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# Osteopontin can act as an effector for a germline mutation of BRCA1 in malignant transformation of breast cancer-related cells

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**Breast cancer-associated 1 (BRCA1) plays an important role in breast cancer initiation and progression through its functions in the cell cycle and DNA repair processes; however, its role in metastatic development in human breast cancer is still poorly understood. We have previously shown that osteopontin (OPN) expression was suppressed by wild-type BRCA1 (Wt.BRCA1) and that a natural mutant allele of BRCA1 (Mut.BRCA1) diminished the effect of Wt.BRCA1 on OPN *in vitro*. In this study, we show that while Wt.BRCA1 suppresses OPN-induced metastasis in a rat syngeneic system, Mut.BRCA1 enhances the development of metastasis through OPN, suggesting that OPN and BRCA1 work closely to regulate metastatic development in the rat. To test whether these findings are relevant to human breast cancer, we have investigated the relationship between BRCA1, OPN, and metastatic properties in human breast cancer-related cells. Using western blot analysis, we show that Wt.BRCA1 suppresses, while Mut.BRCA1 enhances, OPN protein expression; and in parallel that Wt.BRCA1 suppresses, while Mut.BRCA1 enhances, OPN-mediated *in vitro* properties associated with the metastatic state in both MCF-7 and MDA MB435s cells. Overall, these results suggest that Mut.BRCA1 can elicit some of the changes involved in metastatic progression in human breast cancer via the overexpression of OPN. (*Cancer Sci* 2010; 101: 1354–1360)**

Germline mutations in the breast cancer-associated 1 (*BRCA1*) gene have been identified in 15–20% of women with a family history of breast cancer and in 60–80% of women with a family history of both breast and ovarian cancer,[1] whilst female mutation carriers have a lifetime breast cancer risk of 60–80%.[2] These results strongly suggest the importance of studying BRCA1-negative breast cancer. The wild-type BRCA1 (Wt.BRCA1) protein binds to BRCA2, p53, RAD51, and many other proteins involved in cell cycle and DNA damage responses[3,4], but how germline mutations in BRCA1 directly affect the generation of the malignant phenotype and the development of metastasis is largely unknown. One such intermediary in the rat has been suggested to be osteopontin (OPN).[5]

Osteopontin is an extracellular glycophosphoprotein,[6,7] which is important in malignant transformation *in vitro*, enhancing cell properties, such as attachment to extracellular matrixes, migration, and invasion.[8–11] Its production has also been shown to provide prognostic value to breast cancer progression.[12–14] We have previously shown, in the rat mammary cell line Rama 37,[15] that Wt.BRCA1 represses the expression of this estrogen-responsive gene.[5] In this rat cell system, Wt.BRCA1 also inhibits OPN-mediated malignant transformation *in vitro*, while a natural mutant BRCA1 (Mut.BRCA1), which is associated with familial breast cancer,[16] lacks this OPN suppressive effect and impedes Wt.BRCA1 suppression of expression of OPN.[5] We now investigate the effects of Wt. and the Mut.BRCA1 on the metastatic potential of the rat mammary cells in syngeneic rats *in vivo* and upon OPN-mediated cellular properties indicative of malignant transformation of human breast cancer-related cell lines *in vitro*. The Mut.BRCA1 promotes metastasis in rats *in vivo* and malignant transformation of human breast cancer-related cells *in vitro* by increasing the expression of OPN.

## Materials and Methods

**Plasmids and oligonucleotides.** Expression vectors for human Wt.BRCA1 and Mut.BRCA1 in the Rc/CMV vector have been described previously.[5] The Mut.BRCA1 encodes a point mutation (Ala-1708 $\rightarrow$ Glu) in its C-terminal region, This is a germline mutation associated with very early onset familial breast cancer.[16]

**Cell lines and cell culture.** See the Appendix S1.

**Stable and transient transfections.** Permanently expressing OPN cells were produced by transfection of the expression vector for OPN, for OPN antisense mRNA (as-OPN), or the empty expression vector pBK-CMV into Rama 37, MCF-7, and MDA MB435s cells, as described previously[5] (see Appendix S1).

**Western blotting for proteins.** Detection of BRCA1 or OPN proteins were performed using a rabbit polyclonal antibody to BRCA1 which can detect both human and rat (sc-7867; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a mouse monoclonal antibody (mAb) MBIII $B_{10}$ to OPN (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA)[13] as described previously.[5]

**Soft-agar, cell-substrate adhesion, and Matrigel invasion assays.[17]** See the Appendix S1.

**Assays for metastasis.** Stable transfectants were harvested by treatment with EDTA/trypsin solutions, and washed and resuspended in PBS at $10^7$ cells/mL. Two $\times$ $10^6$ cells in 0.2 mL were injected s.c. through the skin into the right inguinal fat pads of 6- to 10-week-old syngeneic female Furth–Wistar rats (Ludwig–Wistar OLA strain), usually 20 rats/group.[15] Rats were autopsied after 12 weeks, and tumors and relevant tissues, particularly the lungs and lymph nodes, were examined for gross metastases. Primary tumors and other tissues of abnormal appearance including all lungs were fixed in methacarn (methanol, inhibisol, acetic acid: 6, 3 ,1), embedded in paraffin-wax, sectioned, and stained with hematoxylin–eosin. Five microscopic fields from two sections for the primary tumor and for the lungs were assessed for microscopically visible metastases by the independent observers, as described previously.[18,19]

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Immunocytochemical staining for BRCA1 and for OPN was performed as described previously.[5,13] Photographs were recorded in a Polyvar microscope (Reichert, Depew, NY, USA) fitted with Hoya 80A plus 80B filters (Hoya, Santa Clara, CA, USA) on Kodak Gold 200ASA color film (Kodak, New York, NY, USA).

