Expression and DNA methylation changes in human breast epithelial cells after bisphenol A exposure

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Abstract. It has been suggested that xenoestrogens, a group of agents termed endocrine disruptors, may contribute to the development of hormone-dependent cancers, such as breast and endometrial cancers. We previously demonstrated that the xenoestrogen, bisphenol A (BPA), was able to induce the transformation *in vitro* of human breast epithelial cells. The normal-like human breast epithelial cell line, MCF-10F, formed tubules in collagen (3-D cultures), although after treatment with BPA (10^{-5} M and 10^{-6} M BPA) the cells produced less tubules (73% and 80%, respectively) and some spherical masses (27% and 20%, respectively). In the present study, expression and DNA methylation analyses were performed in these cells after exposure to BPA. These cells showed an increased expression of *BRCA1*, *BRCA2*, *BARD1*, *CtIP*, *RAD51* and *BRCC3*, all of which are genes involved in DNA repair, as well as the downregulation of *PDCD5* and *BCL2L11* (*BIM*), both of which are involved in apoptosis. Furthermore, DNA methylation analysis showed that the BPA exposure induced the hypermethylation of *BCL2L11*, *PARD6G*, *FOXP1* and *SFRS11*, as well as the hypomethylation of *NUP98* and *CtIP* (*RBBP8*). Our results indicate that normal human breast epithelial cells exposed to BPA have increased expressions of genes involved in DNA repair in order to overcome the DNA damage induced by this chemical. These results suggest that the breast tissue of women with *BRCA1* or *BRCA2* mutations could be more susceptible to the effects of BPA.

Introduction

Bisphenol A (BPA) is an environmental contaminant due to the fact that it is a monomer that is polymerized to manufacture polycarbonate plastic and epoxy resins. Polycarbonate plastic is used to make baby and water bottles, dental fillings and sealants; epoxy resins are used as coatings on the inside of almost all food and beverage cans (1,2). Thus, BPA leaches into food and beverages through the use of tin cans and polycarbonate plastic containers. The rate of leaching increases when polycarbonate is scratched and discolored (3-5). Decades of continuous release of free BPA into food, beverages and the environment have resulted in a widespread human exposure to this chemical.

BPA is lipophilic and it has been detected in breast adipose tissue samples (6). It has also been detected in human urine at concentrations of ≥0.1 μg/l (3-5,7-12). BPA has also been found in maternal plasma (3.1 ng/ml), fetal plasma (2.3 ng/ml; ~10nM), placental tissues (1-104.9 ng/g) and amniotic fluid (8 ng/ml), indicating that there is a significant exposure of pregnant women and their fetuses to BPA (8,11).

In vivo studies have shown that, in rodents, early-life exposure to BPA results in persistent alterations in mammary gland morphogenesis and increased susceptibility to tumorigenesis (13,14). In rats, maternal exposure to BPA during lactation has been shown to decrease time to first tumor latency and increase the number of 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumors in their female offspring (15). However, there is less evidence of the carcinogenic activity of BPA when administered to adult animals. Studies using human breast cancer cells have shown inconsistent data as regards the mitogenic, apoptotic and transcriptional properties of BPA (16-19). This inconsistency is attributed to the lack of the linear dose-dependence of BPA, which often shows a 'U'- or an inverted 'U'-shaped curve (20).

In a previous study, we investigated the effect of BPA on the normal-like human breast epithelial cell line, MCF-10F (21). These cells form tubules in collagen (3-D cultures) resembling the ducts of the normal mammary gland (22). We showed that the treatment of MCF-10F cells with 10^{-5} M or 10^{-6} M of BPA was able to decrease the formation of tubules (73% and 80%, respectively) and increase the formation of spherical masses in collagen (27% and 20%, respectively), an indication of cell transformation (21). The objective of the present study was to investigate the expression and DNA methylation changes in MCF-10F cells after BPA exposure.

Materials and methods

Cells and treatments. MCF-10F is a normal-like human breast epithelial cell line that is estrogen receptor α (ERα)- and proges-

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terone receptor (PgR)-negative. MCF-10F was maintained in DMEM: F12 medium (1:1 Gibco-BRL, Gaithersburg, MD, USA) supplemented with 5% horse serum (Gibco), 100 ng/ml cholera toxin (ICN Biomedicals, Cleveland, OH, USA), 10 μg/ml insulin (Sigma-Aldrich, St. Louis, MO, USA), 0.5 μg/ml hydrocortisone (Sigma-Aldrich), 20 ng/ml epidermal growth factor (Gibco), 1.05 mM $CaCl_2$ and antibiotics (penicillin, 100 U/ml; streptomycin, 100 μg/ml; amphotericin, 0.25 μg/ml; Sigma-Aldrich). BPA (from Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO) and the cells were treated with 10^{-5} M or 10^{-6} M BPA continuously for 2 weeks as described previously (21). As controls, MCF-10F cells were not treated and maintained in the regular medium or treated with 0.284% DMSO (21). After BPA treatment, the cells were expanded and RNA or DNA were isolated for expression and DNA methylation studies, respectively.

Expression arrays. RNA was isolated from the cells using RiboPure™ kit (Life Technologies, Carlsbad, CA, USA). The genome-wide gene expressions were performed using the Human Genome U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA, USA). The arrays were performed in duplicate for the MCF-10F cells treated with 10^{-6} M and 10^{-5} M BPA and for the control (cells without BPA treatment). After hybridization, the chips were scanned using GeneChip Scanner 3000.

