BRMS1 inhibits expression of NF-κB subunit p65, uPA and OPN in ovarian cancer cells

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Summary

Background: Breast cancer metastasis suppressor 1 (BRMS1) is a potent metastasis suppressor of various types of malignancies, including melanoma and ovarian cancer. Unfortunately, the clinical data regarding its role as a true metastatic suppressor and its efficacy as a prognostic marker and therapeutic target remain controversial. This study was designed to investigate the effect of BRMS1 on the invasion and metastasis of human ovarian cancer cells and its potential underlying mechanisms. *Materials and Methods:* BRMS1 small interfering RNAs (siRNAs) or control siRNAs were transfected into the OVCAR3 human ovarian cancer cell line. Invasion and migration activities were assessed using the Transwell invasion and migration assay. Protein levels of nuclear factor-κB (NF-κB) subunit p65, osteopontin (OPN) and urokinase-type plasminogen activator (uPA) were evaluated by Western blot, immunofluorescence and immunocytochemistry methods. *Results:* Successful knockdown of BRMS1 was verified by quantitative real-time RT-PCR and Western blot. The invasion and migration capacities of OVCAR3 cells were significantly enhanced in the BRMS1-silenced group, compared to controls ($p < 0.05$). Silencing of BRMS1 significantly induced the expression of NF-κB subunit, p65, uPA, and OPN proteins. *Conclusions:* BRMS1 inhibits expression of p65, uPA and OPN protein. In turn, this leads to inhibition of ovarian cancer cell invasion and metastasis. This study unveils a potential novel mechanism by which BRMS1 inhibits metastasis of ovarian cancer cells.

Key words: BRMS1; Ovarian cancer cells; Metastasis; uPA; OPN; NF-κB/p65.

Introduction

Ovarian carcinoma is the leading cause of death from gynecologic malignancies worldwide, and has a dismal overall five-year survival rate (30%). The inhibition of invasion and metastasis of ovarian cancer cells is critical to improve the 5-year survival rate. Cancer metastasis is a multi-factor and multi-step process involving complex gene regulations and interactions. The molecular and biochemical mechanisms underlying cancer progression and metastasis remain poorly understood; although, metastasis suppressor genes, such as Non-metastatic 23 (Nm23), Kang ai 1 (KAI1), Raf kinase inhibitory protein (RKIP) and breast cancer metastasis suppressor 1 (BRMS1), have been defined as key modulators of the process [1-4]. As one of the metastasis suppressors, BRMS1 has the ability to inhibit the metastatic potential of cancer cells in vivo without affecting tumorigenicity [5–7]. Recent studies have shown that the nuclear factor-κB (NF-κB) transcription factor positively regulates urokinase-type plasminogen activator (uPA) expression, as well as several other genes implicated in angiogenesis and tumor metastasis [8–10]. One study found that BRMS1 suppressed osteopontin (OPN) gene expression, at least in part, through inhibition of the NF-κB signaling pathway in breast cancer cells [11]. In this study, we investigated whether BRMS1 is capable of suppressing ovarian cancer metastasis and the possible underlying mechanisms of this process. Our results indicate that BRMS1 inhibits the invasion and migration of human ovarian cancer cells through inhibiting expression of the NF-κB subunit p65, uPA and OPN.

Materials and Methods

Cell culture, chemicals, and reagents

The human ovarian cancer cell line, OVCAR3, was obtained from the American Type Culture Collection. Cells were cultured with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 1% penicillin and streptomycin, in an atmosphere of 5% CO_2 at 37°C. Lipofectamine 2000 and TRIzol reagent were used to conduct all transfections.

siRNAs transfection

Targeted small interfering RNAs (siRNAs) for BRMS1 (NM: 015399.3) were synthesized. Three BRMS1-siRNAs were designed to select the most efficient silencing. The three siRNAs were as follows: siRNA1: 5'-CCAUACAUCGUGUACAUGCUU-3'; siRNA2: 5'-GAAGCAGUUCUCGGAGCUAAA-3'; siRNA3: 5'-UUCGUACUUAUUCCUGAUCACAUCC-3'. Negative control siRNA was also synthesized: NC-siRNA: 5'-UUCUCCGAACGUGUCACGUTT-3'. The cells were divided into three groups: experimental group, transfected with BRMS1-siRNA; negative control group with NC-siRNA; and blank control group. Mixtures of siRNA-liposome duplexes were transiently transfected into OVCAR3 cells using Lipofectamine 2000 according to the manufacturer's instructions. FAM-siRNA was used to optimize transfection efficiency. A range of concentrations (0–100 nM) of the BRMS1-siRNAs were tested and inhibition of BRMS1 mRNA expression was confirmed by quantitative reverse transcription (qRT)-PCR after 48-hour transfection.

