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Expression of the Breast Cancer Metastasis Suppressor 1 (BRMS1) maintains *in vitro* chemosensitivity of breast cancer cells

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Abstract

The Breast Cancer Metastasis Suppressor 1 (BRMS1) belongs to an expanding category of proteins called *metastasis suppressors* that demonstrate *in vivo* metastasis suppression while still allowing growth of the orthotopic tumor. Since BRMS1 decreases either the expression or function of multiple mediators implicated in resistance to chemotherapy (NF-κB, AKT, EGFR), we asked whether breast carcinoma cells expressing BRMS1 could be sensitized upon exposure to commonly used therapeutic agents that inhibit some of these same cellular mediators as BRMS1. In this report, we demonstrate that chemosensitivity of breast cancer cells is preserved in the presence of BRMS1. Further, BRMS1 does not change expression of AKT isoforms or PTEN, implicated in chemoresistance to common drug agents. Overall, our data with two different metastatic breast cancer cell lines indicates that BRMS1 expression status may not interfere with the response to commonly used chemotherapeutic agents in the management of solid tumors such as breast cancer. Since tumor protein expression analysis increasingly guides therapy decisions, our data may be of clinical benefit in disease management including profiling for BRMS1 expression before start of therapy.

Keywords

breast cancer metastasis suppressor 1 (BRMS1); chemoresistance; 3D-culture; breast cancer; metastasis

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Conflict of Interest statement

The authors declare no conflicts of interest.

Introduction

The Breast Cancer Metastasis Suppressor 1 (BRMS1) belongs to an expanding class of proteins called *metastasis suppressors* that demonstrate *in vivo* metastasis suppression while allowing growth of the orthotopic tumor [1–3]. BRMS1 functions as a metastasis suppressor in animal models of breast [4], melanoma [5], ovarian carcinomas [6]. Recent studies with clinical samples have indicated a correlation between loss of BRMS1 expression and poor prognosis in a subset of patients [7–9]. Experimentally, loss of metastasis suppressors, including BRMS1 may be reversed using therapeutic agents [10,11] suggesting use of BRMS1 and other metastasis suppressors as markers and a potential adjuvant role of such "re-expression therapy" in the management of metastasis. Experimentally, BRMS1 expression increases susceptibility to anoikis which is proposed to contribute, in part, to metastasis suppression [12,13]. BRMS1 is part of the Sin3-HDAC chromatin remodeling complexes [14,15] that regulate gene expression and which could potentially alter chemotherapeutic responses [16]. Consequently, BRMS1 regulates expression of several signaling intermediates including epidermal growth factor receptor [17], osteopontin [18,19], phosphatidylinositol (4,5) bisphosphate (PtdIns(4,5)P_2) [20], urokinase plasminogen activator [21], fascin [6], and connexins [22]. Further, BRMS1 regulates nuclear factor-kappa B (NF-κB) activity [21] and AKT phosphorylation [17] in response to exogenous stimuli implicated in chemoresistance in a number of cancer models [23–25]. Recently, Rivera and colleagues suggested that BRMS1 expression may increase chemosensitivity as a consequence of downregulation of 14-3-3-γ, sorcin, and Hsp27 [26].

Taken together, since BRMS1 decreases either the expression or activity of multiple mediators implicated in resistance to chemotherapy (e.g. NF-κB, AKT, EGFR) and increases susceptibility to anoikis, we asked whether breast carcinoma cells expressing BRMS1 could respond differently upon exposure to commonly used therapeutic agents in the treatment of breast cancer. In this report, using multiple approaches we evaluated that chemosensitivity of breast cancer cells is preserved in the presence of BRMS1. Further, BRMS1 does not change expression of AKT isoforms or PTEN, implicated in chemoresistance to common drug agents. Information from these studies may be potentially used in the clinic in stratifying patients and designing treatment courses in the management of metastatic disease.

