RESEARCH PAPER

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RAD50 targeting impairs DNA damage response and sensitizes human breast cancer cells to cisplatin therapy

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In tumor cells the effectiveness of anti-neoplastic agents that cause cell death by induction of DNA damage is influenced by DNA repair activity. RAD50 protein plays key roles in DNA double strand breaks repair (DSBs), which is crucial to safeguard genome integrity and sustain tumor suppression. However, its role as a potential therapeutic target has not been addressed in breast cancer. Our aim in the present study was to analyze the expression of RAD50 protein in breast tumors, and evaluate the effects of RAD50-targeted inhibition on the cytotoxicity exerted by cisplatin and anthracycline and taxane-based therapies in breast cancer cells. Immunohistochemistry assays on tissue microarrays indicate that the strong staining intensity of RAD50 was reduced in 14% of breast carcinomas in comparison with normal tissues. Remarkably, RAD50 silencing by RNA interference significantly enhanced the cytotoxicity of cisplatin. Combinations of cisplatin with doxorubicin and paclitaxel drugs induced synergistic effects in early cell death of RAD50-deficient MCF-7, SKBR3, and T47D breast cancer cells. Furthermore, we found an increase in the number of DSBs, and delayed phosphorylation of histone H2AX after cisplatin treatment in RAD50-silenced cells. These cellular events were associated to a dramatical increase in the frequency of chromosomal aberrations and a decrease of cell number in metaphase. In conclusion, our data showed that RAD50 abrogation impairs DNA damage response and sensitizes breast cancer cells to cisplatin-combined therapies. We propose that the development and use of inhibitors to manipulate RAD50 levels might represent a promising strategy to sensitize breast cancer cells to DNA damaging agents.

Introduction

DNA-damaging drugs represent one of the main treatment strategies in human cancer.[1] Platinum-based agents such as cisplatin, and taxanes including doxorubicin and paclitaxel, are the mainstay of chemotherapy for ovarian, testicular, esophageal, stomach, bladder, lung, breast, and head and neck cancers.[2,3] These drugs function as DNA damaging agents, topoisomerase inhibitors, and microtubules stabilizers thereby interfering with the ability of cancer cells to divide and survive.[4] Particularly, cisplatin is a bi-functional agent, which forms intra-chain and inter-chain bonds in DNA strands generating double strand breaks (DSBs). At the cellular level, cisplatin causes inhibition of DNA replication and transcription, cell cycle arrest and apoptosis-induced cell death.[5] However, DNA damage produced by cisplatin does not always result in killing cells, because several distinct cellular responses conducing to enhanced DNA repair are activated to overcome the genotoxic insults.[6,7] Importantly, DNA damage response is not only activated in response to chemotherapy; it has been established that proper activation of DNA damage repair represents a biological anti-cancer barrier in human pre-cancerous lesions in early stages of tumorigenesis.[8-10]

The effectiveness of anti-cancer agents that act causing extensive DSB and cell death is largely influenced by DNA repair pathways. In tumor cells, enhanced DNA repair induces chemoresistance preventing the accumulation of lethal DNA damage from cytotoxic agents.[7] These observations provided the basis for the use of DNA repair inhibitors to improve the therapeutic effects of DNA-damaging drugs.[11-14] Homologous recombination and non-homologous end joining represent key cellular mechanisms to repair DNA damage. The heterotrimeric MRE11-RAD50-NBS1

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Table 1. Clinical features of breast tumors analyzed for RAD50 expression by immunohistochemistry in tissue microarrays

Characteristics	Description	Number (n = 50)
Classification	HER2	4
	Luminal A	29
	Luminal B	3
	Triple negative	8
	Unknown	6
Receptors expression	Estrogen receptor	36
	Progesterone receptor	23
	HER2/neu	7
Histology	Infiltrating ductal carcinoma	34
	Infiltrating lobular carcinoma	2
	Invasive lobular carcinoma	3
	Invasive mucinous carcinoma	3
	Mixed carcinoma	4
	Others	5
Tumor size	1–2 cm	21
	2–3 cm	23
	>3 cm	6

(MRN) protein complex is a critical component in both pathways for sensing, signaling, and repairing DSBs.[15,16] In addition, the MRN complex participates in checkpoint activation, cell cycle arrest, telomeres length maintenance, and DNA replication.[17] Specifically, RAD50 is an ATP-modulated cross-linker protein that acts as a bridge at the junction of DSBs, facilitating the recognition and processing of DNA ends by MRE11 exonuclease to initiate DNA damage repair. Several genetic studies highlighted the value of MRN complex in breast cancer susceptibility. MRE11 and RAD50 have been described as risk genes associated to genomic instability in hereditary breast cancer.[18] Other studies showed that polymorphisms in MRN genes were also associated with risk of breast and ovarian cancer.[19,20] In addition, germline mutations of MRE11 suggested that it is a novel breast cancer susceptibility gene in non-BRCA1/2 families.[21] Furthermore a reduced expression of MRN complex has been reported in breast tumors,[21,22] and predicted a poor effect of radiotherapy in patients with early breast cancer.[23] Two previous studies have explored RAD50 as a potential therapeutic target in head and neck cancer squamous cell carcinoma.[24,25] However, the role of RAD50 as a sensitizer for DNA-damaging agents has not been addressed in breast cancer. In the present study, we evaluated the effect of RAD50 inhibition on DNA damage response, and cytotoxicity of cisplatin-combined therapies in breast cancer cells. Our results highlighted the promising role of RAD50 as a novel molecular target in breast cancer which deserves further investigation.

Results

RAD50 expression is deregulated in breast tumors

Here, the expression of RAD50 protein in 50 locally invasive breast tumors and 28 normal breast tissues was analyzed by immunohistochemistry on tissue microarrays (TMA). Clinical characteristics of breast tumors are summarized in **Table 1**. None of the patients recruited in this study received any anti-neoplastic therapy prior to tumor resection. Staining of RAD50 in TMA experiments was scored as strong (3), moderate (2), and weak–null staining (0–1). Results showed a strong nuclear staining for RAD50 in 43%, moderate in 29%, weak in 24%, and null in 4% of breast tumors. In normal tissues a strong staining for RAD50 in 57%, moderate in 21%, and weak in 21% was found (**Fig. 1**). Cytoplasmic staining was detected in less than 3% of cells and no significant differences were found between normal and tumor cells. Compared with normal tissues, the strong staining intensity of RAD50 was reduced in 14% of breast tumors. A comparison of combined staining scores (strong and moderate) showed that RAD50 expression was also reduced in 6% of breast carcinomas relative to normal tissues.

Knockdown of RAD50 expression by RNA interference

To determine if targeted inhibition of RAD50 could be a suitable strategy to sensitize breast cancer cells to anti-neoplastic agents, first we evaluated its expression in order to select a cell line model for further analysis. Western blot assays using specific antibodies and total proteins from MDA-MB 231, MCF-7, and ZR-75-1 breast cancer cell lines showed that RAD50 was expressed at similar levels in the three cell lines (**Fig. 2A**). β-actin used as a control, did not show significant changes in expression among cell lines. From these experiments we selected epithelial MCF-7 cells as they do not readily undergo apoptosis following DNA damage, and because they have been previously used as a suitable cell model for sensitization to therapy by knockdown of related DNA repair genes.[26] Then, we designed three specific short hairpin RNAs (dubbed as shRAD50.3, shRAD50.4, and shRAD50.5) targeting the human *RAD50* gene (**Table 2**). In addition, we used two shRNAs sequences previously used for *RAD50* silencing in head and neck cancer,[27] and fibrosarcoma cells,[28] denoted here as shRAD50.1 and shRAD50.2. The five constructs were individually introduced into MCF-7 cells and RAD50 expression was analyzed by RT-PCR and western blot at 48 h after transfection. Results showed that shRAD50.3, shRAD50.4 and shRAD50.5 sequences effectively downregulated the *RAD50* mRNA and protein expression (**Fig. 2B–D**). Densitometric analyses of immunodetected bands showed that shRAD50.4 and shRAD50.5 were the most effective interfering RNA sequences since they suppressed RAD50 protein expression by 96% and 98%, respectively (**Fig. 2E**). Thus we selected both sequences for further shRNA-based gene silencing experiments. No significant changes were observed in the expression of *GADPH* mRNA and β-actin protein used as controls.

