Rassf3 is responsible in part for resistance to mammary tumor development in *neu* transgenic mice

ISABELLE C. JACQUEMART, ALISON E.B. SPRINGS and WEN Y. CHEN

Department of Biological Sciences, Clemson University, Clemson, SC 29634;
Oncology Research Institute, Greenville Hospital System, Greenville, SC 29605, USA

Received August 20, 2008; Accepted October 21, 2008

DOI: 10.3892/ijo_00000177

Abstract. MMTV/*neu* transgenic mouse line is a well-documented model for studying HER2/neu-related breast cancer. Approximately 80% of these mice develop mammary tumors by 11 months of age, whereas a small percentage appears to have naturally acquired resistance to HER2/neu tumorigenesis. To identify factors responsible for tumor resistance in these transgenic mice, comparative genetic profiling was used to screen alterations in gene expression in the mammary gland. A novel gene, the RAS association domain (RalGDS/AF-6) family 3 *(Rassf3)*, which belongs to a family of RAS effectors and tumor suppressor genes, was identified. Data indicated 1) that *Rassf3* is overexpressed in mammary gland of tumor-resistant MMTV/*neu* mice compared to tumor-susceptible MMTV/*neu* littermates or non-transgenic mice, and 2) *Rassf3* is significantly up-regulated in *neu*-specific mouse mammary tumors compared to adjacent normal tissues. *In vitro* overexpression of RASSF3 inhibited cell proliferation in HER2/neu positive human and mouse breast cancer cell lines, possibly through induction of apoptosis. A novel MMTV/*Rassf3-neu* bi-transgenic mouse line, overexpressing *Rassf3* and *neu* genes in mammary glands, was established. Mammary tumor incidence in bi-transgenic mice was delayed compared to their MMTV/*neu*$^{+/-}$ littermates. These data suggest that *Rassf3* may influence mammary tumor incidence in MMTV/*neu* transgenic mice.

Correspondence to: Dr Wen Y. Chen, Oncology Research Institute, Greenville Hospital System, 900 West Faris Road, Greenville, SC 29605, USA
E-mail: wchen@ghs.org

Abbreviations: MG, mammary gland; T, tumor; MMTV, mouse mammary tumor virus; HER2, human epidermal growth factor receptor 2; RASSF, Ras association domain (RalGDS/AF-6) family; R-MG, mammary gland from tumor-resistant MMTV/*neu* transgenic mice; S-NMG, normal mammary gland from tumor-susceptible MMTV/*neu* transgenic mice

Key words: HER2/neu mammary tumorigenesis, MMTV/*neu* transgenic mice, tumor suppressor gene, *Rassf3*

Introduction

HER2/neu is overexpressed in 20-30% of primary human breast cancers (1,2) and is associated with a poor clinical outcome. The *HER2/neu* proto-oncogene encodes a transmembrane glycoprotein, termed ErbB2, which belongs to the human epidermal growth factor receptor family. Receptor activation and consequent signal transduction involves hetero- or homo-dimerization utilizing various ErbB receptor combinations. ErbB2 has no known ligand but forms active receptor complexes with co-receptors of the ErbB family. Excess ErbB2 receptors on the cell surface can lead to spontaneous formation of activated ErbB2 homodimers. The transforming potential of ErbB2 is closely related to its intrinsic tyrosine kinase activity (3). Upon dimerization, the protein kinase of each ErbB receptor monomer transphosphorylates a distinct set of tyrosine residues in the intracellular domain of its dimer partner (4) and the resulting phosphotyrosines serve as docking sites for proteins that contain conserved Src homology (SH) 2 domain or phosphotyrosine-binding domain (PTB). Two major downstream signaling pathways activated by the ErbB family are the RAS-RAF-MAPK pathway and the PI3K-AKT survival pathway (5), which lead to activation of transcription factors that mediate cellular responses.

There is considerable evidence that aberrant RAS activation and signaling may promote breast cancer development (6,7). RAS can be activated by persistent upstream signaling from EGFR, ErbB2 or estrogen receptors which are often over-expressed in breast cancer (7). RAS proteins belong to a large superfamily of small GTPases which are signal transduction proteins (20-25 kDa). The RAS family includes the most common H-RAS, K-RAS, and N-RAS proteins which can interact with a wide array of effectors through the *RAS* binding domain (RBD) to stimulate diverse cytoplasmic signaling pathways (8). Although RAS proteins are usually associated with loss of growth control and tumorigenic transformation (9), increasing evidence has demonstrated that RAS proteins have the ability to activate a variety of growth-inhibiting pathways including apoptosis and cell cycle arrest (10-13). These contrasting activities suggest that activation of oncogenes such as RAS can promote conflicting biological processes depending on the interaction with and activation of distinct effectors, cell type, context, signal nature and intensity and tissue origin (14). Four members of the Ras

association domain (RalGDS/AF-6) family, RASSF1, RASSF2, RASSF4 (AD037) and NORE1 (or RASSF5) have been identified as RAS effectors and tumor suppressors which may be involved in the pathways mediating RAS growth-inhibitory effect (15-18).

Overexpression of unactivated *neu* in the mammary epithelium results in appearance of focal mammary adenocarcinomas with a latency of 120-337 days that metastasize with high frequency to the lung. For line N#202, 50% of female virgin mice develop mammary tumors by 7 months (t_{50}=205 days) and ~80% by 11 months or older (3). The latency in tumor development in the MMTV/*neu* transgenic mice reflects the multistep nature of tumorigenesis and implies that additional genetic events beyond *neu* overexpression are required for mammary tissue transformation leading to tumor formation (3). To date, the majority of studies utilizing MMTV/*neu* transgenic mice have focused on the mechanism by which overexpression of HER2/*neu* oncogene in epithelial cells initiates tumorigenesis in the 80% of mice which develop tumors prior to 11 months of age.

In the present study, we used MMTV/*neu* virgin female transgenic mouse model but focused on the small proportion (~20%) of transgenic mice which do not develop mammary tumors by 11 months despite overexpression of the *neu* oncogene. These mice appear to have naturally acquired resistance to HER2/neu-tumorigenesis. This resistance to HER2/neu-tumorigenesis may not be complete resistance to tumor formation, but it is marked by a significant delay in tumor latency. We hypothesized that there may be one or more tumor suppressor genes that are activated or induced in the mammary glands of these tumor-resistant mice. Comparative microarray technology was used to identify gene expression alterations in the mammary gland of tumor-resistant MMTV/*neu* transgenic mice and to identify candidate genes which may be responsible for resistance to HER2/neu mammary tumor development. Among the genes differentially expressed, we identified a member of the RASSF family, mouse *Rassf3*, which shares ~40% and 44% identity at the amino acid level with hRASSF1A and hRASSF1C protein isoforms, respectively. We found that *Rassf3* is up-regulated in the mammary gland of 11-month-old tumor-resistant female MMTV/neu mice (R-MG) compared to tumor-susceptible mice (S-NMG) that bear tumors or age-matched non-transgenic mice (C). The *Rassf3* gene was therefore selected as a candidate gene for further characterization of its role in HER2/neu breast cancer through *in vitro* and *in vivo* studies.

Materials and methods

Animal models. The mice used in this study were of FVB background. The MMTV/*neu* transgenic mouse line (N#202) was purchased from The Jackson Laboratory (Bar Harbor, ME). This transgenic mouse line was developed by Guy *et al* (3) and is well characterized. Transgenic mice express inactivated rat *neu* oncogene under the transcriptional control of the MMTV promoter. The kinetics of tumor formation of this model reveal that ~50% of virgin females develop mammary tumors by 205 days (T_{50}=205) and ~80% by 337 days (3). A similar tumor rate was observed after expanding the N#202 line in our animal facility. According to the experimental design, virgin female MMTV/*neu* transgenic mice were monitored for tumor development. Age-matched female littermates were divided into 2 groups: tumor-susceptible mice (S) that were bearing mammary tumors and tumor-resistant mice (R) that had no palpable tumor and microscopic-free of precursor lesions (Fig. 1A). A distinction was made between tumor tissue (T) and normal mammary gland (S-NMG) excised from a tumor-bearing mouse (S). Non-transgenic FVB mice were used as control (C).