Materials and methods

Cell culture

MDA-MB-231 and MDA-MB-435 breast adenocarcinoma cells [27] were transfected with a lentiviral vector construct expressing BRMS1 under the control of a cytomegalovirus promoter [13]. MDA-MB-231/435 vector transfectants (231/435), and 231^{BRMS1}/435^{BRMS1} were cultured in a 1:1 mixture of Dulbecco's-modified essential medium (DMEM) and Ham's F-12 medium supplemented with 1% non-essential amino acids, and L-glutamine (Invitrogen, Carlsbad, CA) and containing 5% fetal bovine serum (cDMEM-F12). 231 and 231^{BRMS1} cells were passaged using 0.125% trypsin and 2 mM EDTA solution (Invitrogen, Carlsbad, CA) and 435 and 435^{BRMS1} cells were passaged using 2 mM EDTA in Ca^{2+}/Mg^{2+}- free PBS. Cell lines were confirmed to be free of Mycoplasma contamination using PCR (TaKaRa, Japan). No antibiotics or antimycotics were used.

Chemotherapeutic agents

Doxorubicin, vincristine were dissolved in water and 5-fluorouracil (5-FU), paclitaxel were dissolved in dimethyl sulfoxide. Stock solutions of doxorubicin (10 mM), vincristine (1 mM) were stored at 4 C and 5-FU (500 mM), paclitaxel (1 mM) were stored at −20°C according to manufacturer's instructions. For final drug concentrations, solutions were serially diluted in media and added to wells. The highest doses of doxorubicin, vincristine, 5-FU, and paclitaxel

used for assays were 20 μM, 1 μM, 2000 μM and 1 μM respectively. All drugs were purchased from Sigma-Aldrich, St. Louis, MO and were used within one week of preparation.

Clonogenic assay

Cells (231/231^{BRMS1} and 435/435^{BRMS1}) were passaged and allowed to proliferate to 70% confluence in 10 cm dishes for at least 2 passages to ensure log growth before harvesting for seeding. Cells were seeded in triplicate at a density of 1000 cells/well onto 6-well plates (Corning) in a final volume of 2 ml media and allowed to attach overnight. The following day, drugs were added at the indicated final concentrations in a volume of 2 ml media and incubated with cells for 4 h. Drug containing media was aspirated gently after 4 h and replaced with 5 ml fresh pre-warmed medium in each well [28]. Cells were left undisturbed in a humidified incubator at 37°C for 7 days and colonies formed were counted by staining with a 1:3 solution of acetic acid:methanol containing 0.01% crystal violet (Carnoy's fixative). Experiments were performed in triplicate and repeated at least twice independently.

MTT indirect assay for proliferation

Exponentially growing 231/231^{BRMS1} and 435/435^{BRMS1} cells were plated at a density of 2500 cells/well in 24-well plates in quadruplicate and allowed to attach overnight. The next day, drugs were added at the indicated concentrations in a final volume of 500 μl and cells were exposed to drugs for 4 h. After 4 h, drug containing media was gently aspirated and replaced with 1 ml of fresh medium and cells were incubated undisturbed in a humidified incubator until the time of assay. Every second day beginning the day of drug addition, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO) was added to the media in each well at a final concentration of 0.5 mg/ml and incubated with cells for 3 h. Following incubation with MTT, medium was aspirated gently and 500 μl of DMSO was added to each well and plates were shaken on a horizontal shaker for 30 minutes to dissolve the formazan crystals. Absorbance was read in a plate reader at 490 nm and experiments were repeated independently at least twice.

Chemosensitivity in 3D-culture

Exponentially growing 231/231^{BRMS1} and 435/435^{BRMS1} cells were plated at a density of 5000 cells/well on 8-well chamber slides (Nunc, Nalgene). Before plating cells, each well was coated with a Matrigel cushion (40 μl). The final concentration of Matrigel above the cushion layer was adjusted to 10%. Media containing cells (200 μl) was mixed with a 20% Matrigel suspension in cold media containing drug at 2X of the indicated concentrations (200 μl). The two suspensions were mixed together gently to ensure equal distribution of cells and drugs and incubated in a humidified incubator at 37°C. Cells were incubated for nine days and colonies formed were counted. Experiments were repeated independently at least twice.

