SPANDIDOS PUBLICATIONS

Phytoestrogens regulate the expression of genes involved in different biological processes in *BRCA2* knocked down MCF-7, MDA-MB-231 and MCF-10a cell lines

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Abstract. Breast cancer is a public health problem in the Western countries. Several studies have shown that *BRCA2*, like *BRCA1* oncosuppressors, are strongly involved in hereditary and sporadic mammary carcinogenesis. It has also been suggested that soy has a protective effect against breast cancer in Asia and, more particularly, phytoestrogens such as daidzein and genistein. Thus, phytoestrogens may have an impact on the expression of *BRCA2* gene, and there is a possible link between *BRCA2* and genes acting around the *BRCA2*. To focus on these processes, we set up the *BRCA2* specific knockdown by RNA interference in two breast tumor cell lines (MCF-7 and MDA-MB-231) and also in a non-tumorigenic breast cell line (MCF-10a). After inhibition of *BRCA2* expression, cells were maintained in different conditions and treated with either daidzein or genistein or left untreated. Microarray analysis of mRNAs isolated from the *BRCA2* knocked down MCF-7, MDA-MB-231, and MCF-10a cell lines after being treated with phytoestrogens showed 35 differentially expressed genes between positive-ERß cells and negative-ERß cells. After genistein or daidzein treatments, *BRCA1* was found to be up-regulated when knocked down with *BRCA2*-siRNA MCF-7 and *BRCA2* was found to be up-regulated when knocked down with *BRCA2*-siRNA MDA-MB 231 cells. In MCF-10a, we observed a significant decrease in *BAX* and *BCL2* expressions with a greater effect of daidzein. We also found an increase in *BRIP* expression between genistein and daidzein treatment knocked down with *BRCA2*-siRNA MCF-7 and MDA-MB-231 cell lines.

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Introduction

Despite many advances in early detection, prevention and treatment, the incidence of breast cancer is increasing and remains a leading cause of mortality among women. It has been shown that many factors are involved in carcinogenesis. Genetic, hormonal and environmental factors are all important in breast cancer risk. The genetic factor is the mutation in *BRCA1* or *BRCA2* oncosuppressors (usually hereditary) and hazardous mutations that can lead to a decrease in the *BRCA1* and/or *BRCA2* expression (1,2). Hormonal change in the female endocrine system occuring at different stages of life is also an important factor. Finally, environmental factors, which widely vary by geographical region, effect the onset of breast cancer. Indeed, it has been found that the incidence of breast cancer is higher in the West when compared to Asia (3). Following a study on migrant people, the influence of diet on the development of breast cancer in women was revealed. Hence, many studies have emphasized the role of soy consumption in preventing breast cancer, and especially phytoestrogens contained in the food (4). Genistein and daidzein are the main soybean phytoestrogens that have the specificity of having a chemical structure very close to the human estrogen and, therefore to the estrogenic activity (5,6). Thus, we investigated the effects of genistein and daidzein on the expression of the *BRCA2* onco-suppressor following its reduction or elimination by specific RNA interference in human breast cells lines (MCF-7, MDA-MB-231 and MCF-10a). Small inhibitory RNA (SiRNA) technology results in degradation of the mRNA for the target gene, and can be used to specifically reduce gene expression, in this case *BRCA2*. Knocked down *BRCA2* breast cell lines were then tested by pangenomic microarrays

for their response to concomitent treatment with phytoestrogens and capacity to modify gene targeting and effects on whole genone transcriptomic expression.

Materials and methods

Cell cultures. MCF-7 cells (ATCC), originating from invasive breast carcinoma, were cultured in RPMI-1640 (Invitrogen SARL, 95613 Cergy Pontoise, France) containing 15% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 20 μg/ml gentamycin and 0.04 U/ml insulin at 37˚C. They were kept in a humidified atmosphere of 5% CO_2. This cell line has a positive estrogen-receptor status ($ER\alpha^+/ß^+$). MDA-MB-231 cells (ATCC) were grown in L-15 Leibovitz (Invitrogen SARL) supplemented with 2 mM L-glutamine, gentamycin (20 μg/ml) and 15% heat-inactivated FBS at 37˚C and without CO_2 atmosphere. This cell line has an $ER\alpha^-/ß^+$ status. The MCF-10a cell line (ATCC) was established from fibrocystic breast disease. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 (Invitrogen SARL) with 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 0.01 mg/ml insulin and 500 ng/ml hydrocortisone and 15% heat-inactivated horse serum. Cells were grown in a 37˚C humidified atmosphere with 5% CO_2. This cell line has a negative estrogen receptor status for each receptor isoform ($ER\alpha^-/ß^-$).