RAD50 abrogation enhances the cytotoxicity of cisplatin in vitro

The effects of RAD50 silencing in cell viability after cisplatin treatment were evaluated. MCF-7 cells were submitted to

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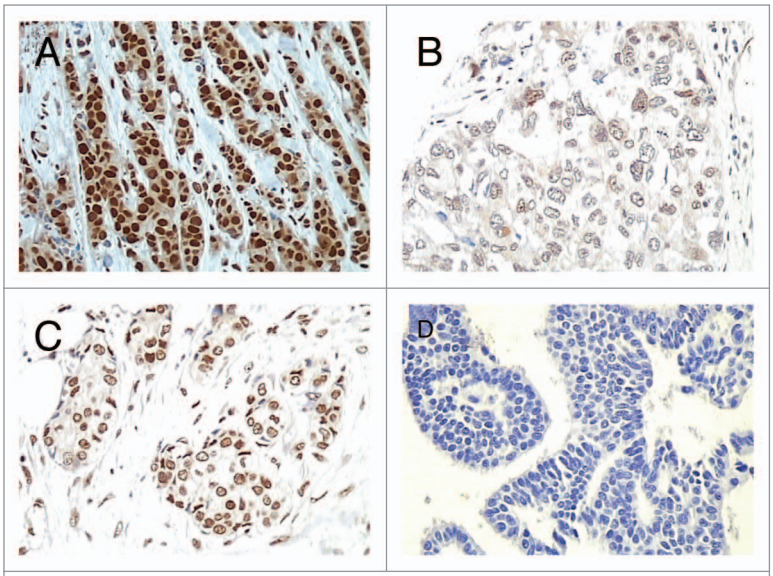
four interventions as follows: non-transfected (control), treated with shRad50.4 or shRad50.5, treated with cisplatin alone (25.8 μM), and treated with both shRad50.4 or shRad50.5 and cisplatin. Data showed that cells treated with shRad50.4 or shRad50.5 exhibit a notorious delay in cell growth at 24 h and 48 h in comparison with control (**Fig. 3A**), which evidenced the importance of RAD50 for sustained cell proliferation. MCF-7 cells treated with cisplatin alone exhibit a marked reduction in cell proliferation at 24 h and 48 h. Remarkably, combined therapy using shRAD50.4 and cisplatin induced extensive cell death at 36 and 48 h (97% and 99%, respectively) in comparison with control. Importantly, this cytotoxic effect was more pronounced in RAD50-deficient cells in comparison with cells treated with cisplatin alone at 12 h. Combined shRAD50.4 and cisplatin therapy produced an early synergistic effect because at 12 h after treatment we found ~80% in cell death in comparison with control, which is significantly higher that cisplatin monotherapy (~20%). Consistently, a similar behavior was observed in MCF-7 cells transfected with shRAD50.5 sequence and treated with cisplatin (**Fig. 3A**). These results indicate that RAD50 abrogation effectively potentiated the early cytotoxic effects of cisplatin in vitro.

RAD50 inhibition potentiates the cytotoxicity of cisplatin-combined therapies

We next analyzed the effects of RAD50 inhibition on the cytotoxicity of cisplatin in combination with an anthracycline and taxane drugs. Doxorubicin and paclitaxel were used at 2.5 μM and 0.2 μM, respectively, which correspond to the calculated IC_{50} in MCF-7 cells (data not shown). MCF-7 cells were submitted to four interventions as described above. Results showed that cisplatin/doxorubicin combination was more effective at early time as it induced a significant increase in cell death of RAD50-deficient cells at 12 h (40%) in comparison with non-transfected MCF-7 cells and treated with cisplatin/doxorubicin (**Fig. 3B**). No significant differences between treatments were found at 24 h and 48 h. This effect in cell viability was similar in MCF-7 cells transfected with shRAD50.4 or shRAD50.5 and treated with cisplatin/doxorubicin combination. Consistently, we observed similar cytotoxic effects when cisplatin/paclitaxel combination was used in RAD50-deficient cells and non-transfected MCF-7 cells (**Fig. 3C**). These data indicate that RAD50 knockdown produced an early sensitization of MCF-7 cells to cisplatin-combined treatments.

To extend these initial findings, we analyzed the MDA-MB-231 and BT20 triple negative breast cancer cells. Both cell subtypes were submitted to four interventions as described above. Results showed that RAD50 abrogation induced a generalized cell death at 12 h in both cancer cell lines, thus they were no suitable for further evaluation of the effects of RAD50 inhibition on the cytotoxicity exerted by cisplatin (data not shown). These findings are in agreement with previous observations indicating that triple negative breast cancer cells are very sensitive to inhibitors of DNA damage repair. Then, we analyzed the SKBR3 and T47D breast cancer cells which are classified as HER2 and estrogen receptors positive, respectively. Both cell lines were individually transfected with shRAD50.4 and analyzed by western blot 48 h after transfection. Results showed that shRAD50.4 sequence effectively suppressed the expression of RAD50 at protein level (**Fig. 4A**). Then, SKBR3 and T47D cells were submitted to drugs treatments. Our results indicate that SKBR3 and T47D cells treated with cisplatin alone exhibit a significant reduction in cell viability at 12 h and 48 h in comparison to controls (**Fig. 4B and C**). Combined cisplatin/doxorubicin and cisplatin/paclitaxel therapies induced an early synergistic cytotoxic effect (12 h) in both SKBR3 and T47D RAD50-deficient cells relative to cisplatin monotherapy (**Fig. 4B and C**), although the effect was less pronounced in comparison with MCF-7 cells. No significant differences between treatments were found at 36 h and 48 h.



E

Staining scores	Tumors (%) n=50	Normal (%) n=24
Negative	0	0
Strong	21 (43)	16 (57)
Moderate	14 (29)	6 (21)
Weak	13 (24)	6 (21)
Null	2 (4)	0

Figure 1. Immunostaining of tumor breast tissues analyzed for RAD50 expression by tissue microarrays. Representative images for RAD50 expression in breast tumors. Immunostaining was defined as (**A**) high, (**B**) moderate, (**C**) weak, and (**D**) null. Magnification is 400×. (**E**) Summary of levels of expression of RAD50 in breast carcinomas based on the immunohistochemical scoring described in materials and methods.

