Detoxification: A Novel Function of BRCA1 in Tumor Suppression?

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Our studies found that BRCA1 levels negatively correlate with DNA adducts induced by Benzo(a)pyrene (BaP). Pulse-chase experiments showed that the increase in BaP-induced DNA adducts in BRCA1 knockdown cells may not be associated with BRCA1’s function in nucleotide excision repair activity; rather, it may be associated with its function in modulating transcriptional regulation. BRCA1 knockdown in MCF-10A cells significantly attenuated the induction of CYP1A1 following BaP treatment indicating that the increase in BaP-induced adducts in BRCA1 knockdown cells is not CYP1A1 dependent. However, our study shows that BRCA1 defective cells may still be able to biotransform BaP by regulating other CYP enzymes, including CYP1B1. Knockdown of BRCA1 also severely affected the expression levels of two types of uridine diphosphate glucorunyltransferase (UGT1A1 and UGT1A9) and NRF2. Both UGTs are known as BaP-specific detoxification enzymes, and NRF2 is a master regulator of antioxidant and detoxification genes. Thus, we concluded that the increased amount of BaP-induced DNA adducts in BRCA1 knockdown cells is strongly associated with its loss of functional detoxification. Chromatin immunoprecipitation assay revealed that BRCA1 is recruited to the promoter/enhancer sequences of UGT1A1, UGT1A9, and NRF2. Regulation of UGT1A1 and UGT1A9 expression showed that the induction of DNA adducts by BaP is directly affected by their expression levels. Finally, overexpression of UGTs, NRF2, or ARNT significantly decreased the amount of BaP-induced adducts in BRCA1-deficient cells. Overall, our results suggest that BRCA1 protects cells by reducing the amount of BaP-induced DNA adducts possibly via transcriptional activation of detoxification gene expression.

Key Words: BRCA1; carcinogen-DNA adduct; xenobiotic stress; detoxification; Benzo(a)pyrene (BaP).

Growing evidence indicates that exposure to certain environmental factors increases cancer risk. However, epidemiologic studies have not yet demonstrated clear correlations, in part, because human genetic variations may significantly affect individual susceptibility. Exposure to certain classes of chemicals including polycyclic aromatic hydrocarbons (PAHs) increases breast cancer risk (Dunnick et al., 1995). Benzo(a)-pyrene (BaP) and 7,12-dimethylbenz(a)anthracene (DMBA) are well-known potent carcinogenic PAHs, which are frequently used to induce mouse skin and/or mammary gland tumors (Luch, 2005). They require metabolic activation to exert mutagenic and carcinogenic effects (Kouri et al., 1982; Shou et al., 1994). For example, BaP biotransformations are frequently characterized as either phase I (metabolic activation) or phase II (conjugation and detoxification) (Williams, 1959; Xu et al., 2005). In phase I, nonreactive BaP is activated into more polar and water soluble intermediate metabolites (e.g., dione and dihydrodiol) that generate reactive oxygen species and further oxidize to BaP-7,8-diol-9,10-epoxide (BPDE), which can damage DNA and/or other macromolecules. In phase II, these intermediate metabolites are conjugated into more easily excreted forms by reactions such as glucuronidation, sulfation, and glutathione conjugation (Williams, 1959; Xu et al., 2005).

Aryl hydrocarbon receptor (AhR) is a phase I transcription factor, which can bind the xenobiotic responsive element (XRE) and direct transcription of several cytochrome P450s (CYPs) and UDP-glucuronosyltransferases (UGTs) (Nebert et al., 2000; Shen and Whitlock, 1992). Nuclear factor (erythroid-derived 2)-like 2 (NRF2) is a phase II transcription factor and induces cytoprotective enzymes such as glutathione S-transferase (GST), several UGTs and multidrug resistance proteins (Köhle and Bock, 2007).

BRCA1 is a tumor suppressor in ovarian and breast cancers (Rosen et al., 2003). Various functions of BRCA1 have been identified including cell cycle progression, DNA repair, ubiquitination (as an E3 ubiquitin ligase), and transcription regulation (O’Donovan and Livingston, 2010). BRCA1 plays a role in the repair of DNA double-strand breaks (Lee et al., 2000; Zhong et al., 2001).
which include homologous recombination repair and microhomology-dependent DNA repair (Moynahan et al., 2001; Zhong et al., 2002). A function of BRCA1 in nucleotide excision repair (NER) of UV irradiation-induced DNA damage has been proposed (Hartman and Ford, 2002), but its NER activity on carcinogen-induced DNA adducts has not been well documented. BRCA1 affects transcription regulation activity by interacting with multiple proteins including transcription factors (e.g., P53, ERx, c-Myc). We recently reported that BRCA1 modulates transcription regulation of stress-associated transcription factors such as HIF-1α, AhR/ARNT, and NRF2 (Bae et al., 2004; Kang et al., 2006a, 2006b, 2008a).

