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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.*,

1999), 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, ERα, 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 SUM1315MO2 (BRCA1 185delAG) are two human breast cancer cell lines with mutations in the BRCA1 gene (Elstrodt *et al*., 2006) and were purchased from Asterand Co. (Detroit, MI). The growing media and conditions of MCF-10A, SUM149PT, and SUM1315MO2 cell lines have been described in elsewhere (Elstrodt *et al*., 2006). Benzo(a)pyrene (BaP) and DMBA were purchased from Sigma and dissolved in dimethyl sulfoxide (DMSO). The [^{3}H]BaP and [γ-^{32}P]ATP radioisotopes were obtained from GE Healthcare (Piscataway, NJ).

siRNAs and DNA expression plasmids. The control (nontargeting scrambled), BRCA1, XPA, ERCC1, UGT1A1, UGT1A9 (-1 and -2), and NRF2 siRNAs were purchased from Dharmacon, Inc. (Lafayette, CO). The following sequences of siRNAs were used for: Control-siRNA 5′-GAC GAG CGG CAC GUG CAC A-3′, BRCA1-siRNA 5′-GAA GGA GCT TCC ATC ATT C-3′, XPA-siRNA 5′-GCA AAT GGC TTC TAT CGA A-3′, UGT1A9-siRNA-1 5′-GCA CAA GTA CGA AGT ATA T-3′, UGT1A9-siRNA-2 5′-GCG AAC 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).

[^{3}H]BaP-induced DNA adducts. Radioactivity of the [^{3}H]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.

[^{32}P]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, 0.15mM 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 [^{32}P] in the mixture ([γ-^{32}P]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 NaH_2PO_4 (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 NaH_2PO_4, 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 NaH_2PO_4 (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 (Vadhanam *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-UGT1A1 (BD Gentest, San Jose, CA), anti-CYP1A1 (Affinity BioReagents, Golden, CO), anti-CYP1B1 (BD Gentest), anti-flag (M2, Sigma-Aldrich, St Louis, MO), anti-GFP (Abcam, Cambridge, MA), and anti-β-actin (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 72 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 CGG GAG GAA AAT GGG TAG TTA-3′ and reverse 5′-TGT GCC AAG GGT GAA TGA TGA AAG-3′, UGT1A1 forward 5′-CTC TTA AAT TTG AAG TTT ATC CTG T-3′ and reverse 5′-ACC ACA ACA CCA TTT TCT CC-3′, UGT1A9 forward 5′-TGC TCA ATG GAA AGC ACA AG-3′ and reverse 5′-CTG GCA CGA TTC CAA AAA CT-3′, NRF2 forward 5′-AAA CCA CCC TGA AAG CAC AG-3′ and reverse 5′-AGC GGC TTG AAT GTT TGT CT-3′, CYP1A1 forward 5′-CTT GGA CCT CTT TGG AGC TG-3′ and reverse 5′-CGA AGG AAG AGT GTC GGA AG-3′, CYP1B1 forward 5′-CAC CAA GGC TGA GAC AGT GA-3′ and reverse 5′-GAT GAC GAC TGG GCC TAC AT-3′, β-actin forward 5′-GCT ATC CCT GTA CGC 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 shRNA (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 chromatins were 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 real-time PCR. The following primer sequences were used to amplify XRE-containing promoter regions; UGT1A9 promoter (−2230 ~ −2126 from translation start site), 5′-CTG GTC 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 TCT CAC CAG AAC AAA C-3′ (forward) and 5′-TAC CCT CTA GCC ATT CTG-3′ (reverse) (Yueh and Tukey, 2007); NRF2 promoter (−2878 ~ −2749 from translation start site), 5′-CAG AAG ACT TAC TTG ATG ATA-3′ (forward) and 5′-TGA GGC AGG AGA ATT GCT TGA-3' (reverse).

Mammary gland organ culture. Female FBV Brca1$^{wt/wt}$ and Brca1$^{co/co}$ 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 aldosterone, 1 μg/ml luteotropic hormone, and 0.1 μg/ml hydrocortisone. After 2 days, mammary glands were transferred to fresh media and treated with 5nM [^{3}H]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 [^{3}H]BaP for 24 h. After removing free [^{3}H]BaP, fresh media were added. Then, genomic DNA was isolated using Wizard SV Genomic DNA purification system (Promega, Madison, WI) after 0, 0.5, 1, 2, 4, or 8 h of medium change, then the amount of [^{3}H]BaP-DNA adduct was measured by liquid scintillation counter.

