AD_____

Award Number: DAMD17-00-1-0156

TITLE: Rb Associated Protein 46: Roles in Progression of Proliferative Breast Cancer

PRINCIPAL INVESTIGATOR: Moshe Yamin, Ph.D.
Zhaoyi Wang, Ph.D.

CONTRACTING ORGANIZATION: Beth Israel Deaconess Medical Center
Boston, Massachusetts 02215

REPORT DATE: July 2003

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2003	Final (15 Jun 2000 - 14 Jun 2003)

4. TITLE AND SUBTITLE
Rb Associated Protein 46: Roles in Progression of Proliferative Breast Cancer

5. FUNDING NUMBERS
DAMD17-00-1-0156

6. AUTHOR(S)
Moshe Yamin, Ph.D.
Zhaoyi Wang, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Beth Israel Deaconess Medical Center
Boston, Massachusetts 02215

E-Mail: zywang@creighton.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Our research program is focused on Rb associated protein 46 (RbAp46), a novel tumor suppressor gene that we recently identified and cloned. Our major goal is to understand the role of RbAp46 in the early development of breast cancer. During the project period (2000-2003), we accomplished all of the tasks proposed in our application. We demonstrated RbAp46 tumor suppressor function in the early development of breast cancer, revealing cellular and molecular mechanisms by which RbAp46 functions as a tumor suppressor.

Using MCF10AT3B cells derived from a xenograft model of human proliferative breast disease, we discovered that RbAp46 overexpression strongly inhibits abnormal growth and tumorigenicity of neoplastigenic MCF10AT3B cells. RbAp46 overexpression also facilitates stress-induced apoptosis in MCF10AT3B cells through consistent activation of the JNK/SAPK pathway. We also found that restoration of RbAp46 expression in breast cancer cells strongly inhibits malignant features of these tumor cells. Importantly, we found that inducible RbAp46 expression in established breast cancer xenografts strongly suppressed progressive growth of breast cancer *in vivo*. Furthermore, we demonstrated that constitutive RbAp46 expression in MCF10AT3B cells inhibits estrogen-stimulated growth of these cells *in vitro* and tumorigenicity *in vivo*. Finally, we revealed that several signaling pathways, including the JNK/SAPK pathway and the β-catenin pathway, are involved in RbAp46 function as a potent tumor suppressor.

14. SUBJECT TERMS
Estrogen, Cell cycle, and Growth Inhibition

15. NUMBER OF PAGES
35

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION:

Our research program is focused on Rb associated protein 46 (RbAp46), a novel tumor suppressor gene that we recently identified and cloned. Our major goal is to understand the role of RbAp46 in the early development of breast cancer. During the project period (2000-2003), we accomplished all of the tasks proposed in our application. We demonstrated RbAp46 tumor suppressor function in the early development of breast cancer, revealing cellular and molecular mechanisms by which RbAp46 functions as a tumor suppressor.

Using MCF10AT3B cells derived from a xenograft model of human proliferative breast disease, we discovered that RbAp46 overexpression strongly inhibits abnormal growth and tumorigenicity of neoplastigenic MCF10AT3B cells. RbAp46 overexpression also facilitates stress-induced apoptosis in MCF10AT3B cells through consistent activation of the JNK/SAPK pathway. We also found that restoration of RbAp46 expression in breast cancer cells strongly inhibits malignant features of these tumor cells. Importantly, we found that inducible RbAp46 expression in established breast cancer xenografts strongly suppressed progressive growth of breast cancer *in vivo*. Furthermore, we demonstrated that constitutive RbAp46 expression in MCF10AT3B cells inhibits estrogen-stimulated growth of these cells *in vitro* and tumorigenicity *in vivo*. Finally, we revealed that several signaling pathways, including the JNK/SAPK pathway and the β-catenin pathway, are involved in RbAp46 function as a potent tumor suppressor.

With the support of this grant, our study provided important information about the function of the novel tumor suppressor gene RbAp46 in the early development of breast cancer, and the molecular and cellular events in early development of human breast cancer.

BODY:

Task 1: Determine the effects of constitutive RbAp46 expression on the progression of human proliferative breast disease in the MCF10AT xenograft model (Months 1-12).

1. Establishment of stable MCF10AT3B cell lines expressing exogenous RbAp46. (From Approved Statement of Work).

2. Analysis of growth characteristics in the stable MCF10AT3B cell lines that express high levels of exogenous RbAp46. (From Approved Statement of Work).

3. In vivo xenograft experiments are being performed. (From Approved Statement of Work).

The results generated from the experiments proposed in Task 1 were recently published (Li *et. al.* 2003 as the appendix 1). We summarize the key findings and their importance below.

(1). Overexpression of RbAp46 suppresses tumorigenicity of MCF10AT3B cells.

The MCF10AT3B cells are epithelial cells derived from MCF10A cells, a cell line that originates from a spontaneous immortalization of non-malignant human mammary gland (Miller *et. al.*, 1993). MCF10A cells transfected with T24 Ha-ras mutant (designated as MCF10AT) acquired the ability to

grow as xenografts in the dorsal flank of nude mice. MCF10AT3B cell line was established from a third generation transplant. When xenografted into nude mice, MCF10AT3B cells form atypical hyperplasia, carcinoma in situ and invasive carcinoma (Dawson *et. al.* 1996). This is a widely used and well-characterized model for the early development of breast cancer suitable for *in vitro* and *in vivo* assays.

We have established stable cell lines that express exogenous RbAp46. The RbAp46-expressing MCF10AT3B cells and the CMV-MCF10AT3B control cells transfected with empty vector were maintained in medium plus 5% fetal calf serum.

A typical feature associated with malignantly transformed cells is anchorage-independent growth. We determined the ability of stable MCF10AT3B cell lines that overexpress RbAp46 to form colonies in soft-agar. The RbAp46-expressing cells formed~45% fewer colonies in soft-agar than parental MCF10AT3B cells and CMV-MCF10AT3B control cells **(see the table I in the appendix 1)**. These results indicate that overexpression of RbAp46 inhibits the ability of MCF10AT3B cells to grow anchorage-independently in soft-agar.

To examine the effect of RbAp46 expression on tumorigenicity of MCF10AT3B cells *in vivo*, two established cell lines from each expression vector were injected in the mammary fatpad of 12 nude mice. Forty weeks after injection, tumor like nodules were detected at about 50% of the injected sites of parental cells and control cells transfected with empty vector. However, tumors were totally absent in the mice injected with RbAp46-transfected cells **(see table II in the appendix 1)**. This data indicates that RbAp46 strongly suppresses tumorigenicity of neoplastigenic MCF10AT3B cells in nude mice.

(2). Overexpression of RbAp46 sensitizes MCF10AT3B cells to apoptosis induced by stress-stimuli.

We examined whether overexpression of RbAp46 enhanced apoptotic cell death in cells following their transfer to media with a low concentration of serum or media without hydrocortisone. In contrast to control cells, RbAp46-expressing cells cultured in low serum or in the absence of hydrocortisone exhibited a dramatic increase in apoptotic cell death **(see the figures 2 and 3 in the appendix 1)**. These data strongly demonstrate that although high levels of RbAp46 expression alone do not induce apoptosis, a significant increase in cell apoptosis occurs when combined with stress-stimuli.

(3). Constitutive activation of the JNK/SAPK pathway in RbAp46-expressing MCF10AT3B cells.

Apoptosis signaling through the JNK/SAPK stress response pathway is triggered by UV irradiation, osmotic stress, and in response to cytokines and growth factors. The JNK/SAPK pathway was implicated as a major apoptotic pathway following DNA damage and in response to Fas ligand and tumor necrosis factor-α (TNF-α) (Chen *et. al.* 1996; Rosette and Karin, 1996).

To determine if the JNK/SAPK pathway is involved in enhanced apoptosis of RbAp46-expressing MCF10AT3B cells, Western blot analysis, using anti-phospho JNK antibody, was performed on control and RbAp46-expressing cells. We observed a constitutively phosphorylated

form of JNK/SAPK in RbAp46-expressing cells (**see the figure 4 in the appendix 1**), indicating a consistent activation of the JNK/SAPK pathway in these cells. Thus, overexpression of RbAp46 may sensitize cells to a potent apoptotic response through consistent activation of the JNK/SAPK pathway.

(4). Constitutive activation of GADD45 in RbAp46-expressing MCF10AT3B cells.

The data described above demonstrated that RbAp46 sensitized cells to apoptosis induced by stress-stimuli, possibly through constitutive activation of JNK/SAPK pathway. Growth arrest- and DNA damage-inducible (GADD) genes belong to a subgroup of genes that are both rapidly induced by DNA-damaging agents and coordinately induced in growth-arrested cells. To examine *GADD45* gene expression in RbAp46-expressing cells, Western blot analysis of proteins from RbAp46-expressing cells and control cells was performed. GADD45 expression levels were dramatically increased in RbAp46–expressing cells compared with control cells (**see the figure 5 in the appendix 1**). This data suggested that overexpression of RbAp46 may mimic the signals of these environmental stimuli and induce expression of GADD45.

Task 2: Determining the effects of RbAp46 on transformed phenotypes of breast cancer cells (Months 13-24).

4. Determining if RbAp46 inhibits transformed phenotypes of breast cancer cell lines such as anchorage-independent growth and tumor formation in nude mice. Establishment of Tet-inducible retrovirus system (From Approved Statement of Work).

(1). Constitutive expression of exogenous RbAp46 reverts transformed phenotypes of breast cancer cells. ***This work is in press in "Anticancer Research" (see the appendix 2).***

We used three human breast cancer cell lines, MDA-MB-231, MDA-MB-436 and MCF7 as tumor models to study the effect of RbAp46 expression on transformed phenotypes of human breast cancer cells. We established a number of cell lines that expressed exogenous RbAp46 and three of each transfectant are maintained for research. We also generated three control cell lines transfected with the empty expression vector for each breast cancer cell line.

We then tested the effects of RbAp46 on transformed phenotypes of these breast cancer cells using two types of experiments. First, we determined the ability of these established cell lines that constitutively express RbAp46 to form colonies in soft-agar. Two hundred cells of each established cell lines were seeded in 60-mm dishes in replicates of five plates for each cell line. After incubation for 3 weeks, colonies with more than 30 cells were counted and scored as anchorage-independent. The RbAp46-expressing cells formed 10-15% fewer colonies in soft-agar than vector-control cells transfected with the empty vector (**see the table 1 in the appendix 2**). These results

indicate that high levels of RbAp46 expression inhibit the ability of these breast cancer cells to grow anchorage-independently in soft-agar.

Second, we examined the effect of RbAp46 on tumorigenicity of the three breast cancer cell lines in nude mice. Each of the established cell lines were injected subcutaneously into three nude mice (at two sites each, using 2 X 10^6 cells per site). Fourteen days after injection, tumors were detected at most sites injected with vector-control cells. However, tumor formation was significantly decreased both in number and in sizes in the mice injected with RbAp46-expressing cells (**see the figure 3 in the appendix 2**). Taken together, these data indicated that RbAp46 strongly suppresses the transformed phenotypes of MDA-MB-231, MDA-MB-436 and MCF7 breast cancer cells.

(2). Establishment of Tet-inducible retrovirus system.

In our progress report for year two, we reported the establishment of a tetracycline-regulated expression system of RbAp46 in MCF7 breast cancer cells. We found tightly regulated induction of RbAp46 expression (**Fig. 1A in the appendix 3**). Western blot analysis did not detect expression of the HA-tagged RbAp46 in the presence of tetracycline. Withdrawal of tetracycline led to induction of exogenous RbAp46 expression, detectable within 5 hours. The maximal induction of the transfected RbAp46 was about 27-fold.

To determine the effect of induced RbAp46 expression on growth of MCF7 cells, the cells were seeded at 1 X 10^4 cells/well and counted every other day after tetracycline withdrawal. Induction of RbAp46 resulted in a normal growth rate for about 2 days, followed by a slower growth rate (~2 fold) compared with the control cells (**Fig. 1B in the appendix 3**). This data strongly suggested that induced expression of RbAp46 inhibits growth of MCF7 breast cancer cells.

5. Tumor formation assay of Tet-inducible cell lines in nude mice will be performed. (From Approved Statement of Work).

(1). Induction of RbAp46 expression suppresses progressive growth of breast tumor xenografts in nude mice.

MCF7 is an established breast cancer cell line that forms tumor in nude mice. We inoculated MCF7 cells carrying Tet-inducible recombinant viruses of RbAp46 into nude mice. The first group of mice was fed with water only immediately after injection to induce RbAp46 expression from the beginning of experiment. The second group of mice was fed with water containing Tetracycline (2 mg/ml) for four weeks when diameters of tumor xenografts reached 4-5 mm^3, and then with water only to induce RbAp46 expression. The third group of mice was continuously fed with water containing Tetracycline (2 mg/ml) from the beginning to the end of experiment to suppress exogenous RbAp46 expression. Tumor growth was monitored weekly. After eight weeks, all mice were sacrificed, and tumors were excised and

weighed. The mice from the first group formed the smallest tumors compared to other two groups (**Fig. 2A**), consistent with our previous finding that constitutive expression of RbAp46 strongly inhibits tumor formation in nude mice (Guan *et. al.* 2001 and Li *et. al.* 2003). Furthermore, in the second group, the induced expression of RbAp46 suppressed progressive growth of established tumor xenografts compared to the tumors from the third group of mice (**Fig. 2A in the appendix 3**). Analysis of the data using a student's t-test showed that the tumor mass reduction in the second group of mice with induced RbAp46 expression compared with control mice was statistically significant ($p < 0.05$). However, although RbAp46 induction resulted in a slow-down of progressive growth of established tumor, tumor regression was not observed (**Fig. 2B in the appendix 3**).

(2). Induced expression of RbAp46 trigged apoptosis.

To further investigate whether induction of RbAp46 could induce apoptotic cell death in MCF7 cells, fluorescent activated cell-sorting (FACS) analysis was performed in propidium iodide stained cells with induced RbAp46 expression. While we did not observe any cell population changes in specific cell cycle phases, we observed an increase of cell population in the sub-G1 phase (**Fig. 3B in the appendix 3**). Electrophoretic analysis of DNA from dying cells revealed chromatin fragmented into a nucleosomal ladder characteristic of apoptosis (**Fig. 3C in the appendix 3**). These dying cells also exhibited the biochemical characteristics of apoptosis; the activation of caspase-3 and the cleavage of poly ADP-ribose polymerase (PARP), a substrate of activated caspase-3, were observed in the cells after induction of RbAp46 (**Fig. 3A in the appendix 3**), indicating that RbAp46 induction triggers programmed cell death in MCF7 cells.

(3). Induction of RbAp46 activates the JNK pathway.

As described above, we have found that the JNK pathway is consistently activated in RbAp46-expressing cells . To confirm this result, Western blot analysis was performed to demonstrate that RbAp46 induction led to phosphorylation of endogenous SEK1/MKK4 (**Fig. 4A in the appendix 3**), the common upstream regulator of the JNK pathway, followed by phosphorylation of JNK (**Fig. 4A in the appendix 3**). Phosphorylation occurred at a level higher than that observed following UV irradiation, a well described inducer of JNK and comparable with the level induced by Methyl methanesulfonate (MMS), a DNA alkylating agent known to induce the JNK pathway (**Fig. 4A in the appendix 3**). JNK kinase activity was also activated as demonstrated by the increased phosphorylation of the JNK substrate c-Jun (**Fig. 4A in the appendix 3**). Induction of RbAp46 did not result in nonspecific activation of the other MAP kinase pathways, as shown by the unaltered tyrosine phosphorylation of MAP kinases such as ERK and p38 (**Fig. 4B in the appendix 3**).

(4). Activation of JNK is required for apoptosis induced by RbAp46.

To determine whether activation of JNK is required for RbAp46 mediated apoptosis, a dominant-negative mutant of JNK1 (JNK1dn) encoded by pcDNA3-Flag-JNK1 (APF) was transiently introduced into cells, and RbAp46 expression was induced by tetracycline withdrawal. JNK1dn expression inhibits

the endogenous JNK activity, shown by the loss of c-Jun phosphorylation (**Fig. 5A in the appendix 3**) and reduced apoptosis trigged by RbAp46 induction (**Fig. 5B in the appendix 3**). These data suggest that JNK kinase activity is required for RbAp46-mediated apoptosis.

Task 3. Determining the effects of constitutive-expression of RbAp46 on estrogen-stimulated progression of proliferative breast disease. (Months 25-36).

6. Determining the effects of constitutive expression of RbAp46 on growth characteristics of estrogen-stimulated MCF10AT cells *in vitro*. (From Approved Statement of Work).

7. Determining the effects of constitutive-expression of RbAp46 on estrogen-stimulated progression of proliferative breast disease *in vivo*. (From Approved Statement of Work).

Despite widespread agreement that estrogens are involved in breast cancer etiology, there is uncertainty as to the precise role of estrogen in the biology of breast cancer progression as well as the mechanisms of estrogen action early in human mammary tumorigenesis. As proposed in our application, we planned to study the effects of constitutive expression of exogenous RbAp46 on estrogen-stimulated cell growth *in vitro* and the progression of proliferative breast disease *in vivo*.

(1). Constitutive expression of RbAp46 suppresses estrogen-stimulated growth of MCF10AT3B cells.