**Luciferase reporter assay.[5]** See the Appendix S1.

## Results

**Effect of permanent transfection of BRCA1 on metastasis *in vivo* in a syngeneic rat model system.** Rama 37 (R37) stable transfectants R37/pBK-CMV, R37/OPN, R37/Wt.BRCA1, R37/Mut.BRCA1, R37/as-OPN, R37/OPN/as-OPN, R37/as-OPN/Mut.BRCA1, and R37/OPN/Wt.BRCA1 were produced from pooled clones of transfectants. When injected into syngeneic rats, there was no significant difference in the incidence of tumors for any of the transfected pooled cells (Fisher's exact test, $P \geq 0.16$, Table 1). Only R37/OPN ($P = 0.00001$) and R37/Mut.BRCA1 ($P = 0.0004$) pooled cells induced a significant increase in the occurrence of metastases (65% and 50%, respectively) when injected into the rats compared to the control R37/pBK-CMV pooled cells (0%). No metastases were found when the rats were injected with R37/Wt.BRCA1 cells (Table 1). When the syngeneic rats were injected with R37/OPN cells transfected with expression vector for Wt.BRCA1, the incidence of metastasis was significantly reduced ($P = 0.003$) to 9.5% (Table 1). Moreover, R37/OPN/asOPN and R37/asOPN/Mut.BRCA1 cells injected into the syngeneic rats produced significantly fewer metastases than their OPN-overexpressing counterparts (12% and 6%, respectively; $P = 0.004$; 0.006). Indeed, the number of rats with metastases produced in this condition was not significantly different from the vector control-transfected cells. The difference in the incidence of metastases between R37/OPN and R37/Mut.BRCA1 cells ($P = 1$) and between any of R37/pBK-CMV, R37/Wt.BRCA1, R37/OPN/as-OPN, and R37/as-OPN/Mut.BRCA1 cells ($P \geq 0.32$) was not significant (Table 1). The majority of the metastases occurred in the lungs (Fig. 1a), with a few metastases in the lymph nodes, but no metastatic deposits were observed in other organs.

**Table 1. Incidence of tumors and metastases produced by transfected rat mammary cells**

| Transfected cells* | Tumor incidence (%)† | Incidence of metastasis (%)‡ |
|---|---|---|
| R37/pBK-CMV | 20 of 20 (100) | 0 of 20 (0) |
| R37/OPN | 17 of 20 (85) | 11 of 17 (65)** |
| R37/Wt.BRCA1 | 25 of 25 (100) | 0 of 25 (0) |
| R37/Mut.BRCA1 | 20 of 20 (100) | 10 of 20 (50)** |
| R37/OPN/as-OPN | 17 of 20 (85) | 2 of 17 (12) |
| R37/as-OPN/Mut.BRCA1 | 18 of 20 (90) | 1 of 18 (6) |
| R37/OPN/Wt.BRCA1 | 21 of 22 (96) | 2 of 21 (9.5) |

*Pools of clones of Rama 37 (R37) cells transfected with the expression vector alone pBK-CMV (R37/pBK-CMV) or with the expression vectors for the following: osteopontin (OPN) (R37/OPN), wild-type breast cancer-associated 1 (BRCA1) (R37/Wt.BRCA1), mutant BRCA1 (R37/Mut.BRCA1), osteopontin and antisense construct to OPN mRNA (R37/OPN/as-OPN), antisense construct to OPN mRNA and mutant BRCA1 (R37/as-OPN/Mut.BRCA1), and osteopontin and wild-type BRCA1 (R37/OPN/Wt.BRCA1). †Tumor incidence = number of tumors/number of rats inoculated. Tumor incidence of all the cells transfected with the expression vectors for different proteins was not significantly different from that of vector alone–transfected cells (Fisher's exact test, $P \geq 0.16$). ‡Incidence of metastasis = number of rats with lung metastases/number of rats with tumors. **Significantly different from R37/pBK-CMV expression vector alone controls and from R37/Wt.BRCA1, R37/OPN/as-OPN, R37/as-OPN/Mut.BRCA1, and R37/OPN/Wt.BRCA1 cells (Fisher's exact test, $P \leq 0.03$). There was no significant difference between R37/OPN and R37/Mut.BRCA1 cells ($P = 1$), and between R37/pBK-CMV, R37/Wt.BRCA1, R37/OPN/as-OPN, R37/as-OPN/Mut.BRCA1, and R37/OPN/Wt.BRCA1 cells (Fisher's exact test, $P \geq 0.32$).