XRE promoter construction and reporter gene assay. PCR reactions were performed using pGL3-control vector as a template. All DNA sequences for primers to amplify XRE for this study were used as follows; pGL3: 5′-ACC AAC AGT ACC GGA ATG CC-3′; UGT1A1 XRE wt (1 copy): 5′-CGG ACT TGA AGC TTG GCC AGC ACG CAA TGA ACA TTA CGC GTG CTA GCC CGG GC-3′; UGT1A1 XRE mt (1 copy): 5′-CGG ACT TGA AGC TTG GCC AGA CCG CAA TGA ACA TTA CGC GTG CTA GCC CGG GC-3′; UGT1A9 XRE wt (2 copies): 5′-AGT ATA ATG GCG TGA TCT AGT ATA ATG GCG TGA TCT TAC GCG TGC TAG CCC GGG C-3′; UGT1A9 XRE mt (2 copies): 5′-AGT ATA ATG GCG GTA TCT AGT ATA ATG GCG GTA TCT TAC GCG TGC TAG CCC GGG C-3′; NRF2 XRE wt (2 copies): 5′-AGT GCA GTG GCG TGA TCT AGT GCA GTG GCG TGA TCT TAC GCG TGC TAG CCC GGG C-3′; NRF2 XRE mt (2 copies): 5′-AGT GCA GTG GCG GTA TCT AGT GCA GTG GCG GTA TCT TAC GCG TGC TAG CCC GGG C-3′. All underlined DNA sequences represent either putative wt or mutant XRE. PCR products were subcloned into the pCR2.1-TOPO vector (Invitrogen), and XRE-containing regions were excised using SacI and HindIII restriction enzymes to be ligated into the pGL3-control vector. Luciferase activity was measured using a luminometer, and the luminescence was normalized for relative transfection efficiency by measuring β-galactosidase activity of a cotransfected reporter plasmid (Kang *et al*., 2006a).

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 [^{3}H]BaP for 24 h. Overexpression of wt BRCA1 in MCF-10A, SUM149PT, and SUM1315MO2 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 at 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 [^{3}H]BaP concentration in MCF-10A (Fig. 2a). To confirm this result, we used an independent assay method, the *in vitro* [^{32}P]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 [^{32}P]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

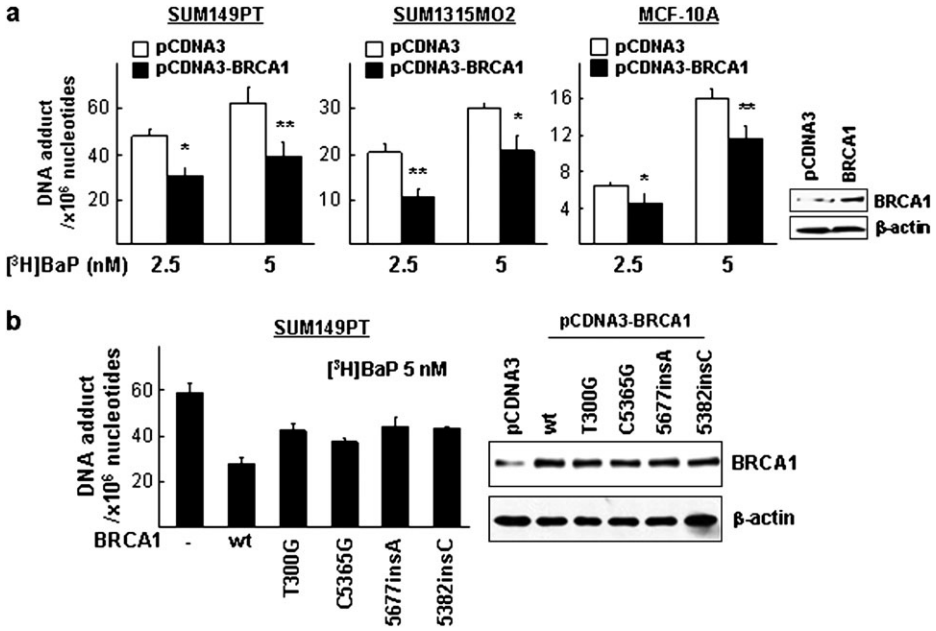

FIG. 1. Overexpression of BRCA1 decreases the amount of DNA adducts induced by BaP. (a) wt BRCA1 overexpression decreases the amount of DNA adducts induced by [^{3}H]BaP in human BRCA1 mutated breast cancer cell lines, SUM149PT and SUM1315MO2, as well as the nearly normal-like human breast cell line, MCF-10A. All three breast cell lines were transfected with expression vectors (pCDNA3 vs. pCDNA3-BRCA1) overnight and were cultured for an additional 24 h in 2.5 or 5nM of [^{3}H]BaP. Analysis by WB demonstrated wt BRCA1 overexpression in MCF-10A cells. (b) Exogenously expressed wt BRCA1 or mutant BRCA1(s) differentially affect(s) the amount of DNA adducts induced by BaP in SUM149PT cells. Cells transfected with various BRCA1 expression vectors were treated with 5nM of [^{3}H]BaP for 24 h, and radioactivity levels were determined. Analysis by WB was done using an anti-BRCA1 antibody to assure that the differential effects on the amount of BaP-induced adducts were not due to differential BRCA1 expression levels. β-Actin was used as a loading control. Student's *t*-tests were applied for statistical significance; (*) is $p < 0.05$ and (**) indicates $p < 0.01$.