The MCF10AT3B cells that express exogenous RbAp46 and control cells that were transfected with empty vector were maintained in F12 medium plus 5% horse serum. To determine the effects of constitutive expression of RbAp46 on estrogen-stimulated cell growth, the RbAp46-expressing cells and control cells were washed three times with PBS and changed to medium containing 5% Charcol/Dextran treated serum at a density of 1×10^4 cells per well for twenty four hours. 10^{-9} M of 17β-estrodial (E2) was added to the medium. After incubation with E2 for 2, 4, 6 and 8 days, cells were trypsinized and counted. We found that constitutive expression of RbAp46 inhibited the estrogen-stimulated growth rate of MCF10AT3B cells at physiology concentration of estrogen (1×10^{-9} M) (**Fig. 6 *in the appendix 3***).

(2). Constitutive-expression of RbAp46 inhibits estrogen-stimulated progression of proliferative breast disease in vivo.

Control MCF10AT3B cells and MCF10AT3B cells that express exogenous RbAp46 were suspended after treatment with 0.025% edetate sodium and 0.05% trypsin in a Ca^{2+}- and Mg^{2+}- free balanced salt solution. A total of 1×10^7 cells were resuspended in 0.1 ml of Matrigel (Collaborative Research, Bedford, MA) and inoculated subcutaneously into the mammary fatpad of ovariectomized female nude mice (6 weeks old, strain CDI nu/nu, Charles River Breeding Laboratory), seven days after subcutaneous implantation of 1.7mg/60-day release E2 (treated; 12 mice) or placebo (control; 12 mice) pellets (Innovative Research, FL). Animals were palpated for tumor formation at the injection site once a week, beginning five weeks after injection. All mice

were sacrificed at 8 weeks after injection. Tumors from the injection sites were removed and weighed.

We found that in the absence of estrogen, constitutive expression of RbAp46 totally abolished tumor formation of MCF10AT3B cells (**Fig. 7 in the appendix 3**), consistent with our previous finding that RbAp46 inhibits tumorigenicity of MCF10AT3B cells *in vivo* (Li *et. al.* 2003). However, estrogen treatment enhanced the tumorigenicity of MCF10AT3B cells, whereas constitutive expression of RbAp46 strongly reduced the progression of tumors formed by MCF10AT3B cells (**Fig. 7 in the appendix 3**). These results demonstrated that estrogen promotes progression of human proliferative breast disease and constitutive expression of RbAp46 strongly inhibits estrogen-stimulated progressive growth of human proliferative breast disease.

Other findings that are pertinent to the proposed Tasks:

(1). Overexpression of RbAp46 in MCF10AT3B cells induces an epithelial-mesenchymal transition (reported in the progress report for year one).

In addition to the growth inhibition and sensitization to apoptosis observed in RbAp46 over-expressing MCF10AT3B cells, a dramatic change of phenotype from epithelial cells to fibroblast-like cells was also observed in RbAp46-expressing cells. Western blot analysis and indirect immunofluoresence staining results demonstrated that the E-Cadherin, P-Cadherin, α-Catenin, β-Catenin and γ-Catenin (markers for epithelial cells) were significantly down-regulated in the RbAp46-expressing cells. N-Cadherin and Vimentin (markers for mesenchymal cells) were up-regulated in these cells. In addition, our experiments also demonstrated that the RbAp46-transfected MCF10AT3B cells retained some epithelial markers, such as epithelial specific antigen (ESA) and epithelial membrane antigen (EMA), indicating their epithelial origin.

(2). RbAp46 facilitates GSK3β-dependent protein degradation of β-catenin (reported in the progress report for year two).

To elucidate the molecular mechanisms by which RbAp46 inhibits transformed phenotypes of breast cancer cells, we examined several signaling pathways in RbAp46-transfected MCF7 cells. We found that the expression levels of β-catenin protein were dramatically downregulated in the RbAp46-transfected cells as demonstrated by Western blot analysis whereas no significant change was observed in its mRNA levels, suggesting that the steady state levels of β-catenin protein were decreased in RbAp46-transfected cells.

β-catenin is an important regulator of the Wnt signal transduction cascade. In the absence of Wnt signaling, cytoplasmic β-catenin is associated with APC-Axin/conductin-GSK-3β complex and normally rapidly degraded through proteasome pathway after being phosphorylated by GSK-3β at serine/threonine residues.

It is well established that the peptide aldehyde ALLN (*N*-acetyl-Leu-Leunorleucinal) and MG132 inhibit proteasome-mediated proteolysis, which leads to an accumulation of proteins that are usually metabolized by this pathway. Upon treatment with ALLN and MG132, the protein levels of β-catenin in RbAp46-transfected cells were increased to a level similar to that of control cells, and the cells treated by ALLN clearly showed higher molecular weight forms of β-catenin, suggesting that the β-catenin protein was destabilized in RbAp46-transfected cells.

To further examine if GSK-3β mediated β-catenin degradation was upregulated in RbAp46-transfected cells, we further determined the expression levels of GSK-3 in RbAp46-transfected cells. We found that the protein levels of both GSK-3α and 3β were dramatically upregulated in RbAp46-transfected MCF7 cells compared with the control cells. Concomitantly, β-catenin was significantly phosphorylated at Ser31/37/Thr41 residues, but not at Thr41/Ser45 residues, indicating that RbAp46 may upregulate the GSK-3-dependent ubiquitination and proteolysis pathway that regulate the steady state levels of β-catenin are regulated.

Activation of the Wnt cascade results in an inactivation of GSK-3β and an accumulation of cytosolic β-catenin, which then translocates to the nucleus, binds to transcription factors of the Lef/Tcf family, and activates the transcription of a variety of target genes including c-myc and cyclin D. To confirm β-catenin was functionally downregulated in RbAp46-transfected MCF7cells, Lef/Tcf-dependent nuclear signaling was measured using the TOP-FLASH reporter construct that contains the Lef/Tcf binding site. As a negative control, the FOP-FLASH reporter vector, in which the Lef/Tcf binding sites have been mutated, was also used. The results showed that β-catenin-Lef/Tcf signaling is significantly inhibited in RbAp46-transfected MCF7 cells compared with the control cells.

KEY RESEARCH ACCOMPLISHMENTS:

(1). Constitutive expression of RbAp46 inhibits abnormal cell growth and tumorigenic phenotype of MCF10AT3B cells, a cell line derived from a xenograft model of human proliferative breast disease.

(2). Constitutive expression of RbAp46 sensitizes MCF10AT3B cells to apoptosis induced by stress-stimuli through consistent activation of JNK/SAPK pathway.

(3). Constitutive expression of RbAp46 induces epithelial-mesenchymal transition.

(4). Overexpression of RbAp46 inhibits transformed phenotypes of breast cancer cells.

(5). Constitutive expression of RbAp46 downregulates the β-catenin/Tcf–mediated signaling pathway.

(6). Induced expression of RbAp46 suppresses progressive growth of established breast tumor xenografts in nude mice.

(7). Induced expression of RbAp46 triggers JNK pathway-dependent cell apoptosis.

(8). Constitutive expression of RbAp46 inhibits estrogen-stimulated abnormal cell growth and enhanced tumorigenesis of MCF10AT3B cells.

REPORTABLE OUTCOMES:

Publications:

1. Li, G-C., Chen, G-C., Guan L-S. and Wang, Z-Y. "RB-associated protein (RbAp46): a Novel Tumor Suppressor" [Oral Presentation] *Cold Spring Harbor Meeting on Cancer Genetics & Tumor Suppressor Genes*, 2000.
2. Li, G-C., Guan, L-S., Chen G-C. and Wang, Z-Y "Constitutive expression of RbAp46 in MCF10AT3B epithelial cells induces epithelial-mesenchymal transition" [Late-Breaking Abstract] *Proceedings Supplement of the American Association for Cancer Research.* 2001, 75: 726.
3. Li, G-C., Guan, L-S., Chen G-C. and Wang, Z-Y. "Rb-Associated Protein 46 (RbAp46) Inhibits Cell Growth, Facilitates Stress-Induced Apoptosis and Induces Epithelial-Mesenchymal Transition in Neoplastigenic Mammary Gland Epithelial Cells" *Proceedings Supplement of the American Association for Cancer Research.* 2002, 76: 726.
4. Li, G-C., Guan, L-S. and Wang, Z-Y. "Overexpression of RbAp46 Facilitates Stress-Induced Apoptosis and Suppresses Tumorigenicity of Neoplastigenic Breast epithelial cells" *Int. J. Cancer.* 2003,105: 762-768 .
5. Zhang, T-F., Yu, S-Q., Guan, L-S. and Wang, Z-Y. "Constitutive Expression of Rb Associated Protein 46 (RbAp46) Suppresses Transformed Phenotypes of Breast Cancer Cells" *Anticancer Res. In press.*
6. Zhang, T-F., Yu, S-Q., Guan, L-S. and Wang, Z-Y. "Inducible Expression of RbAp46 Activates c-Jun NH2-terminal Kinase Dependent Apoptosis and Suppresses Progressive Growth of Tumor Xenografts in Nude Mice" *Submitted.*
7. Li, G-C., Guan, L-S. and Wang, Z-Y. "Epithelial-Mesenchymal Transition Induced by RbAp46 is Associated with Destabilization of β-Catenin" *Submitted.*
8. Zhang, T-F., Yu, S-Q., Guan, L-S. and Wang, Z-Y "Constitutive Expression of RbAp46 Inhibits Estrogen-Stimulated Progression of Human Proliferative Breast Disease" *In Preparation.*

Personnel Trained:

Guan Cheng Li
Teng Fei Zhang
Moshe Yamin

Cell lines:

1. RbAp46-transfected MCF10AT3B cells and control cells transfected with empty vector.

2. RbAp46-transfected MCF7 cells and control cells transfected with empty vector.
3. RbAp46-transfected MDA-MB-231 cells and control cells transfected with empty vector.
4. RbAp46-transfected MDA-MB-436 cells and control cells transfected with empty vector.
5. MCF7 cells carrying RbAp46 inducible system and control cells transfected with empty vector.

These stable cell lines provide valuable tools for future study of RbAp46 function in early development of breast cancer. These cell lines are also valuable assets for research community to study molecular and cellular events during progression of proliferative breast disease into malignant breast cancer.

CONCLUSIONS:

During the project period, we discovered that RbAp46 inhibits abnormal cell growth and tumorigenic phenotype of MCF10AT3B cells derived from a model of human proliferative breast disease. These results established that RbAp46, a novel tumor suppressor, has potent growth inhibitory activity and plays an important role in the regulation of both normal and aberrant growth in breast epithelial cells. Dysregulation of this important protein may contribute to early development of human breast cancer. To our knowledge, this is the first tumor suppressor identified to have an inhibitor role in malignant progression of human proliferative breast disease.

We also found that constitutive expression of RbAp46 strongly inhibits transformed phenotypes of breast cancer cells *in vitro* and *in vivo*. These results indicate that the RbAp46 not only inhibits progression of human proliferative breast disease but also suppresses malignant features of established breast cancer cell lines. These data thus further demonstrate that RbAp46 is a tumor suppressor that is involved in development of human breast cancer.

Finally, we have found that constitutive expression of RbAp46 slows down estrogen-stimulated cell growth *in vitro* and progression of proliferative breast disease *in vivo*. These results provided important information about the role of estrogen in the biology of breast cancer progression and the molecular mechanisms of estrogen action in human mammary tumorigenesis. Future study of this model of estrogen-stimulation will greatly advance our understanding of the role of estrogen in human breast cancer etiology.

In addition to the important findings described above, we revealed several molecular mechanisms by which RbAp46 functions as a tumor suppressor. We have found that sensitizing cells to apoptosis is one of the molecular mechanisms by which RbAp46 suppresses tumorigenic progression of human proliferative breast disease. We have also found that both the JNK/SAPK and Caspase 3 signaling pathway are involved in cell apoptosis induced by RbAp46. These data were also confirmed by the inducible system of RbAp46 in a different cell line. These data provided both critical information about the molecular mechanisms by which RbAp46 functions and revealed a

direction for the future study on the cellular and molecular events during malignant progression of human proliferative breast disease.

Furthermore, we have found that RbAp46 downregulated the β-catenin/Tcf signaling pathway through activation of GSK-3β. This finding is of great biological importance. β-catenin is an important regulator of oncogene Wnt signaling cascade. Cytoplasmic β-catenin levels are important, because dysregulation of β-catenin accumulation as a result of mutations in APC protein and β-catenin itself leads to constitutive activation of Wnt-1/β-catenin/Tcf -mediated transcription, which is associated with a variety of human cancers. The relationship between the β-catenin signaling pathway and breast cancer development has been suggested before. However, experimental evidence establishing this relationship has not been documented. Our finding that β-catenin protein was destabilized in RbAp46-transfected cells suggested that RbAp46 inhibits tumorigencity of breast cancer through downregulation of the Wnt-1/β-catenin/Tcf signaling pathway. These results thus suggest that the Wnt-1/β-catenin/Tcf signaling pathway is involved in early development of human breast cancer. These results also provided important information on the cellular regulation of the Wnt-1/β-catenin/Tcf signaling pathway during tumorigenesis of a variety of human cancers.

As described above, RbAp46 is a potent growth inhibitor that plays an important role in maintaining normal growth in mammary gland cells. Interestingly, we also found that overexpression of RbAp46 in epithelial cells induces a significant epithelial-mesenchymal transition. This finding is of great biological significance. Epithelial-mesenchymal inter-conversion is a fundamental mechanism of normal morphogenesis during development and plays an important role in the metastatic cascade of carcinoma, a major event in the transition from non-invasive tumors to invasive malignant carcinomas. However, an understanding of the molecular mechanisms that lead to this transition has been elusive. The fact that RbAp46 can induce epithelial-mesenchymal transition indicates that RbgAp46 is able to block cell-cell contacts through the inhibition of epithelial genes. Further investigation of molecular mechanisms by which RbAp46 induces epithelial-mesenchymal conversion will not only greatly advance our understanding of this important step of tumor development, but will also lay the foundation for novel approaches to interfere with progression of breast cancer.

In summary, our research has provided strong evidence that RbAp46 is a novel tumor suppressor involved in regulation of normal cell growth in mammary gland, and that dysregulation of RbAp46 expression may contribute to human breast cancer tumorigenesis. Our research also suggested a possible therapeutic approach based on restoration of RbAp46 expression in the treatment of human cancer.

REFERENCES:

Chen, Y.R., Wang, X., Templeton, D., Davis, R.J. and Tan, T.H. (1996), *J. Biol. Chem.* 271: 31929-31936.

Dawaon, P.J. Wolman, S.R. Tait, L. Heppner, G.H. and Miller, F.R. (1996), *Am. J. Pathl.* 148: 313-319.

Guan, L-S. Li, G.C. Chen, G.C. Liu, L.Q. and Wang, Z-Y. (2001), *Intl. J. Cancer.* 93: 333-338.

Li, G-C., Guan, L-S. and Wang, Z-Y. (2003), *Intl. J. Cancer.* 105: 762-768.

Miller, F.R. Soule, H.D. Tait, L. Pauley, R.J. Wolman, S.R. Dawson, P.J. and Heppner, G.H. (1993). *J. Natl. Cancer. Inst.* 85: 1725-1732.

Rosette, C. and Karin, M. (1996), *Science* 275: 1194-1197.

Coux O, Tanaka K, Goldberg A.L. (1996), *Annu. Rev. Biochem.* 65: 801-847.

Int. J. Cancer: **105,** 762–768 (2003)

Publication of the International Union Against Cancer

OVEREXPRESSION OF RbAp46 FACILITATES STRESS-INDUCED APOPTOSIS AND SUPPRESSES TUMORIGENICITY OF NEOPLASTIGENIC BREAST EPITHELIAL CELLS

Guan-Cheng LI, Li-Shuang GUAN and Zhao-Yi WANG*

Division of Hematology and Oncology, Department of Medicine, Beth Israel-Deaconess Medical Center, Harvard Medical School, Boston, MA, USA

We have found previously that the retinoblastoma (Rb) suppressor associated protein 46 (RbAp46) is a gene upregulated by the Wilms' tumor suppressor, WT1, and functions as a potent growth inhibitor. To investigate the effect of RbAp46 overexpression on early development of breast cancer, we established stable cell lines from neoplastigenic breast epithelial cells, MCF10AT3B, a cell line derived from a model of human proliferative disease, to constitutively express exogenous RbAp46. We have found that expression of RbAp46 suppressed colony formation of MCF10AT3B cells in soft-agar, and inhibited tumor formation of these cells in nude mice. Expression of RbAp46 sensitized MCF10AT3B cells to apoptosis induced by serum deprivation and hydrocortisone withdrawal. Furthermore, we have found that the c-Jun NH2-terminal kinase (JNK) pathway and GADD45, a growth arrest- and DNA damage-inducible gene, are constitutively activated in RbAp46-expressing cells. Our data suggested that high levels of RbAp46 expression inhibit the tumorigenicity of neoplastigenic breast epithelial cells by facilitating JNK-dependent apoptotic cell death. Our data also suggested that dysregulation of RbAp46 gene may be involved in the early development of breast cancer.