Methylated DNA immunoprecipitation-on-chip (MeDIP-on-chip). MeDIP-on chip consists of an immunocapturing approach for enriching methylated DNA in combination with detection by DNA microarray. DNA was isolated from cells treated with 10^{-5} M, 10^{-6} M BPA, and the control MCF-10F cells (without BPA treatment) using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA). The DNA was fragmented (150-500 bp) by sonication and methylated DNA was immunoprecipitated with a monoclonal antibody against 5-methylcytidine (Eurogentec, San Diego, CA, USA) (23). Methylated fragments were amplified using the GenomePlex Whole Genome Amplification kit (WGA, Sigma-Aldrich) (23). Double-stranded DNA was treated by a combination of UDG and APE1 that specifically recognizes the dUTP residues and breaks the DNA into fragments. Targets were then labeled with Affymetrix labeling reagent and terminal deoxynucleotidyl transferase (TdT) for 1 h. The mixtures were hybridized to the Human promoter 1.0R Array (Affymetrix) that comprises more than 4.6 million probes tiled to interrogate more than 25,500 genes; it interrogates 7.5 kb upstream and 2.5 kb downstream of transcription start sites. The methylation arrays were performed in duplicate for the cells treated with 10^{-6} M BPA and in triplicate for the cells treated with 10^{-5} M BPA.

Table I. Expression values of certain genes in MCF-10F cells treated with 10^{-6} M BPA.

Gene symbol	GenBank	Fold change
ALDH1A3	NM_000693	20.82
AURKA	NM_003600	23.43
AURKB	AB011446	4.99
BARD1	NM_000465	4.50
BCL11A	AF080216	0.13
BCL2L11 (BIM)	AA629050	0.12
BCL2L13	AA156605	0.42
BOLA3	AI380704	3.58
BRCA1	NM_007295	24.25
BRCA2	NM_000059	6.82
BRCC3	X64643	5.43
CD24	X69397	9.25
CD44	AL552534	0.18
CEACAM1	X16354	317.37
CtIP (RBBP8)	NM_002894	2.06
ERBB3	NM_001982	6.73
FN1	AK026737	11.96
HDAC5	NM_005474	0.02
HDAC8	AF212246	6.06
HDAC9	NM_014707	0.11
ID2	AI819238	17.03
IL18R1	NM_003855	0.04
JAG1	U73936	0.35
JAG2	Y14330	0.26
LOX	NM_002317	0.29
MTSS1	NM_014751	0.02
MUC1	AF348143	3.81
MUC16	NM_024690	124.50
MUC20	AW084511	21.26
MYCBP	D50692	15.78
PARD6G	AI817448	0.06
PDCD5	AI817145	0.04
RAD51	NM_002875	36.25
RARRES1 (TIG1)	NM_002888	1016.92
RARRES3 (TIG3)	NM_004585	3.97
SFRP1	AF017987	0.34
SLIT2	AF055585	0.22
SMAD5	AF010601	0.19
TSPAN5	AA059445	0.02
TWIST1	X99268	0.09
VIM	AI922599	0.02

Genes that are at least 2-fold up- or downregulated (fold change) are indicated.

Data analyses. The mRNA expression arrays were analyzed using the Bioconductor 'limma' package. Briefly, the raw data were normalized using the 'rma' method (24). Differentially expressed genes were identified using empirical Bayes methods implemented into the limma package (25). The criteria for significance are determined by a fold change of >2 and a Benjamini-Hochberg false discover rate of <5%. The functional significance of up- or downregulated genes in the BPA-treated cells were analyzed using Ingenuity Pathways Analysis software (IPA) version 5.0 (Ingenuity Systems, Redwood City, CA, USA). The differentially expressed genes were uploaded into IPA to identify significantly enriched canonical pathways. The significance of a canonical pathway is controlled by the p-value calculated using the right-tailed Fisher's exact test for 2x2 contingency tables.

The MeDIP-on chip data were analyzed using CisGenome Software, an integrated tool package for tiling array and ChIP-seq analysis (26). Two-sample analyses were performed using BPA-treated cells versus MCF-10F control cells (without BPA treatment) precipitated using the antibody against 5-methyl-

