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Decreased DNA double-strand break repair and enhanced chromosomal radiosensitivity in irradiated non-tumorigenic human breast epithelial cells with a partial BRCA1 or BRCA2 knockdown

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Abstract. It has previously been demonstrated that peripheral blood lymphocytes of healthy women carrying *BRCA1* or *BRCA2* mutations exhibit increased chromosomal radiosensitivity, which is characterized by an enhanced formation of micronuclei. These results suggest that the deficient repair of DNA double-strand breaks may also occur in breast epithelial cells of women exhibiting a reduced expression of wild-type BRCA1/BRCA2 proteins due to the presence of germline mutations in *BRCA1/2* genes. The aim of this study was to further investigate *in vitro* the effects of the reduced expression of BRCA1 and BRCA2 in MCF10A human non-tumorigenic breast epithelial cells, tentatively mimicking the phenotype of heterozygous cells of carriers of *BRCA1/2* mutations. By lentivirus-mediated RNA interference, the stable reduction of BRCA1 and BRCA2 expression at the mRNA and protein level was achieved, thus generating the BRCA1i and BRCA2i cell lines. In these cells, homologous recombination was impaired, as significantly lower yields of RAD51 foci were obtained following exposure to 2 Gy ionizing radiation compared to the control MCF10A cells (BRCA1i cells, 58% reduction; BRCA2i cells, 64% reduction). Moreover, in the BRCA1i and BRCA2i cells, a dose-dependent increase in micronuclei was observed compared to the cells which were not subjected to gene knockdown. Cell viability was also affected by partial BRCA1/2 knockdown. On the whole, the findings of this study indicated that in cells with a reduced BRCA1 or BRCA2 expression, the impairment of homologous recombination resulted in a >50% decrease in RAD51 foci following irradiation and increased chromosomal abnormalities (micronuclei). These findings suggest that the healthy breast tissue of *BRCA1/2* mutation carriers may be prone to neoplastic transformation upon exposure to diagnostic or therapeutic radiation, and that the RAD51 foci assay may be useful for the assessment of the functionality of HR repair and radiosensitivity in these women.

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Abbreviations: BN, binucleated cells; BRCA1i, MCF10A cells in which BRCA1 was knocked down; BRCA2i, MCF10A cells in which BRCA2 was knocked down; DSB, double-strand break; HR, homologous recombination; HRP, horseradish peroxidase; IR, ionizing radiation; MN, micronucleus/micronuclei; PARP, poly(ADP-ribose) polymerase; PARPi, PARP inhibitor; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PI, propidium iodide; RNAi, RNA interference; SSB, single-strand break

Key words: Rad51, BRCA1, BRCA2, DNA repair, homologous recombination, ionizing radiation, radiosensitivity, micronuclei

Introduction

The risk of developing breast cancer markedly increases in individuals carrying a germline mutation in the *BRCA1* or *BRCA2* caretaker genes, which are activated in the DNA damage response machinery and which exert their activity in homologous recombination (HR). HR is a DNA double-strand break (DSB) repair pathway, activated in the S and G2 phases of the cell cycle following exposure to genotoxic agents, such as ionizing radiation (IR) (1,2).

It has been hypothesized that individuals harboring a germline mutation in the *BRCA1* and *BRCA2* genes may exhibit enhanced radiosensitivity and may thus be exposed to an increased carcinogenic risk following exposure to IR

for therapeutic or diagnostic purposes (3-19). The authors have recently demonstrated an increased radiosensitivity and micronucleus formation in peripheral blood lymphocytes of healthy women carrying *BRCA1* or *BRCA2* mutations (18,19). These results, obtained in peripheral blood lymphocytes, suggested that the deficient repair of DNA DSBs might also occur in mammary epithelial cells of women exhibiting a reduced expression of wild-type BRCA proteins.

Hence, the main aim of this *in vitro* study was to assess the DNA DSB repair capacity and the formation of chromosomal abnormalities in irradiated, non-tumorigenic human mammary epithelial cells exhibiting a decreased expression of wild-type BRCA1/2 proteins, as may occur in the case of the healthy mammary tissue of women harboring heterozygous *BRCA1/2* mutations. In the MCF10A non-tumorigenic human mammary epithelial cell line, by lentivirus-mediated RNA interference, the partial reduction of the expression levels of BRCA1 and BRCA2 was achieved to levels which may functionally mimic those of mammary cells in heterozygous *BRCA* mutation carriers. In these cells, the repair capacity following irradiation was investigated using the RAD51 foci assay, which specifically detects DNA DSB repair by the homologous recombination pathway. A deficient repair capacity was phenotypically confirmed by analyzing chromosomal abnormalities with the micronucleus assay and by cell viability testing.