Key words: *RbAp46; tumor suppressor; apoptosis; JNK; breast cancer*

The retinoblastoma (Rb) suppressor associated protein 46 (RbAp46) was first identified as a protein in HeLa cells that binds to an Rb affinity column.[1] The cDNA encoding RbAp46 was later cloned.[2,3] The predicted amino acid sequence contains 4 typical WD repeats that end with WD residues and 4 non-typical WD repeats that end with WN, FD or YD.[3] Homologues of RbAp46 have been cloned from tomato and *Arabidopsis thaliana*, sharing 65% amino acid sequence homogeneity with their human counterpart.[4] The predicted amino acid sequence of human RbAp46 exhibits a strong homology with MSI1, a protein characterized as a negative regulator of the Ras signal transduction pathway in *Saccharomyces*.[3,5] Overexpression of human RbAp46 or MSI1 suppresses heat-shock sensitivity and decreases cellular cAMP levels in a constitutively active $RAS2^{Val\text{-}19}$ mutant yeast strain.[3,5] Recently, the *C. elegans lin*-53 gene, which shares 70% amino acid homogeneity with human RbAp46, was shown to antagonize the Ras-signaling pathway in *C. elegans* and to play a critical role in vulval induction.[6]

RbAp46 has been shown to be a component of the mSin3 histone deacetylase (HDAC) complex,[7] which is involved in the transcriptional repression mediated by growth-related transcription factors. RbAp46 is also known as the histone acetyltransferase (hat) Type B subunit 2 in which RbAp46 binds selectively to H2A and H4 histones and greatly stimulates hat activity.[8] RbAp46 is found as a subunit of NuRD, a multi-subunit complex containing chromosome-remodeling activity.[9] Thus, RbAp46 functions as a core-histone-binding subunit that targets chromatin assembly factors, chromatin-remodeling factors, histone acetyltransferase and deacetylase to their histone substrates in chromatin.

Previously, we isolated RbAp46 as a downstream target gene of the Wilms' tumor suppressor gene product, WT1.[10] We have further found that overexpression of RbAp46 inhibits transformed phenotype of adenovirus-transformed human embryonic kidney 293 cells.[11] It has been discovered recently that RbAp46 specifically interacts with the BRCT domain of breast cancer and ovarian cancer susceptibility gene BRCA1 and modulates its transcriptional transactivation activity.[12,13] These results suggest that RbAp46 regulates the function of BRCA1 as a tumor suppressor and dysregulation of RbAp46 may contribute to the early development of breast cancer.

Human breast cancer evolves in a progressive stepwise sequence from benign hyperplasia through atypical hyperplasia to carcinoma *in situ* and eventually to fully malignant, invasive tumors with the potential to metastasize. In the human female breast, a spectrum of pathologic change has been termed proliferative breast disease.[14] The progression of histopathological features of proliferative breast disease has been associated with increased risk for the development of invasive carcinoma.[14] Women with the most severe forms of proliferative breast disease, atypical hyperplasia, have a 4- to 5-fold increased risk of developing breast cancer.[14] Thus, the study of proliferative breast disease is likely to provide important information about the molecular and cellular events in the early development of breast cancer.

The human cell line MCF10A originated from spontaneous immortalization of non-malignant breast epithelium.[15] MCF10A cells transfected with T24 Ha-ras mutant (designated as MCF10AT) acquired the ability for xenograft growth in the dorsal flank of nude mice. The MCF10AT3B cell line was established from a hyperplastic lesion found in the third generation transplant of nude mice. When xenografted into nude mice, the MCF10AT3B cells form atypical hyperplasia, carcinoma *in situ* and invasive carcinoma.[16] Long-term transplant of MCF10AT3B cells in nude mice produces lesions of various morphological types and grades, resembling the morphological characteristics of human proliferative breast disease as well as *in situ* and invasive carcinoma.[15]

We show that constitutive expression of RbAp46 inhibits clonogenicity of MCF10AT3B cells in soft-agar and tumorigenicity of these cells in nude mice. We also show that RbAp46 facilitates stress-induced apoptosis in these cells. Furthermore, we found that the c-jun N-terminal kinase pathway and GADD45, a growth arrest- and DNA damage-inducible gene, are constitutively acti-

Grant sponsor: National Institute of Health; Grant number: 84328; Grant sponsor: American Cancer Society; Grant number: RPG-00272-02-MGO; Grant sponsor: Department of Defense Breast Cancer Research Program; Grant number: DAMD17-00-10156.

*Correspondence to: The Division of Hematology and Oncology, Department of Medicine, Beth Israel-Deaconess Medical Center, 21-27 Burlington Av., Boston, MA 02215. Fax: +617-632-0821.
E-mail: zwang2@bidmc.harvard.edu.

Received 21 June 2002; Revised 25 November 2002, 30 January 2003; Accepted 27 January 2003

DOI 10.1002/ijc.11148
Published online 7 April 2003 in Wiley InterScience (www.interscience.wiley.com).

vated in RbAp46-expressing cells, providing a molecular mechanism for RbAp46 function.

MATERIAL AND METHODS

Cell culture and DNA transfection

MCF10AT3B cells were obtained from Karmanos Cancer Institute (Detroit, MI) and maintained at 37°C in a 10% CO_2 atmosphere in DMEM/F12 medium supplemented with 5% horse serum, L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), hydrocortisone (0.5 μg/ml), insulin (10 μg/ml), EGF (2 ng/ml), cholera toxin (0.1 μg/ml) and fungizone (0.5 μg/ml). Cells were plated at a density of 1×10^5 cells per 60-mm dish and transfected 24 hr later with RbAp46 expression vector driven by the cytomegalovirus (CMV) promoter using the FuGene6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN). The RbAp46 expression vector was constructed by cloning a 1.9-kb *BamHI–KpnI* cDNA fragment of RbAp46 from pBluescript into the *Bgl*II and *Kpn*I sites of mammalian expression vector pCB6+ as described before.[10] Empty vector was also transfected into cells to serve as a control. Forty-eight hours after transfection, the cells were replated and selected with 250 μg/ml of Zeocin (Invitrogen, Carlsbad, CA) for 2 weeks. The medium was changed every 3 days until colonies appeared. Individual clones were then isolated and expanded to confirm expression of RbAp46 by Western blot and Northern blot analysis. Parental cells and cells transfected with the empty vector were analyzed as controls.

RNA extraction and northern blot analysis

Total cellular RNA was isolated using Trizol (Gibco/BRL Life Technologies, Carlsbad, CA), according to the manufacturer's instruction. Ten micrograms of total RNA was separated by electrophoresis on a 1.2% formamide/formaldehyde gel and blotted onto a nylon membrane (Hybond-N, Amersham Pharmacia Biotech, Piscataway, NJ). The blots were prehybridized for 1 hr and hybridized for 2 hr in Quick-Hybridization solution (Amersham Pharmacia Biotech) at 65°C. The probes included a 120 bp DNA fragment containing part of the CMV IE promoter and polylinker sequence 5′ upstream of the RbAp46 cDNA, a 1.9 kb full-length cDNA of RbAp46, and a β-actin DNA probe from CLONTECH. The DNA probes were labeled with ^{32}P dCTP and a Rediprime II DNA labeling kit (Amersham Pharmacia Biotech). The blots were washed twice with 2× SSC and 0.1% SDS for 15 min at room temperature, and twice with 0.1× SSC and 0.1% SDS for 15 min at 55°C. Blots were autoradiographed using intensifying screens at −70°C overnight. The same membranes were stripped and reprobed with a labeled β-actin DNA probe to confirm equal loading.

Western blot analysis

Cells were washed with PBS and lysed with lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.25 mM EDTA, pH 8.0, 0.1% SDS, 1% Triton X-100, 50 mM NaF, 1 mM PMSF and 10 μg/ml aprotinin and leupeptin). After adjustment to the same total protein content, cell lysates were analyzed by Western blot analysis. The cell lysates were boiled for 5 min in SDS gel loading buffer and separated on a 10% or 6% SDS-PAGE gel. After electrophoresis, the proteins were transferred to a PVDF membrane (Millipore). The membranes were probed with various primary antibodies, HRP-conjugated secondary antibodies, and visualized with enhanced chemiluminescence (ECL) detection reagents (Amersham Pharmacia Biotech). The same membranes were stripped and reprobed with antibody against β-actin to confirm equal loading.

The antibody against activated form of Caspase-3 (AAP-113) was obtained from StressGen Biotechnologies (Victoria, BC) and anti-PARP (Ab-2) antibody from Oncogene Research (Boston, MA). Antibodies used to analyze activation of MAP kinase pathway including p-SAPK/JNK (Thr 183/Tyr 185); p-SEK1/MKK4 (Thr 261); p-c-Jun (Ser 63) and p-c-Jun (Thr 73) were all purchased from Cell Signaling Technology (Beverly, MA). Antibody against β-actin was purchased from Sigma (St. Louis, MO). Anti-GADD45 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell survival assays

To exclude the possibility of clonal variation, equal number of cells from 3 clones of RbAp46-expressing cells and control cells were mixed before cell survival assay, cells were plated 5×10^4 cells/well in 12-well plates and were treated with serum deprivation or hydrocortisone withdrawal for 3 days. Each well was washed vigorously twice with PBS to eliminate dead cells. Viable cells were fixed and stained for 10 min in 250 μl of 0.02% crystal violet in a solution of 20% ethanol/0.74%formaldehyde/80%water. The optical density (OD) of stained cells was measured at a wavelength of 590 nm with a spectrophotometer after they were treated with 1 ml of methanol. A background value of crystal violet staining for wells containing only culture medium was subtracted from each OD measurement. Assays were carried out in quadruplicate. The percentage of survival cells was calculated as follows: survival rate (%) = OD (treated group)/OD (control group) × 100%.

Fluorescent activated cell-sorting analysis and DNA fragmentation analysis

Fluorescent activated cell-sorting (FACS) analysis was carried out with a Coulter EPICS Profile I, and data from 60,000 cells per sample were analyzed with ModFitLT version 2.0 software. The gate was selected for single cell within a normal size range. The cells were seeded at 5×10^4 cells/dish and cultured with different concentration of serum for 72 hr, then fixed in 95% ethanol. Before FACS analysis, the cells were stained with 10 μg/ml of propidium iodide (PI) and treated with 100 μg/ml of RNase A at 37°C for 30 min. To exclude the possibility of clonal variation, equal number of cells from 3 clones of RbAp46-expressing cells and control cells were mixed right before FACS analysis. The PI signals were used as a measure for DNA content and hence cell cycle stage. For DNA fragmentation analysis, DNA was isolated using a DNA ladder kit from the Roche Molecular Biochemicals and electrophoresed on a 2% agarose gel.

Soft-agar colony formation assay and tumor formation in nude mice

To determine anchorage-independent growth in soft-agar, 200 cells from each of the stable cell lines were suspended in 3 ml of 3.5% (W/V) agar containing 1× DMEM/F12 medium plus 30% FCS. The cells were then overlaid onto a 0.7% (W/V) agar containing 1× DMEM/F12 medium plus 30% FCS in 5 replica dishes. After 4 weeks, colonies with ≥20 cells were scored as positive using an inverted microscope.

Tumor formation was assayed in 4~5-week-old female athymic nude mice (strain Ncr nu/nu; Sprague-Dawley, Indianapolis, IN) by the mammary fat-pad injection of 1×10^7 cells in 200 μl Matrigel (BD Biosciences, Palo Alto, CA). Groups of 12 mice were injected using 2 individual clones from each transfectant. Animals with tumors were examined weekly, beginning at 30 days after the injections, to monitor tumor growth for 40 weeks.

RESULTS

Expression of RbAp46 inhibits tumorigenicity of MCF10AT3B cells.

To investigate the effects of high levels of RbAp46 expression on the early development of breast cancer, we established stable cell lines that express exogenous RbAp46 from MCF10AT3B, a cell line derived from a xenograft model of human proliferative disease.[15] The individual cell clones were isolated and expanded. We established several clonal cell lines that expressed exogenous RbAp46 at high levels, 3 of which are described in detail here (RbAp46-4, -7 and -9). We also generated 3 control cell lines transfected with the empty expression vector (CMV-2, -5 and -6).

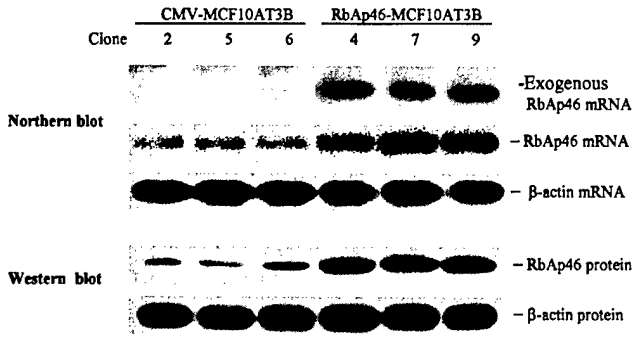


FIGURE 1 – Northern blot and Western blot analysis of RbAp46 expression in the control MCF10AT3B cells transfected with empty vector (CMV-2, -5, and -6) and RbAp46 transfected MCF10AT3B cells (RbAp46-4, -7 and-9). For Northern blot analysis, DNA probes included a 120 bp DNA fragment containing part of the CMV IE promoter and polylinker sequence 5′ upstream of the RbAp46 cDNA that detects only the exogenous RbAp46, and an 1.9 kb full-length cDNA of RbAp46 that detects both exogenous and endogenous RbAp46. The filter was also hybridized to an β-actin DNA probe for a loading control. For Western blot analysis, the filter was probed with anti-RbAp46 antibody, and an anti-β-actin antibody as a control.

The clonally isolated colonies were examined by Northern blot analysis using a DNA sequence unique to exogenous RbAp46 as a probe. A distinct 2.0 kb transcript was highly expressed in RbAp46-transfected cells (Fig. 1). Western blot analysis using polyclonal antibody raised against the final 15 amino acids of RbAp46 confirmed that the levels of RbAp46 (~50 kDa) protein were about 4~5 fold higher in these cloned cells than the endogenous RbAp46 level in the control cells transfected with empty vector (Fig. 1).

We then explored the effects of RbAp46 on the tumorigenic activity of MCF10AT3B cells using 2 types of experiments. First, we determined the ability of these established cell lines to form colonies in soft-agar. Two hundred cells from each established cell line were seeded in 60-mm dishes in replicates of 5 dishes for each cell line. After incubation for 4 weeks, colonies with more than 20 cells were counted and scored as anchorage-independent. The RbAp46-expressing cells formed ~50% fewer colonies in soft-agar than parental cells and control cells transfected with the empty vector (Table I). Analysis of the data using a Student's *t*-test showed that the reduction in the number of colonies formed by RbAp46-expressing cells compared to control cells was statistically significant ($p < 0.05$). In addition to the decreased number of colonies, we noted a significant decrease in the average size of colonies formed by cell lines expressing high levels of RbAp46 (data not shown). This result indicates that high levels of RbAp46 expression inhibit anchorage-independent growth of MCF10AT3B cells.

Second, we examined the effect of RbAp46 on the tumorigenicity of MCF10AT3B cells in nude mice. Two established cell lines from each expression vector were injected in the mammary fat-pad of 12 nude mice. Forty weeks after injection, tumor like nodules were detected at about 50% of the injected sites of parental cells and control cells transfected with empty vector. When the host animals were sacrificed and the tumor like lesions resected, a portion of each was prepared for histological examination. All lesions showed distended ducts filled with uniform cells containing enlarged nuclei, relatively abundant cytoplasm and sharp cell boundaries (data not shown), corresponding to a Grade 4 lesion of proliferative breast disease, *i.e.*, carcinoma *in situ*.[16] Tumors were totally absent in the mice injected with RbAp46-expressing cells (Table II). This data indicates that RbAp46 strongly suppresses the tumorigenic ability of neoplastigenic MCF10AT3B cells in nude mice.

TABLE I – SOFT-AGAR COLONY FORMATION OF RBAP46-EXPRESSING CELLS

Cells	colonies (n)
CMV-2	33.4 ± 5.1
CMV-5	30.6 ± 4.2
CMV-6	32.2 ± 6.1
RbAp46-4	12.8 ± 3.1
RbAp46-7	15.4 ± 2.9
RbAp46-9	16.6 ± 3.6

[1]Number of colonies with ≥20 cells per dish formed in soft-agar colony formation assay. Five replicates of each transfectant were cultured for 4 weeks and colonies were counted. The difference in the number of colonies is statistically significant for all pairings ($p < 0.05$).

Expression RbAp46 facilitates stress-induced apoptosis.

When grown in normal serum media (5% horse serum), we noticed that some RbAp46-expressing cells were floating in the culture dish, suggesting that high levels of RbAp46 may affect cell survival. To pursue this further, we examined cells with exogenous RbAp46 following their transfer to media containing low concentrations of serum. In contrast to the control cells, RbAp46-expressing cells incubated in low serum exhibited a dramatic increase of cell death (Fig. 2*a*). In low concentrations of serum, many RbAp46-expressing cells exhibited morphological characteristic of apoptosis such as retraction of cellular processes, nuclear condensation, and loss of adherence to the tissue culture dish (data not shown). It has been reported previously that hydrocortisone is vital to normal growth of MCF10A cells in culture and hydrocortisone withdrawal will induce cell apoptosis in MCF10A cells.[17] Because MCF10AT3B cells was derived from MCF10A cells,[15] we tested if hydrocortisone withdrawal would induce apoptosis in MCF10AT3B cells. Hydrocortisone withdrawal markedly induces apoptosis in RbAp46-expressing cells at a level higher than that exhibited by control cells (Fig. 2*b*).