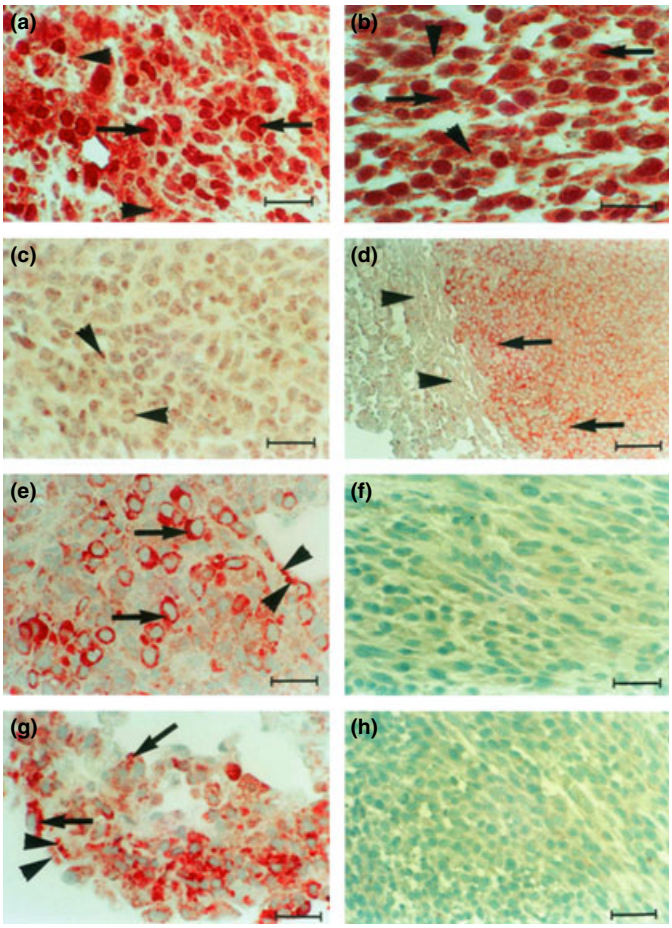


**Fig. 1.** Immunocytochemical staining of primary tumors and metastases produced by pools of Rama 37 (R37) transfectants in the rat. (a) Lung metastases produced by R37/Mut.BRCA1 (mutant breast cancer-associated 1) pooled cells incubated with antibodies to BRCA1 showing strong red nuclear (arrow) and weaker red cytoplasmic (arrowhead) staining. (b) Primary tumor produced by R37 antisense osteopontin (as-OPN)/Mut.BRCA1 pooled cells incubated with antibodies to BRCA1 showing strong red nuclear (arrow) and weaker red cytoplasmic staining (arrowhead). (c) Primary tumor produced by R37/OPN pooled cells showing little nuclear and a very weak reddish cytoplasmic staining (arrowhead) for BRCA1. (d) Cannon ball lung metastasis produced by R37/Mut.BRCA1 pooled cells incubated with antibodies to OPN showing strong red staining of the malignant cells (arrow) and no staining of the parenchymal lung tissue (arrowhead). (e) More diffuse lung metastasis produced by R37/Mut.BRCA1 pooled cells incubated with antibodies to OPN showing strong cytoplasmic (arrowhead) and pericellular (arrow) red staining of the malignant cells. (f) Primary tumor produced by R37/Wt.BRCA1 pooled cells showing no staining for OPN. (g) More diffuse lung metastasis produced by R37/OPN pooled cells showing cytoplasmic (arrows) and pericellular (arrowheads) red staining of the malignant cells for OPN. (h) Primary tumor produced by R37/as-OPN/Mut.BRCA1 pooled cells showing no staining for OPN. Magnification: (a, c, e, f) ×580; (b) ×720; (d) ×230; (g,h) ×460. Bar: 20 μm (a–c, e,f); 50 μm (d); 25 μm (g,h). In all sections cell nuclei were counterstained blue by hemalum.

The histological appearance of primary tumors and any metastases from all of the six groups were similar, primarily consisting of spindle cells admixed with more cuboidal, epithelial-like cells (Fig. 1a,b). Immunocytochemical staining for BRCA1 confirmed that R37/Mut.BRCA1 and R37/as-OPN/Mut.BRCA1 cells overproduced a BRCA1-related molecule in the lung metastases (Fig. 1a) and primary tumors (Fig. 1b) in

comparison with primary tumors produced by injection of R37/OPN cells (Fig. 1c). The resultant staining was predominantly nuclear with lesser staining in the cytoplasm in the Mut.BRCA1-transfected cells (Fig. 1a,b), whereas BRCA1 staining in the R37/OPN cells was much weaker and mainly cytoplasmic (Fig. 1c). The R37/Mut.BRCA1 cells produced immunocytochemically detectable OPN in both cannon ball (Fig. 1d) and more diffuse (Fig. 1e) lung metastases, whereas no immunologically detectable OPN was observed in the primary tumors produced by R37/Wt.BRCA1 cells (Fig. 1f). The cannon ball and the more diffuse lung metastases produced by R37/Mut.BRCA1 cells (Fig. 1e) and by R37/OPN cells were similar (Fig. 1g); the staining for OPN was cytoplasmic and pericellular with no staining of cellular nuclei (Fig. 1e,g). In contrast, R37/as-OPN/Mut.BRCA1 cells produced primary tumors that stained weakly for OPN (Fig. 1h). These results suggest that the primary tumors and the resultant metastases contain the genetic modifications we have introduced *in vitro* and are a result of the injection of the corresponding Rama 37 derivatives.