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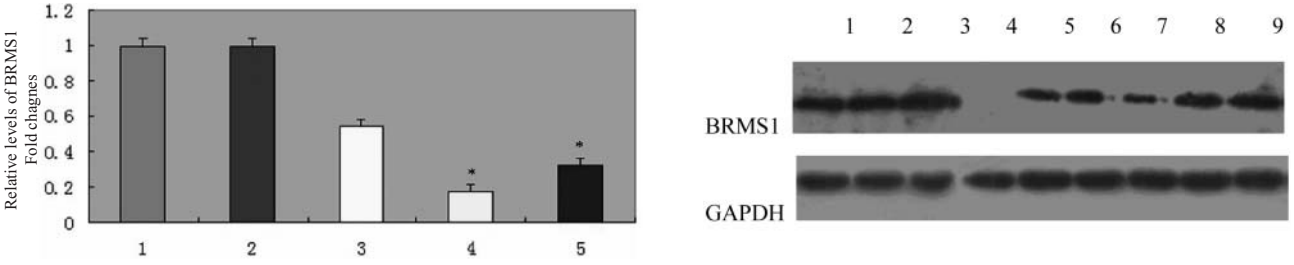


Figure 1. — A) BRMS1-siRNA3 silenced BRMS1 mRNA in a time-dependent manner. Total RNA was isolated from OVCAR3 cells transfected with BRMS1-siRNA3 for up to three days. The expression level of BRMS1 mRNA was determined by qRT-PCR and normalized to that of β-actin. Bars: 1, Blank control; 2, Negative control; 3, 24 hours after BRMS1-siRNA3 transfection; 4, 48 hours after BRMS1-siRNA3 transfection; 5, 72 hours after BRMS1-siRNA3 transfection. * indicates significant differences as compared to the control (48 and 72 hours: $p < 0.05$). B) BRMS1-siRNA3 silenced BRMS1 protein in a time-dependent manner. Cellular protein was collected from cells transfected with BRMS1-siRNA3 for up to three days. The expression of BRMS1 was determined by Western blot and normalized to that of GAPDH. Lanes: 1, 24 hours after BRMS1-siRNA3 transfection; 2, Negative control at 24 hours; 3, Blank control at 24 hours; 4, 48 hours after BRMS1-siRNA3 transfection; 5, Negative control at 48 hours; 6, Blank control at 48 hours; 7, 72 hours after BRMS1-siRNA3 transfection; 8, Negative control at 72 hours; 9, Blank control at 72 hours. The densitometry results are listed at the bottom. * indicates statistically significant differences as compared to the control ($p < 0.05$).

RNA extraction and qRT-PCR

Total RNA was isolated from cells using the TRIzol reagent according to the manufacturer's protocol, and cDNA was synthesized from the RNA using the Prime-Script RT reagent kit according to manufacturer's instructions. Oligonucleotide primers for qRT-PCR of BRMS1 and β-actin (internal control) were designed as follows: BRMS1 (forward): 5'-GCGGAGCCTCAAGATTCGCAT-3'; BRMS1 (reverse): 5'-CTCTCCAGGTGCTGTTTGGCT-3'; β-actin (forward): 5'- ATCTGGCACCACACCTTCTAC-3'; β-actin (reverse): 5'-CAGCCAGGTCCA GACGCAGG-3'. PCR was performed using SYBR Premix Ex Taq according to the manufacturer's instructions. BRMS1 expression levels were normalized to that of β-actin. Cycling conditions were: 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds, and 60°C for 30 seconds. Fluorescence was quantified as a threshold cycle (Ct) value. All experiments were performed in duplicate and repeated twice. The Stratagene Mx3000P real-time PCR System software was used to monitor the amplification process and to determine the Ct for each reaction. The differences between the mean Ct values of BRMS1 and β-actin were denoted as ΔCt and the difference between ΔΔCt and the ΔCt value was calculated. The 2-ΔΔCt gave the relative quantification value of expression. PCR products were observed on ethidium bromide-stained gels.