To further confirm that cell death induced by serum deprivation is apoptotic, fluorescent activated cell-sorting (FACS) analysis was carried out in propidium iodide stained cells. Although we did not observe any change of cell population in a specific phase of the cell cycle, we observed a dramatic increase of cell population in the sub G1 phase in the RbAp46-expressing cells at low concentration of serum for 3 days (Table III). Electrophoretic analysis of DNA from these dying cells revealed the fragmentation of chromatin into a nucleosomal ladder characteristic of apoptosis (Fig. 3*a*). These dying cells also exhibited the biochemical characteristics of apoptosis; the activation of caspase-3 and the cleavage of poly ADP-ribose polymerase (PARP), a substrate of activated caspase-3, were also observed in RbAp46-expressing cells at low concentrations of serum (Fig. 3*b,c*). These data strongly indicate that serum deprivation induces apoptosis in RbAp46-expressing MCF10AT3B cells, and even though high levels of RbAp46 expression alone do not induce significant apoptosis, when combined with stress-stimuli, leads to a significant increase in apoptotic cell death.

Constitutive activation of the JNK signaling pathway in RbAp46-expressing MCF10AT3B cells

To probe the molecular mechanism by which RbAp46 facilitates stress-induced apoptosis, we examined several apoptosis signaling pathways. We found that phosphorylation levels of endogenous SEK1/MKK4, the common upstream regulator of JNK pathway,[18] were higher in RbAp46-expressing cells compared to control cells (Fig. 4*a*). The phosphorylation level of JNK was also enhanced in RbAp46-expressing cells (Fig. 4*b*), at a level comparable to that observed after UV irradiation (data not shown), a well-described inducer of JNK.[19] Furthermore, JNK kinase activity was also activated as demonstrated by the increased phosphorylation of the

TABLE II – TUMORIGENICITY OF RBAP46-EXPRESSING MCF10AT3B CELLS IN NUDE MICE[1]

Cells	Tumor formed
MCF10AT3B	6/12
CMV-2	6/12
CMV-6	7/12
RbAp46-4	0/12
RbAp46-9	0/12

[1]The tumor formation ability of the parental cells (MCF10AT3B), the control cells (CMV-2 and 6), and RbAp46-expressing cells (RbAp46-4 and -9) was tested by subcutaneously injecting 1×10^7 cells at each site of nude mouse. Animals with tumors were monitored weekly starting 30 days following the injections. Tumor formed represents the number of tumors divided by the number of mice.

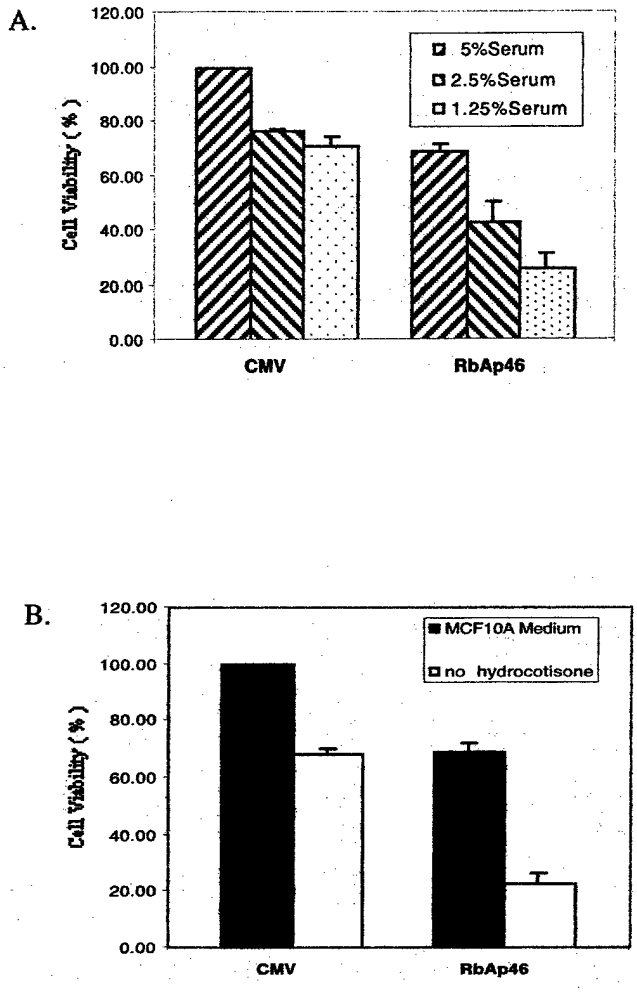


FIGURE 2 – RbAp46 expression facilitates cell death induced by stress stimuli. (*a*) Cells were cultured in different concentrations of serum as indicated for 3 days. (*b*) Cells were cultured in medium with or without hydrocortisone for 48 hr. Cell survival assays were carried out as described in Material and Methods.

JNK substrate c-Jun (Fig. 4*c*). Expression of RbAp46 did not result in nonspecific activation of the other MAP kinase pathways, as shown by the unaltered tyrosine phosphorylation of MAP kinases such as ERK and p38 (data not shown). These results indicated that the JNK signaling pathway is constitutively activated in RbAp46-expressing cells, which may provide an explanation for the sensitivity of RbAp46-expressing cell to apoptosis induced by stress stimuli.

RbAp46 induces expression of GADD45

The data described above demonstrated that RbAp46 sensitized cells to apoptosis induced by serum stimuli, possibly through constitutive activation of JNK pathway. Growth arrest- and DNA damage-inducible (GADD) genes belong to a subgroup of genes that are not only rapidly induced by DNA-damaging agents but are coordinately induced in growth-arrested cells.[20] Two studies have shown recently that GADD45 may play a role in apoptosis via activation of the JNK signaling pathway. Takekawa and Saito[21] showed that GADD45 interacts with MTK1/MEKK4, an upstream activator of JNK pathway, to induce JNK-mediated apoptosis. Harkin *et al.*[22] also showed that BRCA1 triggered JNK-dependent apoptosis is associated with transcriptional induction of GADD45. These results together with our data suggest a possibility that expression of GADD45 may be induced in cells with high levels of RbAp46.

To examine expression of the *GADD45* gene in RbAp46-expressing cells, Western blot analysis was carried out with proteins from RbAp46-expressing cells and control cells. The levels of GADD45 expression were markedly increased in RbAp46–expressing cells compared to control cells (Fig. 5*a*). The levels of GADD45 induced by RbAp46 were comparable to that induced by UV irradiation and higher than that induced by Methyl methanesulfonate (MMS), a DNA alkylating agent known to induce GADD45 in all cells tested (Fig. 5*b*). This data suggested that overexpression of RbAp46 may mimic the signals of these environmental stimuli and induce expression of GADD45.

DISCUSSION

We used MCF10AT3B cells as a model system to study the effect of RbAp46 overexpression on the early development of human breast cancer. Our results from both *in vitro* and *in vivo* assays demonstrate that high levels of RbAp46 expression strongly inhibit the tumorigenic phenotype of neoplastigenic MCF10AT3B cells.

We found recently that RbAp46 is upregulated more than ten-fold in WT1-expressing cells.[10] It has been reported that WT1 suppresses tumorigenicity of Wilms' tumor cells,[23] and osteosarcoma cells.[24] WT1 also inhibits growth and tumorigenicity of ras-transformed NIH 3T3 fibroblasts.[25] Like WT1, the growth of several types of tumor cells was greatly inhibited by overexpression of RbAp46.[10,11] We provide evidence to demonstrate that RbAp46 is able to suppress the transformed phenotype of neoplastigenic MCF10AT3B cells. Taken together, these results are consistent with the hypothesis that RbAp46 mediates some of the tumor suppressor functions of WT1.

In studies of tumor suppression, a common phenomenon known as tumor suppressor resistance (TSR) is usually observed in which tumor growth in nude mice is partially inhibited by introduction of a tumor suppressor.[25] Our result that expression of RbAp46 totally abrogates the tumor formation in nude mice suggests that RbAp46 has a strong ability to inhibit tumor formation or that RbAp46 may function in a different mechanism from the tumor suppressors previously described. The exact mechanism of inhibition of tumor formation by RbAp46 remains to be elucidated. Because RbAp46 sensitizes cells to apoptosis induced by low concentration of serum, we speculate that RbAp46 may enhance apoptosis of tumor cells implanted in nude mice before angiogenesis is established at the tumor.

Furthermore, we found that RbAp46 sensitizes MCF10AT3B cells to apoptosis induced by serum deprivation and hydrocortisone withdrawal. We also found that RbAp46 sensitizes MCF10AT3B cells to apoptosis induced by TNF-α and Fas ligand treatment (data not shown). RbAp46-expressing cells do not exhibit significant cell death under normal conditions, whereas RbAp46 enhances apoptosis induced by other signals. Apparently, in the serum deprivation case, the growth-promoting factors in serum induce the expression of positive activities that overcome

TABLE III – CELL-CYCLE PROFILE OF RBAP46-EXPRESSING CELLS[1]

Cells	Cell fraction (%) at 5% Serum				Cell fraction (%) at 1.25% Serum			
	G0/G1	G2/M	S	Apoptosis	G0/G1	G2/M	S	Apoptosis
CMV-2	51.84	16.31	31.85	0.22	76.10	11.15	12.75	4.85
CMV-5	42.55	25.18	32.27	0.38	80.75	9.99	9.26	2.26
RbAp46-4	57.94	14.84	27.22	2.83	87.27	9.29	3.44	26.44
RbAp46-9	56.28	20.36	23.36	4.03	89.52	7.37	3.11	25.27

[1]The cell cycle distribution was determined by staining for DNA content with propidium iodide, followed by FACS analysis. The control cells and RbAp46-expressing MCF10AT3B cells were cultured in normal and low concentrations of serum for 3 days. Apoptosis portion of cells is indicated. The experiment was repeated 3 times with similar results, and the result from 1 experiment is shown here.

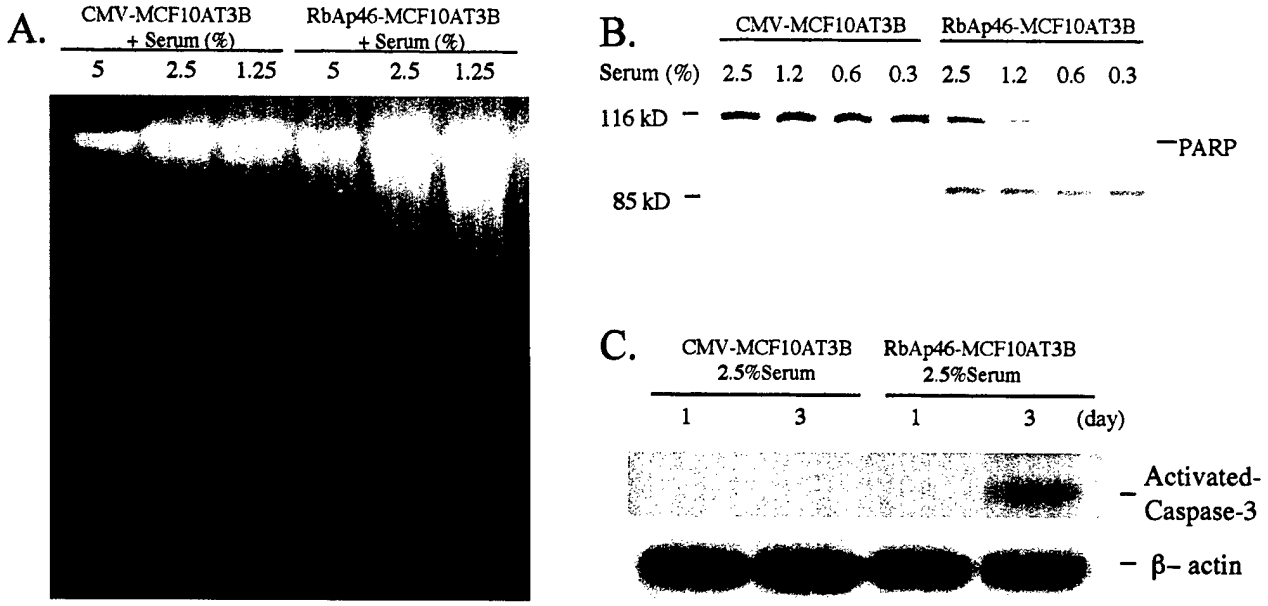


FIGURE 3 – RbAp46 sensitizes cells to apoptosis induced by low concentration of serum. (*a*) DNA fragmentation assay. Cells grown at different concentrations of serum as indicated for 3 days were harvested and cellular DNA was extracted and analyzed by electrophoresis on a 1.8% agarose gel. (*b*) Enhanced PARP cleavage in RbAp46-expressing cells after cultured in different concentrations of serum for 3 days. Western blot analysis of proteins from cells with overexpression of RbAp46 was carried out with an anti-PARP antibody that detects intact (116 kDa) and cleaved (85 kDa) products. (*c*) Caspase-3 activation in RbAp46-expressing cells grown in low concentrations of serum for 3 days. Cell lysates were prepared, and Western blot analyses were carried out using anti-caspase-3 and anti-actin antibodies. Activated caspase-3 (17 kDa) was indicated.

the negative effect of RbAp46. It is also possible, however, that high levels of RbAp46 can actually induce apoptosis, and cells that express RbAp46 at high levels may die of apoptosis during the drug selection period.

We further demonstrated that the JNK stress-response pathway is constitutively activated in RbAp46-expressing cells. The phosphorylation levels of SEK1/MKK4, an upstream activator of JNK pathway, and JNK were enhanced greatly in RbAp46-expressing cells compared to control cells. The kinase activity of JNK was also activated because the phosphorylation levels of its substrate c-jun were enhanced in RbAp46-expressing cells. The JNK pathway is activated by environmental stresses (*e.g.*, UV, γ-irradiation, methyl methanesulfonate [MMS], osmotic stress, and the translation inhibitor anisomycin) and has been implicated recently as a major apoptotic pathway after DNA damage and in response to Fas ligand and tumor necrosis factor α.[19] Overexpression of RbAp46 appears to mimic these signals in response to various environmental stress stimuli and constitutively activate the JNK signaling pathway. The constitutive activation of JNK pathway in RbAp46-expressing cells apparently makes cells more sensitive to apoptosis induced by stress signals.

We have established recently a tetracycline-inducible RbAp46 expression system in Saos-2 cells, an osteosarcoma cell line that lacks both functional p53 and Rb. Inducible expression of RbAp46 activated the c-Jun N-terminal kinase (JNK) signaling pathway and potentiate apoptosis in Saos-2 cells (unpublished data). Thus, we have an inducible system that can be used to study the molecular mechanism by which RbAp46 activates the JNK signaling pathway and sensitizes cells to apoptosis.

The ability to sensitize cells to apoptosis is not unique to RbAp46. It has been reported that expression of gene products, such as cyclin D,[27,28] myc,[29–31] or E1A[32,33] also sensitize cells to undergo apoptosis after serum deprivation, but do not induce cell death under optimal growth conditions. It also has been shown recently that over-expression of cyclin G enhances apoptotic cell death induced by treatment with TNF-α, RA or low serum.[34] It will be interesting to examine whether these proteins share a similar pathway as RbAp46.

The molecular mechanism by which RbAp46 activates the JNK-signaling pathway is unclear. A possible mechanism for the activation of JNK pathway by RbAp46 is provided by the experimental data that the expression levels of GADD45 were increased in RbAp46-expressing cells. GADD45 has been reported recently to bind and activate MAPKKK MTK1/MEK4, an upstream regulator of JNK, triggering JNK-dependent apoptosis.[21] In addition, it has been reported that forced expression of BRCA1 induced JNK-dependent apoptosis is also associated with upregulation of GADD45.[22] Thus, it is possible that RbAp46 activates the JNK

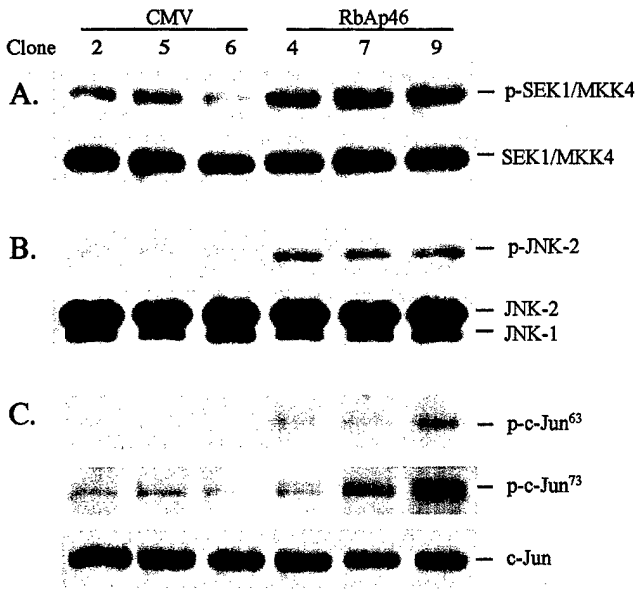


FIGURE 4 – JNK/SAPK signaling pathway is constitutively activated in RbAp46-expressing cells. The different clones of RbAp46-transfected MCF10AT3B cells and empty vector transfected cells were maintained in medium containing 5% serum, Western blot analyses were carried out with (*a*) anti-phospho-SEK1/MKK4 and anti-SEK1/MKK4; (*b*) anti-phospho-JNK and anti-JNK; and (*c*) anti-phospho-c-Jun and anti-c-Jun antibodies.

signaling pathway through upregulation of GADD45. The role of GADD45 in mediating the stress-induced JNK activation remains controversial. Stress-induced JNK activation is not altered in gadd45-deficient embryonic fibroblasts,[35,36] suggesting that GADD45 is not required for the JNK activity. Therefore, it is possible that RbAp46 employ different mechanisms to activate the JNK signaling pathway.

