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The carboxyl-terminal of BRCA1 is required for subnuclear assembly of RAD51 after treatment with cisplatin but not ionizing radiation in human breast and ovarian cancer cells

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Abstract

BRCA1 plays an important role in maintaining genomic stability through its involvement in DNA repair. Although it is known that BRCA1 and RAD51 form distinct DNA repair subnuclear complexes, or foci, following environmental insults to the DNA, the role of BRCA1 in this process remains to be characterized. The purpose of the study was therefore to determine the role of BRCA1 in the formation of RAD51 foci following treatment with cisplatin and ionizing radiation. We found that although a functional BRCA1 is required for the subnuclear assembly of BRCA1 foci following treatment with either ionizing radiation or cisplatin, a functional BRCA1 is required for RAD51 foci to form following treatment with cisplatin but not with ionizing radiation. Similar results were obtained in SKOV-3 cells when the level of BRCA1 expression was knocked down by stable expression of a retrovirus-mediated small-interfering RNA against BRCA1. We also found that the carboxyl-terminal of BRCA1 contains uncharacterized phosphorylation sites that are responsive to cisplatin. The functional BRCA1 is also required for breast and ovarian cancer cells to mount resistance to cisplatin. These results suggest that the carboxyl-terminal of BRCA1 is required for the cisplatin-induced recruitment of RAD51 to the DNA-damage site, which may contribute to cisplatin resistance.

Keywords: BRCA1; Rad51; Cisplatin; γ-Radiation; Breast and ovarian cancer

Women with germline mutations in the *BRCA1* gene are particularly susceptible to breast cancer and ovarian cancer [1,2]. *BRCA1* is considered a tumor suppressor gene because the tumors that arise in these women exhibit loss of heterozygosity, which is consistent with Knudson's two-hit hypothesis with regard to the recessive tumor suppressor gene [3]. The *BRCA1* gene encodes a 1863-amino acid, 220 kDa nuclear phosphoprotein with multiple functional domains, including the carboxyl-terminal domain, which consists of two tandem *BRCA1* carboxyl-terminal repeats (BRCT) [2,4,5], each of which is approximately 100 amino acid long. The BRCA1 protein participates in a large number of cellular processes, including DNA replication, cell cycle checkpoint control, transcriptional regulation, protein ubiquitination, apoptosis, and chromatin remodeling [6–18]. BRCA1 is also part of a large complex of proteins involved in DNA-damage recognition and repair [15]. In particular, it interacts with multiple DNA repair/recombination proteins, including RAD51, the RAD50/MRE11/NIBRIN complex, Bloom's helicase, a DEAH helicase BACH1, and Fanconi's D2 protein [15,19–21]. This interaction of BRCA1 with the DNA repair protein complex maintains genomic stability and may at least in part account for its role as a tumor suppressor. Its particular role in DNA repair is to maintain genomic stability. Lack of the BRCA1 protein in mice causes them to die early in embryogenesis as a result of a high level of genetic instability and cell proliferation arrest resulting from activation of the p53/p21 pathway [22].

RAD51 is a nuclear protein showing homology to the yeast protein, RecA, which is involved in homologous

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recombination and double-strand DNA break repair [23]. Upon exposure to ionizing radiation, RAD51 oligomerizes on DNA, which promotes pairing and strand exchange between the homologous DNAs. Also in response to ionizing radiation, RAD51 has been shown to form into distinct subnuclear complexes, or foci, which are likely to represent functional multimeric forms of RAD51 that promote DNA repair [24]. These RAD51 foci represent a critical step in the assembly of DNA repair complexes in the damaged sites, and both BRCA1 and BRCA2 have been shown to possibly play a critical role in this process [6,7,25]. However, the underlying mechanism in this process is not clear. In human cancer cells, BRCA2 but not BRCA1 is required for RAD51 foci to form after ionizing irradiation [26], whereas in murine cells, BRCA1 is required for RAD51 foci to form after treatment with either cisplatin or ionizing radiation [27]. Genetic studies of yeast cells have shown that RAD51 foci assembly requires several other proteins, including RAD52, RAD55, and RAD57, which are also part of RAD51 foci [28]. Xrcc3 is required for RAD51 foci formation and double-strand DNA repair in Chinese hamster cells [29]. These collective findings therefore suggest that the formation of RAD51 foci is a complicated biochemical process involving multiple proteins. In addition, RAD51 knockout mice displayed embryonic lethality and sensitivity to ionizing irradiation [30,31]. Further, RAD51 mutants showed a phenotype similar to that of BRCA1 mutants, which suggests that BRCA1 is involved in the homologous recombination pathway. These findings therefore suggest that both RAD51 and BRCA1 play a critical role in the maintenance of genetic stability [22].