Knock down of BRCA2 using siRNA and transfection and phytoestrogen treatment. BRCA2-specific siRNA were purchased from Santa Cruz Biotechnology (sc-29825). The siRNA sequences were 5'-CCA AGG AUG UUC UGU CAA Att-3', 5'-CAA GCU ACA UAU UGC AGA Att-3', and 5'-GAA ACG GAC UUG CUA UUU Att-3'. MCF-7, MCF-10a, and MDAMB-231 cells were grown for 24 h in 6-well plates. At 50% of confluence, cells were transfected with siRNA (final concentration 50 nM) diluted in the siRNA transfection reagent (sc-29528, Santa Cruz Biotechnology) according to the manufacturer's protocol. The negative control was made from a cell culture grown under the same conditions, but without siRNA. For transfected cells, after a 24-h incubation period, they were cultivated in their respective medium without antibiotics. The phytoestrogen treated-cells were incubated for 48 h after transfection, in the presence of 18.5 μM genistein or 78.5 μM daidzein. These phytoestrogen concentrations correspond to the IC50 (7), they were previously assessed by flow cytometry. Finally, RNA extractions were carried out after a 72-h incubation period corresponding to the beginning of the transfection with the siRNA and followed by the phytoestrogen treatment of the cell lines.

RNA extraction. After 72 h of treatment, the total RNA isolated from cells transfected or non-transfected previously in a 6-well plate was extracted using 1 ml RNA-PLUS (MP Biomedicals) in each well according to the manufacturer's protocol. Six wells were pooled for each condition. The RNA quality was checked by electrophoresis using a Bioanalyzer 2100 with RNA 8000 Nanodrop LabChip® and BioSizing A.02.11 software (Agilent Technologies). The samples were then stored in liquid nitrogen.

Reverse transcription and quantitative PCR of BRCA2 mRNA expression. RNAs were diluted to 1.6 g/μl in water DEPC. The reverse transcription was performed using the First-Strand cDNA Synthesis Kit (Amersham Biosciences), according to the manufacturer's instructions. In each cell line, we assayed by quantitative PCR, the expression of *BRCA2* mRNA in transfected siRNA-specific BRCA2 cells and in transfected cells treated with genistein and daidzein by comparison to controls of non-transfected cells. cDNA (5 ng/μl) was diluted in water treated for PCR. We added 5 μl of this solution to 20 μl Mix reaction, composed of 62.5 nM probe, 50 nM forward and reverse primers for 18S RNA, 250 nM probe and 500 nM primers specific for BRCA2, and 12.5 μl PCR Mix (Universal PCR Master Mix Taqman, Roche) that consisted of dNTP, the DNA polymerase and buffer reaction 2X. PCR reaction was performed for 40 cycles divided into 15-sec denaturations at 95˚C and 1-min hybridization-elongation at 60˚C. Two independent total RNA extractions were done as two independent reverse transcriptions with a RNA extraction. The data were generated in triplicate and expressed as mean ± SD. The results were analyzed with significant p-value differences between control and assays.

Oligo microarray, RNA labeling and hybridization. Whole Human Genome Microarray Kit, 4 x 44 K (G4112F) were provided by Agilent Technologies. We amplified and labeled 200 ng of total RNA using Low RNA Input Fluorescent Linear Amplification Kit PLUS, two-color (5188-5340, Agilent) according to the manufacturer's instructions. Amplified RNA was labeled by fluorescent probes with Cy5-CTP for the treated samples and Cy3-CTP for the control samples corresponding to control cells without any treatment or knock down and competitively hybridized to pangenomic arrays. Hybridization was carried out at 60˚C overnight. Slides were washed using a wash buffer kit (5188-5327, Agilent) and a stabilization solution (5185-5979, Agilent Technologies). The fluorescent intensities were obtained with a DNA microarray scanner (G2565CA, Agilent Technologies) and signal quantitative values were calculated using the internal feature extraction software (Agilent Technologies). For each treatment, 3 microarrays were done and analyzed for MDA-MB-231 and MCF-10a and 4 microarrays for MCF-7.