Western blot

Cellular proteins were extracted from the transfected cells and protein concentration was quantified by using the BCA protein assay kit. Whole cell protein extracts were separated on 12% SDS-PAGE gels and then transferred onto polyvinylidene difluoride membranes. After transferring, gels were stained with Coomassie Blue to confirm equal loading and transfer efficiency. Blocking of non-specific binding was carried out with 5% skim milk in TBST (Tris-buffered saline with 0.05% Tween-20). The primary antibodies used included human mouse monoclonal antibody against BRMS1 and uPA (1:700), human rabbit polyclonal p65 and OPN antibodies (1:400), and anti-GAPDH antibody (1:10000). The blots were incubated overnight at 4°C. After washing, the secondary antibody anti-mouse HRP-conjugate (1:4000) and an anti-rabbit HRP- conjugate (1:5000) were added to the membrane and incubation carried out for one hour. Immunoreactivities to BRMS1, uPA, p65, OPN, and GAPDH proteins were visualized using an Immobilon Western Chemiluminescent HRP Substrate.

Cell invasion and migration assays

In vitro cellular invasion and migration assays were performed by determining the ability of cells to invade through a synthetic basement membrane. Briefly, transfected OVCAR3 cells (5×104 per well in serum-free media) were plated in the top chamber of a Transwell plate and serum-containing media (10%) was added to the bottom chamber as a chemoattractant. Cells were then incubated at 37°C and allowed to invade through the matrigel barrier for 24 hours. Afterwards, filters were removed, fixed and stained with Crystal Violet Staining Solution. Non-invading cells were removed from the top portion and invading cells that had transversed to the underside of the filter were enumerated using an inverted microscope. Experiments were performed for three times, and a minimum of ten grids (100x) were counted per filter.

Immunocytochemical assay

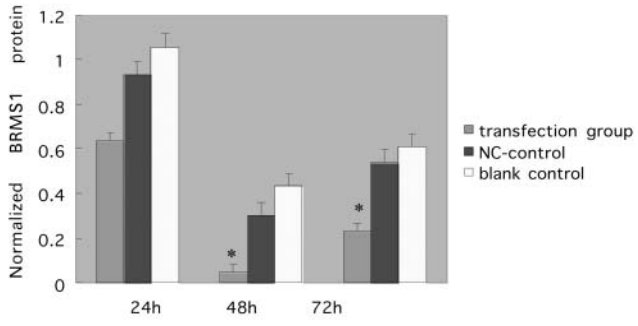
Immunocytochemical staining was carried out by using the SABC kit, according to the instructions from the manufacturer. Briefly, cells transfected with control or BRMS1siRNAs for 24 hours were plated onto coverslips in six-well plates. Afterwards, cells were fixed with 4% paraformaldehyde for 20 minutes, immersed in 0.5% Triton X-100 for 20 minutes, and blocked with 5% BSA for 30 minutes at 37°C. The slides were incubated overnight at 4°C with uPA or OPN antibody (1:100), followed by incubation with HRP-conjugated goat anti-mouse/rabbit secondary antibodies (1:100) for 30 minutes. Coloration was achieved through staining with Diaminobenzidine (DAB). After a series of washes, hematoxylin counterstaining was performed and slides were mounted with neutral gum for imaging (400x) by an inverted microscope.

Immunofluorescence assay

The above-described slides were incubated overnight with OPN antibody (1:100) at 4°C, followed by incubation for one hour with fluorescent secondary antibodies FITC-goat anti-rabbit IgG (1:100). Fluorescent images were obtained using a DM IRBE laser-scanning confocal microscope.