Materials and methods

Cell lines. Mycoplasma-free MCF10A cells (cat. no. CRL-10317, freshly obtained from ATCC) were cultured in monolayers using equal volumes of DMEM-glutamax and F12-glutamax (Life Technologies; Thermo Fisher Scientific) supplemented with 5% fetal calf serum (Invitrogen; Thermo Fisher Scientific), antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin, Invitrogen; Thermo Fisher Scientific), 10 μg/ml insulin (Sigma-Aldrich), 0.5 μg/ml hydrocortison (Sigma-Aldrich) and 20 ng/ml epidermal growth factor (Peprotech). Experiments were performed on cells in which BRCA1 and BRCA2 were knocked down (these cells are referred to as BRCA1i and BRCA2i cells). RNA interference (RNAi) was achieved by stable transduction with lentiviral vectors harboring DNA sequences encoding short hairpin RNAs specific for BRCA1 or BRCA2. In brief, lentiviral particles were constructed using pLKO.1-puro vectors (Addgene). The RNAi sequences for *BRCA1* and *BRCA2* were 5'-GCCCACCTAATTGTACTGAAT-3' and 5'-TACAATGTA CACATGTAACAC-3', respectively. A negative control cell line, transduced with an empty pLKO.1-puro lentiviral vector (hereafter referred to as the control) was also established. This is an acknowledged limit of this study, as scrambled sequence transduction would have been preferable, though according to previous experience, this procedure may target unintended mRNAs. Moreover, empty vectors allow for the determination of the effects of transduction on cell response and gene expression (20). The transduction of the MCF10A cells was achieved by the addition of 1 μg/ml DNA, TurboFect (1.5 μg/ml, Thermo Fisher Scientific) and polybrene (1 μg/ml, Sigma-Aldrich) to a 30% confluent culture. Cells were grown in puromycin-supplemented DMEM medium (2 μg/ml, Life Technologies; Thermo Fisher Scientific) for 15 days to obtain stably transduced cell lines. Notably, stable BRCA1 knockdown by retrovirus-mediated RNAi does not alter the non-tumorigenic phenotype of MCF10A cells (21). According to our personal experience, BRCA1/2 knockdown neither induces transformation *in vitro*, nor tumorigenesis *in vivo*.

Reverse transcription-quantitative PCR (RT-qPCR) analysis. *BRCA1* and *BRCA2* mRNA knockdown was evaluated by RT-qPCR analysis. Total RNA was extracted using RNeasy Mini kits (Qiagen Benelux), following the manufacturer's instructions, without optional DNase treatment. The RNA concentration and quality were determined using a DropSense96 kit (Trinean) before removing contaminating DNA with the Heat&Run gDNA removal kit (Articzymes). Reverse transcription was achieved with the iScript cDNA synthesis kit, following the manufacturer's instructions. A total of 5 μl of qPCR reaction contained 10 ng cDNA (total RNA equivalents), 2.5 μl SsoAdvanced universal SYBR-Green supermix (Bio-Rad) and 2.5 μM forward and reverse primers. Cycling and detection was performed on a Lightcycler LC480 (Roche) with 2 min denaturation at 95˚C followed by 45 cycles with 5 sec at 95˚C, 30 sec at 60˚C and 1 sec at 72˚C. Melting curve analysis was performed to test for non-specific amplification. Three biological repeats were performed for each amplification. The analysis of relative gene expression was performed using a qBase+ platform (Biogazelle), developed by Hellemans *et al* (22), based on the $\Delta\Delta$Cq method by Livak and Schmittgen (23).

Western blot analysis. BRCA1 and BRCA2 protein knockdown was evaluated by western blot analysis. Protein extraction was performed in subconfluent cultures of the control, BRCA1i and BRCA2i cells using a tris-EDTA lysis buffer containing 1% NP-40 and 1% protease inhibitor (Sigma-Aldrich). For each sample, 50 μg protein were loaded together with 25% SDS sample buffer (Thermo Fisher Scientific) and 10% dithiothrietol (DDT, Sigma-Aldrich) on a 3-8% Tris-Acetate gel (Novex; Thermo Fisher Scientific), and run for 5 h at 25 mA. Proteins were transferred to a methanol-pretreated PVDF membrane in Tris/glycine blotting buffer enriched with 10% methanol for 16 h at 30 V. Following standard blocking, the membrane was incubated overnight at 4˚C with a primary antibody (rabbit polyclonal anti-BRCA1, cat. no. 07-434, diluted 1:1,000, or monoclonal anti-BRCA2, cat. no. OP95, diluted 1:500; Millipore), together with mouse monoclonal α-actinin (cat. no. 05-384, diluted 1:30,000 Millipore). The membranes were washed and incubated for 2 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-rabbit-HRP, Perbio, cat. no. 31460, diluted 1:1,000, or goat anti-mouse-HRP, cat. no. 31432, Thermo Fisher Scientific, diluted 1:1,000). Visualization was achieved with a chemoluminescence kit (Thermo Fisher Scientific), and analysis of relative expression at the protein level was performed using a shareware ImageJ software (version 1.52q, bundled with Java 1.8.0_172; developer: NIH).

RAD51 foci assay. The HR pathway is active in the S and G2 phases of the cell cycle to repair DSBs induced by IR (2,24-26), and relies on RAD51, whose recruitment to a DSB site is mediated by BRCA1 and BRCA2. RAD51 recruitment can be

typically detected in the form of nuclear *foci* upon immunostaining (25,27). The RAD51 irradiation-induced foci assay has been widely used to detect HR defects both in cancer biopsy samples and in non-tumor cells of BRCA1/2 breast cancer patients (28-30).