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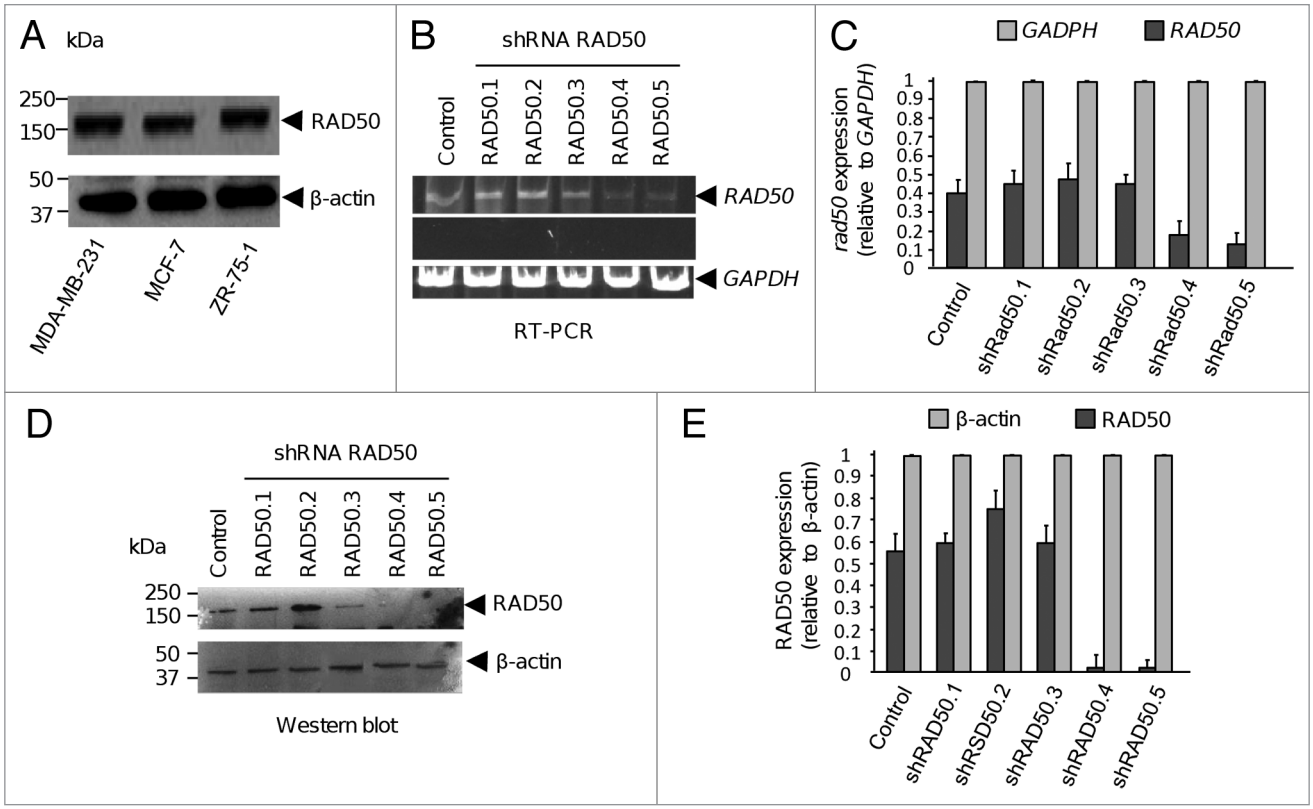

Figure 2. RAD50 expression and shRNA-mediated RAD50 silencing in breast cancer cells. (**A**) Western blot assays for RAD50 expression in MCF-7, MDA-MB-231, and ZR-75-1 breast cancer cells. β-actin was used as internal loading control. (**B**) RT-PCR assays for RAD50 knock-down using three shRNAs in MCF-7 cells. *GAPDH* gene was amplified as internal control. (**C**) Densitometric analysis of bands from B. (**D**) Western blot assays for RAD50 protein in MCF-7 cells. Total proteins isolated from shRAD50-transfected cells were resolved by SDS-PAGE, transferred to PVDF membrane, and blotted with anti-RAD50 antibodies. β-actin protein was detected as internal loading control. (**E**) Densitometric quantification of immunodetected bands depicted in (**D**). In both RT-PCR and western blot assays, pixels corresponding to *GAPDH* mRNA and β-actin protein expression, respectively, were taken as 100% and used to normalize RAD50 expression. Representative data are shown and densitometric data represent the mean of three independent assays ± SD.

Increased DNA damage in RAD50-deficient cells was associated with delayed H2AX phosphorylation

Cisplatin induces DSBs resulting in apoptosis and cell death. We evaluated the extent of in situ fragmented DNA in RAD50-deficient cells using TUNEL assays. MCF-7 cells were transfected with shRAD50.4 and DNA damage was analyzed at 30 min, 3 h, and 12 h after cisplatin treatment. Results showed that both non-treated and shRAD50.4 transfected cells had low levels of fragmented DNA (**Fig. 5A and E**). In contrast, RAD50 silenced-cells treated with cisplatin exhibit a time-dependent and higher increase in DNA damage (**Fig. 5F–H**) in comparison with MCF-7 cells treated with cisplatin alone (**Fig. 5B–D**), being more evident at 12 h after treatment.

Then, we sought if RAD50 suppression impairs the cellular response to cisplatin induced-DSBs through the quantification of the phosphorylated form of H2AX histone (γH2AX), which represents an early marker of DNA damage. Western blot assays in non-transfected MCF-7 cells or transfected with shRad50.4 and treated with cisplatin were performed. As expected, γH2AX was detected at high levels in control cells after 30 min of treatment (**Fig. 6A**). In contrast, RAD50-deficient cells exhibit very low levels of γH2AX in the absence of drug. However, γH2AX levels were increased in a time-dependent manner in response to cisplatin treatment (**Fig. 6B**). After 3 h and 12 h of treatment, γH2AX levels increased from 3.4- to 12.9-fold. Notably, proper phosphorylation of HA2X in RAD50-deficient cells was impaired as they taken up to 12 h to reach the γH2AX levels observed 30 min after cisplatin treatment in non-transfected cells. Then, we studied the cellular localization of γH2AX using immunofluorescence and laser confocal microscopy. As shown in **Figure 6C–E**, the presence of γH2AX in nuclear foci representing DSB repair sites became evident after 30 min of cisplatin treatment. In contrast, the apparition of γH2AX in nuclear foci was delayed in RAD50-deficient cells since they were detected until 3 h and 12 h after cisplatin treatment (**Fig. 6G–I**) in comparison to cells treated with shRAD50.4 alone (**Fig. 6F**). These data are in agreement with western blot assays and suggest that RAD50 silencing induced a delay in the early phosphorylation of H2AX histone.

RAD50-deficient cells exhibit a significant increase in chromosomal aberrations

The extent of chromosomal aberrations in MCF-7 cells with diminished RAD50 expression was evaluated through the

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Table 2. Nucleotide sequences of short-harping interfering RNAs specific for RAD50 gene