In this study, we investigated whether BRCA1 has a protective function against carcinogen-induced DNA damage. We found that abnormal BRCA1 levels or BRCA1 dysfunction lead to detectably increased DNA damage after exposures to certain xenobiotics (perhaps even after short exposures). In this paper, we further investigate and discuss its potential molecular mechanisms.

MATERIALS AND METHODS

Cell lines and reagents. A nearly normal-like human breast cell line, MCF-10A, was obtained from American Type Culture Collection (Manassas, VA). SUM149PT (BRCA1 2288delT) and SUM1352MO2 (BRCA1 185delAG) are two human breast cancer cell lines with mutations in the BRCA1 gene (Eilstrodt et al., 2006) and were purchased from Asterand Co. (Detroit, MI). The growing media and conditions of MCF-10A, SUM149PT, and SUM1352MO2 cell lines have been described in elsewhere (Eilstrodt et al., 2006). Benzo(a)pyrene (BaP) and DMBA were purchased from Sigma and dissolved in dimethyl sulfoxide (DMSO). The [3H]BaP and [32P]ATP radioisotopes were obtained from GE Healthcare (Piscataway, NJ).

siRNAs and DNA expression plasmids. The control (non-targeting scrambled), BRCA1, XPA, ERCC1, UGT1A1, UGT1A9 (1- and 2), and NRF2 siRNAs were purchased from Dharmacon, Inc. (Lafayette, CO). The following sequences of primers were used; BRCA1 forward 5'-GCC TTC TAT CAG ATA A-3', reverse 5'-AAA CCA CAC AGT CTT C-3', XPA-siRNA 5'-GCA AAT GGC TTC TAT CGA A-3', UGT1A1-siRNA 5'-GCA CAA GCA GAT AGA TAT T-3', UGT1A1-siRNA 2 5'-GGA AAC ACG ATA CTT G-3', NRF2-siRNA 5'-GAG TAT GAG CTG GAA AAA C-3'. Smart pool siRNAs were used for UGT1A1- and ERCC1-siRNA. The shRNA-coding DNA vectors (pSuper-control and pSuper-BRCA1) were kindly provided by Dr Khanna (Fabbro et al., 2004). The UGT1A1 and UGT1A9 expression plasmid DNA were purchased from Origene Technology, Inc. (Rockville, MD).

[3H]BaP-induced DNA adducts. Radioactivity of the [3H]BaP-induced DNA adducts was measured by liquid scintillation counter (Beckman Coulter, Brea, CA) with the equal amounts of DNA (10 μg). Each radioactivity value was subtracted by the nontreated background value to calculate the specific radioactivity (cpm of adducts/cpm of total nucleotides × 1/dilution factor) and represents the number of DNA adducts per 10^6 nucleotides.

[32P]Postlabeling assays. This assay is a highly sensitive method to detect and measure the amount of DNA adducts induced by carcinogens. MCF-10A cells were transfected with 4 μg of BRCA1-shRNA for 72 h and treated with BaP (5μM) or DMBA (1 or 5μM) for 24 h. Isolated genomic DNA (10 μg) was incubated with a digestion mixture (10 units/ml micrococcal nuclease, 1.1 unit/ml spleen phosphodiesterase, 25mM CaCl_2, and 75mM sodium succinate [pH 6.0]) at 37°C for 4 h. After digestion, 50mM ammonium formate (pH 7.1) was added. The solid phase extraction column (Varian, Inc., Harbor City, CA) was equilibrated with methanol. After the sample was loaded onto the column, it was washed with 50mM ammonium formate (pH 7.1). The DNA adducts were eluted with 50% methanol and were completely dried using a SpeedVac. Then the nuclease P1 mixture (2 μg/μl nuclease P1, 0.15M zinc chloride, 62.5mM sodium acetate [pH 5.0]) was added and incubated at 37°C for 1 h. After incubation, 500mM Tris base was added, and reaction mixture was dried. The adducts were labeled with [32P] in the mixture (1γ-32P]ATP [10 μCi/μl], T4 polynucleotide kinase, and apyrase) at 37°C for 30 min. The labeled adducts were spotted on a thin layer chromatography (TLC) sheet and developed in the following buffers. The BaP-DNA adducts (Fig. 2b) were developed direction (hereafter, D1) (bottom to top) in 1.7M NaH2PO4 (pH 5.8) overnight; D3 (bottom to top) in 3.4M lithium formate and 6.4M urea (pH 3.5) for 4 h; and D4 (left to right) in 0.8M NaH2PO4, 0.5M Tris-HCl, and 7.5M urea (pH 3.5) for 4 h (Pan et al., 2006). The DMBA-DNA adducts (Fig. 2c) were developed D1 (bottom to top) in 1M NaH2PO4 (pH 6.0) for overnight; D3 (bottom to top) in 0.7M ammonium hydroxide for 4 h; and D4 (left to right) in isopropanol/4M ammonium hydroxide (1:5:1) for 4 h (Vadhvanam et al., 2003). After development, the TLC was dried and exposed to x-ray film. The adduct spots were excised and placed in scintillation vials to measure radioactivity using a liquid scintillation counter (Beckman Coulter).