The MMTV/*Rassf3* transgenic mouse line was established for this study. Mouse (723 bp) *Rassf3-His Tag* cDNA was subcloned into pMSG expression vector (pMSG/*Rassf3-His Tag*) and fused to MMTV-LTR promoter. The 3,180 bp MMTV-*Rassf3-His Tag* cDNA fragment was microinjected into fertilized mouse single-cell embryos of FVB mice (The Jackson Laboratory), following standard protocol (19). The tyrosinase gene, responsible for the agouti fur color was co-injected with the transgene of interest to have a color marker for transgene presence. MMTV/*Rassf3* transgenic mice founder line #13 was identified as transgene-positive and expanded. Animals were maintained in barrier facilities and cared for in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines and policies. Both male and female MMTV/*Rassf3* transgenic mice presented no apparent overt phenotype up to currently 21 months of age. MMTV/*Rassf3* heterozygotic male mice were cross-bred with MMTV/*neu* homozygous females to produce a MMTV/*Rassf3-neu* bi-transgenic line. All offspring were heterozygous for MMTV/$neu^{+/-}$ transgene and 50% were positive for the MMTV/*Rassf3* transgene and had the agouti fur color.

Transgene incorporation was confirmed by PCR analysis using genomic tail DNA and primers specific for the transgene. The forward primer (5'-TGT TTG TGT CTG TTC GCC AT-3') was designed to anneal to a region on the MMTV promoter sequence whereas the reverse primer (5'-TTT GCA GAG TTC CAT CTG CAC-3') was designed to anneal to a region located in the middle of the *Rassf3* sequence. Transgene incorporation was checked for every colored mouse from F1, F2 and F3 generations as well as for certain white littermate mice as negative control. Virgin female MMTV/*Rassf3-neu* bi-transgenic mice and virgin female MMTV/$neu^{+/-}$ littermates were checked weekly for the development of mammary tumors. Kaplan-Meier survival curves were computed and statistical differences were calculated using the Log-rank test.

Affymetrix microarray. Mammary gland tissues were dissected from 11-month-old mice (n=3 per group) (S-NMG, R-MG and C). For tumor-susceptible mice (S), typically, a piece of normal mammary gland was collected from the opposite site of the tumor-bearing gland and named 'S-NMG'. S-NMG samples were not histologically confirmed but selected by a careful visual examination under dissecting microscope to remove all potential lesions from the collected samples. Only a piece of the whole mammary gland was used for RNA or protein extractions. Total RNA was extracted and pooled per group to control for individual variations between mice. RNA samples were purified with Qiagen RNeasy cleanup procedure following the manufacturer's instructions (Qiagen, Valencia, CA). The purity and quantity of each sample was assessed on

an Agilent Technologies bioanalyzer and the RNA 6000 nano lab chip, following the manufacturer's instructions (Agilent Technologies, Santa Clara, CA). Keck Affymetrix Resource Group at Yale University (http://info.med.yale.edu/wmkeck/affymetrix) carried out sample processing (cDNA and cRNA preparation), array hybridization and array data analysis.

The GeneChip mouse expression MOE430A (Affymetrix) was employed for analysis and provided ~22,000 probe sets representing transcripts and variants from >14,000 well-characterized mouse genes. The Affymetrix microarray 5.0 (MAS) software was used to inspect hybridization artifacts and to detect changes in gene expression. Microsoft Excel and GeneSpring GX (Agilent Technologies) software programs were used for further data analysis. The microarray analysis was repeated with new total RNA samples prepared from a second set of mice in order to obtain two sets of data. Despite careful sample preparation, the two Affymetrix experiments conducted at different times through commercial source did not show significant overlap. There was a substantial overlapping in down-regulated genes as compared to that of up-regulated genes. We speculate that the large variation between the two microarray results may be due to the fact that some of the mammary gland tissues from tumor-resistant mice were in various pre-tumor stages microscopically due to the presence of *neu* oncogene. Nonetheless, based upon the literature review, we selected *Rassf3* as the target for this study. Table I presents the first Affymetrix experiment, which indicated *Rassf3* expression difference.

RNA isolation and RT-PCR analyses. Tissues were homogenized using a Polytron PT1200 motorized homogenizer (Polytron; Bad Wildbad, Germany) and RNA was isolated by TRIzol (Invitrogen, Carlsbad, CA) and chloroform extraction (Fisher Scientific, Pittsburgh, PA)/isopropyl alcohol precipitation (Sigma, St. Louis, MO). RNA was quantified by UV spectrophotometry and was reverse transcribed using the Reverse transcription-PCR kit (Promega, Madison, WI) with appropriate primers. RT-PCR analysis was carried in a Gene Amp PCR system 9700 (Applied Biosystems, Foster City, CA); conditions used were: 45 min at 48˚C, 1 min at 95˚C, 35 cycles: 1 min at 94˚C, 30 sec at 54˚C, 1 min at 72˚C, and a final extension step at 72˚C for 6 min. The PCR conditions for the *Rassf3* gene were slightly different and consisted of 35 cycles: 1 min at 94˚C, 1.5 min at 68˚C, and a final extension step at 72˚C for 6 min.

Specific pairs of primers were designed for each gene: *mRassf3* (5'-GCTAGCATGAGCAGCGGCTACAGCAG-3' and 5'-ACCGGTGCCGGGCTTCCACACCTCGC-3'), *mSocs2* (5'-TTGACTCATCTCCCATGACC-3' and 5'-GCTGCATTCGGAGATAGTCT-3'), *mEtv1* (5'-GGAGCAGAATGGATGGATTTT-3' and 5'-GGGTTACTCATGTTAGTACAC-3') and G3PDH (5'-CCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3'). RT-PCR products were resolved on 1% agarose gel. Amplification of the G3PDH housekeeping gene was performed simultaneously to demonstrate equal starting amounts of total RNA. RT-PCR analyses on total RNA samples from pairs of human tumors and adjacent normal tissues purchased from Ambion Inc. (Austin, TX), were conducted in duplicate with specific human *RASSF3* primers (5'-CTAGCATGAGCAGCGGCTACAGCAG-3' and 5'-GTCGACTTAATCAGGCTTCCACACCT-3'). The number of PCR cycles was limited to 25. Gel electrophoresis photographs were analyzed with the Kodak 1D image analysis software (Eastman Kodak Company Molecular Imaging Systems; Rochester, NY) to compare the mean intensity of the bands. The mean intensity was set to 100% for the normal breast tissue. The data were calculated as relative percentage of *RASSF3* expression in the different samples and represent the mean ± SEM of two independent experiments.

Cell lines. SKBR3, BT-474, MDA-MB-453, BT-483, MDA-MB-134, T-47D, MCF-7, MDA-MB-468, MDA-MB-436 and MDA-MB-231 human breast cancer cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained as recommended by ATCC. HC11 murine mammary cell line was kindly provided by Dr Ameae Walker (University of California, Riverside, CA) and were maintained in RPMI Medium 1640 supplemented with 10% FBS, 1 mM sodium pyruvate, 10 μg/ml insulin and 10 ng/ml EGF (Cambrex, East Rutherford, NJ). MCNeuA and N202Fb3 murine mammary cells, provided by Dr Mike Campbell (University of California, San Francisco, CA), were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS and 1 mM sodium pyruvate.

Protein isolation and immunoblot analyses. Cell monolayers were washed with cold phosphate buffer saline (PBS) and lysed in lysis buffer (50 mM Tris·HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA, pH 8.0) containing protease inhibitors (1 μg/ml aprotinin; 1 μg/ml leupeptin; 170 μg/ml PMSF; 180 μg/ml sodium orthovanadate) according to standard protocols. Protein content was measured using the Coomassie Plus Protein assay reagent (Pierce, Rockford, IL). Mammary gland, brain or mammary tumor tissues were excised, frozen on dry ice or immediately homogenized in lysis buffer (50 mM Tris·HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA, pH 8.0) containing protease inhibitors (1 μg/ml aprotinin; 1 μg/ml leupeptin; 1 μg/ml pepstatin; 170 μg/ml phenylmethylsulphonyl fluoride (PMSF); 180 μg/ml sodium orthovanadate; 50 mM sodium fluoride), using a Polytron PT1200 motorized homogenizer. Lysates were processed as described above.

For immunoblotting, samples containing equal amounts of protein (50-100 μg) were mixed with SDS-PAGE sample buffer and heated at 100˚C for 5 min, separated using 12% SDS-PAGE (Bio-Rad Laboratories, Hercules, CA) and transferred to a Hybond nitrocellulose (Amersham Biosciences, Piscataway, NJ) according to standard protocol. Membranes were blocked in tris-buffered saline (TBS) containing 5% non-fat powdered milk and 0.05% Tween-20 for 1-2 h (TBS-T) before overnight incubation with the primary antibody in TBS-T/5% milk at 4˚C with gentle agitation. Membranes were washed with TBS-T for 5 min, incubated with a secondary antibody in TBS-T/5% milk for 2 h at room temperature and washed 3 times with TBS-T before incubation with the ECL™ Western blotting detection reagents (Amersham Biosciences), as per the manufacturer's instructions. To visualize banding, membranes were exposed to Kodak

Biomax MR film (Fisher Scientific) and developed with a Konica SRX-101A processor (Konica Minolta Medical Imagining, Wayne, NJ).

