BRCA1 Deficiency Exacerbates Estrogen-Induced DNA Damage and Genomic Instability

Kienan I. Savage1, Kyle B. Matchett1, Eliana M. Barros1, Kevin M. Cooper2, Gareth W. Irwin1, Julia J. Gorski1, Katy S. Orr2, Jekaterina Vohhodina1, Joy N. Kavanagh1, Angelina F. Madden1, Alexander Powell1,2, Lorenzo Manti3, Simon S. McDade1, Ben Ho Park4, Kevin M. Prise1, Stuart A. McIntosh1, Manuel Salto-Tellez1, Derek J. Richard5, Christopher T. Elliott6, and D. Paul Harkin1

Abstract

Germline mutations in BRCA1 predispose carriers to a high incidence of breast and ovarian cancers. BRCA1 functions to maintain genomic stability through critical roles in DNA repair, cell-cycle arrest, and transcriptional control. A major question has been why BRCA1 loss or mutation leads to tumors mainly in estrogen-regulated tissues, given that BRCA1 has essential functions in all cell types. Here, we report that estrogen and estrogen metabolites can cause DNA double-strand breaks (DSB) in estrogen receptor-α-negative breast cells and that BRCA1 is required to repair these DSBs to prevent metabolite-induced genomic instability. We found that BRCA1 also regulates estrogen metabolism and metabolite-mediated DNA damage by repressing the transcription of estrogen-metabolizing enzymes, such as CYP19A1, in breast cells. Finally, we used a knock-in human cell model with a heterozygous BRCA1 pathogenic mutation to show how BRCA1 haploinsufficiency affects these processes. Our findings provide pivotal new insights into why BRCA1 mutation drives the formation of tumors in estrogen-regulated tissues, despite the general role of BRCA1 in DNA repair in all cell types. Cancer Res; 74(10); 2773–84. ©2014 AACR.

Introduction

BRCA1 is a tumor suppressor protein that functions to preserve genomic stability by regulating key cellular processes, including homologous recombination (HR)-mediated DNA repair, cell-cycle checkpoint control, transcriptional regulation, chromatin remodeling, and postreplicative repair (1–3). Despite playing a role in processes essential to all cells, BRCA1 mutation predisposes to tumors predominantly in estrogen-regulated tissues, such as the breasts and ovaries. Indeed, germline mutations in a single BRCA1 allele confer a lifetime risk of up to 90% of developing breast cancer and 30% to 40% of ovarian cancer (4, 5).

Several observations suggest that estrogen has an important role in the development of BRCA1-dependent breast cancer. Pre- or postmenopausal oophorectomy in BRCA1 mutation carriers significantly reduces the risk of breast cancer onset and recurrence (6–8). Furthermore, pregnancy increases the risk of early-onset breast cancer in BRCA1 mutation carriers, in contrast with noncarriers for whom pregnancy is protective (9). It has also been reported that BRCA1 represses the expression of CYP19A1 (aromatase), which converts androgens to bioactive estrogens (10). Thus BRCA1 loss may increase CYP19 expression and subsequent estrogen production, further driving tumorigenesis (11).

Estrogen is postulated to promote tumorigenesis directly through stimulation of the estrogen receptor-α (ER-α) and the downstream activation of promitogenic transcriptional programs. However, this is confounded by observations that approximately 70% to 80% of BRCA1-mutated breast tumors are ER-α negative (12, 13). Furthermore, BRCA1 drives ER-α expression, suggesting the role of estrogen in BRCA1-dependent tumor, development may be independent of ER-α (14). Consistent with this, estradiol (E2; the predominant estrogen) induces tumor formation in ER-α knockout mice (15). In these mice, reduction of endogenous E2, by either oophorectomy or treatment with aromatase inhibitors, delayed tumorigenesis, whereas the ER-α antagonist fulvestrant had no effect (15).

