# The Role of *RASSF1A* in Uveal Melanoma

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**Purpose.** *RASSF1A* inactivation in uveal melanoma (UM) is common and methylation-induced. We investigated the effect of *RASSF1A* re-expression on the UM phenotype in vivo and in vitro.

**Methods.** The phenotypic effect of methylation-induced inactivation of *RASSF1A* in UM was explored using a stable *RASSF1A*-expressing UM-15 clone. *RASSF1A* expression was assessed using QRT-PCR. Proliferation was evaluated in vitro using MTT assays. Additionally, athymic NOD/SCID mice were injected subcutaneously or intraocularly with *RASSF1A*-expressing and –non-expressing UM-15 clones, and euthanized when tumors reached a volume of 1500 $mm^3$, or at 56 or 46 days, respectively. Tumor tissues, eyes, and livers were analyzed histologically.

**Results.** In vitro analysis confirmed the lack of *RASSF1A* expression and full methylation of the *RASSF1A* promoter region in the UM-15 cell line, which was reversible following treatment with 5-Aza-2-deoxycytidine. Cells expressing exogenous *RASSF1A* showed slower proliferation than controls and regained sensitivity to cisplatin. Compared to mice injected with control cells, mice treated with UM-15 cells expressing exogenous *RASSF1A* did not acquire intraocular tumors, and their subcutaneous tumors were relatively delayed and small. Neither group had liver metastases.

**Conclusions.** UM cells reduced tumorigenicity in the presence of activated *RASSF1A. RASSF1A* apparently has an important role in the development of UM, and its reactivation might be applied in the development of new treatments. (*Invest Ophthalmol Vis Sci.* 2012;53:2611–2619) DOI:10.1167/iovs.11-7730

Uveal melanoma (UM) is the most common form of primary eye cancer in adults, with an annual incidence of 6–7 cases per million per year.[1] It accounts for 80% of all noncutaneous melanomas and 13% of all deaths caused by melanoma. The tumor carries up to 50% 5-year mortality from metastasis.[2]

Uveal and cutaneous melanomas originate from the same precursor cell, the melanocyte, which migrates from the neural crest to the respective sites during embryonic development.[3,4] However, their biological and clinical behaviors differ.[2] Additionally, although alterations of chromosomes 1 and 6 are common to both tumors, aberrations, such as monosomy of chromosome 3 and gain of 8q, in addition to other aberrations, typically are found only in UM.[5–7] Following the identification of late genetic events in UM progression and metastasis, such as loss of chromosome 3,[8] researchers directed attention to the early initiating events leading to malignant transformation and development of a clinically detectable tumor.[9] Studies revealed that a mutation in the alpha subunit of the heterotrimeric G gene (*GNAQ*) was present in almost half of all UMs examined,[9–15] and that UM metastatic spread was related to mutations in the BRCA associated protein 1 (*BAP1*) gene on chromosome 3.[16] However, more data were needed to clarify the still poorly characterized tumorigenesis of UM.[9]

Aberrant promoter hypermethylation of CpG islands is thought to have an important role in the inactivation of tumor suppressor genes (TSGs) in cancer.[17,18] In a study of 86 metastatic specimens of cutaneous melanoma, Hoon et al. identified 4 TSGs that frequently were inactivated: retinoic acid receptor-beta2 (*RAR-beta2*, 70%), RAS association domain family protein 1A (*RASSF1A*, 57%), O6-methylguanine DNA methyltransferase (*MGMT,* 34%), and death-associated protein kinase (*DAPK*, 19%).[19] Hypermethylation of *MGMT, RASSF1A,* and *DAPK* was significantly lower in primary melanomas than in metastatic melanomas, whereas the rate of *RAR-beta2* hypermethylation was 70% in both types. Prompted by these data, we sought, in an earlier study, to elucidate the role of epigenetic events in UM by investigating the methylation status of these genes and the newly described methylator phenotype TSG panel.[20] The results showed that *RAR-beta2, MGMT,* and *DAPK* promoter hypermethylation was uncommon in UM, and all samples were negative for the CpG methylator phenotype. Similar findings also were reported by another group.[21]

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**Table 1.** List of All Primers and Probes Used in This Study

| | Forward 5′-3′ | Probe 5′-3′ |
|---|---|---|
| MSP primers: | | |
| *RASSF1A*_UM (unmethylated) | CCC ATA CTT CAA CTT TAA AC | |
| *RASSF1A*_M (methylated) | GCG TTG AAG TCG GGG TTC | 6FAM-ACA AAC GCG AAC CGA ACG AAA CCA-TAMRA |
| β-Actin (MSP) | TGG TGA TGG AGG AGG TTT AGT AAG T | ACC ACC ACC CAA CAC ACA ATA ACA AAC ACA |
| Expression primers: | | |
| *RASSF1A* | GCA GTG CGC GCA TTG CAA GT | |
| GAPDH | ACCACAGTCCATGCCATCAC | |

However, promoter hypermethylation for the *RASSF1A* gene was present in a significant proportion of the samples, in accordance with the study of Maat et al.[22]

Besides cutaneous melanoma, the *RASSF1A* promoter gene is known to be extremely common in cancers of the breast, head and neck, and lung.[23] In general, an epigenetic mechanism underlies its aberrant methylation, as opposed to somatic inactivating mutations, which occur rarely.[23] Furthermore, *RASSF1A* lies on the 3p21.3 region of chromosome 3, which frequently is rearranged in UM, making it a candidate TSG in this tumor.[24,25] The *RASSF1A* gene contains two CpG islands that are susceptible to inactivating methylation, spanning the promoter and the first exon gene regions.

The aim of the our study was to examine the influence of exogenous expression of *RASSF1A* on the UM cell phenotype in vitro and in vivo.

