Breast Cancer Metastasis Suppressor 1 Inhibits Gene Expression by Targeting Nuclear Factor-κB Activity

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Abstract
Breast cancer metastasis suppressor 1 (BRMS1) functions as a metastasis suppressor gene in breast cancer and melanoma cell lines, but the mechanism of BRMS1 suppression remains unclear. We determined that BRMS1 expression was inversely correlated with that of urokinase-type plasminogen activator (uPA), a prometastatic gene that is regulated at least in part by nuclear factor-κB (NF-κB). To further investigate the role of NF-κB in BRMS1-regulated gene expression, we examined NF-κB binding activity and found an inverse correlation between BRMS1 expression and NF-κB binding activity in MDA-MB-231 breast cancer and C8161.9 melanoma cells stably expressing BRMS1. In contrast, BRMS1 expression had no effect on activation of the activator protein-1 transcription factor. Further, we showed that suppression of both constitutive and tumor necrosis factor-α-induced NF-κB activation by BRMS1 may be due to inhibition of IκBα phosphorylation and degradation. To examine the relationship between BRMS1 and uPA expression in primary breast tumors, we screened a breast cancer dot blot array of normalized cDNA from 50 breast tumors and corresponding normal breast tissues. There was a significant reduction in BRMS1 mRNA expression in breast tumors compared with matched normal breast tissues (paired t test, P < 0.0001) and a general inverse correlation with uPA gene expression (P < 0.01). These results suggest that at least one of the underlying mechanisms of BRMS1-dependent suppression of tumor metastasis includes inhibition of NF-κB activity and subsequent suppression of uPA expression in breast cancer and melanoma cells. (Cancer Res 2005; 65(9): 3586-95)

Introduction
Breast cancer metastasis suppressor 1 (BRMS1) is a known metastasis suppressor gene, and high expression of BRMS1 in both breast cancer and melanoma cell lines significantly reduces their metastatic potential without affecting primary tumor growth (1). BRMS1 is a nuclear protein that contains an imperfect leucine zipper motif and coiled-coiled domains (1), suggesting that it may function as part of a transcriptional complex. Recent studies suggest that BRMS1 inhibits metastasis in part through gene regulation via interaction with histone deacetylases (HDAC; refs. 2, 3). However, the underlying mechanism of BRMS1 metastasis gene regulation remains unclear.

An important component of metastatic dissemination is the proteolysis and degradation of the extracellular matrix (ECM), and among the key mediators of ECM remodeling is the urokinase-type plasminogen activator (uPA), a serine protease that stimulates the conversion of inactive plasminogen to the broad-spectrum protease plasmin (4, 5). Plasmin mediates cellular invasion directly by degrading members of the matrix proteins (6, 7) and indirectly by activating matrix metalloproteinases (MMP; ref. 8). Several groups have shown that uPA plays a critical role in tumor metastasis (9) and that elevated levels of uPA as well as its inhibitor plasminogen activator inhibitor-1 (PAI-1) are strong indicators of poor prognosis in breast cancers (10–14).

Recent studies have shown that the nuclear factor-κB (NF-κB) transcription factor positively regulates uPA expression as well as several other genes implicated in angiogenesis and metastasis (15–17). NF-κB is a heterodimeric transcription factor composed predominantly of p50 (NF-B1) and p65 (RelA) subunits and is activated by the IKK complex (18–20). NF-κB is sequestered in the cytoplasm through its interaction with inhibitors of NF-κB (IκB). Following certain stimuli, a cascade of events leads to the phosphorylation of IκB by the IKK complex and subsequent degradation of IκB through ubiquitination. This in turn leads to the liberation of NF-κB and its translocation to the nucleus where it transactivates NF-κB-responsive genes (21–24). NF-κB is constitutively activated in many cancers, and several studies suggest that it plays a critical role in apoptosis, deregulation of cell growth, and propensity of tumors to metastasize (16, 17, 25–32).

The present study was done to determine mechanisms underlying the suppression of metastasis by BRMS1 in human breast cancer and melanoma cells. We show that highly invasive MDA-MB-231 breast cancer and C8161.9 melanoma cell lines show constitutive NF-κB activity and that BRMS1 expression inversely correlates with suppression of constitutive and tumor necrosis factor-α (TNF-α)–induced activation of NF-κB-dependent uPA gene expression. Furthermore, BRMS1 seems to suppress NF-κB activity by blocking IκBα phosphorylation and degradation. Finally, we show a general inverse correlation between BRMS1 and uPA expression in primary breast tumors. These data suggest that BRMS1 regulates metastatic potential at least in part through the down-regulation of NF-κB-dependent metastasis-related gene expression.