Cisplatin is a widely used chemotherapeutic agent and is particularly used in the treatment of ovarian cancer, testicular cancer, and other solid tumors. After cisplatin enters the cells, its chloride ligands are replaced by water, which forms aquated species that react with the nucleophilic sites on the DNA, in particular with the linker regions associated with the histone H1 protein [32–34]. Notably, the DNA-damaging agent creates bulky lesions that can be removed by nucleotide excision repair [35–37] or form interstrand cross-links that can be corrected by double-strand break repair [38]. Similar to ionizing radiation, the interstrand cross-linking agent mitomycin also induces RAD51 foci formation, suggesting that RAD51 is involved in the common DNA repair pathway induced by different DNA-damaging agents [17,39]. BRCA1 has been shown to bind to RAD51 and co-localize with RAD51 foci following ionizing irradiation [7]. However, it is unclear whether a functional BRCA1 is essential for this process, in particular whether it is required in differing ways for different types of DNA-damaging agents to produce their effects.

The purpose of the study we report here was therefore to examine the role of BRCA1 in the formation of RAD51 foci following treatment with cisplatin and ionizing radiation. Our results showed that RAD51 foci formation depends on the presence of a functional BRCA1 following treatment with cisplatin but not with ionizing radiation. This showed that BRCA1 may function differently depending on the type of DNA-damaging signal. Detailed information on these differences would be useful in the development of therapies that exploit the effects of the BRCA1 protein and in the development of therapies for radiation- and cisplatin-resistant cancers.

Materials and methods

Cell cultures. Cells of the SNU-251 endometrioid ovarian cancer cell line were cultured as described previously [40], after which they were maintained in RPMI 1640 medium (Gibco-BRL/Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The human ovarian cancer cell lines DOV-13, SKOV-3, SKOV-3 (pcDNA3), SKOV-3 (vector), and SKOV-3 (small-interfering RNA [siRNA] BRCA1), and the human breast cancer cell lines MCF-7 and HCC1937 were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cancer cell lines DOV-13, SKOV-3, MCF-7, and HCC1937 were purchased from the American Type Culture Collection (Manassas, VA). All cells were grown at 37 °C in a humidified atmosphere that included 5% CO_2.

DNA damage induced by ionizing radiation and cisplatin. The procedure to induce DNA damage through the application of ionizing radiation was performed as described previously [27]. Cells were grown on coverslides for the experiment in which foci formation was induced by ionizing irradiation. Cells showing exponential growth phase were irradiated at 12 Gy and then immediately put into an incubator, where cultures were allowed to continue for 8 h prior to fixation and staining. For the experiment in which foci formation was induced by cisplatin and the experiments examining the time course of the response to cisplatin, cells were washed three times with serum-free medium before and after incubation with serum-free medium containing 10 μM cisplatin for 1 h at 37 °C. After this, cells were allowed to grow in complete medium for 3 h and then were stained or began to be collected at different times for Western blot analyses. For the experiments examining the responses of cells to different doses of cisplatin, cells were treated as above with various concentrations of cisplatin and then collected after 24 h of treatment.

Immunofluorescent analysis. For the immunofluorescence studies, coverslides were fixed in methanol at −80 °C for 5 min and then washed with 1× phosphate-buffered saline. A previously described procedure was used for staining the slides [41]. After permeabilization with 0.2% Triton X-100 and TBST (50 mM Tris, pH 7.4; 150 mM NaCl; and 0.05% Tween 20), cells were blocked with normal serum and then incubated with a monoclonal antibody against human RAD51 (Santa Cruz Biotechnology, Santa Cruz, CA) or BRCA1 (Ab-1; Oncogene Research, Cambridge, MA) followed by a second antibody conjugated with either fluorescein isothiocyanate or Texas red-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology). Finally, cell nuclei were stained with 4′,6-diamidino-2-phenylindole, and the cells were mounted. A confocal microscope (Leica Microsystem, Bannockburn, IL) was used to acquire the microscopic images, and Adobe Photoshop software (Adobe Systems, San Jose, CA) was used to process the images.

Construction of BRCA1 siRNA retroviral vector. The strategy we used to construct a retroviral BRCA1 siRNA expression vector was similar to that described in previous publications [42,43]. Two complementary DNA oligonucleotides against nucleotides 103–129 in the BRCA1 coding region (indicated in bold) were synthesized: GG**TCACAGTGTCCTTTATG TA**ttcaagagata**CATAAAGGACACTGTGA**TTTTTGGAAA and AGCTT TTCCAAAAA**TCACAGTGTCCTTTATG**tatctcttgaa**TACATAAAGGACT GTGA**CC. The *Hin*dIII restriction enzyme sites in the oligonucleotides are underlined. The oligonucleotides in the loop are indicated by lowercase letters (also see Fig. 2A). The pLNCX-U6 retroviral expression vector was cut by *Apa*I, blunted by T4 DNA polymerase, and then cut by *Hin*dIII. Equal amounts of two complementary DNA oligomers were mixed, boiled at 95 °C for 5 min, and slowly cooled to room temperature. The annealed,

double-stranded siRNA BRCA1 was ligated above the pLNCX-U6 vector flanked by the blunted end and *Hin*dIII to create the pLNCX (siRNA BRCA1) retroviral expression vector.

Retroviral infection. We used a previously described retroviral transfection procedure from our laboratory [42,43]. Briefly, a package cell line, AmphoPack 293, was infected with pLNCX (BRCA1 siRNA) by using the calcium phosphate method. After a 2-day infection, viruses were collected to infect the SKOV-3 cells twice. Cells were recovered by growing them in fresh culture medium for 24 h and then were selected by growing them in a neomycin-containing medium for 14 days.