Primary antibodies were used at the following dilutions: 1:1000 anti-phospho-HER2/*neu* (Lab Vision; Fremont, CA) for cell lysate and 1:400 anti-phospho-HER2/*neu* (Santa Cruz Biotechnology, Santa Cruz, CA) for tissue homogenate; 1:1000 anti-HER2/*neu* (EMD Biosciences; Darmstadt, Germany); 1:1000 anti-RAS (Cell Signaling Technology); 1:10,000 anti-ß-actin (Millipore, Billerica, MA); 1:10,000 anti-ß-tubulin (Sigma). The secondary antibodies, goat anti-mouse IgG- and goat anti-rabbit IgG-horseradish peroxidase-conjugates were obtained from Bio-Rad Laboratories and used at 1:2000. A custom polyclonal antibody to mouse RASSF3 protein was produced by Proteintech Group Inc. (Chicago, IL) (http://www.PTGLab.com). Anti-RASSF3 antibody [RASSF3-Nt (S-1228-1)] was produced from the following antigenic peptide sequence N'-MSSGYSSLEEDEDFFFTART-C and used at a dilution of 1:500 for immunoblot analyses. Control RASSF3 protein was produced in the lab using a previously published protocol (34) and used to test the specificity of the RASSF3-Nt antibody. The RASSF3 protein was immunoblotted as indicated above at different concentrations to determine the minimal amount of RASSF3 protein detected by this customized antibody.

Transient transfection, cell viability and apoptosis assay. SKBR3, BT-474, MCNeuA and HC11 cells were seeded to reach 60-80% confluency after 24 h. Cells were starved for 2 h with Opti-MEM® reduced serum medium (Invitrogen) before transfection with 2 or 4 μg of DNA plasmids [pcR3.1 vector, pcDNA3.1/*Rassf3*, pcDNA3.1/H-RAS *(G12V)* or pcDNA3.1/H-RAS *(S17N)*] using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's recommendations. Total amount of transfected DNA was maintained constant between compared samples. For cell viability counts, transfected cell lines were harvested 48 h after transfection, counted and re-seeded at equal concentration. After 24 h, viability was assessed by MTS/PMS solution (Promega). Cell viability was calculated as a percentage of control, corresponding to pcR3.1 vector transfected cells. The data are represented as the mean ± standard error of the mean (SEM) for all experiments. Transfection assays were performed in triplicate for BT-474, MCNeuA and HC11 cell lines and repeated 8 times for SKBR3 cell line.

For apoptosis assays, SKBR3 cells were seeded and transiently transfected with 2 μg of pcR3.1 vector, 4 μg of pcR3.1 vector, 2 μg of pcDNA3.1/*Rassf3* and 4 μg of pcDNA3.1/*Rassf3* as described above. Untransfected SKBR3 cells and camptothecin-treated SKBR3 cells (50 μM; 24 h) (Sigma) were used as negative control and positive control for induction of apoptosis, respectively. Each sample was prepared in triplicate. After 48 h, non-adherent apoptotic/dead cells and viable adherent cells were collected from each well and centrifuged at 310 x g for 5 min at 4˚C. Cell pellets were resuspended in growth media, diluted in Guava® ViaCount® reagent (Guava Technologies, Hayward, CA) and analyzed after 5 min incubation with a Guava personal cytometer using the Guava CytoAnalysis software. The amount of live/healthy cells, apoptotic and dead cells were expressed as a percentage of the total cellular population for each sample. Data are expressed as mean ± SEM and are representative of two experiments.

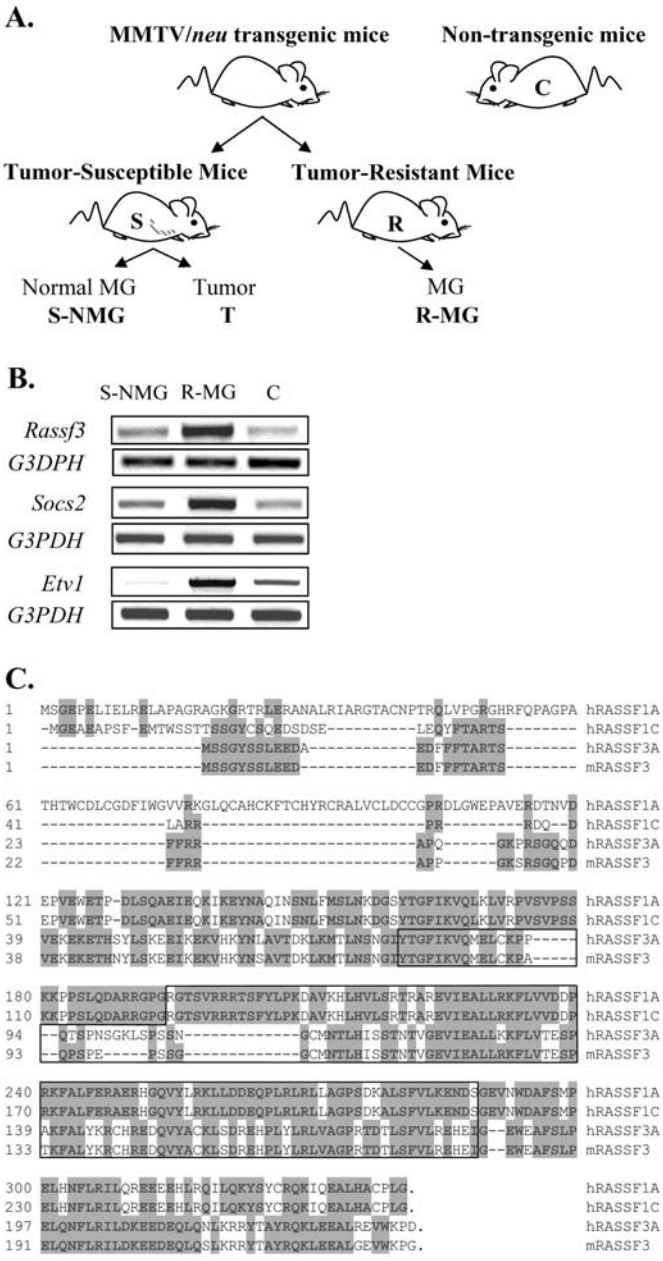

Figure 1. Nomenclature of mouse groups and microarray data confirmation for selected genes by RT-PCR analysis. (A) Three groups of virgin female mice were defined as follows: 1) Tumor-susceptible MMTV/*neu* transgenic mice that developed mammary tumors (S), 2) Tumor-resistant MMTV/*neu* transgenic mice that did not develop tumors (R) and 3) non-transgenic FVB mice used as control (C). Mammary gland isolated from the S group is defined as susceptible normal mammary gland (S-NMG). (B) Total RNA was isolated from the mammary glands of three 11-month-old R and S mice and of three age-matched C mice, then pooled into one sample per mouse group to conduct RT-PCR analyses for *Rassf3, Socs2, Etv1* genes and *G3PDH* as a loading control. The gels shown from pooled samples are representatives of individual mammary gland samples. (C) The alignment of hRASSF1A (GenBank NM_007182), hRASSF1C (GenBank NM_170713), hRASSF3 (GenBank BC100951) and mRASSF3 (GenBank NM_138956) were generated with the MegAlign program from Lasergene (DNASTAR) using the ClustalW algorithm. Identical amino acids are highlighted in gray and boxes define the RA domains.