Immunoblotting

Cells grown to approximately 80% confluence on 100 mm dishes were rinsed 2X with ice-cold PBS cells and lysed in a buffer containing 25 mM Tris-HCl (pH 7.4), 50 mM β-glycerol phosphate, 0.5 mM EDTA, 5% glycerol, 0.1% Triton X-100, 1 mM sodium orthovanadate, 1 mM benzamidine, and protease inhibitor cocktail containing aprotinin, leupeptin, and phenylmethylsulfonyl fluoride (Roche, Indianapolis, IN). Protein concentration was determined using a Bradford colorimetric assay (Pierce, Rockford, IL). Protein was denatured with Laemmli's buffer at 95°C for 5 min and lysate equal to 50 μg total protein was loaded onto each well. Proteins were separated using either 8% or 12% SDS-PAGE gels and resolved proteins were transferred to PVDF membranes. Membranes were incubated in Tris-buffered saline containing 0.05% Tween-20 and 5% fat-free dry milk for 1 h at room temperature. Membranes were incubated with primary antibodies to AKT1, AKT2, AKT3, PTEN (Cell

Signaling, Danvers, MA) overnight at 4°C and subsequently with HRP-conjugated secondary antibody at room temperature for 2 h. Gels were either run separately for each protein or membranes were stripped with stripping buffer (Pierce, Rockford, IL) and re-probed. Signals were visualized using ECL (Amersham Biosciences, Piscataway, NJ) following manufacturer's instructions. Blots were probed with an anti-β-actin antibody (Sigma, St. Louis, MO) to confirm equal protein loading.

Results

BRMS1 expression does not affect clonogenicity in response to chemotherapeutic agents

Clonogenic assays were used to determine dose-responses over a minimum of four log concentration range for each drug with 231/231^{BRMS1} and 435/435^{BRMS1} cells. IC_{50} values and corresponding confidence intervals were determined from the dose-response curves generated using Prism software (GraphPad Software, La Jolla, CA) (Fig. 1, Table 1). A trend towards BRMS1-expressing cells forming fewer colonies was observed with most drugs in 231 as well as 435 cells, although statistical significance was not reached. IC_{50} values were similar in BRMS1-expressing *vs.* vector transfected cells (Fig. 1, Table 1).

Expression of BRMS1 does not affect proliferation of breast cancer cells in the presence of chemotherapeutic agents

Consistent with previous results, expression of BRMS1 did not affect proliferation of either 231 or 435 cells [4,13]. Addition of chemotherapeutic agents inhibited the proliferation of vector transfected cell lines in a dose-dependent manner as measured by MTT assays. Following exposure to chemotherapeutic agents, BRMS1 expressing cells showed a dose-dependent decrease in proliferation that paralleled decreases observed with the vector transfectants (Fig. 2).

Breast cancer cells expressing BRMS1 are equally sensitive to chemotherapeutic agents in 3D-culture

Cells grown in 3D-culture exhibit distinct structural, behavioral phenotypes and drug responses compared to 2D-culture [29–31]. Further, responses to chemotherapy appear to be distinct in 2D- *vs.* 3D-culture [32]. Therefore, we tested whether the response of BRMS1- expressing cells to chemotherapeutic agents was modified under 3D-culture conditions. Consistent with observations that cells in 3D-culture display differential drug responses [29–31], IC_{50} values obtained were different from those obtained in clonogenic assays (Fig. 3A, Table 1, and Table 2). A dose-dependent decrease in colony formation was observed in 435 and 435^{BRMS1} cells. BRMS1-expressing cells tended to form fewer colonies in 3D-culture compared to 435 cells, however, the results were variable and IC_{50} differences did not reach statistical significance (Fig. 3A, Table 2). Similar results were obtained with 231 and 231^{BRMS1} expressing cells (Table 2). Although not quantified, colony sizes between vector transfectants and BRMS1-expressing cells with or without addition of chemotherapeutic agents were also not appreciably different, although colony size decreased with increasing drug concentrations.