Sample preparation. The cells were switched to puromycin-free culture DMEM medium (Life Technologies) at the beginning of each experiment. Cells (n=200,000) were seeded in 2 ml culture medium in 6-well plates. Approximately 24 h after seeding, the cells were examined for subconfluency. To achieve a maximum number of cells in the S and G2 phases of the cell cycle, the cells were synchronized by the addition of the DNA polymerase inhibitor, aphidicolin (1 μg/ml, Sigma-Aldrich) to the culture medium for 24 h. The cells were subsequently washed with PBS (1.78 g/l Na_2HPO_4; 0.42 g/l KH_2PO_4; 7.2 g NaCl, VWR) and incubated at 37˚C for 8 h with fresh culture medium.

Cell cycle analysis. The MCF10A control cells (empty vector-transduced) were harvested at various time points following synchronization to evaluate the percentage of cells in each phase of the cell cycle. Cell permeabilization was achieved by fixation in 95% ethanol at -20˚C and the DNA was subsequently stained with propidium iodide (PI, Sigma-Aldrich) in a hypotonic staining buffer containing 0.1% Sodium citrate, 0.3% Triton X-100, 0.01% PI and 0.002% ribonuclease A (all reagents were from Sigma-Aldrich). The PI cell content was analyzed on a FACSCanto™ (BD Biosciences). Cells of interest were selected based on forward and side scatter area patterns. A non-synchronized sample was used as reference.

Irradiation and olaparib treatment. At 3 h following aphidicolin removal, the cells were irradiated with 2 Gy 220 kV-13 mA X-rays to generate DSBs (SARRP unit, XSTRAHL Ltd.). The cells were subsequently incubated at 37˚C for 5 h for optimal RAD51 foci formation. To determine this optimal time point, different time points varying between 2 and 8 h were previously tested (data not shown).

Since cells defective in HR are sensitive to poly(ADP-ribose) polymerase (PARP) inhibitor (PARPi) drugs (31-33), the PARPi, olaparib, was used to enhance the number of DSBs in S-phase cells. To part of the cultures, 5 μM olaparib (Bio-Connect) was added to the medium 1 h prior to irradiation, in order to block the repair of radiation-induced single-strand breaks (SSBs). RAD51 foci formation was also evaluated in untreated cell cultures and in cultures exposed to olaparib alone. In total, 8 repeats were performed for each of the 4 experimental conditions (irradiated or sham-irradiated cells, with or without olaparib treatment).

The induction of DNA DSBs by IR was assessed by gamma-H2AX staining as previously described (34) (Fig. S1). Inhibition by olaparib was confirmed by the PARP activity following exposure to H_2O_2 in the presence of olaparib, as previously described (35) (Fig. S2).

RAD51 foci immunostaining. Prior to RAD51 foci staining, the cells were harvested, cytospinned on polylysine-coated slides (VWR) and fixed in 3% paraformaldehyde (Sigma-Aldrich) for 20 min. The slides were washed twice in PBS and antigen retrieval was achieved by incubation for 20 min in heated (95˚C) citrate buffer (0.02% citric acid, pH 6, Sigma-Aldrich). The slides were subsequently washed and incubated with a blocking serum containing 1% BSA (Roche), 5% goat serum (Dako) and 0.2% Tween-20 (Sigma-Aldrich) in PBS. The slides were then incubated overnight at 4˚C with a RAD51 H-92 rabbit primary antibody (dilution: 1:2,000, Santa Cruz Biotechnology, cat. no. sc-8349), washed with PBS containing 3% Tween-20 (Sigma-Aldrich), and incubated for 30 min at room temperature with a secondary antibody (goat anti-rabbit; dilution: 1:1,000, Thermo Fisher Scientific; cat. no. A32731). Finally, the slides were washed in PBS/3% Tween-20 and mounted with 200 ng/ml DAPI in fluoromount (Sigma-Aldrich).

The slides were scanned using the Metacyte software module on a Metafer4 scanning platform (Axio Imager, Metasystems) at a x63 magnification. This software module enables automatic cell detection and foci counting according to set parameters, resulting in an unbiased data acquisition. The number of RAD51 foci was automatically scored in at least 500 cells for each experimental condition, and expressed as the number of RAD51 foci per cell (RAD51 foci/cell).

Micronucleus (MN) assay. Chromosomal damage was assessed with the MN assay as previously described (36). Briefly, the cells were seeded in 2 ml culture medium in 6-well plates (200,000 cells/well) 1 day prior irradiation. Subconfluent cultures of exponentially dividing cells were irradiated with doses of 0.2, 0.5, 1, 2 and 4 Gy, and cytochalasin B (2.25 μg/ml; Sigma-Aldrich) was immediately added to block cytokinesis. The cells were maintained at 37˚C in a humidified 5% CO_2 atmosphere incubator for 16 h. Sham-irradiated cultures were included in each experiment. The cells were then harvested and subjected to a cold hypotonic shock with 0.075 M KCl, followed by overnight fixation in 3/1/4 methanol/acetic acid/ringer solution (ringer: 9 g/l NaCl, 0.42 g/l Kcl and 0.24 g/l $CaCl_2$). Subsequently, the cells were fixed in a 3:1 methanol/acetic acid solution. For further analysis, the cells were stained with DAPI (200 ng/ml; Sigma-Aldrich). The slides were scanned at 10 X magnification with the MSearch software module of the Metafer 4 scanning system and the MNScore software (Metasystems). The automated image analysis system selects BN cells and determines the number of MN for each BN cell. BN cells and MN were manually examined for false positives and negatives. Two slides for each culture were automatically scanned and approximately 400 BN cells were scored in each slide. All experiments were performed in duplicate. Each experiment was repeated thrice.