## Methods

The study protocol was approved by the national and institutional review boards. The study was conducted in adherence with the Declaration of Helsinki.

### Cell Lines

The UM-15 cell line and all 20 paraffin-embedded UM samples were classified and provided by the Laboratory of Ophthalmic Pathology, Hadassah-Hebrew University Medical Center, Jerusalem.

[Karyotype for UM-15 cell line: 53 ~ 56 < 2n > ,XY,+X,+der(1;13)(p10;q10),+2,+der(3)t(3;5)(p13;q15),i(5)(p10)x2,+i(5)(q10),add(8)(p22)x2,der(11)t(1;11)(p15;q10),dup(14)(q11.2q22),+del(16)(q10),der(19)t(3;19)(p13;p13.3),der(20)t(17;20)(q21;q13.3),+22,+add(22)(q13)(cp16)].

### Immunohistochemistry Staining for RASSF1A

UM-15 cells were seeded on 22 × 22 mm glass coverslips in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and grown to 50–60% confluence. The cells then were transfected with 1 μg of each expression plasmid and incubated for 14–18 hours. Following incubation, the cells were fixed in 3% paraformaldehyde solution (PFA) for 20 minutes at room temperature and permeabilized using 0.5% Triton X-100 solution for 10 minutes. Coverslips were blocked with 3% horse serum for 30 minutes at room temperature. They then were incubated with primary mouse monoclonal [3F3] anti-*RASSF1A* antibody (ab23950; Abcam, Cambridge, UK) in a humidifying chamber for 1 hour at room temperature, and stained with Cy5- and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (1:100; Jackson Immunoresearch, West Grove, PA) for 1 hour at room temperature. Histological sections of kidney tissue were used as a positive control for *RASSF1A* expression.

### Fluorescence In Situ Hybridization (FISH) for Monosomy 3

Using standard techniques, FISH was performed with pericentromeric chromosome enumeration probes (CEP3) labeled with SpectrumOrange (Abbott Molecular, Abbott Park, IL). The UM-15 cell line was counterstained with 4′,6-diamidino-2-phenylindole Antifade.

### DNA Extraction

DNA was extracted from microdissected tumor material and the UM-15 cell line, as described previously.[20] In brief, 10-mm section slides stained with hematoxylin and eosin were reviewed by a pathologist. Thereafter, areas containing only tumor were separated by microdissection from 5 consecutive 10-mm unstained paraffin sections of each block using a No. 11 surgical blade. Following deparaffinization, the microdissected tissues were incubated overnight in 1% sodium dodecyl sulfate (SDS) and Proteinase K 0.5 mg/ml. DNA was purified by phenol-chloroform extraction and ethanol precipitation, and dissolved in 50 mL of distilled water.

### Bisulfite Modification

Methylation detection using the bisulfite treatment method has been described previously.[20] In brief, 1–2 μg of genomic DNA were denatured in sodium hydroxide (NaOH, 0.3 M) for 15 minutes at 37°C. Cytosines were sulfonated in sodium bisulfite 3.12 M (Sigma, St. Louis, MO), and hydroquinone 5 mM (Sigma) for 16 hours at 50°C. The DNA samples then were purified (Wizard DNA Clean-Up System; Promega, Madison, WI), desulfonated in NaOH (0.3 M), precipitated in ethanol, and suspended in water.

### Methylation-Specific PCR (MSP)

The primers and probes used in the MSP study are shown in Table 1. The 3′DNA was sequenced by mixing bisulfite-treated DNA (100 ng) with 50 pmoles of each primer in a reaction buffer (50 μL) containing dNTPs (200 μM each) and AmpliTaq Gold Taq polymerase (Applied Biosystems, Inc., Foster City, CA) at 95°C for 10 minutes followed by 94°C for 30 seconds, 55°C for 30 seconds, and 74°C for 1 minute, for 35 cycles. The PCR products were analyzed on 1.5% agarose gel.

### Real-Time Quantitative Methylation-Specific PCR (QMS-PCR)

The sodium-bisulfite-treated genomic DNA was analyzed with the ABI Prism 7900 Sequence Detection System (Applied Biosystems), as described previously.[20] Amplifications were carried out in 96-well plates, each containing patient samples and water blanks, and positive and negative controls. For the positive controls, we used DNA extracted from leukocytes of healthy individuals that were methylated in vitro with SssI methyltransferase (New England Biolabs Inc., Beverly, MA); serial dilutions of this DNA were used to construct the standard

**Table 1.** Extended

| Reverse 5′-3′ |
|---|
| GGT GTT GAA GTT GGG GTT TG |
| CCC GTA CTT CGC TAA CTT TAA ACG<br>AAC CAA TAA AAC CTA CTC CTC CCT TAA |
| AGG CTC GTC CAC GTT CGT GT<br>TCC ACC ACC CTG TTG CTG TA |

curves on each plate. The relative degree of methylation of each DNA sample was defined as the ratio between the value of the gene of interest and the value of the internal reference gene (gene of interest:reference gene × 1000), as described previously.[26] For the negative control, we used DNA extracted from a healthy individual. The primers used in the QMS-PCR study are shown in Table 1. The 3′DNA was sequenced by mixing bisulfite-treated DNA (100 ng) with 50 pmoles of each primer in a reaction buffer (50 μL) containing dNTPs (200 μM each) and AmpliTaq Gold Taq polymerase (Applied Biosystems) at 95°C for 10 minutes followed by 94°C for 30 seconds, 55°C for 30 seconds, and 74°C for 1 minutes, for 35 cycles. The PCR products were separated on 1.5% agarose gel.