Primer name	Sequence	Nucleotide position in *RAD50* gene	References
Rad50.1 S	5′-GATCCGCAGA CTTAGACAGG ACCCTTCAAG AGAGGGTCCT GTCTAAGTCT GCTTTTTTGG AAA-3′	1940–1959	27
Rad50.1 AS	3′-GCGTCTGAAT CTGTCCTGGG AAGTTCTCTC CCAGGACAGA TTCAGACGAA AAAACCTTTT CGA-5′	1940–1959	27
Rad50.2 S	5′-GATCCGGAGA AGGAAATACC AGAATTCAAG AGATTCTGGT ATTTCCTTCT CCTTTTTTGG AAA-3′	2630–2649	28
Rad50.2 AS	3′-GCCTCTTCCT TTATGGTCTT AAGTTCTCTA AGACCATAAA GGAAGAGGAA AAACCTTTTC GA-5′	2630–2649	28
Rad50.3 S	5′-GATCCGCATC GATCAGTGCT CAGAGTTCAA GAGACTCTGA GCACTGATCG ATGTTTTTTG GAAA-3′	4354–4375	This report
Rad50.3 AS	5′-AGCTTTTCCA AAAAACATCG ATCAGTGCTC AGAGTCTCTT GAACTCTGAG CACTGATCGA TG CG-3′	4354–4375	This report
Rad50.4 S	5′-GATCCATGCA GTGTTAGCTC CCTGTTCAAG AGACAGGGAG CTAACACTGC ATTTTTTTGG AAA-3′	4381–4402	This report
Rad50.4 AS	5′-AGCTTTTCCA AAAAAATGCA GTGTTAGCTC CCTGTCTCTT GAACAGGGAG CTAACACTGC ATG-3′	4381–4402	This report
Rad50.5 S	5′-GATCCGGCGG AATTATAACT ACCGTTCAAG AGACGGTAG TTATAATTCC GCCTT TTTT GGAAA-3′	4014–4035	This report
Rad50.5 AS	5′-AGCTTTTCCA AAAAAGGCGG AATTATAACT ACCGTCTCT TGAACGGTAG TTATAATTCC GCC G-3′	4014–4035	This report

analysis of chromosomes in metaphase. Data showed the presence of similar low frequencies of aberrations per cell in non-transfected and transfected cells with scramble control sequence (0.33 and 0.72 respectively, **Fig. 7A and B**). Remarkably, a higher frequency of chromosomal aberrations (3.04) including chromatid breaks, chromosome fragments, as well as radial figures such as tri and tetraradials, was observed after cisplatin treatment (**Fig. 7C**). Interestingly, in RAD50-deficient cells we found an increase in chromosome and chromatid-type breaks (up to 1.49 aberrations per cell, **Fig. 7D**), indicative of failures in repair of basal DNA damage. The most striking response was observed when combined treatments were performed. Cisplatin plus scramble sequence resulted in an additive response, with a frequency of 3.2 aberrations per cell (**Fig. 7E**). However, RAD50-deficient cells treated with cisplatin showed a dramatical increase of chromosomal alterations up to 6.4 aberrations per cell, which is approximately twice of the expected additive response (**Fig. 7F**). These findings indicate that RAD50 abrogation and cisplatin induced a synergism effect observed as chromosomal damage accumulation. Furthermore it became apparent that in RAD50-deficient cells treated with cisplatin, there was a marked decrease in the number of metaphase cells so in two of the three replicates it was not possible to achieve the complete number of 25 metaphases required for the analysis. In such cultures we also observed mitosis with a countless number of chromosomal aberrations, similar to pulverized metaphases. **Figure 7G** summarizes the frequency of chromosomal aberrations average induced by treatments. These results indicate that RAD50 silencing produced a significant increase in the chromosomal damage induced by cisplatin.

Discussion

The ultimate goal of therapy is to improve the rates of patients' survival; therefore identification of molecular factors involved in sensitization of tumor cells to current chemotherapy represents a challenge in cancer research.

Survival in breast cancer patients is improving mostly related to early diagnosis. However, at least 40% of the early cases present recurrent disease following surgery adjuvant systemic therapy. Although hormonal treatment is preferred for advanced breast cancer, chemotherapy is also required for hormone receptor-negative and hormone refractory disease.[29] Anthracycline and cisplatin combinations have been commonly used to treat breast cancer. In addition, taxanes became the standard treatment for these patients following anthracycline failure. Cisplatin is used as first line therapy in several malignancies; however its clinical efficacy in most cancers is limited by its toxicity and development of resistance. These findings indicate that novel therapeutic combinations and molecular targets should be implemented to improve survival in breast cancer patients.

In tumor cells, the critical role of DNA repair machinery in preventing the accumulation of lethal DNA damage by chemotherapeutic agents is related with tumor chemoresistance, and increased DNA repair is also associated with poor outcome of patients. Targeting of DNA repair pathways by specific inhibitors, beside the DNA damage generated by chemotherapeutic agents, results in a synergistic effect and cell death.[30] Specifically, targeting of MRN complex may result in impaired DNA repair and increased cytotoxicity of ionizing radiation and chemotherapy. RAD50 is a fundamental protein in homologous recombination

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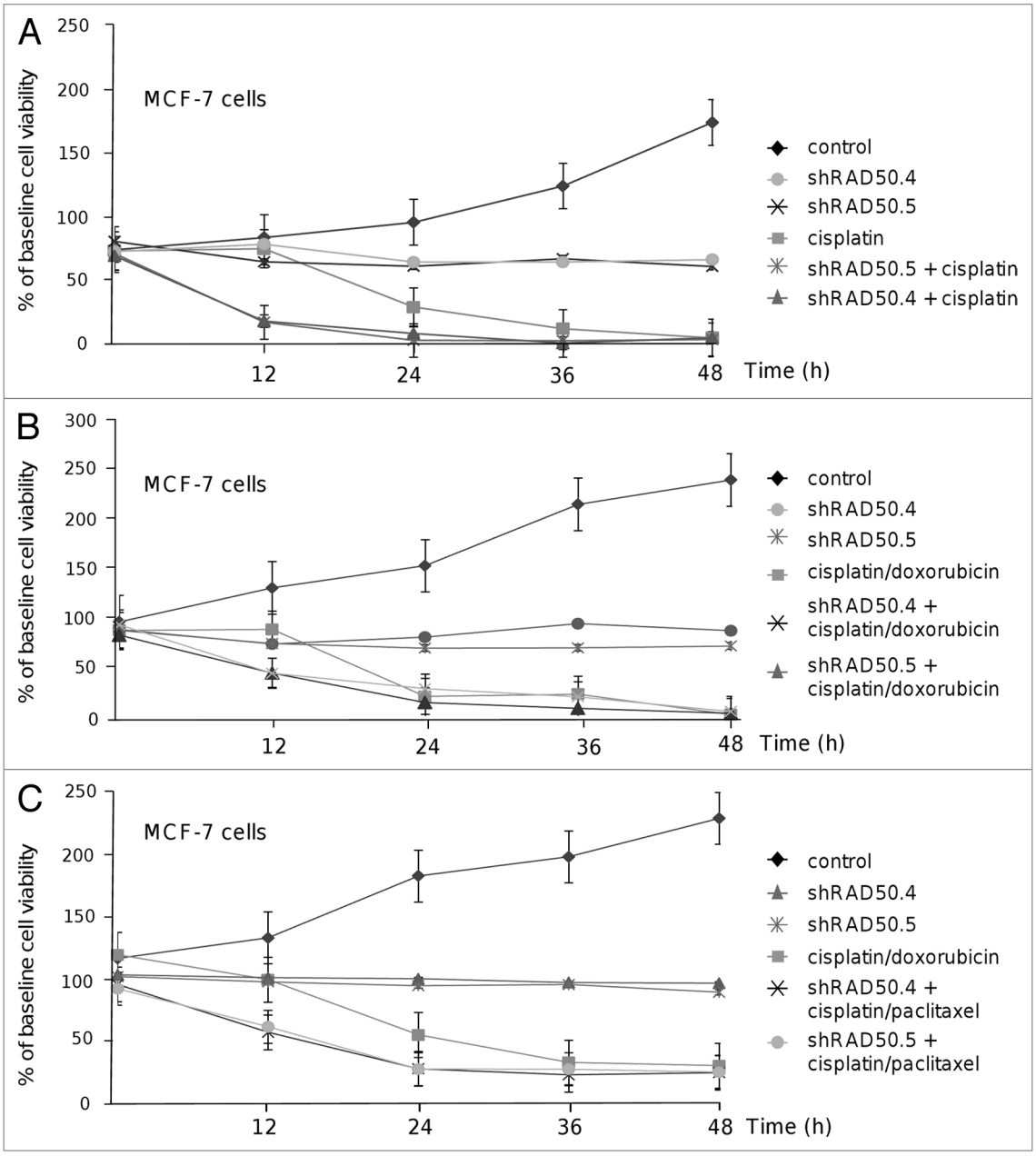

Figure 3. RAD50 silencing by RNA interference sensitizes MCF-7 breast cancer cells to combined chemotherapeutic treatment. Cell growth curves of MCF-7 cells transfected with shRAD50.4 or shRAD50.5 and treated with (**A**) cisplatin (25.8 μM), (**B**) cisplatin plus doxorubicin (2.5 μM), and (**C**) cisplatin plus paclitaxel (0.2 μM). Cell viability was evaluated after 12, 24, and 48 h of treatment. Non-transfected and shRAD50-transfected cells without treatment were used as controls. Graphs show the mean value of three independent experiments ± SD.