Cell viability assay. A protocol described previously was used for this assay (37,38). Briefly, following irradiation (doses of 0.5, 1, 2, 3, 4, 6 and 8 Gy), the cultures were further incubated for 4 days at 37˚C until sham-irradiated plates nearly reached confluence. The cells were fixed for 10 min in a solution of buffered formalin (3.7%), washed with PBS (pH 7.3) and stained with a 0.01% crystal violet solution (Sigma-Aldrich). The stain was dissolved overnight in 1 ml 10% sodium dodecyl sulfate (SDS). The optical density of the samples was measured with a spectrophotometer at 590 nm. All cell viability assays were performed in quadruplicate. Each experiment was repeated 3 times.

Statistical analysis. Differences in mean RAD51 foci, in micronucleus counts and in cell viability data were analyzed by one-way analysis of variance (ANOVA). The Tukey's range test was applied to perform post-hoc analysis of the significance of comparisons. A 5% alpha error threshold (P-value <0.05) was applied to all analyses. Statistical inference was performed using the R software environment. For the Tukey post-hoc test, the *multcompView* package in R was used.

Results

Effects of RNAi. The lentivirus-mediated RNA interference of *BRCA1* and *BRCA2* was confirmed at the RNA and protein levels by RT-qPCR analysis and western blot analysis. Compared to the control cells, 45 and 35% reductions in the *BRCA1* and *BRCA2* mRNA levels were achieved, respectively (Fig. S3). Notably, in experiments assessing knockout specificity, it was demonstrated that the knockdown of BRCA1 in the BRCA1i cells did not affect the mRNA levels of *BRCA2* (97% of controls), and the knockdown of BRCA2 in the BRCA2i cells did not affect the mRNA levels of *BRCA1* (97% of controls). The quantification of the protein knockdown using ImageJ software revealed an estimated 70% reduction of BRCA1 in BRCA1i cells, and an estimated 51% BRCA2 reduction in BRCA2i cells (Fig. 1).

Cell cycle analysis. The results of cell cycle analysis in non-synchronized cells, and in synchronized cells at various time points following aphidicolin removal are shown in Fig. 2. As an example, the histogram charts of all time points for one repeat are illustrated in Fig. 2A-E. The mean percentage (4 repeats) of cells in each phase of the cell cycle in a non-synchronized sample, and in synchronized cells at various time points (0, 2, 3 and 8 h) following aphidicolin removal is shown in Fig. 2F.

Synchronization with aphidicolin increased the number of cells in the S phase of the cell cycle. Immediately following aphidicolin synchronization, approximately 70% of the cells were at the beginning of the S phase, compared to 30% in non-synchonized cells. At 2 and 3 h following aphidicolin removal, the lowest number of cells in the G1 phase was achieved, while the number of cells in the S phase remained between 60 and 70%, compared to the non-synchronized cultures. At 8 h following aphidicolin synchronization, the cells shifted towards the G2 and M phases of the cell cycle. Since the number of cells in the late S phase peaked at 2 and 3 h following synchronization, these time points were selected for the addition of olaparib (2 h following the removal of aphidicolin) and irradiation (3 h following the removal of aphidicolin) to maximize the effects of these agents on RAD51 foci formation.

RAD51 foci formation. Representative examples of the induction of RAD51 foci by radiation in the nuclei of MCF10A cells, with or without a knockdown of BRCA1 or BRCA2 are illustrated in Fig. 3. The mean number of RAD51 foci per cell in synchronized cell cultures, assessed in the 4 tested conditions (irradiated or sham-irradiated, with or without olaparib) is shown in Fig. 4. The significance values for all comparisons are listed in Table SI.

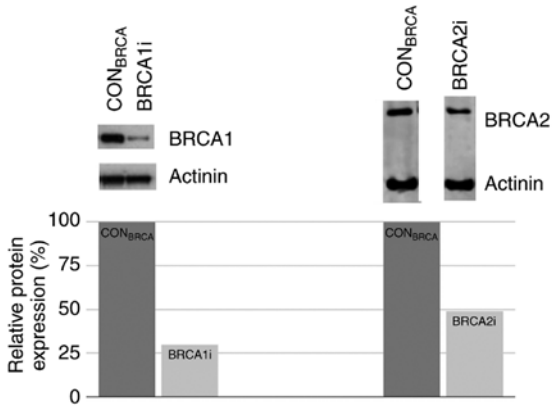

Figure 1. Western blot analysis of BRCA1 and BRCA2 protein levels in BRCA1i, BRCA2i and control (CON_{BRCA}) cell lines. Actinin was used as a protein loading control. The bar chart shows the relative protein expression (%), assessed using ImageJ software.

Control MCF10A cells. Compared to the cells not irradiated and not exposed to olaparib, exposure to IR induced marked and significant increases in the mean RAD51 foci/cell, both in the presence (~2.3-fold) or absence (~1.9-fold) of olaparib (Fig. 4, blue bars and Table SI).