BRMS1 does not change expression of AKT isoforms or PTEN

Since chemoresistance has been correlated with increased expression of AKT, expression of AKT isoforms in vector-transfected *vs.* BRMS1-expressing cells was tested by immunoblotting. Basal protein levels of AKT1 and AKT2 did not change significantly in BRMS1 expressing cells or changes were not consistent between cell lines (Fig. 3B). AKT3 was undetectable in either cell line or BRMS1-expressing cells. Since BRMS1-expressing cells have >95% lower levels of PtdIns(4,5)P2 [20], we also examined whether PTEN (catalyzes

conversion of PtdIns(3,4,5)P2 to PtdIns(4,5)P2) expression was altered by BRMS1 expression. PTEN protein expression was unchanged in BRMS1 expressing cells.

Discussion

Innate or acquired chemoresistance to therapeutic agents remains a challenge in the management of cancer and especially metastasis. Multi-drug therapy is often targeted at the heterogeneous residual cancer cells, usually following surgical resection, with the hope of preventing recurrence. In addition to standard-of-care drugs, the choice of particular chemotherapeutics is often guided by histo- and/or expression analysis of various markers - dependent on cancer type [33]. Expression profiling of tumors before initial chemotherapy is becoming increasingly common and has aided in the development of targeted therapy including Herceptin® and Avastin®.

Histochemical analysis reveals that clinically, loss of BRMS1 is correlated with poor prognosis in a subset of breast carcinomas [7–9]. Further, BRMS1 expression is lost in higher grade ovarian [6] and supraglottic laryngeal carcinomas [34]. At the molecular level, increased AKT and NF-κB activity have been demonstrated to contribute to chemoresistance [23–25]. BRMS1 selectively decreases AKT phosphorylation in response to growth factors [17] and downregulates NF-κB activity [21]. Although we show here that BRMS1 does not modulate PTEN expression, PtdIns(4,5)P_2 is decreased by >95% in BRMS1-expressing cells [20]. Since BRMS1 downregulates a number of mediators of chemoresistance, and since BRMS1 expression appears to be important in determining metastatic fate, these data provided a compelling rationale to test the hypothesis that BRMS1 would increase chemosensitivity of breast cancer cells. To our surprise, BRMS1 expression did not alter chemosensitivity of breast cancer cells in multiple *in vitro* assays.

Chemotherapeutic agents in this study were selected based on their known mechanisms of action, and their ability to antagonize or act through some of the same pathways mediated by BRMS1. For example, doxorubicin has been demonstrated to decrease levels of PtdIns(4,5)P_2[35] while vincristine mediates cell death, in part, by activation of pro-apoptotic genes through NF-κB [36,37]. Previous data indicates that BRMS1 expression did not alter *in vitro* proliferation or rate of *in vivo* tumor growth [4,13,38,39]. Halogenated pyrimidines including 5-FU are metabolized to derivatives that replace thymidylate in actively dividing cells [40]. The anthracycline antibiotic doxorubicin (adriamycin) forms a trimer-complex with topoisomerase-II and DNA and also intercalates with DNA in dividing cells preventing DNA replication. The relative lack of effect of 5-FU and doxorubicin on both colony formation and proliferation of BRMS1-expressing cells may therefore be explained by the lack of change in proliferation rate by BRMS1 *in vitro*, although a comparable dose-dependent decrease in assay end-points is observed. Doxorubicin is also reported to decrease PtdIns(4,5)P_2 levels [35] (as BRMS1 does), however, no potentiation of growth inhibition was observed when BRMS1-expressing cells were exposed to doxorubicin. The vinca alkaloid vincristine and the diterpenoid paclitaxel both bind β-tubulin and disrupt microtubule polymerization and disassembly respectively. BRMS1 has recently been shown to downregulate β-tubulin 6 (TUBB6) expression by microarray analysis [41]. In the presence of downregulation of TUBB6, BRMS1-expressing cells show no apparent disruption in cell-cycle progression (data not shown). Inhibition of NF-κB is known to sensitize tumor cells to microtubule disrupting agents such as paclitaxel and vincristine [42]. Reports also indicate that activation of NF-κB following vincristine exposure is perhaps required for inducing pro-apoptotic genes [36,37]. The apparent lack of change in survival of BRMS1-expressing cells may reflect antagonistic signaling through NF-κB. Expression analysis of common mediators of chemoresistance including AKT isoforms and PTEN also did not show consistent changes in BRMS1-

expressing cells suggesting that the lack of increased sensitivity to drugs tested may not involve these mediators.

