

The *RASSF1A* Tumor Suppressor Gene Is Inactivated in Prostate Tumors and Suppresses Growth of Prostate Carcinoma Cells¹

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ABSTRACT

We analyzed expression status of the recently identified tumor suppressor gene *RASSF1A* in primary prostate carcinomas and in prostate cell lines. We found complete methylation of the *RASSF1A* promoter in 63% of primary microdissected prostate carcinomas (7 of 11 samples). The remaining 4 samples (37%) were partially methylated, possibly because of contamination with normal cells. No promoter methylation was observed in matching normal prostate tissues. High levels of *RASSF1A* transcript and no methylation of *RASSF1A* promoter were found in explanted primary normal prostate epithelial and stromal cells. Complete silencing and methylation of *RASSF1A* promoter was observed in five widely used prostate carcinoma cell lines, which acquired the ability to grow in culture spontaneously, including LNCaP, PC-3, ND-1, DU-145, 22Rv1, and one primary prostate carcinoma immortalized by overexpression of the human telomerase catalytic subunit (RC-58T/hTERT). However, no silencing of *RASSF1A* was found in four other prostate carcinoma cell lines, which were adapted for cell culture after transformation with human papillomaviral DNA. Suppression of cell growth *in vitro* was demonstrated after the reintroduction of *RASSF1A*-expressing construct into LNCaP prostate carcinoma cells. Our data implicate the *RASSF1A* gene in human prostate tumorigenesis.

INTRODUCTION

The RAS family of proto-oncogenes plays a fundamental role in signal transduction pathways involved in cellular proliferation and survival, interacting with other regulatory circuits of cell growth and death. In each cell type, the final outcome of the RAS-mediated signals depends on a particular intracellular environment, which includes a diverse array of effector proteins (1). Many known RAS effectors are oncoproteins on their own and, when overexpressed, may cause reduction of growth factor dependency, promote loss of contact inhibition, resistance to apoptosis, or other features of the tumor phenotype. Less is known about RAS effectors possessing tumor suppressor properties. Recently, a new family of genes encoding RAS-binding proteins, *RASSF1*, has been identified within the critical lung and breast cancer deletion region at 3p21.3 (2, 3). Two GC-rich promoters were found, producing *RASSF1A* and *RASSF1C* transcripts. The *RASSF1A* gene is silenced by highly selective aberrant methylation of promoter A in a large fraction of lung (2, 4, 5), breast (4, 5), ovarian (4), nasopharyngeal (6), kidney (7, 8), gastric (9),

bladder tumors (10, 11), and in neuroblastomas and pheochromocytomas (12). *RASSF1A* gene was also shown to be mutated in 9.5% of primary nasopharyngeal carcinomas (6). In bladder carcinomas, *RASSF1A* hypermethylation was correlated with poor prognosis (11). Re-expression of the *RASSF1A* transcript in lung and clear renal carcinoma cells reduced colony formation and suppressed anchorage-independent growth (2, 5, 7). Both *RASSF1A* and *RASSF1C* proteins possess the RAS-binding domain, which binds RAS in a GTP-dependent manner *in vivo* and *in vitro* (13). This domain was shown to mediate apoptotic response within the *ras*-signaling pathway in the 293-T human fetal kidney cells (13). In the present study, we investigated the expression of *RASSF1A* in primary prostate carcinomas in normal prostate stromal and epithelial cells using laser capture microdissection technique. *RASSF1A* expression was also analyzed in spontaneously, *hTERT*³- and HPV-immortalized prostate tumor cell lines and in primary explanted normal and tumor prostate cells. Finally, the *RASSF1A* gene was re-expressed in LNCaP prostate carcinoma cells to assess its growth suppression activity.

MATERIALS AND METHODS

Laser Capture Microdissection. Prostatectomy specimens were fixed overnight in 70% ethanol and processed in increased concentrations of ethanol and xylene, then embedded in paraffin, cut into glass slides, dewaxed, and stained with eosin. Areas corresponding to cancer or normal tissues were dissected (14), placed in proteinase K solution, and incubated overnight at 55°C. After that, the samples were incubated at 95°C for 8 min.