Statistical analysis

Results were calculated as means ± standard deviation (SD). Intergroup differences were analyzed by Student's t-test and *p*-



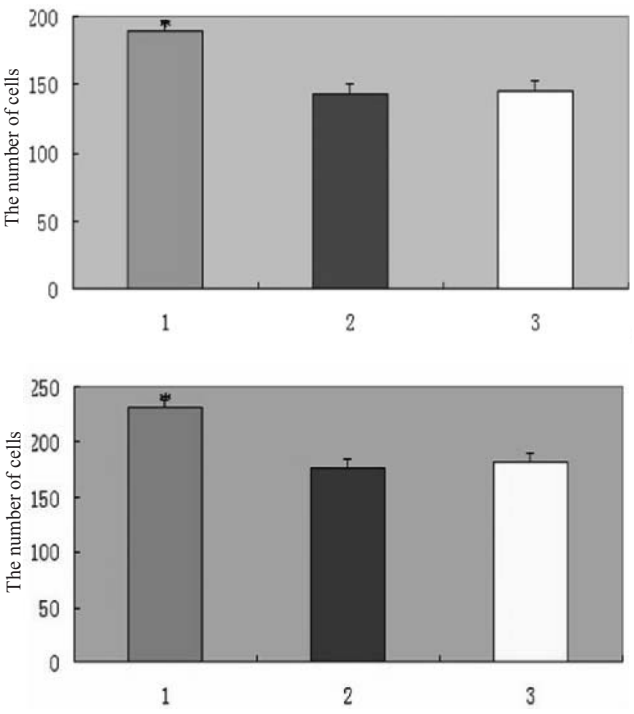


Figure 2. — The effects of siRNA silencing of BRMS1 on the invasion and migration of OVCAR3 cells. (a) Cells were transfected with control or BRMS1-siRNA3 for 48 hours. Afterwards, cell invasion assays were performed to determine the ability of cells to invade through a synthetic basement membrane. (b) Cells were transfected with control or BRMS1siRNA3 for 24 hours and allowed to invade through a matrigel barrier for an additional 24 hours. After incubation, filters were fixed and stained with Crystal Violet staining solution. The non-invading cells were removed and invaded cells on the underside of the filter were enumerated using an inverted microscope. Bars: 1, 48 hours after BRMS1-siRNA3 transfection; 2, Negative control; 3, Blank control. * indicates statistically significant differences as compared to the control ($p < 0.05$).

values < 0.05 were considered statistically significant. SPSS v16.0 was used for all statistical procedures.

Results

Knockdown of BRMS1 by siRNAs

Cells were transfected with three different BRMS1 siRNAs for 48 hours followed by qRT-PCR examination of BRMS1 mRNA expression using BRMS1-specific primers. Only one of the BRMS1 siRNAs (siRNA3) remarkably inhibited BRMS1 mRNA expression (reduced by 82.3%; Figure 1a). The significant reduction of BRMS1 protein expression by siRNA3 was confirmed by Western blot (Figure 1b).

Silencing of BRMS1 enhances the invasion and migration of ovarian cancer cells

Using the matrigel invasion assay we examined the number of invading cells in the three groups (experimental, NC-siRNA, and blank control). BRMS1-siRNA3 cells had 190 ± 8.5 cells with invasive capacity, which was more than either NC-siRNA (144±7.8) or blank control (146 ± 6.8) (Figure 2a). Likewise, BRMS1-siRNA3 cells were more likely to migrate than the NC-siRNA or blank control cells (231 ± 8.9 vs. 177 ± 9.7 and 182 ± 7.9, respectively) (Figure 2b). These results revealed that BRMS1 silencing led to significantly increased cell invasion and migration ($p < 0.05$), suggesting BRMS1 plays a role as a suppressor of invasion and migration of ovarian cancer cells.

Silencing of BRMS1 up-regulates the expression of p65, uPA, and OPN

The authors next used the BRMS1 siRNA knockdown system to test whether BRMS1 could influence the expression of NF-κB signal and uPA and OPN gene expression. Real-time PCR analysis demonstrated that silencing of BRMS1 dramatically up-regulated the expression of NF-κB subunit p65 and uPA ($p < 0.05$) in a time-dependent manner; maximal induction occurred at 72 hours post-siRNA treatment (Figure 3).

The authors then examined the link between the expression level of OPN and BRMS1. They found that the expression of OPN in the BRMS1-silenced cells was dramatically higher than that in the NC-siRNA control cells, as evidenced by immunofluorescence (Figures 4a-b) and by immunocytochemistry (Figures 4d-e). The results indicated that BRMS1 inhibited expression of OPN in ovarian cancer cells.