**Effect of BRCA1 on OPN gene expression in human breast cancer cells.** To investigate whether our findings in rat mammary epithelial cells are applicable to human breast cancer development, we modulated the expression levels of Wt. BRCA1, Mut.BRCA1, and OPN in human breast cancer cell lines MDA MB435s and MCF-7. Immunoblots using a polyclonal antibody to the BRCA1 N-terminal region indicated similar elevated levels of BRCA1 protein in MDA MB435s/Wt.BRCA1 and MDA MB435s/Mut.BRCA1 cells (5- and 5.2-fold, respectively) over the control untransfected MDA MB435s cells (Fig. 2a). A similar and approximately equal pattern of BRCA1 levels were observed in MCF-7/Wt and Mut.BRCA1 cells (3.4- and 3.2-fold, respectively) (Fig. 2c).

OPN protein levels were found to be reduced fivefold in MDA MB435s/Wt.BRCA1 cells but increased 3.4-fold in MDA MB435s/Mut.BRCA1 cells compared to control MDA MB435s cells (Fig. 2a). Mut.BRCA1 expressing MCF-7 cells produced 8.3-fold higher OPN protein levels than MCF-7 cells alone (Fig. 2c). When the MDA MB435s and MCF-7 cells were stably transfected with an expression vector for OPN, they produced a 2.5- ($P = 0.006$) and 3.9- ($P = 0.0001$) fold increase in OPN protein, for the MDA MB435s/OPN and MCF-7/OPN cells, respectively (Fig. 2b,d). In the BRCA1 expression vector-transfected, OPN-overexpressing cells, Wt.BRCA1 inhibited OPN protein by approximately 13- ($P = 0.001$) and 3- ($P = 0.0002$) fold in MDA MB435s/OPN and in MCF-7/OPN cells, respectively, while Mut.BRCA1 produced little ($P = 0.03$) or no ($P = 0.49$) significant effect on OPN protein levels (Fig. 2b,d). These data were obtained with pools of transfected cell clones, but similar results were obtained with two single clones of transfected cells (not shown). In all cases, BRCA1 and OPN protein levels were normalized against constitutively expressed β-actin in the same protein samples (Fig. 2a–d). Transfection of MDA MB435s and MCF-7 cells with an expression vector for as-OPN reduced OPN protein levels by 76% ($P < 0.0001$) and 20% ($P = 0.03$), respectively, compared to untransfected cells (Fig. 2b,c). Transfection of MDA MB435s/as-OPN and MCF-7/as-OPN cells with an expression vector for Mut.BRCA1 produced no ($P \geq 0.57$) significant increase in levels of OPN (Fig. 2b,c). Transient transfection of MDA MB435s/Wt.BRCA1 and MCF-7/Wt.BRCA1 cells with an expression vector for Mut.BRCA1 significantly induced OPN protein expression by 18- ($P = 0.0001$) and 13- ($P = 0.0001$) fold, respectively, restoring them to levels observed with Mut.BRCA1 (Fig. 2a–d). These results suggest that OPN expression can be regulated in a negative and positive manner by Wt. and Mut.BRCA1, respectively, in human breast cancer-related cell lines in culture.

We have previously shown, in a rat mammary cell line, that BRCA1 binds to transactivating transcription factors of OPN, such as Pea3 and c-Jun, to down-regulate OPN expression, while mutant BRCA1 up-regulates OPN.[5] To investigate whether Wt.BRCA1 and Mut.BRCA1 behave similarly in human breast cancer, we cloned the human OPN promoter into pGL-3 luciferase reporter construct and co-transfected this construct with vector control, Wt.BRCA1 expression vector, or both Wt.BRCA1 and Mut.BRCA1 expression vectors. Similar to our previous findings, Wt.BRCA1 suppressed the human OPN promoter by 29 ± 2% ($P = 0.009$), while Mut.BRCA1 abolished the suppressive effect of Wt.BRCA1 on the human OPN promoter (Fig. 2e). This result suggests that BRCA1 suppresses OPN expression by inhibiting OPN promoter activity and that this suppression was reversed by Mut.BRCA1. Recent studies on MDA MB435s cells have revealed uncertainty regarding the breast origin of this cancer cell line.[20,21] We therefore confirmed the results in another breast cancer cell line, MDA MB468, which expresses OPN, though in relatively low levels compared to MDA MB435 cells (data not shown). Overexpression of Wt.BRCA1 in MDA MB468 cells resulted in increased mRNA and protein levels of BRCA1 (Fig. S1). Overexpression of Wt.BRCA1 on MDA MB468 cells resulted in a significant 91% ($P < 0.0001$) reduction in mRNA levels (Fig. S1b), as well as a significant 23% ($P = 0.02$) reduction in protein levels of OPN (Fig. S1c). These results confirm that Wt.BRCA1 plays an important role in expression of OPN in human breast cancer-related cells.