## cDNA Preparation

Total RNA was isolated using TRIzol reagent (Invitrogen, Life Technologies, Carlsbad, CA), according to the manufacturer's protocol. Before cDNA synthesis, RNA was treated with DNase I. The cDNA synthesis was carried using a random hexamer (Amersham Biosciences, Buckinghamshire, UK) and Moloney murine leukemia virus (M-MLV)-reverse transcriptase (Promega).

## Analysis of mRNA Expression of RASSF1A by Quantitative Real-Time Polymerase Chain Reaction (QRT-PCR)

Two-stage QRT-PCR was used to evaluate *RASSF1A* expression. *RASSF1A* cDNA input levels were normalized against human glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and analyses were done with the Sequence Detection System (Prism 7000; Applied Biosystems). The primers for *RASSF1A* and GAPDH are shown in Table 1.

Reactions were performed in a 20-μL volume containing 4 μL cDNA, 0.5 μM each of the forward and reverse primers, and buffer included in the Master Mix (SYBR$^R$ Green I; Applied Biosystems). Cycling conditions consisted of an initial denaturation step of 95°C for 10 minutes followed by 40 cycles of 1-minute denaturation at 95°C and 1 minute of annealing and extension at 60°C. Duplicate QRT-PCR reactions were performed for each gene to minimize individual tube variability, and an average was taken for each time point.

## Re-Expression of RASSF1A by 5-Aza-2-Deoxycytidine Treatment

UM-15 cells were treated by different concentrations (1, 3, 5, 10 μM) of the demethylating agent 5-Aza-2′-deoxycytidine (5-Aza-CdR) for 4 days, $5 \times 10^6$ cells for each concentration. The cells were fed with fresh medium supplemented daily with the drug. Control cells received mock treatment (no treatment). At the end of the treatment period, the medium was removed, and the expression pattern of *RASSF1A* was tested. The methylation status was analyzed by QRT-PCR, as described above.

## Construction of RASSF1A-Expressing UM-15 Clone

The construction of a stable *RASSF1A* clone was performed as described previously.[27,28] In brief, a UM-15 cell line showing no *RASSF1A* expression was used for the transfection study. Human wild-type *RASSF1A* cDNA (kindly provided by Reinhard Dammann, Institute for Human Genetics, Martin Luther University, Halle-Wittenberg, Halle/Saale, Germany) was cloned into pcDNA3.1. The *RASSF1A*-containing plasmid and the empty vector were introduced into the UM-15 cells using Lipofectamine 2000 (Invitrogen). A stable clone of *RASSF1A* transfectants was obtained by sustained G418 selection (50 μg/mL). The expression of exogenous *RASSF1A* was confirmed by quantitative QRT-PCR. The same cell line transfected with empty pcDNA3.1 vector served as the control.

## Cell Proliferation Assays

The proliferation rate of melanoma cells treated with cisplatin was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma). UM cell lines were cultured in 96-well plates ($1X10^6$ cells/well) with and without cisplatin (0.25 or 0.5 μg/mL) for 24 hours ($N = 3$ for each cell line). The cells then were treated with a 20 μL aliquot of MTT (5 mg/mL) for 4 hours and solubilized in 200 μL of dimethyl sulfoxide (DMSO). Absorbance was measured spectrophotometrically at 570 nm. The inhibition rate (IR) of cell growth was calculated by the following formula: IR = 1 – (value in experimental groups/value in control group) × 100%.

## Mice

We used 59 athymic NOC/SCID mice 5–6 weeks old (44 female, 15 male) in the study. The mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in a barrier facility (kindly provided by Nadir Askenasy, Frankel Laboratory, Center for Stem Cell Research, Schneider Children's Medical Center of Israel, Petach Tikva, Israel) under a 14-hour light/10-hour dark cycle with standard chow and water ad libitum. The mice were managed in accordance with the NIH Guidelines on Laboratory Animal Welfare and the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmology and Vision Research. All protocols were approved and monitored by the Animal Care Committee of Rabin Medical Center.

## Subcutaneous Cell Transplantation

Of the mice studied 18 were deeply anesthetized with ketamine 80 mg/kg and Xylazine 4 mg/kg (Kepro B.V., Barneveld, The Netherlands) and divided into 3 equal groups, each of which received $1 \times 10^6$ cells, as follows: (1) *RASSF1A*-pcDNA3.1-transfected UM-15 cells (*RASSF1A* expression was validated before injection), (2) parental UM-15 cells transfected with empty pcDNA3.1 vector (control 1), and (3) non-transfected parental UM-15 cells (control 2).

The resulting tumors were measured with calipers 3 times per week. On the basis of these values, tumor volume was calculated using the modified ellipsoidal formula: 1/2(length × width$^2$).[29] The mice were euthanized 56 days after cell transplantation, and the liver tissue, eyes, and subcutaneous tumors were analyzed histologically and molecularly for *RASSF1A* mRNA expression. This experiment was done in duplicate.

## Intraocular Cell Transplantation

As described previously, UM-15 cells with (group 1) or without (groups 2, 3) exogenous *RASSF1A* expression were transplanted intravitreally to the right eye of athymic NOD/SCID mice ($n = 4$ in each group). The left eye served as a control. Briefly, mice were placed under anesthesia, and the cells ($5 \times 10^5$ cells/3.0 μL) were injected with a syringe (Hamilton, Reno, NV) fitted with a 27-gauge needle. After 46 days, the