The endogenous conversion of estrogen to genotoxic metabolites has been reported as an alternative, potentially ER-α independent mechanism for estrogen-dependent breast tumorigenesis. Estrogen is hydroxylated to form the catechol estrogens 2-hydroxyestradiol (2-OHE1(E2)) and 4-hydroxyestradiol...
(4-OHE1(E2)), a process that is catalyzed by a number of cytochrome (CYP) P450 enzymes, including CYP1A1, CYP1A2, CYP1B1, and CYP3A4. The catechol estrogens are further oxidized (by the same enzymes) into semiquinone and quinone forms, the latter of which can react with DNA to form adducts. Interestingly, urinary levels of 2-OHE2 and 4-OHE2 are elevated in patients with breast cancer compared with healthy controls (16), and 4-OHE2 concentrations have been reported to be up to three times higher in breast cancer biopsies compared with normal breast tissue (17). Moreover, in vivo studies have demonstrated that exogenous 2-OHE2 and 4-OHE2 can induce kidney and uterine cancers in mice (18, 19). The DNA adducts induced by these metabolites produce apurinic sites in the DNA that require repair, error-prone repair of which can lead to A-T to G-C mutations in DNA in the form of G.T heteroduplexes (20–22). Furthermore, high levels of depurinated estrogen adducts have been observed in serum and urine samples from patients with breast cancer and women with a strong family history of breast cancer (23, 24). It has been suggested that these depurinating adducts are repaired through the nucleotide excision repair (NER) and base excision repair pathways; however, a study that examined chromosomal aberrations in DT40 cells after treatment with 4-OHE2 observed no difference between wild-type cells and cells depleted of XPA, a key protein in NER (25, 26). In contrast, there were enhanced chromosomal breaks following 4-OHE2 treatment of rad51- and ku70-mutant DT40 cells, both of which are required for repair of double-strand breaks (DSB) by HR and non-homologous end joining, respectively. This suggests that estrogen metabolites may produce DNA DSBs.

The idea that estrogen metabolites may cause DNA DSBs, coupled with the role of BRCA1 in DSB repair, lead us to hypothesize that BRCA1-deficient cells may be more susceptible to estrogen metabolite-induced DNA damage and subsequent genomic instability. We therefore examined whether estrogen and its metabolites 2-OHE2 and 4-OHE2 can cause DSBs in human breast cells and examined the role of BRCA1 in both the induction and repair of estrogen metabolite-induced DNA damage.

Materials and Methods

Cell lines

MCF7 and MCF10A cells were obtained from the American Type Culture Collection and maintained according to the recommended instructions. MCF10A BRCA1+/− 185delAG and matched control BRCA1+/- cells were generated as previously described (27). All cell lines were verified by short tandem repeat profiling.

siRNAs

siRNAs were obtained from Qiagen and reverse transfected into cells using RNAiMAX (Invitrogen) to a final concentration of 10 nmol/L. See Supplementary Materials and Methods for sequences.

Immunofluorescence microscopy

Cells were transfected with siRNAs as above and incubated for 48 hours. Cells were then plated onto coverslips and treated with E2, 2-OHE2, or 4-OHE2 (Sigma), or mock treated with vehicle and incubated for indicated time points. Cells were then fixed and stained with γ-H2AX (Millipore), 53BP1 (Millipore), cyclin A (SCBT), or pATMSer1981 (Cell Signaling Technology) primary antibodies and imaged using a Nikon Eclipse Ti microscope, using a ×60 objective.

Comet assays

Neutral comet assays were carried out using the Cell Biolabs SCGE Kit. Comets were scored using CometScore (TriTek Corp.).

Western blotting

Western blotting was carried out as previously described (28).

Metaphase spreads, FISH staining, and chromosomal aberrations

Analysis was carried out as described previously (29).

Quantitative real-time PCR analysis

Quantitative real-time PCR (qRT-PCR) was carried out using Roche LightCycler 480 Real-Time ready catalogue assays for each gene (ACTB, CYP1A1, CYP1A2, CYP3A4, CYP1B1, COMT, and NQO1) as per the manufacturer’s instructions. A matched qRT-PCR reaction was carried out using the RT-ve control for each sample ensuring no genomic DNA contamination.

Chromatin immunoprecipitations

Chromatin immunoprecipitations (ChiP) were performed as described previously (28). See Supplementary Materials and Methods for complete protocol and primer sequences.

Immunohistochemical staining of CYP1A1

Immunohistochemical staining was performed using a fully automated BondMax immunostainer with a polymer-based peroxidase detection system. [CYP1A1 (B4)] SCBT primary antibody was used at a dilution of 1:50.

Ultra performance liquid chromatography/tandem mass spectrometry

Samples were extracted using liquid–liquid extraction with diethyl ether, followed by dansyl chloride derivatization as described by Xu and colleagues and analyzed using ultra performance liquid chromatography/tandem mass spectrometry (UPLC/MS-MS). See Supplementary Materials and Methods for complete protocol (30).

Isolation and culture or primary breast progenitor cells

See Supplementary Materials and Methods for complete protocol. Ethical approval to obtain primary breast tissue was granted through the Northern Ireland Biobank. Tissue was dissociated and mammospheres cultured as previously described (31) in ultralow attachment 75 cm² flasks for 7 days. Mammosphere cultured cells were then dissociated and plated into Lab-Tek II CC3 treated chamber slides (Nunc) in the media above.
Results