**Effect of BRCA1 on anchorage-dependent and independent growth in human breast cancer cells.** Transfectants of MDA MB435s and MCF-7 cells overexpressing Wt.BRCA1, Mut.BRCA1, or OPN showed little or no changes in their respective proliferation rates in culture (data not shown). When MDA MB435s or MCF-7 cells were transfected with an expression vector for Wt.BRCA1 and assayed in soft agar, the colony number per plate was reduced by 63% (Student's *t*-test, $P = 0.0001$) (Fig. 3a) and 20% ($P = 0.074$) (Fig. 4a), respectively. Overexpression of Mut.BRCA1 produced no change in the number of colonies formed in MDA MB435s cells (Fig. 3a), while it significantly increased the number of colonies formed in MCF7 cells by 6.5-fold ($P = 0.0004$) (Fig. 4a). Similarly, overexpression of OPN produced only little change in the number of colonies formed in MDAMB 435s (Fig. 3a), but it increased the number of colonies formed by 7.5-fold in MCF-7 cells ($P = 0.0001$) (Fig. 4a). Furthermore, Wt.BRCA1, but not Mut.BRCA1, suppressed the colony-forming ability of OPN-overexpressing MDA MB435s (Fig. 3a) and MCF7 cells (Fig. 4a). When the expression of OPN was silenced by antisense OPN, there was a significant 5.3-fold decrease in the number of colonies formed in MDA MB435s cells ($P = 0.0001$) (Fig. 3a) but not in MCF7 cells (Fig. 4a), which express only low levels of endogenous OPN. Transfection of MDA MB435s/as-OPN or MCF-7/as-OPN cells with an expression vector for Mut.BRCA1 did not affect colony formation ($P = 0.75$, 0.9, respectively) (Figs 3a,4a). However, transient transfection of MDA MB435s/Wt.BRCA1 or MCF-7/Wt.BRCA1 cells with an expression vector for Mut.BRCA1 significantly increased the number of colonies formed by 3- ($P = 0.0001$) or 9- ($P = 0.0003$) fold (Figs 3a,4a), respectively, restoring them to similar levels of MDA MB435s or MCF-7/OPN cells. Similarly MDAMB 468 cells overexpressing Wt.BRCA1 produced 41% fewer colonies ($P = 0.03$, Fig. S1d) compared to the vector control cells.

**Effect of BRCA1 on adhesion and invasion in human breast cancer cells.** When parental MDA MB435s cells were transfected with an expression vector for Wt.BRCA1, the resultant cell adhesion and invasion values were reduced by 2.4-fold (Student's *t*-test, $P = 0.0001$) and 2.6-fold ($P = 0.006$),


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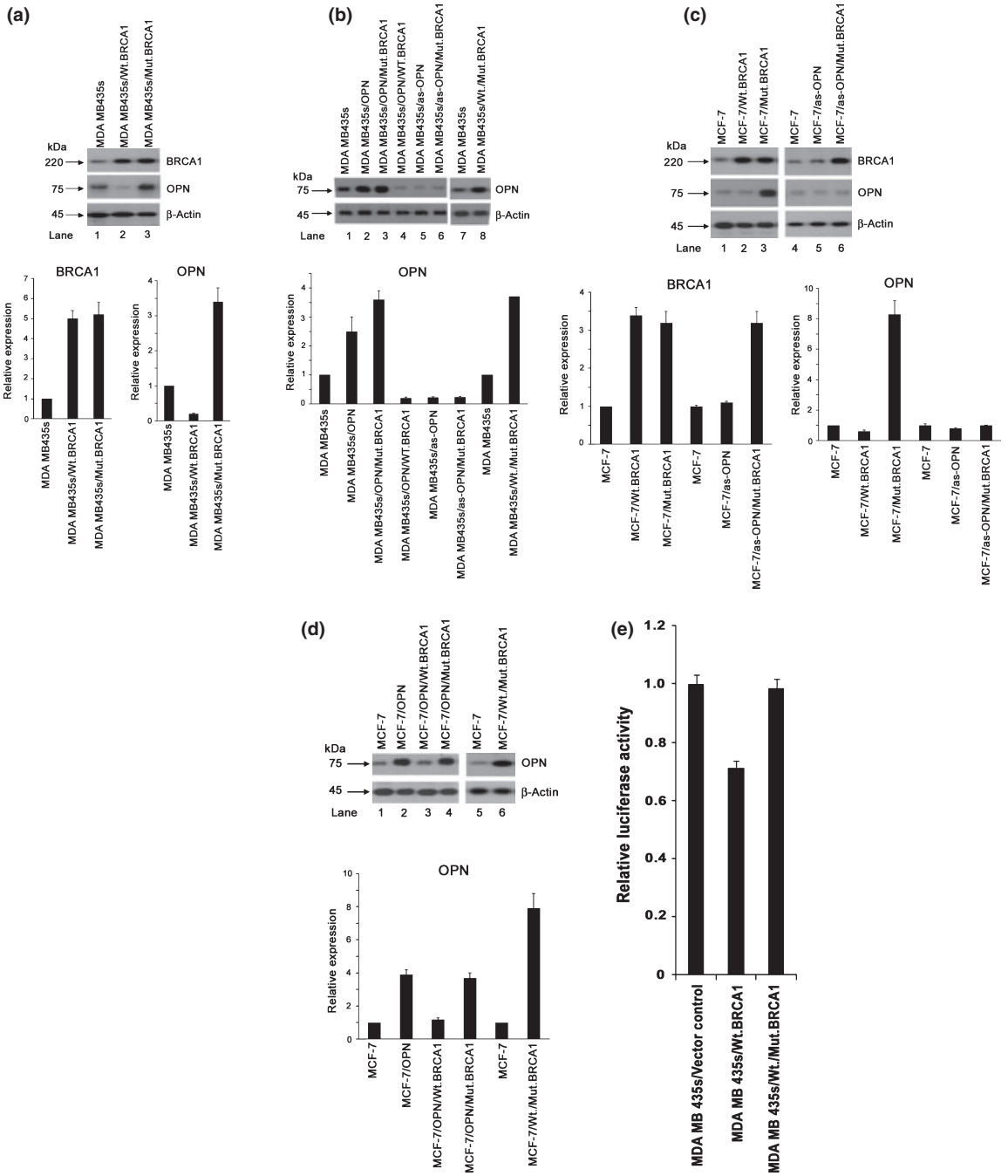