Overall, our data with two different breast cancer cell lines indicates that BRMS1 expression status does not modify the response to commonly used chemotherapeutic agents although the mechanisms of actions of these drugs and BRMS1 intersect at various levels of signaling. Since tumor protein expression analysis increasingly guides therapy decisions, and since loss of BRMS1 is predictive of metastatic outcome [7], our data may be of translational benefit in directing the course of disease management including profiling for BRMS1 expression before start of therapy.

Acknowledgments

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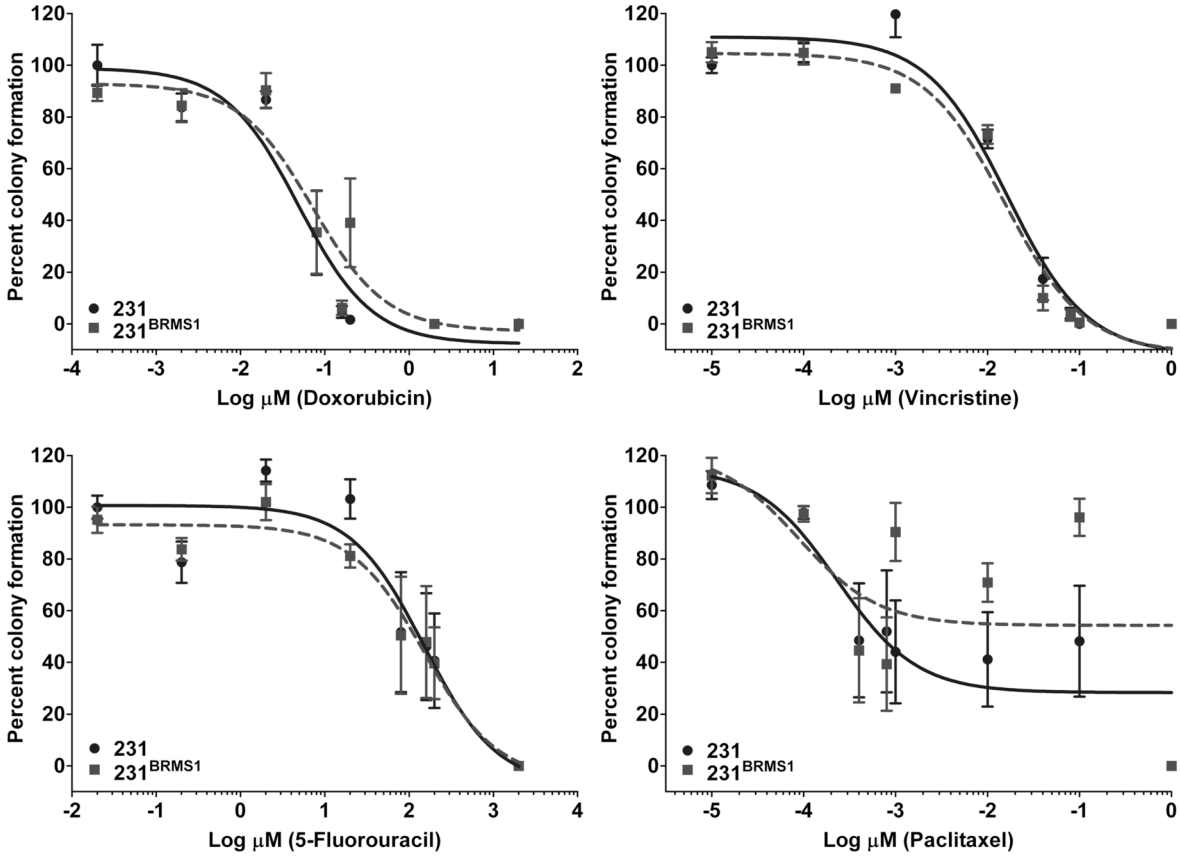

Figure 1.