**Estrogen metabolites induce DNA DSBs in breast cells**

To determine whether estrogen metabolites can generate DNA DSBs, we first assessed whether the parent hormone, E2, induces DSBs in breast cells. Normal like, ER-α-negative MCF10A breast cells were treated with either E2 or mock treated for 3 hours and stained with the DSB markers 53BP1 and γH2AX. Treatment with E2 resulted in a significant increase in 53BP1 and γH2AX foci, respectively, compared with controls (P < 0.001; Fig. 1A and B). To investigate whether...
estrogen metabolites cause DSBs, we treated MCF10A cells with the metabolic intermediates 2-OHE2 and 4-OHE2 for 3 hours and stained with 53BP1 and γH2AX. Similar to E2 treatment, we found that both metabolites induced a significant increase in DSB foci (P < 0.01; Fig. 1C and D). These findings were also confirmed in the ER-α-positive breast cancer cell line, MCF7, indicating that E2 metabolite-induced DSB induction is independent of ER-α (Supplementary Fig. S1A and S1B).

To further confirm that 2-OHE2 and 4-OHE2 induce DSBs, we performed neutral comet assays. Indeed, MCF10A cells treated with 2-OHE2 and 4-OHE2 demonstrated a greater percentage of tail DNA, tail moment, and tail length, indicative of increased DSBs (Fig. 1E and F and Supplementary Fig. S1C and S1D).

DNA DSBs are known to result in activation of the ATM kinase, which is activated through autophosphorylation of ATM at serine-1981. In keeping with this, 2-OHE2 and 4-OHE2 treatment resulted in a 2.27-fold and 3.37-fold increase in ATM phosphorylation (Supplementary Fig. S1G and S1H). This suggests that the DNA damage induced by estrogen metabolites is highly likely to be DSBs. This was visualized at multiple time points following 3 hours of 2-OHE2 or 4-OHE2 treatment (Supplementary Fig. S2C and S2D). In addition, given that E2 metabolites induce DNA damage during S phase, taken together with the known role for BRCA1 in the postreplicative repair of bulky DNA adducts, we assessed whether depletion of BRCA1, using two independent siRNAs, caused replication fork stalling, marked by residual ps4/8 RPA32 foci (3). Consistent with a role for BRCA1 in repairing E2-metabolite-induced DNA damage in S phase cells, we observed a dramatic increase in ps4/8 RPA32-positive cells upon BRCA1 depletion following 2-OHE2 and 4-OHE2 treatment (Fig. 2D).

We next examined whether 2-OHE2 and 4-OHE2 treatment also induced chromosomal instability in BRCA1-depleted cells. We assessed chromosomal aberrations in control and BRCA1-depleted MCF10A cells 24 hours following treatment with 2-OHE2 and 4-OHE2. Structural rearrangements were visualized and quantified in metaphase spreads using chromosome 1 and 2 FISH staining (Fig. 2E and F). BRCA1 depletion resulted in a marked increase in chromosomal aberrations following both 2-OHE2 and 4-OHE2 treatment, demonstrating that E2 metabolite treatment induces genomic instability in BRCA1-deficient cells. Intriguingly, when we examined DSB production in these cells immediately following 2-OHE2 and 4-OHE2 treatment (3 hours), we found that 2-OHE2 and 4-OHE2 treatment resulted in a significant increase in 53BP1 and γH2AX foci in BRCA1-depleted cells compared with control cells (Fig. 2G and H and Supplementary Fig. S2E). In addition, similar to that demonstrated earlier, E2 metabolite-mediated DNA damage occurred specifically in S-G2 phase of the cell cycle in BRCA1-depleted cells (Supplementary Fig. S2F). Because of the relatively short treatment time (3 hours), it is unlikely that this increase in DSBs observed in BRCA1-depleted cells is solely due to defective DNA repair. To confirm that this occurred at early time points and was not confounded by the dose and/or time points of E2-metabolite treatment used, we assessed γH2AX marked DNA damage in control and BRCA1-depleted cells following treatment with 1 nm, 10 nm, 100 nm, and 1 μmol/L E2, 2-OHE2 and 4-OHE2 at various time points (Supplementary Fig. S3A–S3C). This revealed increased DNA damage in BRCA1-depleted cells at all doses and treatment time points assessed.