**Fig. 2.** Expression studies in MDA MB435s and MCF-7 pooled transfectants. Breast cancer-associated 1 (BRCA1) and osteopontin (OPN) protein expression levels were recorded by immunoblot for wild-type (Wt.BRCA1), mutant BRCA1 (Mut.BRCA1), and OPN in either (a,b) MDA MB 435s-related cells or in (c,d) MCF-7-related cells. A representative blot of three experiments is shown. Bands were quantified using densitometric analysis, normalized against β-actin and standardized with respect to the level of their parental control cells. The average fold increase ± SD for three different experiments is shown on a histogram. (e) Osteopontin promoter activity was measured by firefly luciferase reporter activity normalized to Renilla luciferase activity with same cells (see 'Material and Methods').

respectively. Transfection of MDA MB435s with an expression vector for Mut.BRCA1 produced no significant difference in cell adhesion and invasion (Fig. 3b,c). Transfection of MDA MB435s cells with an expression vector for OPN increased cell adhesion and invasion by a modest 1.5- and 1.3-fold, respectively ($P = 0.001$, $P = 0.01$) (Fig. 3b,c). Transfection of MDA MB435s/OPN cells with an expression vector for Wt.BRCA1, but not that for Mut.BRCA1, reduced cell adhesion and invasion by 2.9- and 2.8-fold, respectively ($P = 0.0003$, $P = 0.0002$) (Fig. 3b,c). In contrast to MDA MB435s cells, transfection of MCF-7 cells with an expression vector for Mut.BRCA1, but not that for Wt.BRCA1, increased cell adhesion by 2.7-fold ($P = 0.0001$) and invasion by 5.1-fold ($P = 0.001$) (Fig. 4b,c). Moreover, transfection of MCF-7 cells with an expression vector for OPN increased cell adhesion and invasion by 2.8- and 5.7-fold, respectively ($P = 0.002$, 0.001). Further transfection of these MCF-7/OPN cells with an expression vector for Wt.BRCA1 reduced cell adhesion and invasion by 2.5- and 4.2-fold, respectively ($P = 0.002$, $P = 0.0005$) (Fig. 4b,c). Similarly, MDA MB468 cells overepressing Wt.BRCA1 were 22%

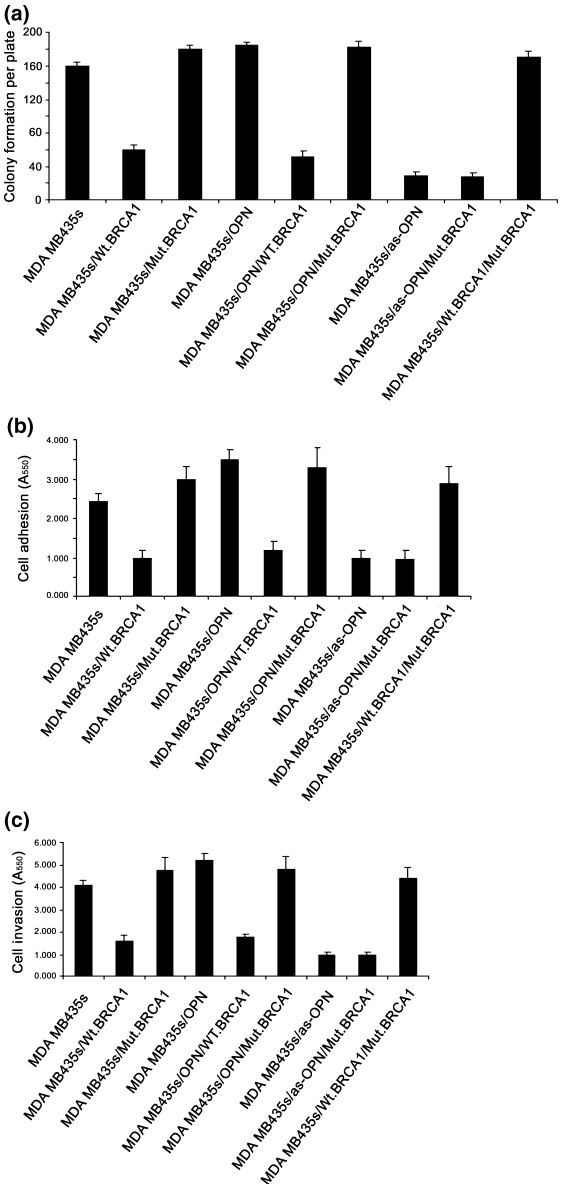


**Fig. 3.** Effects of overexpression of breast cancer-associated 1 (BRCA1), mutant BRCA1 and osteopontin (OPN) on properties associated with the malignant state of MDA MB435s cells *in vitro* were investigated by (a) soft agar, (b) cell adhesion, and (c) cell migration assays as described in the Material and Methods.