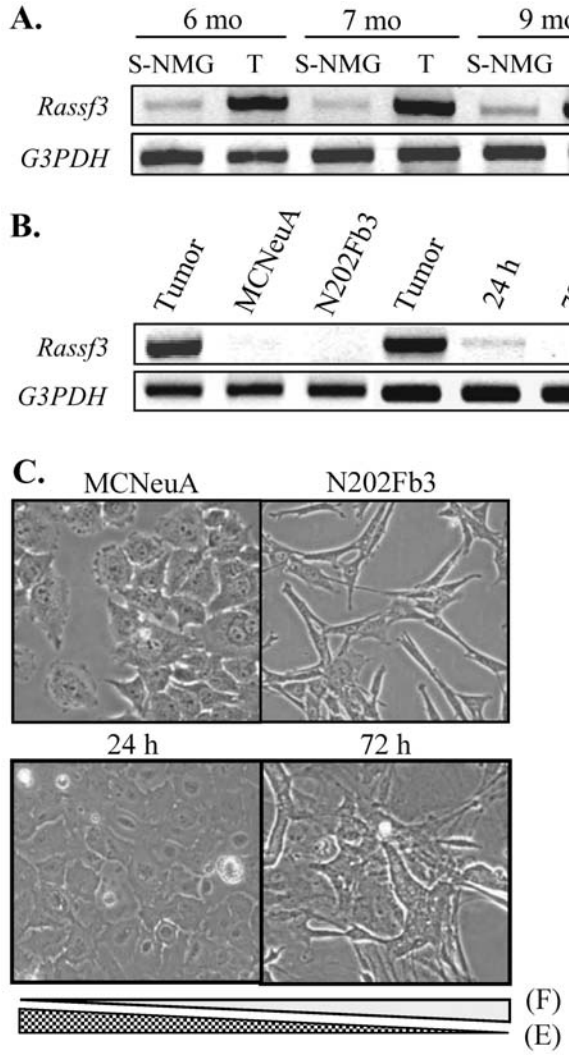

Figure 2. Rassf3 expression is higher in mammary tumors than in adjacent normal mammary tissues but absent in HER2/neu mammary tumor-derived cells. (A) Total RNA was isolated from pairs of mammary tumors (T) and adjacent normal tissues (S-NMG) of MMTV/*neu* transgenic mice that developed tumors at the age of 6, 7, 9 and 11 months (mo) to conduct RT-PCR analyses with appropriate primers for *Rassf3* and *G3PDH* as a loading control. (B) Total RNA was isolated from a primary HER2/neu mouse mammary tumor tissue (T), from established epithelial cells (MCNeuA) and fibroblasts (N202F3) derived from a HER2/neu mouse mammary tumor and from the primary cultured cells of a HER2/neu mammary tumor mix at 24 h (passage 1) and 72 h (passage 3) for RT-PCR analyses. (C) The cell morphology of MCNeuA and N202F3 cells and of primary cultured cells at 24 and 72 h post-passages was recorded under a microscope to demonstrate the different cell populations (F, fibroblast; E, epithelium).

Results

Identification and confirmation of differentially expressed genes by cDNA microarray analysis. The approach utilized sought for genes that exhibited an opposing pattern of expression between the mammary glands of tumor-resistant mice (R-MG) and the mammary glands of tumor-susceptible mice (S-NMG) (Fig. 1A). Particular attention was accorded to genes up-regulated in R-MG but down-regulated in S-NMG (Table I). We focused on genes involved in signal transduction and on genes which could interact with proteins involved in the HER2/neu receptor tyrosine kinase signaling pathway. Three genes were selected to confirm microarray data by semi-quantitative RT-PCR analyses on total RNA samples isolated from mammary glands of the three groups of mice. Two of these genes encode for intracellular proteins involved in signal transduction and correspond to the *Socs2* gene, a well-characterized suppressor of cytokine signaling (20), and the novel *Rassf3* gene which belongs to the Ras association domain family (RalGDS/AF-6) gene (21). The third gene is *Etv1* which belongs to the ETS family of transcription factors (22). The *Rassf3, Socs2* and *Etv1* genes are overexpressed in mammary glands of tumor-resistant mice (R-MG) compared to tumor-susceptible mice (S-NMG) and control mice (C), which confirmed the microarray data (Fig. 1B). *Rassf3* expression level was also validated by RT-PCR in individual mammary glands from 11-month-old tumor-resistant mice (R-MG) and tumor-susceptible mice (S-NMG) from a different set of mice. *Rassf3* was up-regulated in 3 out of 5 individual R-MG mammary glands but totally absent in individual S-NMG mammary glands (5 out of 5) (data not shown).

Shared homology between RASSF family members. Sequences were aligned with ClustalW algorithm (Fig. 1C). The mouse (m) RASSF3 (232 amino acids; M.W. 26.7 kDa) and human (h) RASSF3 (247 amino acids; M.W. 28.6 kDa) sequences share 94% identity at the amino acid level, whereas the hRASSF1A (340 amino acids) and hRASSF1C (270 amino acids) isoforms share 81% identity at the amino acid level and differ mainly at the N-terminus. The mRASSF3 and hRASSF3 both share ~40% and 44% identity with hRASSF1A and hRASSF1C proteins, respectively. The proteins contain a highly conserved Ras association (RalGDS/AF-6) (RA) domain at the C-terminus defined by the boxes in Fig. 1C.

Rassf3 expression is higher in mammary tumor than in normal adjacent tissue. Total RNA from four pairs of mammary tumors (T) and adjacent normal tissues (S-NMG) of MMTV/*neu* mice that developed tumors at the age of 6, 7, 9 or 11 months was used for RT-PCR analyses to study the expression patterns of *Rassf3* (Fig. 2A). Surprisingly, *Rassf3* expression levels were consistently higher in mammary tumors (T) than

Table I. cDNA microarray analysis.[a]

Gene name		GenBank	Fold change
Signal transduction			
Growth factors and growth factor receptors			
Mia	Melanoma inhibitory activity 1	NM_019394	3.2
Tm4sf3	Tetraspanin 8	BC025461	2.1
Transcription factors			
Cited4	Cbp/p300-interacting transactivator, with Glu/Asp-richcarboxy-terminal domain, 4	BC025116	2.2
Etv1	Ets variant gene 1	NM_007960	2.2
Tcfap2c	Transcription factor AP-2, γ	BC003778	2.1
Etv5	Ets variant gene 5	BG966751	2.1
Hey1	Hairy/enhancer-of-split related with YRPW motif 1	BG966751	2.0
Intracellular signaling molecules			
Socs2	Suppressor of cytokine signaling 2	NM_007706	2.8
Rassf3	Ras association (RalGDS/AF-6) domain family 3	BC011511	2.4
Cell tissue structure			
Cytoskeleton			
Mylpc	Myosin, light polypeptide 2, regulatory, cardiac, slow	NM_010861	5.8
Tncc	Troponin C, cardiac/slow skeletal	NM_009393	3.6
Tpm3	Tropomyosin 3, γ	NM_022314	3.2
Homer2	Homer homolog 2 *(Drosophila)*	AB017136	2.9
Tnnt1	Troponin T1, skeletal, slow	NM_011618	2.9
Mylc	Myosin, light polypeptide 3	X67685	2.9
Myh7	Myosin, heavy polypeptide 7, cardiac muscle, ß	NM_080728	2.5
Myh2	Myosin, heavy polypeptide 2, skeletal muscle, adult	BC008538	2.2
Tnni1	Troponin I, skeletal, slow 1	NM_021467	2.1
Extracellular matrix protein, cell adhesion			
Col9a1	Procollagen, type IX, α1	NM_007740	3.7
Col11a1	Procollagen, type XI, α1	NM_007729	3.3
Ceacam10	CEA-related cell adhesion molecule 10	NM_007675	3.0
Npnt	Nephronectin	AW553512	3.0
Comp	Cartilage oligomeric matrix protein	NM_016685	2.2
Ceacam1	CEA-related cell adhesion molecule 1	NM_011926	2.1
Protein modification, proteases and proteases inhibitor			
Papln	Papilin, proteoglycan-like sulfated glycoprotein	BC005747	2.4
Spag5	Sperm associated antigen 5	BM208112	2.4
Timp4	Tissue inhibitor of metalloproteinase 4	BI788452	2.0
Treansporter			
Electron transporter			
2410043F08Rik	Filamin binding LIM protein 1	NM_133754	2.3
Ion transporter			
Kcnn4	Potassium intermediate/small conductance calcium-activatedchannel, subfamily N, member 4	NM_008433	2.9
Kcnk1	Potassium channel, subfamily K, member 1	NM_008430	2.0
Other transporter			
Aqp5	Aquaporin 5	NM_009701	4.6
Slc29a1	Solute carrier family 29 (nucleoside transporters), member 1	AF305501	3.0
Lman1	Lectin, mannose-binding, 1	AK011495	2.0
Slc21a2	Solute carrier organic anion transporter family, member 2a1	NM_033314	2.0

Table I. Continued.