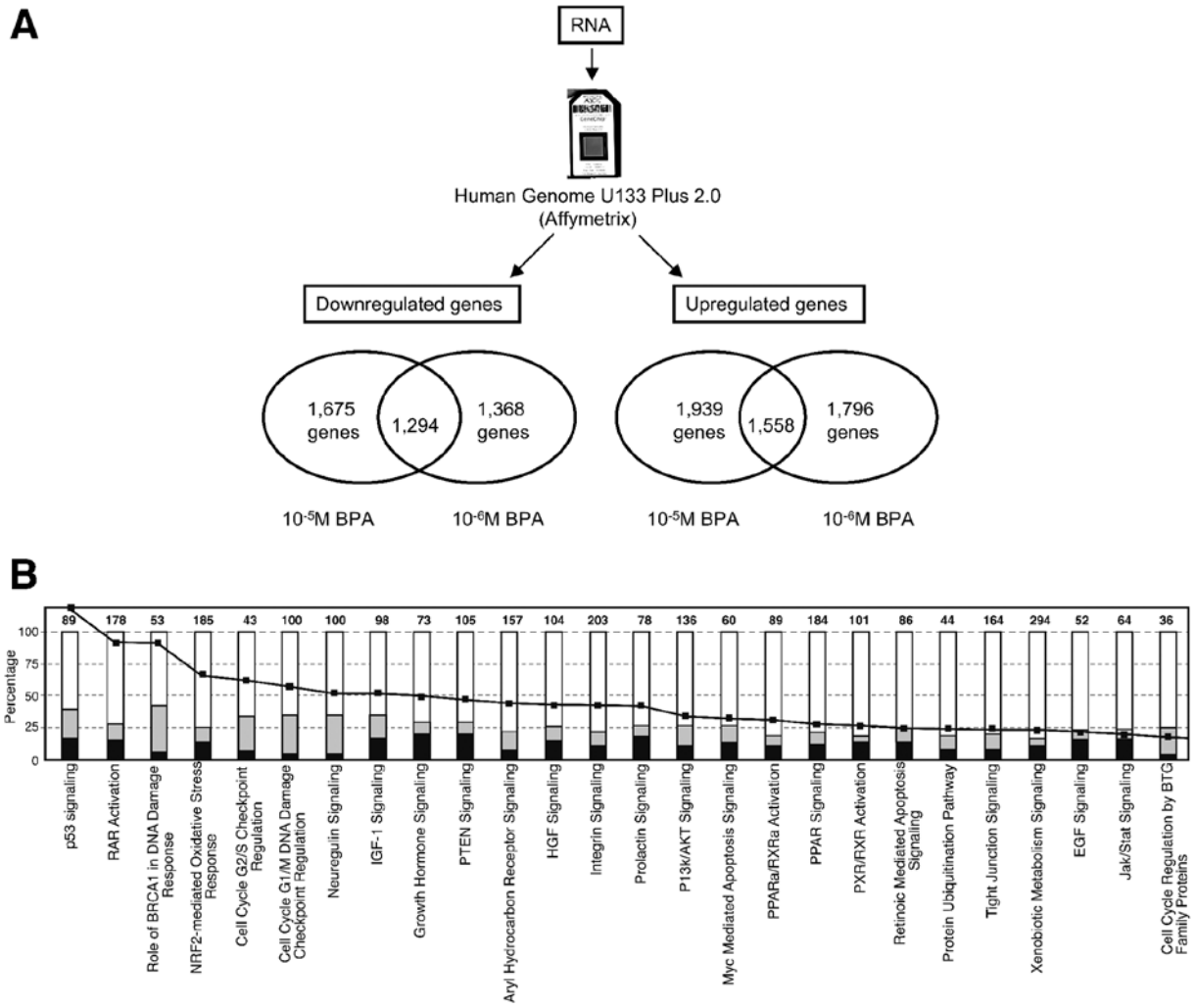

Figure 1. Expression studies of the MCF-10F cells exposed to BPA. (A) RNA was isolated from the cells treated with 10^{-5} M and 10^{-6} M BPA and expression analyses were performed using the Human Genome U133 Plus 2.0 arrays (Affymetrix). (B) Canonical pathways enriched with deregulated genes in MCF-10F cells exposed to 10^{-6} M BPA. Black, number of downregulated genes; gray, number of upregulated genes; black line, log (p-value).

cytidine. Data were quantile normalized before the comparison. The ChIP-chip peak calling was detected with the Model-based Analysis of Tiling-array (MAT) algorithm (27) integrated in CisGenome. MAT was run with the following parameters to capture regions of increased signal intensity: a bandwidth of 300, a maximum gap of 300, a max run of insignificant probes within a region of 5, a minimum region length of 100, a minimal number of significant probes within a region of 5 and window p-value cut-off of 0.0001. Hyper- or hypomethylated regions were determined by the signals of BPA-treated cells which were significantly higher or lower than those of the MCF-10F control cells. The MAT library and mapping files based on the March 2006 Human Genome Assembly (HG18) were used to link ChIP Hits to RefSeq genes. Briefly, the files containing transcription start site (TSS) and end-point were linked to the RefSeq table with accession numbers. Chromosomal positions were then used to associate ChIP hits with RefSeq gene IDs. Specifically, hits falling within a window of -0.5 to +2.5 kb of a given RefSeq TSS were annotated as being associated with that gene. To illustrate the impact of methylation on gene expression, the hyper- or hypo-methylated genes were compared to the down- or upregulated genes determined by the expression array. The raw data were submitted to the NCBI GEO data base with accession numbers GSE26884 and GSE27865 for the expression and methylation arrays, respectively.

Results

Expression studies of the human breast epithelial cells after BPA exposure. We characterized the cells exposed to 10^{-5} M or 10^{-6} M BPA by expression studies using microarrays. MCF-10F cells exposed to 10^{-5} M or 10^{-6} M BPA showed 3,614 and 3,164 deregulated genes (up- or downregulated compared to the MCF-10F control), respectively (Fig. 1A). We found a total of 1,675 genes downregulated in the cells exposed to 10^{-5} M BPA and 1,368 genes downregulated in the cells exposed to 10^{-6} M BPA; from these genes 1,294 genes were found to be downregulated with both BPA concentrations (Fig. 1A). A total of 1,939 genes were upregulated in the cells treated with 10^{-5} M BPA and 1,796 genes were upregulated in the cells exposed to 10^{-6} M BPA; from these genes, 1,558 genes were upregulated in the cells treated with both concentrations (Fig. 1A).