[^{3}H]BaP from the culturing media. BaP-induced DNA adducts were removed at the similar rate in both control-siRNA and BRCA1-siRNA transfected cells (Fig. 3a). Extension of detection time points (12 or 24 h after washing out the [^{3}H]BaP) also showed a same pattern (data not shown). Similar experiments were performed in XPA, ERCC1, or XPA + ERCC1 knockdown cells. There were significantly decreased rates in the amount of DNA adduct removal (Figs. 3b–d), indicating that NER proteins are at least, in part, responsible for repairing DNA damage induced by BaP. These data also suggest that BRCA1 may not reduce BaP-induced DNA adducts via NER activity. Our results indicate both NER and non-NER (e.g., detoxification) activities may be involved in limiting DNA adducts induced by BaP.

BRCA1 Regulates Genes Encoding Metabolic Activation/ 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, sulfotransferase (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 were found in BRCA1 knockdown cells (Fig. 5). Time course studies demonstrated that BaP increased UGT1A1 and UGT1A9 mRNA and protein

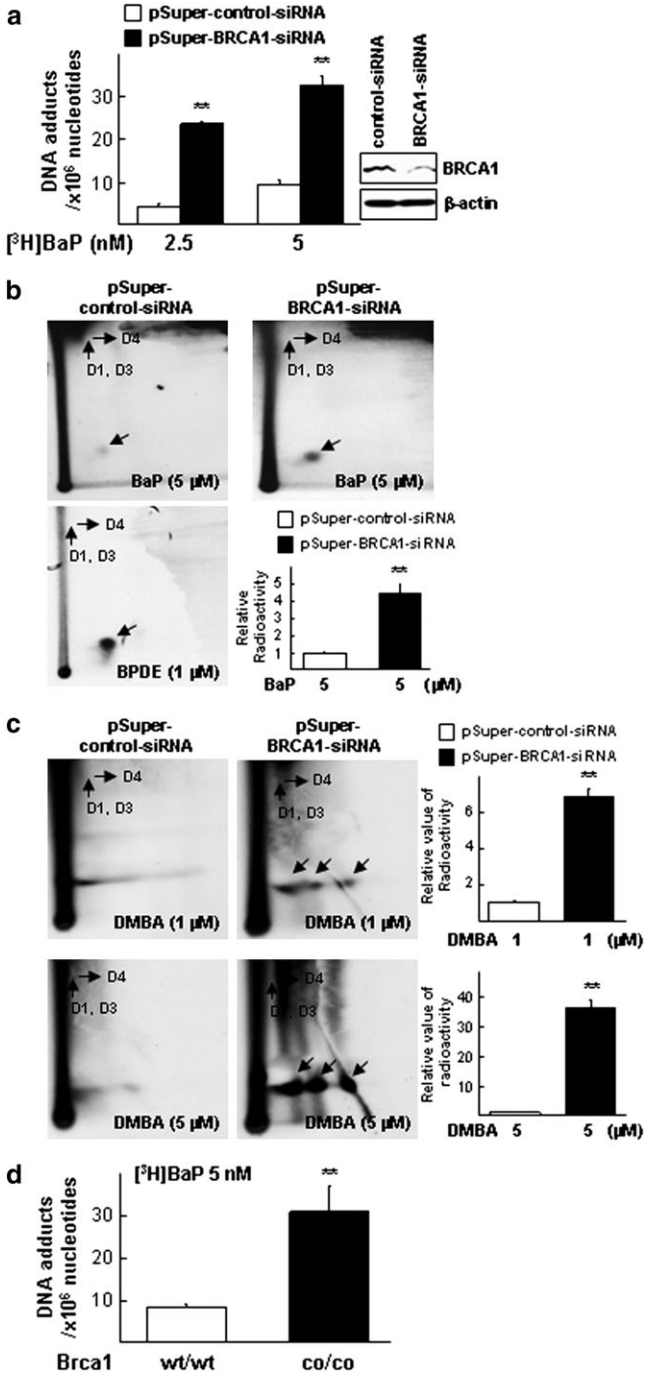

FIG. 2. BRCA1 knockdown or knockout increases the amount of DNA adducts induced by carcinogens (BaP or DMBA). (a) BRCA1 knockdown increases the amount of DNA adducts induced by [^{3}H]BaP in MCF-10A cells. Cells were transfected with shRNA coding vector (pSuper-control vs. pSuper-BRCA1) for 72 h and then treated with 2.5 or 5 nM of [^{3}H]BaP for an additional 24 h. (b) The effect of BRCA1 knockdown on the amount of adducts induced by BaP was measured by the [^{32}P]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, [^{32}P]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 [^{3}H]BaP in cultured whole mammary glands of Brca1 conditional knockout mice (co/co). The third mammary glands from BRCA1$^{wt/wt}$ (wt/wt) and BRCA1$^{co/co}$ (co/co) mice were treated with 5 nM of [^{3}H]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 $p < 0.01$.

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 5μM 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

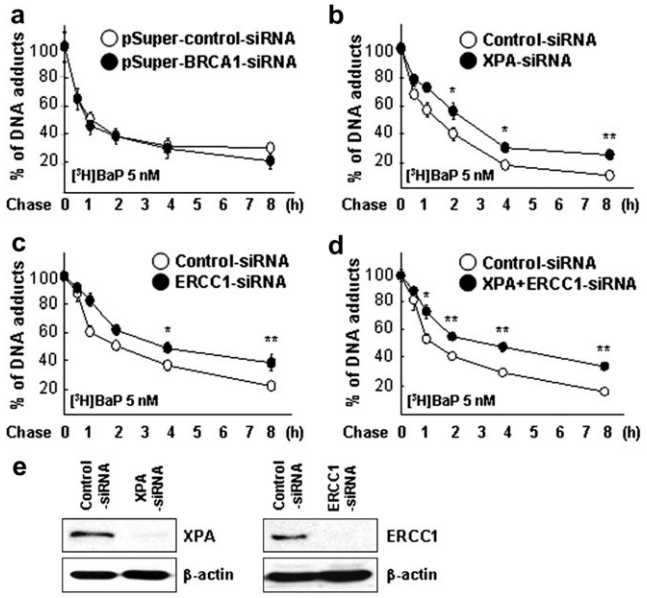

FIG. 3. Kinetics of BaP-DNA adducts removal in BRCA1 and NER knockdown cells. (a) Pulse-chase experiments were used to monitor the effect of BRCA1 status on the removal of DNA adducts induced by [^{3}H]BaP. MCF-10A cells were transfected with the shRNA containing vector for 72 h and treated with 5nM of [^{3}H]BaP for 24 h. Cells were then washed and incubated in fresh media for the indicated times and the remaining levels of [^{3}H]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 [^{3}H]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.

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 [^{3}H]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 [^{3}H]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