Microarray analysis. Signal intensity ratios (Cy3/Cy5) of each gene were converted into a logarithmic value (log2) with LimmaGUI 1.7.0 using R 2.2.0 (8). Then, Edward's background correction was performed. This adjustment substracts local and off-spot signal from each spot's foreground with a log-linear interpolation of lower-intensity spots designed to produce positive net values. Normalizations were performed within and between-array via a global lowess method (9). This was based on local estimation of intensities and a regression calculation weighted toward similar spots. Negative controls were removed from the data set. Using a differential expression cut-off of 2-fold and hierarchical clusters obtained with TIGR MeV version 4.2.02 software using a clustering method, a complete-linkage Pearson correlation was performed. Clusters were colored in red for up-regulation and in green for down-regulation in comparison to control.

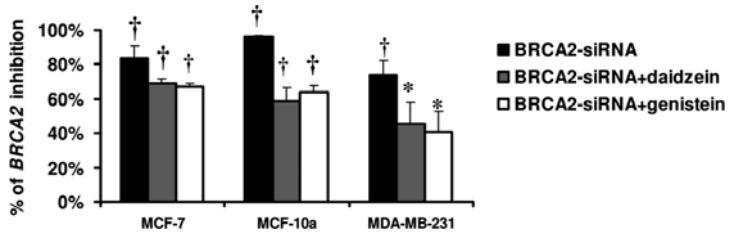

Figure 1. Effect of BRCA2 depletion on gene targeting, expressed as the percent of BRCA2 inhibition measured by RT-qPCR in MCF-7, MCF-10a and MDA-MB-231 breast cell lines after daidzein or genistein 72-h treatments by comparison to control cells without any treatment corresponding to 0% of inhibition. Values are means ± standard error of three replicate experiments. Student's t-test was performed (*P<0.05; †P<0.01).

Table I. Gene average of *BRCA1* and *BRCA2* after genistein or daidzein treatment in MCF-7, MDA-MB-231 and MCF-10a knocked down with *BRCA2*-siRNA.

	BRCA1			*BRCA2*		
	Genistein	Daidzein	Adjusted p-value	Genistein	Daidzein	Adjusted p-value
MCF-7	2.0±0.07	2.6±0.18	0.004[a]	5.8±2.2	6.4±2.1	0.6 (n.s.)
MDA-MB-231	1.4±0.4	2.4±0.1	0.05 (n.s.)	3.0±1.06	9.4±1.4	0.007[a]
MCF-10a	1.5±0.4	2.5±0.3	0.1 (n.s.)	1.5±0.4	2.5±0.2	0.1 (n.s.)

Rate calculation. In order to find if there is a difference between ER-positive α or ß and ER-negative α or ß cell lines, we first performed a hierachical clustering and a t-test was used to find significantly different expression of genes between the treatments. We used the Pearson correlation and a cut-off of 2-fold. A p-value was calculated to show if there was a significant difference between each condition. Therefore, significantly expressed genes were classified according to implicated biological pathways by using EASE version 2.0 software (10) and Gene Ontology (GO) database.

Results

Knock down of BRCA2 transcript levels in cell lines by Q-PCR. Quantification was carried out for MCF-7, MCF-10a and MDA-MB-231 human breast cell lines in order to measure the specific *BRCA2*-siRNA inhibition on *BRCA2* expression. Relative gene expression was determined using the comparative CT (threshold cycle) method consisting of normalizing the number of target gene copies to the endogenous reference gene (*18S* rRNA), designated as the calibrator. The amount of mRNA in transfected cell lines, whether treated or not with phytoestrogens was then normalized to control cells without any intervention. In Fig. 1, results are presented with the mean ± standard deviation with three replications. MCF-7, MCF-10a and MDA-MB-231 cells, after transfection by specific *BRCA2*-siRNA, revealed 84, 96 and 74% inhibition in *BRCA2* expression, respectively as compared to control cells. These results were statistically significant with p<0.01 according to the Student's t-test (11).