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and non-homologous end-joining mechanisms to repair DSB in drug-induced DNA damage;[31] hence, the inhibition of RAD50 expression may disturb DBSs repair and it may contribute to sensitize tumor cells to DNA-damaging agents.

In this study, we analyzed the expression of RAD50 in breast tumors. The expression of MRN genes has been found deregulated in some human cancers. Previous studies reported reduced MRN levels in breast cancer patients from Scandinavian[8,23] and French populations.[22] However, striking differences were found in these studies, which indicates that progression and heterogeneity of breast tumors may dictate the expression of MRN proteins. To contribute to the better understanding about the role of DNA repair in cancer, here we analyzed the expression of RAD50 in locally invasive breast tumors from a Mexican cohort of patients. Our data from MAT experiments showed that RAD50 expression was decreased in 14% of breast tumors. A comparison of combined staining scores (strong and moderate) showed that RAD50 expression was also reduced in 6% of breast tumors relative to normal tissues. These results have a similar tendency to those previously reported for RAD50

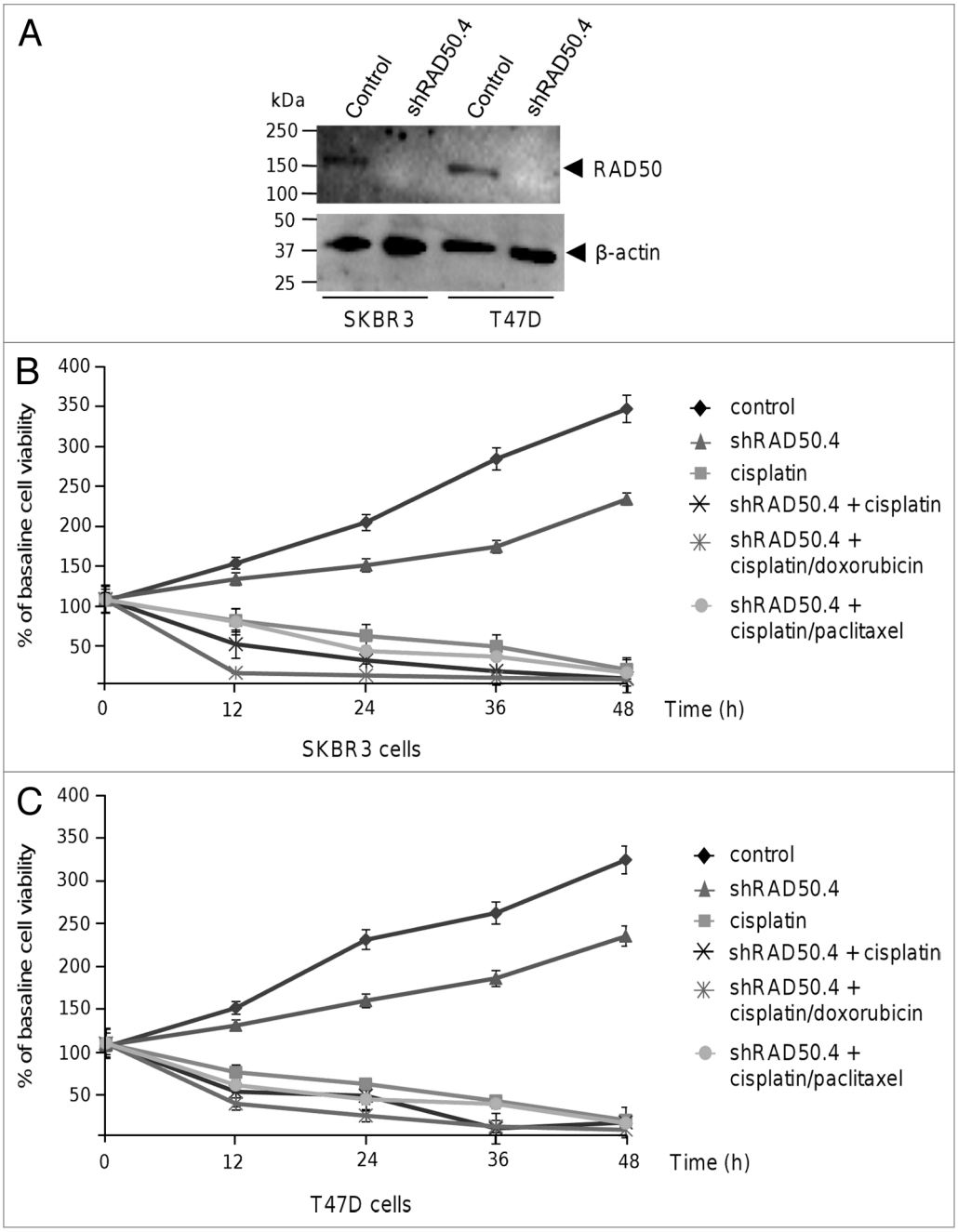

Figure 4. RAD50 silencing by RNA interference sensitizes SKBR3 and T47D breast cancer cells to combined therapy. (**A**) Western blot assays for RAD50 in SKBR3 and T47D cells. Total proteins isolated from non-transfected and shRAD50.4-transfected cells were resolved by SDS-PAGE, transferred to PVDF membrane and blotted with anti-RAD50 antibodies. β-actin was detected as internal loading control. (**B and C**) Cell growth curves of (**B**) SKBR3 and (**C**) T47D cells transfected with shRAD50.5 and treated with cisplatin (25.8 μM), cisplatin plus doxorubicin (2.5 μM), and cisplatin plus paclitaxel (0.2 μM). Cell viability was evaluated after 12, 24, and 48 h of treatment. Non-transfected and shRAD50-transfected cells were used as controls. Graphs show the mean value of three independent experiments ± SD.

in breast tumors. Angele and colleagues reported reduced levels of RAD50 in 28% of tumors, whereas Bartkova and coworkers reported RAD50 suppression in 4% of sporadic non BRAC1/2 breast tumors.[21-23] It is important to note that in these previous reports and in the present study, no patients exhibiting resistance to cytotoxic DNA damaging agents were included. Thus the contribution of RAD50 expression to therapy resistance remains to be addressed.

In this study, we evidenced that targeted inhibition of RAD50—a key protein involved in the repair of DSBs—has synergistic effects on the cytotoxic effects produced by cisplatin-based therapy in MCF-7 breast cancer cells.