The functional enrichment of up- or downregulated genes in the BPA-exposed cells was analyzed using IPA software. Gene

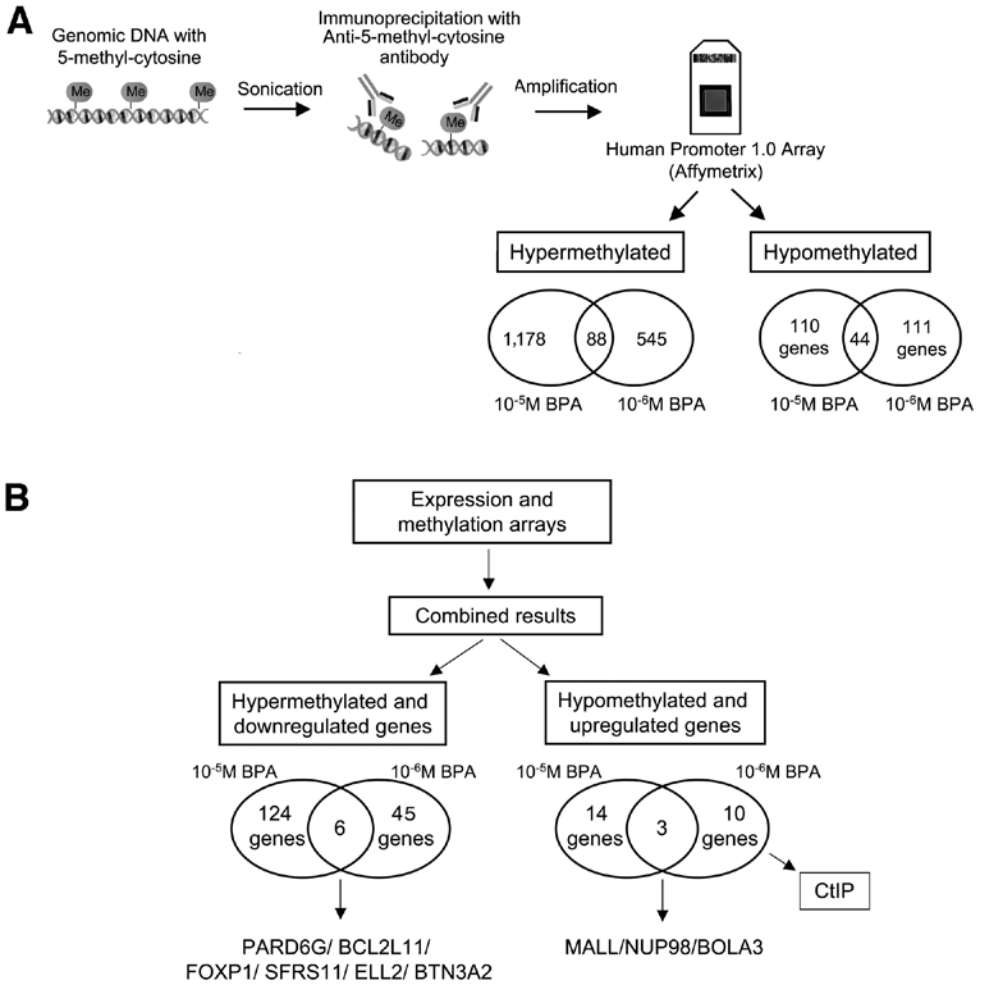

Figure 2. DNA methylation studies in MCF-10F cells exposed to BPA. (A) DNA was isolated from the cells and fragmented by sonication. Methylated DNA was isolated using an antibody against 5-methylcytidine and amplified, followed by hybridization using a promoter microarray to identify regions with altered methylation in the promoters. The Human Promoter 1.0R Arrays were used and hyper- and hypomethylated promoters were identified. Hypermethylated targets were sequences with significantly increased signals in the cells exposed to BPA relative to the control without treatment immunoprecipitated using the antibody against 5-methylcytidine. (B) Combined results from the expression and DNA methylation arrays: the list of genes that were found hypermethylated by MeDIP-on-chip and the list of genes found downregulated by expression arrays were compared. Left, the number of genes hypermethylated and downregulated is indicated for the cells exposed to 10^{-5} M and 10^{-6} M BPA. The same was done for genes hypomethylated and upregulated. Right, the number of genes hypomethylated and upregulated is indicated for the cells treated with 10^{-5} M and 10^{-6} M BPA.

networks and canonical pathways representing key genes were identified. The canonical pathways more affected in the cells exposed to BPA were the DNA damage response, p53 signaling (activated by genotoxic or non-genotoxic stress), the retinoic acid receptor activation, and the neuregulin signaling pathways (Fig. 1B). Cells treated with 10^{-5} M or 10^{-6} M BPA showed decreased expressions of *PDCD5* and *BCL2L11* (also known as *BIM*), both of which are involved in apoptosis, and an increased expression of *BRCA1*, *BARD1*, *CtIP* (also known as *RBBP8*), *RAD51* and *BRCC3*, all of which are involved in DNA repair.