The exposure of sham-irradiated control cells to olaparib did not significantly increase the number of foci/cell. Conversely, in the irradiated control cells, exposure to olaparib caused a small, yet significant increase in RAD51 foci/cell (~1.2-fold; Fig. 4, blue bars and Table SI).

BRCA1i and BRCA2i cells. Compared to their respective controls, a significantly lower yield of RAD51 foci was observed in the irradiated BRCA1i and BRCA2i cells, exposed (BRCA1i cells, 49% reduction; BRCA2i cells, 60% reduction) or not (BRCA1i cells, 58% reduction; BRCA2i cells, 64% reduction) to olaparib (Fig. 4 and Table SI).

Compared to the BRCA1i or BRCA2i cells not irradiated and not exposed to olaparib, exposure to IR did not induce statistically significant increases in the mean RAD51 foci/cell, both in the presence or absence of olaparib (Fig. 4 and Table SI).

Micronucleus assay. The spontaneous MN yield (mean number of micronuclei per 1,000 sham-irradiated binucleated cells ± SD) for the control, BRCA1i and BRCA2i cells was 28.1±2.5; 31.7±4.2 and 26.0±1.7, respectively. There was no significant difference in the spontaneous MN values between the BRCA1i or BRCA2i cell lines and control MCF10A cells.

The MN dose-response curves in the control cells, and in the BRCA1i and BRCA2i cells are shown in Fig. 5. Compared to the irradiated controls, significantly higher MN yields were obtained in both the BRCA1i and BRCA2i irradiated cell lines. The BRCA1i cells were the most radiosensitive, resulting in a steeper, quasi-linear dose-response curve. The P-values for all the comparisons are listed in Table SI.

Cell viability assay. Cell viability/survival curves of knockdown cell lines together with the control cell line are shown in Fig. 6. The BRCA1i and BRCA2i irradiated cells exhibited a dose-dependent decrease in survival, when compared to the