Clonogenic survival assay. A previously described clonogenic survival assay method was followed [27]. Briefly, $1–2 \times 10^3$ cells were plated onto 35-cm^2 plates for 24 h before exposure to cisplatin at various doses for 1 h. After this, the medium was changed to complete medium, and the cells were incubated at 37 °C in an atmosphere containing 5% CO_2 for 8–10 days. Colonies were then counted after staining with crystal violet. Each sample was prepared in triplicate, and each experiment was repeated three times.

MTT assays. The MTT assay was performed as previously described by Zhou et al. [40]. Briefly, cells were grown for 24 h on a 6-well plate before transient transfection. The plasmid pcDNA3 vector (wild-type BRCA1) [40] or pcDNA3 vector (2 μg) was transiently transfected into the cells by using Fugene 6 (Roche Applied Science, Indianapolis, IN). The green fluorescent protein expression plasmid was used to assess transfection efficiency. After 24 h of transfection, cells were diluted and plated onto a 96-well plate containing various doses of cisplatin for 24 h. The viability of cells was determined by using WTS-1 kits (Promega, Madison, WI).

Immunoblotting. The immunoblotting procedures used were those described in detail by Zhou et al. [40]. Briefly, cells were washed with ice-cold phosphate-buffered saline and lysed in 1× lysis buffer (50 mM Tris–HCl, pH 8.0; 120 mM NaCl; and 0.5% Nonidet P-40), 50 mM NaF, 1 mM sodium orthovanadate, 100 μg/ml phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 10 μg/ml leupeptin for 1 h on ice. Protein extracts were run on a 6% sodium dodecyl sulfate–polyacrylamide gel and transferred to a nitrocellular membrane (Bio-Rad, Hercules, CA). The membrane was incubated with dried skim milk for 1 h and then blotted with a primary mouse anti-BRCA1 monoclonal antibody (1:100, Ab-1; Oncogene Research) or RAD51 (1:1000). The membranes were washed three times for 10 min each with Tris-buffered saline (0.9% sodium chloride and 20 mM Tris–HCl, pH 7.4) supplemented with 0.05% Tween 20 (TBST). The peroxidase-conjugated horse anti-mouse antibody (1:5000, Amersham Pharmacia Biotech, Piscataway, NJ) was added, and the membranes were then washed with TBST three times for 10 min each. An enhanced chemiluminescence kit was used to detect the signals (Amersham Pharmacia Biotech).

Immunoprecipitation with anti-tyrosine antibody and phosphatase treatment. SKOV3 cells were cultured in 10 cm tissue to 60% confluence and treated with or without 10 mM cisplatin for 24 h and the cell lysates (up to 1 mg protein) were incubated with 5 μg anti-*p*-tyrosine antibody (Zymed Laboratories, San Francisco, CA) at 4 °C for 4 h. Twenty microliters of protein A–Sepharose beads was added to the lysates and rotated at 4 °C overnight. The immune complexes were collected, washed four times, and inoculated with 400 units of lamda protein phosphates (λppase) from New England Biolab (Beverly, MA) in 50 μl of reaction mixture at 30 °C for 30 min. The immune complexes were resolved using 6.5% SDS–PAGE gel, transferred to nitrocellular membrane, and exposed to a mouse anti-BRCA1 monoclonal antibody (1:100, Ab-1; Oncogene Research). The signals were detected using same methods described above.

Results

Carboxyl-terminal of BRCA1 is required for BRCA1 foci formation induced by cisplatin and ionizing radiation

To understand the role of BRCA1 in cancer cells after exposure to cisplatin or ionizing radiation, we examined the subnuclear formation of BRCA1 foci after treatment with cisplatin or ionizing radiation. Both the SNU-251 ovarian cancer cells and HCC1937 breast cancer cells expressed the carboxyl-terminal-truncated BRCA1 protein and were used for comparisons with the SKOV-3 ovarian cancer cells, which expressed full-length BRCA1 (Fig. 1A). Approximately 74% and 58% of SKOV-3 cells had BRCA1 foci after ionizing radiation and cisplatin treatment, respectively (Figs. 1B and E). In contrast, there was a great decrease in the proportion of SNU-251 cells with BRCA1 foci (12%) and virtually no such foci detected in HCC1937 cells (0.5%) after ionizing radiation treatment compared with the proportion of SKOV-3 cells with these foci (74%) (Figs. 1B–E). The decrease was even greater for SNU-251 (4%) and HCC1937 (0.5%) cells after cisplatin treatment (Figs. 1C–E). These data showed that the carboxyl-terminal of BRCA1 is critical for BRCA1 foci to form after cisplatin and ionizing radiation treatment.