Materials and Methods
Cell lines. The MDA-MB-231 estrogen receptor–negative human breast cancer cell line is highly metastatic to the lung when injected i.v. (33). The C8161 amelanotic human melanoma cell line is metastatic to the lung when injected s.c., i.d., or i.v. in nude mice (34). The C8161.9 is a highly metastatic clone obtained by limiting dilution cloning of parental C8161 cells (35). MDA-MB-231 and C8161.9 cells were grown in DMEM (Life Technologies,
expression on I
polyacrylamide gels, which were dried and exposed to Kodak BioMax film
hours). Nuclear and cytoplasmic protein extracts were isolated and EMSA
protein extracts were prepared from untreated and TNF-
(0-2 hours) in cells stably expressing exogenous BRMS1. Cytoplasmic
assay (Pierce, Rockford, IL). Nuclear proteins (10
A
were resolved on 12% SDS-
ultra 10K filters (Millipore, Bedford, MA) at 4,000
L) was incubated with 100
A
charged by incubation in 1
/C2, and GAPDH were denoted (\(\Delta C_t\)) for each time
course and the difference between \(\Delta C_t\) and the \(\Delta C_t\) value of the GDPD
was calculated as \(\Delta \Delta C_t\). The log(\(\Delta \Delta C_t\)) gave the relative quantification
value of uPA expression.

Western blot analysis. Exponentially growing cells were rinsed twice
with 1× PBS and lysed in buffer containing 50 mmol/L Tris (pH 8.0),
150 mmol/L NaCl, 1% Triton X-100, and 1× cocktail of proteinase inhibitors
(Boehringer, Mannheim, Germany). Protein concentration was determined
using the BCA protein assay kit. Proteins were separated on 12% SDS-PAGE
gels. The resolved proteins were electroblotted on to PVDF membranes
(Millipore) using transfer buffer containing 48 mmol/L Tris, 39 mmol/L
NaCl, 0.1% SDS at room temperature for 1 hour and finally in 0.1% SSC/0.1%
NaCl at 50°C for 45 minutes. Membranes were exposed overnight at –80°C
to Kodak BioMax film. RNA levels were quantified and normalized against
28S RNA for loading.

Real-time PCR quantification. The following uPA primers were
designed using Primer Express version 1.5 (Applied Biosystems, Foster City,
CA): uPA (forward) 5’-GCGTGCTGAGATCCCTTGTC-3’ and uPA (reverse)
5’-GGATCGTTATACATCGAGGGGCA-3’. A single PCR-amplified band was
observed on ethidium-stained gels using standard PCR conditions. Real-
time PCR experiments were done using the SYBR Green PCR Core kit
(Applied Biosystems) according to the vendor’s instructions and an ABI
7900HT (Applied Biosystems) real-time PCR instrument. uPA expression
levels were calibrated using endogenous glyceraldehyde-3-phosphate
dehydrogenase (GAPDH) expression levels as an internal control. The
following GAPDH primers were designed using Primer Express version 1.5:
GAPDH (forward) 5’-GAAGGTTGAGGTGCTGACT-3’ and GAPDH (reverse)
5’-GGATGGTGATGGTCTGTTT-3’. Cycle conditions were 95°C for 10
minutes (AmpliTaq Gold activation) followed by 45 cycles of 95°C for 15
seconds (denaturation) and 60°C for 60 seconds (annealing and extension)
and fluorescence was quantified as a Ct value. The differences between
the mean Ct values of uPA, IκBα, and GAPDH were denoted (\(\Delta C_t\)) for each time
course and the difference between \(\Delta C_t\) and the \(\Delta C_t\) value of the GAPDH
was calculated as \(\Delta \Delta C_t\). The log(\(\Delta \Delta C_t\)) gave the relative quantification
value of uPA expression.

Transient expression of breast cancer metastasis suppressor 1 and
ssII-Bxc. Transient transfections were done on exponentially growing
cells using LipofectAMINE 2000 (Invitrogen) in serum-free DMEM according
to the manufacturer’s instructions with pcDNA.901BRMS1 and ssII-Bxc
/ssII-Boxic (IκBα is a mutant IκBα containing serine-to-alanine substitutions
at amino acid positions 32 and 36) containing plasmids at a range of
concentrations (0-5 μg). Transfected cells were incubated in serum-free
DMEM for 24 hours and medium was collected for uPA and PAI-1 analysis.

Urokinase-type plasminogen activator activity assay. uPA activity
was assayed using the synthetic substrate Pyro-Glu-Glu-Arg-pNA.HCI
(Chemicon, Temecula, CA) in conditioned medium from MDA-MB-231
and C8161.9 cells following 12-hour treatment with or without TNF-α
(20 ng/mL). Cells were grown in serum-free DMEM over a time course
(0-24 hours) and supernatant was collected and centrifuged at 4,000 × g
for 10 minutes. The supernatant was concentrated 10-fold using Amicon
ultra 10K filters (Millipore, Bedford, MA) at 4,000 × g for 30 minutes.
The supernatant (80 μL) was incubated with 100 μL buffer containing
100 mmol/L Tris and 0.5% Triton X-100 (pH 8.8) and 20 μL substrate
for 24 hours at 37°C in 96-well microplates. Reactions were monitored
at 405 nm using a microplate reader.