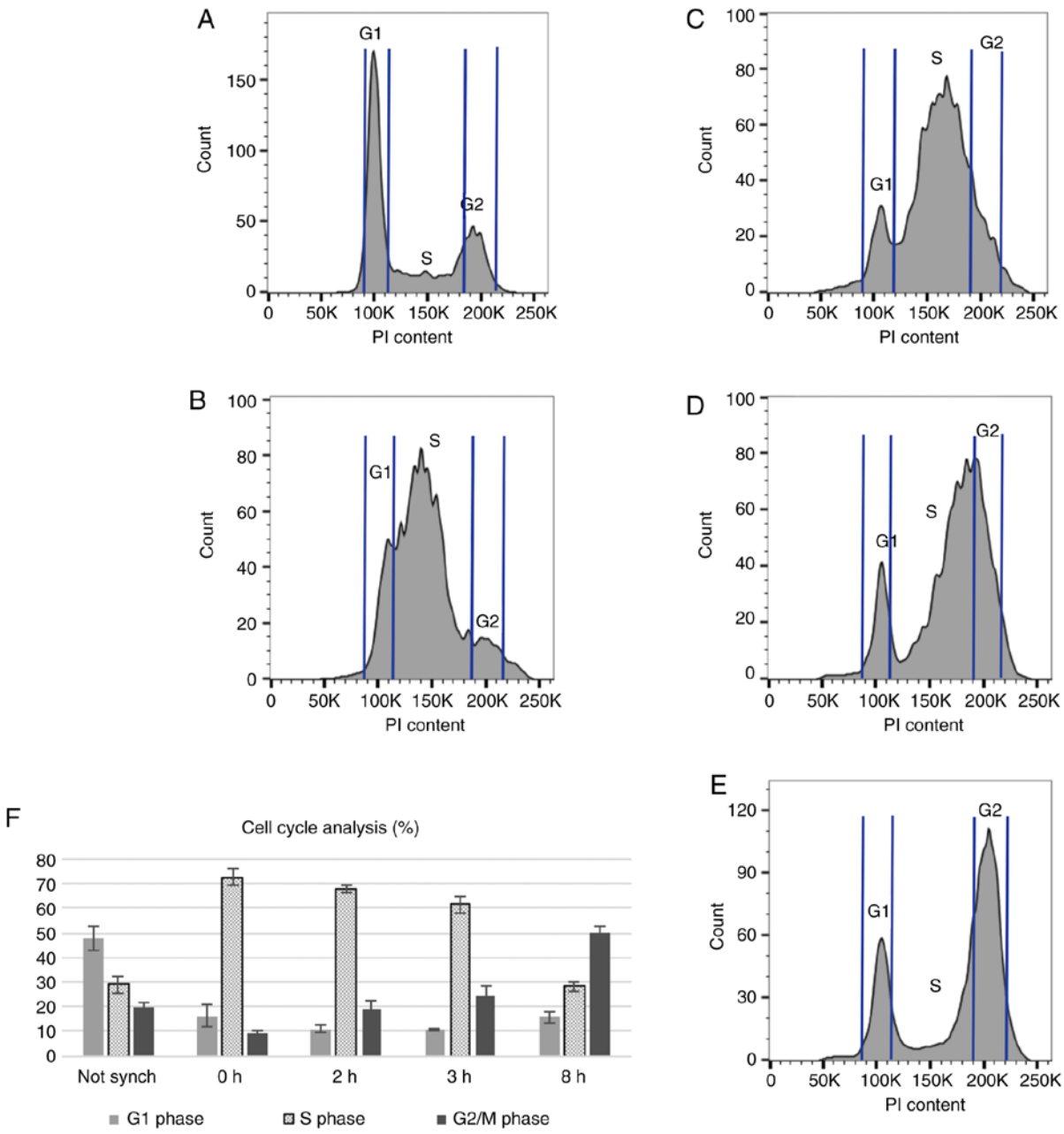

Figure 2. Cell cycle analysis. (A-E) Representative histograms (one repeat) showing the number of MCF10A control cells in each phase of the cell cycle. (A) Non-synchronized control cell culture; (B) cells immediately (0 h) following synchronization, and at (C) 2 h, (D) 3 h and (E) 8 h following synchronization. (F) Mean percentage of cells in each phase of the cell cycle prior to synchronization and at various time points following aphidicolin removal. Cells were stained with propidium iodide (PI). Horizontal axes (linear scale) show the PI content and vertical axes (linear scale) indicate cell counts. Means and standard error bars are based on 4 repeats. Equivalent patterns of cell cycle distribution were found in BRCA1i and BRCA2i cells.

control cells. The P-values for all the comparisons are listed in Table SI.

Discussion

The main aim of this *in vitro* study was to assess homologous recombination repair and radiosensitivity in human mammary cells showing reduced protein levels of wild-type BRCA proteins, a condition that may model the reduced protein expression observed in heterozygous cells of women carriers of *BRCA1*/*BRCA2* mutations. A reduced *BRCA* expression, and in turn, an impaired DNA repair capacity may point to an increased risk of developing radiation-induced carcinogenesis in such women. One of the authors' concerns was that a *BRCA* mutation carrier receiving, for example, repeated mammography screens each year, or adjuvant radiotherapy following surgical removal of a primary lesion, may be at increased risk of developing secondary, radiation-induced neoplasia. Thus, in this study, a dose of radiation was selected which is commonly administered to women receiving a single session of radiotherapy (2 Gy). In the experiments in this study, the non-tumorigenic and non-mammosphere-forming MCF10A cell line was selected, which is often used as *in vitro* model, together, with its *in vitro*-transformed derivative cell lines-to investigate the biology of non-neoplastic human mammary cells (39). These diploid cells have a relatively stable karyotype

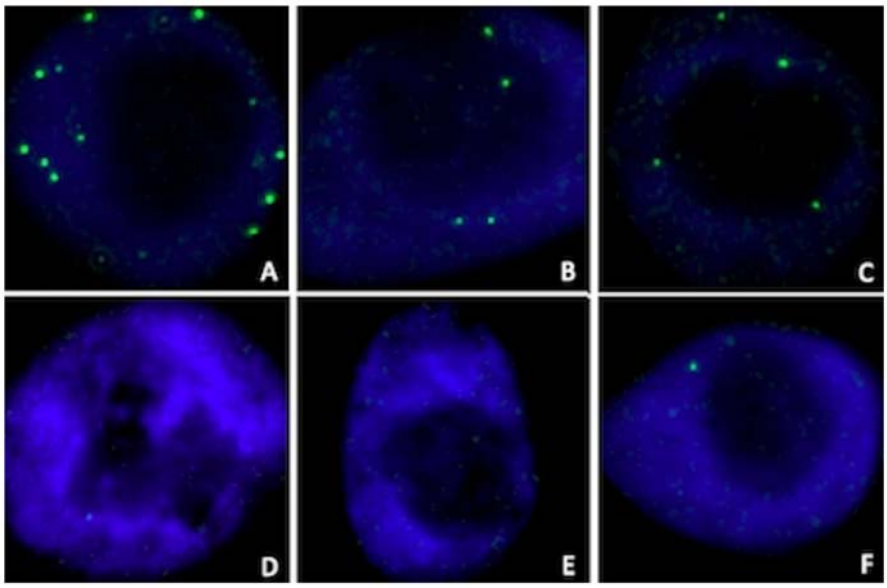

Figure 3. RAD51 foci in the nuclei of (A and D) control MCF10A cells, of (B and E) BRCA1i MCF10A cells and (C and F) BRCA2i MCF10A cells. (A-C) Cells with DAPI-stained nuclei (blue) containing green fluorescent RAD51 foci induced following ionizing radiation. (D-F) Foci staining in sham-irradiated cells. These images were obtained with a Metafer4 equipment (Metasystems).