Similar results were observed for the expression of uPA, by immunocytochemistry (Figures 5a-c); specifically, uPA expression was significantly higher in the BRMS1-silenced cells than that in the NC-siRNA control cells (Figures 5a-b). The results suggested that BRMS1 acts to suppress the expression levels of uPA in OVCAR3 cells.

Discussion

Metastasis is a complex process associated with tumor progression and is comprised of sequential advancement through several biological processes, including tumor an-

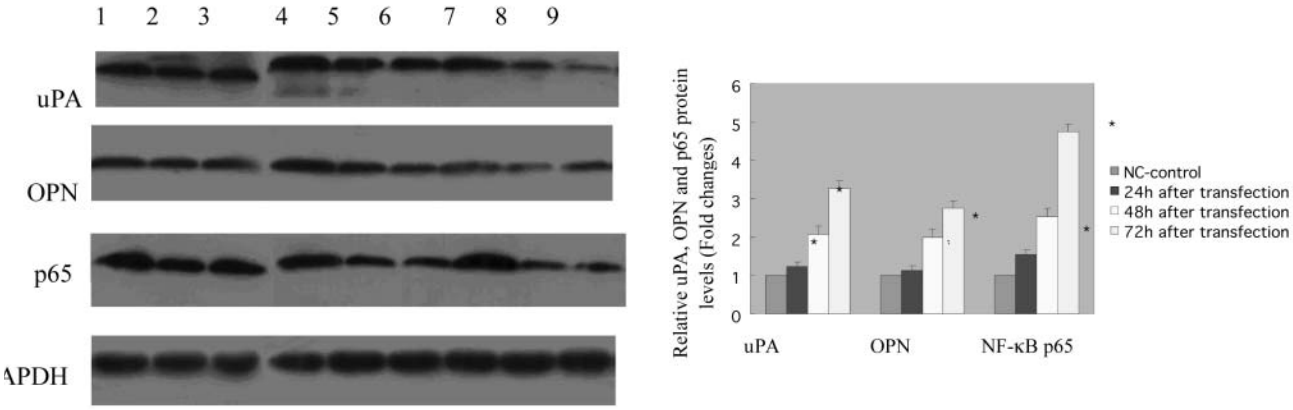


Figure 3. — BRMS1-siRNA3 silencing up-regulated uPA, OPN, and NF-κB/p65 in a time-dependent manner. Cellular proteins were collected from cells transfected with BRMS1-siRNA3 for up to three days. Western Blot detected expression of uPA, OPN, and p65 protein. GAPDH was used as the loading control. The densitometry results are listed at the bottom. * indicates statistically significant differences as compared to the control ($p < 0.05$).