Northern blot analysis. Total RNA was extracted from cell lines with
TRizol reagent (Life Technologies) according to the manufacturer’s
instructions. RNA (15 μg) was separated on 1.5% agarose gels containing
2.2 mol/L formaldehyde, transferred to nylon membranes (Nytran Super
Charge, Schleicher & Schuell, Dassel, Germany), and UV cross-linked.
Probes for BRMS1 and uPA were amplified by PCR from MCF-7- and MDA-
MB-231-derived cDNA, respectively. PCR products were radiolabeled with
[32P]dATP by random priming using a DNA labeling kit (Ambion Inc.,
Austin, TX). Northern blot hybridization was done using QuickHyb
(Clontech Laboratories, Inc. Palo Alto, CA) following the manufacturer’s
instructions. After hybridization, membranes were washed twice in 0.2%
SSC (1 × SSC is 0.15 mol/L NaCl/0.015 mol/L sodium citrate) containing
0.1% (w/v) SDS at room temperature for 1 hour and finally in 0.1% SSC/0.1%
SDS at 50°C for 45 minutes. Membranes were exposed overnight at –80°C
to Kodak BioMax film. RNA levels were quantified and normalized against
28S RNA for loading.

Electrophoretic mobility shift assay and nuclear factor-κB
activation. Electrophoretic mobility shift assay (EMSA) was done to
determine the effect of BRMS1 expression on NF-κB activation (38). Briefly,
nuclear and cytoplasmic proteins were extracted from exponentially
growing cells, and protein concentration was quantified using BCA protein
assay (Pierce, Rockford, IL). Nuclear proteins (10 μg) were incubated at room temperature for 20 minutes with 32P-end-labeled nucleotide derived from a NF-κB binding sequence (5′-AGTTGAGGGACTTCCAGC-3′) from the immunoglobulin gene promoter (38) and the activator protein-1
(AP-1) transcription factor consensus oligonucleotide (5′-CGCTTGAAGTGT-
CATCAGCCA-3′ (Promega, Madison, WI). For TNF-α-dependent NF-κB
activation, exponentially growing cells were rinsed twice with 1× PBS
and treated with TNF-α (20 ng/mL) in serum-free DMEM for various times (0-2
hours). Nuclear and cytoplasmic protein extracts were isolated and EMSA
was done as described above. Samples were resolved on 5% native
polyacrylamide gels, which were dried and exposed to Kodak BioMax film
(Rochester, NY) at –80°C.

IκBα phosphorylation and degradation. The effect of BRMS1
expression on IκBα phosphorylation and degradation with and without
TNF-α (20 ng/mL) treatment was examined in a time-dependent manner
(0-2 hours) in cells stably expressing exogenous BRMS1. Cytoplasmic
protein extracts were prepared from untreated and TNF-α (20 ng/mL)-
treated cells and quantitated and 50 μg were resolved on 12% SDS-
polyacrylamide gels. Following electrophoret, polyvinylidene difluoride
(PVDF) filters were probed with rabbit polyclonal antibodies specific to
IκBα (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-IκBα (Ser27; Cell
Signaling Technology, Inc., Beverly, MA), and IκBα (Sigma-Aldrich, St.
Louis, MO), and immuneoreactive proteins were detected by enhanced
chemiluminescence (ECL, Amersham Biosciences, Piscataway, NJ) accord-
ting to the manufacturer’s instructions.

Transcription of breast cancer metastasis suppressor 1 and
ssII-Bxc. Transient transfections were done on exponentially growing
cells using LipofectAMINE 2000 (Invitrogen) in serum-free DMEM according
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(ssII-Bx is a mutant ssII-Bx containing serine-to-alanine substitutions
at amino acid positions 32 and 36) containing plasmids at a range of
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human ubiquitin cDNA probe as a quantitation control. Filters were washed and exposed to X-ray film. For quantitation of gene expression, the film was scanned using Storm imager (Molecular Dynamics), and densitometric values of the spots were calculated using ImageQuant 1.2 software (Amersham Biosciences, Sunnyvale, CA).

**Statistical analysis.** Gene expression levels determined by PhosphorImager analysis of BRMS1 and uPA in normal and tumor tissues were compared with paired t test using the Statview (J4.02; Abacus Concepts, Berkley, CA) computer program (39). P was set at <0.05.