Western blotting analysis. Whole cell lysates were separated by SDS-PAGE, transferred to PVDF membranes (Millipore, Billerica, MA) for further Western blotting (WB) analysis (Kang et al., 2006a). The anti-BRCA1 (C-20), anti-XPA (FL-273), anti-ERCC1 (FL-297), anti-NRF2 (H-300), and anti-ARNT (H-172) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-UGT1A9 (Novus Biologicals, Littleton, CO), anti-P53 (BD Gentest, San Jose, CA), anti-CYP1A1 (Affinity BioReagents, Golden, CO), anti-CYP1B1 (BD Gentest), anti-FLAG (M2, Sigma-Aldrich, St Louis, MO), anti-APC (Abcam, Cambridge, MA), and anti-Jo-1 (Sigma-Aldrich) antibodies were obtained from different sources. Antibody-protein complexes were visualized by enhanced chemiluminescence (Santa Cruz Biotechnology, Inc.) using x-ray films.

Semiquantitative RT-PCR. MCF-10A cells were pretreated with siRNA (control vs. BRCA1) for 24 h and treated with 5μM of BaP for the indicated time points in figures. Total RNAs were isolated with Trizol solution (Invitrogen, Carlsbad, CA), and cDNA were prepared with Superscript II reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. The following sequences of primers were used; BRCA1 forward 5'-TTG CCG GAG GAA AAT GGG TAG TTA C-3' and reverse 5'-TGC TGT AAC GAG GGT GAA TGA TGA AAG C-3', UGT1A1 forward 5'-CTC TAT AAT TGG AGG TTT ATC CTC T-3' and reverse 5'-ACC ACA ACA CCA CTT TTC CC-3', UGT1A9 forward 5'-TGC TCA ATG GAA AGC ACA AGG C-3' and reverse 5'-CTG GCA GTG CCA AAA AAA CT-3', NRF2 forward 5'-AAA CCA CCC TGA AAG ACC AGC-3' and reverse 5'-AGG GCC GTC TTG ATT GTT CT-3', CYP1A1 forward 5'-CTT GCA CCT TGG AGC TCG-3' and reverse 5'-CCG AGA AGG AAT GTG CCA AGG-3', CYP1B1 forward 5'-CAC CAA GGC TGA GAC AGA-3' and reverse 5'-GAT GAC GAC TGG GCC TAC AT-3', β-actin forward 5'-GCT ATC CCT GGA CTC TG-3' and reverse 5'-ACA TCT GCT GGA AGG TGG AC-3'.

Ethoxyresorufin-O-deethylase activity. CYP1A1 enzymatic activity was measured using a CYP1A1 ethoxyresorufin-O-deethylase (EROD) activity kit (IKZUS Environment, Genova, Italy). MCF-10A cells were transfected with a DNA vector containing siRNA (pSuper-control vs. pSuper-BRCA1) for 72 h and treated with various doses of BaP (0, 1, 2.5, 5μM) for 24 h. Then, cells were washed and incubated with reaction buffers (5μM of 7-ethoxyresorufin and 10μM of dicumarol) for 30 min. Fluorescence was measured every 10 min for 60 min at 37°C with an Ultra 384 fluorometer (Tecan, Switzerland) using 535 nm excitation and 590 nm emission filters as previously described (Kang et al., 2008b).

Chromatin immunoprecipitation assays. MCF-10A cells were transfected with siRNA (control vs. BRCA1) for 72 h and treated with 5μM of BaP for 30 or 60 min. Chromatin immunoprecipitation (ChiP) assays were performed using
a ChIP-IT assay kit (Active Motif, Carlsbad, CA) according to the manufacturer’s instructions. The sheared chromatin was immunoprecipitated with normal mouse IgG (the negative control), anti-BRCA1 (Ab1 + Ab2 + Ab3, EMD chemicals, Gibbstown, NJ), and anti-ARNT (H-172, Santa Cruz Biotechnology, Inc.) antibodies. Immunoprecipitated complexes were collected on Protein A/G beads, and elutes were used as templates for semiquantitative RT-PCR or realtime PCR. The following primer sequences were used to amplify XRE-containing promoter regions; UGT1A9 promoter (−2230 – −2126 from translation start site), 5′-CTG TGC TCG AAC TCC CGA CC-3′ (forward) and 5′-CCT TGG CCT CCC AAA GTG TAG G-3′ (reverse); UGT1A1 promoter (−3399 – −3209 from translation start site), 5′-GTG TTA TTC CAG CAG AAA C-3′ (forward) and 5′-TAC CCT TCA GCC ATT CTG-3′ (reverse) (Yueh and Tukey, 2007); NRE promoter (−2878 – −2749 from translation start site), 5′-CAG AAG ACT TAC TTG ATG ATA-3′ (forward) and 5′-TGA GCC AGG AGA ATT GCT TGA-3′ (reverse).