Cell Lines. Cryopreserved normal human prostate stromal (PrSC passage 4) and epithelial (PrEC passage 2) cells were purchased from Clonetics-BioWhittaker, Inc. (Walkersville, MD). The cells were propagated for an additional two passages and used for DNA and RNA isolation. DU145, LNCaP, and PC3 prostate carcinoma cell lines were purchased from American Type Culture Collection (Manassas, VA). ND-1 prostate carcinoma cell line was obtained from SAIC-Frederick, Inc., Biopharmaceutical Manufacturing and Quality Operations (Frederick, MD). R. M. Sramkoski (Comprehensive Cancer Center, Cleveland, OH) provided the 22Rv1 prostate carcinoma line. HPV-immortalized human prostate cell lines 1512T, 1512N, 1542T, 1542N, 1550T, and 1550N (15); HPV18 C-1 (normal prostate epithelial cells immortalized with HPV18 DNA); Ki-MuSV virus *Ki-ras*-transformed HPV18 C-1 (16); and 129NU5002-1 (17) were described elsewhere. HPV-immortalized prostate carcinoma line RC53T/E6E7 was developed by John S. Rhim.⁴ RC-58T/hTERT prostate carcinoma line adapted to cell culture by infection with a retroviral vector expressing the hTERT was described elsewhere (18).

Cell culture immortalization of the cell lines 1512T, 1512N, 1542T, 1542N, 1550T, 1550N, and RC53TE6E7 was accomplished by transduction of actively proliferating cells with a recombinant retrovirus encoding the E6- and E7-transforming proteins of HPV 16 and the selection marker neomycin phosphotransferase (15). Immortalization of HPV18 C-1 was performed by a

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³ The abbreviations used are: *hTERT*, human telomerase catalytic subunit; RT-PCR, reverse transcription-PCR; HPV, human papillomavirus; tTA, tetracycline-controlled transcriptional activator.

⁴ John S. Rhim, unpublished observations.

plasmid containing a complete single copy of the HPV 18 genome inserted into the pSV2neo plasmid; E7 gene product was identified in HPV18 C-1 cells using antibody (16).

Analysis of Methylation of RASSF1 Promoters. Bisulfite sequencing and methylation analyses for RASSF1A and RASSF1C promoters were described elsewhere (7). RASSF1A and RASSF1C PCR produced fragments of 377 and 224 bp, respectively. However, for methylation analysis of DNA samples obtained after microdissection, a shorter PCR fragment was amplified (190 bp). Primers used for the first PCR were B-RAS-E-5D (GGGTTTTATAGTTTTGTATTTAGGT) and B-RAS-E-3R (CAACTCAATAAACTCAAACCTCCC) at 94°C, 30 s; 58°C, 30 s; 72°C, 30 s; 35 cycles in FailSafe buffer I purchased from Epicentre Technologies (Madison, WI), followed by nested PCR with primers B-RAS-E-4D: TTTAGTTGGATTTTGGGGGAGG and B-RAS-E-7R: TCRCTAACTTTAAACRCTAACAAAC using the same PCR protocol in FailSafe buffer H. Each PCR fragment was digested with BstUI and MspI and run on 4–20% gradient polyacrylamide gel (Invitrogen, Carlsbad, CA).

RASSF1A Expression Analysis. RASSF1A mRNA RT-PCR quantification was performed with PCR primers described previously (7). A portion (0.1 µg) of total RNA per reverse transcriptase reaction was used. For each sample, five reverse transcriptase reactions (5 µl each) were set up using RETROscript kit (Ambion, Inc., Austin, TX) in the presence of different amounts of competing artificial RASSF1A RNA (7) carrying a 20-bp deletion (1, 0.1, 0.01, 0.001, 0.0001, and 0.00001% of total RNA). The competing artificial RASSF1A RNA produced a shorter PCR band. First and nested PCR reactions were carried out in FailSafe buffer H (Epicentre Technologies) at 95°C for 30 s and at 72°C for 40 s. Depending on the amount of PCR product, 20–35 cycles were necessary for each PCR. The amount of RASSF1A mRNA in each sample was estimated by comparison of the relative intensity of the normal and truncated PCR bands after 4–20% gradient PAGE.