Carboxyl-terminal of BRCA1 is required for assembly of RAD51 and BRCA1 complex after cisplatin but not ionizing radiation treatment

It has been reported that BRCA1 could co-localize with RAD51 after exposure to ionizing radiation [6,7,25]. To determine whether BRCA1 is required for the subnuclear assembly of RAD51 after cisplatin treatment, SNU-251, HCC1937, and SKOV-3 cells were double stained with the anti-BRCA1 and anti-RAD51 antibody after ionizing radiation or cisplatin treatment. About 73% of SNU-251 cells and 54% of HCC1937 cells had RAD51 foci after ionizing radiation; 66% of SKOV-3 cells had these foci (Figs. 1B, C, D, and F). This indicates that the carboxyl-terminal of BRCA1 is not required for the subnuclear assembly of RAD51 after ionizing irradiation. In contrast, only 37% of SNU-251 cells and 7% of HCC1937 cells had RAD51 foci after cisplatin treatment, while 55% of SKOV-3 cells had these foci after cisplatin treatment (Figs. 1B, C, D, and F). The co-localization of BRCA1 and RAD51 was detected in 2% of SNU-251 cells and 26% of SKOV-3 cells, as shown in the merged pictures (Figs. 1B, C, D, and F). These results therefore suggest that the carboxyl-terminal of BRCA1 is essential for recruiting RAD51 to the DNA repair site after exposure to the DNA-damaging agent cisplatin, but it is only minimally involved in RAD51 assembly after ionizing irradiation.

Depletion of BRCA1 reduces RAD51 foci formation after cisplatin but not ionizing radiation treatment

To further show that BRCA1 is required for RAD51 foci to form after cisplatin treatment, we sought to reduce the expression of the BRCA1 protein by using an siRNA technique in SKOV-3 cells that normally express high levels of BRCA1 so that the effect of RAD51 subnuclear assembly could be tested against an identical genetic background. In this experiment, we used previously described procedures