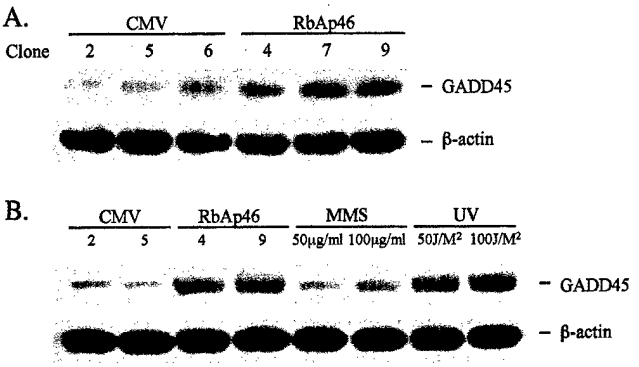


FIGURE 5 – Expression of GADD45 is activated in RbAp46-expressing cells. (*a*) The different clones of RbAp46-transfected MCF10AT3B cells and empty vector transfected cells were cultured in medium containing 5% horse serum, Western blot analysis with anti-GADD45 and anti-actin antibodies were carried out. (*b*) For comparison, the induction of GADD45 by UV irradiation and MMS is also shown.

RbAp46 has been shown recently to exist in the mSin3 complex with human histone deacetylase HDAC1 and HDAC2,[7] which is involved in the transcriptional repression mediated by growth-related transcription factors, such as PLZF protein,[37] LAZ-3 (Bcl-6) oncogene,[38] AML-1 protein,[39] and Mad-Max proteins.[40] Together with our results, these observations suggest that the histone acetylation/deacetylation system plays an important role in the regulation of cell growth, differentiation and apoptosis. Dysregulation of this system may lead to altered expression of growth-related genes and ultimately to uncontrolled cell growth characteristic of tumorigenesis.

ACKNOWLEDGEMENTS

This work was supported by Department of Defense Breast Cancer Research Program DAMD17-00-10156 to Z.-Y. Wang.

REFERENCES

1. Huang S, Lee WH, Lee EY. A cellular protein that competes with SV40 T antigen for binding to the retinoblastoma gene product. Nature 1991;350:160–2.
2. Qian YW, Wang YC, Hollingsworth REJ, Jones D, Ling N, Lee EHP. A retinoblastoma-binding protein related to a negative regulator of Ras in yeast. Nature 1993;364:648–52.
3. Qian YW, Lee EHP. Dual retinoblastoma-binding proteins with properties related to a negative regulator of ras in yeast. J Biol Chem 1995;270:25507–13.
4. Ach RA, Taranto P, Gruissem W. A conserved family of WD-40 proteins binds to the retinoblastoma protein in both plants and animals. PLANT Cells 1997;9:1595–606.
5. Ruggieri R, Tanaka K, Nakafuku M, Kaziro Y, Tohe A, Matsumoto K. MSI1, a negative regulator of the RAS-cAMP pathway in *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 1989;86:8778–82.
6. Lu X, Horvitz HR. lin-35 and lin-53, two genes that antagonize a *C. elegans* Ras pathway, encode proteins similar to Rb and its binding protein RbAp48. Cell 1998;95:981–91.
7. Zhang Y, Iratni R, Erdjument-Bromage H, Tempst P, Reinberg D. Histone deacetylases and SAP18, a novel polypeptide, are components of a human Sin3 complex. Cell 1997;89:357–64.
8. Verreault A, Kaufman PD, Kobayashi R, Stillman B. Nucleosome assembly by a complex of CAF-1 and acetylated histones H3/H4. Cell 1996;87:95–104.
9. Zhang Y, Ng HH, Erdjument-Bromage H, Tempst P, Bird A, Reinberg D. Analysis of the NuRD subunits reveals a histone deacetylase core complex and a connection with DNA methylation. Genes Dev 1999;13:1924–35.
10. Guan L-S, Rauchman M, Wang Z-Y. Induction of Rb-associated protein (RbAp46) by Wilms' tumor suppressor WT1 mediates growth inhibition. J Biol Chem 1998;273:27047–50.
11. Guan L-S, Li G-C, Liu L-Q, Wang Z-Y. Rb-associated protein 46 (RbAp46) suppresses the tumorigenicity of adenovirus-transformed human embryonic kidney 293 cells. Int J Cancer 2001;93:333–8.
12. Yarden RI, Brody LC. BRCA1 interacts with components of the histone deacetylase complex. Proc Natl Acad Sci USA 1999;96:4983–8.
13. Chen G-C, Guan L-S, Yu H-J, Li G-C, Choi K-H, Wang Z-Y. Rb-associated protein 46 (RbAp46) inhibits transcriptional transactivation mediated by BRCA1. Biochem Biophys Res Commun 2001;284:507–14.
14. Page DL, Dupont WD. Anatomic markers of human premalignancy and risk of breast cancer. Cancer 1990;66:1326–35.
15. Miller FR, Soule HD, Tait L, Pauley RJ, Wolman SR, Dawson PJ, Heppner GH. Xenograft model of progressive human proliferative breast disease. J Natl Cancer Inst 1993;85:1725–32.
16. Dawson PJ, Wolman SR, Tait L, Heppner GH, Miller FR. MCF10AT: a model for the evolution of cancer from proliferative breast disease. Am J Pathol 1996;148:313–9.
17. Moran TJ, Gray S, Mikosz CA, Conzen SD. The glucocorticoid receptor mediates a survival signal in human mammary epithelial cells. Cancer Res 2000;60:867–72.
18. Derijard B, Raingeaud J, Barrett T, Wu IH, Han J, Ulevitch RJ, Davis RJ. Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. Science 1995;267:682–5.
19. Davis RJ. Signal transduction by the JNK group of MAP kinases. Cell 2000;103:239–52.
20. Sheikn MS, Hollander MC, Fornance AJ Jr. Role of Gadd45 in apoptosis. Biochem Pharmacol 2000;59:43–5.
21. Takekawa M, Saito H. A family of stress-inducible GADD45-like proteins mediate activation of the stress-responsive MTK1/MEKK4 MAPKKK. Cell 1998;95:521–30.
22. Harkin DP, Bean JM, Miklos D, Song YH, Truong VB, Englert C, Christians FC, Ellisen LW, Maheswaran S, Oliner JD, Haber DA.

Induction of GADD45 and JNK/SAPK-dependent apoptosis following inducible expression of BRCA1. Cell 1999;97:575–86.
23. Haber DA, Park S, Maheswaran S, Englert C, Re GG Hazen-Martin DJ, Sens DA, Garvin J. WT1-mediated growth suppression of Wilms tumor cells expressing a WT1 splicing variant. Science 1993;262: 2057–9.
24. Englert C, Hou X-Y, Maheswaran S, Bennett P, Ngwu C Re GG, Garvin AJ, Rosner MR, Haber DA. WT1 suppresses synthesis of the epidermal growth factor receptor and induces apoptosis. EMBO J 1995;14:4662–75.
25. Luo XN, Reddy JC, Yeyati PL, Idris AH, Hosono S, Harber DA, Licht JD, Atweh GF. The tumor suppressor gene WT1 inhibits ras-mediated transformation. Oncogene 1995;11:743–50.
26. Zhou Y, Li J, Xu K, Hu SX, Benedict WF, Xu HJ. Further characterization of retinoblastoma gene-mediated cell growth and tumor suppression in human cancer cells. Proc Natl Acad Sci USA 1994; 91:4165–9.
27. Janicke RU, Lin XY, Lee FH, Porter AG. Cyclin D3 sensitizes tumor cells to tumor necrosis factor-induced, c-Myc-dependent apoptosis. Mol Cell Biol 1996;16:5245–53.
28. Sofer-Levi Y, Resnitzky D. Apoptosis induced by ectopic expression of cyclin D1 but not cyclin E. Oncogene 1996;13:2431–7.
29. Janicke RU, Lee FH, Porter AG. Nuclear c-Myc plays an important role in the cytotoxicity of tumor necrosis factor alpha in tumor cells. Mol Cell Biol 1994;14:5661–70.
30. Evan GI, Wyllie AH, Gilbert CS, Littlewood TD, Land H, Brooks M, Waters CM, Penn LZ, Hancock DC. Induction of apoptosis in fibroblasts by c-myc protein. Cell 1992;69:119–28.
31. Klefstrom J, Vastrik I, Saksela E, Valle J, Eilers M, Alltalo K. c-Myc induces cellular susceptibility to the cytotoxic action of TNF-α. EMBO J 1994;13:5442–50.
32. Chen MJ, Holskin B, Strickler J, Gorniak J, Clark MA, Johnson PJ, Mitcho M, Shalloway D. Induction by E1A oncogene expression of cellular susceptibility to lysis by TNF. Nature 1987;330:581–3.
33. Lowe SW, Ruley HE. Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. Genes Dev 1993;7:535–45.
34. Okamoto K, Prives C. A role of cyclin G in the process of apoptosis. Oncogene 1999;18:4606–15.
35. Shaulian E, Karin M. Stress-induced JNK activation is independent of Gadd45 induction. J Biol Chem 1999;274:29595–8.
36. Wang X, Gorospe M, Holbrook NJ. gadd45 is not required for activation of c-Jun N-terminal kinase or p38 during acute stress. J Biol Chem 1999;274:29599–602.
37. David G, Alland L, Hong SH, Wong CW, DePinho RA, Dejean A. Histone deacetylase associated with mSin3A mediates repression by the acute promyelocytic leukemia-associated PLZF protein. Oncogene 1998;16:2549–56.
38. Dhordain P, Lin RJ, Quief S, Lantonine D, Kerckaert JP, Evans RM, Albabli O. The LAZ3 (BCL-6) oncoprotein recruits a SMRT/mSIN3A/histone deacetylase containing complex to mediate transcriptional repression. Nucleic Acids Res 1998;26:4645–51.
39. Lutterbach B, Westendorf JJ, Linggi B, Isaac S, Seto E, Hiebert SW. A mechanism of repression by acute myeloid leukemia-1, the target of multiple chromosomal translocations in acute leukemia. J Biol Chem 2000;275:651–6.
40. Ayer DE, Lawrence QA, Eisenman RN. Mad-Max transcriptional repression is mediated by ternary complex formation with mammalian homologs of yeast repressor Sin3. Cell 1995;80:767–76.

Appendix 2

ANTICANCER RESEARCH *23*: xxx-xxx (2003)

No: 4374 - Z
Please mark the appropriate section for this paper
☐ Experimental
☐ Clinical
☐ Epidemiological

Constitutive Expression of Rb Associated Protein 46 (RbAp46) Reverts Transformed Phenotypes of Breast Cancer Cells

TENG-FEI ZHANG[1], SHUI-QING YU[1], THOMAS F. DEUEL[2] and ZHAO-YI WANG[1]

[1]*Cancer Center, Creighton University Medical Center, 2500 California Plaza, Omaha, NE 68178;*
[2]*The Scripps Research Institute, 10550 N. Torrey Pine Rd., LaJolla, CA 92037, U.S.A.*

Abstract. *The retinoblastoma (Rb) suppressor associated protein 46 (RbAp46) is a subunit of chromatin modifying and remodeling complexes. Previously, we found that RbAp46 functions as a potent growth inhibitor. It is also a downstream effector of the Wilms' tumor suppressor, WT1. The findings that expression levels of WT1 were downregulated in breast cancer cell lines and in subsets of primary breast tumors led us to investigate the possible role of RbAp46 in breast cancer tumorigenesis. Here, we found that RbAp46 expression levels were decreased in five established breast cancer cell lines compared to a normal mammary gland epithelial cell line. To investigate the effect of constitutive expression of RbAp46 on the transformed phenotypes of breast cancer cells, we established stable cell lines that constitutively express exogenous RbAp46 using three breast cancer cell lines, MCF-7, MDA-MB-231 and MDA-MB-436. We have found that RbAp46 expression suppressed colony formation of these breast cancer cells in soft-agar, and inhibited tumor formation of these cells in nude mice. Our data demonstrated that constitutive RbAp46 expression suppresses the transformed phenotypes of breast cancer cells, and suggested that dysregulation of RbAp46 expression may contribute to breast cancer tumorigenesis.*

RbAp46 is a component of the mSin3 histone deacetylase (HDAC) complex (1), which is involved in transcription repression mediated by growth-related transcription factors such as the acute promyelocytic leukemia-associated PLZF protein (2), LAZ-3 (Bcl-6) oncogene (3), the acute myeloid leukemia-1 (AML-1) protein (4), and Mad-Max, members of oncogene Myc family (5). RbAp46 is also known as the histone acetyltransferase (hat) type B subunit 2, in which RbAp46 binds selectively to H2A and H4 histones and greatly stimulates hat activity (6). Recently, RbAp46 was found as a subunit of NuRD, a multi-subunit complex with chromosome-remodeling activity (7). Thus, RbAp46 may function as a core-histone-binding subunit that targets chromatin assembly factors, chromatin-remodeling factors, and histone acetyltransferase and deacetylase to their histone substrates in chromatin.

Previously, we isolated RbAp46 as a downstream target gene of the Wilms' tumor suppressor gene product, WT1 (8). We have further found that overexpression of RbAp46 inhibits transformed phenotype of adenovirus-transformed human embryonic kidney 293 cells through sensitizing cells to apoptosis (9). These data suggested that RbAp46 is an important downstream effector of the WT1 pathway and may also function as a tumor suppressor for certain types of human cancer. Recently, others and our laboratory have discovered that RbAp46 specifically interacts with the BRCT domain of breast cancer and ovarian cancer susceptibility gene BRCA1 and modulates its transcriptional transactivation activity (10,11), suggesting a role of RbAp46 in breast cancer tumorigenesis.

Previously, Siberstein *et al.* (12) found that WT1 is expressed in myoepithelial cells of the normal breast duct. However, reduced WT1 staining was seen in 60% of breast tumors, suggesting a correlation between the loss of WT1 expression and mammary carcinogenesis. Laux *et al.* (13) and Huang *et al.* (14) used methylation-sensitive restriction endonucleases to demonstrate aberrant methylation of the WT1 promoter and first intron in breast cancer samples, further suggesting that dysregulation of WT1 is involved in the genesis of breast cancer. These results lead us speculate that appropriate expression of RbAp46, as an important downstream effector of WT1 system, may be required for normal growth of mammary gland cells and dysregulation of RbAp46 may contribute to tumorigenesis of breast cancer.

Here, we showed that RbAp46 expression is downregulated in several established breast cancer cell lines. We also found that constitutive expression of RbAp46 inhibits clonogenicity of MCF-7, MDA-MB-231 and MDA-MB-436 breast cancer cells in soft-agar and tumorigenecity of these cells in nude mice.

Correspondence to: Zhao-Yi Wang, Cancer Center, Criss II, Room 510, Creighton University Medical Center, 2500 California Plaza, Omaha, NE 68178, U.S.A. Tel: (402) 280-4355, Fax: (402) 280-3442, e-Mail: zywang@creighton.edu

Key Words: RbAp46, Tumor suppressor, breast cancer.

0250-7005/2003 $2.00+.40

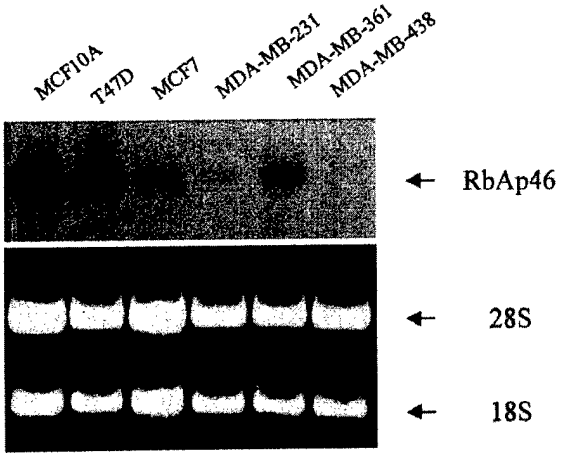


Figure 1. *Northern blot analysis of RbAp46 expression in normal mammary gland epithelial cells, MCF10A, and breast cancer cells, MCF-7, T47D, MDA-MB-231, MDA-MB-361 and MDA-MB-436 cells.*

Materials and Methods

Cell culture and DNA transfection. Cells were maintained in DMEM medium supplemented with 10% fetal calf serum at 37°C in a 10% CO2 atmosphere. Cells were plated at a density of $1x10^5$ cells per 60-mm dish and transfected 24 hours later with a RbAp46 expression vector containing RbAp46 cDNA fused with an influenza hemagglutinin (HA) epitope driven by the cytomagalovirus (CMV) promoter using the FuGene6 transfection reagent (Roche Molecular Biochemicals). Empty vector was transfected into cells to serve as a control. Forty-eight hours after transfection, the cells were replated and selected with 500 μg/ml of G418 (Gibco-BRL) for two weeks. The medium was changed every three days until colonies appeared. Individual clones were then isolated and expanded to confirm expression of RbAp46 by Western blot analysis. Three clones from each cell line were retained for further experiments. Three clones from cells transfected with the empty vector were analyzed as controls.