Gene name		GenBank	Fold change
Metabolism			
Fatty acid biosynthesis			
Scd2	Stearoyl-Coenzyme A desaturase 2	BG060909	2.0
B4galt6	UDP-Gal:ßGlcNAcß 1,4-galactosyltransferase, polypeptide 6	BG066773	2.0
9430020A05Rik	Acyl-CoA synthetase long-chain family member 4	BI153391	2.0
Glycolysis			
Aldo3	Aldolase 3, C isoform	BM941201	3.6
Hk1	Hexokinase 1	NM_010438	3.0
Amino acid metabolism			
Got1	Glutamate oxaloacetate transaminase 1, soluble	AA792094	2.0
Other			
2310047E01Rik	Carbonic anyhydrase 12	AK009873	3.0
Egln3	EGL nine homolog 3 *(C. elegans)*	NM_028133	2.8
4930555L11Rik	Ethanolamine kinase 1	BG066916	2.4
Cp	Ceruloplasmin	BB531328	2.3
Rdh12	Retinol dehydrogenase 12	BC016204	2.1
Ckmt2	Creatine kinase, mitochondrial 2	AK009042	2.1
Other genes			
Xlr3a	X-linked lymphocyte-regulated 3a	NM_011726	3.6
Scrg1	Scrapie responsive gene 1	NM_009136	3.4
- - -	Similar to Shb-like adapter protein, Shf - human	BB798279	2.4
4931430I01Rik	EF hand domain containing 1	BC019531	2.2
- - -	Tumor differentially expressed 1	BM239368	2.1
Emu1-pending	EMI domain containing 1	NM_080595	2.1

[a]List of genes that are overexpressed in the mammary gland of tumor-resistant MMTV/*neu* female transgenic mice compared to tumor-susceptible MMTV/*neu* littermates.

in adjacent normal mammary tissues (S-NMG), independent of age at which the tumor appeared. Sequencing analyses of the *Rassf3* gene cloned from 6 mammary tumors revealed no consensus mutation (data not shown). In addition, this particular expression pattern of the *Rassf3* gene between T and S-NMG tissues was not observed for other members of the *Rassf* family, such as *Rassf1, Rassf2, Rassf4* and *Rassf5* (data not shown).

To further study the expression pattern of *Rassf3* within tumor tissues, expression levels were compared between epithelial cells (MCNeuA) and fibroblasts (N202Fb3), both derived from a *Her2/neu* mammary tumor (23). Interestingly, even though *Rassf3* expression was high in *Her2/neu* primary tumors, *Rassf3* expression appeared to be absent in the two established cell lines (Fig. 2B). *Rassf3* expression pattern in the primary cultured cells from a *Her2/neu* mammary tumor was also examined at different time points post-passage (24 and 72 h). *Rassf3* gene was expressed in primary cells at early time points (24 h), but its expression diminished after 72 h (Fig. 2B). Concurrently, the morphology of the primary culture revealed a switch in cell population composition, corresponding to an increase in fibroblast (F) population and a progressive loss of epithelial cells (E) population (Fig. 2C).

RASSF3 expression is inversely correlated to HER2 expression in human breast cancer cell lines. Ten human breast cancer cell lines were analyzed for HER2 and phosphorylated HER2 (p-HER2) protein levels and for *RASSF3* gene expression level. *RASSF3* gene expression was inversely correlated to HER2 protein levels among the 10 cell lines (Fig. 3A). *RASSF3* expression was minimal or non-detectable in SKBR-3 and BT-474 cell lines, which express high levels of HER2 and p-HER2. On the contrary, *RASSF3* expression levels were high in MDA-MB-231 and MDA-MB-436 cell lines, which did not show expression of HER2 protein. T47D cells showed moderate levels of both endogenous *RASSF3* and HER2.

RASSF3 expression in human tissues is tumor-type specific. To assess *RASSF3* gene expression in human tumors compared to adjacent normal tissues, total RNA samples from five pairs of human tumors and adjacent normal tissues from breast, lung, uterus, colon and cervix were used for RT-PCR analysis (Fig. 3B). *RASSF3* expression was higher in tumor compared to adjacent tissue of the breast and cervix samples ($p<0.05$). *RASSF3* expression level was lower in tumor compared to adjacent tissue of lung, uterus and colon organs. These

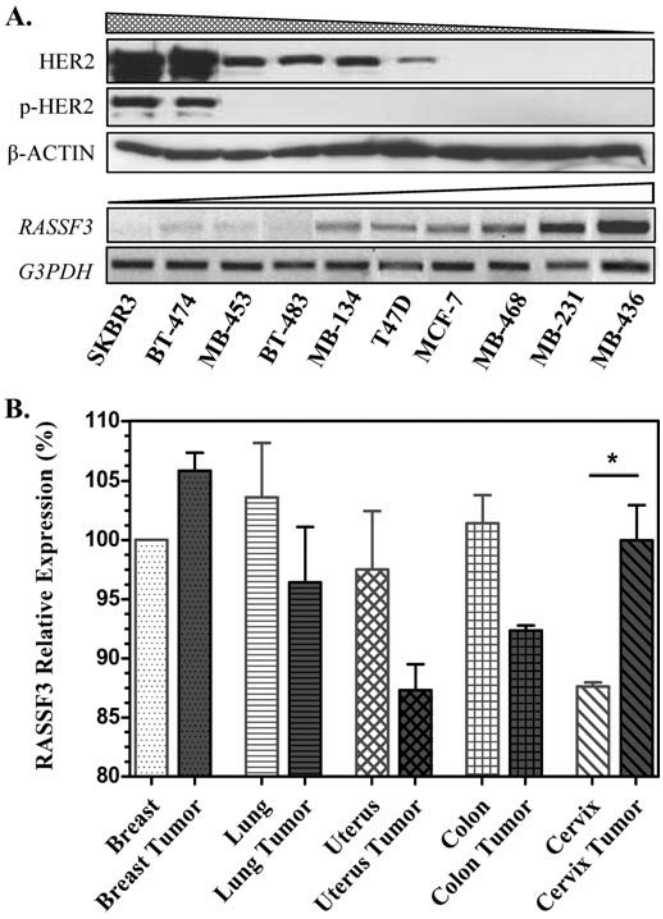

Figure 3. *RASSF3* expression is inversely correlated to HER2 expression levels and is tumor-type specific. (A) HER2 and p-HER2 protein levels, determined by immunoblot analyses, were compared with levels of *RASSF3* mRNA, determined by RT-PCR analyses, in 10 human breast cancer cells. ß-actin and *G3PDH* were used as loading controls. (B) *RASSF3* mRNA levels were determined in pairs of tumor and adjacent normal tissue from human breast, lung, uterus, colon and cervix, by RT-PCR analysis. *G3PDH* was used as a loading control. Kodak 1D image analysis software was used to compare the mean intensity of the bands. Mean intensity was set to 100% for normal breast tissue. Data are presented as percentage and correspond to RASSF3 relative expression in the different tissues. The data represent the mean ± SEM of two independent experiments ($^{*}p<0.05$).

observations indicate that *RASSF3* expression patterns are tumor-type specific.

RASSF3 expression reduces cell viability and induces apoptosis in human breast cancer cell lines. Transient transfection was used to study the effects of *Rassf3 in vitro*. SKBR3 human breast cancer epithelial cell line, which expresses very high levels of HER2 protein, was selected to examine the effects of *Rassf3* expression on cell viability. *Rassf3* expression reduced cell viability in a dose-dependent manner. The inhibitory effect reached a maximum with 2 μg of pcDNA3.1/*Rassf3* plasmid and after 24 h of proliferation. Multiple epithelial cell lines (SKBR3, BT-474, MCNeuA and HC11), which express high levels of HER2 protein, were used to confirm *Rassf3* inhibitory effect. Cells were transfected with 2 μg of pcDNA3.1/*Rassf3* plasmid or vector and cellular viability was measured after 24 h. For this transfection experiment, a novel polyclonal antibody was developed against RASSF3. The specificity was confirmed with a titer test and by immunoblot with varied amounts of RASSF3 protein (data not shown) both test revealing the specificity of the antibody and the ability to distinguish variable amounts of RASSF3. RASSF3 protein expression was confirmed post-transfection in each transfected cell line (Fig. 4A). *Rassf3* transient expression reduced cell viability by 20% in SKBR3, 8% in BT-474, 15% in MCNeuA and 10% in HC11 cells (Fig. 4B).

The effects of RASSF3 and H-RAS on cell viability were compared by transfection with activated *H-RAS* [pcDNA3.1/H-*RAS (G12V)*] or dominant-negative *H-RAS* [pcDNA3.1/H-*RAS (S17N)*] in SKBR3 cells. *Rassf3* reduced cell viability in a highly significant manner compared to activated H-RAS or dominant-negative H-RAS alone ($p<0.01$) which had minimal effects on cell viability (Fig. 4C).