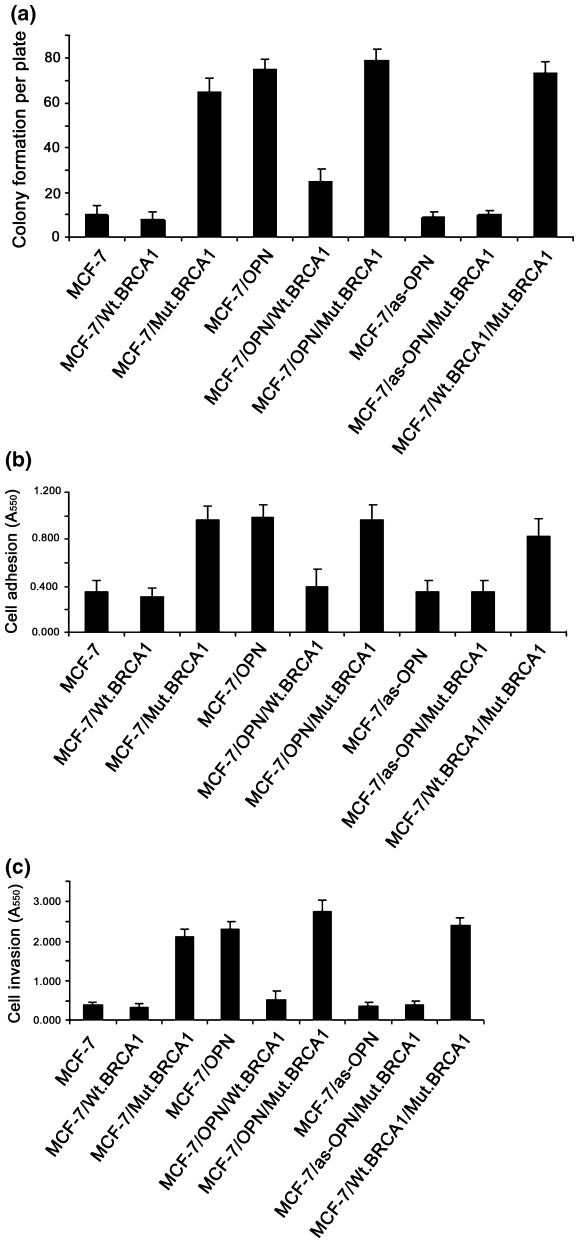


**Fig. 4.** Effects of overexpression of breast cancer-associated 1 (BRCA1), mutant BRCA1, and osteopontin (OPN) on properties associated with the malignant state of MCF-7 cells *in vitro* were investigated by (a) soft agar, (b) cell adhesion, and (c) cell migration assays as described in the Material and Methods.

less adhesive compared to the vector control cells ($P = 0.004$) (Fig. S2e).

When MDA MB435s cells were transfected with the construct for as-OPN, cell adhesion and invasion were reduced by 2.5- and 4.1-fold, respectively ($P = 0.0001$, 0.001) (Fig. 3b,c), while there was no significant effect of the same construct for as-OPN on MCF-7 cell adhesion or invasion ($P \geq 0.9$) (Fig. 4b,c). Transfection of MDA MB 435s/as-OPN or MCF-7/as-OPN cells with an expression vector for Mut.BRCA1 showed no significant changes in cell adhesion or invasion ($P \geq 0.9$) (Figs 3b,c, 4b,c). In contrast, transient transfection of MDA MB 435s/Wt.BRCA1 or MCF-7/Wt.BRCA1 cells with an expression vector for Mut.BRCA1 increased cell adhesion by 2.9- or 2.6-fold, respectively ($P = 0.004$, 0.002) (Fig. 3b,4b) and invasion by 2.7- or 6.8-fold, respectively ($P = 0.002$, 0.001) (Fig. 3c,4c).

## Discussion

Previously, we have shown the interaction *in vitro* between BRCA1 and a well-established inducer of malignant metastatic spread in a rat mammary modal cell line system,[5] OPN. Osteopontin itself is thought to induce metastasis by its activity to promote ''inter alia'' anchorage independent growth, cell adhesion, and cell invasion,[6–11] and its expression in human breast cancer is associated with patient death from metastatic disease.[12–14] In the present study, we have further confirmed the results in the rat model system by observation of induction of metastasis *in vivo* by Mut.BRCA1 but not by Wt.BRCA1. This metastasis-inducing effect of Mut.BRCA1 depends on OPN, since transfection of an as-OPN construct to OPN mRNA into


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R37/Mut.BRCA1 cells significantly reduces the incidence of metastasis to a level similar to that for the R37/vector control cells (Table 1). The changes in incidences of metastasis are unlikely to be due to alterations in cell proliferation rates, as these are not significantly different between all the R37 derivatives described in the present study. Moreover the histology of the lung metastases produced by injection into the rat mammary fat pad of either R37/Mut.BRCA1 or R37/OPN cells and their cellular content/distribution of immunoreactive OPN are very similar (Fig. 1d,e,g), suggesting that the development of metastases is mainly due to overexpression of OPN. Primaries which fail to overexpress OPN also fail to metastasize (Fig. 4f,h). The results from the *in vivo* study suggest that Mut.BRCA1 promotes development of metastases through up-regulation of OPN protein levels and that Wt.BRCA1 counteracts the metastasis-inducing effect of OPN overexpression, suggesting that BRCA1 works closely with OPN in development of metastasis.