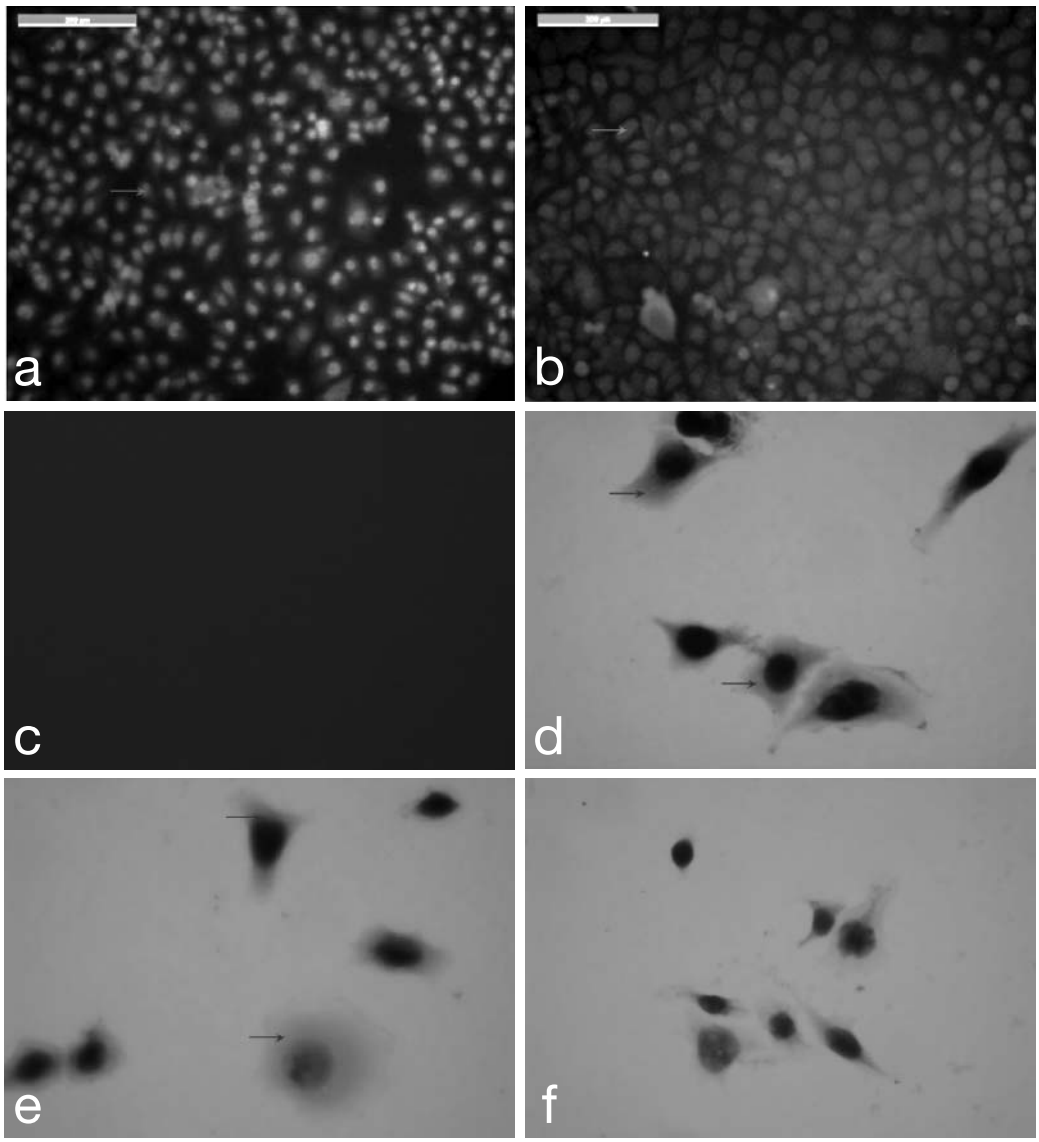


Figure 4. — Silencing of BRMS1 induces OPN protein expression. Cells transfected with BRMS1-siRNA3 were incubated overnight with OPN antibody, followed by incubation with (a-c) fluorescent secondary FITC-goat anti-rabbit IgG or (d-f) HRP-conjugated goat anti-mouse/rabbit secondary antibody. Coloration was achieved through staining with DAB. BRMS1-siRNA3 transfected cells exhibited (a, d) high levels of OPN expression, whereas (b, e) low expression levels were observed in cells transfected with NC-control cells. No expression was detected in the blank control cells (c, f). The OPN was located mainly in the cytoplasm.