Mutation Screening. For mutation screening, RASSF1A coding region was amplified using primers RASSF1A-1D: AGCGCCAAAGCCAGC-GAAGCAGC and RASSF1A-1R: CCCCACTGGCCCTGTCACACTCACA for 35 cycles at 95°C for 30 s and 72°C for 2 min in FailSafe buffer E (Epicentre Technologies). For nested PCR, we used primers RASSF1A-2D: GAAGCAGGGCCCAACCGGGCC and RASSF1A-2R: ACGCACTTGCGCTGCCTGCTGTC in FailSafe buffer D (Epicentre Technologies). The PCR reaction was carried out at 95°C for 30 s, 67°C for 30 s, and 72°C for 2 min. The final PCR products were cloned and sequenced.

Cell Growth Assay. Retroviral vector pLNCtTA-EF1α, encoding a tTA, was used to engineer LNCaP prostate carcinoma cells to constitutively produce tTA (19). The cells were maintained in RPMI 1640 with the addition of 15% FCS, 20 µg/ml hydrocortisone, and 500 µg/ml G418. Wild-type and mutant RASSF1A (Val211Ala) reading frames were subcloned into an episomal tetracycline-regulated vector pETE (19). The constructs were transfected into LNCaP/tTA cells. Stably transfected clones expressing RASSF1A transcript were expanded, seeded into 24-well plates, and grown for ≤15 days. For each line, the cells were trypsinized and counted in triplicate every other day.

RESULTS

RASSF1A Promoter Is Hypermethylated and Silenced in Primary Prostate Tumors. Microdissected tumors and matching normal prostate tissues were obtained from resected tissues of 11 individuals. DNA was isolated and treated with sodium bisulfite as described in “Materials and Methods.” The original target sequence contained one BstUI cutting site. The RASSF1A promoter region was amplified by PCR and digested with BstUI. Only those BstUI CGCG restriction sites that had both cytosines methylated in the original DNA remained preserved after sodium bisulfite treatment and PCR amplification. Therefore, BstUI cuts of the PCR products indicated methylation of the original DNA. To verify complete DNA sequence conversion after bisulfite treatment, the same PCR fragments were digested with MspI. This enzyme recognizes a CCGG sequence that is not preserved in bisulfite-converted DNA, regardless of its methylation status. As expected, MspI cut none of the PCR fragments.

Using this approach, we found no methylation of the RASSF1A

promoter in any of the DNA from normal microdissected tissues (Table 1; Fig. 1). Complete promoter methylation was evident in 7 of 11 tumor samples, as demonstrated by BstUI digest, whereas the remaining 4 DNAs were partially methylated. Therefore, all analyzed tumors exhibited some degree of aberrant RASSF1A promoter hypermethylation, 63% of them being completely methylated.

In a separate experiment, RASSF1A expression was analyzed during the first passage of two primary explanted prostate carcinoma tumors (Table 2). In one tumor (RC-123T), RASSF1A mRNA was not expressed, whereas the matching normal prostate epithelial cells (RC123N) had normal RASSF1A expression. In the second tumor sample (RC114T), we found normal expression of RASSF1A. No mutations were detected in this sample after the RASSF1A reading frame was reverse transcribed, cloned, and sequenced.