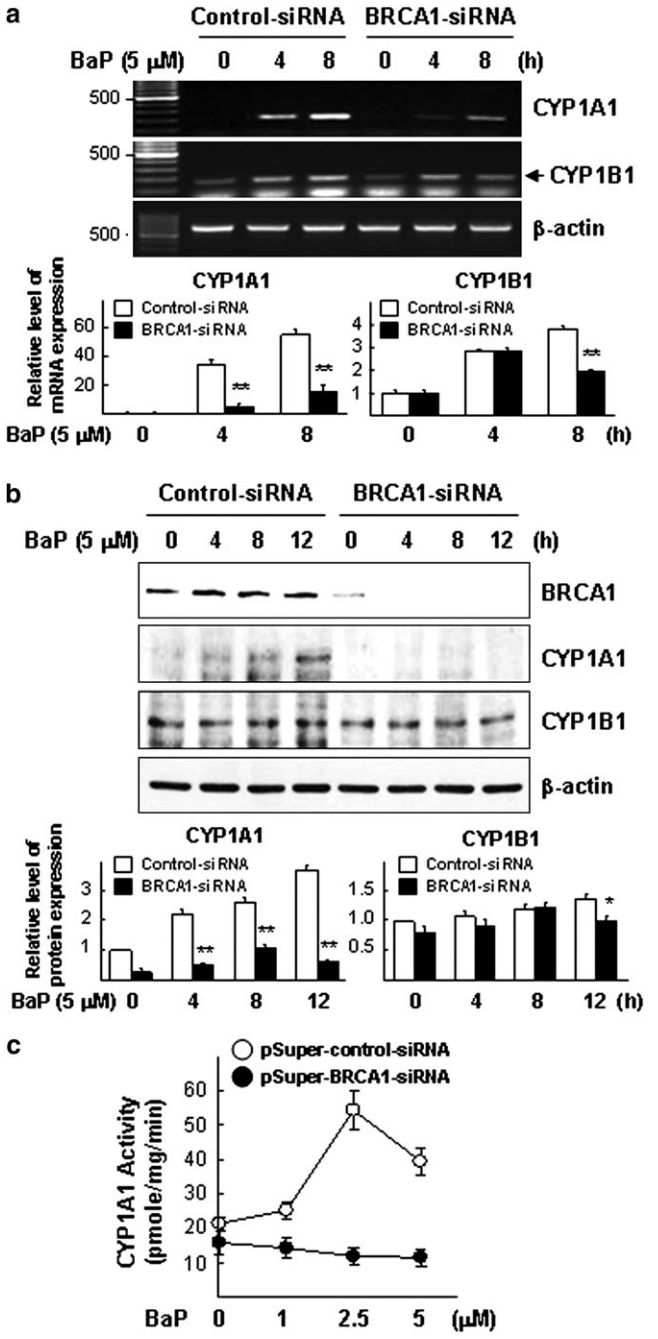

FIG. 4. Effect of endogenous BRCA1 on CYP1A1 and CYP1B1 gene, protein, and EROD activity. MCF-10A cells were pretreated with siRNA (control vs. BRCA1), treated with BaP and harvested for (a) CYP1A1 and CYP1B1 mRNA, (b) CYP1A1 and CYP1B1 protein, and (c) CYP1A1 enzymatic activity. Bar graphs show the 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 $p < 0.01$.

BRCA1 knockdown cells are partially due to the decreased expression of UGT1A1 and UGT1A9, we overexpressed the two UGTs (independently or simultaneously) in BRCA1