**Results**

Breast cancer metastasis suppressor 1 and urokinase-type plasminogen activator gene expression levels are inversely correlated. To provide insight into possible mechanisms underlying BRMS1-regulated metastasis suppression, we examined the expression of uPA, a key metastasis-related gene implicated strongly in breast cancer metastasis. Here, we show an inverse correlation between BRMS1 mRNA expression and uPA mRNA and protein expression (Fig. 1). BRMS1 and uPA mRNA expression was examined in highly metastatic breast cancer cell line MDA-MB-231/pC DNA and melanoma-derived C8161.9/pC DNA cells and compared with mRNA levels in clonally derived stably expressing BRMS1 transfectants MDA-MB-231/BRMS1-11 and MDA-MB-231/BRMS1-13 and C8161.9/BRMS1-6 and C8161.9/BRMS1-11, respectively, which exhibited low metastatic potential (1, 37). Northern blot analysis revealed that uPA mRNA expression was reduced in BRMS1 transfectants compared with parental cells and that the extent of reduction in expression generally correlated with levels of BRMS1 mRNA expression. Secreted uPA protein levels in supernatants were examined from the same cells grown for 24 hours. A similar inverse correlation between BRMS1 mRNA expression and levels of secreted uPA protein was observed. Parental MDA-MB-231 and C8161.9 cells exhibited similar levels of BRMS1 and uPA mRNA expression to MDA-MB-231/pC DNA and C8161.9/pC DNA cells, respectively (data not shown).

NF-κB has been shown previously to regulate uPA expression in MDA-MB-231 cells (16), suggesting that BRMS1 may affect uPA expression at least in part directly or indirectly through interaction with NF-κB. NF-κB is activated in cells in response to several extracellular signals, including treatment with TNF-α. Therefore, to further examine the potential relationship between

![Figure 1. BRMS1 expression and uPA expression are inversely correlated.](image_url)

According to the manufacturer's instructions, briefly, the membrane was prehybridized for 30 minutes with 10 mL ExpressHyb solution (Clontech Laboratories) at 65°C. The 32P-labeled cDNA probes were mixed with 30 μg of Cot-1 DNA (Roche Molecular Biochemicals, Indianapolis, IN), 150 μg of sheared salmon sperm DNA, and 50 μL of 20 × SSC in a total volume of 200 μL. The mixture was heated to 95°C for 5 minutes, incubated at 65°C for 30 minutes, and mixed with 5 mL fresh ExpressHyb solution. The filter was hybridized overnight at 65°C and washed four times at 65°C for 20 minutes with 2× SSC-0.5% SDS, once with 0.2× SSC-0.5% SDS, and finally with 2× SSC for 5 minutes at room temperature. For competitive hybridizations, the filter was stripped at 68°C using probe degradation and removal reagents (Ambion) and hybridized with a human uPA cDNA probe followed by a human ubiquitin cDNA probe as a quantitation control. Filters were washed and exposed to X-ray film. For quantitation of gene expression, the film was scanned using Storm imager (Molecular Dynamics), and densitometric values of the spots were calculated using ImageQuant 1.2 software (Amersham Biosciences, Sunnyvale, CA).

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![Figure 2. A, TNF-α-induced, NF-κB-dependent uPA mRNA expression is suppressed by BRMS1. Total RNA was isolated from exponentially growing cells treated with TNF-α for 0 to 6 hours and total RNA (2 μg) was reverse transcribed. At the indicated time points, gene expression of uPA was analyzed by real-time quantitative PCR and normalized to GAPDH expression as described in Materials and Methods. Relative expression levels of uPA and GAPDH were determined in highly metastatic MDA-MB-231/pC DNA and C8161.9/pC DNA cells and metastasis-suppressed transfectants MDA-MB-231/BRMS1-13 and C8161.9/BRMS1-11 expressing high levels of BRMS1. B, TNF-α induction of NF-κB-dependent uPA protein expression is inhibited by BRMS1. Following exposure of the same cells to increasing concentrations of TNF-α, total secreted uPA protein was determined by Western blot analysis.](image_url)
BRMS1 and NF-κB, we determined whether BRMS1 expression inhibited NF-κB-dependent uPA induction by TNF-α. In Fig. 2A, we show that induction of uPA mRNA expression was reduced and/or delayed in cells expressing high levels of BRMS1 compared with parental MDA-MB-231/pcDNA and C8161.9/pcDNA cells. In these experiments, we examined levels of mRNA expression using real-time PCR amplification following exposure of cells to TNF-α for different lengths of time. uPA gene expression was induced by TNF-α in MDA-MB-231/pcDNA and C8161.9/pcDNA cells as early as 1 hour following exposure, whereas induction was delayed and/or reduced in both MDA-MB-231/BRMS1-13 and C8161.9/BRMS1-11 transfectants. GAPDH was used as an internal control and calibrator in these experiments. Experiments were done in triplicate.