Table I shows genes which were at least 2-fold up- or down-regulated in the cells after exposure to 10^{-6} M BPA. A number of genes involved in DNA damage response were upregulated, such as *BRCA1* (24.25-fold induction), *BRCA2* (6.82-fold), *BRCC3* (5.43-fold), *BARD1* (4.5-fold), *CtIP* (2.06-fold) and *RAD51* (36.25-fold). Other genes downregulated after exposure to 10^{-6} M BPA were: *JAG1* (0.35-fold induction), *JAG2* (0.26-fold), *SMAD5* (0.19-fold), *TWIST1* (0.09-fold), *VIM* (0.02-fold), *TSPAN5* (0.02-fold), CD44 (0.18-fold) and HDAC5 (0.02-fold) (Table I). Exposure to 10^{-6} M BPA induced the overexpression of *RARRES1* (1016.92-fold) and *RARRES3* (3.97-fold), both of which involved in the retinoic acid receptor pathway (Table I). Some upregulated genes were *CEACAM1* (317.37-fold), *ALDH1A3* (20.82-fold), *AURKA* (23.43-fold), *ID2* (17.03-fold), *FN1* (11.96-fold induction) and *CD24* (9.25-fold) (Table I).

DNA methylation studies of MCF-10F cells after BPA exposure. We studied DNA methylation changes in the MCF-10F human breast epithelial cells after exposure to BPA using MeDIP-on-chip (23). Gene regulatory regions that were hypo- or hypermethylated were identified. Hypermethylated targets were sequences with significantly increased signals in the cells exposed to BPA relative to the control cells (MCF-10F growth without treatment). The DNA from the BPA-treated cells and the control cells was immunoprecipitated with the antibody against 5-methyl-cytosine. The cells treated with 10^{-5} M BPA showed 1,178 genes hypermethylated and those treated with 10^{-6} M BPA showed 545 hypermethylated genes; from these

Table II. Downregulated and hypermethylated genes in MCF-10F cells treated with 10^{-5} M BPA.

Gene symbol	Fold change	Gene symbol	Fold change	Gene symbol	Fold change	Gene symbol	Fold change
SGSH	0.50	FADS3	0.42	PURA	0.34	ZNF197	0.22
OGFRL1	0.50	ABCA12	0.42	RBMS3	0.34	MARK1	0.22
IL13RA1	0.49	AFF4	0.42	STK16	0.33	TOMM20	0.22
ANKDD1A	0.49	MLL	0.41	PEX11A	0.32	PARD6G	0.21
UBE2Z	0.49	WWOX	0.41	SENP6	0.32	C1orf21	0.21
PRKAG2	0.49	SLC7A8	0.40	KIAA0182	0.32	BCL2L11 (BIM)	0.20
ZNF488	0.48	MCEE	0.40	PARD3	0.31	PRKCH	0.20
ZC3H11A	0.48	HMHA1	0.40	RNF135	0.31	C7orf31	0.19
KCNAB2	0.48	RIOK3	0.39	STAT5B	0.31	TMEM91	0.19
RHOQ	0.48	ELL2	0.39	ATF6	0.30	MGST1	0.18
DFFB	0.47	C7orf38	0.39	ADARB1	0.30	ANKRD28	0.18
TBC1D8B	0.47	METTL9	0.39	JAK1	0.29	DHRS3	0.18
DGAT2	0.47	FBXO9	0.38	CP	0.29	CCDC11	0.16
ZNF219	0.47	EFHC1	0.38	TMEM67	0.29	TMEM37	0.15
LRCH3	0.47	CD99L2	0.38	FARP1	0.28	HSD11B1	0.15
SH3BP2	0.47	CAPN1	0.38	FAM119B	0.28	IL18R1	0.13
RUFY1	0.47	SFRS11	0.38	VPS41	0.28	LETMD1	0.12
UBE2Q1	0.46	ACVR2A	0.38	TMEM80	0.28	FAM19A2	0.11
SSR2	0.46	ATP9A	0.38	TRIM69	0.27	BBOX1	0.10
CIR	0.46	DUSP1	0.38	GJA3	0.27	EBF1	0.10
GRAMD2	0.46	PPIL6	0.38	IFNGR1	0.27	GBP2	0.08
DNAJC16	0.46	MAP2K5	0.37	GPR177	0.24	SULT1E1	0.07
HHLA3	0.45	RAB13	0.37	BTN3A2	0.24	TSLP	0.07
SMYD3	0.45	PKIA	0.37	PIK3R1	0.24	PLEKHA6	0.07
C5orf 25	0.45	SFXN1	0.37	ZBTB20	0.24	KLHDC8B	0.07
TMLHE	0.45	FGFR1OP2	0.37	TPPP	0.23	PDZK1IP1	0.06
KIAA1244	0.44	MSX2	0.37	PIR	0.23	DCN	0.05
ITSN2	0.44	LYPLAL1	0.36	DUSP16	0.22	SORL1	0.04
MAPT	0.44	KLRK1	0.36	PRKAG2	0.22	ZBTB10	0.03
RNPC3	0.44	GNPTAB	0.35	DSC3	0.22	BCL6	0.03
FOXP1	0.43	ADRBK2	0.34	ITGA4	0.22	GALNTL2	0.003

Hypermethylated genes and their expression values are indicated (fold change).

genes, 88 genes were hypermethylated at both concentrations (Fig. 2A). Hypomethylated targets were sequences that were significantly increased in the control relative to the cells treated with BPA. Using these criteria, we identified 110 genes that were hypomethylated in the cells treated with 10^{-5} M BPA and 111 hypomethylated genes in the cells treated with 10^{-6} M BPA; from these genes, 44 genes were hypomethylated at both concentrations (Fig. 2A).