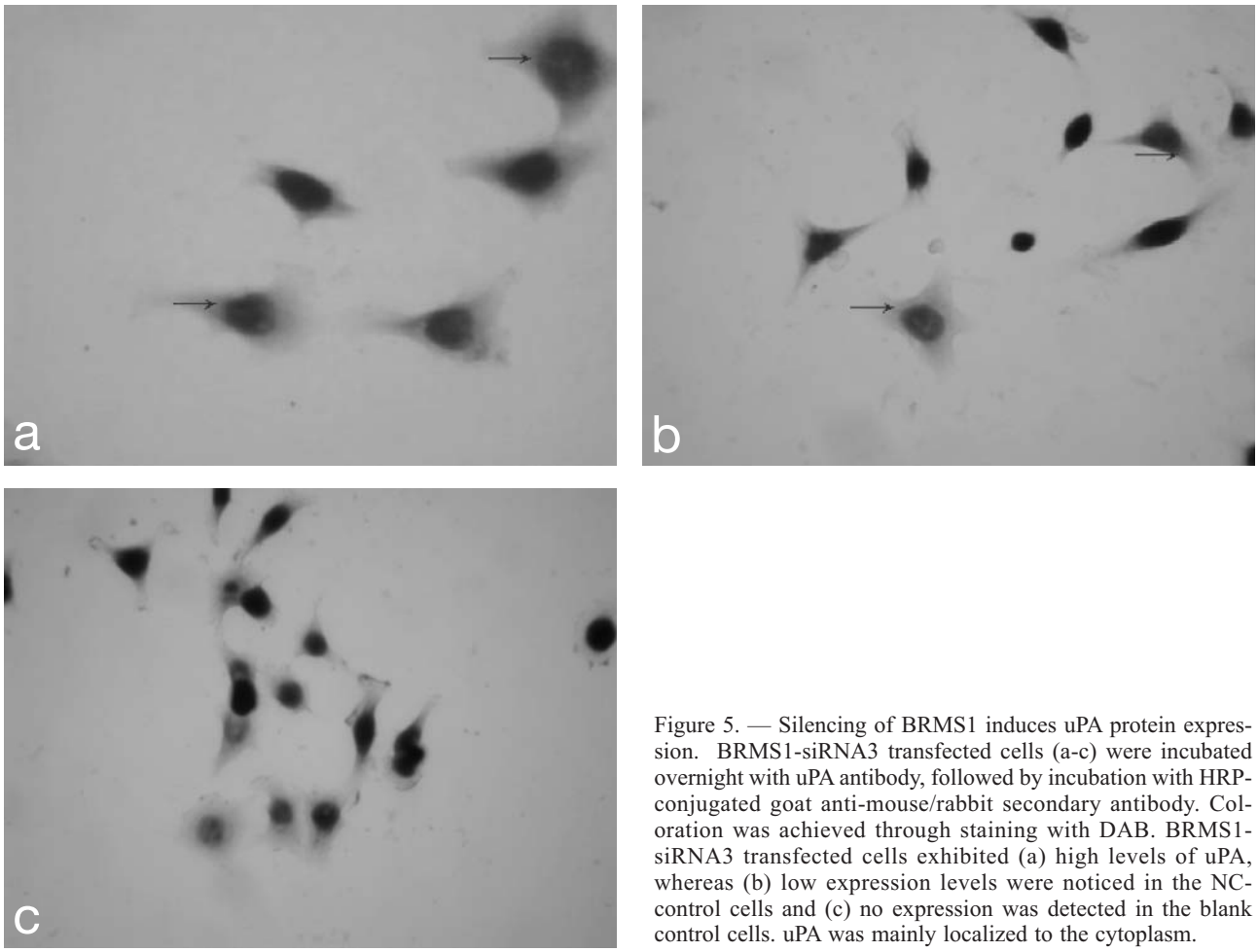


Figure 5. — Silencing of BRMS1 induces uPA protein expression. BRMS1-siRNA3 transfected cells (a-c) were incubated overnight with uPA antibody, followed by incubation with HRP-conjugated goat anti-mouse/rabbit secondary antibody. Coloration was achieved through staining with DAB. BRMS1-siRNA3 transfected cells exhibited (a) high levels of uPA, whereas (b) low expression levels were noticed in the NC-control cells and (c) no expression was detected in the blank control cells. uPA was mainly localized to the cytoplasm.

giogenesis, disaggregation, invasion of the basement membrane (BM) and extracellular matrix (ECM), infiltration into blood vessels, survival in the circulation, extravasation to surrounding tissues, and growth at secondary sites [12]. By gaining a detailed understanding of each of these processes the precise biological profile of cancer growth and metastasis may be elucidated, including key molecules that may represent useful therapeutic targets. Thus, this strategy will also facilitate the development of more efficient targeted therapies for cancer patients.

Ovarian cancer is one of the most common cancers worldwide and leading causes of death from gynecological malignancies. In 2009, approximately 22,000 women were newly diagnosed with ovarian cancer in the USA alone, and an additional 15,000 deaths were attributed to this disease as well [13]. Inhibiting the invasion and metastasis capacities of ovarian cancer is critical to improving patient survival rates. However, our understanding of the mechanisms underlying these critical steps remains incomplete, thereby limiting efforts to improve the survival rate.