To determine the potential mechanism behind the growth-inhibitory properties of *Rassf3*, Guava ViaCount™ assay for cellular viability were performed on transfected SKBR3 cells (Fig. 4D). SKBR3 cells were transfected with 2 or 4 μg of vector and pcDNA3.1/*Rassf3* and collected after 48 h. *Rassf3* transfection caused ~15-20% increase in the number of apoptotic and dead cells compared to non-specific vector transfection. These changes in cells numbers were statistically highly significant ($p<0.01$) with 2 or 4 μg of pcDNA3.1/*Rassf3* plasmid.

MMTV/Rassf3-neu bi-transgenic mice display delayed tumor formation. To study *Rassf3* function *in vivo*, two novel transgenic mouse lines were generated: MMTV/*Rassf3* transgenic and MMTV/*Rassf3-neu* bi-transgenic lines. Incorporation of *Rassf3* transgene was confirmed by PCR analysis on genomic DNA isolated from mouse tail biopsies (Fig. 5A). Tissues from female MMTV/*Rassf3* mice and their non-transgenic littermates were analyzed by RT-PCR for *Rassf3* gene expression which indicated that the *Rassf3* gene is expressed in the mammary gland as well as in the brain, small intestine and muscle of MMTV/*Rassf3* transgenic mice (data not shown). RASSF3 protein was not detected in mammary gland tissues of MMTV/*Rassf3* mice and MMTV/*Rassf3-neu* bi-transgenic mice by immunoblot, although *Rassf3* mRNA was present and amplified by RT-PCR analysis of mammary gland homogenates (Fig. 5B). However, RASSF3 protein was detected in the brain tissue of all MMTV/*Rassf3* transgenic mice and of MMTV/*Rassf3-neu* bi-transgenic mice whereas it was not detected in littermates (Fig. 5B).

To assess the effect of *Rassf3* overexpression on *HER2/neu* mammary tumorigenesis, MMTV/*Rassf3-neu* transgenic mice and MMTV/*neu*$^{+/-}$ littermates were monitored for development of palpable mammary tumors. Comparison of mammary tumor incidence revealed a delay in tumor formation ($p=0.0552$) in bi-transgenic mice (Fig. 5C). The t_{50} of MMTV/*Rassf3-neu* bi-transgenic mice ($n=37$; $t_{50}=262$) was increased by 37 days compared to the t_{50} of MMTV/*neu*$^{+/-}$ littermates ($n=32$; $t_{50}=225$). RASSF3 protein was detected by immunoblot in tumors of MMTV/*Rassf3-neu* bi-transgenic mice and of MMTV/*neu*$^{+/-}$ littermates (Fig. 5D). Expression levels of RASSF3 and p-HER2 proteins appeared to be both higher in tumors from bi-transgenic mice than in tumors from littermates.

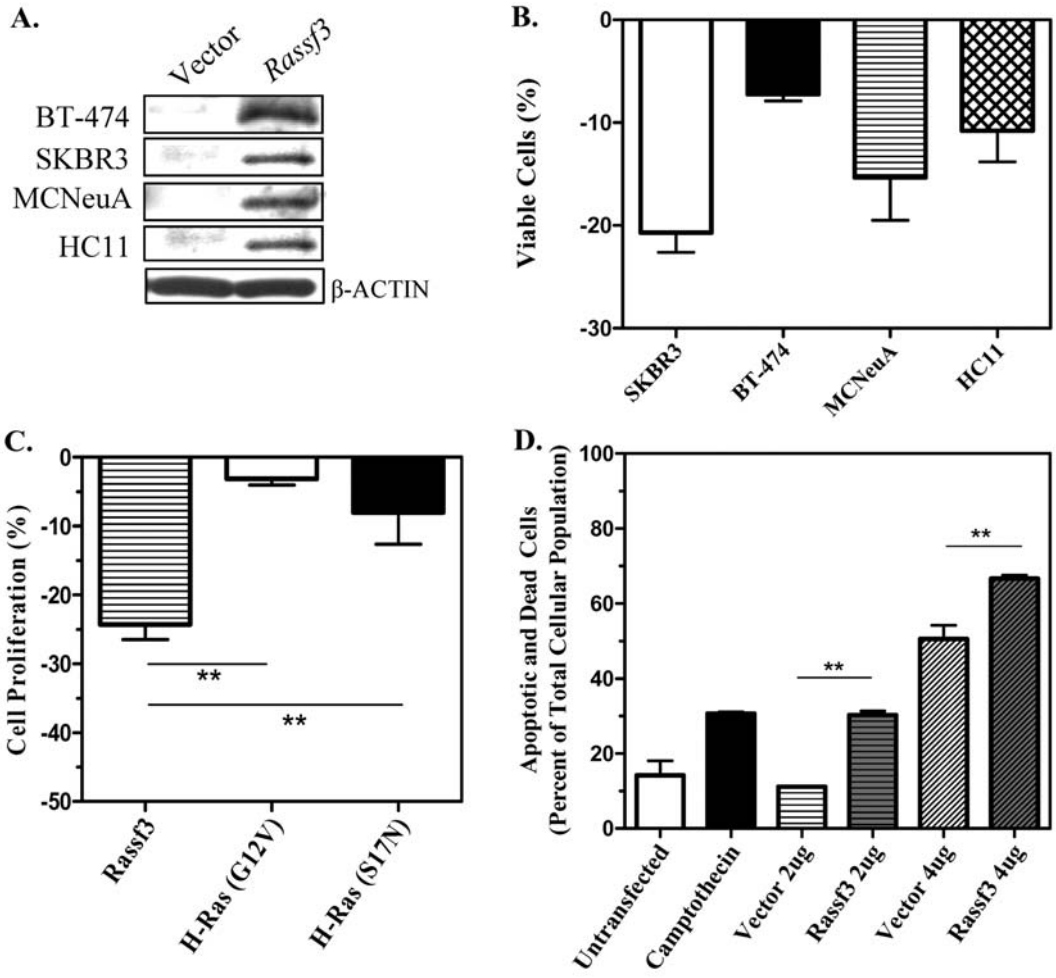

Figure 4. *Rassf3* expression reduces cell viability and induces cell death in SKBR3 human breast cancer cells. (A) RASSF3 protein expression was confirmed by immnoblot analysis using a specific anti-RASSF3 antibody in four cell lines (SKBR3, BT-474, MCNeuA and HC11) expressing high levels of HER2 protein, 24 h after transient transfection with 2 μg of *Rassf3* or vector plasmids. ß-actin was used as a loading control and is representative of all the gels. (B) Forty-eight hours post-transfection, cells were plated at an equal cell concentration and viability was monitored after 24 h. Data are presented as mean ± SEM of a percentage of *Rassf3* inhibition compared to vector. Means are obtained from 8 independent experiments for SKBR3 cells and 3 independent experiments for BT-474, MCNeuA and HC11 cells. (C) SKBR3 cells were transfected with 2 μg of vector, pcDNA3.1/*Rassf3*, pcDNA3.1/*H-RAS (G12V)* or pcDNA3.1/*H-RAS (S17N)*. Cells were plated 48 h post-transfection at an equal cell concentration and viability was monitored after 24 h. Data are presented as a percentage of *Rassf3, H-RAS (G12V)* and *H-RAS (S17N)* effect on cell viability compared to vector. The data correspond to the mean ± SEM of percentages obtained from 4 independent experiments ($^{**}p<0.01$). (D) SKBR3 cells were transiently transfected with 2 or 4 μg of vector and *Rassf3* plasmid and collected after 48 h to run the Guava ViaCount™ assay. Untransfected cells and camptothecin-treated cells were used as negative and positive controls respectively. The amount of apoptotic and dead cells were expressed as a percentage of the total cellular population for each treatment. Data are presented as mean ± SEM of triplicates and are representative of two separate experiments ($^{**}p<0.01$).

Discussion

The novel approach described herein, utilizing MMTV/*neu* transgenic mouse model, focused on naturally acquired tumor-resistant MMTV/*neu* female transgenic mice which either have a significant delay in or resistance to *neu* tumorigenesis. This approach identified *Rassf3* as a candidate tumor suppressor gene which may be involved in this resistance to or the delaying of HER2/neu-initiated mammary tumorigenesis. Our data demonstrated that the *Rassf3* gene is over-expressed in the mammary gland of tumor-resistant MMTV/*neu* mice compared to tumor-susceptible MMTV/*neu* transgenic littermates or non-transgenic FVB mice (Fig. 1B). The *RASSF3* gene is classified as a member of the Ras association domain (RalGDS/AF-6) family gene *(RASSF)*, which contains characterized RAS effectors and tumor suppressor genes (15-18). However, the *Rassf3* gene remains relatively uncharacterized and no functional studies of human *RASSF3* and mouse *Rassf3* genes have been reported to date.