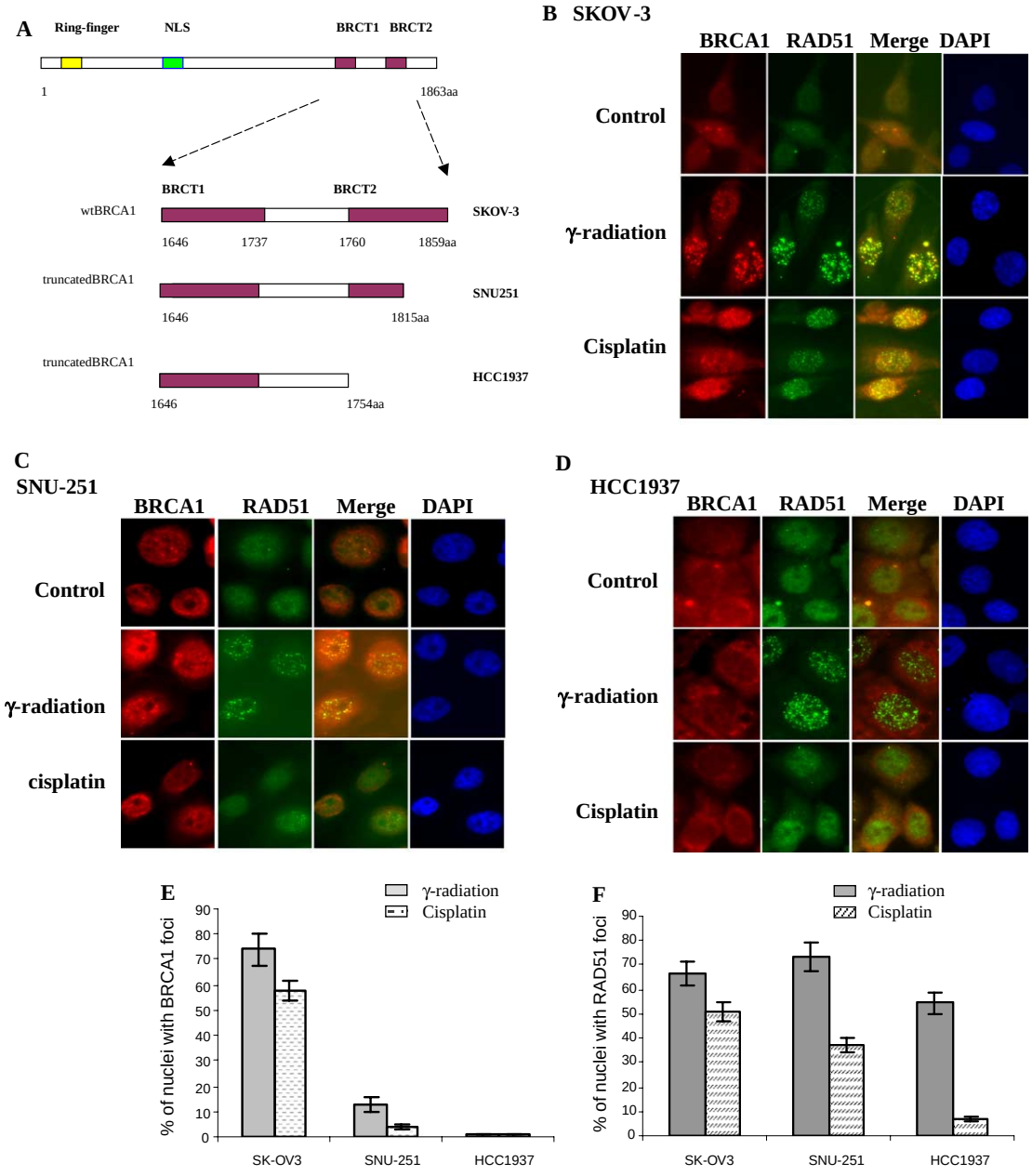

Fig. 1. (A) BRCA1 mutations in SKOV-3, SNU-251, and HCC1937 cells. aa, amino acid; NLS, nuclear localization signal; BRCT1 or BRCT2, BRCA C-terminal tandem repeat 1 or 2. (B–D) Formation of BRCA1 and RAD51 subnuclear foci and co-localization of BRCA1 and RAD51 after ionizing γ-radiation and cisplatin treatment in SKOV-3 (B), SNU-251 (C), and HCC1937 (D) cells. The cells were double stained with anti-BRCA1 antibody and anti-RAD51 antibody. The first column shows BRCA1 subnuclear foci (red dots) after treatment consisting of 15 Gy of ionizing γ-radiation or 10 μM cisplatin for 3 h; the second column shows RAD51 subnuclear foci (green dots); the third column shows merged pictures depicting the co-localization of BRCA1 and RAD51 co-localization (indicated by yellow dots); and the fourth column shows the cell nuclei (blue) stained by 4′,6′-diamidino-2-phenylindole. Control represents no treatment. (E) Histogram shows the percentages of nuclei containing detectable BRCA1 foci after treatment with 15 Gy of ionizing γ-radiation for 8 h or 10 μM cisplatin for 3 h. (F) Histogram shows the percentages of nuclei containing detectable RAD51 foci after treatment with 15 Gy of ionizing γ-radiation or 10 μM cisplatin for 3 h. Two different individuals counted 200 cells of each cell line. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

used in our laboratory to first create a retroviral expression vector expressing siRNA against BRCA1 [42,43]; the schematic of the siRNA against BRCA1 in the retroviral expression vector is shown in Fig. 2A. Western blot analysis confirmed that BRCA1 expression was decreased by about 80% in the SKOV-3 (siRNA BRCA1) cells compared with the level in the SKOV-3 (vector) cells (Fig. 2B). After ionizing radiation, BRCA1 foci were detected in only 15% of SKOV-3 (siRNA BRCA1) cells transfected with siRNA BRCA1 compared with 75% of SKOV-3 (vector) cells containing the empty vector (Fig. 2C). After cisplatin treatment, only 5% of SKOV-3 (siRNA BRCA1) cells showed BRCA1 foci compared with 65% of SKOV-3 (vector) cells. Therefore, BRCA1 foci

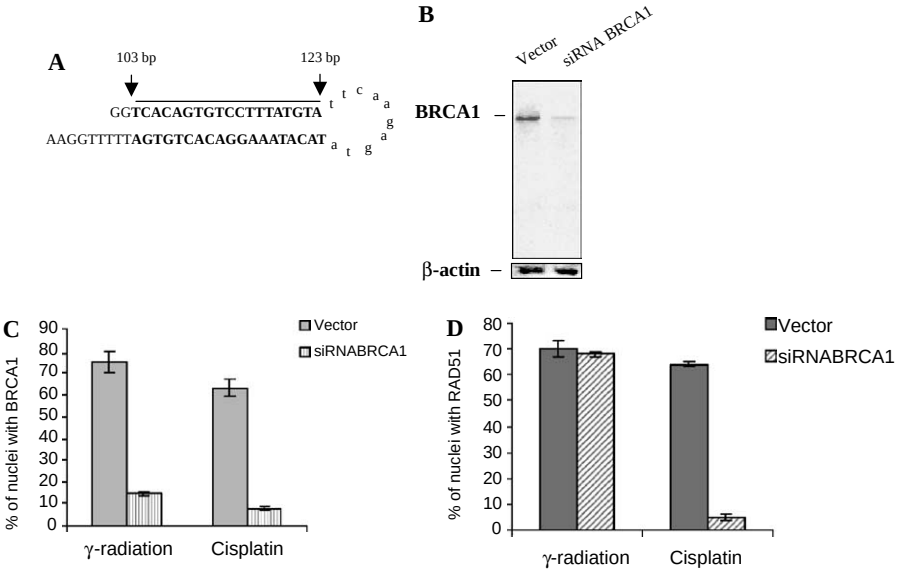

Fig. 2. Depletion of BRCA1 in SKOV-3 cells by siRNA and foci formation following treatment of γ-radiation and cisplatin. (A) Predicted siRNA BRCA1 structure. (B) BRCA1 expression level in SKOV-3 (siRNA BRCA1) and SKOV-3 (vector) cells was analyzed by Western blot. (C,D) SKOV-3 (siRNA BRCA1) and SKOV-3 (vector) cells were treated with either 10 μM cisplatin or 12 Gy of γ-ionizing radiation. The cells were then double stained with anti-BRCA1 antibody and anti-RAD51 antibody, BRCA1 and RAD51 foci were visualized and counted as described in Figs. 1B and C. The relative number of positive foci is shown in histograms.

formation was reduced by about 60–65% by siRNA BRCA1 in SKOV-3 cells after ionizing radiation or cisplatin treatment. However, depletion of the BRCA1 protein in the SKOV-3 cells resulted in an approximate 60% decrease in RAD51 foci formation after cisplatin treatment compared with that in SKOV-3 (vector) cells (Fig. 2D). After ionizing radiation treatment, about 70% of SKOV-3 (siRNA BRCA1) and 68% of SKOV-3 (vector) cells had RAD51 foci (Fig. 2D). These results thus further showed that BRCA1 is required for the recruitment of RAD51 for DNA-damage repair in cells treated with cisplatin but not ionizing radiation.