BRMS1, a tumor metastasis suppressor gene, has been shown to inhibit malignant tumor invasion and metastasis in several cancers, such as melanoma, bladder cancer, and ovarian cancer [14-16]. The anti-invasion and anti-migration properties of BRMS1 have been experimentally evidenced using ovarian cancer cells [14].

Invasion through BM to blood vessels and then to the secondary sites involves secretion of chemokines that enhance tumor cell motility in a certain direction, while proteolytic enzymes contribute to ECM degradation [17]. Degradation of ECM and BM further promotes invasion and metastasis of cancer cells. uPA is one of the serine-specific proteinases involved in ECM degradation. Elevated levels of uPA showed to be associated with cancer cell progression in many types of cancers, including ovarian cancer [18, 19]. The mechanism by this was, at least in part, involved in NF-κB signaling [9, 20, 21].

NF-κB is a family of transcription factors that exist as homo and heterodimers. Several members, including p65, p50, RelB, and c-Rel molecules, have been demonstrated to be involved in the regulation of multiple cellular processes, such as immune response, inflammation, cell proliferation,

and apoptosis [22]. NF-κB is recognized as an anti-apoptotic factor and protects cancer cells from apoptosis in order to facilitate tumor progression. Furthermore, NF-κB promotes transcription of genes that are associated with tumor metastasis, such as Interleukin-1 (IL-1), Interleukin-2 (IL-2), tumor necrosis factor-α (TNF-α), uPA [18, 23], and OPN [20, 24, 25].

Studies have shown that the NF-κB signaling pathway is also involved in tumor metastasis [20, 24, 25]. A recent study suggested that NF-κB could be an effective diagnostic biomarker of early-stage epithelial ovarian cancer (EOC) [26]. NF-κB is known to modulate the expression of genes involved in cancer cell invasion, metastasis, angiogenesis, and apoptosis, and constitutive activation of NF-κB has been clinically observed in several cancer cell types [21,27-29]. The authors demonstrated, here, that BRMS1 significantly reduces NF-κB subunit p65, suggesting a role of this transcription factor in the ovarian cancer malignant process. Consistent with this, another study found that blockade of NF-κB by BRMS1 leads to down-regulation of OPN expression in MDA-MB-435 melanoma cells [29]. Thus, compounds that block NF-κB signal may be useful as potential therapeutic agents to inhibit tumor invasion and migration.

As a member of the ECM family, OPN also regulates expression and activation of matrix metalloproteinases (MMPs), and plays a significant role in ECM degradation and in facilitating cell motility, tumor growth, and metastasis by interacting with integrin receptors. Induced cellular motility by OPN is a major step in cancer metastasis and involves the up-regulation of certain genes known to promote cell invasion [30]. Overexpression of OPN has been clinically detected in many human cancers [31]. Still other studies have indicated that BRMS1 can inhibit OPN expression in breast cancer and melanoma cells, suggesting that this functional interplay might contribute to suppression of cancer metastasis [15, 29, 32].

The present data showed that invasion and migration activities were enhanced dramatically in cells with silenced BRMS1. Meanwhile, these data were consistent with other studies demonstrating that BRMS1 can negatively regulate NF-κB binding activity [11, 29]. Using Western blot, immunofluorescence, and immunocytochemistry, the present authors demonstrated that BRMS1 does indeed act as a tumor suppressor in OVCAR3 cells, likely by inhibiting expression of p65, uPA and OPN. These data indicate a mechanistic profile by which BRMS1 might control ovarian cancer cell survival. Similarly, BRMS1 has been shown to inhibit OPN expression by modulating the activity of NF-κB signaling in breast cancer [29], and decreasing OPN has been suggested to contribute to inhibition of breast cancer metastasis [32]. Cicek *et al.* [21] reported that BRMS1 possibly regulated metastasis by influencing phosphoinositide signaling or recruiting of HDAC1 to NF-κB binding site of the uPA promoter.

In summary, the present findings suggest that BRMS1 inhibits human ovarian cancer cell invasion and metastasis by reducing the expression of NF-κB-p65, uPA and OPN proteins. It is possible the NF-κB mediates the effect of BRMS1 on reduction of uPA and OPN although this needs to be determined in our system. Further studies are expected to explore the detailed mechanism.

Acknowledgments

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