Northern blot and Western blot analysis. For Northern blot analysis, total cellular RNA was isolated using Trizol (Gibco/BRL Life Technologies, Inc.) according to the manufacturer's instruction. Ten μg of total RNA were separated by electrophoresis on a 1. 2 % formamide/formaldehyde gel and blotted onto a nylon membrane (Hybond-N, Amersham Pharmacia Biotech). The blots were prehybridized for 1 hour and hybridized for 2 hours in Quick-Hybridization solution (Amersham Pharmacia Biotech) at 65°C. The probes included a 1.9 kb DNA fragment containing RbAp46 cDNA. The DNA probes were labeled with ^{32}P dCTP and a Rediprime II DNA labeling kit (Amersham Pharmacia Biotech). The blots were washed twice with 2 x SSC and 0.1% SDS for 15 minutes at room temperature, and twice with 0.1 x SSC and 0.1% SDS for 15 minutes at 55°C. Blots were autoradiographed using intensifying screens at -70°C overnight.

For Western blot analysis, cells were washed with PBS and lysed with lysis buffer (50 mM Tris-HCl pH8.0, 150 mM NaCl, 0.25 mM EDTA, pH8.0, 0.1% SDS, 1% Triton X-100, 50 mM NaF, 1mM PMSF and 10 μg/ml aprotinin and leupeptin). After adjusted to the same total protein content, cell lysates were analyzed by Western blot analysis. The cell lysates were boiled for 5 minutes in SDS gel loading buffer and separated on a 10% SDS-PAGE gel. After electrophoresis, the proteins were transferred to a PVDF membrane (Millipore). The membranes were probed with Anti-HA-tag rat monoclonal antibody (3F10) purchased from Roche Molecular Biochemicals, and HRP-conjugated secondary antibodies. Western blots were visualized with enhanced chemiluminescence (ECL) detection reagents (Amersham Pharmacia Biotech). The same membranes were stripped and reprobed with antibody against β-actin to confirm equal loading.

Soft-agar colony formation assay and tumor formation in nude mice. To determine anchorage-independent growth in soft-agar, two hundred cells from each of the stable cell lines were suspended in 3 ml of 3.5% (W/V) agar containing 1 x DMEM/F12 medium plus 30% fetal calf serum. The cells were then overlaid onto a 0.7% (W/V) agar containing 1 x DMEM/F12 medium plus 30% fetal calf serum in five replica dishes. After three weeks, colonies with ≥ 20 cells were scored as positive using an inverted microscope.

Tumor formation was assayed in 4 ~ 5 week old female athymic nude mice (strain Ncr nu/nu; Sprague-Dawley, Indianapolis, IN) by subcutaneously injecting 2 x 10^6 cells suspended in 200 μl of sterile phosphate-buffered saline. Groups of three mice were injected at two sites per mouse using three individual clones from each transfectant. Animals were monitored daily, beginning at two week after the injections, to measure the rate of tumor growth. Relative tumor volume was estimated by multiplying the longest diameter by the square of the shortest diameter and dividing by two.

Results

RbAp46 expression is downregulated in established breast cancer cell lines. Recent experiments suggested the possibility that WT1 pathway may be involved in breast cancer tumorigenesis (7-9). As a functional effector of the WT1 pathway, RbAp46 is likely important for normal cell growth of in mammary gland, while dysregulation of RbAp46 may be involved in development of breast cancer. To test this possibility, we used Northern blot analysis to determine the levels of RbAp46 expression in a normal mammary epithelial cell line MCF10A, and established breast cancer cell lines with varying degrees of malignancy. We found that RbAp46 is highly expressed in MCF10A cells, is expressed at intermediate levels in MCF-7, T47D, and MDA-MB-361 breast cancer cells, and is undetectable in MDA-MB-231 and MDA-MB-436 cells derived from highly malignant breast cancers (Figure 1). This data demonstrates that RbAp46 expression is downregulated in breast cancer cells.

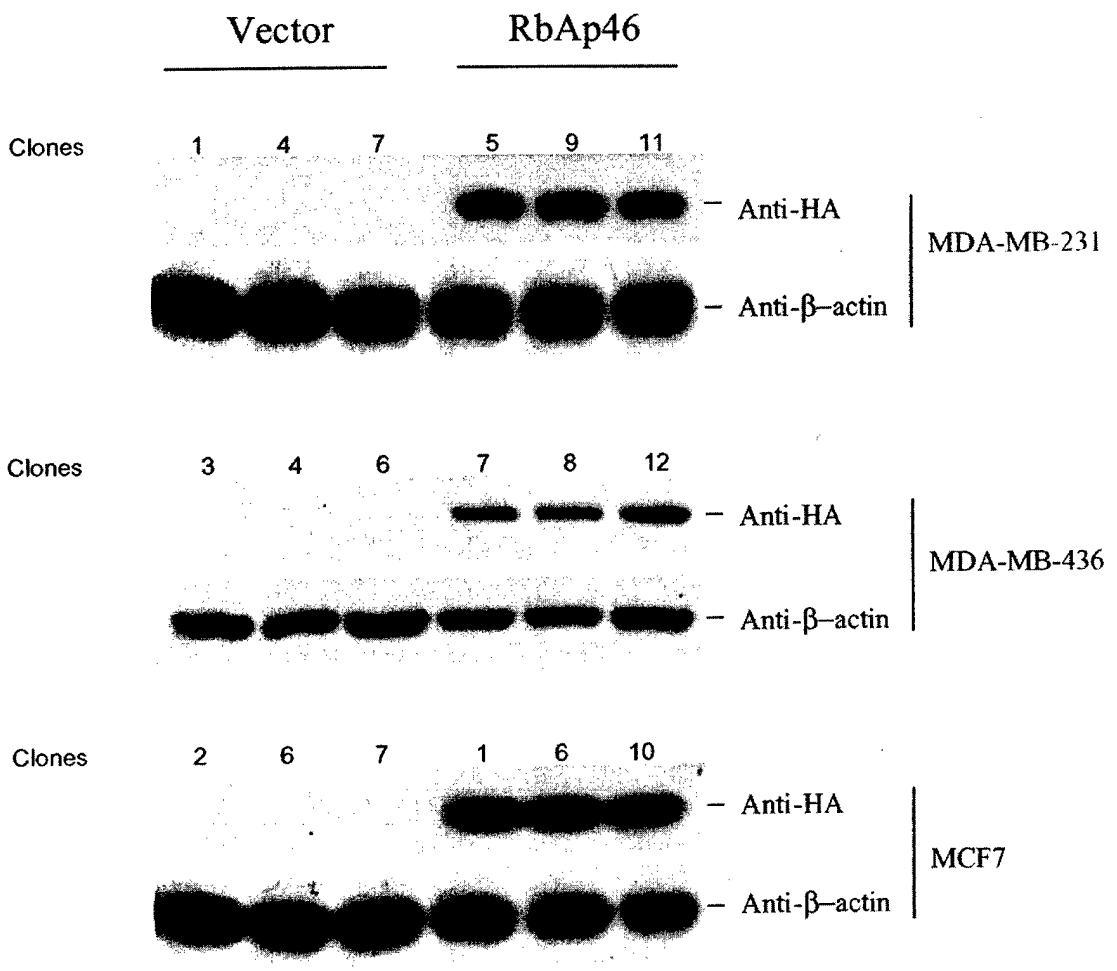


Figure 2. *Western blot analysis of exogenous RbAp46 in the control cells transfected with empty vector, and RbAp46-transfected MCF-7, MDA-MB-231 and MDA-MB-436 cells.*

Table I. *Soft-agar colony formation of RBAP46-expressing breast cancer cell.*

MDA-MB-231		MDA-MB-436		MCF7	
clones	colonies	clones	colonies	clones	colonies
Vector (1)	66±3.3	Vector (3)	78±4.3	Vector (2)	112±1.3
Vector (4)	108±4.1	Vector (4)	84±5.6	Vector (6)	118±2.6
Vector (7)	84±2.4	Vector (6)	69±3.7	Vector (7)	110±1.7
RbAp46 (5)	8±0.8	RbAp46 (7)	23±1.8	RbAp46 (1)	8±1.8
RbAp46 (9)	8±0.8	RbAp46 (9)	19±1.5	RbAp46 (9)	4±0.5
RbAp46 (11)	14±1.1	RbAp46 (12)	20±2.2	RbAp46 (10)	12±1.2

Number of colonies with ≥20 cells per dish formed in soft-agar colony formation assay. Five replicates of each transfectant were cultured for three weeks and colonies were counted. The difference in the number of colonies is statistically significant for all pairings ($p<0.05$).

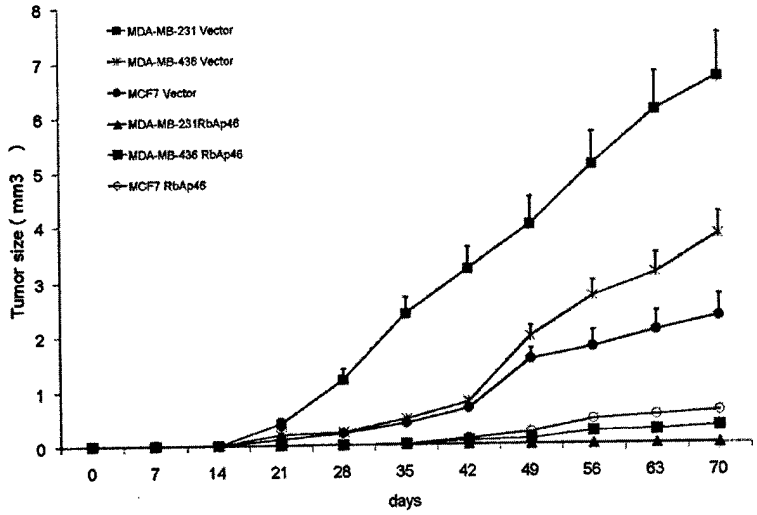


Figure 3. *In vivo tumor formation assay of the control cells and RbAp46-expressing cells in nude mice. The mice were subcutaneously injected with 1 x 10^7 cells at each site from each tranfectant. Relative tumor volume was estimated by multiplying the longest diameter by the square of the shortest diameter and dividing by two. The average of the relative volumes of tumor developing at multiple sites was calculated for each cell line and plotted against the number of week after inoculation.*

Constitutive expression of RbAp46 suppresses clonogenicity of MCF-7, MDA-MB-231 and MDA-MB-436 breast cancer cells in soft-agar. To investigate the effects of constitutive RbAp46 expression on transformed phenotypes of breast cancer cells, we transfected an expression vector containing RbAp46 cDNA tagged with an influenza hemagglutinin (HA) epitope into three established breast cancer cell lines, MCF-7, MDA-MB-231 and MDA-MB-436. The individual cell clones were selected, isolated, and expanded. We established a number of clonal cell lines that constitutively expressed exogenous RbAp46. Three clones from each of the breast cancer cell lines are described here. We also generated three control cell lines transfected with the empty expression vector from each of breast cancer cell lines. Western blot analysis using antibody against HA tag confirmed that exogenous RbAp46 (~50 kDa) protein was readily expressed in these cloned cells (Figure 2).

We then explored the effects of RbAp46 expression on the tumorigenic activity of MCF-7, MDA-MB-231, and MDA-MB-436 cells. We determined the ability of these established cell clones to form colonies in soft-agar. Two hundred cells from each established cell clone were seeded in 60-mm dishes in replicates of five dishes. After incubation for three weeks, colonies with more than 20 cells were counted and scored as anchorage-independent. The RbAp46-expressing breast cancer cells readily form fewer colonies in soft-agar than control cells transfected with the empty vector (Table I). In MCF-7 and MDA-MB-231 cells, RbAp46 expression lead to 10% fewer colonies than control cells. In MDA-MB-436 cells, RbAp46 expression also reduced the number of colonies about 25%. Analysis of the data using a student's *t*-test showed that the reduction in the number of colonies formed by RbAp46-expressing cells compared with control cells was statistically significant ($p<0.05$). In addition to the decreased number of colonies, we noted a significant decrease in the average size of colonies formed by breast cancer cells expressing exogenous RbAp46 (data not shown). These results indicate that constitutive RbAp46 expression inhibits anchorage-independent growth of MCF-7, MDA-MB-231, and MDA-MB-436 breast cancer cells.

Constitutive expression of RbAp46 inhibits tumorigenecity of MCF-7, MDA-MB-23, and MDA-MB-436 breast cancer cells in nude mice. We further examined the effect of RbAp46 expression on the tumorigenicity of MCF-7, MDA-MB-231 and MDA-MB-436 cells in nude mice. Groups of three mice were subcutaneously injected at two sites per mouse using three individual clones from each established breast cancer cell lines. Four weeks after injection, tumors were detected at all injected sites of control cells transfected with empty vector. Tumors were totally absent in sites injected with RbAp46-expressing MDA-MB-231 cells. However, very small nodules were later detected in 1/3 sites injected with RbAp46-expressing MDA-MB-436 cells, and in 1/2 of sites

with RbAp46-expressing MCF-7 cell. The appearance of tumors derived from the RbAp46-expressing breast cancer cells was delayed several weeks compared to tumors derived from control cells (Figure 3). More importantly, the growth rates of tumors that developed from RbAp46-expressing cells were significantly decreased compared to those of the control cells. Figure 3 shows that tumor growth was markedly inhibited in mice injected with RbAp46-expressing breast cancer cells. This data indicates that RbAp46 strongly suppresses the tumorigenic abilities of MCF-7, MDA-MB-231, and MDA-MB-436 breast cancer cells in nude mice.

Discussion and Conclusion

We used MCF-7, MDA-MB-231 and MDA-MB-436 cells as model systems to study the effect of RbAp46 expression on the transformed phenotype of breast cancer cells. We found that expression of RbAp46 is down-regulated in established breast cancer cell lines. Our results from both *in vitro* and *in vivo* assays demonstrated that restoration and/ or constitutive expression of RbAp46 expression strongly inhibit the tumorigenic phenotypes of MCF-7, MDA-MB-231, and MDA-MB-436 breast cancer cells.

Recently, we found that RbAp46 is upregulated more than ten-fold in WT1-expressing cells and RbAp46 (8). It has been reported that WT1 suppresses tumorigenicity of Wilms' tumor cells, and osteosarcoma cells (15,16). WT1 also inhibits growth and tumorigenicity of ras-transformed NIH 3T3 fibroblasts. Like WT1, the growth of several types of tumor cells was greatly inhibited by overexpression of RbAp46 (8,9). In this report, we further demonstrate that RbAp46 is also able to suppress transformed phenotype of MCF-7, MDA-MB-231, and MDA-MB-436 breast cancer cells. Taken together, these results are consistent with the hypothesis that RbAp46 mediates some of the tumor suppressor functions of WT1.

The molecular mechanism by which RbAp46 reverts transformed phenotypes of MCF-7, MDA-MB-231 and MDA-MB-436 cells are not clear. Recently, we found RbAp46 sensitizes human embryonic kidney 293 cells to apoptosis induced by serum deprivation (8,9). However, in RbAp46 expressing breast cancer cells, we did not observe any change of growth rate and signs of cell apoptosis, suggesting that in breast cancer cells, RbAp46 may function in a different mechanism. Recently, it was reported that forced expression of histone deacetylase 5 inhibited tumor cell growth by regulating growth-related genes (18). One hypothesis is that RbAp46 may modulate the activity of histone deacetylase or acetyl transferase to regulate growth-related genes, which will suppress the transformed phentypes of breast cancer cells.

It also has been reported that the Sno protein, a component of macromolecular complex containing co-repressor N-CoR/SMRT, mSin3 and histone deacetylase, functions as a tumor suppressor in mice (19). Heterozygous (sno (+/-)) mice developed spontaneous lymphomas and showed an increased level of tumor formation relative to wild-type mice when exposed to a chemical carcinogen (19). Together with our results, these observations suggest that the histone acetylation/deacetylation system plays an important role in the regulation of cell growth, differentiation and apoptosis. Dysregulation of this system may lead to altered expression of growth-related genes and ultimately to uncontrolled cell growth characteristic of tumorigenesis.

Acknowledgements

This work was supported by National Institute of Health Grant, CA 84328 (Z.Y. Wang), and CA84400 (T.F. Deuel); American Cancer Society grant, RPG-00272-02-MGO and Department of Defense Breast Cancer Research Program DAMD17-00-10156 (Z-Y Wang) and DAMD-17-00-1-0151 (T.F. Deuel).

References

1 Zhang Y, Iratni R, Erdjument-Bromage H, Tempst P and Reinberg D: Histone deacetylases and SAP18, a novel polypeptide, are components of a human Sin3 complex. Cell *89*: 357-364, 1997.

2 David G, Alland L, Hong SH, Wong CW, DePinho RA and Dejean A: Histone deacetylase associated with mSin3A mediates repression by the acute promyelocytic leukemia-associated PLZF protein. Oncogene *16*: 2549-2556, 1998.

3 Dhordain P, Lin RJ, Quief S, Lantonine D, Kerckaert JP, Evans RM and Albabli O: The LAZ3 (BCL-6) oncoprotein recruits a SMRT/mSIN3A/histone deacetylase containing complex to mediate transcriptional repression. Nucleic Acids Res *26*: 4645-4651, 1998.

4 Lutterbach B, Westendorf JJ, Linggi B, Isaac S, Seto E and Hiebert SW: A mechanism of repression by acute myeloid leukemia-1, the target of multiple chromosomal translocations in acute leukemia. J Biol Chem *274*: 651-656, 2000.