Carboxyl-terminal of BRCA1 is involved in BRCA1 phosphorylation induced by cisplatin

To determine whether the carboxyl-terminal of BRCA1 is required to mediate the effect of RAD51 assembly after cisplatin treatment, we examined the phosphorylation of BRCA1 in SKOV-3, SNU-251, and HCC1937 cells, as reflected by the shift in BRCA1 protein size. In SKOV-3 cells, the level of regular BRCA1 gradually decreased and phosphorylated BRCA1 began to be detected after 4 h of exposure to cisplatin (Fig. 3A, lanes 1–5). After 24 h of cisplatin treatment, phosphorylated BRCA1 could

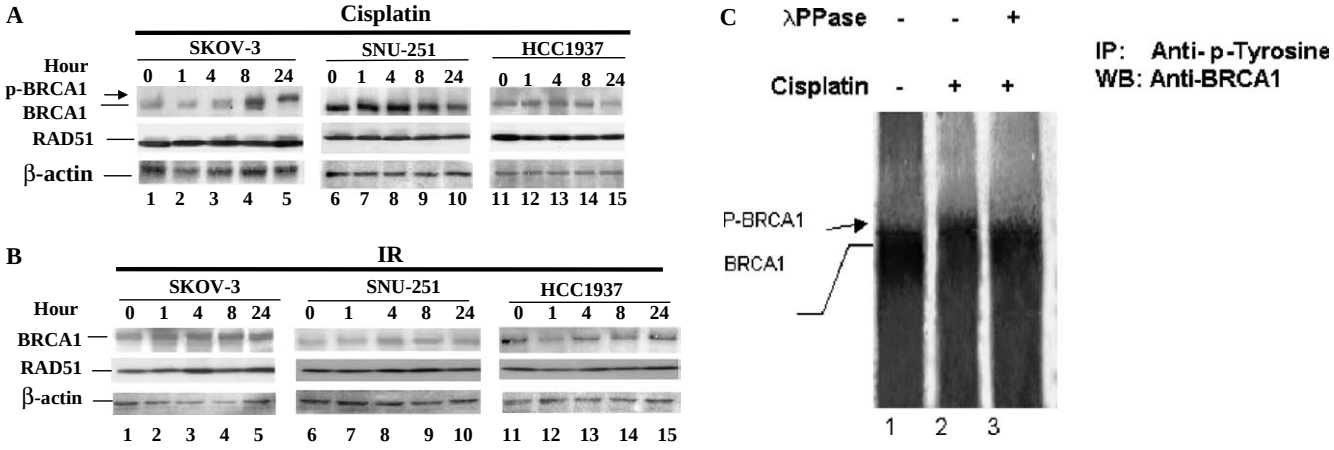

Fig. 3. Carboxyl-terminal of BRCA1 is involved in BRCA1 phosphorylation induced by cisplatin. (A) The expression of BRCA1 and RAD51 was detected using Western blots after SKOV-3, SNU-251, and HCC1937 cells were treated with 10 μM cisplatin (B) or 12 Gy of ionizing γ-radiation (IR) for various times. β-Actin was used as a loading control. (C) The phosphorylated BRCA1 protein can be immunoprecipitated by anti-tyrosine antibody. The cell extracts were first immunoprecipitated with anti-tyrosine antibody following cisplatin treatment. Then the immunoprecipitates were treated with lamda phosphatase and probed with antibody BRCA1 antibody as described in Materials and methods.

be detected and the level of phosphorylated BRCA1 had dramatically increased, suggesting that BRCA1 phosphorylation was induced by cisplatin in a time-dependent manner. However, the mobility shift was completely lost in SNU-251 cells (lanes 6–10) and HCC1937 cells (Fig. 3A, lanes 11–15), but there is no change in the level of RAD51 or its size after cisplatin treatment (Fig. 3A, lanes 1–15). There was also no significant change in the mobility shift of BRCA1 in any of the three cell lines after ionizing irradiation (Fig. 3B). These results suggest that the carboxyl-terminal of BRCA1 contains uncharacterized phosphorylation sites that are specifically induced by cisplatin.

To further characterize the nature of phosphorylation site(s) induced by cisplatin, we immunoprecipitated the cell extracts following the treatment with cisplatin, which were subsequently treated with lamda phosphatase before they were run on the SDS-gel and probed with anti-BRCA1 antibody. As shown in Fig. 3C, anti-tyrosine antibody detected slower migrating band(s) as compared with cell extracts without treatment of cisplatin. The intensity of such slower migrating bands was significantly decreased following the treatment of lamda phosphatase, suggesting that cisplatin most likely induced the phosphorylation of tyrosine residue(s) in the BRCA1 protein.