It has been documented that some genes with tumor suppressor activity, notably TGF-ß, p53 and SOCS tumor suppressors, show a marked increase in their expression in specific cancers (20,24-26). In accordance with this observation, our data indicate that the *Rassf3* gene is significantly up-regulated in *neu*-specific mouse mammary tumors compared to adjacent normal tissues. Overexpression of *Rassf3* gene in mouse mammary tumors may be the result of a cellular defensive response to high levels of HER2/Neu proteins in mammary tumors of MMTV/*neu* transgenic mice (Fig. 2A).

Comparison of HER2 protein and *RASSF3* mRNA levels among multiple human breast cancer cell lines revealed an

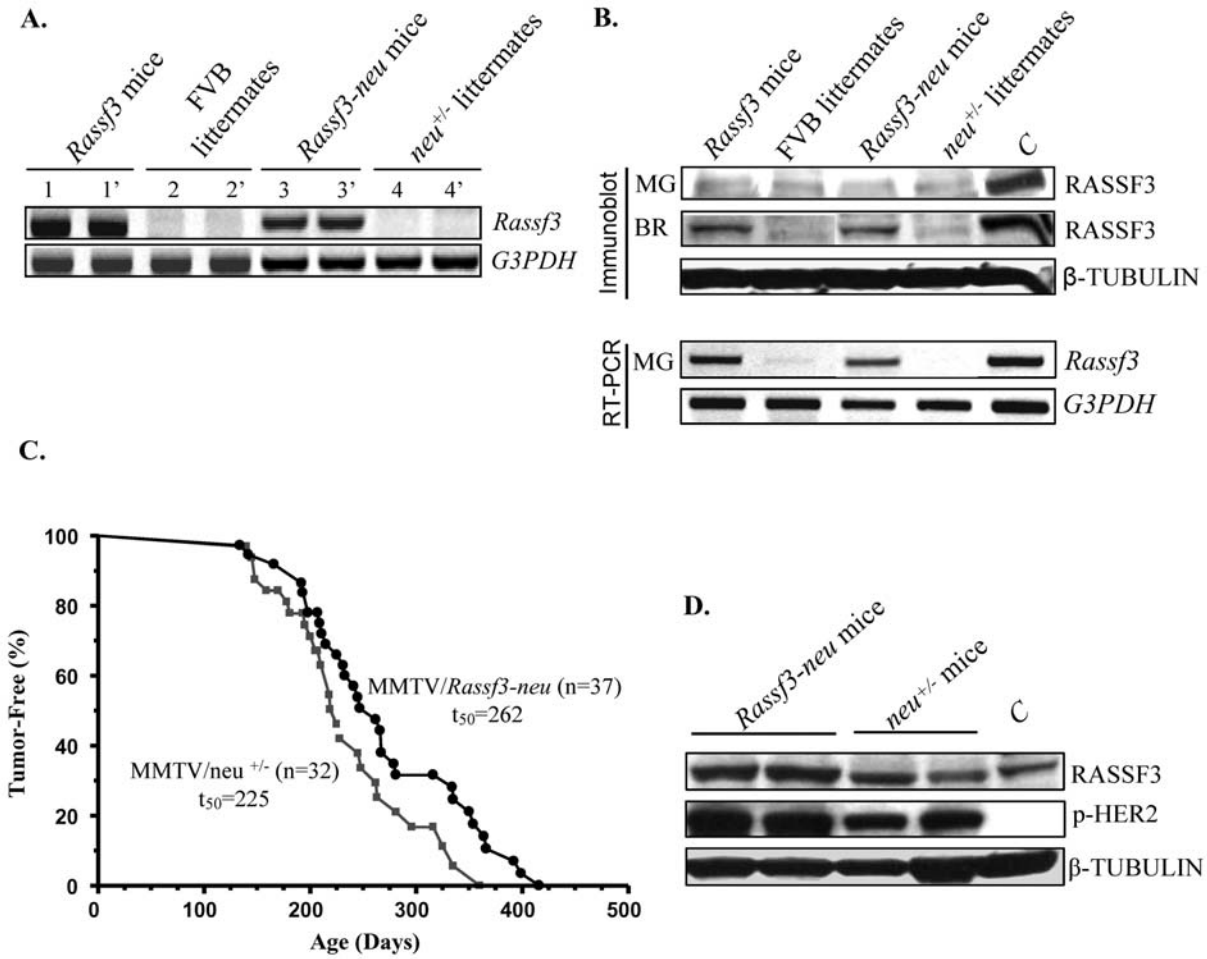

Figure 5. Bi-transgenic MMTV/*Rassf3-neu* mice display increased tumor latency. (A) Transgene incorporation was confirmed by PCR analysis of genomic tail DNA from MMTV/*Rassf3* transgenic mice (1, 1'), FVB non-transgenic littermates (2, 2'), MMTV/*Rassf3-neu* bi-transgenic mice (3, 3') and MMTV/*neu*$^{+/-}$ littermates (4, 4') with a pair of specific primers that span the MMTV promoter and the *Rassf3* cDNA sequence. *G3PDH* was used as a loading control. (B) Tissue lysates were isolated from mammary gland and brain tissues of transgenic mice and their littermates for immunoblot analysis with an anti-RASSF3 antibody and total RNA samples from mammary gland tissues were used for RT-PCR analysis. Purified RASSF3 protein was used as a positive control (C). ß-tubulin and *G3PDH* were used as loading controls. (C) Data are plotted as the percentage of tumor-free female mice (y-axis) as a function of age in days (x-axis). MMTV/*Rassf3-neu* bi-transgenic mice (n=37) and MMTV/*neu*$^{+/-}$ littermates (n=32) are represented by closed circles and closed squares, respectively. The t_{50} of MMTV/*Rassf3-neu* bi-transgenic mice and their littermates correspond to 262 and 225 days, respectively. Log rank test, p=0.0552. (D) Protein lysates were isolated from mammary tumors of MMTV/*Rassf3-neu* bi-transgenic mice and of MMTV/*neu*$^{+/-}$ littermates for immunoblot analysis with anti-RASSF3 and anti-phospho-HER2 antibodies. Purified RASSF3 protein was used as a positive control (C) and ß-tubulin as a loading control.

inverse correlation of expression between these two genes (Fig. 3A). The inverse correlation of expression between an oncogene and a tumor suppressor gene has been reported in different cancers (15). The inverse correlation suggests that *HER2* and *RASSF3* genes have a cooperative role in breast carcinogenesis and that they are involved in the same pathway. The analysis of human tumors and adjacent normal tissues also revealed that *RASSF3* overexpression pattern is dependent on the tissue and cancer types (Fig. 3B). Therefore, *Rassf3* overexpression may become an interesting biomarker in breast cancer.

Most HER2/neu mammary tumors in this study contain high levels of *Rassf3* mRNA (Fig. 2A). Since all tumor samples prepared in this study were tissue homogenates, it is impossible to differentiate the origin of *Rassf3* expression in tumors. The primary culture experiment suggests that *Rassf3* expression in the mouse HER2/neu tumor mix is likely from the epithelium cells rather than the stromal cells since *Rassf3* expression level decreased drastically within days when the population of epithelial cells dropped while the stromal cell population increased after passages (Fig. 2C). The loss of *Rassf3* expression can be explained by the epithelial-mesenchymal transition hypothesis, which is often characterized by the turning-off of genes encoding epithelial markers (e.g. E-cadherins), the loss of epithelial features and the increase in markers of mesenchymal cells. However, we have noted that *Rassf3* expression in established epithelium cells, derived directly from the MMTV/*neu* mouse tumor (Fig. 2B), is barely detectable, which is consistent with the results shown in Fig. 3A where *Rassf3* level is inversely correlated with HER2 level. One possible explanation is that it is well known that breast cancer cells are genetically unstable. Once extracted from the tumor, they can undergo specific genotype/phenotype alterations resulting from long-

term culture in simplified conditions. Nonetheless, more studies are needed to evaluate whether *Rassf3* expression in tumors could be used as a valuable marker to predict the prognoses.