Silencing and Methylation of RASSF1A Gene in “Spontaneously” Immortalized Prostate Cell Lines. To further establish a correlation between RASSF1A transcriptional silencing and hypermethylation of the respective promoter, we analyzed RASSF1A methylation and transcription in a number of primary and immortalized prostate cell lines. No RASSF1A promoter methylation was found in primary explanted normal human stromal prostate cells. In primary normal prostate epithelial cells obtained from the same donor, we found low level promoter methylation (Table 2; Fig. 2). Compared with stromal cells, RASSF1A expression in epithelial cells was slightly decreased. A complete absence of the transcript and heavy methylation of the RASSF1A promoter was found in all “spontaneously immortalized” prostate carcinoma cell lines, including DU 145, LNCaP, PC3, 22Rv1, and ND-1. According to quantitative RT-PCR analysis, the relative amount of the RASSF1A mRNA in each of these samples was well below 0.0001% of total RNA (Fig. 2). We were able to reverse RASSF1A silencing in PC3 cells after treatment with 5 µM 5-aza-2'-deoxycytidine for 3 days (data not shown). Other cell lines were not tested. The RASSF1C promoter remained unmethylated in all examined cell lines (data not shown); therefore, RASSF1A methylation was highly specific in all affected cell lines.

According to recent publications, most of the prostate carcinoma cell lines used in this experiment are authentic and unique. Only the DU 145 cells were shown to share common origin with the ND-1 cell line (20).

The RASSF1A gene was also silenced in another prostate carcinoma cell line, RC-58T/hTERT (18), immortalized by overexpression of hTERT (Table 2).

Absence of RASSF1A Methylation in HPV-immortalized Prostate Cell Lines. Adaptation of prostate carcinoma cells for growth in culture always represents a significant challenge. To alleviate this problem, the primary tumor cells are often “immortalized” by transfection with SV40 virus or an HPV DNA. We tested the expression of the RASSF1A gene in four prostate carcinoma cell lines, adapted to grow in cell culture by transfection with the DNA, which expresses E6/E7-transforming proteins of HPV serotype 16 or 18 (11, 16). Unexpectedly, all four tested HPV-transformed cell lines (1512T, 1542T, 1550T, and RC53T/E6E7) expressed normal levels of RASSF1A mRNA (Table 2). Similar amounts of RASSF1A mRNA (from 0.001 to 0.005% of total RNA) were present in matching HPV-immortalized cell lines derived from the normal epithelial cells of the same patients (1512N, 1542N, and 1550N).

Table 1 RASSF1A methylation in primary prostate normal and tumor samples

	Unmethylated	Partially methylated	Methylated
Normals	11	0	0
Tumors	0	4	7

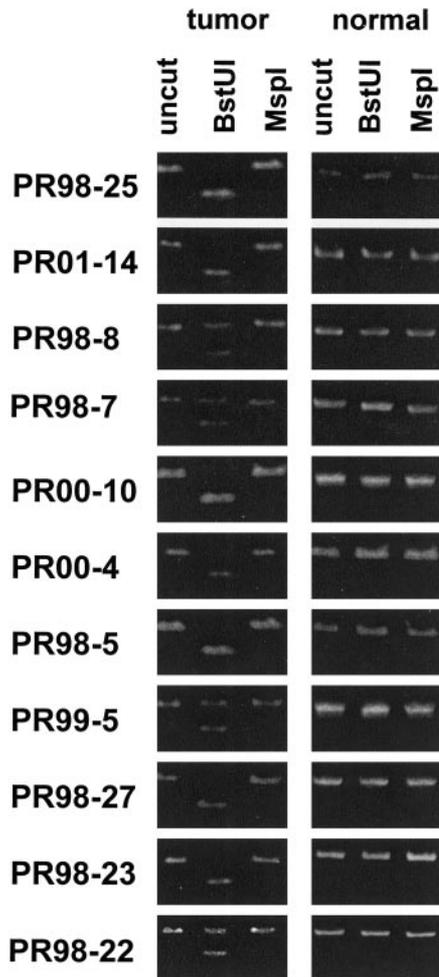


Fig. 1. Methylation status of the RASSF1A promoter in prostate tumors and matching normal prostate tissues. *BstUI*, indicates promoter methylation in tumors.