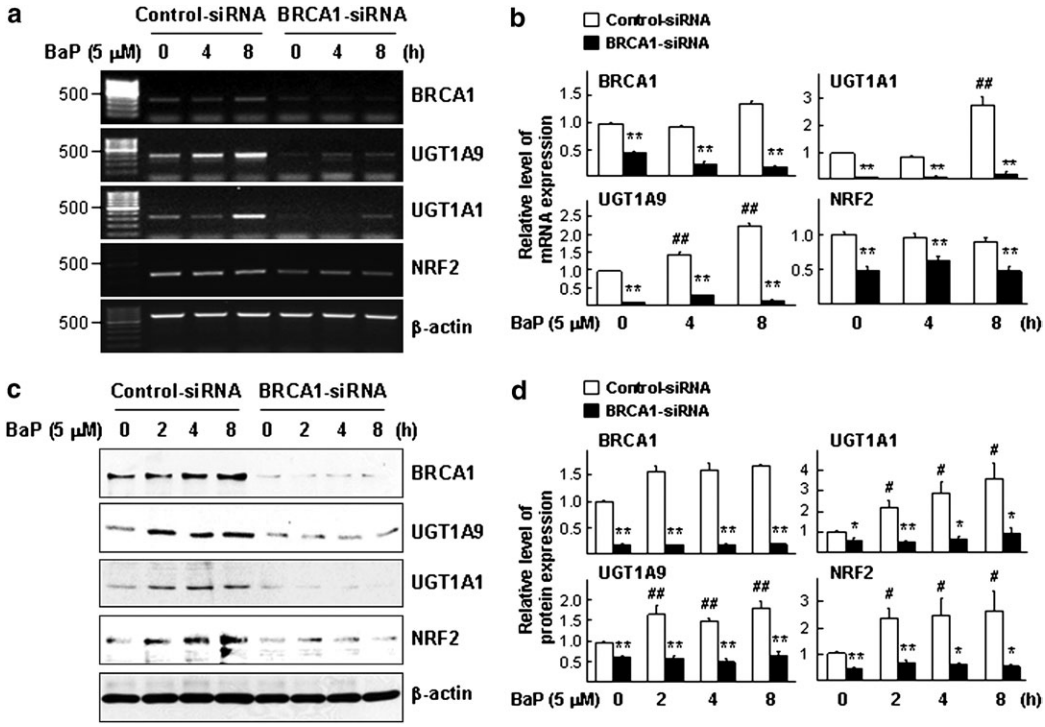

FIG. 5. BRCA1 regulates the expression of endogenous UGT1A1, UGT1A9, and NRF2. Differential gene expression of UGT1A1, UGT1A9, or NRF2 in the presence and absence of endogenous BRCA1 was determined by semiquantitative RT-PCR and WB analysis. (a) BRCA1 levels affect the mRNA level of UGT1A1, UGT1A9, and NRF2 following BaP. Cells were pretreated with siRNA (control vs. BRCA1) for 72 h, treated with 5μM of BaP, and harvested at the indicated time points. The agarose gel image is a representative of three independent experiments. (b) Mean ± SE values from three independent semiquantitative RT-PCR assays were plotted to compare relative level of each transcript. (c) Cells were pretreated with siRNA (control vs. BRCA1) for 72 h, treated with 5μM of BaP (0, 2, 4, or 8 h), and harvested for WB analysis. (d) Mean ± SE values from three independent WB analysis were plotted to show relative intensity values of each band as determined by densitometry. Student's *t*-tests were applied for statistical significance. Symbols (*) and (**) indicate $p < 0.05$ and $p < 0.01$ (control- vs. BRCA1-siRNA in the same concentration of BaP), respectively; Symbols ($^{\#}$) and ($^{\#\#}$) indicate $p < 0.05$ and $p < 0.01$ (no treatment vs. BaP treatment in control-siRNA), respectively.

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 MCF-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.

Restoring ARNT and NRF2 Significantly Decreases the Amount of DNA Adducts Induced by BaP in BRCA1 Knockdown Cells

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 knockdowned 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