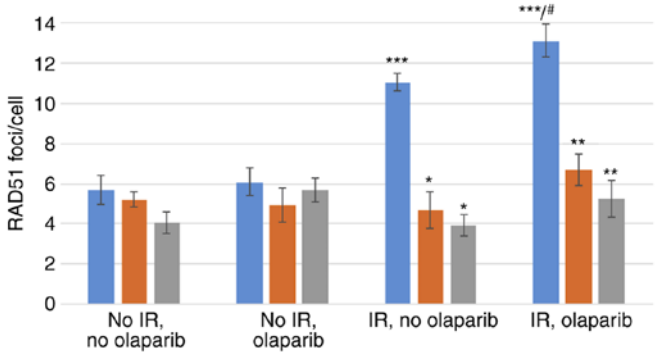

Figure 4. Mean number of RAD51 foci per cell (RAD51 foci/cell) in aphidicolin-synchronized MCF10A control (blue bars), BRCA1i (orange bars) and BRCA2i (grey bars) cell lines. Cells were irradiated (IR) or sham-irradiated (no IR), and exposed/not exposed to the PARP inhibitor olaparib (olaparib/no olaparib). Error bars indicate the standard errors of the means, based on 8 repeats. *P<0.00001 vs. irradiated controls not exposed to olaparib, **P=0.0001 (BRCA1i) or **P<0.00001 (BRCA2i) vs. irradiated controls exposed to olaparib, ***P<0.00001 vs. non-irradiated controls not exposed to olaparib, $^{\#}$P<0.05 vs. irradiated controls not exposed to olaparib. Statistical analysis was carried out using one-way ANOVA with Tukey's post-hoc test and the detailed results are shown in Table SI.

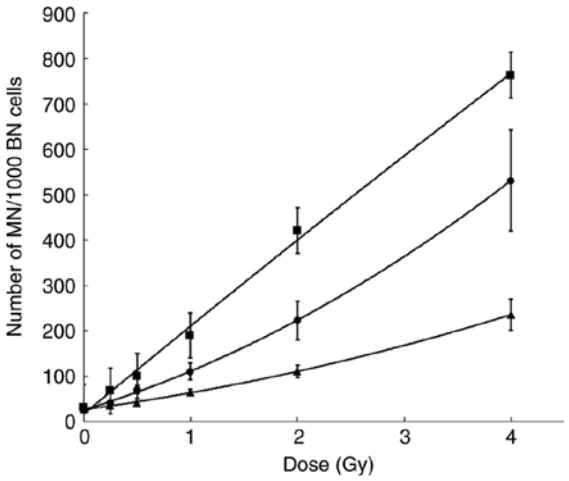

Figure 5. Micronucleus assay in BRCA1i (■), BRCA2i (●) and control (▲) MCF10A cells. Dose-response curves of cells exposed to 0.25, 0.5, 1, 2 and 4 Gy ionizing radiation. Data points are the mean values (± SD) of 3 independent experiments performed in duplicate. With the exception of the 0 Gy (BRCA1i and BRCA2i lines) and 0.25 Gy doses (BRCA2i line only), all data points of both curves (■,●) indicate significant differences compared to the control data points (▲) at the same radiation dose (detailed results of statistical analysis are shown in Table SI). Statistical analysis was carried out using one-way ANOVA with Tukey's post-hoc test. MN, micronuclei; BN, binucleate.

and form *in vitro* acinar structures that recapitulate many aspects of mammary gland architecture.

The cells were synchronized in the S and G2 phases for an optimal evaluation of the DNA repair capacity by homologous recombination, a pathway which involves both BRCA1 and BRCA2. DSBs were induced by exposure to ionizing radiation. To increase the number of DSBs, olaparib was also used, which is a PARPi that transforms radiation-induced single-strand breaks into DSBs during the S phase of the cell cycle.

As expected, HR was involved in the repair of radiation-induced DSBs in synchronized MCF10A cells, as shown by the highly significant increase of RAD51 foci yields in irradiated control cells, compared to their sham-irradiated counterparts. Conversely, exposure to IR did not increase the yield of RAD51 foci, either in BRCA1i or in BRCA2i cells. In fact, significantly lower yields of RAD51 foci were observed in the BRCA1i and BRCA2i cell lines compared to the irradiated control cells. The lack of induction of RAD51 foci in the BRCA1i and BRCA2i cells may suggest that the knockdown may phenotypically mimic *BRCA* haploinsufficiency in breast epithelial cells, where the presence of a single wild-type allele may result in increased DNA

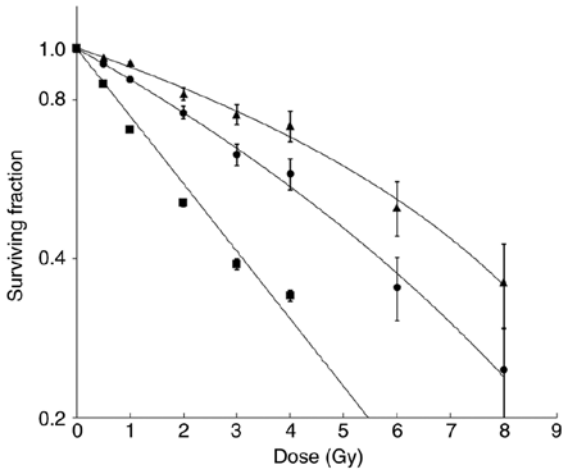

Figure 6. Cell viability assay of BRCA1i (■), BRCA2i (●) and control (▲) MCF10A cells. Dose-response curves of cells exposed to 0.5, 1, 2, 3, 4, 6 and 8 Gy ionizing radiation. Data points are the mean values (± SD) of 3 independent experiments performed in duplicate. With the exception of the 2 Gy and 8 Gy (BRCA2i line only), all data points of both curves (■,●) indicate significant differences compared to the control data points (▲) at the same radiation dose (detailed results of statistical analysis are shown in Table SI). Statistical analysis was carried out using one-way ANOVA with Tukey's post-hoc test. A logarithmic graph is shown.

damage resulting from deficient HR repair, as suggested by Sedic and Kuperwasser (40).