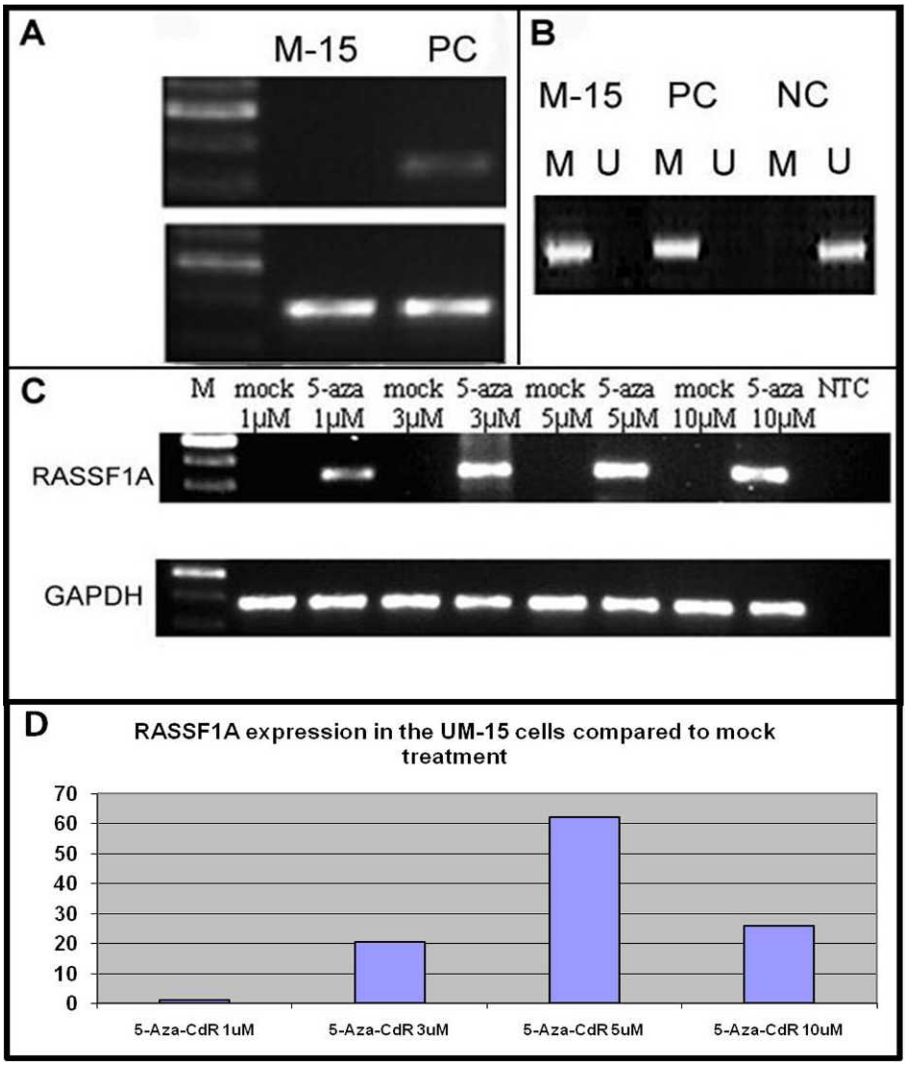


**Figure 1.** *RASSF1A* expression. (**A**) *RASSF1A* expression is detected only in the positive control (PC, RNA extracted from peripheral blood of a healthy individual) and not in the UM-15 cell line (M-15). (**B**) *RASSF1A* methylation is detected in the UM-15 cell line and the positive control (PC, SSSI-treated DNA) but not in the negative control (NC, DNA from a healthy individual). (**C**) Following *RASSF1A* reactivation with increasing concentrations of 5-Aza-CdR, *RASSF1A* expression increases accordingly. 5-aza, 5-Aza-2′-deoxycytidine; 5-Aza-CdR, mock-no treatment, without the active agent 5-aza; GAPDH, reference gene, glyceraldehyde-3-phosphate dehydrogenase. (**D**) QRT-PCR results showing the relative expression levels of *RASSF1A* following increasing doses of 5-Aza-2′-deoxycytidine (5-Aza-CdR) treatment in the UM 15 cell line.

mice were euthanized, and histologic analysis was performed on eye and liver (for metastasis) tissues under a light microscope. This experiment was done in duplicate.

### Statistical Analysis

Group differences in subcutaneous tumor size were analyzed by a square-root transformation and then a log transformation of the data to achieve a Gaussian distribution. A $P$ value of less than 0.05 was considered statistically significant.

## Results

### In Vitro Experiments

**RASSF1A Methylation Status and Expression in UM-15 Cell Line (Figs. 1–3).** Promoter hypermethylation of the *RASSF1A* gene was detected in the UM-15 cell line (Fig. 1B). In addition, analysis of the promoter methylation status in 20 UM samples revealed that all were methylated.

There was no mRNA transcription of the *RASSF1A* gene in the UM-15 cells. *RASSF1A* re-expressed after treatment with 5-Aza-CdR for 4 days (Figs. 1A, 1C). On analysis by QRT-PCR, the level of *RASSF1A* expression correlated with the increasing doses of 5-Aza-CdR demethylating agent up to 5 μM (Figs. 1C, 1D, 3A). Accordingly, there was no expression of the *RASSF1A* protein in the UM-15 cells, and it re-expressed after treatment with 5-Aza-CdR for 4 days (Fig. 2).