BRMS1 does not modify survival in clonogenic assays in response to chemotherapeutic agents. 231/231^{BRMS1} and 435/435^{BRMS1} cells were plated at a density of 1000 cells/well of a 6-well plate and allowed to attach overnight. The next day, cells were exposed to drugs at the indicated concentrations for 4 h followed by replacement of drug containing medium with fresh medium. Cells were left undisturbed in a humidified incubator for 7 days at 37°C. At the assay end point, colonies formed were stained and counted. Similar dose-response curves were generated for 435/435^{BRMS1} cells (data not shown).

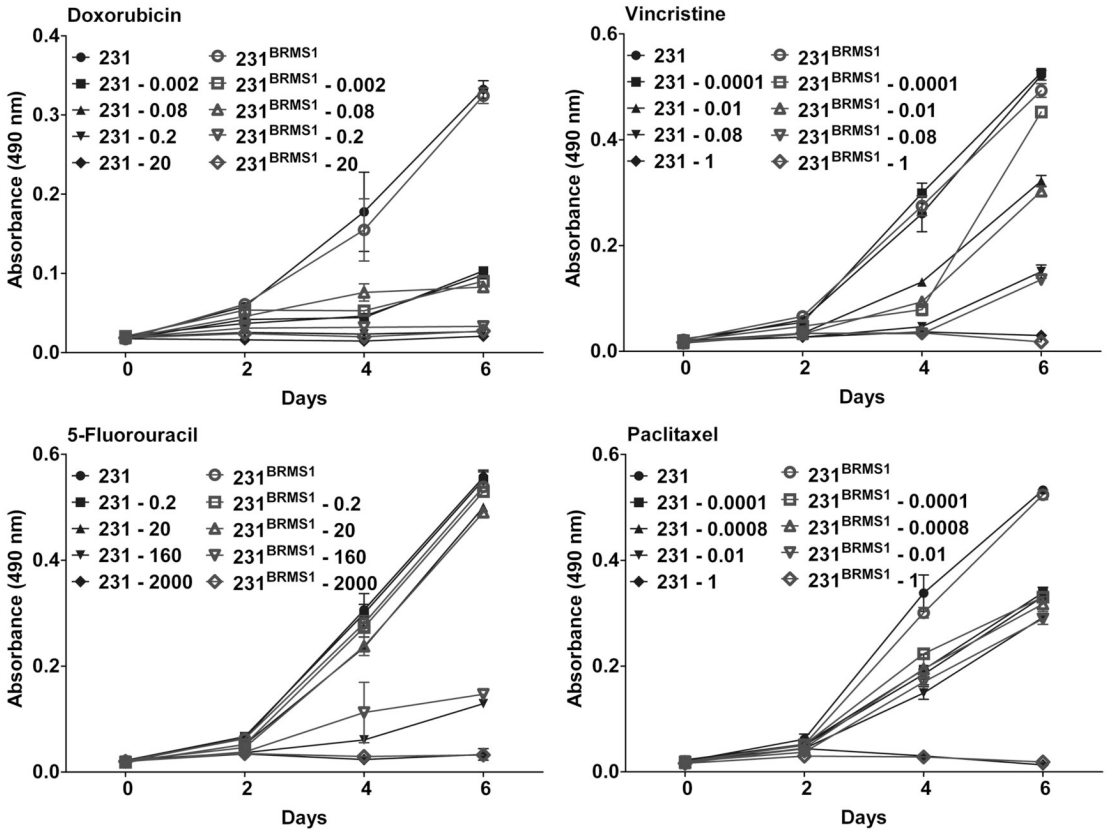

Figure 2.

BRMS1 does not change proliferation of breast cancer cells in response to chemotherapeutic agents. 231/231^{BRMS1} and 435/435^{BRMS1} cells were plated at density of 2500 cells/well of 24-well plates and allowed to attach overnight. The following day, cells were exposed to drugs for 4 h and media was replaced with fresh medium. At each assay point, MTT was added to each well at a final concentration of 0.5 mg/ml and incubated for 3 h. Formazan crystals were dissolved in DMSO and absorbance was recorded on a plate reader at 490 nm. Similar curves were generated for 435/435^{BRMS1} cells (data not shown).