5 Ayer DE, Lawrence QA and Eisenman RN: Mad-Max transcriptional repression is mediated by ternary complex formation with mammalian homologs of yeast repressor Sin3. Cell *80*: 767-776, 1995.

6 Verreault A, Kaufman PD, Kobayashi RA and Stillman B: Nucleosome assembly by a complex of CAF-1 and acetylated histones H3/H4. Cell *87*: 95-104, 1996.

7 Zhang Y, Ng HH, Erdjument-Bromage H, Tempst P, Bird A and Reinberg D: Analysis of the NuRD subunits reveals a histone deacetylase core complex and a connection with DNA methylation. Genes Dev *13*: 1924-1935, 1999.

8 Guan L-S, Rauchman M and Wang Z-Y: Induction of Rb-associated protein (RbAp46) by Wilms' tumor suppressor WT1 mediates growth inhibition. J Biol Chem *273*: 27047-50, 1998.

9 Guan L-S, Li G-C, Liu L-Q and Wang Z-Y: Rb-associated protein 46 (RbAp46) suppresses the tumorigenicity of adenovirus-transformed human embryonic kidney 293 cells. Int J Cancer *93*: 333-338, 2001.

10 Yarden RI and Brody LC: BRCA1 interacts with components of the histone deacetylase complex. Proc Natl Acad Sci USA

96: 4983-8, 1999.

11 Chen G-C, Guan L-S, Yu H-J, Li G-C, Choi K-H and Wang Z-Y: Rb-associated protein 46 (RbAp46) inhibits transcriptional transactivation mediated by BRCA1. Biochem Biophys Res Commun *284*: 507-14, 2001.

12 Silberstein GB, Van Horn K, Strickland P, Roberts CT Jr and Daniel CW: Altered expression of the WT1 wilms tumor suppressor gene in human breast cancer. Proc Natl Acad Sci USA *94*: 8132-8137, 1997.

13 Laux DE, Curran EM, Welshons WV, Lubahn DB and Huang TH: Hypermethylation of the Wilms' tumor suppressor gene CpG island in human breast carcinomas. Breast Cancer Res Treat *56*: 35-43, 1999.

14 Huang T H, Laux DE, Hamlin BC, Tran P, Tran H and Lubahn DB: Identification of DNA methylation markers for human breast carcinomas using the methylation-sensitive restriction fingerprinting technique. Cancer Res *57*: 1030-1034, 1997.

15 Haber DA, Park S, Maheswaran S, Englert C, Re GG Hazen-Martin DJ, Sens DA and Garvin J: WT1-mediated growth suppression of Wilms tumor cells expressing a WT1 splicing variant. Science *262*: 2057-9, 1993.

16 Englert C, Hou X-Y, Maheswaran S, Bennett P, Ngwu C Re GG, Garvin AJ, Rosner MR and Haber DA: WT1 suppresses synthesis of the epidermal growth factor receptor and induces apoptosis. EMBO J *14*: 4662-75, 1995.

17 Luo XN, Reddy JC, Yeyati PL, Idris AH, Hosono S, Harber DA, Licht JD, and Atweh GF: The tumor suppressor gene WT1 inhibits ras-mediated transformation. Oncogene *11*: 743-50, 1995.

18 Huang Y, Tan M, Gosink M, Wang KK and Sun Y: Histone deacetylase 5 is not a p53 target gene, but its overexpression inhibits tumor cell growth and induces apoptosis. Cancer Res *62*: 2913-2922 (2002).

19 Shinagawa T, Dong HD, Xu M, Maekawa T and Ishii S: The sno gene, which encodes a component of the histone deacetylase complex, acts as a tumor suppressor in mice. EMBO J *19*: 2280-22891 (2000).

Received June 16, 2003
Accepted July 2, 2003

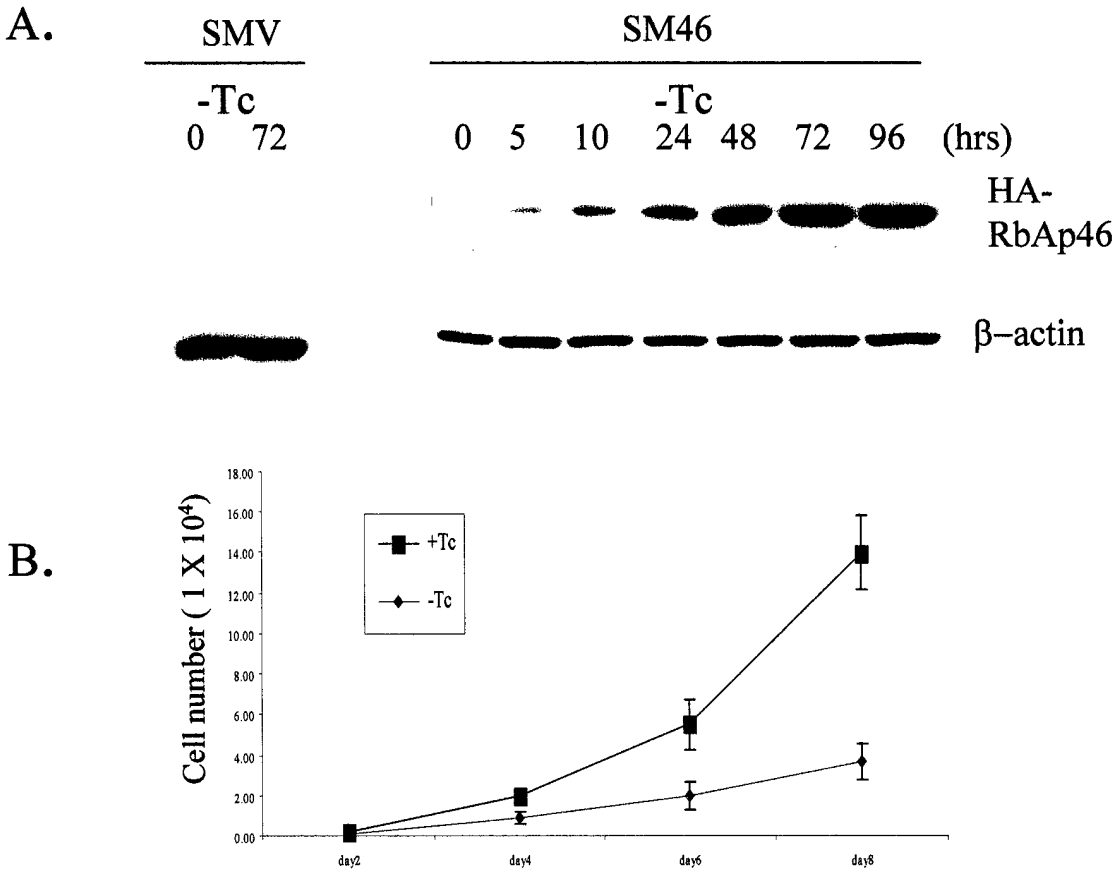
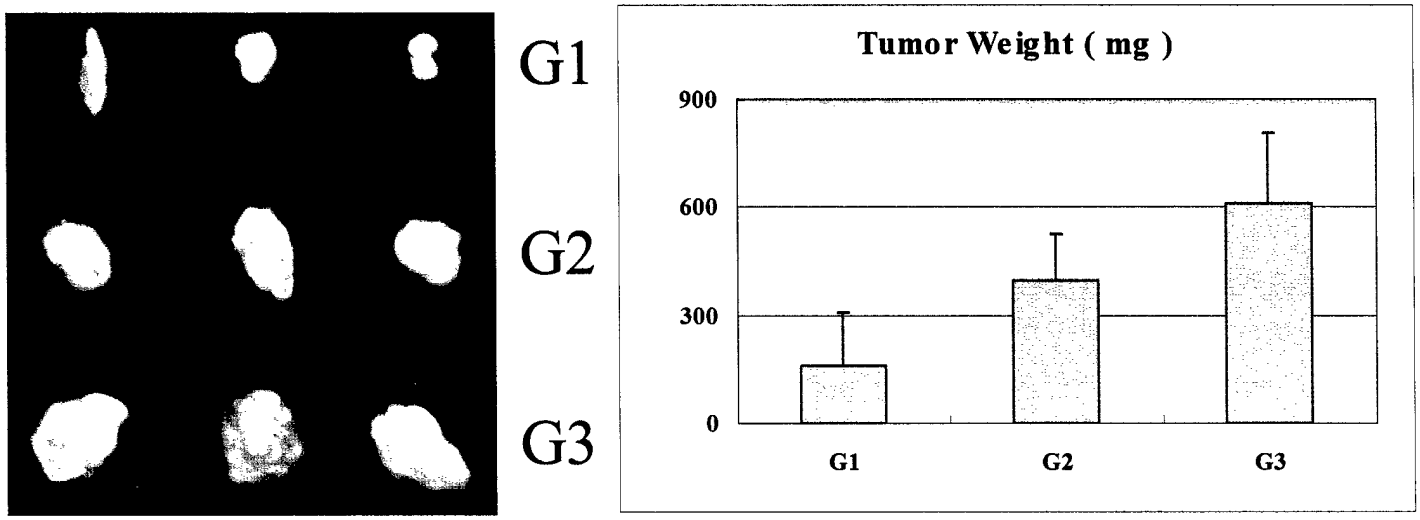


Figure 1. Inducible expression of RbAp46 inhibits MCF7 cell growth. **A.** Inducible expression of RbAp46 in MCF7 cells. Western blot analysis of protein extracts prepared from cells infected with RbAp46 containing retrovirus (SM46) and cells infected with empty retrovirus (SMV) growing in the presence (+Tc) or absence (-Tc) of tetracycline for time periods indicated. Anti-HA tag antibody was used to probe the inducible expression of the HA-tagged RbAp46, showing a tightly regulated inducible expression of RbAp46. β-actin was also probed as a loading control. **B.** Reduction of the growth rate of SM46 cells in the absence of tetracycline. The cells were seeded at 1 X 10^4 cells/well and counted daily in the absence of tetracycline. Data shown represent the mean of three experiments with standard deviations.

A.



B.

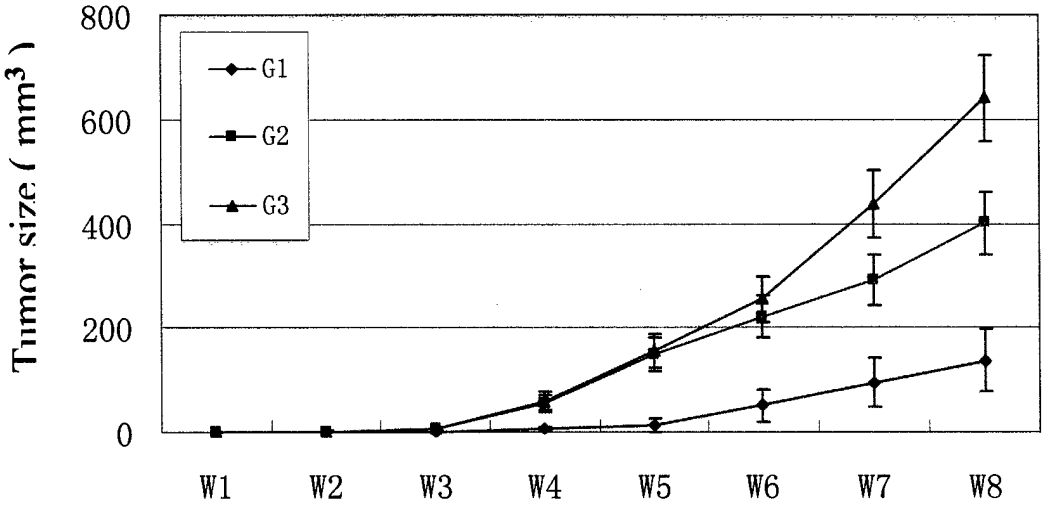
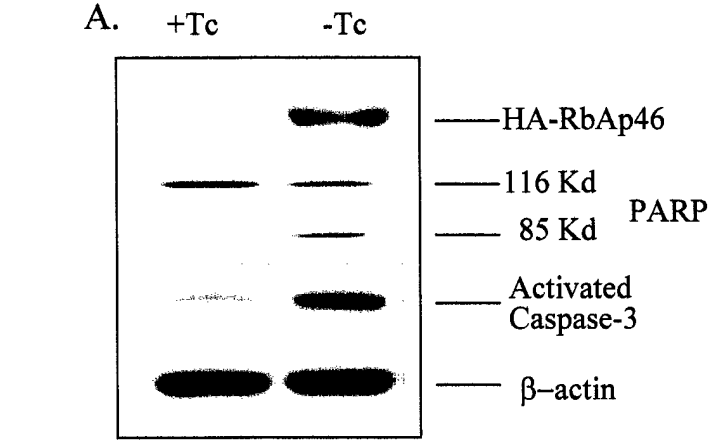
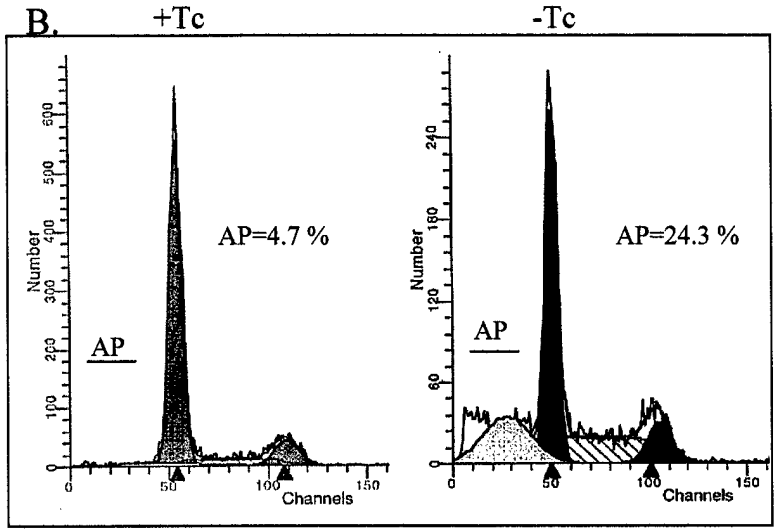


Figure. 2. Antitumoral activity of RbAp46 *in vivo*. Nude mice were injected subcutaneously with 5 X 10^5 MCF7 cells carrying inducible RbAp46 system. Mice were divided into three groups; group 1 (G1): mice fed with Tc-free water; group 2 (G2): mice fed with Tc-containing (2mg/ml) water for four weeks and then with Tc-free water; group 3 (G3): mice fed with Tc containing (2mg/ml) water. **A.** Representative tumors and tumor weight of three group mice. **B.** the tumors were measured once a week in two perpendicular diameters. Tumor volume was estimated by multiplying the longest diameter by the square of the shortest diameter and dividing by two.





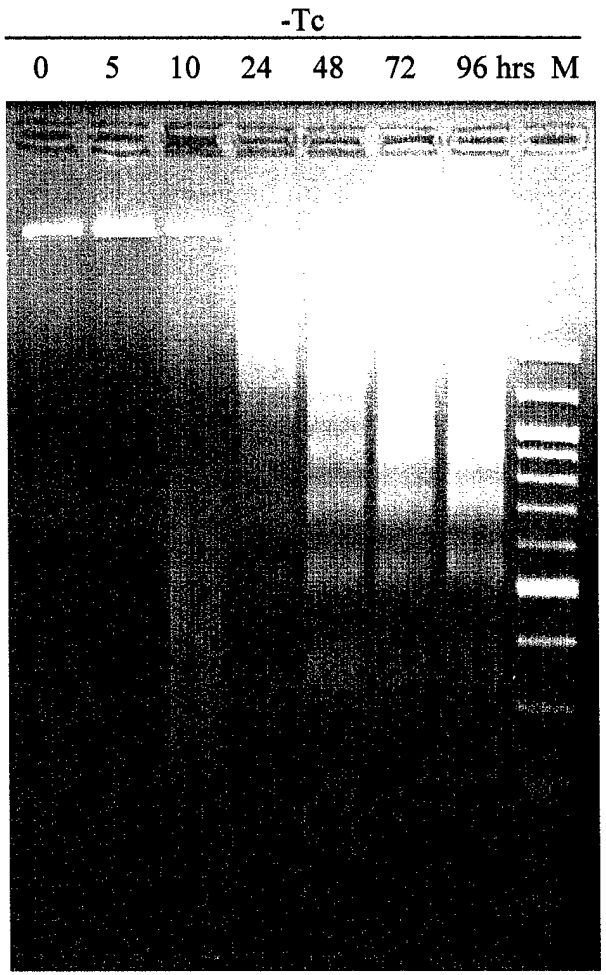


Figure 3. Induction of RbAp46 triggers apoptosis. **A**. Activation of caspase-3 and PARP cleavage in Sm46 cells after tetracycline withdrawal. Western blot analysis of proteins from cells with inducible expression of RbAp46 (24 hr after tetracycline withdrawal). PARP cleavage was assessed with an anti-PARP antibody which detected intact (116 kDa) and cleaved (85 kDa) products. Activated Caspase-3 (17 kDa) was detected by an anti-Caspase-3 antibody. **B**. FACS analysis of ST46 cells grown in the presence or absence of tetracycline. In the presence of tetracycline (+Tc) or twenty-four hours after tetracycline withdrawal (-Tc), cells were fixed, stained with propidium iodide, and analyzed by flow cytometry. The fraction of apoptotic cells is indicated as Ap. **C**. DNA fragmentation assay of cells with inducible expression of RbAp46.