**UM Cell-Line Phenotype.** The provided karyotype of the UM-15 cell line did not show monosomy of chromosome 3. We confirmed this observation using FISH analysis of chromosome 3 copy number. To explore the effect of *RASSF1A* re-expression on the UM-15 cell-line phenotype, cells were transfected with a plasmid containing the ORF sequence of human *RASSF1A*. The stable clone was maintained in complete medium supplemented with G418. Total RNA was isolated and subjected to QRT-PCR. The results confirmed that the clone was expressing the transfected *RASSF1A* gene (Fig. 3). The expression of *RASSF1A* in cell lines transfected with the *RASSF1A*-containing plasmid was greater by 21,076-fold than in

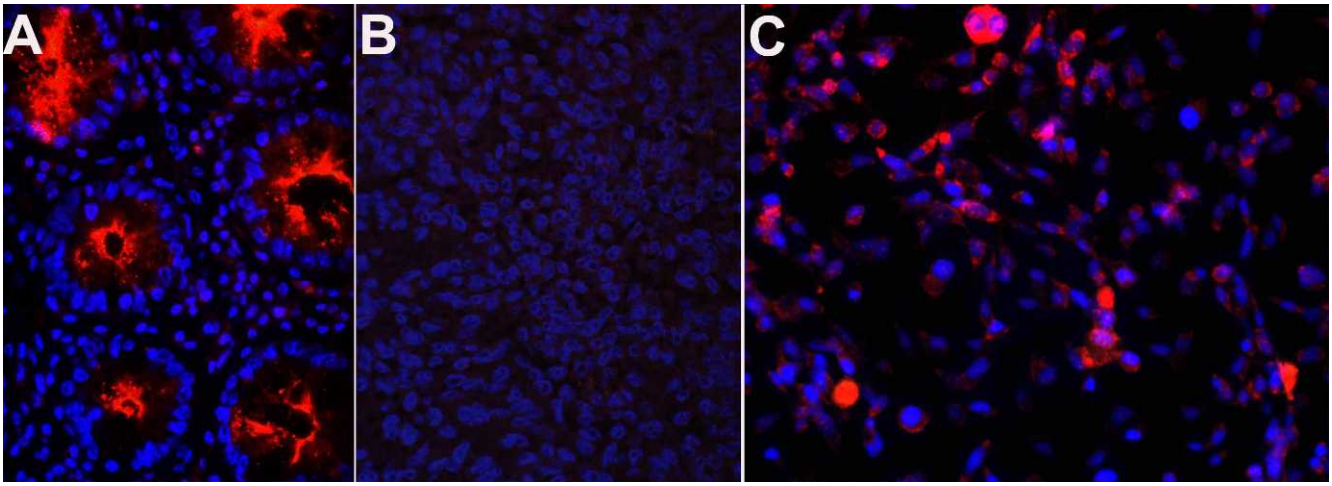


**FIGURE 2.** Immunohistochemistry staining for *RASSF1A* protein in the UM-15 cell line before and after treatment with 5-Aza-CdR. (**A**) Human kidney tissue was used as positive control staining for *RASSF1A* (*red staining*). (**B**) Untransfected UM-15 cell line showed negative staining for *RASSF1A*. (**C**) Following the addition of the demethylating agent 5-Aza-CdR, immunostaining of UM-15 cell line is positive (*red staining*) in all cells.

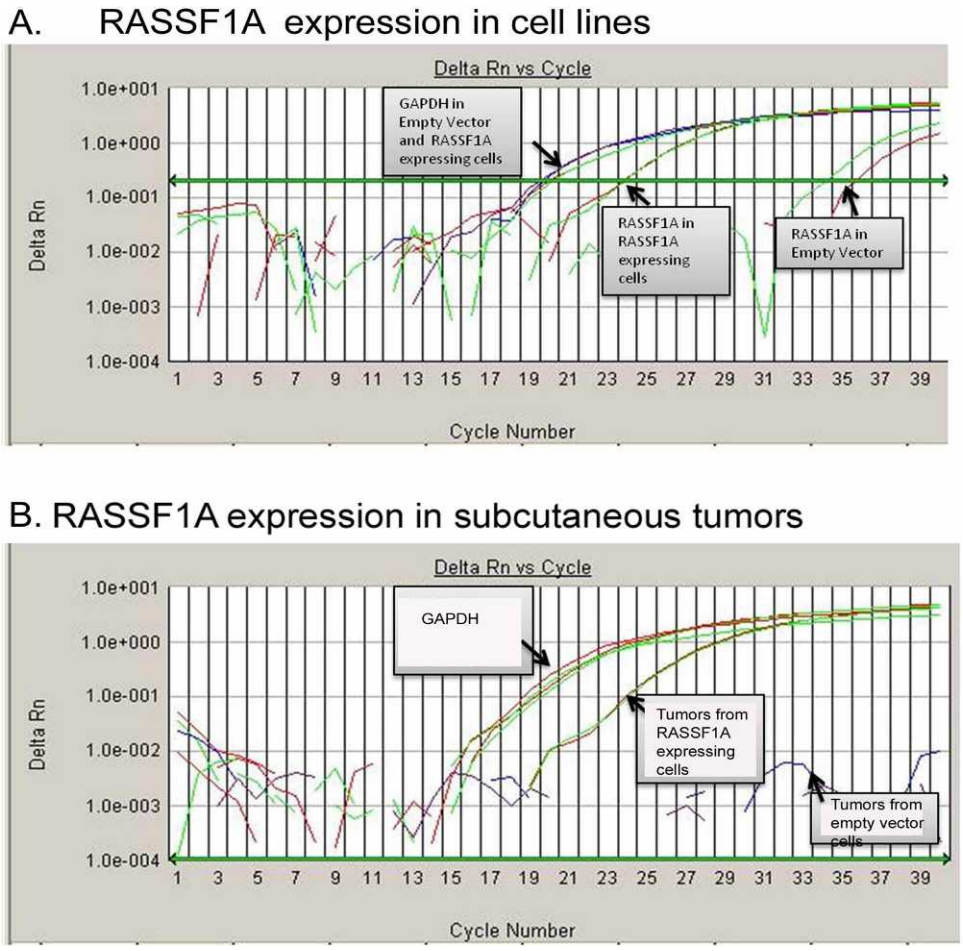


**FIGURE 3.** *RASSF1A* expression in cell lines and subcutaneous tumors. (**A**) *RASSF1A* is expressed (24 Ct cycles) in exogenous cell line transfected with *RASSF1A* containing plasmid; *RASSF1A* is not expressed (<34 Ct cycles) in the cell line transfected with empty vector. *GAPDH* served as the reference gene for both. (**B**) Same results for subcutaneous tumors originating from the *RASSF1A*-expressing cell line (24 Ct cycles); negative results for tumors originating from the cell line transfected with empty vector.