Loss of BRCA1 increased cellular sensitivity to cisplatin

To examine the effect of wild-type and mutant BRCA1 on the cellular response to cisplatin, we compared the survival of SKOV-3 (vector), MCF-7, and DOV-13 cells, which contain wild-type BRCA1, with the survival of SNU-251 and HCC1937 cells, which contain mutant BRCA1. In addition, we compared the survival of SKOV-3 cells with their isogenic counterpart, created by retrovirus-mediated siRNA interference, and the survival of SNU-251 cells with their isogenic counterpart, created by the re-introduction of wild-type BRCA1. Clonogenic assay was performed to analyze cellular sensitivity to cisplatin. The results showed that the HCC1937, SNU-251, and SKOV-3 (siRNA BRCA1) cells were 2- to 3-fold more sensitive to cisplatin at very low concentrations and about 50-fold more sensitive to cisplatin at a 3 μM concentration than were SKOV-3 (vector), MCF-7, and DOV-13 cells (Fig. 4A). Similar results were seen for SNU-251 cells with the re-introduced wild-type BRCA1. That is, as shown by the MTT assay, the cells containing wild-type BRCA1 were more resistant to cisplatin than were SNU-251 cells containing the empty vector (Fig. 4B). Thus, it appears that the carboxyl-terminal of BRCA1 is important for this molecule to protect cells from the cytotoxic effect of cisplatin and plays an important role in resistance to cisplatin.

Discussion

Our findings showed that BRCA1 is required for BRCA1 foci to form after treatment with either ionizing radiation or cisplatin; however, functional BRCA1 is not required for the formation of RAD51 foci after ionizing irradiation, but it is essential for the formation of RAD51 foci after cisplatin treatment. Our data also suggested that the carboxyl-terminal of BRCA1 is required to mediate such an effect following cisplatin treatment and contains uncharacterized cisplatin phosphorylation sites, most likely involving tyrosine residue(s). Two other groups [44,45] also observed that BRCA1 is critical for RAD51 foci formation following cisplatin treatment in human cancer cells. In addition, it appears that BRCA1 is required for RAD51 foci formation in murine cells following treatment with either cisplatin or ionizing radiation [27], but the RAD51 foci formation induced by cisplatin or ionizing radiation in human cells appears to result from the activation of two distinct pathways: in the case of cisplatin, the pathway requires functional BRCA1; in the case of ionizing radiation, the pathway requires functional BRCA2 [26]. These two pathways are diagrammed in Fig. 5.

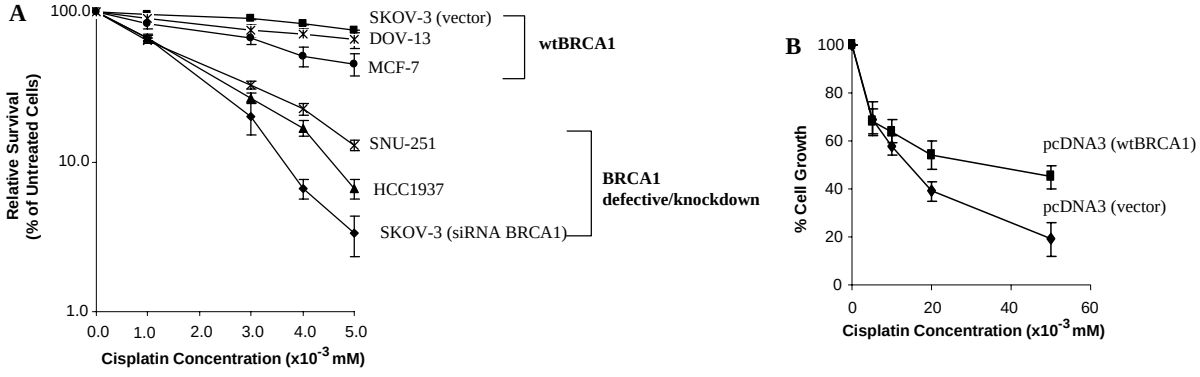

Fig. 4. Sensitivity of wild-type and mutant BRCA1 cells to cisplatin. (A) Cells were exposed to 10 μM cisplatin for 1 h and then allowed to grow in complete medium for 10–14 days, after which colonies were counted. Each sample was prepared in triplicate, and experiments were repeated at least once. (B) The plasmid pcDNA3 (wtBRCA1) containing the wild-type BRCA1 cDNA was transiently transfected into SNU-251 cells. Forty-eight hours after transfection, the cells were treated with 10 μM cisplatin for 24 h. The percentage of surviving cells was measured by the MTT assay. Cells transiently transfected with a pcDNA3 empty vector were used as the control. Error bars indicate the standard error (SE). Where error bars are not shown, the SEs were too small to show on the graph.