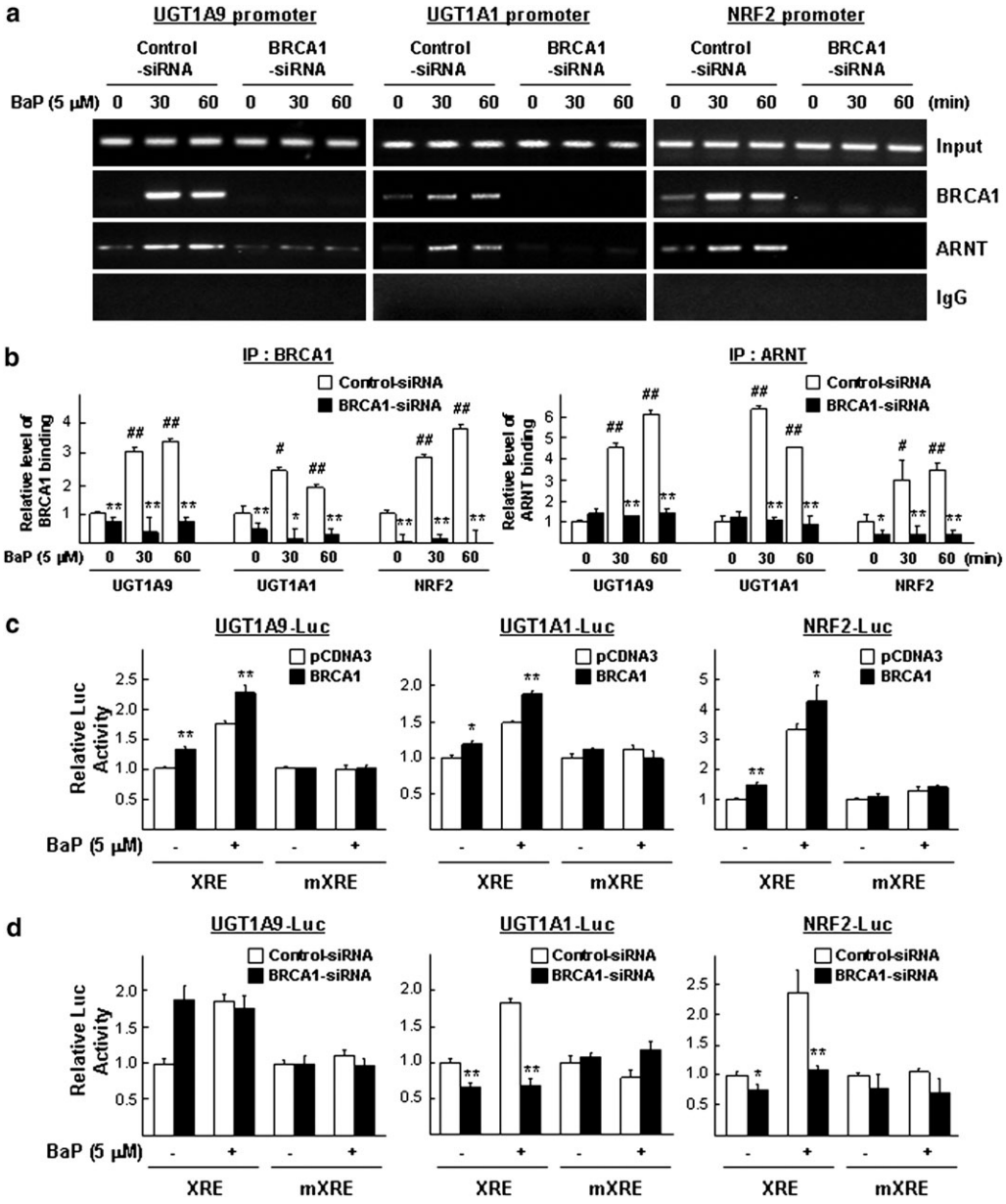

FIG. 6. BRCA1 is recruited to UGT1A1, UGT1A9, and NRF2 promoter/enhancer sequences. (a) ChIP assays were performed to determine the effect of BRCA1 knockdown on promoter occupancy by UGT1A1, UGT1A9, and NRF2. Fragmented genomic DNA from MCF-10A cells, which had been pretreated with siRNA for 72 h and then treated with 5μM BaP for 30 or 60 min, was immunoprecipitated by anti-BRCA1 and anti-ARNT antibodies. (b) RT-PCR of (a) results. Student's *t*-tests were applied for statistical significance; ($^{\#}$) and ($^{\#\#}$) indicate $p < 0.05$ and $p < 0.01$ (no treatment vs. BaP treatment in control-siRNA), respectively and (*) $p < 0.05$ and (**) $p < 0.01$ (control-siRNA vs. BRCA1-siRNA), respectively. (c) The effects of BRCA1 overexpression on BaP-induced UGT1A1, UGT1A9, and NRF2 promoter reporter activity (XRE and mutant XRE [mXRE]). Cells were transfected with the reporter gene and expression vector and treated with either DMSO or BaP for 24 h. (d) Effects of BRCA1 knockdown on BaP-induced reporter activity of UGT1A1, UGT1A9, and NRF2 promoter. Cells were pretreated with siRNA for 48 h, transfected with a reporter plasmid, and then treated with 5μM BaP for 24 h. Student's *t*-tests were applied for statistical significance; (*) and (**) indicate $p < 0.05$ and $p < 0.01$ (control-siRNA vs. BRCA1-siRNA), respectively.