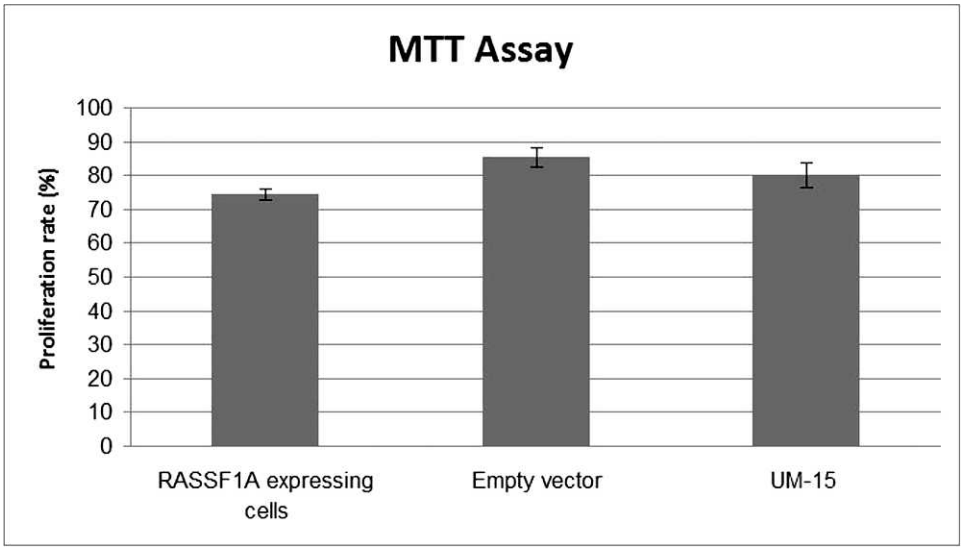


**Figure 4.** MTT assay results showing decreased proliferation rate in the presence of cisplatinum for the *RASSF1A*-expressing cell line compared to the non-*RASSF1A*-expressing (UM-15 and empty vector) cells.

the original UM-15 cell line and greater by 30,083-fold than in the empty plasmid transfected cell line.

**Cell Proliferation and Cisplatin Cytotoxicity.** Cell proliferation was estimated by comparing the initial and final amount of cells. The cells with exogenous expression of *RASSF1A* had a slower rate of proliferation than both the nontransfected cells and the cells transfected with empty vector plasmid. Given earlier findings of the relative resistance of UM cells (as well as skin melanoma) to cisplatin treatment in vivo,[30,31] we investigated the cisplatin resistance of UM cells expressing exogenous *RASSF1A*. On MTT assay, the UM-15 nontransfected (original) cells and the cells containing empty vector were more resistant to cisplatin than the cells expressing *RASSF1A*, with twice the proliferation rate (85% and 84% vs. 69% for *RASSF1A*-expressing cells, Fig. 4).

## In Vivo Studies

**Subcutaneous UM-15 Cell Transplantation.** *Tumor Size.* Subcutaneous tumors developed in all 3 groups ($n = 17$). Tumor growth was slower in mice injected with the *RASSF1A*-expressing cell line ($n = 5$, detected by day 39) than in the mice injected with parental UM ($n = 6$, detected by day 32) or empty vector ($n = 6$, detected by day 28, Fig. 5A). The tumors in the last group, which grew more slowly, also were smaller (NS). Macroscopically, all of the tumors in the parental UM and empty vector groups were elevated, smooth, elastic, and darkly pigmented. Two of the 5 tumors in the *RASSF1A*-expressing cells were amelanotic.

*Tumor Histology.* Histologic study revealed melanoma epithelioid cells with a low mitotic index (Fig. 5B). When exogenous expressing UM-15 cell line was injected, the tumor expressed *RASSF1A* (Fig. 5C, $n = 2$). No metastases were detected in the liver of any of the mice in the study.

***RASSF1A* Expression Level in Subcutaneous Tumors.** QRT-PCR analysis showed no *RASSF1A* expression in tumor tissue from mice injected with UM-15 paternal cells ($n = 6$) or empty vector ($n = 6$). As expected, *RASSF1A* was expressed in all subcutaneous tumors that grew from *RASSF1A*-expressing UM-15 cells ($n = 5$, Figs. 3B, 5C).

**Intraocular UM Cell Transplantation.** *Tumor Evaluation.* At 46 days after cell transplantation, mice injected with the stable *RASSF1A*-expressing clone ($n = 4$) showed no tumor on fundus examination or transscleral light illumination. Mice injected with cells containing empty vector ($n = 4$) or nontransfected parental cells ($n = 4$) acquired intraocular tumors.

*Ocular Histology.* Following enucleation, the presence of tumors was confirmed by transscleral light illumination, before the eyes were embedded in paraffin. Hematoxylin and eosin staining revealed epithelioid cells with low mitotic index in all retinal layers, with cells invading the sclera. In mice intravitreally injected with *RASSF1A*-expressing UM-15 cells, no tumor was detected funduscopically or histologically (Fig. 6A). However, histologic sections from eyes injected intravitreally with cells transfected with empty vector or parental UM cells revealed tumors invading all retinal layers and the sclera (Figs. 6B, 6C).

## Discussion

Our study shows that *RASSF1A* expression suppresses UM tumorigenesis and *RASSF1A* is silenced in the UM-15 cell line. Re-expression of *RASSF1A* in UM-15 cells reverses the tumoral behavior, as indicated by the slower proliferation rate and regain of sensitivity to cisplatin.