Discrimination according to the ER cell status. With pangenomic microarrays, using TIGR MeV version 4.2.02 software (The Institute for Genomic Research), hierarchical clustering was performed and followed with a t-test analysis. We carried out 2 discriminations according to estrogen receptor isoform α (Fig. 2A) or ß (Fig. 2B). For each discrimination, we defined 2 groups. The first group was cells exhibiting ERα (MCF-7) or not exhibiting it (MDA-MB-231; MCF-10a). The second group was cells exhibiting ERß (MCF-7; MDA-MB-231) or not exhibiting it (MCF-10a). Results showed 888 differentially expressed genes between positive-ERα cells and negative-ERα cells (data not shown) and 35 differentially expressed genes between positive-ERß cells and negative-ERß cells (Fig. 2). Moreover, we found 3 common differentially expressed genes between the 2 discriminations: ATP11A, GBP3, STK16.

Discrimination according to isoflavone treatment. We also performed discrimination according to genistein or daidzein treatment in MCF-7 (Fig. 3A), MDA-MB-231 (Fig. 3B) and MCF-10a (Fig. 3C) previously knocked down with *BRCA2*-siRNA. For MCF-7, hierarchical clustering showed 256 differentially expressed genes between genistein and daidzein treated cells. For MDA-MB-231, we observed 95 differentially expressed genes between genistein and daidzein treated cells and 392 differentially expressed genes between genistein and daidzein treated MCF-10a cells. Among these genes, we found 14 common differentially expressed genes between the three cell lines: *AES*, *BRIP1*, *FAF1*, *HNRPA3*, *HOMER1*, *MAP4K5*, *MAPK3*, *PPAP2C*, *PPAT*, *RAP2B*, *RPS2*, *TP53*, *ZNF442*, *ZNF443* (Fig. 3).

Significant results were observed in *BRCA1* and *BRCA2* expression after genistein or daidzein treatment (Table I) and respectively in MCF-7 and MDA-MB-231 (Fig. 4). In MCF-7, we noted a 2-fold change ± 0.07 of *BRCA1* expression after genistein treatment and a 2.6-fold change ± 0.18 after daidzein treatment (Fig. 4). Nevertheless, *BRCA2* expression was not influenced in MCF-7 after phytoestrogen supplementation (Table I). In MDA-MB-231, *BRCA1* expression was not

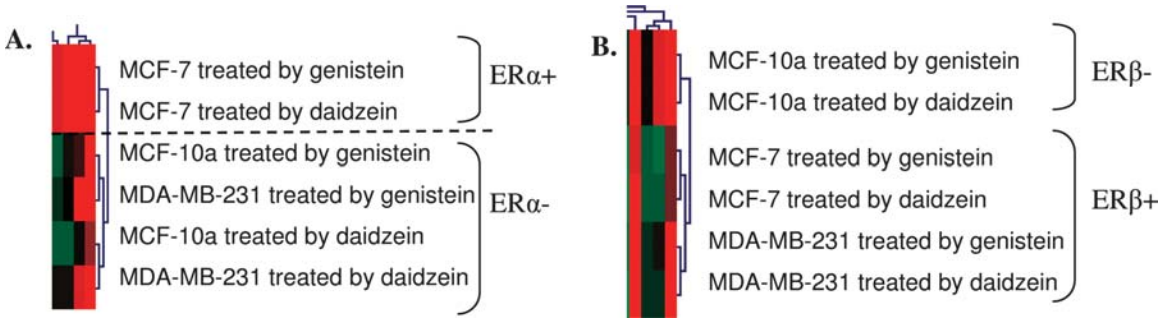

Figure 2. Hierarchical clustering after ER discrimination according ERα (A) or ERß (B) between MCF-7 ($ER\alpha^{+}/ß^{+}$), MDA-MB-231 ($ER\alpha^{-}/ß^{+}$) and MCF-10a ($ER\alpha^{-}/ß^{-}$) knocked down with *BRCA2*-siRNA.

Discrimination according to ERß cell status: 35 differentially expressed genes between positive-ERß cells (MCF-7) and negative-ERß cells (MDA-MB-231 and MCF-10a). In bold type, common genes (3) expressed according to both ERα and ERß discriminations. Significant expressed genes were discriminated using a t-test and we confirmed our results by calculating adjusted p-value using the SAM method (Significant Analysis of Microarray) to attest significant effect between genistein and daidzein treatment. Gene ontology (GO) was used to identify implicated biological process.