We next determined whether BRMS1 affected dose-dependent TNF-α induction of NF-κB–dependent uPA protein expression and secretion (Fig. 2B). MDA-MB-231/pcDNA and C8161.9/pcDNA and BRMS1 transfectants were exposed to increasing amounts of TNF-α for 24 hours. Western blot analyses revealed a TNF-α dose-dependent increase in the level of secreted uPA protein in MDA-MB-231/pcDNA and C8161.9/pcDNA cells, which was substantially reduced in both MDA-MB-231/BRMS1-13 and C8161/BRMS1-11 transfectants expressing high levels of BRMS1. TNF-α induced uPA protein expression as early as 6 hours in MDA-MB-231 cells and as early as 3 hours in C8161.9 cells (Fig. 3A). BRMS1-expressing MDA-MB-231 and C8161.9 cells showed substantially reduced uPA protein expression compared with parental cells. In addition, we also examined the effect of BRMS1 on TNF-α–induced uPA activity using

Figure 3. Constitutive and TNF-α–dependent NF-κB activation of uPA activity is inhibited by BRMS1 expression. TNF-α–dependent NF-κB activation of uPA activity was examined in MDA-MB-231/pcDNA, C8161.9/pcDNA, and BRMS1-expressing cells treated with TNF-α (20 ng/ml) for 0 to 12 hours in serum-free medium. A, BRMS1-expressing cells show a marked reduction in TNF-α–dependent uPA protein secretion compared with parental cells in both MDA-MB-231 and C8161.9 cells. Relative levels of secreted uPA protein were detected by Western blot analysis. B, BRMS1-expressing cells show a marked reduction in TNF-α–dependent uPA enzyme activity compared with parental cells at all doses of TNF-α. uPA activity was determined using a synthetic substrate Pyro-Glu-Gly-Arg-pNA. HCl in MDA-MB-231, C8161.9, and BRMS1-expressing cells. Relative levels of uPA activity in parental and BRMS1 transfectants are shown graphically.

Figure 4. NF-κB DNA binding activity but not AP-1 binding activity is inhibited by BRMS1 expression. Nuclear proteins were extracted from exponentially growing MDA-MB-231/pcDNA, C8161.9/pcDNA, and corresponding BRMS1 transfectants treated with or without TNF-α (20 ng/ml) for increasing lengths of time in serum-free medium. Nuclear proteins (10 μg) were incubated with 32P-end-labeled NF-κB oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGG-3′) from the immunoglobulin gene promoter and an AP-1 consensus oligonucleotide (5′-CGCTTGATGAGTCAGCCGGAA-3′) and EMSA was done. NF-κB and AP-1 DNA binding activities were examined in the same nuclear extracts. NS, nonspecific interaction. Left, MDA-MB-231/pcDNA and transfectant MDA-MB-231/BRMS1-13. NF-κB DNA binding activity but not AP-1 binding activity was reduced and delayed in BRMS1-expressing cells. Right, C8161.9/pcDNA and transfectant C8161.9/BRMS1-11. As with MDA-MB-231, in C8161.9 cells, NF-κB DNA binding activity but not AP-1 binding activity was substantially reduced and delayed in BRMS1-expressing cells.
Breast cancer metastasis suppressor 1 inhibits IκB phosphorylation and degradation. Phosphorylation and subsequent proteolytic degradation of IκBα facilitates NF-κB translocation to the nucleus where NF-κB in turn binds to consensus sequences on promoters and activates NF-κB-regulated genes. We therefore examined whether constitutive IκB phosphorylation and degradation was reduced by BRMS1 expression. We found that there was a reduction in IκBα phosphorylation in transfectants compared with MDA-MB-231/pDNA and C8161.9/pDNA cells (Fig. 5), consistent with the reduction in constitutive NF-κB binding activity seen in these cells.

To determine whether BRMS1 inhibition of constitutive and TNF-α–induced NF-κB binding was due to the inhibition of proteolytic activity, we did EMSA. We determined that constitutive NF-κB binding activity was suppressed in BRMS1 transfectants compared with MDA-MB-231/pDNA and C8161.9/pDNA cells (Fig. 4) and nontransfected parental cells (data not shown). In general, the level of suppression of NF-κB binding activity inversely correlated with the level of BRMS1 mRNA expression in both MDA-MB-231 and C8161.9 cells. Nuclear protein extracts were prepared from exponentially growing cells following growth of cells for 24 hours in serum-free medium and treatment with or without TNF-α and subjected to EMSA. Activation of NF-κB DNA binding was observed within 20 minutes of treatment with TNF-α in both parental breast cancer and melanoma cell lines, whereas BRMS1 transfectants showed a marked delay and reduction in TNF-α–induced NF-κB activation (Fig. 4).