*RASSF1A* is a tumor suppressor gene and is inactivated commonly through promoter hypermethylation in many human cancers, including UM.[20,21] Our finding of *RASSF1A* methylation in UM-15 cells is in agreement with studies in other cell lines derived from primary tumors of breast and ovarian cancer.[32,33] In addition, the methylation of p16INK4a was shown to be more common in UM cell lines than in other primary tumors.[34–38] These observations suggest that tumors with *RASSF1A* methylation are more oncogenic and more likely to be established as cell lines. They also are consistent with the report of loss of heterogeneity of the primary tumor in cell-line cultures.[39]

The *RASSF1A* transcript encodes a COOH-terminal RAS-association domain[40] and serine residue 131, which serves as a putative phosphorylation target for the ataxia-telangiectasia mutation (ATM). Thus, *RASSF1A* could act as a link between the RAS and ATM-regulated pathways.[41] Shivakumar et al. reported that *RASSF1A* functions as a negative regulator of cell proliferation.[42] It apparently blocks cell-cycle progression from

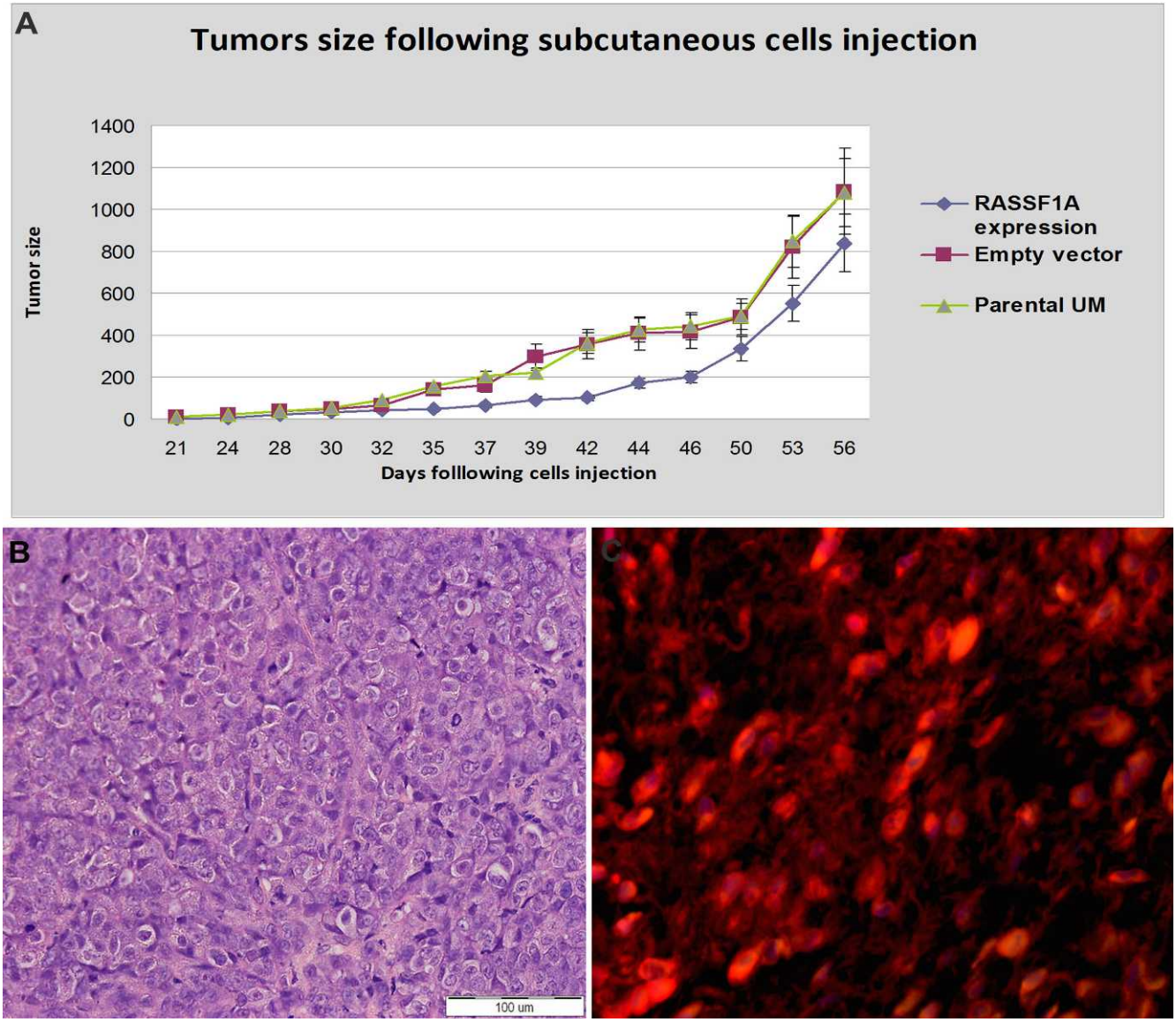


**Figure 5.** Subcutaneous tumor size (mm) following injection of 3 cell lines: *RASSF1A*-expressing cells, cells transfected with empty vector, and original UM-15. (**A**) All mice injected subcutaneously had tumors ($n = 17$). Tumors were detected by day 28 in all mice injected with empty vector ($n = 6$), by day 32 in mice injected with parental UM ($n = 6$), and by day 39 in mice injected with *RASSF1A*-expressing cell line ($n = 5$). Tumor growth was slower in mice injected with *RASSF1A*-expressing cells than in the other groups (NS). (**B**) Histologic findings of melanoma epithelioid cells with a low mitotic index from a subcutaneous tumor originated from original (non *RASSF1A*-expressing) UM cells. (**C**) *RASSF1A* positive immunostaining of subcutaneous tumor originated from *RASSF1A*-expressing UM cells ($n = 2$, *red*, ×40).