Ectopic expression of *Rassf3* reduced cell viability of various cell lines including SKBR3 and BT474 human breast cancer cell lines, MCNeuA mouse cancer cell line and HC11 mouse cell line (Fig. 4B), all of which are characterized by expression of high levels of HER2 protein. The growth-inhibitory effect of *Rassf3* was compared to an activated form of H-RAS and a dominant negative form of H-RAS in SKBR3-transfected cells. It has been shown that differences in effector binding and signaling exist between the different RAS isoforms for the RASSF proteins (8,27); therefore, we used the H-RAS isoform that has been shown to specifically interact with RASSF1, since RASSF3 gene shares more homology with this member of the RASSF family. *Rassf3* growth-inhibition was greater than that of the dominant negative form of H-RAS response in SKBR3-transfected cells (Fig. 4C). Investigation of the mechanism by which *Rassf3* promotes growth inhibition in SKBR3 cells suggested that *Rassf3* promotes cell apoptosis (Fig. 4D) which supports a role for *Rassf3* as a tumor suppressor gene.

To date, in the RASSF family, only RASSF1A animal model was reported (28). A mouse knockout for *Rassf1a*, where the *Rassf1a* was specifically inactivated, revealed that *Rassf1a*$^{+/-}$ and *Rassf1a*$^{-/-}$ were prone to spontaneous tumorigenesis with advanced age (18-20 months) and were more susceptible to chemical carcinogen-induced tumor formation. The tumors included lung adenomas, lymphomas and breast carcinomas. These data reinforced the role of RASSF1 gene as a tumor suppressor. In this study, to elucidate the role of *Rassf3* in HER2/neu-initiated breast cancer, MMTV/*Rassf3* transgenic mice were cross-bred with MMTV/*neu* transgenic mice to produce MMTV/*Rassf3-neu* bi-transgenic mouse line. This novel MMTV/*Rassf3-neu* bi-transgenic mouse model permitted the determination of *Rassf3* influence on *HER2/neu* mammary tumor formation *in vivo*. Despite the fact that RASSF3 protein in our established MMTV/*Rassf3* transgenic mouse line was detected mainly in the brain tissue but minimally present in the mammary gland, *Rassf3* expression still delayed tumor onset in the bi-transgenic mouse line (Fig. 5C). We believe that if RASSF3 expression levels and activity were increased in the mammary gland of the bi-transgenic mouse line, the delay in tumor onset would have been more significant. It is interesting to note that RASSF3 and p-HER2 protein levels in mammary tumors of MMTV/*Rassf3-neu* bi-transgenic mice and MMTV/*neu*$^{+/-}$ littermates are positively correlated (Fig. 5D). We speculate that high levels of RASSF3 found in tumors are not the causal factor but are the result of a cellular/host defensive response. It appears that RASSF3 expression is induced in response to HER2 activity in HER2/neu-positive tumors.

In summary, this study presented a novel approach for studying the well-documented MMTV/*neu* transgenic mouse model by focusing on naturally acquired tumor-resistant MMTV/*neu* female transgenic mice. We provide evidence suggesting an important role for the *Rassf3* gene in the process of HER2/neu-initiated mammary tumorigenesis. Furthermore, we report the first functional study for the *Rassf3* gene using *in vitro* and *in vivo* models. The findings suggest that the *Rassf3* gene exhibits the properties of a RAS effector and tumor suppressor gene. However, the molecular mechanism of growth-inhibition of the *Rassf3* gene and its particular role in HER2/neu tumor initiation and progression needs further investigation.

References

1. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A and McGuire WL: Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235: 177-182, 1987.
2. Slamon DJ, Godolphin W, Jones LA, *et al*: Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science 244: 707-712, 1989.
3. Guy CT, Webster MA, Schaller M, Parsons TJ, Cardiff RD and Muller WJ: Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. Proc Natl Acad Sci USA 89: 10578-10582, 1992.
4. Lodish H BD, Berk A, Zipursky SL, Matsudaira P: Molecular Cell Biology. W.H. Freeman and Co., New York, 2001.
5. Bazley LA and Gullick WJ: The epidermal growth factor receptor family. Endocr Relat Cancer 12 (Suppl 1): 17-27, 2005.
6. Clark GJ and Der CJ: Aberrant function of the Ras signal transduction pathway in human breast cancer. Breast Cancer Res Treat 35: 133-144, 1995.
7. von Lintig FC, Dreilinger AD, Varki NM, Wallace AM, Casteel DE and Boss GR: Ras activation in human breast cancer. Breast Cancer Res Treat 62: 51-62, 2000.
8. Rodriguez-Viciana P, Sabatier C and McCormick F: Signaling specificity by Ras family GTPases is determined by the full spectrum of effectors they regulate. Mol Cell Biol 24: 4943-4954, 2004.
9. Lowy DR, Johnson MR, DeClue JE, *et al*: Cell transformation by ras and regulation of its protein product. Ciba Found Symp 176: 67-80, 1993.
10. Chen CY and Faller DV: Direction of p21ras-generated signals towards cell growth or apoptosis is determined by protein kinase C and Bcl-2. Oncogene 11: 1487-1498, 1995.
11. Chen CY, Liou J, Forman LW and Faller DV: Differential regulation of discrete apoptotic pathways by Ras. J Biol Chem 273: 16700-16709, 1998.
12. Khokhlatchev A, Rabizadeh S, Xavier R, *et al*: Identification of a novel Ras-regulated proapoptotic pathway. Curr Biol 12: 253-265, 2002.
13. Shao J, Sheng H, DuBois RN and Beauchamp RD: Oncogenic Ras-mediated cell growth arrest and apoptosis are associated with increased ubiquitin-dependent cyclin D1 degradation. J Biol Chem 275: 22916-22924, 2000.
14. Cox AD and Der CJ: The dark side of Ras: regulation of apoptosis. Oncogene 22: 8999-9006, 2003.
15. Agathanggelou A, Cooper WN and Latif F: Role of the Ras-association domain family 1 tumor suppressor gene in human cancers. Cancer Res 65: 3497-3508, 2005.
16. Eckfeld K, Hesson L, Vos MD, Bieche I, Latif F and Clark GJ: RASSF4/AD037 is a potential ras effector/tumor suppressor of the RASSF family. Cancer Res 64: 8688-8693, 2004.
17. Hesson L, Dallol A, Minna JD, Maher ER and Latif F: NORE1A, a homologue of RASSF1A tumour suppressor gene is inactivated in human cancers. Oncogene 22: 947-954, 2003.
18. Vos MD, Ellis CA, Elam C, Ulku AS, Taylor BJ and Clark GJ: RASSF2 is a novel K-Ras-specific effector and potential tumor suppressor. J Biol Chem 278: 28045-28051, 2003.
19. Wagner TE, Hoppe PC, Jollick JD, Scholl DR, Hodinka RL and Gault JB: Microinjection of a rabbit beta-globin gene into zygotes and its subsequent expression in adult mice and their offspring. Proc Natl Acad Sci USA 78: 6376-6380, 1981.
20. Raccurt M, Tam SP, Lau P, *et al*: Suppressor of cytokine signalling gene expression is elevated in breast carcinoma. Br J Cancer 89: 524-532, 2003.
21. Tommasi S, Dammann R, Jin SG, Zhang Xf XF, Avruch J and Pfeifer GP: RASSF3 and NORE1: identification and cloning of two human homologues of the putative tumor suppressor gene RASSF1. Oncogene 21: 2713-2720, 2002.

22. Goel A and Janknecht R: Concerted activation of ETS protein ER81 by p160 coactivators, the acetyltransferase p300 and the receptor tyrosine kinase HER2/Neu. J Biol Chem 279: 14909-14916, 2004.
23. Campbell MJ, Wollish WS, Lobo M and Esserman LJ: Epithelial and fibroblast cell lines derived from a spontaneous mammary carcinoma in a MMTV/neu transgenic mouse. In Vitro Cell Dev Biol Anim 38: 326-333, 2002.
24. Oft M, Heider KH and Beug H: TGFbeta signaling is necessary for carcinoma cell invasiveness and metastasis. Curr Biol 8: 1243-1252, 1998.
25. Gonzalez-Palacios F, Sancho M, Martinez JC and Bellas C: Microvessel density, p53 overexpression, and apoptosis in invasive breast carcinoma. Mol Pathol 50: 304-309, 1997.
26. Rowland BD and Peeper DS: KLF4, p21 and context-dependent opposing forces in cancer. Nat Rev Cancer 6: 11-23, 2006.
27. Yan J, Roy S, Apolloni A, Lane A and Hancock JF: Ras isoforms vary in their ability to activate Raf-1 and phosphoinositide 3-kinase. J Biol Chem 273: 24052-24056, 1998.
28. Tommasi S, Dammann R, Zhang Z, *et al*: Tumor susceptibility of Rassf1a knockout mice. Cancer Res 65: 92-98, 2005.