Studies investigating the influence of BRCA1 and BRCA2 on RAD51 foci formation and HR function have been previously performed using non-human, non-mammary cell lines such as CHO, DT40 and mouse embryonic stem cells. All studies have demonstrated an impaired function of HR pathways, resulting in a reduction in RAD51 foci formation in heterozygous cells (30,41-44).

The results of studies focusing on the effects of BRCA1 on both RAD51 foci formation and HR have been less univocal (43,44). In a study using lymphoblastoid cell lines of heterozygous *BRCA1* mutation carriers, Vaclová *et al* could not directly demonstrate a decrease in RAD51 foci formation, compared to the controls, at 4 h following exposure to 10 Gy IR. However, they did observe a significant increase in staining intensity of γH2AX foci in the same heterozygous *BRCA1* cells compared to controls 4 h following irradiation, thus suggesting an increase in the number of DSBs. They also argued that this result implies impaired HR in mutation carriers (29). Pathania *et al* did not detect a reduction in radiation-induced RAD51 foci in human mammary epithelial cells containing a *BRCA1* mutation exposed to 10 Gy compared to control cells. However, the combined exposure to UV and IR did yield a significant reduction of RAD51 foci (45).

Notably, with one exception, these studies were performed in non-mammary cells. The authors of this study believe that experiments performed herein modeled more closely the cellular makeup of human breast cells.

Moreover, none of these studies considered the variation of HR-based DSB repair throughout the different phases of the cell cycle. It was demonstrated that 48% of non-synchronized cycling MCF10A cells were in the G1 phase of the cell cycle, a phase during which HR cannot be activated due to the absence of the homologous sister chromatid. In the synchronized cell cultures, at the moment of irradiation approximately 90% of cells were in the S or G2 phases of the cell cycle, during which HR activation is maximal for repair of DSBs (2,46).

In order to activate the HR pathway more extensively, olaparib was added to the cultures prior to irradiation. A small, yet significant increase was observed in the number of RAD51 foci in irradiated, olaparib-treated control cells compared to cells not exposed to olaparib. However, PARP inhibition did not significantly increase the yield of RAD51 foci in irradiated BRCA1i or BRCA2i cells. The limited effect of olaparib treatment in the experiments in this study may be due to an exhaustion of HR capacity by aphidicolin synchronization and exposure to IR. Furthermore, it should be considered that PARP fulfills a number of different functions in the DNA damage response, including detection and signaling of DSBs and stabilization of stalled replication forks (47,48). It was hypothesized that in the experiments herein, PARP trapping, initiated by olaparib at the SSB site (47,49), may have partly impaired HR activation once the SSB is transformed in a DSB due to the replication fork collapse.

To investigate whether the impairment of HR shown by a decrease in RAD51 foci would result in increased chromosomal abnormalities, we have performed the micronucleus assay. Compared to controls, a marked and dose-dependent increase of micronucleus formation was observed in irradiated cells harboring a BRCA1 or BRCA2 knockdown. This effect was more pronounced in BRCA1i cells likely because BRCA1 plays a broader role in DNA DSB repair, which includes the non-homologous end-joining pathway (1,2). This pathway is active also in the G1 phase of the cell cycle, and the micronucleus assay was performed in cycling MCF10A cells, of which a fraction of >40% was in the G1 phase.

The impaired DNA repair capacity in BRCA1i and BRCA2i cells also resulted in decreased cell survival compared to controls. In addition, in this case, the BRCA1i cells were affected by radiation to a greater extent, when compared to the BRCA2i cells.

In conclusion, this study demonstrated that in cells containing a knockdown for either BRCA1 or BRCA2, the HR pathway is impaired, resulting in a >50% reduction of RAD51 foci, in significant increases in MN yields and in decreased cell viability following exposure to ionizing radiation, compared to the cells not subjected to knockdown. As in both BRCA1i and BRCA2i cell lines <50% of the protein is retained after lentiviral knockdown, the results obtained in the MCF10A cell line may mimic the situation in healthy carriers of a germline *BRCA1/2* mutation. Therefore, the assessment of RAD51 foci in heterozygous *BRCA1* and *BRCA2* mutation carriers may be a useful strategy to measure radiosensitivity and HR capacity in these subjects. However, as the results of this study are preliminary, this hypothesis is currently being tested in breast epithelial cells and lymphocytes of *BRCA1/BRCA2* women who may present impaired expression of BRCA due to the presence of germline heterozygous mutations in different functional domains of both genes. Information from these investigations will also be crucial to direct further research, ultimately aiming at improving protection strategies in subjects showing increased risk for radiation-induced carcinogenesis.

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Availability of data and materials

Data and materials are available to colleagues who shall make written request of them. The source of such data and material should be acknowledged in published articles.

Authors' contributions

AV and AB were involved in the conceptualization of the study. AB, MFP, JD, MVH, BV, JV, KBMC and AS were involved in the investigative aspects of the study. JP and AV were involved in the study methodology. AB, MVH and GP were involved in data analysis. AB was involved in the writing of the original draft. AV, KBMC and GP were involved in the writing, reviewing and editing of the manuscript. AV and KBMC were involved in manuscript supervision. AV was involved in project administration and funding acquisition.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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