The AP-1 transcription factor has also been reported to regulate uPA gene expression in MDA-MB-231 cells on treatment with insulin-like growth factor-I (40). To confirm the specificity of suppression of NF-κB binding activity by BRMS1, we also determined the effect of BRMS1 expression on the DNA binding activity of AP-1 (Fig. 4). BRMS1 expression had no effect on AP-1 DNA binding activity in the presence or absence of TNF-α.
Transient expression of breast cancer metastasis suppressor 1 and ssI-Bcα and inhibitor studies. To more directly evaluate the effect of BRMS1 on the expression of uPA through NF-κB activity, we did transient transfections of BRMS1 cDNA. The effect of increasing concentrations of BRMS1 cDNA on uPA expression was compared with the effect of inhibiting NF-κB through mutant IκBα (ssI-Bcα). To accomplish this, we transiently expressed increasing concentrations of BRMS1 cDNA or ssI-Bcα in MDA-MB-231 and C8161.9 cells. Western blot analyses were done on proteins isolated from serum-free medium 24 hours following transient transfection. Increasing concentrations of BRMS1 and ssI-Bcα both led to a similar concentration-dependent reduction in uPA protein expression (Fig. 7) and uPA activity (data not shown) compared with untreated MDA-MB-231 and C8161.9 cells. PAI-1 expression levels were also examined and were unaffected by BRMS1 and ssI-Bcα expression. Transfection efficiencies for MDA-MB-231 and C8161.9 were 37% and 43.4%, respectively.

Overexpression of IκBα kinase β activates nuclear factor-κB and induces urokinase-type plasminogen activator expression in the absence or presence of breast cancer metastasis suppressor 1 expression. To more directly assess whether BRMS1 down-regulates uPA expression through inhibition of NF-κB signaling, we artificially activated NF-κB signaling by overexpressing IKKβ. This was achieved by infecting cells with adenovirus expressing IKKβ or GFP as a negative control. We monitored IκBα degradation and NF-κB-regulated uPA and cyclooxygenase-2 (COX-2) expression in infected parental and BRMS1 cells (Fig. 8). We found that overexpression of IKKβ led to IκBα degradation and induction of degradation of IκBα, we examined cytoplasmic IκBα phosphorylation and degradation by Western blot analysis using IκBα and phospho-IκBα antibodies (Fig. 5). Whereas IκBα phosphorylation and degradation was seen over time in MDA-MB-231/pDNA cells, there was a notable reduction in IκBα phosphorylation and degradation in MDA-MB-231/BRMS1-13 cells. We observed little difference in total IκBα levels between C8161.9 and C8161.9/BRMS1-11 cells following TNF-α stimulation (Fig. 5). However, a prolonged IκBα degradation was consistently observed in parental C8161.9 compared with C8161.9/BRMS1-11 cells. In addition, we observed reduced IκBα phosphorylation in C8161.9/BRMS1-11 compared with parental C8161.9 cells.

We also examined the effect of BRMS1 on TNF-α–induced p65 phosphorylation and translocation of p65/p50 proteins to the nucleus. Consistent with the effect of BRMS1 expression on IκBα phosphorylation and degradation, we observed a time-dependent, TNF-α–dependent induction of p65 phosphorylation and nuclear translocation of p65/p50 as early as 20 minutes in MDA-MB-231/pDNA cells (Fig. 6) and MDA-MB-231 cells (data not shown). In contrast, p65 phosphorylation and p65/p50 translocation were almost completely abolished in MDA-MB-231/BRMS1-13 cells even following treatment with TNF-α. C8161.9/BRMS1-11 cells also showed a suppression of p65 phosphorylation and p65/p50 translocation to the nuclear fraction when treated with TNF-α compared with C8161.9/pDNA cells, although the level of suppression was not as robust as that seen in MDA-MB-231/BRMS1-13 cells (Fig. 6).
of uPA and COX-2 expression, suggesting that IKKβ is one of the major factors controlling NF-κB signaling. These data support a role for BRMS1 in the regulation of uPA through upstream inhibition of NF-κB signaling.