Normal expression of the *RASSF1A* transcript was also detected in a set of prostate epithelial cell lines immortalized by transfection with HPV-18 DNA and expressing the HPV-18 E7 gene product. This panel includes the original immortalized human prostate epithelial cell line HPV18 C-1, Ki-*ras*-transformed HPV18 C-1 cells, which acquired

the ability to grow in nude mice (16), and a malignant subclone of HPV18 C-1 (129NU5002-1) selected after multiple exposure to the chemical carcinogen *N*-nitroso-*N*-methylurea (17). No *RASSF1A* mutations were found in the 129NU5002-1 derivative of the HPV18 C-1 (Table 2).

Re-expression of *RASSF1A* Gene in LNCaP Cells Suppresses their Growth *in Vitro*. LNCaP cells constitutively producing tTA tetracycline *trans*-activator (19) were transfected with the episomal pETE tetracycline-regulated vector, carrying a wild-type or mutated (Val211Ala) *RASSF1A* cDNA (7). These episomal vectors provide more reproducible and uniform expression of transgenes. Hygromycin-resistant cells expressing high levels of *RASSF1A* mRNA from the plasmid (>0.005% of total RNA) were assessed for growth on plastic surfaces in the absence of doxycycline. The original LNCaP cells and the clone encoding the mutant *RASSF1A* protein had virtually identical growth curves. In contrast, growth of the cells expressing wild-type *RASSF1A* protein was suppressed >10-fold (Fig. 3).

When 5 µg/ml doxycycline were added to the growth medium, the level of *RASSF1A* mRNA decreased but remained detectable in all transgenic clones. Under these conditions, it was compatible to the amount of *RASSF1A* mRNA, which was found in normal prostate epithelial cells (0.001% of total RNA; Fig. 2). Therefore, for every clone maintained in the presence of doxycycline, we observed growth curves identical to those obtained in the media without doxycycline (data not shown).

Renal cell carcinoma line KRC/Y was less sensitive to low levels of *RASSF1A* mRNA re-expression. Similar 10-fold growth suppression was achieved in these cells only when *RASSF1A* expression exceeded 0.005% of total RNA (7).

DISCUSSION

We found aberrant hypermethylation of *RASSF1A* promoter in microdissected primary prostate carcinomas but not in matching normal prostate tissues. In 7 of 11 primary prostate tumors, the *RASSF1A* promoter was completely methylated. We estimate, in the remaining 4 tumors with partial *RASSF1A* methylation, contamination of the microdissected tissue with normal cells may reach as high as 20%. These cells may produce the PCR bands corresponding to unmethylated *RASSF1A*, making the percentage of methylated samples even higher.

As a consequence of frequent *RASSF1A* promoter methylation, the

Table 2 *RASSF1A* inactivation in prostate cell lines

Sample name	Immortalization	Tumorigenicity	RASSF1A Methylation	RASSF1A Expression	RASSF1A Mutations
RC-114T	No	ND, ^a tumor cells	ND	+	None
RC-123T	No	ND, tumor cells	ND	-	ND
RC-123N	No	ND, normal PrEC	ND	+	ND
Clonetics PrSC	No	ND, normal cells	-	+	ND
Clonetics PrEC	No	ND, normal cells	-	+	ND
DU 145	Spontaneous	Tumorigenic	+	-	ND
LNCaP	Spontaneous	Tumorigenic	+	-	ND
PC-3	Spontaneous	Tumorigenic	+	-	ND
22Rv1	Spontaneous	Tumorigenic	+	-	ND
ND-1	Spontaneous	Tumorigenic	+	-	ND
RC-58T/hTERT	hTERT	ND, tumor origin	ND	-	ND
RC53T/E6E7	By HPV	ND, tumor origin	ND	+	None
1512T	By HPV	ND, tumor origin	-	+	ND
1512N	By HPV	ND, from PrEC	-	+	ND
1542T	By HPV	ND, tumor origin	-	+	ND
1542N	By HPV	ND, from PrEC	-	+	ND
1550T	By HPV	ND, tumor origin	-	+	ND
1550N	By HPV	ND, from PrEC	-	+	ND
HPV18 C-1	By HPV	Nontumorigenic	ND	+	ND
Ki- <i>ras</i> /HPV18 C-1	By HPV	Tumorigenic	ND	+	None
129NU5002-1	By HPV	Tumorigenic	ND	+	None

^a ND, not determined; PrSC, normal human prostate stromal cell; PrEC, normal human prostate epithelial cell.