As hypermethylation is related to gene downregulation and hypomethylation is related to increased gene expression (28), the expression and DNA methylation data were superimposed. The downregulated and hypermethylated genes by BPA exposure were identified (Fig. 2B). In the cells treated with 10^{-5} M BPA, 124 genes were found to be hypermethylated and downregulated (Fig. 2B). In the cells treated with 10^{-6} M BPA, 45 genes were found hypermethylated and downregulated (Fig. 2B). As indicated in Fig. 2B, 6 genes were found to be hypermethylated and downregulated in the cells treated with both 10^{-5} M or 10^{-6} M BPA: *PARD6G*, *BCL2L11* (or *BIM*), *FOXP1*, *SFRS11*, *ELL2* and *BTN3A2*.

In Table II, the 124 genes which were downregulated and hypermethylated in the cells after exposure to 10^{-5} M BPA are indicated; some of these genes were *PARD6G* (0.21-fold induction), *BCL2L11* (0.2-fold), *FOXP1* (0.43-fold), *SFRS11* (0.38-fold), *ELL2* (0.39-fold) and *BTN3A2* (0.24-fold). Other genes downregulated and hypermethylated were *STAT5B* (0.31-fold induction), *WWOX* (0.41-fold induction) and *SULT1E1* (0.07-fold induction). Table III shows the 45 genes downregulated and hypermethylated in the cells after being exposed to 10^{-6} M BPA. Some of these genes were *PARD6G* (0.16-fold induction), *BCL2L11* (0.18-fold), *FOXP1* (0.38-fold), *SFRS11* (0.22-fold), *ELL2* (0.31-fold) and *BTN3A2* (0.28-fold). Other genes downregulated and hypermethylated were *RHOU* (0.02-fold induction), *TWIST1* (0.11-fold induction), and *SFRP1* (0.39-fold induction).

Similarly, hypomethylated and upregulated genes by BPA were identified (Fig. 2B). In the cells exposed to 10^{-5} M BPA, 14 genes were found to be hypomethylated and upregulated; in the cells treated with 10^{-6} M BPA, 10 genes were found to be hypomethylated and upregulated (Fig. 2B). From these genes,

Table III. Downregulated and hypermethylated genes in MCF-10F cells treated with 10^{-6} M BPA.

Gene symbol	Fold change	Gene symbol	Fold change	Gene symbol	Fold change	Gene symbol	Fold change
NIPA1	0.50	SYNE1	0.38	UACA	0.24	MRPL39	0.14
EDA	0.49	NPL	0.37	RAB4A	0.24	KLF9	0.13
RERE	0.48	C17orf69	0.36	SFRS11	0.22	TWIST1	0.11
SLC25A28	0.47	TPCN2	0.35	KIAA0564	0.21	RNF128	0.10
FAM19A1	0.45	TOP1MT	0.35	BCL2L11 (BIM)	0.18	DST	0.10
MGEA5	0.44	TMEM47	0.33	KLHL13	0.18	TOX	0.07
TLR3	0.44	ELL2	0.31	IGF1R	0.17	TTC7B	0.04
ZHX2	0.41	PCDHB14	0.29	RPL37	0.16	NXN	0.04
OSBPL6	0.40	BTN3A2	0.28	PARD6G	0.16	RHOU (WRCH1)	0.02
NUDT2	0.40	TARSL2	0.27	GPC4	0.15	FHL1	0.01
SFRP1	0.39	LONRF1	0.27	GAB2	0.15	SOD2	0.01
FOXP1	0.38						

Hypermethylated genes and their expression values (fold change) are indicated.

Table IV. Hypomethylated and upregulated genes in MCF-10F cells treated BPA.

Gene symbol	Gene title	Fold change
Cells treated with 10^{-5} M BPA		
MALL	Mal, T cell differentiation protein-like	67.65
NUP98	Nucleoporin 98 kDa	9.65
ARHGAP11A	Rho GTPase activating protein 11A	7.67
BOLA3	BolA homolog 3 (*E. coli*)	5.43
CA2	Carbonic anhydrase II	5.10
GPR172A	G protein-coupled receptor 172A	4.20
CCDC80	Coiled-coil domain containing 80	3.76
BID	BH3 interacting domain death agonist	3.53
NT5E	5'-Nucleotidase, ecto (CD73)	3.23
SHC4	SHC (Src homology 2 domain containing) family, member 4	2.73
C12orf30	Chromosome 12 open reading frame 30	2.62
CCDC90A	Coiled-coil domain containing 90A	2.31
FAM86A	Family with sequence similarity 86, member A	2.31
SYNCRIP	Synaptotagmin binding, cytoplasmic RNA interacting protein	2.25
Cells treated with 10^{-6} M BPA		
MALL	Mal, T cell differentiation protein-like	72.00
RPL27A	Ribosomal protein L27a	13.93
GDA	Guanine deaminase	7.46
NUP98	Nucleoporin 98 kDa	5.39
TIAM1	T cell lymphoma invasion and metastasis 1	4.44
BCMO1	β-carotene 15,15'-monooxygenase 1	4.35
BOLA3	BolA homolog 3 (*E. coli*)	3.10
DDX52	DEAD (Asp-Glu-Ala-Asp) box polypeptide 52	3.01
CtIP (RBBP8)	Retinoblastoma binding protein 8	2.06
PPME1	Protein phosphatase methylesterase 1	2.04

Hypomethylated genes and their expression values (fold change) are indicated.

