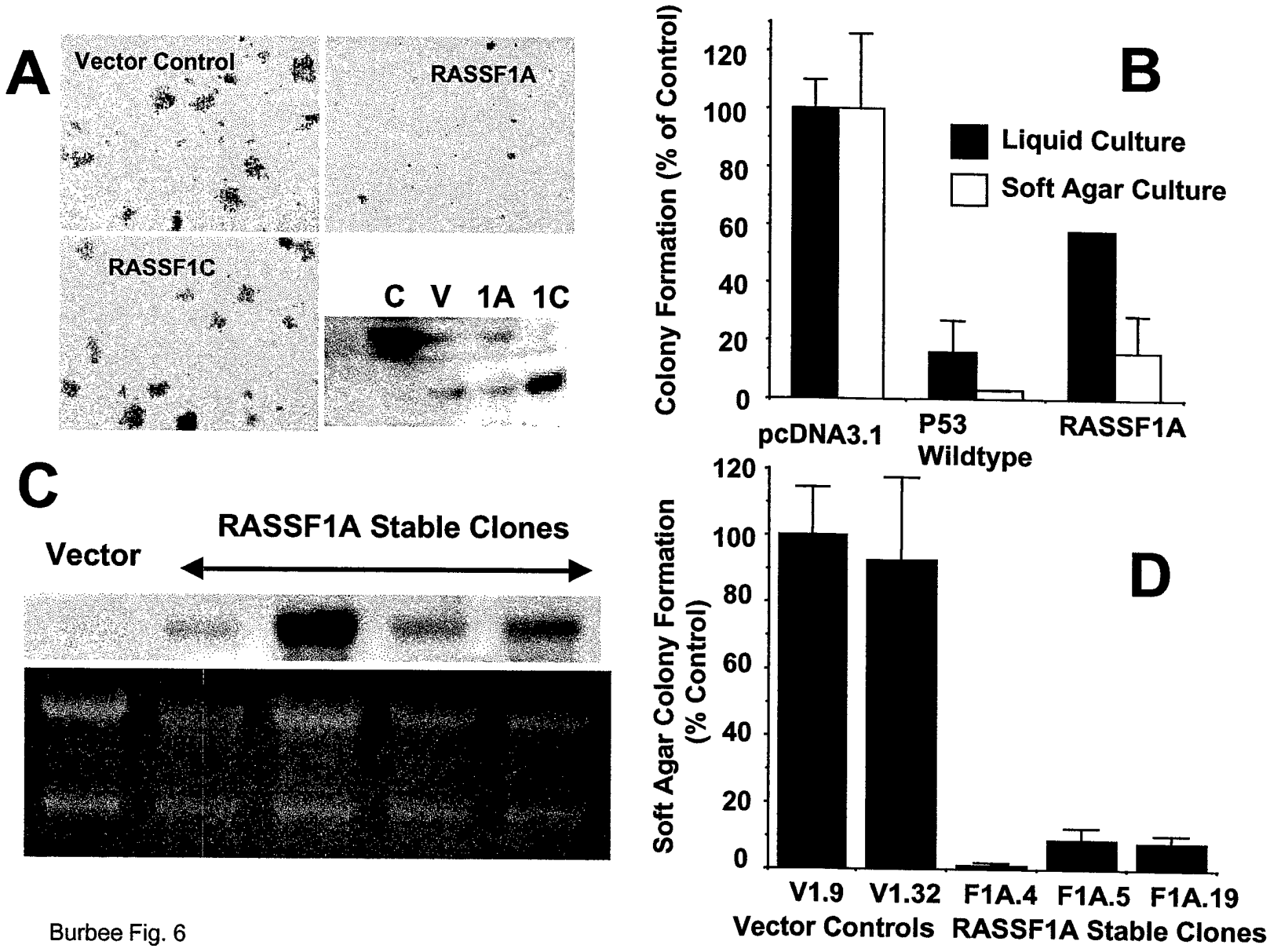
Primary Breast Cancers



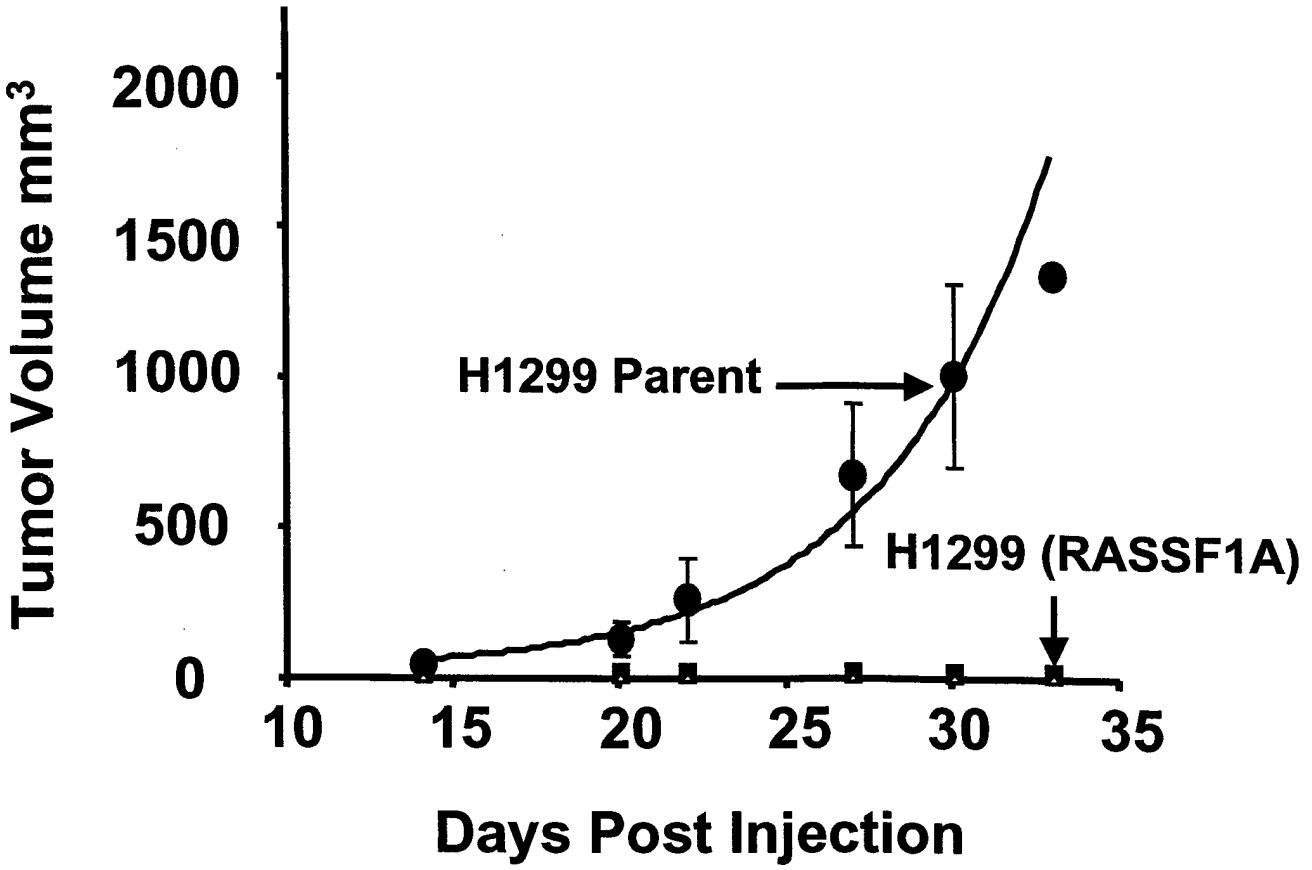
Burbee Figure 4


Kaplan-Meier Survival Curve
Cumulative survival
1.0
0.8
0.6
0.4
0.2
0.0
RASSF1A Unmethylated
RASSF1A Methylated
0
20
40
60
80
100
Months

Burbee Fig. 5


A
Vector Control
RASSF1A
RASSF1C
C V 1A 1C
B
Colony Formation (% of Control)
120
100
80
60
40
20
0
Liquid Culture
Soft Agar Culture
pcDNA3.1
P53 Wildtype
RASSF1A
C
Vector
RASSF1A Stable Clones
D
Soft Agar Colony Formation (% Control)
120
100
80
60
40
20
0
V1.9
V1.32
F1A.4
F1A.5
F1A.19
Vector Controls
RASSF1A Stable Clones

Burbee Fig. 6



Burbee Fig. 6E