Taken together, these data indicate that loss of BRCA1 expression results in delayed repair kinetics but also increased levels of DNA damage following treatment with E2 metabolites. To further confirm this, we examined induction and repair of DNA DSBs following 2-OHE2 and 4-OHE2 treatment in BRCA1-
mutant MDA-MB-436 cells stably transfected with either empty vector (EV) or a BRCA1 expression plasmid (Supplementary Fig. S4A–S4C). Like, BRCA1-depleted cells, this revealed increased DNA damage induction at short time points (3 hours) in the BRCA1-deficient cells (Supplementary Fig. S4B). Similarly, defective repair of 2-OHE2 and 4-OHE2-induced DSBs was observed in BRCA1-deficient cells in comparison with BRCA1 reconstituted cells (Supplementary Fig. S4C).
Given that BRCA2 is also involved in HR-mediated DSB repair and mutations in this gene also predispose to tumors in the breast and ovaries, we examined the effect of BRCA2 depletion on 2-OHE2 and 4-OHE2-induced DNA damage in both MCF10A and MCF7 cells. Intriguingly, BRCA2 depletion resulted in slightly increased DSBs following treatment with 2-OHE2 and 4-OHE2 for 3 hours, which seemed to remain unrepaird at 24 hours following recovery from 2-OHE2 and 4-OHE2 treatment (Supplementary Fig. S5). Nevertheless, the increased level of DNA damage observed following treatment with 2-OHE2 and 4-OHE2 in BRCA2-depleted cells was minimal in comparison with that observed in BRCA1-depleted cells. In addition, the defective repair of these DSBs in BRCA2-depleted cells is consistent with BRCA2's role in HR-mediated DSB repair.

**BRCA1 regulates the expression of estrogen-metabolizing enzymes**

We have previously proposed that BRCA1 may transcriptionally repress the expression of estrogen metabolizing genes. Specifically, we have demonstrated that CYP1A1, the enzyme responsible for conversion of E2 to 2-OHE2 and semiquinone/quinone metabolites, is repressed by BRCA1 in a number of breast cancer cell lines (32). In addition, BRCA1 has been found to transcriptionally activate a number of detoxification/anti-oxidant genes, including NAD(P)H quinone oxireductase 1 (NQO1), which reduces genotoxic quinones to nonreactive hydroquinones (33). This led us to hypothesize that the exacerbated DNA damage observed in BRCA1-depleted cells at short time points following 2-OHE2 and 4-OHE2 treatment may be due to, at least in part, increased estrogen metabolism in these cells, mediated by upregulated expression of estrogen-metabolizing enzymes, and/or downregulated expression of detoxification enzymes such as NQO1. To assess this, we examined the expression of a panel of estrogen-metabolizing and detoxification genes, including CYP1A1, CYP1B1, CYP1A2, CYP3A4, NQO1, and COMT, using qRT-PCR, in MCF10A and MCF7 cells transfected with control and BRCA1-depleting siRNAs (Fig. 3A and B). This revealed that CYP1A1 and CYP3A4 are consistently upregulated upon BRCA1 loss, suggesting that BRCA1 represses the expression of these genes. In addition, NQO1 was consistently downregulated upon BRCA1 depletion in both cell lines. We also examined the expression of these genes in BRCA1-mutant MDA-MB-436 cells, stably transfected with EV or BRCA1 Fig. 3C). Ectopic expression of BRCA1 in these cells repressed expression of CYP1A1, but had a limited effect on CYP3A4 and NQO1 expression.

To assess whether BRCA1 regulates the transcription of these genes directly, we performed BRCA1 ChIP-qPCR assays from MCF10A cells, using primers specific to the promoter regions of CYP1A1, CYP3A4, NQO1, and COMT as a negative control (Fig. 3D). We observed enrichment of BRCA1 at all of these promoters with the exception of COMT, which is neither transcriptionally regulated by BRCA1 nor promoter bound by BRCA1.

To confirm that this occurs in BRCA1-deficient tumors, we assessed the levels of CYP1A1, the most highly deregulated gene upon BRCA1 loss, using immunohistochemistry (IHC) in a panel of 21 BRCA1-mutant and 75 BRCA1 wild-type breast tumors. Intratumoral CYP1A1 expression was scored by a pathologist and an independent scorer as very weak/absent (1, moderate = 2, or strong = 3 (Fig. 3E and F)). This revealed that the mean expression of CYP1A1 expression is significantly upregulated in this cohort of BRCA1-mutant tumors compared with BRCA1 wild-type sporadic tumors (P = 0.0007).

Given that we observed a slight increase in E2 metabolite-induced DNA damage in BRCA2-depleted cells treated with 2-OHE2 and 4-OHE2, we also assessed the role of BRCA2 in regulating the expression of CYP1A1 in both MCF10A and MCF7 cells (Supplementary Fig. S6A and S6B). This revealed no significant difference in CYP1A1 expression between control and BRCA2-depleted cells.

**BRCA1 suppresses estrogen metabolite-mediated DNA damage by suppressing estrogen metabolism**

As BRCA1 depletion results in increased levels of CYP1A1 and CYP3A4 enzymes and decreased NQO1 expression, we hypothesized that the levels of 2-OHE2 and 4-OHE2 and 2,3/3,4 quinone products would be increased in BRCA1-depleted cells compared with control cells following E2 treatment. Quinone and semiquinone metabolites are extremely unstable and have a very limited half-life making them difficult to quantify. In contrast, 2-OHE2 and 4-OHE2 (which are generated by CYP1A1 and CYP3A4 in breast cells) are relatively stable. We therefore developed an UPLC/MS-MS method for the detection of 2-OHE2 and 4-OHE2. The method was optimized for baseline chromatographic separation and the accurate identification and quantification of both isomeric forms of the hydroxyl-estradiol metabolite (Fig. 4A). Using this method, we found that the relative concentration of 2-OHE2 and 4-OHE2 was significantly higher in BRCA1-depleted cells compared with control cells (P < 0.05; Fig. 4B).