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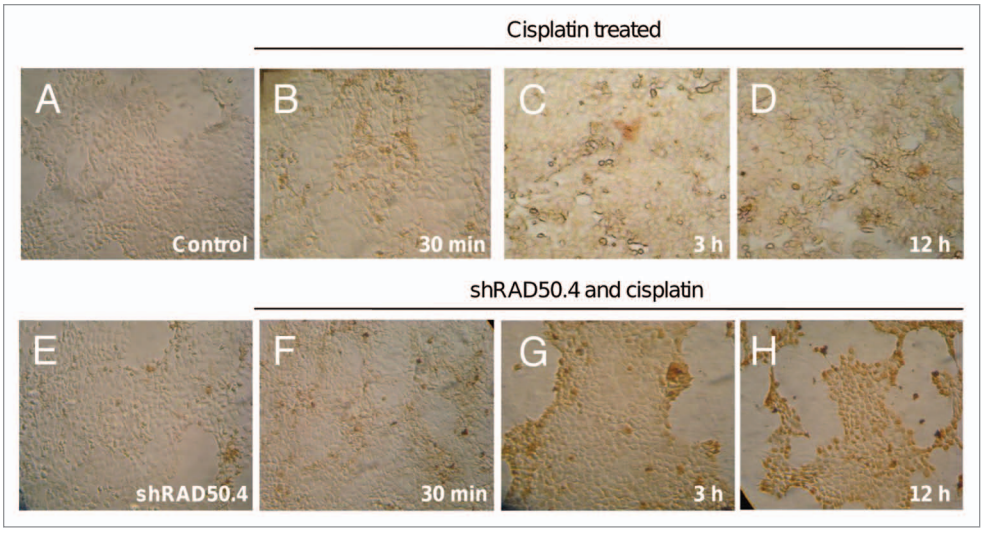

Figure 5. RAD50 suppression enhances cisplatin-induced DNA damage. TUNEL assays in (**A**) non-treated control MCF-7 cells, (**B–D**) treated with cisplatin alone, (**E**) transfected with shRAD50.4, and (**F–H**) treated with shRAD50.4 and cisplatin. Cells were analyzed 30 min (**B and F**), 3 h (**C and G**), and 12 h (**D and H**) after cisplatin treatments. Cells with nuclear staining were taken as positive in TUNEL reaction. Magnification is 60×.

Combination therapy with cisplatin–doxorubicin or cisplatin–paclitaxel persistently suppressed cell growth within 12 h of treatment in comparison with cisplatin treatment alone ($P < 0.05$). RAD50 abrogation was therefore, capable of potentiating the cytotoxic effect of cisplatin-combined therapies in breast cancer.

The chemosensitizing effect of RAD50 abrogation may be due to interruption of proper DNA damage response. Consistently, we observed that the enhanced cytotoxicity induced by RAD50 knockdown was associated with increased number of DSBs, and downregulation of DNA repair signaling. Furthermore, TUNEL assays showed a significant increase in cisplatin-induced DNA damage in RAD50-deficient cells. Cisplatin activates DNA damage signaling, which is characterized by a cascade of phosphorylation events. One of the earliest events in the cellular response against DNA damage is the phosphorylation of serine 139 of histone H2 variant H2AX (γH2AX) which is used as an early indicator of chemotherapy response, and it becomes a powerful tool to monitor DNA DSBs and clinical response to DNA targeted therapies in cancer research.[32] Here we evidence alterations in this key mechanism of DNA damage response and signaling in RAD50-deficient cells. Results from western blot and immunofluorescence assays indicate that early signaling and activation of DNA damage repair in response to cisplatin was affected after RAD50 abrogation, as we found a delay in H2AX phosphorylation in RAD50-deficient cells in comparison to control cells. Remarkably, we found an increase in chromosome and chromatid-type breaks (up to 1.49 aberrations per cell) in RAD50-deficient cells, indicative of failures in repair of basal DNA damage. We note that after transfection of shRAD50.5 sequence, the number of chromosomal aberrations increased. Notably, when the RAD50-silenced cells were treated with cisplatin, the increase in the frequency of chromosomal aberrations either rejoined or breaks, was greater than the sum of both independent treatments, indicating a synergistic effect. All together these effects are likely to have contributed to deficient cell growth and the enhanced cytotoxicity of cisplatin-combined therapies observed in RAD50-deficient cells.

In conclusion our data suggested that interruption of DNA repair signaling is an efficient strategy to sensitize breast cancer cells to chemotherapeutic treatment. These observations provide the basis for the rational use of DNA repair inhibitors to improve the therapeutic effects of DNA-damaging drugs. Therefore, the development of novel inhibitors to manipulate RAD50 levels might represent an efficient strategy to sensitize breast cancer cells to DNA damaging anti-neoplastic agents, which warrants further investigation in animal models.

Materials and Methods

Clinical tumor samples

Human primary tumors and normal breast tissues were selected following the regulations approved by the Institute of Breast Diseases-FUCAM ethics committee, and in accordance with the Helsinki Declaration of 1975. None of the patients recruited in this study received any antineoplastic therapy prior to surgery. After tumor resection, specimens were embedded in Tissue-Tek and snap frozen in liquid nitrogen at –80 °C until analysis. Pathologist confirmed the existence of 80% tumor cells in specimens. Tumors were classified according to hormonal receptors and HER2 status.

Tissues microarrays

High throughput analysis of 50 tumors (**Table 1**) and 24 normal breast tissues was performed by immunohistochemistry in a home-made tissue microarray (TMA, Tissue Microarrayer ATA100 Chemicon) as previously described.[33] Briefly, sections of tumoral and non-tumoral specimens were incubated overnight at 4 °C with anti-RAD50 antibodies clone13B3/2C6 (Santa Cruz Biotechnology sc-56209, 1:50) followed by incubation with universal secondary antibodies for 15 min and detection using Trek avidin-HRP for 10 min and DAB (3,3'-diaminobenzidine tetrahydrochloride)-substrate chromagen solution (Detection System, StarTrek, HRP universal kit Biocare Medical, STUHRP700 H, L10). Nuclei were stained with Mayer's hematoxylin before imaging, and slides were mounted with Permont