3 were hypomethylated and upregulated in both cells: *MALL*, *NUP98* and *BOLA3* (Fig. 2B and Table IV). In the cells treated with 10^{-5} M BPA, *MLL*, *NUP98* and *BOLA3* showed a 67.65-, 9.65- and 5.43-fold induction, respectively (Table IV). In the cells treated with 10^{-6} M BPA, *MLL*, *NUP98* and *BOLA3* showed a 72-, 5.39- and 3.10-fold induction, respectively (Table III); these cells also showed an upregulation and hypomethylation of *CtIP* (2.06-fold induction).

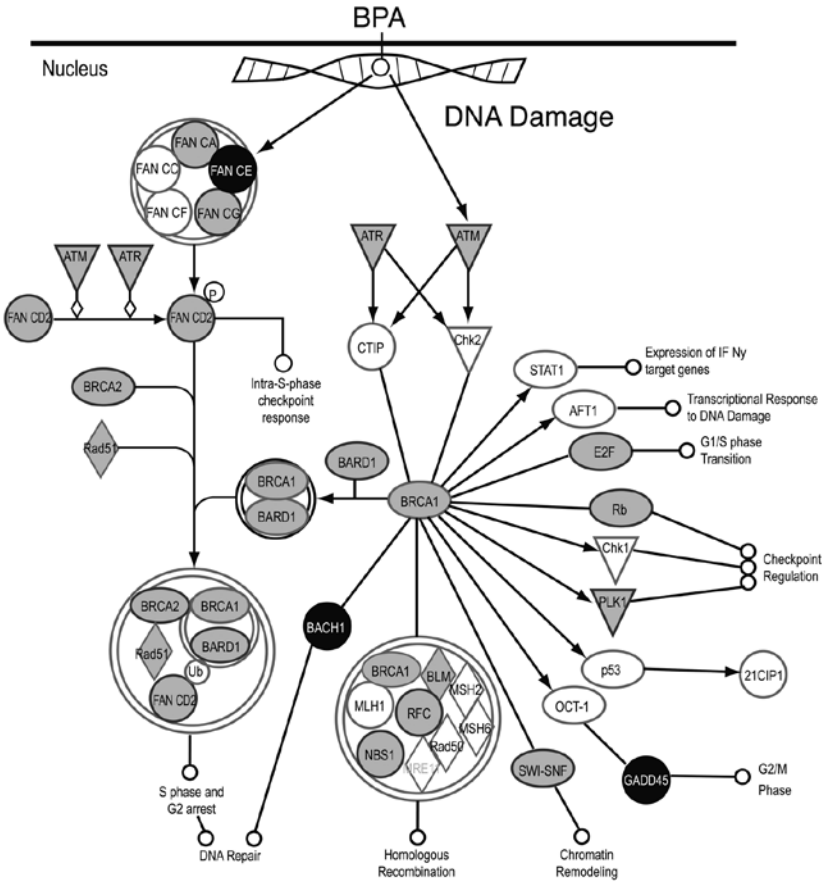

Figure 3. DNA repair genes were induced in the normal breast epithelial cells after being exposed to BPA. The MCF-10F cells showed increased expression of *BRCA1*, *BARD1*, *BRCA2* and RAD51 after being exposed to 10^{-6} M BPA. Upregulated genes are shown in gray. Downregulated genes are shown in black.

Discussion

The normal-like human breast epithelial cell line, MCF-10F, showed increased expressions of genes involved in DNA repair (*BRCA1*, *BARD1*, *CtIP*, *RAD51* and *BRCC3*) and decreased expressions of genes involved in apoptosis (*PDCD5* and *BCL2L11*) after being exposed to BPA for 2 weeks. These cells also showed hypermethylation of different genes such as *BCL2L11*, *PARD6G*, *FOXP1*, *SFRS11*, and hypomethylation of *CtIP* (or *RBBP8*) and *NUP98*.

Epigenetic changes derived from exposure to endocrine disruptors have been described in several tissues and organisms (29,30) although, this is the first demonstration that BPA induces DNA methylation changes in genes related to apoptosis and DNA repair in human breast epithelial cells. *BCL2L11* (or *BIM*) has the ability to trigger apoptosis in various cells, such as epithelial and neuronal cells (31); the fact that this gene was hypermethylated after BPA treatment suggests that apoptosis was inhibited in the cells after exposure to BPA. *CtIP* was hypomethylated and upregulated after BPA treatment; CtIP is involved in double-strand break (DSB) repair and plays a role in DNA-damage-induced cell cycle checkpoint control at the G2/M transition (32). MCF-10F cells treated with BPA also showed changes in the DNA methylation pattern of partitioning defective 6 homolog γ (*PARD6G*) which is an adapter-protein involved in asymmetrical cell division and cell polarization processes.