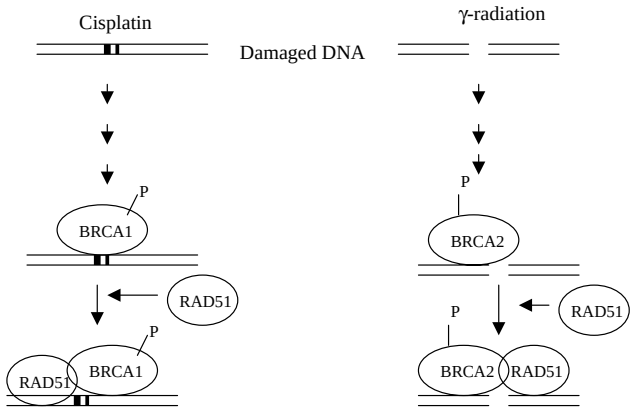

Fig. 5. Schematic models of BRCA1 and BRCA2 in the assembly of RAD51 foci formation following cisplatin and ionizing γ-radiation treatment. Following insult of cisplatin, the DNAs were cross-linked, while γ-radiation resulted in double-stranded breaks. These two different damage signals will attract BRCA1 and BRCA2 to the damage sites, respectively, which was followed by the binding of RAD51.

BRCA1 has recently been shown to also function in the same pathway with Fanconi's anemia proteins [19]; this anemia is a cancer susceptibility syndrome that is highly sensitive to the interstrand cross-linking agents mitomycin and cisplatin. In terms of the mechanism, BRCA1 is crucial for recruiting Fancd2 to the MMC-induced DNA-damage repair pathway [19]. The RING domain is commonly associated with ubiquitin-ligase activity, which polymerizes ubiquitin on the target protein for destruction by the ubiquitin-mediated proteolytic pathway. Fanconi's anemia subtype D2 is the target for this ligase activity, but in this case, BRCA1 may not be directly involved in the mono-ubiquitination of Fancd2 [19]. It must be emphasized, however, that although BRCA1 and not BRCA2 is required for cisplatin-mediated RAD51 assembly to take place, both BRCA1 and BRCA2 appear to be involved in the DNA repair induced by cisplatin. Similar to BRCA1 in the pathway of Fanconi's anemia, BRCA2 was identified as the gene product responsible for a different subtype of Fanconi's anemia, Fanc-B1 and Fanc-D [46]. The precise role of BRCA1, the BRCA2, and Fanconi's anemia gene products in DNA repair is not clear at this time. It is possible that the Fanconi's anemia, BRCA1, and BRCA2 gene products are a bridge to the assembly of the DNA repair machine involving RAD51. Extensive biochemical characterization and in vitro reconstitution experiments with appropriate substrates may need to be performed before the mechanistic details can be fully understood.

Our data suggested that the second BRCT repeat is essential for the subnuclear assembly of BRCA1 and RAD51 complexes following treatment with cisplatin. This region also contains cisplatin-specific phosphorylation sites, which are likely to play a critical role in this process. Recently, a BRCT repeat was shown to be a phosphopeptide-binding module that is responsible for the phosphorylation-dependent recruitment of proteins into DNA repair complexes [20,21]. Because several previously reported phosphorylation sites that are responsive to ionizing radiation and UV light [47–51] are located before the BRCT repeat, the second repeat is likely to be a novel uncharacterized phosphorylation site that is responsive to cisplatin and that is different from these previously reported ones. Although less likely, it is still possible that cisplatin causes phosphorylation at the same sites as those induced by ionizing radiation or UV light but that deletion of the second repeat results in a conformational change in the protein and makes these sites unresponsive to 'ataxia telangiectasia mutated' (ATM) and ataxia telangiectasia and Rad3-related protein (ATR) kinases. Further defining the phosphorylation sites in this region may therefore shed new light on the mechanism of RAD51 foci formation.

The signal transduction pathway that leads to the phosphorylation of BRCA1 following treatment with cisplatin is not clearly understood. Cisplatin may activate or partially activate ATM and ATR pathways similar to those induced following exposure to ionizing radiation or UV. This is supported by the finding that both ATM and ATR are responsible for the phosphorylation of BRCA1 after exposure to ionizing radiation, UV light, and mitomycin [47,50]. However, it has also been reported recently that cross-linking agents activate the ATR-CHK1 and ATR-NBS1-Fanc-d2 branch pathways [52]. Although it is well accepted that these pathways play a role in the phosphorylation of BRCA1, the molecular pathways used by BRCA1 in the formation of RAD51 following treatment with cisplatin are still unknown at this time. It will therefore be important to examine the status of the phosphorylation of BRCA1 in light of this newly described branch pathway that is responsive to cisplatin.

In conclusion, our data showed that RAD51 repair complex assembly requires a functional BRCA1 following treatment with cisplatin, probably due to the phosphorylation of the second BRCT repeat. Fully understanding how BRCA1 functions in the DNA damage and repair induced by cisplatin should also help explain the resistance of ovarian cancers to cisplatin, an extremely common and frustrating situation in the treatment of patients with ovarian cancer. Understanding such a signaling pathway will allow us to identify patients who will not benefit from this treatment, thereby saving them from undergoing unnecessary treatment. It will be also possible to change the therapeutic response by molecular intervention to disable BRCA1 during treatment with cisplatin.

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and the M. D. Anderson Cancer Center *SPORE in Ovarian Cancer*.

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