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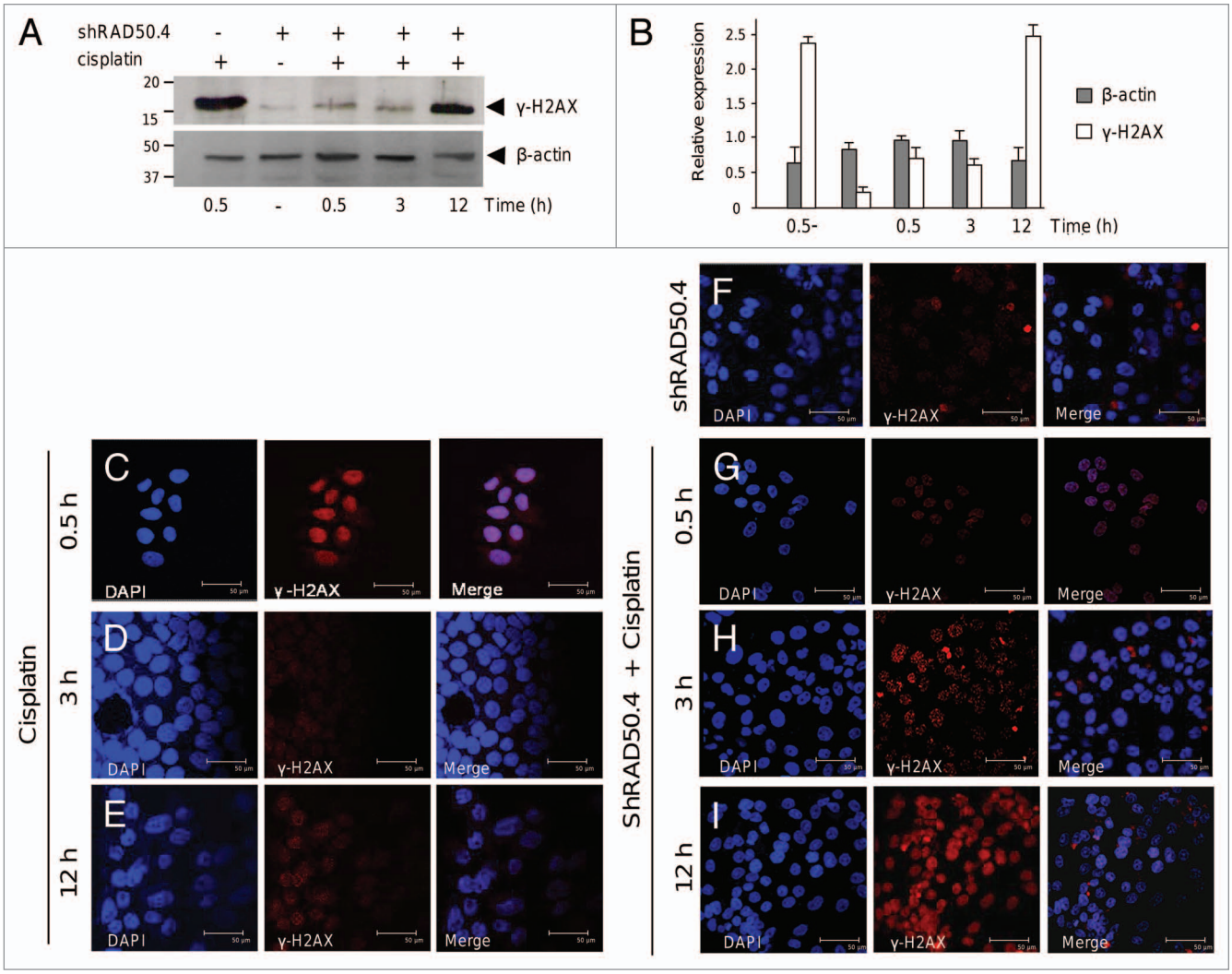

Figure 6. Delayed phosphorylation of histone H2AX (γH2AX) in RAD50-deficient breast cancer cells. (**A**) Western blot assays for γH2AX protein in MCF-7 cells transfected with shRad50.4 and analyzed at 0.5, 3, and 12 h after cisplatin treatment. β-actin was used as internal loading control. (**B**) Densitometric analysis of immunodetected bands depicted in (**A**). Graph shown the results of three independent experiments ± SD (**C–I**) Immunofluorescence assays and confocal microscopy for cellular localization of γH2AX in MCF-7 cells. γH2AX antibodies were coupled to alexa fluor-647 (red channel). Cell nuclei were stained with DAPI (blue channel). γH2AX and DAPI immunostaining was visualized in cells treated with cisplatin alone (**C–E**), transfected with shRAD50.4 (**F**), transfected with shRAD50.4 and treated with cisplatin (**G–I**) at the indicated times.

reactive. Staining intensity of protein was scored as negative (0), weak (1), moderate (2), and strong (3).

Cell lines and cultures

Human breast carcinoma cell lines were obtained from the American Type Culture Collection. MCF-7 (ATCC: HTB-22), MDA-MB-231 (ATCC: HTB-26), and ZR-75-1 (ATCC: CRL-1500), SKBR3 (ATCC: HTB-30), T47D (ATCC: HTB-133), BT20 (ATCC: HTB-19) were grown in Dulbecco's modification of Eagle's minimal essential medium (DMEM), supplemented with 10% fetal bovine serum and penicillin–streptomycin (50 unit/mL; Invitrogen). Cells were maintained in a humidified incubator with 5% CO_2 at 37 °C.

Western blot assays

Protein extracts were obtained using TNT buffer (50 mM TRIS-HCl pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 5 mM EDTA) supplemented with complete proteases inhibitor cocktail (Roche Molecular Biochemicals). Protein extracts (35 μg) were separated by 10% SDS-PAGE and electrotransferred to nitrocellulose membrane (BioRad). After blocking with 5% nonfat dry milk and 0.05% Tween-20 in PBS pH 7.4 overnight at 4 °C, membranes were probed with RAD50 antibodies (1:400) overnight at 4 °C. For detection, membranes were incubated with peroxidase-conjugated goat anti-mouse secondary antibodies (Molecular Probes Invitrogen G-21040, 1:5000) in 5% nonfat dry milk and 0.05% Tween-20 in PBS pH 7.4 and immunocomplexes were developed using the ECL chemiluminescence system (Amersham Pharmacia Biotech). Membranes were subjected to striping and re-blotting with β-actin monoclonal antibodies (Santa Cruz Biotechnology sc-8432, 1:1000).

Reverse transcription and PCR assays

RNA (1 μg) obtained with Trizol (Invitrogen) was mixed with 5 mM $MgCl_2$, 50 mM KCl, 10 mM TRIS-HCl, pH 8.3,

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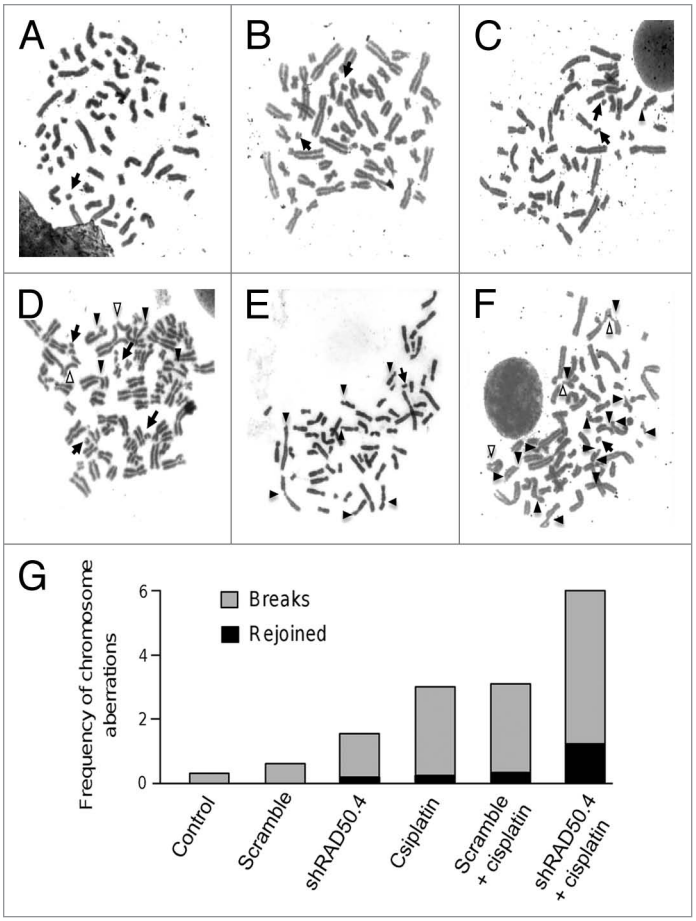

Figure 7. RAD50 suppression increases the number of chromosomal aberrations. Chromosomes observed in (**A**) non-treated cells, (**B**) transfected with scramble sequence, (**C**) treated with cisplatin (25.8 μM), (**D**) transfected with shRad50.4, (**E**) treated with scramble plus cisplatin, and (**F**) treated with shRad50.4 and cisplatin. Black arrowheads show chromatid breaks, arrows show chromosome fragments and white arrowheads show chromosomes aberrations and exchange radial figures. (**G**) The chart shows the frequency of chromosomal aberrations average from three assays induced by different treatments. The black bars show the rejoined chromosome aberrations such as radial figures and dicentrics, while gray bars indicate chromosome and chromatid breaks.