Gene name	GO biological process	Adjusted p-value
AQP8	Water transport	0.0047
ATP11A	Ion transport	0.0066
CCL17	Inflammatory response	0.0042
CD1D	Antigen presentation	0.0040
CDKN2A	Cell cycle arrest	0.0094
CYP2A7	Ion transport	0.0070
DMP1	Ossification	0.0065
ECE2	Regulation of G-protein	0.0050
EDG3	Positive regulation of cell proliferation	0.0021
FLJ20035	Regulation of translation	0.0049
GBP2	Immune response	0.0092
GBP3	Immune response	0.0031
GM2A	Glycolipid catabolism	0.0015
GPR103	Regulation of G-protein	0.0019
GRIN3A	Ion transport	0.0040
HCN1	Potassium sodium ion transport	0.0082
IL1A	Regulation of cell cycle	0.0036
KIAA1706	Differenciation	0.0098
KLHL4	Actin cytoskeleton organization and biogenesis	0.0034
OR2C1	Regulation of G-protein	0.0089
POLB	DNA repair	0.0070
PRKACA	Protein amino acid phosphorylation	0.0026
RER1	Retrograde (Golgi to ER) transport	0.0073
RYR1	Calcium ion transport	0.0044
SCN3B	Sodium ion transport	0.0066
SCNN1D	Sodium ion transport	0.0088
SERPINB5	Cell motility	0.0081
SLC38A4	Amino acid transport	0.0042
SORBS1	Cell-matrix adhesion	0.0053
STK16	Protein amino acid phosphorylation	0.0092
TAT	Biosynthesis	0.0083
TCF1	Regulation of transcription from Pol II promoter	0.0058
TCF7	Immune response	0.0084
TNFSF12	Angiogenesis	0.0041
TRPM3	Ion transport	0.0051

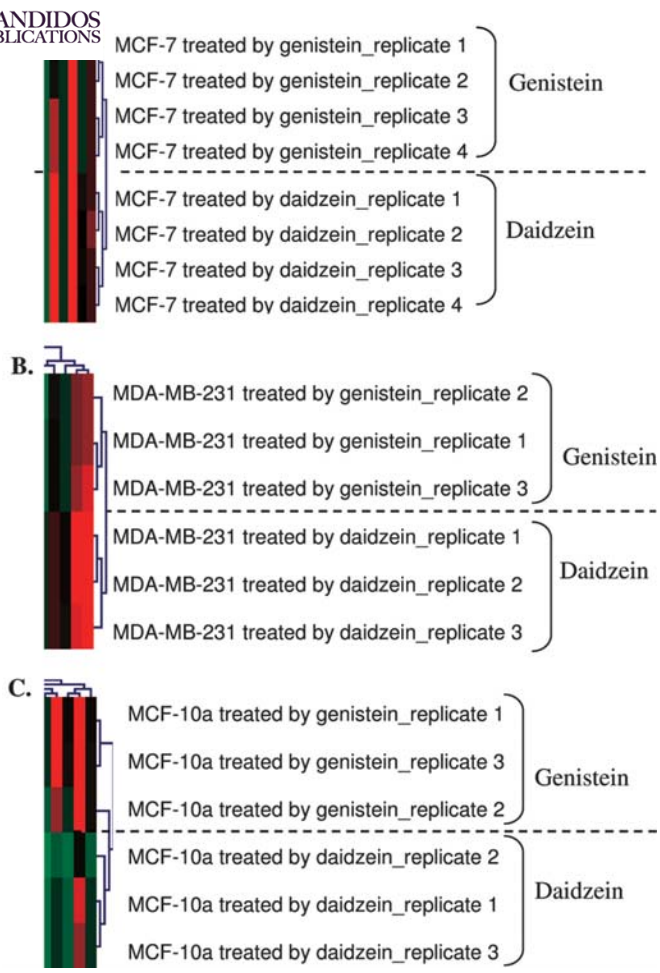

Figure 3. Hierarchical clustering after discrimination according to genistein or daidzein treatment in MCF-7 (A), MDA-MB-231(B) and MCF-10a (C) knocked down with *BRCA2*-siRNA.

Discrimination according to phytoestrogen treatment: 14 common differentially expressed genes between MCF-7, MDA-MB-231 and MCF-10a cells knocked down with BRCA2-siRNA. Significant expressed genes were discriminated using a t-test and an adjusted p-value was calculated using the SAM method to attest significant effect between genistein and daidzein treatment. Gene ontology (GO) was used to identify implicated biological process.