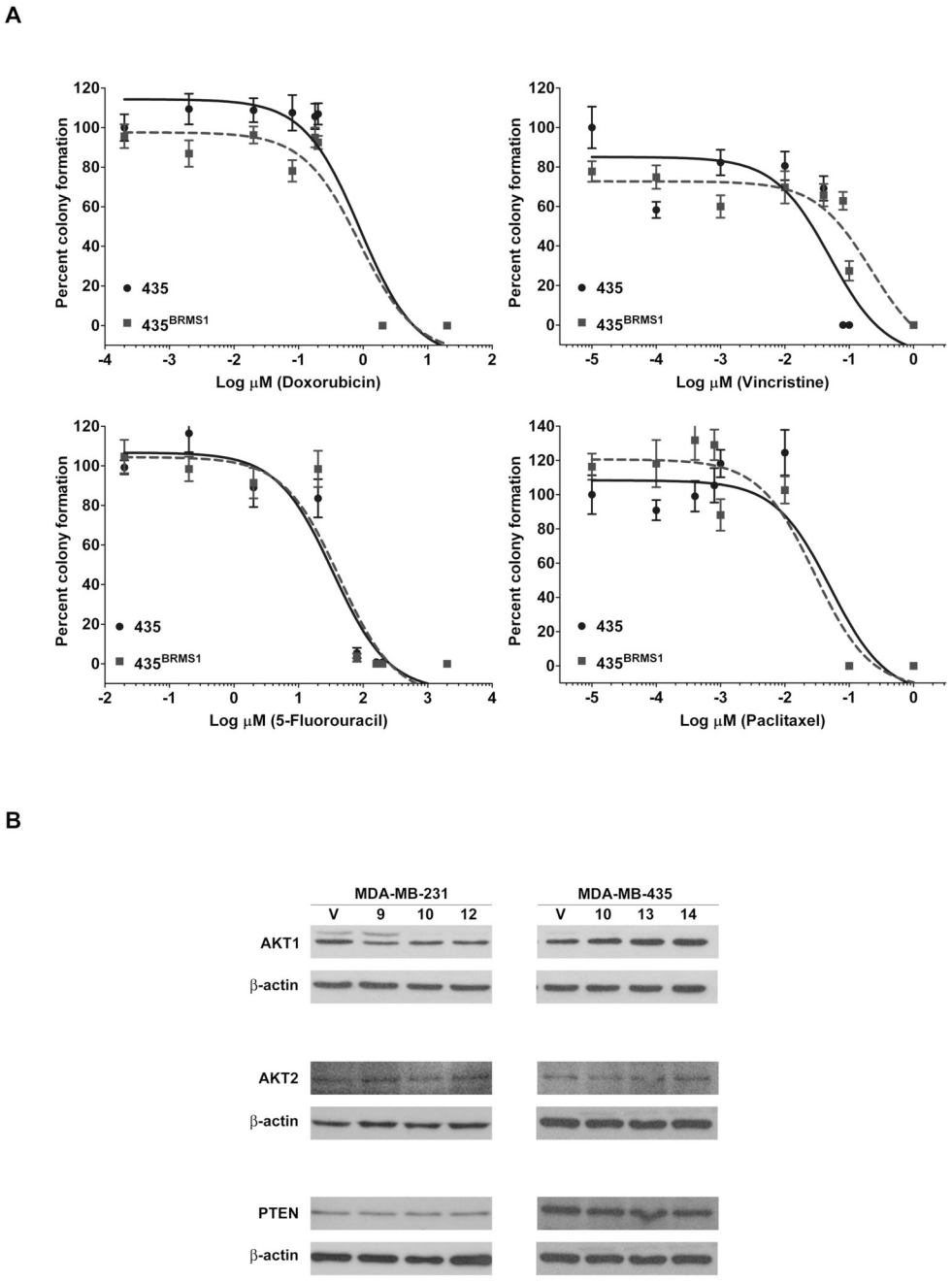

Figure 3.