Mammary gland organ culture. Female FBV Brca1<sup>−/−</sup> and Brca1<sup>−/−/co</sup> mice carrying the MMTV-Cre were maintained in accordance with institutional guidelines approved by the Georgetown University Animal Care and Use Committee. Mice (3 weeks old) were primed with hormone pellets (estrogen + progesterone) (Innovative Research of America, Sarasota, FL) for 14 days. The third mammary glands were removed and floated on gelatin sponges in 6-well plates containing Waymouth’s MB 752/1 media, 100 unit/ml antibiotic-antimycotic, 5 μg/ml insulin (bovine, lyophilized), 0.1 μg/ml aldobetone, 1 μg/ml luteotonic hormone, and 0.1 μg/ml hydrocortisone. After 2 days, mammary glands were transferred to fresh media and treated with 5nM [3H]BaP for 24 h, and DNA-adduct formation was analyzed.

Pulse-chase assays. Cells were transfected with specific siRNAs (BRCA1, XPA, ERCC1, UGT1A1, or UGT1A9-2) for 72 h and then treated with 5nM of [3H]BaP for 24 h. After removing free [3H]BaP, fresh media were added. Then, the amount of [3H]BaP-DNA adduct was measured by liquid scintillation counter.

To test if wild-type (wt) BRCA1 protein levels affect the amount of DNA adducts induced by [3H]BaP for 24 h, we performed pulse-chase experiments where the kinetics of DNA damage induced by BaP was monitored (Fig. 1a). Next, in SUM149PT cells, the effect of wt BRCA1 overexpression was compared with the overexpression of four different mutant BRCA1 proteins (T300G, C5365G, 5677insA, 5382insC). None of the mutant BRCA1 proteins were as effective as wt BRCA1 in reducing the amount of BaP-induced adducts (Fig. 1b).

RESULTS

BRCA1 Levels Affect the Amount of DNA Adducts Induced by Carcinogens (BaP or DMBA)

To test if wild-type (wt) BRCA1 protein levels affect the amount of BaP-induced DNA adducts, cells were transfected with DNA (empty vector vs. wt BRCA1) and treated with [3H]BaP for 24 h. Overexpression of wt BRCA1 in MCF-10A, SUM149PT, and SUM135MO2 cells significantly decreased the amount of DNA adducts induced by BaP (Fig. 1a). Next, in SUM149PT cells, the effect of wt BRCA1 overexpression was compared with the overexpression of four different mutant BRCA1 proteins (T300G, C5365G, 5677insA, 5382insC). None of the mutant BRCA1 proteins were as effective as wt BRCA1 in reducing the amount of BaP-induced adducts (Fig. 1b). We further investigated whether knockdown of endogenous BRCA1 increases the amount of DNA adducts induced by BaP. Indeed, BRCA1 knockdown significantly increased the amount of DNA adducts induced by BaP according to [3H]BaP concentration in MCF-10A (Fig. 2a). To confirm this result, we used an independent assay method, the in vitro [3P]postlabeling assay using TLC. Knockdown of BRCA1 significantly and reproducibly increased the amount of adducts induced by BaP (about 4.5-fold) (Fig. 2b, the upper right panel). As a positive control, BPDE (Fig. 2b, the lower left panel) was used. The intensity of adduct spots detected by the [3P]postlabeling assay was significantly higher in BRCA1 knockdown cells than control cells (Fig. 2b). When another carcinogen, a known mammary gland tumor-inducing chemical, DMBA, was used, larger effects on DNA adducts were found (about 7-fold in 1μM of DMBA and 36-fold in 5μM of DMBA) (Fig. 2c). These results reinforce the conclusion that BRCA1 levels influence the amount of adducts induced by either BaP or DMBA. To test whether the increased BaP-induced adducts could also occur in mammary gland tissue of Brca1 conditional knockout mice, we used a whole mammary gland organ culture assay. We found a 3- to 4-fold increase in the amount of DNA adducts induced by BaP in the Brca1 knockout mice compared with controls (Fig. 2d).

BRCA1 Regulates the Amount of BaP-induced DNA Adducts in NER-Independent Manner

Although BRCA1 has been shown to function in the NER pathway following UV irradiation (Hartman and Ford, 2002), there are limited reports linking BRCA1 mutations to defective NER. Because few studies showed that DNA damage induced by BPDE can be repaired by NER (Kennedy et al., 2005; Motykiewicz et al., 2002; Shi et al., 2004), we tested whether the increased amount of DNA adducts induced by BaP in BRCA1 knockdown cells is due to the lack of NER activity. Accordingly, we examined whether knockdown of the genes encoding NER enzymes (XPA or ERCC1) with their specific siRNAs can affect the amount of adducts induced by BaP. Only slight increases (about 1.2- to 1.5-fold induction) in the amount of BaP-induced adducts were observed in ERCC1 knockdown or both XPA and ERCC1 knockdown compared with control-siRNA treated cells (data not shown). Next, we performed pulse-chase experiments where the kinetics of DNA adduct removal/repair was monitored after removing the...