As CYP1A1 was the most robustly upregulated estrogen-metabolizing gene upon BRCA1 depletion, we sought to examine the role of this enzyme in estrogen-dependent DNA damage. To investigate this, BRCA1 and CYP1A1 were codelected in MCF10A cells and DNA DSBs assessed 3 hours following treatment with E2 (Fig. 4C and D). BRCA1 and CYP1A1 depletion was assessed by qRT-PCR (Supplementary Fig. S6C and S6D). Strikingly, CYP1A1 depletion lead to a marked reduction in estrogen-mediated DSBs in BRCA1-depleted cells (P < 0.001), suggesting that estrogen-mediated DNA damage in BRCA1-depleted cells is, at least in part, due to increased CYP1A1 levels in these cells.

This suggests that BRCA1, apart from mediating repair of E2 metabolite-mediated DNA damage, transcriptionally regulates estrogen-metabolizing enzymes and subsequently represses estrogen metabolism, thereby protecting cells against estrogen-induced DNA damage. This is particularly important in breast and ovarian cells, which are exposed to much higher levels of estrogen than other tissues within the body (34). Nevertheless, to confirm whether this mechanism may drive genomic instability in nonbreast cells, we assessed E2 metabolite-induced DNA damage in HEK293, kidney cells (Supplementary Fig. S6E and S6F). This revealed that, although 2-OHE2 and 4-OHE2 are capable of inducing DNA DSBs in these cells,
Figure 3. BRCA1 transcriptionally regulates estrogen metabolizing genes. A and B, qRT-PCR determined expression of genes involved in estrogen metabolism in control (siSCR) and BRCA1-depleted (siBRCA1) MCF10A (A) and MCF7 (B) cells. Gene expression was normalized to ACTB expression and is shown relative to expression in control (siSCR) cells. Bars, mean relative expression ± SEM from three independent experiments. Significance of changes in gene expression was assessed using Student two-tailed t-test. ***, P < 0.001. C, qRT-PCR determined expression of genes as above in the BRCA1-deficient MDA-MB-436 cells stably transfected with either empty vector (+EV) or a BRCA1 expression plasmid (+BRCA1). Significance of changes in gene expression was assessed using Student two-tailed t-test. **, P < 0.01; ***, P < 0.001. D, BRCA1 ChIP qPCR using primers targeting the promoters of COMT (not regulated by BRCA1), CYP1A1, CYP3A4, and NQO1. Quantified amounts of immunoprecipitated DNA were normalized to inputs and reported relative to the amount quantified at a nonspecific control region. Bars, mean fold enrichment ± SEM from three independent experiments. E, IHC determined expression of CYP1A1 in BRCA1 mutant and matched BRCA1 wild-type breast tumors. CYP1A1 expression in each tumor was scored as 0 = absent, 1 = low, 2 = moderate, or 3 = high. Significance of changes in gene expression was assessed using Student two-tailed t-test. ***, P < 0.001. F, representative images of moderate and high CYP1A1 staining in BRCA1 wild-type and BRCA1 mutant breast tumors.
they induce much lower levels of DSBs in comparison with breast cells. In addition, 2-OHE2 and 4-OHE2 treatment (3 hours) did not induce increased levels of DSBs in BRCA1-depleted cells (Supplementary Fig. S6E). Moreover, we observed similar low levels of DNA damage in both BRCA1- and BRCA2-depleted cells. In contrast, consistent with the role of BRCA1 and BRCA2 in DSBR repair, both BRCA1 and BRCA2 were required for repair of E2 metabolite-induced DNA damage in these cells (Supplementary Fig. S6F). Given that much lower levels of DNA damage were observed in these cells and that we did not observe any increased DNA damage in BRCA1-depleted cells in comparison with control, or BRCA2-depleted cells, we hypothesized that BRCA1 may not regulate the expression of CYP1A1 in these cells. Intriguingly, we were unable to detect any CYP1A1 transcript in these cells, suggesting that these cells may not metabolize estrogen at the same rate as breast cells (data not shown).