BRMS1 maintains colony formation in 3D-culture, expression of AKT isoforms and PTEN. **A)** 435/435^{BRMS1} cells were plated at a density of 5000 cells/well on 8-well chamber slides (Nunc, Nalgene) along with the indicated concentration of drugs in 10% Matrigel and incubated in a humidified incubator at 37°C for seven days. After nine days, colonies formed were visualized under a light microscope and counted. Cell clusters visually identified as containing >50 cells were counted as colonies. Similar dose-response curves were generated for 231/231^{BRMS1} cells (data not shown). **B)** Cells lysates were obtained from 231, multiple 231^{BRMS1} clones (9, 10, 12) and 435, multiple 435^{BRMS1} clones (10, 13, 14) and immunoblotted for AKT isoforms and PTEN. β-actin was used as a loading control. No

consistent changes in expression of AKT isoforms or PTEN were observed in BRMS1-expressing cells.

Table 1

Effect of chemotherapeutic agents on clonogenicity of breast cancer cells expressing BRMS1

	IC_{50} (μM)			
Drug	**231**	**231^{BRMS1}**	**435**	**435^{BRMS1}**
Doxorubicin	0.049 (0.027–0.086)	0.071 (0.033–0.153)	0.122 (0.078–0.190)	0.114 (0.065–0.199)
Vincristine	0.016 (0.010–0.025)	0.015 (0.010–0.021)	0.001 (0.0002–0.01)	0.001 (4.4e-006–0.472)
5-Fluorouracil	150.2 (54.06–417.4)	151.6 (56.10–409.5)	663 (486.7–903.1)	476.9 (142.3–1599)
Paclitaxel	0.0002 (4.5e-005–0.001)	8.81e-005 (6.9e-006–0.001)	0.029 (0.011–0.074)	0.012 (0.004 to 0.034)

$231/231^{BRMS1}$ and $435/435^{BRMS1}$ cells were seeded in triplicate at a density of 1000 cells/well in 6-well plates in a final volume of 2 ml media and allowed to attach overnight. The following day, drugs were added at the indicated final concentrations in a volume of 2 ml media and allowed contact with cells for 4 h. Drug containing media was aspirated after 4 h and replaced gently with 5 ml fresh pre-warmed medium in each well. Cells were left undisturbed in a humidified incubator at 37°C for 7 days and colonies formed were counted by staining with a 1:3 solution of acetic acid:methanol containing 0.01% crystal violet (Carnoy's fixative). Cell clusters containing >50 cells were counted as colonies. Experiments were performed in triplicate and repeated at least twice independently. Numbers in parentheses represent 95% confidence interval values. IC_{50} values represent concentrations of drugs resulting in a 50% reduction in colony number.

Table 2

Effect of chemotherapeutic agents on colony formation of BRMS1-expressing breast cancer cells in 3D-culture

	$IC_{50}(\mu M)$			
Drug	**231**	**231^{BRMS1}**	**435**	**435^{BRMS1}**
Doxorubicin	0.277 (0.177–0.431)	0.544 (0.333–0.891)	0.895 (0.507–1.580)	0.881(0.511–1.521)
Vincristine	0.017 (0.010–0.027)	0.032 (0.019–0.054)	0.05 (0.026–0.096)	0.22 (0.104–0.465)
5-Fluorouracil	43.35 (27.94–67.26)	65.09 (41.81–101.3)	33.97 (20.15–57.25)	42.98 (25.27–73.11)
Paclitaxel	0.028 (0.017–0.048)	0.043 (0.023–0.079)	0.051 (0.021–0.124)	0.029 (0.013–0.063)

Exponentially growing 231/231^{BRMS1} and 435/435^{BRMS1} cells were seeded at a density of 5000 cells/well on 8-well chamber slides (Nalgene) in 10% Matrigel. Media containing cells (200 μl) was mixed with a 20% Matrigel suspension in cold media containing drug at 2x of the indicated concentrations (200 μl). The two suspensions were mixed together gently to ensure equal distribution of cells and drugs and incubated in a humidified incubator at 37°C. Cells were incubated for nine days and colonies formed were counted. Experiments were performed in triplicate and repeated at least twice independently. Numbers in parentheses represent 95% confidence interval values. IC_{50} values represent concentrations of drugs resulting in a 50% reduction in colony number.