BRCA1-Regulated Detoxification

Because the increased amount of BaP-induced DNA adducts in BRCA1 knockdown cells cannot be explained by its DNA repair activity, we examined whether BRCA1 regulates genes involved in metabolic activation and/or detoxification of BaP. BRCA1 is known to regulate the expression of xenobiotic stress-inducible genes such as CYP1A1 and CYP1B1 in MCF-7, an estrogen receptor positive breast cancer cell line (Kang et al., 2006a). Both CYP1A1 and CYP1B1 are known to be important in metabolic activation in vitro. However, in vivo, in a Cyp1a1 knockout mouse study, a potential important role for CYP1A1 in detoxification rather than metabolic activation of BaP is shown (Uno et al., 2004). In our study, we found that in MCF-10A cells (estrogen receptor alpha negative), endogenous BRCA1 regulates expression of CYP1A1 mRNA, protein, and enzymatic activity (Fig. 4). On the other hand, another cytochrome p450 enzyme, CYP1B1 mRNA and protein expression were less affected by BRCA1 knockdown (Figs. 4a and 4b). Several enzymes including GST, sulfo transferring (SLT), and UGT families are reported to detoxify BaP metabolites (Fang et al., 2002). We were particularly interested in UGT1A1, UGT1A9, and NRF2 genes, all of which contain XRE on their promoters or enhancers. The two UGT genes, UGT1A1 and UGT1A9, have been reported as the only two major hepatic enzymes among UGTs that detoxify BaP metabolites (Fang et al., 2002). The mRNA of NRF2 has been reported to be regulated by 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin via AhR/ARNT (Miao et al., 2005), and its protective function against various carcinogens has been well documented (Kwak and Kensler, 2010). As expected, significant decreases in basal expression level of UGT1A1, UGT1A9, and NRF2 genes, all of which contain XRE on their promoters or enhancers. The two UGT genes, UGT1A1 and UGT1A9, have been reported as the only two major hepatic enzymes among UGTs that detoxify BaP metabolites (Fang et al., 2002). The mRNA of NRF2 has been reported to be regulated by 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin via AhR/ARNT (Miao et al., 2005), and its protective function against various carcinogens has been well documented (Kwak and Kensler, 2010). As expected, significant decreases in basal expression level of UGT1A1, UGT1A9, and NRF2 genes, all of which contain XRE on their promoters or enhancers. The two UGT genes, UGT1A1 and UGT1A9, have been reported as the only two major hepatic enzymes among UGTs that detoxify BaP metabolites (Fang et al., 2002). The mRNA of NRF2 has been reported to be regulated by 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin via AhR/ARNT (Miao et al., 2005), and its protective function against various carcinogens has been well documented (Kwak and Kensler, 2010).
expression in the presence of BRCA1, but the induction was attenuated in BRCA1 knockdown cells (Fig. 5). In comparison, BaP induces NRF2 protein levels without increasing its mRNA levels, and the induction of NRF2 protein is also significantly attenuated in BRCA1 knockdown cells (Fig. 5c). Together, these experiments show that BRCA1 plays an important role in the regulation of xenobiotic stress-inducible gene expression, which influences the levels of DNA adducts induced by carcinogens.

Abrogation of BRCA1 Decreases ARNT Binding to XRE and BaP-Induced XRE Promoter Reporter Activity

Because BRCA1 physically binds ARNT and enhances xenobiotic stress-induced gene regulation activity (Kang et al., 2006a), we investigated whether BRCA1 and/or ARNT can be found on XRE-containing promoter/enhancer regions of UGT1A1, UGT1A9, and NRF2 using ChIP assay. To demonstrate that BRCA1 is recruited to XRE regions with ARNT following BaP treatment and that BaP-mediated ARNT recruitment to XRE is affected by the level of endogenous BRCA1, cells were pretreated with siRNA (control vs. BRCA1) and treated with 5 μM of BaP for 30 or 60 min. As expected, we found that BRCA1 and ARNT proteins were significantly recruited to the endogenous genomic UGT1A1, UGT1A9, and NRF2 promoter after BaP treatment, but these recruitments were significantly decreased in BRCA1 knockdown cells (Figs. 6a and 6b). These results suggest that BRCA1 may be functionally and physiologically important for UGT1A1, UGT1A9, and NRF2 transcriptional regulation. In order to test whether BRCA1 affects gene expression via XRE binding, we constructed luciferase reporters containing wt or mutant XREs from the three genes. Reporter gene assays show that overexpression of BRCA1 enhances the basal and BaP-mediated wt XRE-containing promoter activity of all three genes (Fig. 6c). On the other hand, no activation was found in all three reporters containing mutant XRE sequence, suggesting that BRCA1 and BaP activate the transcription of XRE-containing genes. Meanwhile, knockdown of BRCA1 significantly decreased the BaP-induced XRE reporter activity of UGT1A1 and NRF2 (Fig. 6d). Interestingly, the UGT1A9-XRE-Luc reporter activity rather increased in BRCA1 knockdown cells regardless of BaP treatment. This indicates