**Estrogen metabolite-mediated DNA DSBs are exacerbated in BRCA1 heterozygous breast cells**

Taken together, our data suggest that in BRCA1-deficient breast cells, deregulated estrogen metabolism results in increased levels of genotoxic metabolites resulting in increased DNA damage, which, coupled with defective DNA repair, leads to genomic instability, a key hallmark of cancer initiation and progression. However, clinical evidence suggests a direct role for estrogen in breast cancer development in BRCA1 mutation carriers, suggesting that heterozygous loss of BRCA1 may result in haploinsufficiency in at least one of BRCA1’s functions. Indeed, a number of studies have reported increased sensitivity to ionizing radiation in BRCA1 carrier/heterozygous cells, suggesting that DNA DSB repair may be impaired in carriers (35). In addition, a recent study by Konishi and colleagues, using somatic cell gene targeting to introduce the common pathogenic BRCA1 mutation 185delAG into a single...
BRCA1 allele in MCF10A cells, demonstrated that this heterozygous BRCA1 mutation confers impaired HR-mediated DSB repair, hypersensitivity to genotoxic stress, and increased genomic instability (27). We therefore set out to determine whether heterozygous mutation of BRCA1 affects estrogen metabolite-mediated DNA damage and estrogen metabolism.

Using the same cell line model developed by Konishi and colleagues, heterozygous BRCA1 185delAG (BRCA1+/−) and control (BRCA1+/+) MCF10A cells were treated with 2-OHE2 and 4-OHE2 for 3 hours and the media replaced with normal media for 24 hours, before fixing and staining for 53BP1 and γH2AX. Consistent with a defect in BRCA1 function, a significant number of unresolved DNA damage foci were visible in 2-OHE2- and 4-OHE2-treated BRCA1+/− cells in comparison with control BRCA1+/+ cells (P<0.001; Fig. 5A). Surprisingly, the level of unrepaird DNA DSBs was similar to that observed in BRCA1-depleted MCF10A cells, suggesting that BRCA1 haploinsufficiency imparts a major defect in repair of E2 metabolite-mediated DNA damage. We next examined whether estrogen metabolites generate more DSBs in BRCA1+/− cells. Indeed, although short-term (3 hours) 2-OHE2 and 4-OHE2 treatment induced DNA damage in BRCA1+/− cells, significantly more DNA DSB foci were observed in BRCA1+/− cells (P<0.01; Fig. 5B). In support of this, we treated normal primary breast progenitor cells, isolated from breast tissue obtained from a woman undergoing elective breast reduction, as well as BRCA1+/− primary breast progenitor cells from a BRCA1 mutation carrier undergoing a risk-reducing mastectomy, with both 2-OHE2 and 4-OHE2 and examined both DNA damage induction at 3 hours posttreatment, as well as their ability to repair E2 metabolite-induced DNA damage 24 hours following treatment with 2-OHE2 and 4-OHE2. In keeping with our previous findings, 2-OHE2 and 4-OHE2 treatment for 3 hours induced more DNA damage in the BRCA1+/− mammary progenitor cells compared with the BRCA1 wild-type cells and this DNA damage was not repaired as efficiently in the BRCA1+/− cells compared with the BRCA1 wild-type cells (Fig. 5C and D). Taken together these data suggest that BRCA1-depleted cells, BRCA1+/− cells may have increased rates of estrogen metabolism as well as defective repair of E2 metabolite-induced DNA damage.

To examine this, we assessed the expression levels of the same panel of estrogen metabolizing and detoxification enzymes in BRCA1+/− and BRCA1+/+ cells. Indeed, as in BRCA1-depleted cells, CYP1A1 and CYP3A4 were upregulated in BRCA1+/− compared with BRCA1+/+ cells (Fig. 5E). Intriguingly, NQO1 expression was maintained at similar levels in both cell lines, suggesting that BRCA1 haploinsufficiency does not negatively impact all BRCA1-regulated transcriptional targets. Finally, consistent with the defective DSB repair observed in these cells following 2-OHE2 and 4-OHE2 treatment, we found that treatment with either of these metabolites induced genomic instability in BRCA1+/− cells but not BRCA1+/+ cells (Fig. 5F).

Discussion

One of the most perplexing features of BRCA1 biology is that despite playing a central role in the DNA damage response and DSB repair pathways in all cells, mutation carriers predominantly develop tumors in the breast and ovaries: both estrogen-driven tissues exposed to high levels of estrogen. Here, we show that treatment with both E2 and the E2 metabolites 2-OHE2 and 4-OHE2 induces DNA DSBs in human breast cancer cells in an ER-α independent manner. We also found that E2 metabolite-mediated DSBs occur specifically in S phase cells, suggesting that induction of these lesions is coupled to DNA replication. We hypothesize that E2 metabolite adducted DNA bases represent replication barriers, which lead to replication fork stalling during DNA synthesis. Indeed, a number of studies have shown that 4-hydroxyequilenin (4-OHEN), a metabolite of the equine estrogen equilenin (which is almost identical to 4OHE-2 in humans), causes identical DNA adducts to those caused by 4-OHE2 and that these adducts cause replication fork stalling (36, 37).