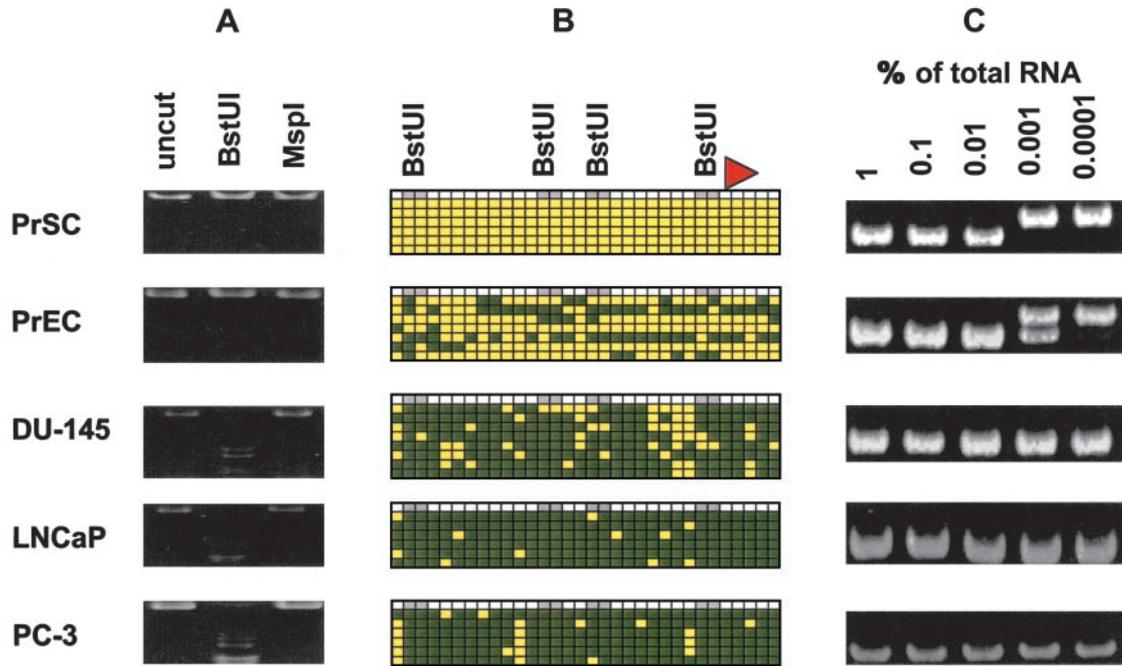


Fig. 2. Promoter methylation and *RASSF1A* expression in prostate normal and tumor cells. *A*, combined bisulfite-restriction analysis of the *RASSF1A* promoter in normal and tumor prostate cells. Genomic DNA was treated with bisulfite, *RASSF1A* promoter was amplified by PCR, and the product was digested with *Bst*UI or *Msp*I. *B*, bisulfite sequencing of the PCR-amplified products from *A*. For methylation analysis, PCR fragments were subcloned, and several plasmid clones were sequenced. Each horizontal row corresponds to one plasmid clone and contains 32 yellow and green squares representing unmethylated and methylated CpG dinucleotides, respectively. Gray squares delineate CpG dinucleotides within *Bst*UI recognition sites. *C*, quantification of the *RASSF1A* transcript by RT-PCR. The amount of the competitor *RASSF1A* RNA added to each reverse transcriptase reaction is indicated.

gene is often silenced in freshly explanted primary prostate tumors but not in normal prostate epithelial or stromal cells. Silencing and hypermethylation of the *RASSF1A* was also found in all five widely used permanent prostate carcinoma cell lines, which were adapted to cell culture spontaneously and in an *hTERT*-transformed line.