FIG. 2. BRCA1 knockdown or knockout increases the amount of DNA adducts induced by carcinogens (BaP or DMBA). (a) BRCA1 knockdown increases the amount of DNA adducts induced by [3H]BaP in MCF-10A cells. Cells were transfected with shRNA coding vector (pSuper-control vs. pSuper-BRCA1) for 72 h and then treated with 2.5 or 5 nM of [3H]BaP for an additional 24 h. (b) The effect of BRCA1 knockdown on the amount of adducts induced by BaP was measured by the [32P]post labeling method. MCF-10A cells transfected with DNA vectors containing shRNA for 72 h were treated with 5 μM of BaP for 24 h. Genomic DNA was isolated, [32P]post labeled, and analyzed by a TLC. BPDE was used as a positive control. (c) The effect of BRCA1 knockdown on the amount of DMBA-induced adducts. A similar experiment as in (b) was performed except with DMBA. The radioactivity of BaP- or DMBA-induced DNA adduct spots from BRCA1 knockdown cells was measured by scintillation counter and compared with controls as mean of relative radioactivity ± S.E. (d) The increase in DNA adducts induced by [3H]BaP in cultured whole mammary glands of Brca1 conditional knockout mice (co/co). The third mammary glands from BRCA1 wt/wt (wt/wt) and BRCA1co/co (co/co) mice were treated with 5 nM of [3H]BaP for 24 h and then assayed to measure the amount of DNA adducts. The data presented in (a) and (d) are as the mean of the number of DNA adducts per 10^6 nucleotides ± S.E of three independent experiments. Student’s t-tests were applied for statistical significance; (*) is p < 0.05 and (**) indicates p < 0.01.
that either (1) BRCA1 binding to UGT1A9 (shown by ChIP assay) may not be as crucial as UGT1A1 or NRF2 or (2) unidentified factors activated by BRCA1 knockdown may stimulate the UGT1A9 promoter reporter. Thus, we found that BRCA1 binds to the XRE-containing promoter region of UGT1A1, UGT1A9, and NRF2 and regulates their promoter reporter activities in an XRE-dependent manner.

**Restoring UGT1A1 and UGT1A9 Decreases the Amount of Adducts Induced by BaP in BRCA1 Knockdown Cells**

The function of UGTs in detoxification has been studied mainly in *in vitro* enzymatic assays. In order to demonstrate the intracellular BaP-detoxification functions of UGT1A1 and UGT1A9, cells were pretreated with siRNA (control, UGT1A1, or UGT1A9) for 72 h and treated with [3H]BaP for 24 h. Increased amounts of BaP-induced adducts were found in either UGT1A1 (Fig. 7a) or UGT1A9 (Fig. 7b) knockdown cells. Because there were no differences in the [3H]BaP removing rate (Fig. 7c), we believe that the increased amount of adducts induced by BaP in UGT knockdown cells is due to the loss of their detoxifying function. To further confirm whether the increase in BaP-induced adducts in BRCA1 knockdown cells are partially due to the decreased expression of UGT1A1 and UGT1A9, we overexpressed the two UGTs (independently or simultaneously) in BRCA1

**FIG. 3.** Kinetics of BaP-DNA adducts removal in BRCA1 and NER knockdown cells. (a) Pulse-chase experiments were used to monitor the effect of BRCA1 status on the removal of DNA adducts induced by [3H]BaP. MCF-10A cells were transfected with the shRNA containing vector for 72 h and treated with 5nM of [3H]BaP for 24 h. Cells were then washed and incubated in fresh media for the indicated times and the remaining levels of [3H]BaP adducts were measured. (b–d) Pulse-chase experiments were performed as in (a) to monitor the effect of XPA (b), ERCC1 (c), or XPA + ERCC1 (d) knockdowns (using chemical siRNA) on the removal of [3H]BaP-induced DNA adducts. (e) Knockdowns of each gene (XPA or ERCC1) were confirmed by WB analysis. Student’s *t*-tests were applied for statistical significance; (*) and (**) indicate *p* < 0.05 and *p* < 0.01, respectively.