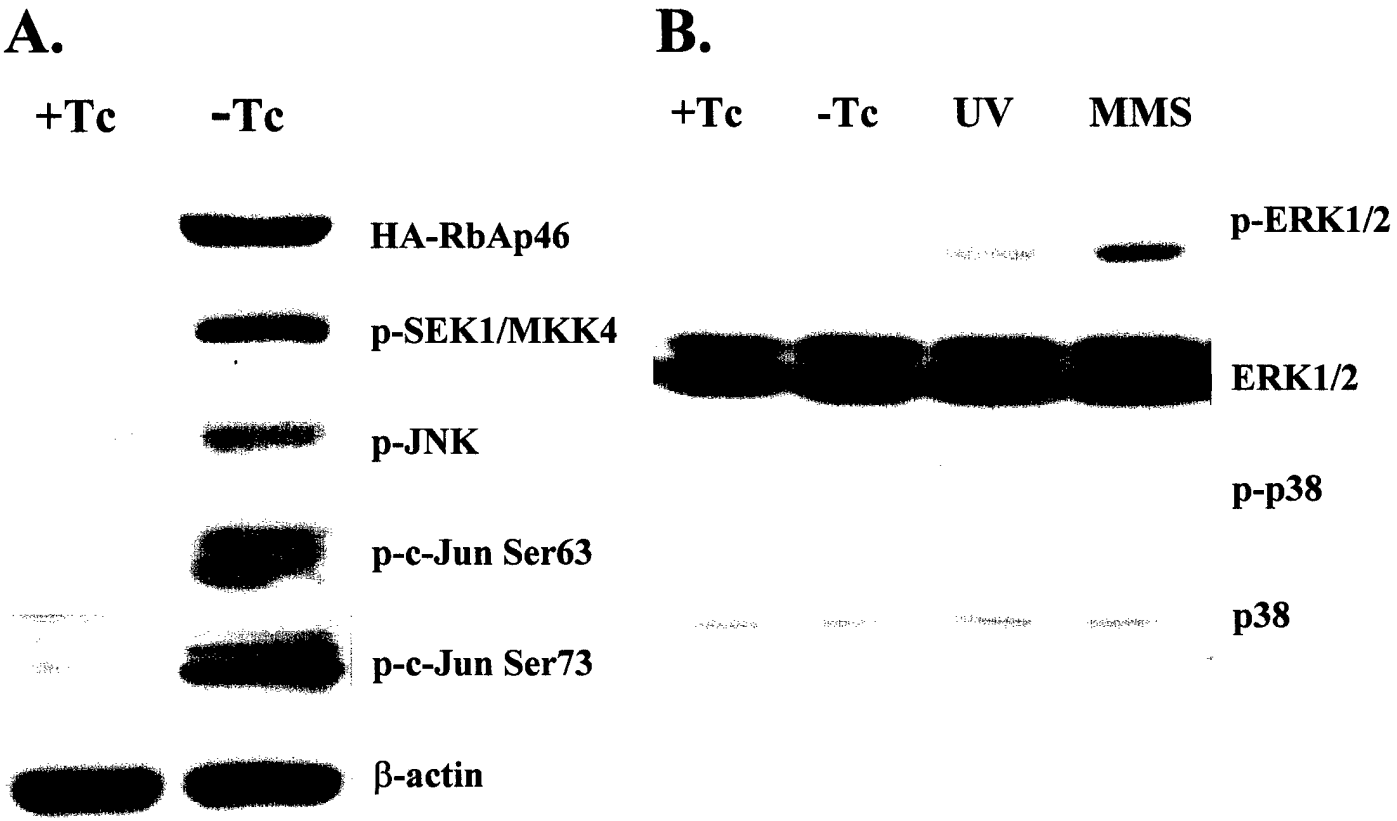


Figure 4. RbAp46 induces the activation of the JNK pathway. **A.** Phosphorylation of endogenous JNK pathway following induction of RbAp46. Western blot analysis, using anti-phosphorylation specific antibodies, of proteins from SM46 cells at 24 hours after withdrawal of tetracycline. Western blotting using anti-SEK1/MKK4, JNK and c-jun antibodies was also performed to demonstrate comparable amounts of the proteins (data not shown). **B**. Absence of RbAp46-mediated activation of other MAP kinase pathways. Western blot analysis of cellular lysates from SM46 cells grown in the presence (+Tc) or absence (24 hr) of tetracycline (-Tc), using antibodies against p42 and p44 MAP kinase (ERK1 and ERK2), p38 MAP kinase and their phosphorylated forms. For comparison, their activation is shown following treatment of cells with UV irradiation and MMS.

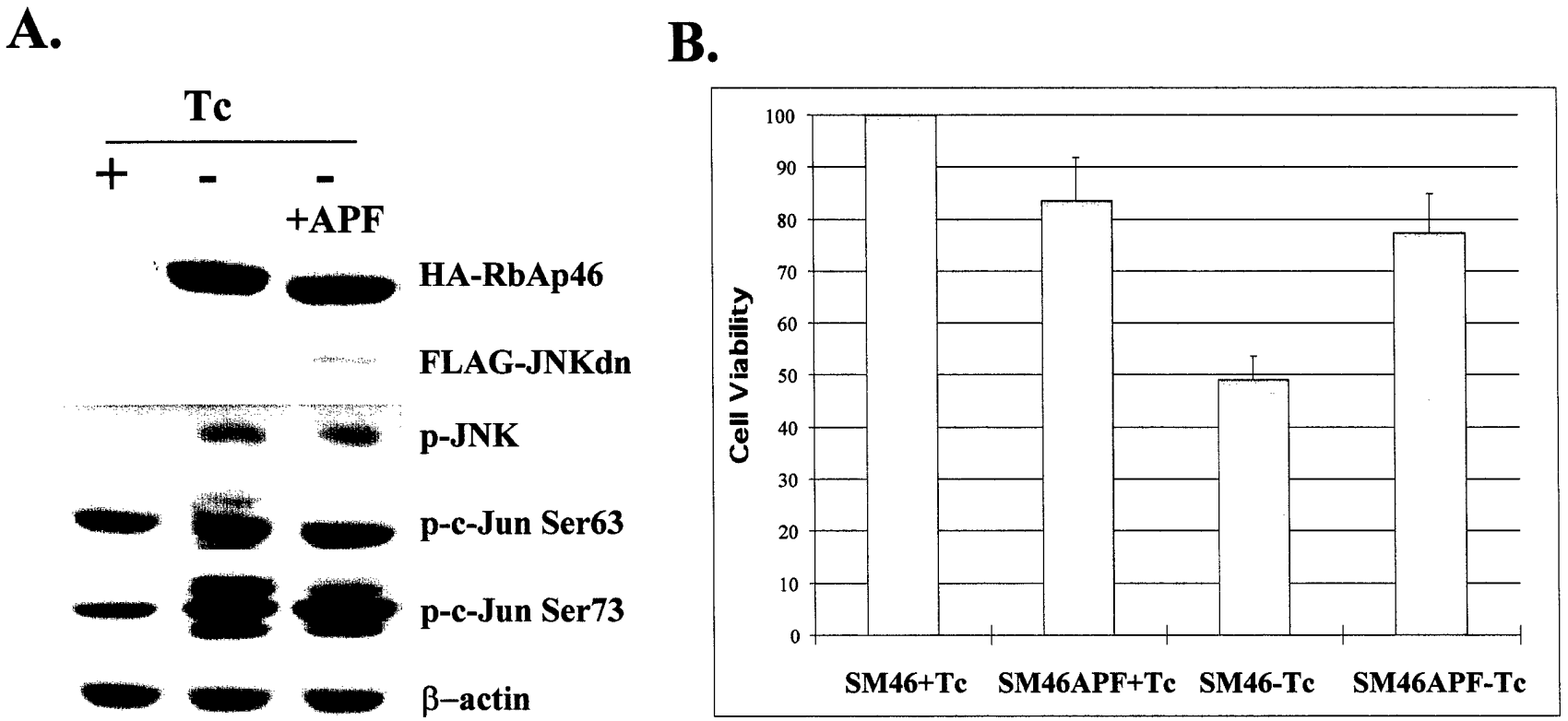


Figure 5. RbAp46-mediated apoptosis is reduced by a dominant-negative mutant of JNK1 (JNK1dn). **A**. Western blot analysis of the JNK activity following transfection of the JNK1dn in the presence or absence of tetracycline. Expression vector containing a Flag-tagged JNK1dn, pcDNA3 JNKAPF, was transiently transfected into SM46 cells and cells were grown in the presence or absence of tetracycline for 48 hrs. Cell lysates were analyzed by Western blot analysis with anti-Flag-tag, and anti-phosphorylation -specific c-Jun (Ser 63 and Ser 73) and JNK antibodies. **B.** Cell survival assays of SM46 cells transfected with or without JNK1dn in the presence or absence of tetracycline. SM46+Tc and SM46APF+Tc: SM46 cells transfected with empty vector or pcDNA3JNKAPF respectively in the presence of Tc; SM46-Tc and SM46APF-Tc: SM46 cells transfected with empty vector or pcDNA3JNKAPF respectively in the absence of Tc.

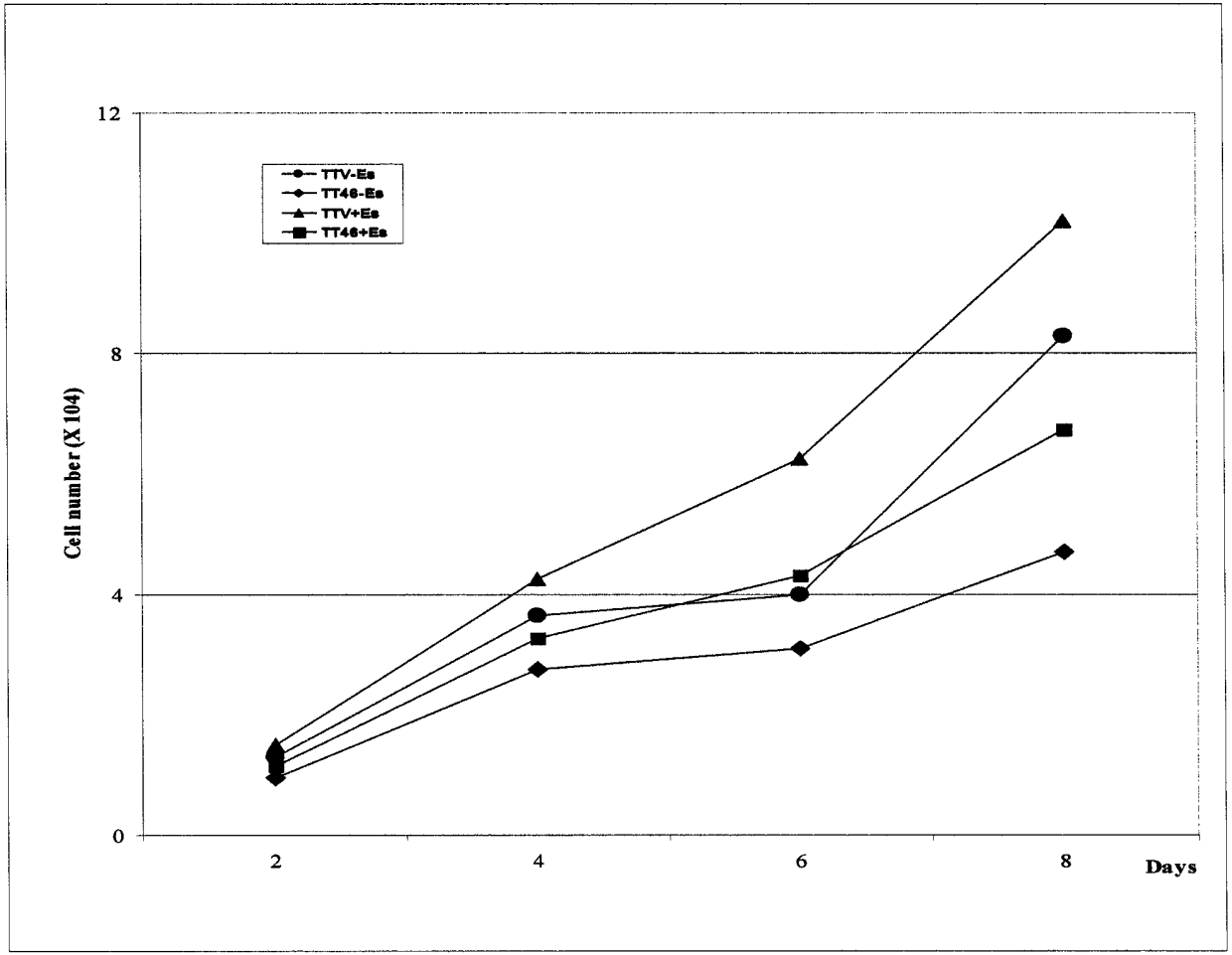


Figure. 6. RbAp46 inhibits MCF10AT3B cell growth-stimulated by estrogen. The MCF10AT3B cells that express exogenous RbAp46 (TT46) and control cells that were transfected with empty vector (TTV) were maintained in F12 medium plus 5% horse serum. The TT46 and TTV cells were washed three times with PBS and changed to medium containing 5% Charcol/Dextran treated serum at a density of 1 X 10^4 cells per well for twenty-four hours. One X 10^{-9} M of 17β-estrodial (E2) was added to the medium. After incubation with E2 for 2, 4, 6 and 8 days, cells were trypsinized and counted.

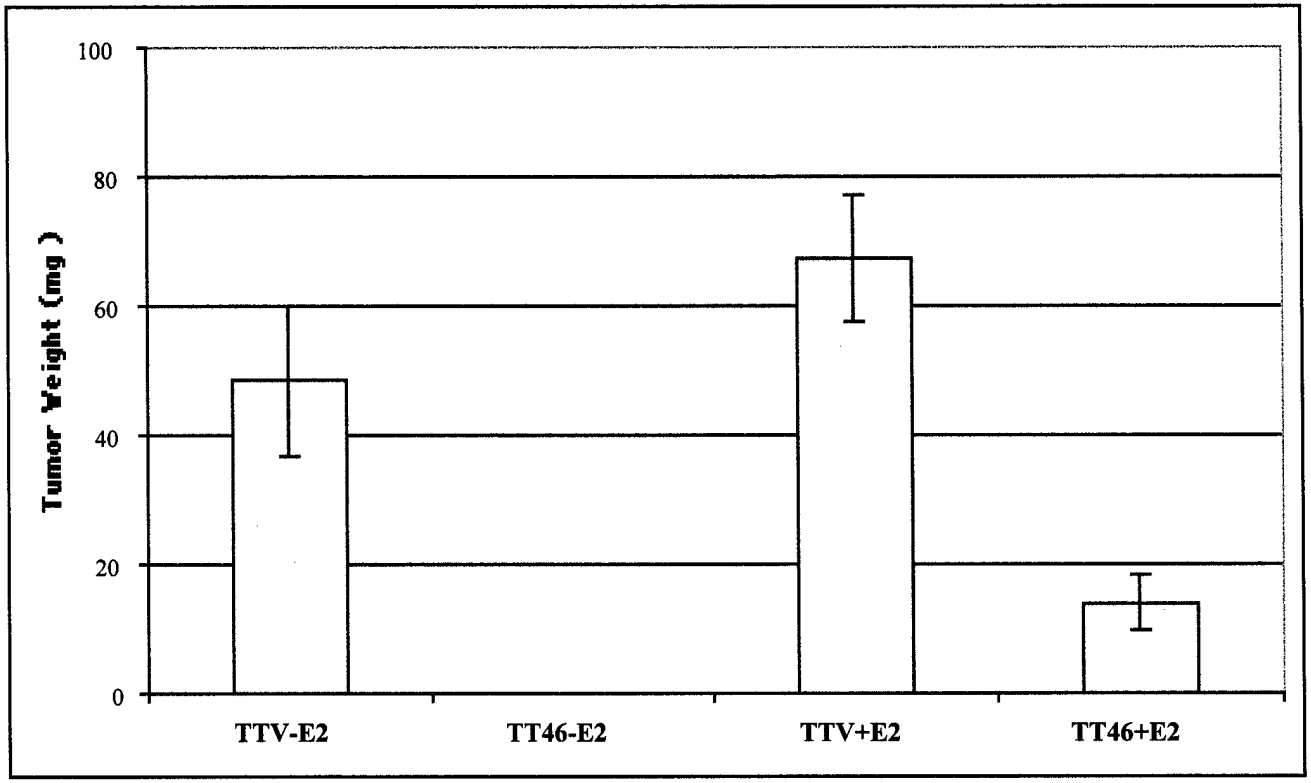


Figure 7. RbAp46 inhibits tumorigenicity of MCF10AT3B cells promoted by Estrogen *in vivo*. Control MCF10AT3B cells (TTV) and MCF10AT3B cells that express exogenous RbAp46 (TT46) was inoculated subcutaneously into the mammary fatpad of ovariectomized female nude mice seven days after subcutaneous implantation of 1.7mg/60-day release E2 (treated; 12 mice) or placebo (control; 12 mice) pellets.. All mice were sacrificed at 8 weeks after injection. Tumors from the injection sites were removed and weighed.