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

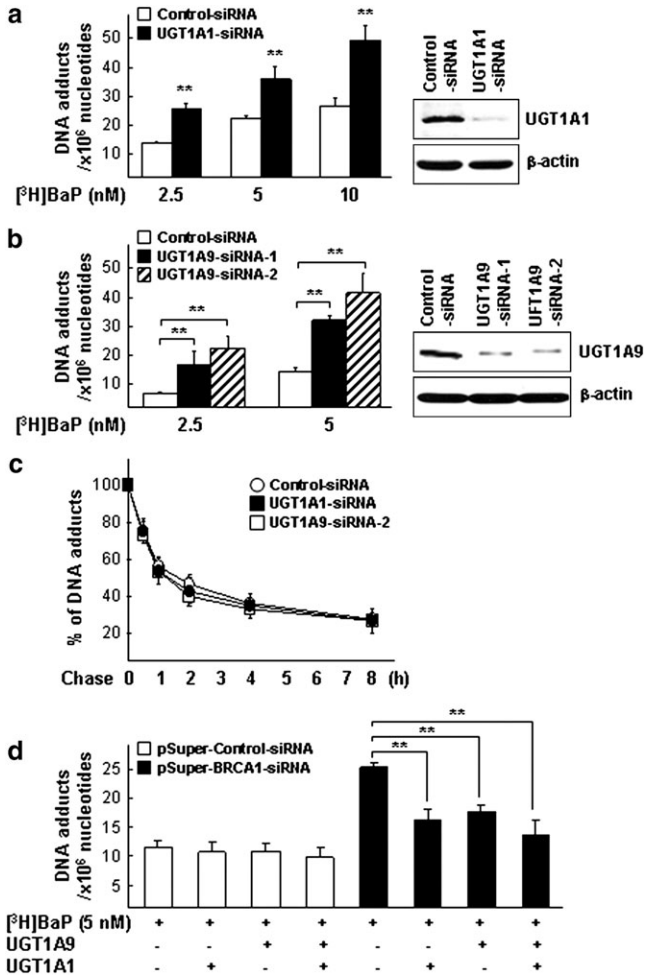

FIG. 7. UGT1A1 or UGT1A9 regulates the amount of DNA adducts induced by BaP. (a, b) Effect of UGT1A1- or UGT1A9-siRNA on the amount of BaP-induced adducts. Cells were pretreated with siRNAs for UGT1A1 (a) or UGT1A9 (b) for 72 h and treated with 2.5, 5, or 10nM of [^{3}H]BaP for 24 h. Knockdown of UGTs was confirmed by WB analysis (right panels). (c) Kinetics of removal of DNA adducts induced by [^{3}H]BaP in UGT1A1 or UGT1A9 knockdown cells. Pulse-chase experiments were performed to determine effects of UGT1A1 or UGT1A9 knockdown on the removal of [^{3}H]BaP-DNA adducts. (d) Restoring UGT1A1 and/or UGT1A9 genes decreased the amount of BaP-induced DNA adducts in BRCA1 knockdown MCF-10A cells. Cells transfected with shRNA-coding vector (control vs. BRCA1) for 48 h were transfected with 1 μg of expression vectors for either UGT1A1 or UGT1A9 or both for an additional 24 h. The transfected cells were further treated with 5nM of [^{3}H]BaP for 24 h, and the amount of DNA adducts was measured. Student's *t*-tests were applied for statistical significance; (**) indicates $p < 0.01$ (a, b, and d).