**FIG. 4.** Effect of endogenous BRCA1 on CYP1A1 and CYP1B1 gene, protein, and EROD activity. MCF-10A cells were pretreated with siRNA (control vs. BRCA1), treated with BaP and harvested for (a) CYP1A1 and CYP1B1 mRNA, (b) CYP1A1 and CYP1B1 protein, and (c) CYP1A1 enzymatic activity. Bar graphs show the results of semiquantitative RT-PCR (a) and WB (b) results of three independent experiments quantified by densitometry. Student’s *t*-tests were applied for statistical significance; (*) is *p* < 0.05 and (**) indicates *p* < 0.01.
knockdown cells and found that each of the UGTs can significantly decrease the amount of adducts induced by BaP (Fig. 7d). These findings strongly suggest that the intracellular amounts of these UGTs are important for determining the extent to which mutagenic BaP metabolites are able to induce DNA damages, at least, in MC F-10A cells. When the two UGTs were transfected simultaneously, no additive or synergistic effects on reducing BaP-induced adducts in BRCA1 knockdown cells were found (Fig. 7d). In these experiments, we confirmed that the two BaP-detoxification enzymes or NRF2 are important in the BaP-DNA metabolite detoxification process. In addition, we found that the increased amount of BaP-induced adducts in BRCA1 knockdown cells are partially due to the reduced levels of UGTs, NRF2, and ARNT expression.

Because knockdown of BRCA1 caused the decrease in basal expression or induction of ARNT and NRF2 protein (Fig. 8a), we determined to test whether restoring ARNT or NRF2 could decrease the amount of BaP-induced DNA adducts in BRCA1 knockdown cells. When either ARNT or NRF2 was overexpressed in BRCA1 knockdown cells, the amount of DNA adducts induced by BaP was significantly decreased (Figs. 8b and 8c), suggesting that these two transcription factors are important in controlling the amount of BaP-induced DNA adducts. In addition, we found significantly increased amounts of BaP-induced DNA adducts when ARNT or NRF2 was knocked down by siRNAs in MCF-10A cells (data not shown).

DISCUSSION

Our findings suggest that BRCA1 levels affect the amount of DNA adducts induced by carcinogens such as BaP or DMBA and that this effect is potentially a function of transcriptional
regulation of genes involved in metabolic activation and/or detoxification and not NER.

Because activated xenobiotics are capable of damaging DNA, our results raised the possibility that BRCA1 defects may play a more immediate, acute, and direct role in generating carcinogen-induced mutations by locally increasing the potency of carcinogens. This possibility has not been previously systematically investigated because it is generally assumed that the acute short-term DNA damaging and/or mutagenic effects of a fixed concentration from xenobiotic
Carcinogens (e.g., BaP) are not significantly affected by BRCA1 defects. However, our results open a new avenue of investigation with respect to BRCA1’s role in reducing carcinogen-induced short-term DNA damage.

Currently, measurement of increased levels of carcinogen-DNA adduct formation is a generally acceptable marker for carcinogen studies (Kriek et al., 1993; Poirier, 1997). Thus far, BPDE has been widely used to study the relationship between carcinogens and DNA repair because BPDE, an ultimate metabolic form of BaP, directly binds DNA and causes DNA damage. Studies have demonstrated that deficient DNA repair capacity (particularly NER) is associated with increased breast cancer risk using BPDE as a DNA damage inducer (Kennedy et al., 2005; Motykiewicz et al., 2002; Shi et al., 2004). In this study, we used BaP as a carcinogen since we were particularly interested in environmental factors to which we are routinely being exposed and that require biotransformation to become active carcinogens.

Although it is known that BRCA1 plays a role in repairing DNA double-stranded breaks by homologous recombination, few studies have documented the potential of BRCA1 in DNA single-strand break repair (Hartman and Ford, 2002; Saha et al., 2010). Studies have demonstrated that BRCA1 activity participates in transcription-coupled repair, a subpathway of NER (Abbott et al., 1999; Le Page et al., 2000) whose mechanism is very complex and not well understood (Feng et al., 2002). BRCA1 is also associated with the human chromatin remodeling complex (SWI/SNF-related complex), which can affect gene expression and/or DNA repair (Bochar et al., 2000). A recent study showed that treatment with histone deacetylase inhibitor, trichostatin A, increases radiation-induced DNA damage and slows down the repair kinetics using HCC1937, a BRCA1 mutant cell line (Zhang et al., 2007), suggesting a potential role of BRCA1 in chromatin remodeling. This evidence combined with the data from this study underscores the necessity of further studies to investigate the exact role(s) of BRCA1 in influencing the level of DNA adducts induced by carcinogens.

In this study, we have shown that BRCA1 is required for normal induction or maintenance of constitutive levels of genes coding for both metabolic activation enzymes (e.g., CYP1A1) and detoxification enzymes (e.g., UGTs). However, under carcinogenic stress, such as BaP, it is puzzling as to why there are increased amounts of DNA adducts in BRCA1-defective cells or tissues. Although CYP1A1 has the greatest capacity for initiation of BaP metabolism, CYP1B1 also plays a major role in the metabolic activation pathway (Kim et al., 1998). Because a substantial amount of CYP1B1 protein still expresses even after BRCA1 knockdown in MCF-10A cells, BaP may be metabolically activated via either CYP1B1 or other CYP1 family of proteins or unidentified metabolic enzymes in these cells. On the other hand, a significant delay or defect in eliminating increased amounts of BaP-induced adducts observed in BRCA1 knockdown cells can correlate with decreased expression and induction of detoxification enzymes and NRF2. Perhaps, the tight coupling and sequential activation of detoxification genes following metabolic activation gene expression may be important for an adequate detoxification process.