0.25 mM each dNTP, 40 U RNase inhibitor, 0.5 μM oligo-dT primer, and 50 U Superscript II (Invitrogen). The mix reaction was incubated for 45 min at 42 °C. An internal fragment of *RAD50* gene was PCR amplified from cDNA using PCR buffer supplemented with 2.5 mM $MgCl_2$, 0.5 μM primers (Fw: 5′-AATTGGCATT AGGGCGAC-3′; Rv: 5′-TCCTCAGCAT CCCGAAAT-3′) and 1 U Taq DNA polymerase (Invitrogen). The *GAPDH* transcript was amplified as an internal control. Amplified fragments were visualized by electrophoresis in agarose gels stained with ethidium bromide.

Inhibition of RAD50 expression in breast cancer cell lines

Short hairpin RNAs (shRNAs) design

Three oligonucleotides (21–23 nt) encoding shRNAs targeting the *RAD50* gene were designed (**Table 2**). To minimize the possibility of shRNAs off targeting effects, a nucleotide BLAST search was performed. In addition, we used two shRNAs sequences previously reported for *RAD50* silencing.[27,28] The oligonucleotides codifying for specific shRNAs were cloned into pSilencer 5.1 U6 retro plasmid (Ambion) and sequences were confirmed by automatic sequencing. The resulting plasmids express the shRAD50.1, shRAD50.2, shRAD50.3, shRAD50.4, and shRAD50.5 sequences. The effectiveness of each construct in gene silencing was tested by transient transfection of MCF-7 cells followed by RAD50 detection using RT-PCR and western blotting at 48 h post-transfection.

Transfection assays

MCF-7 cells (3×10^5) were seeded into a six well tissue culture plate and grown at 80% confluence. The culture medium was removed and cell monolayers were transfected with 5 μg of pSilencer-RAD50 plasmids using lipofectamine 2000 reagent (Invitrogen, 12566014). Then, transfected cells were incubated at 37 °C in presence of CO_2 for 24 h and 48 h, and harvested for further assays.

Cell treatments

MCF-7, SKBR3, T47D, MDA-MB-231, and BT20 breast cancer cells (2.5×10^5) were plated on six-well dishes, grown at 37 °C for 24 h and treated with increasing concentrations of drug (cisplatin: 0, 5, 50, 100, 200, and 300 μM; doxorubicin: 1, 2, 3, 4, 5, and 6 μM, and paclitaxel: 1, 2, 3, 4, and 5 μM) for 24 h. Then, cell viability was analyzed by trypan blue exclusion test using a TC10 automated cell counter (Bio Rad). Half inhibitory concentration (IC50) was determined using the dose-response curve fitting model. To assess the chemosensitivity of shRAD50.4 and shRAD50.5 transfected MCF-7 cells (5×10^3), they were treated with cisplatin (25.86 μM), cisplatin/doxorubicin (25.8 μM/2.5 μM), or cisplatin/paclitaxel (25.8 μM/0.2 μM) and cell viability was analyzed at 12, 24, 36, and 48 h post-treatment as described above. Experiments were performed three times by triplicate.

Analysis of DNA damage

TUNEL assays

MCF-7 cells (3.5×10^5) were incubated with cisplatin (25.8 μM) for 30 min, 3 h, and 12 h. Cells were fixed with 4% paraformaldehyde for 45 min, washed three times with PBS buffer, blocked with 3% methanol-H_2O_2 solution for 60 min at 37 °C and washed three times. Then cells were permeabilized with PBS-2%Triton-X100 for 2 min on ice, and DNA damage was quantified using the in situ cell death POD system (Roche). Briefly, 450 μL labeling solution were mixed with 50 μL terminal deoxynucleotidyl transferase enzyme in a sterile tube, 50 μL of the mixture were added to each well, and cells were incubated for 1 h at 37 °C protected from light. Then, 25 μL converter-POD solution were added and cells were incubated for 30 min at 37 °C. Finally, 50 μL DAB substrate was added and incubated for 10 min. Slides were mounted with PBS/glycerol solution and analyzed in bright field optical microscope. Assays were performed by triplicate.

Immunodetection of phosphorylated H2AX histone (γH2AX)

Proteins extracts were obtained from pSilencer-RAD50 transfected MCF-7 cells treated with cisplatin (25.8 μM) for 30 min,

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3, and 12 h. Proteins were separated by 15% SDS-PAGE and electrotransferred to a nitrocellulose membrane (BioRad). After blocking with 5% BSA/PBS-tween 0.5% solution, membranes were incubated at room temperature for 2 h with phospho-Histone H2AX Ser139 monoclonal antibody (Cell Signaling 20E3, 1:7000). Membranes were then washed with PBS-Tween 0.05% and incubated at 37 °C for 1 h with goat anti-rabbit horseradish peroxidase secondary antibodies (Zymed 62-6120, 1:5000). Bands were revealed by ECL Plus western blotting system (Amersham).

Cellular immunolocalization of γH2AX

MCF-7 cells transfected with pSilencer-RAD50.4 were grown on coverslips for 24 h, fixed with 4% paraformaldehyde and permeabilized with 0.2% Tween-20 in PBS. Slides were blocked for 1 h in PBS supplemented with 10% fetal bovine serum and 1% BSA, and incubated overnight at 4 °C with phospho-Histone H2A.X Ser139 monoclonal antibody (1:100). Then slides were incubated for 1 h with the anti-rabbit monoclonal antibody coupled to Alexa Fluor 647 (Molecular Probes, Invitrogen; 1:1000). Nuclei were counterstained using 1 μg/mL of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Molecular Probes, Invitrogen). Samples were mounted using vecta-shield (Roche) and visualized by fluorescence confocal microscopy.

Analysis of chromosomal aberrations

Drug treatments

MCF-7 cells (3.5×10^6) cells in exponentially growing cultures were treated with cisplatin (25.8 μM) for 24 h, and then colcemid (Sigma-Aldrich) was added at 0.1 ug/mL for 60 min to obtain mitotic cells. After treatment, cell viability was determined with trypan blue stain. Cultures with more than 80% of viability were processed to assess chromosomal damage. Experiments were performed by triplicate.

Chromosomes analysis

Non-transfected and transfected MCF-7 cells with shRAD50.4 were treated with a hypotonic solution of 0.075 mM KCl for 20 min at 37 °C, fixed with cold 3:1 methanol/glacial acetic acid and dropped onto glass slides to obtain metaphase spreads. Slides were stained with Wright/Giemsa, and coded by an independent individual to ensure blind scoring. Twenty-five metaphase spreads were analyzed for each condition and chromosomal aberrations were scored, including chromatid and chromosome breaks, fragments, tri-, and quadri-radial chromosomes as well as other structural abnormalities. The average chromosomal aberrations per cell and standard errors were calculated from three biological replicates and statistical significance determined.

Statistical analysis

Experiments were performed three times by triplicate and results were represented as mean ± SD. One-way analysis of variance (ANOVA) followed by the Tukey test was used to compare the differences between means. A $P < 0.05$ was considered as statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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