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 underscore 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 (e.g., UGTs) 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

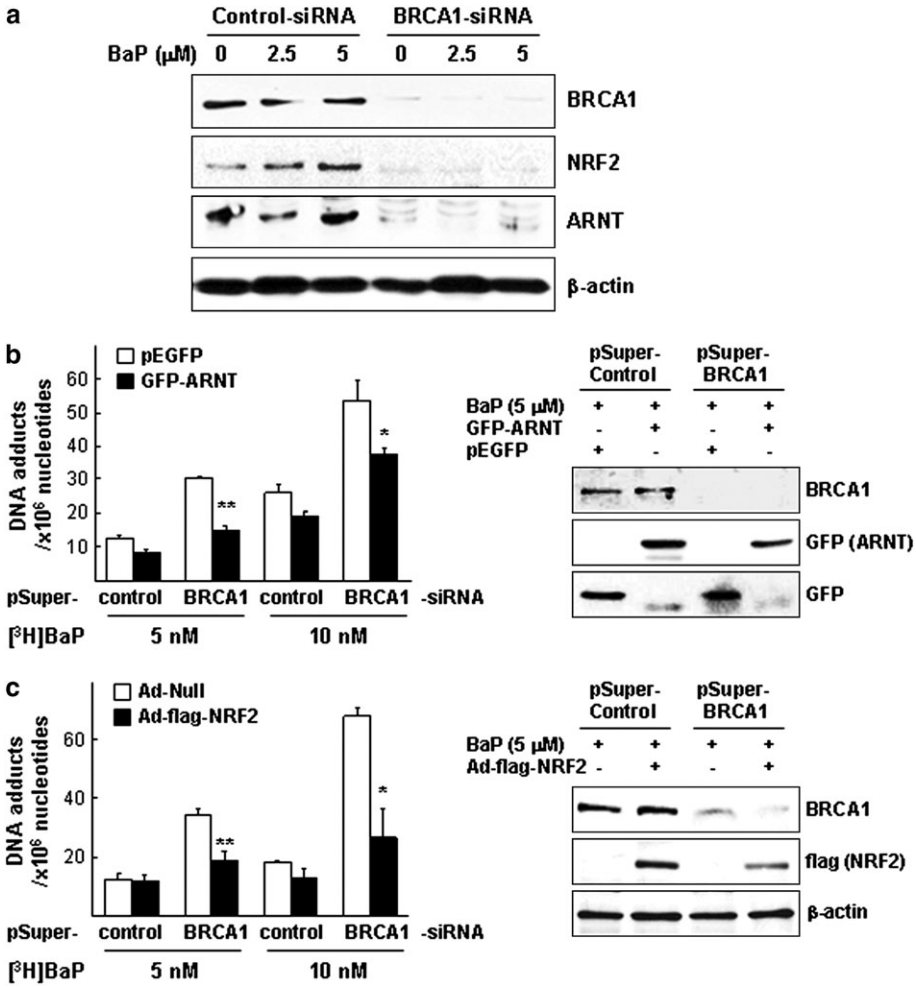

FIG. 8. Effects of restoring ARNT or NRF2 on BaP-induced DNA adducts in BRCA1 knockdown cells. (a) Cells were pretreated with siRNA (control vs. BRCA1), treated with BaP (0, 2.5, and 5μM), harvested, and confirmed by WB analysis using anti-BRCA1, anti-NRF2, and anti-ARNT antibodies. (b) Overexpression 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}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}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.

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 *in vitro* (Kwak and Kensler, 2010).

Similarly, we demonstrated the significance of Brca1 levels in determining the amount of BaP-induced DNA adducts only in *ex vivo* experiments using Brca1$^{wt/wt}$ and Brca1$^{co/co}$ mice. There are only a few reports on the function of Brca1 in carcinogenesis in *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$^{\Delta 11/\Delta 11}$p53$^{(+/-)}$) (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 *in vivo* carcinogenesis model will elucidate the complete function of BRCA1 in environmental risk.

An *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

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 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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