Gene name	GO biological process	Adjusted p-value
AES	Regulation of transcription	0.0016
BRIP1	DNA repair	0.0064
FAF1	Apoptosis	0.0093
HNRPA3	RNA-nucleus export	0.0053
HOMER1	Regulation of synapse	0.0093
MAP4K5	Response to stress	0.0041
MAPK3	Regulation of cell cycle	0.0097
PPAP2C	Germ-cell migration	0.0056
PPAT	Glutamine metabolism	0.0003
RAP2B	Small GTPase mediated signal transduction	0.0045
RPS2	Protein biosynthesis	0.0057
TP53	DNA repair	0.0027
ZNF442	DNA repair	0.0005
ZNF443	Apoptosis	0.0068

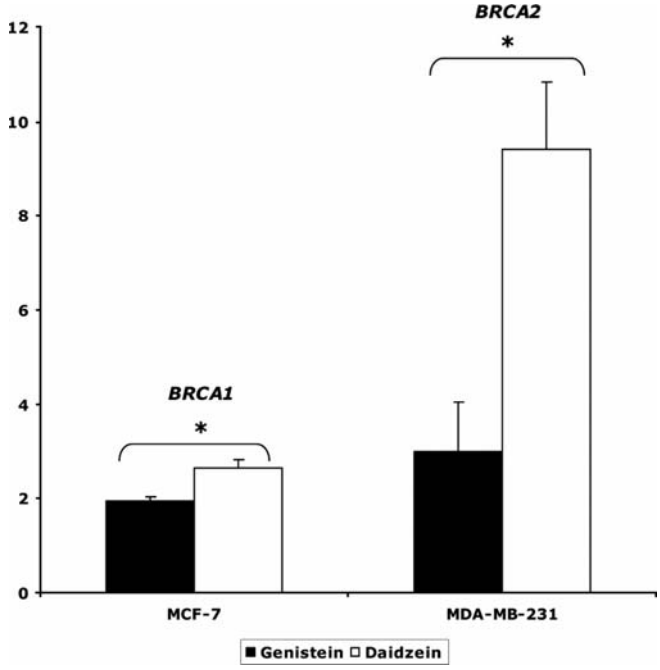

Figure 4. Differences in *BRCA1* and *BRCA2* fold change between genistein and daidzein treatment, respectively in MCF-7 or MDA-MB-231 knocked down with *BRCA2*-siRNA.

affected either (Table I), but *BRCA2* expression was increased by 3±1.06 after genistein supplementation and increased by 9.4±1.4 with daidzein (Fig. 4). There was no significant effect on *BRCA1* and *BRCA2* expression after genistein or daidzein treatment in the fibrocystic breast cell line MCF-10a (Table I) but we observed a significant decrease in *BAX* and *BCL2* expression (Fig. 5A). Moreover, we noted a higher effect of daidzein than genistein on *BAX* and *BCL2* expression (Fig. 5B). We also found a differential *BRIP* expression between genistein and daidzein treatment, more importantly with daidzein (Fig. 6) in MCF-7 and MDA-MB-231 cell lines.

Discussion

Our objective was to understand the effects of the transcient ablation of *BRCA2* gene expression in human breast cell lines (MCF-7, MDA-MB-231 and MCF10a) combined with phytoestrogen treatment (18.5 μM genistein or 78.5 μM daidzein), also, to investigate the consequence on the whole genome by pangenomic microarrays.

Other researches have previously performed studies on the molecular consequences of transient or stable *BRCA2* deficiency in human breast cell lines (12) and in mice using siRNA (13).

Tripathi and Chaudhuri (12) reported observations on the effect of transient depletion of *BRCA2* gene expression on human mammary epithelial cells (HMEC) and BT549 human breast carcinoma cells. They performed microarray analysis of the mRNAs isolated from these *BRCA2* knocked down cells as well and showed a limited number of 14 significant genes that are down-regulated as an immediate consequence.