Unexpectedly, the gene was expressed in all studied HPV-immortalized lines. The extent of the prostate HPV infections and their role, if any, in prostate tumorigenesis remains highly controversial (21). In one study, seropositivity against HPV-18 was associated with a 2.6-fold increase in the risk of developing prostate cancer. Our data point to a possible correlation between HPV infection and *RASSF1A* expression. This correlation could reflect a functional interaction between the cellular *RASSF1A* and the viral E6/E7 proteins, which may

play important roles in both neoplastic transformation and immortalization of the prostate epithelial cells. In this case, the high rate of *RASSF1A* methylation in primary prostate cancer would imply that HPV is only involved in a limited number of the natural prostate tumors.

Evidence for causal association between HPV and a subset of head and neck cancers was recently reported (22). Therefore, similar reverse correlation between HPV infections and *RASSF1A* methylation may exist for this type of cancer.

The HPV infections are recognized as a major contributor to the development of cervical neoplasia. HPV DNA was detected in 93% of the cervical tumors worldwide (23). Interestingly, *RASSF1A* methylation was not identified in primary cervical tumors. Currently, this tumor type remains the only reported one in which *RASSF1A* methylation has not been found (4). However, HPV presence was not assessed in that particular study. Simultaneous assessment of *RASSF1A* inactivation and HPV presence in cervical carcinomas may reveal this putative correlation.

We assessed *RASSF1A* status in a panel of prostate cell lines of the same origin transformed either by the introduction of activated *Ki-ras* (16) or by a chemical carcinogen, *N*-nitroso-*N*-methylurea (129NU5002-1 cells; Ref. 17). Cells (129NU5002-1) acquired tumorigenic phenotype without the involvement of RAS proteins. In both tumorigenic cell lines, no *RASSF1A* dysregulation was found (Table 2).

In our final experiment, the *RASSF1A* expression construct was reintroduced into a prostate carcinoma cell line in which the endogenous *RASSF1A* gene was silenced. Re-expression of the wild-type, but not mutant, *RASSF1A* suppressed the growth of the prostate carcinoma cell line LNCaP *in vitro*. Prostate LNCaP cells were significantly more sensitive to low levels of *RASSF1A* expression compared with a KRC/Y renal carcinoma cell line (7). A >10-fold growth suppression of LNCaP was observed after reintroduction of the *RASSF1A* transgene.

Our data implicate *RASSF1A* gene in human prostate tumorigenesis.

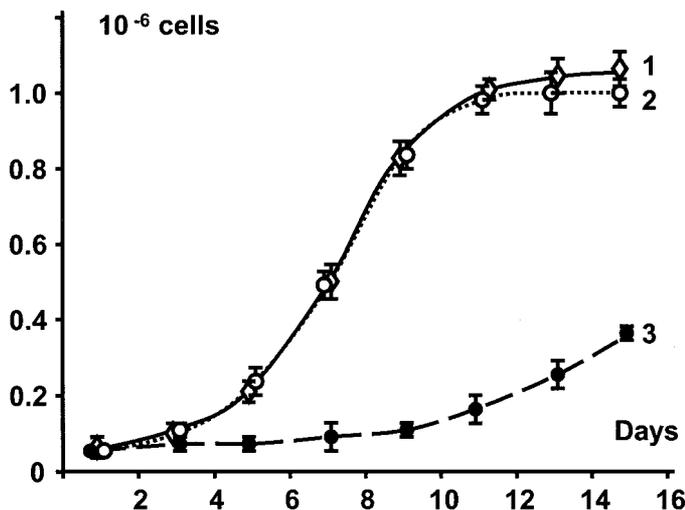


Fig. 3. Growth inhibition of LNCaP cells by *RASSF1A* gene. 1, original LNCaP cells; 2, LNCaP transfected with mutant *RASSF1A*; 3, LNCaP cells transfected with wild-type *RASSF1A*.

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The *RASSF1A* Tumor Suppressor Gene Is Inactivated in Prostate Tumors and Suppresses Growth of Prostate Carcinoma Cells

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