In general, stalled replication forks do not collapse and form DSBs, but are instead stabilized by the ATR kinase through the signaling and recruitment of a plethora of checkpoint signaling and repair proteins, resulting in resolution of the stalled fork through a HR-mediated repair process involving BRCA1. However, recent studies have shown that E2 inhibits ATR signaling, suggesting that E2 and its metabolites may lead to replication fork stalling and subsequent fork collapse and DSB formation through the combined effect of replication fork stalling and ATR inhibition (38). Further to this, BRCA1 has been shown to be required for both resolution of stalled replication forks as well as HR-mediated repair of DSBs caused following stalled fork collapse (39). BRCA1 is also required for the removal and repair of bulky base adducts, a mechanism through which BRCA1 may suppress adduct-induced mutagenesis (3). Consistent with this, we observed a dramatic increase in pS4/8 RPA32-positive cells, upon BRCA1 depletion in 2OHE2- and 4OHE2-treated cells.

Interestingly, we found that BRCA1 depletion also resulted in increased levels of E2 metabolite-induced DNA damage, even at very early time points. This suggested that BRCA1 may also play a more direct role in regulating the physical levels of DNA damage induced by estrogen metabolites. Previous data from our laboratory had indicated that BRCA1 loss leads to upregulation of CYP1A1, a major regulator of E2 metabolism in breast tissues (32). We therefore tested whether BRCA1 may also regulate the expression of other estrogen metabolizing enzymes, thereby regulating the levels of estrogen-derived metabolites. This analysis revealed that BRCA1 directly represses the transcription of CYP1A1 and CYP3A4 and promotes the expression of the NAD(P)H:quinone oxidoreductase, NQO1. We confirmed, using IHC in a cohort of BRCA1-mutant and matched BRCA1 wild-type tumors, that CYP1A1, the major enzyme involved in conversion of E2 to 2-OHE2 in breast tissues, is significantly upregulated in BRCA1-mutant tumors. This is consistent with the increased levels of both 2-OHE2 and 4-OHE2 observed in BRCA1-depleted cells. We also demonstrated that depletion of CYP1A1 significantly reduces the amount of DNA damage induced in BRCA1-depleted cells exposed to short-term E2 treatment, confirming that E2-mediated DNA damage in BRCA1-depleted cells is, at least in part, due to increased estrogen metabolism. Intriguingly, when examining the impact of BRCA2 on estrogen metabolite-induced DNA damage, we found that although BRCA2 is
Figure 5. BRCA1 heterozygosity leads to increased estrogen metabolite-mediated DNA damage, defective DNA repair, and genomic instability, and loss of repression of estrogen-metabolizing enzymes. A and B, quantification of γH2AX marked DNA DSBs in BRCA1 wild-type (BRCA1+/+) and BRCA1 heterozygous 185delAG (BRCA1+/−) MCF10A cells 24 hours after treatment with 2-OHE2 or 4-OHE2 (A) or immediately following 3 hours of treatment with 2-OHE2 or 4-OHE2 (B). Bars, mean number of foci per cell ± SEM from three independent experiments (>200 cells were counted per experiment). Significance of changes in foci numbers was assessed using Student two-tailed t test. **, P < 0.01; ***, P < 0.001. C and D, quantification of γH2AX marked DNA DSBs as above in BRCA1 wild-type (BRCA1+/+) and BRCA1 heterozygous primary breast progenitor cells 24 hours after treatment with 2-OHE2 or 4-OHE2 (C) or immediately following 3 hours of treatment with 2-OHE2 or 4-OHE2 (D). Significance of changes in foci numbers was assessed using Student two-tailed t test. **, P < 0.01. E, qRT-PCR determined expression of genes involved in estrogen metabolism in BRCA1 wild-type (BRCA1+/+) and BRCA1 heterozygous 185delAG (BRCA1+/−) MCF10A cells. Bars, mean relative expression ± SEM from three independent experiments. Significance of changes in gene expression was assessed using Student two-tailed t test. ***, P < 0.001. F, quantification of chromosomal aberrations in metaphase spreads from BRCA1 wild-type (BRCA1+/+) and BRCA1 heterozygous 185delAG (BRCA1+/−) MCF10A cells 24 hours after treatment with 2-OHE2 or 4-OHE2. Bars, mean aberrations per metaphase ± SEM from three independent experiments (>100 metaphases were scored per experiment). Significance of changes in aberration numbers was assessed using Student two-tailed t test. ***, P < 0.001.
required for the repair of these breaks, loss of BRCA2 does not lead to deregulated estrogen metabolism and the associated increased DNA damage. Perhaps this explains why BRCA2 mutations are less penetrant than BRCA1 mutations in predisposing carriers to breast and ovarian cancers.