To investigate whether our findings are applicable to the human cancer metastatic development, we have tested our hypothesis also in the human cell lines MDA MB435s and MCF7. Although both MDA MB435s and MCF-7 cell lines were derived from breast cancers, the MDA MB435s cells are more aggressive than MCF-7 cells, insofar as MDA MB435s cells possess the ability to form metastases in immunodeficient mice,[22] an ability which the unsupplemented MCF-7 cells normally lack *in vivo*.[23] Knockdown of OPN in MDA MB435s cells reduces, and overexpression of OPN in MCF-7 cells increases, colony formation, cell adhesion, and invasion *in vitro*, suggesting that OPN plays an important role in metastatic behavior in these two cell lines at least *in vitro*.

The inhibitory effect of Wt.BRCA1 on *in vitro* malignant properties of MCF-7/OPN cells and MDA MB435s cells is probably exerted by a lowering of the level of OPN protein. The fact that transfection of MDA MB435s/OPN and MDA MB435s cells with an expression vector for Wt.BRCA1 reduces OPN and *in vitro* malignant properties to similar levels suggests that expression from the endogenous and transgenes for OPN are both suppressed by Wt. BRCA1. It is postulated that BRCA1 may regulate OPN protein levels post-transcriptionally, since post-transcriptional regulation of OPN expression has been reported previously.[24,25] However, we have shown that Wt.BRCA1 suppresses the endogenous mRNA for OPN by 91% in the MDA MB468 cells (Fig. S1b) and that the promoter activity of human OPN is reduced by 29% when transfected into MDA MB435s cells (Fig. 2e). These results suggest that much of the reduction in production of OPN protein may be due to inhibition of transcription of the endogenous *OPN* gene possibly at its promoter, as we have found in detail in the rat mammary model systems.[5]

To gain insight into the mechanism whereby BRCA1 suppresses and Mut.BRCA1 reverses and stimulates OPN transcription, we have shown in previous publications in the rat mammary cells that Wt.BRCA1 is able to suppress estogen receptor-α (ERα), c-jun, and Pea3 transcription factor transactivation of the rat OPN promoter-reporter construct.[5] The human OPN promoter isolated here contains recognition sequences for these same transcription factors (El-Tanani M.K., unpublished results), suggesting that these factors would also be operational on the human OPN promoter *in vitro*.[26,27] This suggestion is consistent with our previous reports where the expression of human OPN was significantly associated with the presence of these transcription factors in primary breast carcinomas *in vivo*.[26,27] The surprising result that Wt.BRCA1 also suppresses expression of exogeneously produced OPN in both MDA MB435s and MCF-7 cells (Fig. 2b,d) may be due to the fact that the OPN transgene has been placed under the control of the CMV promoter which contains a major core of 11 and three recognition sequences for c-jun and Ets transcription factors, respectively.[5] Moreover, we have suggested that BRCA1 may inhibit OPN expression ''inter alia'' by specifically binding to these three transcription factors, as demonstrated in broken cell extracts and in that Mut.BRCA1 inhibits this process by its capability of binding to Wt.BRCA1 in a dominant negative manner.

In conclusion, human and rat breast cancer-related cell lines have been used in the present study to show that Mut.BRCA1 can promote metastatic progression both *in vitro* and *in vivo*, respectively, via the overexpression of a metastasis-inducing protein, OPN. This result is consistent with the appearance of enhanced expression of OPN in a group of familial primary breast cancers with mutant BRCA1, in comparison with that found in sporadic breast cancers containing wild-type BRCA1.[5] Moreover, we have found that Mut.BRCA1 promotes metastatic progression *in vitro* regardless of the expression status of Wt.BRCA1. Single copies of mutated BRCA1 and BRCA2 have been shown to confer as high as an 80% likelihood of developing breast cancer[28] while mutant BRCA1 allele has been shown to function in a dominant negative manner.[5,29] Our results suggest that Mut.BRCA1 might promote metastatic progression of human breast cancer cells through suppressing the Wt.BRCA1-inhibited OPN expression in a dominant negative manner.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Appendix S1**. Supplementary Materials and Methods.

**Fig. S1.** The suppressive effect of breast cancer-associated 1 (BRCA1) on osteopontin (OPN) was confirmed in another breast cancer cell line MDA MB468, which expresses OPN but low levels of BRCA1. (a) BRCA1 mRNA expression levels were increased in MDA MB468/Wt.BRCA1 compared to vector alone-transfected MDA MB468 cells. Osteopontin (b) mRNA and (c) protein levels were reduced in Wt.BRCA1-transfected MDA MB468 cells. Both (d) colony formation and (e) cell adhesion were reduced in MDA MB468 cells with higher levels expression of BRCA1.

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