To study this, we used RNA interference in human breast cells (MCF-7, MCF-10a, and MDA-MB-231) to down-

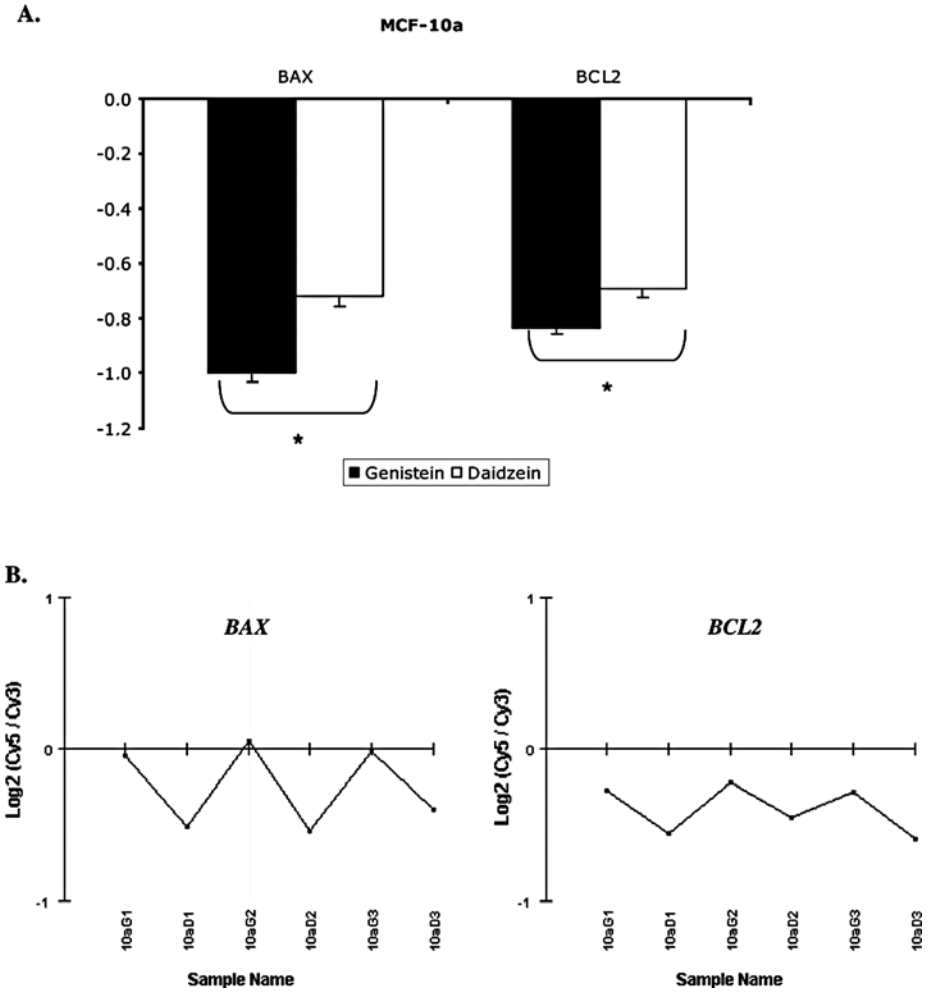

Figure 5. Fold change (A) and Log2 expression (B) of BAX and BLC2 between genistein and daidzein MCF-10a treated cells after the knocked down with *BRCA2*-siRNA.

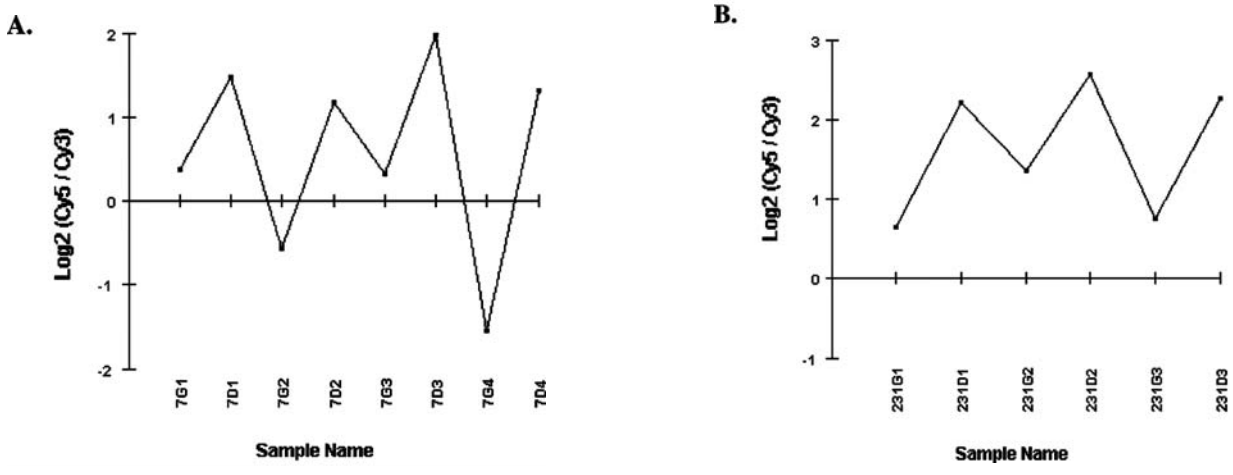

Figure 6. Log2 expression of *BRIP* in MCF-7 (A) and MDA-MB-231 (B) cell lines after genistein or daidzein treatment.