the G1 to the S phase by controlling the entry at the retinoblastoma restriction point and inhibiting cyclin D1 protein accumulation at the post-transcriptional level.[42] Therefore, hypermethylation-induced loss of *RASSF1A* expression could lead to a reduction in G1/S-phase cell-cycle control. In epithelial cells derived from lung and breast tumor, the reintroduction of *RASSF1A* expression resulted in growth arrest that was correlated with reduced cyclin D1 protein accumulation; by contrast, iRNA-mediated inhibition of *RASSF1A* expression resulted in abnormal accumulation of native cyclin D1.[42]

Using an immortalized cell line, the same researchers reported that oncogenic RAS did not alter *RASSF1A*-induced growth-inhibitory effects.[42] However, in human mammary epithelial cells, the *RASSF1A* effects dominated the oncogenic RAS effects. Thus, loss of *RASSF1A* may be a determining step for oncogenic transformation in the absence of RAS-activating mutations. Moreover, overexpression of *RASSF1A* promotes the formation of stable microtubules and blocks RAS-activated genomic instability, suggesting a potential role of the *RASSF1A* protein in the maintenance of spindle function and genomic stability.[43]

*RASSF1A* has been reported to inhibit tumor formation in nude mice.[42] Therefore, to analyze further the role of *RASSF1A* reactivation in vivo, we compared tumorigenesis between mice injected subcutaneously or intraocularly with UM-cells expressing/not expressing reactivated *RASSF1A*. Intraocular tumors developed only in the absence of *RASSF1A* expression.

*RASSF1A* gene is located on chromosome 3p21.3, and its absence or inactivation has been suggested as a contributing factor for UM tumor formation and progression.[22] *RASSF1A* methylation, frequently observed in UM tumors, could be the second hit in the classical model for TSG inactivation when coupled with monosomy 3, which is the change observed most

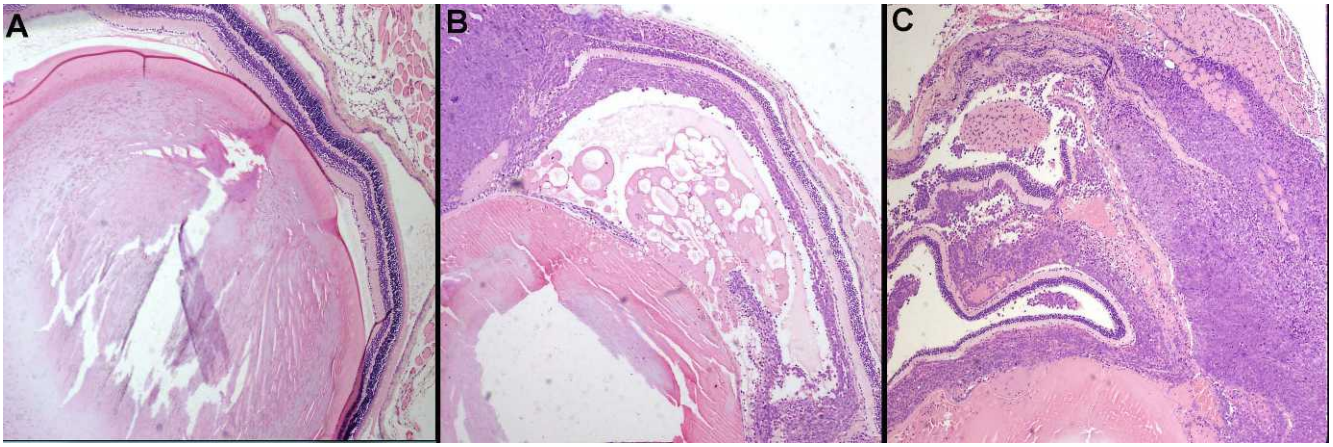


**Figure 6.** (**A**) Normal retina. No tumors are detected following intraocular injection of UM-15 cell expressing exogenous *RASSF1A* (×5, $n = 4$). (**B**) Histological section of an eye following intravitreal injection of parental UM cells (×5, $n = 4$). Note the tumor invading all retinal layers. (**C**) Histological section of an eye injected with UM cells transfected with empty vector ($n = 4$). Note the large invasive melanoma.

consistently in UM. The lack of intraocular tumor formation after intraocular injection of *RASSF1A*-expressing cell lines might point to an increased relevance of *RASSF1A* to the development of UM in the eye.

The UM-15 cell line showed lack of monosomy of chromosome 3 and, although we confirmed the cell line karyotype by FISH, we could not rule out duplications of the remaining chromosome 3. Therefore, the study is limited in measurement of the effect of monosomy 3 and its relation to *RASSF1A* expression on intraocular tumor development.

Epigenetic modification of gene expression is an important mechanism in tumor development, and may be reversed by specific treatment. The frequent methylation of the *RASSF1A* gene in UM cell lines (91%) suggests that *RASSF1A* also has a role in UM pathogenesis.[22] Loss of one copy of chromosome 3 (monosomy 3) has been reported in approximately 50% of all UMs and is associated with the metastatic behavior of the tumor. Given the location of *RASSF1A* on the p21.3 region of chromosome 3, it might serve as a TSG whose silencing by methylation represents the second hit after monosomy occurs. Although methylation of *RASSF1A* may not be held wholly responsible for UM development, it could be a contributing factor in tumor formation and progression. This assumption is supported by the high methylation in the primary tumor. One study of the correlation of UM methylation and survival yielded positive findings, though only at trend level.[22] The same study also suggested that the *RASSF1A* protein is a potential tumor marker in UM.

In conclusion, *RASSF1A* has an important role in the biologic behavior of UM cells and the development of UM, specifically in the eye. Its activation might be applied to the development of future treatments.

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