Our results show that BaP induces CYP1A1, CYP1B1, UGT1A1, and UGT1A9 mRNA expression without altering
NRF2 mRNA expression levels. On the other hand, BaP does increase NRF2 protein expression level. This may be due to a posttranslational modification of NRF2, which has been reported as a major mechanism of its regulation in oxidative stress responses \textit{in vitro} (Kwak and Kensler, 2010).

Similarly, we demonstrated the significance of Brca1 levels in determining the amount of BaP-induced DNA adducts only \textit{in ex vivo} experiments using Brca1\textsuperscript{wt/wt} and Brca1\textsuperscript{co/co} mice. There are only a few reports on the function of Brca1 in carcinogenesis in \textit{in vivo} models. Dr Deng’s group has shown that oxidative stress and a carcinogen (methyl-N-amylnitrosamine) allows the formation of esophagus and forestomach cancer in mice that are homozygous for full-length Brca1 deletion and heterozygous for a p53-null mutation (Brca1\textsuperscript{$\Delta 11/\Delta 11$}p53\textsuperscript{+/-}) (Cao et al., 2007). Because breast and ovary are the major organs affected by BRCA1 defects, it will be worthy to extend our studies with a mammary gland-specific laboratory carcinogen such as DMBA. In addition, mammary gland-specific conditional Brca1 knockout mice can be used to determine whether the specific carcinogen, DMBA, induces mammary gland tumors. Thus, future development of an \textit{in vivo} carcinogenesis model will elucidate the complete function of BRCA1 in environmental risk.

An \textit{in vivo} model will be profoundly useful in evaluating the human condition. The effect of cigarette smoking on the risk of breast cancer among carriers of BRCA1 and/or BRCA2

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**FIG. 8.** Effects of restoring ARNT or NRF2 on BaP-induced DNA adducts in BRCA1 knockdown cells. (a) Cells were pretreated with siRNA (control vs. BRCA1), treated with BaP (0, 2.5, and 5\textmu M), harvested, and confirmed by WB analysis using anti-BRCA1, anti-NRF2, and anti-ARNT antibodies. (b) Overexpression of ARNT in BRCA1 knockdown cells decreases the amount of adducts induced by BaP. Cells transfected with shRNA coding vector (pSuper-control vs. pSuper-BRCA1) for 48 h were transfected with GFP-tagged ARNT (vs. pEGFP as a empty vector) and then treated with $[^{3}$$\text{H}]$BaP for 24 h. (c) Restoring NRF2 reduced the amount of DNA adducts in BRCA1 knockdown cells. Cells were transfected with shRNA as in (a) and infected with adenovirus (Ad)-flag-NRF2 (vs. Ad-Null) and then $[^{3}$$\text{H}]$BaP-DNA adducts were measured. Student’s t-tests were applied for statistical significance; (*) and (**) indicate $p < 0.05$ and $p < 0.01$, respectively. Far right panels of (b) and (c) show the results of WB analysis after restoring either GFP-ARNT or Ad-flag-NRF2 in BRCA1 knockdown cells.
mutation is still controversial. Some studies showed reduced (Brunet et al., 1998) or no effects on breast cancer risk (Ginsburg et al., 2009). However, a recent epidemiology study showed that smoking is significantly associated with increased breast cancer risk when BRCA mutant women smoked for 5 or more years before the age of 50 (Breast Cancer Family Registry, 2008). The frequency of BRCA1 mutations among human breast cancer patients is not very high (less than 5%), but the majority of women with familial breast cancer harbor a mutation either in BRCA1 or BRCA2. More importantly, emerging evidence suggests that a high frequency of hypermethylation on either in BRCA1 or BRCA2. More importantly, emerging evidence suggests that a high frequency of hypermethylation on the BRCA1 promoter significantly decreases the expression level of BRCA1 mRNA and protein in sporadic breast cancer (Ali et al., 2011; Galizia et al., 2010; Jing et al., 2010; Mueller and Roskelley 2003; Rice et al., 2000). A recent study shows that 36.7% (25 of 68 tumors) of sporadic breast cancer have hypermethylated BRCA1 promoters (Veeck et al., 2010). Thus, a large portion of sporadic breast cancers express low levels of BRCA1 and could be susceptible to environmental factors that require fully functional BRCA1 for detoxification. Thus, future studies of all aspects of BRCA1 function will elucidate its role in environmental risk for all women.

SUPPLEMENTARY MATERIAL

Supplementary data are available online at http://toxsci. oxfordjournals.org/.

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