It has been suggested that BPA is a weak carcinogen (33). Evidence of the estrogenic effects of BPA has been reported in a number of studies showing that it activates ERs α and β (34,35), although the affinity of BPA is at least 10,000-fold less than estrogen for both receptors (36). It has been proposed that BPA, similar to certain estrogen metabolites, can react with DNA to cause mutations that can lead to cancer initiation (33,37-41). One mechanism by which estrogen and BPA initiates breast cancer is by generating adducts that can produce a variety of DNA modifications that, if not countered by DNA repair, can lead to cell transformation. It has been shown that BPA is able to form DNA adducts *in vitro* and *in vivo* (42-45); BPA can be converted to bisphenol O-quinone (46) and, the BPA semiquinone and/or quinone intermediates may be the ultimate DNA binding metabolites. In the present study, the ERα-negative breast epithelial cell line, MCF-10F, was used indicating that in these cells other mechanisms independent of the ER were responsible for the biological effect of BPA.

In addition to DNA adduct formation, oxidative stress could be another reason for the alterations produced by BPA in the DNA (47). Oxidative DNA lesions include the oxidation of nucleotidic bases, modifications to the sugar moiety of DNA which may result in base-loss abasic (apurinic/apyrimidinic) sites and/or strand breakage (single and DSBs), DNA intra-strand adducts, and DNA-protein crosslinks, all of which are cytotoxic and some can be mutagenic (48,49). The results from the present study revealed that after BPA treatment, the cells

showed an upregulation of genes involved in DNA repair, suggesting that BPA produced DNA DSBs and that the normal breast epithelial cells increased the expression of DNA repair genes to overcome the damage (Fig. 3). The normal-like human breast epithelial cell line, MCF-10F, showed an increased expression of *BRCA1*, *BARD1*, *CtIP*, *RAD51* and *BRCC3*, all of which are genes involved in DNA repair, after exposure to BPA.

The human BRCA1 is a nuclear polypeptide consisting of 1,863 amino acids and it contains several functional domains that interact directly or indirectly with a variety of molecules, including tumor suppressor, oncogenes, DNA damage repair proteins, cell cycle regulators, and transcriptional activators and repressors (Fig. 3) (50). BRCA1 exists as a heterodimer with BARD1 and most of the functions of BRCA1 have been attributed to occur in association with BARD1 (51,52). Disruption of the BRCA1-BARD1 interaction would impair the cell cycle checkpoint control as well as DNA repair functions of BRCA1 which could lead to tumorigenesis. BRCA1 ubiquitinates its phosphorylation-dependent partner, CtIP (RBBP8), and this reaction plays a role in the G2/M checkpoint control upon DNA damage (53). RAD51, also involved in DNA-damage repair, interacts with BRCA1 (54). Our results showed that *BRCA2* was downregulated in the cells treated with 10^{-6} M BPA; the BRCA2 protein, which has a function similar to that of BRCA1, also interacted with RAD51. By influencing DNA damage repair, BRCA1, BRCA2, and RAD51 play a role in maintaining the stability of the human genome. BRCC3 encodes a subunit of the BRCA1-BRCA2 containing complex (BRCC), which is an E3 ubiquitin ligase; this protein is also thought to be involved in the cellular response to progression through the G2/M checkpoint. Our results demonstrated that normal breast epithelial cells treated with BPA showed an increased expression of *BRCA1*, *BARD1*, *CtIP*, *RAD51* and *BRCC3*; all of which are involved in DNA repair. This supports the hypothesis that BPA can initiate breast cancer by generating adducts or reactive oxygen species (ROS) that can produce a variety of DNA modifications that, if not countered by DNA repair, can lead to cell transformation.

Our results suggest that the loss of *BRCA1* could lead to an increased sensitivity to BPA as it was shown by Jones *et al* (55). We isolated primary breast epithelial cells from a *BRCA1* carrier; these cells were treated continuously for 1 week with medium containing 10^{-5} M or 10^{-6} M BPA and, at the end of the treatment the ductulogenic and invasion assays were performed. These *BRCA1* mutant cells treated with BPA formed an increased number of spherical masses in collagen and showed increased invasion (data not shown). Our results suggest that women that carry *BRCA1* mutations could be more susceptible to the effects of BPA.

Human exposure to BPA is widespread and studies have shown detectable levels of BPA ranging from 0.2 to 10 ng/ml (~0.5-40 nM) in adult and fetal human serum (56). Although the doses of 10^{-5} M and 10^{-6} M BPA that were used in our studies were higher compared to the concentrations found in serum samples, the cells were exposed for 2 weeks in contrast to humans that are exposed to low doses for longer periods of time.

In conclusion, our results show that BPA induces the expression of genes related to DNA repair in normal human breast epithelial cells. The upregulation of *BRCA1*, *BRCA2*, *RAD51*, *BARD1* and *BRCC3* expression was induced after BPA exposure in MCF-10F cells. This suggests that in BRCA1 carriers, BPA exposure could lead to increased frequency of DNA mutations. Our results suggest that loss of *BRCA1* could lead to an increased sensitivity to BPA. Furthermore, this is the first study demonstrating that BPA induces DNA methylation changes in human breast epithelial cells.

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