We have also demonstrated that 2-OHE2 and 4-OHE2 treatment leads to increased DSB production in MCF10A cells and primary breast cells harboring a pathogenic heterozygous BRCA1 mutation, and that like BRCA1-depleted cells, these cells are unable to repair E2 metabolite-mediated DNA damage, leading to increased genomic instability. Importantly, we found that BRCA1 heterozygous mutant cells also have upregulated levels of CYP1A1 and CYP3A4, suggesting that increased estrogen metabolism may contribute to E2-mediated DNA damage in these cells. Consistent with this, higher levels of urinary excreted 2-OHE2 and 4-OHE2 have been observed in BRCA1 carriers compared with healthy control women with no BRCA1 mutation (40).

Taken together, these findings suggest that exposure to estrogen and its subsequent metabolism in BRCA1-deficient breast cells is capable of driving genomic instability, a well-defined early event in breast cancer development. Given that estrogen levels in normal/benign breast tissue are known to be six to seven times that of circulating estrogen levels, our findings suggest a mechanism through which BRCA1 carriers, through enhanced production of DNA-damaging estrogen metabolites, may acquire the genetic alterations that initiate neoplastic transformation in breast tissue (34). Similarly, levels of estrogen in ovarian tissues greatly exceed that of circulating estrogen, suggesting that this model may also explain the substantially increased risk of ovarian cancer in BRCA1 carriers (41).

A phase III trial termed, Prevention of Breast Cancer by Letrozole in Postmenopausal Women carrying a BRCA1/2 Mutation (LIBER), (ClinicalTrials.gov number, NCT00673335) is currently enrolling postmenopausal women for treatment with letrozole, an aromatase inhibitor, to evaluate its ability to prevent the development of breast cancer in patients with a BRCA1/2 mutation. Our results coupled with the finding that aromatase levels are substantially higher in prophyllactic mastectomy and oophorectomy tissue from BRCA1 carriers (10), provides further mechanistic data to support this approach.

However, aromatase inhibitors may have little preventative effect in premenopausal women, in whom the majority of BRCA1-linked tumors develop, and in whom estrogen production occurs predominantly in the ovaries through an aromatase-independent biosynthesis pathway. In these women, oophorectomy has been shown to reduce the risk of breast cancer by up to 60% (42). Taking our findings into account, it may also be worth considering the use of aromatase inhibitors as an additional chemopreventative strategy in premenopausal women, who have undergone risk-reducing oophorectomy without mastectomy.

Finally, in premenopausal women who have opted not to undergo risk-reducing oophorectomy or mastectomy, luteinizing hormone releasing hormone agonists, may prove useful as chemopreventative agents. These drugs cause reversible ovarian suppression/ablation and are currently used in combination with tamoxifen or aromatase inhibitors for the treatment of premenopausal women with ER-α-positive breast cancer (43, 44).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: K.I. Savage, S. McIntosh, C.T. Elliot, D.P. Harkin
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.I. Savage, K.B. Matchett, E.M. Barros, K.M. Cooper, G.W. Irwin, K.S. Orr, J.N. Kavanagh, A. Powell, L. Manti, S. McIntosh, M. Salto-Tellez, D.J. Richard
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K.I. Savage, K.B. Matchett, E.M. Barros, K.M. Cooper, G.W. Irwin, A. Powell, L. Manti, S.S. McDade, D.P. Harkin
Writing, review, and/or revision of the manuscript: K.I. Savage, K.B. Matchett, E.M. Barros, K.M. Cooper, G.W. Irwin, J.J. Gorski, A. Powell, S.S. McDade, B.H. Park, K.M. Prise, S. McIntosh, D.P. Harkin
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K.I. Savage, J. Voh Hodgina, A.F. Madden, B.H. Park
Study supervision: K.I. Savage, D.P. Harkin

Acknowledgments
The authors thank the Northern Ireland Biobank for providing breast tumor sections and fresh normal breast tissues.

Grant Support
This work was supported by grants from Cancer Research UK (CS268/A8121; D.P. Harkin, K.B. Matchett, and E.M. Barros), the Research and Development Office Northern Ireland (G.W. Irwin), and Cancer Focus Northern Ireland (K.I. Savage).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 10, 2013; revised January 31, 2014; accepted February 22, 2014; published OnlineFirst March 17, 2014.

References


BRCA1 Deficiency Exacerbates Estrogen-Induced DNA Damage and Genomic Instability


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-13-2611

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2014/03/17/0008-5472.CAN-13-2611.DC1

Cited articles
This article cites 44 articles, 13 of which you can access for free at:
http://cancerres.aacrjournals.org/content/74/10/2773.full#ref-list-1

Citing articles
This article has been cited by 5 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/74/10/2773.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.