regulate *BRCA2*. Genistein and daidzein 72-h treatments were added to this transitory loss of function, and we performed a global gene expression profiling by DNA microarray analysis. The isoflavones were added to observe their effect on the *BRCA2* ablation. The breast cell lines used here exhibit different ER status, so we compared gene expression changes to ER status as well as to genistein or daidzein treatment. After a treatment discrimination, the MCF-7 (ERα^{+}/ß$^{+}$) cell line showed a significant up-regulation of the onco-suppressor *BRCA1* transcript after *BRCA2* specific knock down and additional treatment by either genistein or daidzein isoflavones. These results were in comparison to the control

Under the same analysis conditions, we found also a slight up-regulation of *BRCA2* mRNA in MDA-MB-231 ($ER\alpha^-/ß^+$) cell line, and modulations of the apoptosis-related genes *BAX* and *BCL2* in MCF-10a ($ER\alpha^-/ß^-$) cell line. Moreover, discrimination according to phytoestrogen treatment revealed 14 common differentially expressed genes between the three cell lines, including the *BRIP1* and *P53* genes.

Chromosome aberrations in *BRCA2*-deficient cells have been attributed to loss of normal control by *BRCA2* of the eukaryotic strand exchange protein RAD51 required during the S phase for DNA repair and homologous recombination (14,15). Abnormal cytokinesis has been also suggested to explain chromosomal instability and aneuploidy in cells deficient of BRCA2 (16). Our results suggest a potential role of phytoestrogens to limit genome instability in *BRCA2* deficient cells by promoting *BRCA1* and *BRCA2* expressions.

On the basis of its interaction with *BRCA1*, the tumor suppressor *BRIP1* is also considered a potential breast cancer susceptibility gene. The DEAH helicase family member BRIP1 was first identifed as a 130-kDa protein directly interacting with the C-terminal (BRCT) domains of BRCA1; hence, the name BRIP1 (BRCA1-interacting protein). The BRIP1/BRCA1 interaction was found to contribute to the DNA repair activity of BRCA1 (17). In our study, *BRIP1* was found significantly overexpressed after genistein and daidzein treatments under *BRCA2* knock down conditions in comparison to the control group. Recently, it has been reported that BRCA1/BRCA2-associated BRIP1 activation was required for progression through the S phase, and it has been shown that depletion of *BRIP1* as well as *BRCA1* by RNA interference, resulted in delayed G_1/S progression (18). Our results showed an up-regulation of *BRIP1* after phytoestrogen treatments under *BRCA2* knock down conditions, and also suggested a plausible link between *BRCA2*, *BRIP1* and phytoestrogen pathways. Further investigation will certainly help to gain insights into the role of *BRIP1* in the BRCA-breast cancer pathways and its tumor suppression function.

In our study, we obtained a down-regulation of *BCL2* and *BAX* gene expressions after phytoestrogens treatments under *BRCA2* knockdown conditions. In a recent study, Castro *et al* reconstructed the evolutionary scenario that linked apoptosis with genome stability pathways in a functional human gene/protein association network (19). They demonstrated how genome stability and apoptosis were co-opted during evolution recruiting genes that merge both systems. They found the apoptosis-related *BAX* and *BCL2* genes as important to cancer as the *BRCA1*, *BRCA2*, and *P53* genes. Our results corroborate their analysis and suggest a link between apoptosis, BRCA-breast cancer and phytoestrogen pathways.

In conclusion, our results showed a limited number of genes that are modulated as an immediate consequence of the decrease of *BRCA2* and phytoestrogen treatments. A number of important breast cancer genes (*BRCA1*, *BRCA2*) and apoptosis-related genes (*BAX*, *BCL2*) have also been modulated by this transitory oncosuppressor knock down and sensitivity revealed to the phytoestrogen supplementations. Taken together, these results suggested a potential chemopreventive effect of phytoestrogens in promoting apoptosis and maintenance of genome stability.

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