Cancer profiling array: expression of breast cancer metastasis suppressor 1 and urokinase-type plasminogen activator in breast tumors. To determine the relationship between BRMS1 and uPA expression in human cancers, we analyzed a cancer profiling array containing cDNA from 241 human tumor (T) and corresponding normal (N) tissues from individual patients was probed consecutively with 32P-labeled cDNA probes of BRMS1, uPA, and ubiquitin and relative expression levels were quantitated using PhosphorImager. A, we observed a generally consistent reduced expression of BRMS1 only in breast tumors compared with normal tissues, suggesting that the BRMS1 may only affect breast cancer progression (melanoma was not represented on the arrays). B, breast tumor and normal cDNAs in expanded view to show the inverse correlation between expression levels of BRMS1 and uPA. Ubiquitin is shown as a control for cDNA loading. C, a scatter plot analysis shows a >2-fold reduction in BRMS1 mRNA expression in ~90% of the breast tumor samples. X axis, all corresponding breast tumor (●) and normal tissue pairs (○) labeled 1 to 50; Y axis, relative PhosphorImager values of BRMS1 and uPA hybridization. D, graphical summary. A general inverse correlation between BRMS1 and uPA mRNA expression is observed in breast tumors. BRMS1 showed reduced mRNA expression levels in 90% of the tumors versus matched normal tissues (P < 0.0001). In contrast, 88% of the breast tumors showed higher uPA mRNA expression levels than corresponding normal tissues (P < 0.01).
profiling array containing normalized cDNA from tumors and corresponding normal tissues of 241 subjects with different cancers. Included in the array were cDNAs of 50 breast tumors and matched normal breast tissues. We hybridized the array in turn with human BRMS1, uPA, and ubiquitin cDNA probes, and relative expression levels were quantitated using a PhosphorImager (Fig. 9A). We observed a generally consistent reduced expression of BRMS1 only in breast tumors compared with normal tissues, suggesting that loss of BRMS1 expression may be a specific marker for breast cancer progression (melanoma was not represented on the arrays; Fig. 9B). Scatter plot analysis revealed a >2-fold reduction in BRMS1 mRNA expression levels in ~90% of the breast tumor samples compared with matched normal tissues (Fig. 9C). We observed an inverse correlation between BRMS1 and uPA expression in breast tumors with a reduction in BRMS1 mRNA expression levels in 90% (P < 0.0001) of the tumor versus matched normal tissues. In contrast, 88% (P < 0.01) of the breast tumors showed higher uPA mRNA expression levels than corresponding normal tissues (Fig. 9D).

Discussion

NF-κB is known to modulate the expression of genes involved in cancer cell invasion, metastasis, angiogenesis, and apoptosis, and constitutive NF-κB activation has been reported in several different cancers (26, 30, 31, 41–45). This study is the first to implicate NF-κB signaling in the suppression of the metastatic phenotype by the BRMS1 metastasis suppressor gene. We show an inverse correlation between BRMS1 expression and that of the NF-κB-regulated gene uPA in both highly metastatic MDA-MB-231 breast cancer and C8161.9 melanoma cell lines, with elevated BRMS1 expression leading to a reduction in levels of both secreted uPA protein and uPA enzyme activity in these cells. We also show an inverse correlation between BRMS1 expression and both constitutive and TNF-α–induced NF-κB binding activity in these cells. Phosphorylation and degradation of IκBα facilitates NF-κB translocation to the nucleus where it binds to promoter consensus sequences to activate NF-κB-regulated genes, and we show an inverse correlation between BRMS1 expression and levels of constitutive and TNF-α–induced IκBα phosphorylation and degradation. This leads to decreased nuclear translocation of NF-κB p50 and phospho-p65 subunits that would in turn be expected to reduce expression of NF-κB-regulated genes, such as uPA. Related to these findings, the expression of the KISS-1 metastasis suppressor gene has been reported to inhibit NF-κB-regulated MMP9 gene expression, suggesting that inhibition of NF-κB signaling may be a common target of metastasis suppressor genes (46).

The underlying mechanism of regulation of NF-κB signaling by BRMS1 remains to be elucidated. However, our finding that BRMS1 expression leads to the inhibition of IκBα phosphorylation and degradation and subsequently to a reduction of p65 and p50 nuclear translocation in both MDA-MB-231 breast cancer and C8161.9 melanoma cells suggests that constitutive and TNF-α–induced NF-κB suppression by BRMS1 could be through the classic NF-κB IKK pathway. To more directly assess the role of BRMS1 in the inhibition of NF-κB signaling, we activated NF-κB through overexpressing IKKβ. NF-κB activation through IKKβ overexpression resulted in increased uPA expression in both parental and BRMS1–expressing cells, supporting a role for BRMS1 in the upstream inhibition of NF-κB signaling. BRMS1 is reported to be a nuclear protein, and this result suggests that BRMS1 may have additional cellular functions to those described here.

The AP-1 transcription factor has also been reported to regulate the expression of uPA and cell migration of MDA-MB-231 cells (16). Phosphatidylinositol 3-kinase has been shown to regulate expression of uPA through the activation of both AP-1 and NF-κB transcription factors (16). However, we found that BRMS1 expression did not affect AP-1 activity in either MDA-MB-231 or C8161.9 cells, implying that NF-κB and not AP-1 is the major transcription factor responsible for uPA expression in MDA-MB-231 and C8161.9 cells.

NF-κB, like many other inducible transcription factors, regulates gene expression at least in part through histone acetylation-deacetylation reactions (47). The p65/RelA subunit of NF-κB has been shown to activate transcription by interacting alternatively with multiple coactivators or corepressor complexes. Among the corepressors, three members of the HDAC class I family of proteins (HDAC1, HDAC2, and HDAC3) modulate NF-κB transcriptional activity (48–50). TNF-α–induced, NF-κB–dependent gene expression has been reported repressed by HDAC1 and HDAC2 through a direct association between HDAC1 and the p65 subunit of NF-κB (48). HDAC2 seems to exert its influence through its interaction with HDAC1 (48). Recent studies show that BRMS1 interacts with retinoblastoma binding protein-1 and a complex that includes at least seven members of the mammalian Sin3-HDAC complex, including HDAC1 and HDAC2 (3). These data would suggest that BRMS1 could modulate dissociation of NF-κB from its consensus DNA sequence in part through its interaction with HDAC1 and HDAC2. However, our data support an upstream role for BRMS1 in NF-κB signaling by inhibiting IκBα phosphorylation/degradation. Further studies will be needed to determine whether the effects of BRMS1 on NF-κB signaling and histone acetylation-deacetylation reactions are related and how these two pathways may interact to ensure the ability of BRMS1 to suppress metastasis.

We observed a strong correlation between expression of BRMS1 and uPA, a key mediator of ECM remodeling, in both breast cancer and melanoma cells. uPA stimulates the conversion of inactive plasminogen to the broad-spectrum serine protease plasmin (4, 5). This in turn mediates cellular invasion by degrading members of the matrix proteins, such as fibronectin, collagen, and laminin, and by activating MMPs, including MMP2, MMP3, and MMP9 (6–8). Through this action, uPA may promote tumor cell migration and invasion. Although the BRMS1–expressing cells exhibit significantly lower uPA activity, we have shown previously that they do not show significantly reduced invasive abilities (1, 51). This is probably due to redundancies of proteolytic activities of many different proteinases in cancer cells, and it is important to note that uPA and other proteinases likely play a role in many different stages of metastasis besides invasion (52). Proteinases, including uPA, are produced by both tumor and stromal cells. Depending on the tumor, the proportions produced by each compartment vary. Our study does not address modifications in stromal production of uPA but only that produced directly by the breast cancer cells. Modification of uPA production by stromal cells in a BRMS1–expressing microenvironment is possible but has not been measured. The data presented here do not delimit the exact step(s) in the metastatic cascade affected by BRMS1 or uPA. Nonetheless, the roles of both molecules are important in metastasis and our data suggest that they converge to some extent.
Elevated expression of uPA has been reported in colon (53), lung (54), prostate (55), and breast (56) cancers, and several studies have implicated uPA in cancer metastasis, particularly that of the breast (10–14, 57). Using a commercial cDNA dot blot assay, we observed a general inverse correlation between BRMS1 and uPA expression in breast tumor samples with a decrease in BRMS1 mRNA levels seen in 90% of the breast cancers compared with their corresponding normal tissues ($P < 0.0001$). Conversely, 88% of the breast tumor samples showed higher uPA mRNA levels compared with their corresponding normal tissues ($P < 0.01$).

This general inverse correlation between BRMS1 and uPA expression exists in vitro and in vivo in breast cancer cells implies that an important mechanism of BRMS1-mediated metastasis suppression in breast cancer results from its inhibition of NF-$\kappa$B-mediated uPA expression. The resulting reduction in uPA expression would be expected in turn to lead to reduced ECM remodeling and a lower metastatic potential. Despite this observation, we observed no consistent reduction in expression of BRMS1 in any other cancers (melanomas were not represented on the cDNA dot blots). This suggests that the effect of BRMS1 on suppression of metastasis may be cancer specific. That we observed a general inverse correlation between BRMS1 and uPA expression in the majority of primary breast tumors suggests that BRMS1 may also play a role in the growth of primary breast cancer, although in vivo tumorigenesis assays showed a limited effect of BRMS1 expression on primary tumor growth. Further studies will be needed to determine the effect of reduced BRMS1 expression, if any, on primary breast tumor growth.

In summary, we showed a correlation between BRMS1 expression and inhibition of both constitutive and TNF-$\alpha$-induced NF-$\kappa$B activity in both highly invasive MDA-MB-231 breast cancer and CS1861.9 melanoma cell lines, which seems to be through the inhibition of I$\kappa$B phosphorylation and degradation. We also showed an inverse correlation between BRMS1 expression and TNF-$\alpha$-induced activation of the NF-$\kappa$B-dependent gene uPA expression. These studies suggest that at least some of the ability of BRMS1 to suppress breast cancer and possibly melanoma metastasis may be due to its ability to inhibit NF-$\kappa$B-dependent regulation of genes important in the metastatic process, including uPA. Although the mechanism of inhibition of NF-$\kappa$B activation has not been determined, our data suggest that this inhibition may involve the classic IKK pathway.

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