Genomics & Informatics Vol. 3(4) 154-158, December 2005

X-linked Gene Expression Profiles by RNAi-Mediated BRCA1 Knockdown in MCF7 Cells

Min-Ae Song[1], Jung-Hoon Park[1], Hee-Jeong Ahn[3], Jung-Jae Ko[2] and Suman Lee[1]*

[1]Functional Genomics Lab, [2]CHA Research Institute, Bundang Campus, Pochon CHA University College of Medicine, [3]Department of Pathology, Bundang CHA Hospital, Pochon CHA University College of Medicine, 222 Yatap-Dong, Bundang-Gu, Sungnam-Si, Gyeonggi-Do 463-836, Korea

Abstract

Germ-line mutations of the BRCA1 gene confer an increased risk for breast and ovarian cancers. BRCA1 in female cells is directly related with the maintenance of the inactive X chromosome (Xi). The effect by the loss of the BRCA1 function on the X chromosome gene expression remains unclear in cancer cells. We attempted to investigate the expression pattern of the X-linked genes by performing BRCA1 knockdown via RNA interference in the MCF 7 breast cancer cell line. The transcriptional and translational levels of BRCA1 were decreased over 95% in the MCF 7 cells after BRCA1 knockdown. The expression patterns of one hundred ninety X-linked genes were profiled by the X chromosome-specific cDNA arrays. A total of seven percent of the X-linked genes (14/190) were aberrantly expressed by over 2-fold in the MCF7-BRCA1 knockdown cells, which contained two up-regulated genes (2/190, 1%) and 12 down-regulated genes (12/190, 6.3%). It is interesting that 72% of the aberrantly expressed X-linked genes were located on the Xq (10/14,) region. Our data suggests that BRCA1 may not be important to maintain X chromosome inactivation in cancer because the BRCA1 knockdown did increase the expression of the only one percent of X-linked genes in the human breast cancer cells.

Keywords: BRCA1; RNA interference (RNAi); X chromosome-specific cDNA microarrays; MCF7

Introduction

Breast cancer is a complex disease and a variety of risk factors are involved in conjunction with the observed the BRCA1 germline mutations in familial breast cancer. Women with a mutation in one allele of the BRCA1 gene have higher rates of breast, ovarian and other cancers (Easton *et al.*, 1993). The hereditary breast cancers have a different gene expression pattern than do the sporadic cancers (Hedenfalk *et al.*, 2003).

BRCA1 has multiple functions for DNA repair, maintenance of genomic integrity, transcription regulation and cancer- suppression (Venkitaraman 2002). BRCA1 mutation can regulate the expression of the various genes such as oestrogen-receptor, ERBB2, p53 and cyclin D1 in familial cancer (Narod *et al.*, 2004). However, BRCA1 mediated transcriptional regulation is not well understood. The overexpression of BRCA1 induced the genes related with the DNA damage-response, the apoptotic pathway, the estrogen-response and the cytokine signal transduction pathway (Welcsh *et al.*, 2002). The expression pattern of the downstream genes according to the loss of BRCA1 is not clear. BRCA1 may change the other gene expressions by direct or indirect protein interactions with RNA polymerase II, helicase A and etc. (Rosen *et al.*, 2003).

BRCA1 in female cells is directly related with the maintenance of the inactive X chromosome (Xi) (Ganesan *et al.*, 2004). BRCA1 may be related to the expression of the X chromosome genes. Xi-specific transcript (XIST) is located within the X-inactivation center (XIC) in the female somatic cells, and it plays a crucial role in Xi. Silencing of Xi is initiated at one location on the XIC. The coating of XIST on Xi is supported by BRCA1 because BRCA1 defect cancers show the Xi chromatin structural defects (Ganesan *et al.*, 2002). The role of BRCA1 as related with the XIST location in breast cancer suggests that the BRCA1 is a key point to maintain the Xi in breast cancer (Sirchia *et al.*, 2005).

To study the differential expression pattern of X-linked genes in BRCA1-associated breast cancers, we attempted to investigate the change of expression of the 190 X-linked genes by inducing BRCA1 knockdown in a human breast cancer cell line having the wild type BRCA1, MCF7 by performing an X chromosome specific cDNA microarray. Microarray technology has been used for the analysis of genome-wide gene expression profiles, and it is a powerful tool for analyzing the variety genes expression of samples.

*Corresponding author: Email suman@cha.ac.kr, Tel +82-31-725-8372, Fax +82-31-725-8398
Accepted 25 November 2005

Materials and Methods

BRCA1 RNA interference (RNAi) expression vector

The mammalian expression vector, pSuper.retro.puro. (Oligoengine, Seattle, WA) was used for examining the expression of RNAi in MCF7 cells. The construct was generated by annealing the following complementary oligonucleotides. The synthesized oligonucleotides were forward, 5`-G ATC CCC **GAA AGT ACG AGA TTT AGT C** TT CAA GAG A**GA CTA AAT CTC GTA CTT TC**T TTT TGG AAA-3`, and reverse, 5'-AG CTT TTC CAA AAA **GAA AGT ACG AGA TTT AGT** CTC TCT TGA A**GA CTA AAT CTC GTA CTT TC**G GG-3', and they were inserted into the pSUPER vector at the Bgl II and HindIII sites (Fabbro *et al.*, 2004)

Cell culture and transfection

Human breast cancer MCF7 cells were obtained from American Type Culture Collection (Rockville, MD). Cells were grown in RPMI-1640 medium (Gibco BRL, Cergy Pontoise, France), and this was supplemented with 10% fetal bovine serum, 1% penicillin and 1% streptomycin. The cells were seeded onto 60 cm^2 dishes and then transfected at 70-80% confluence with 8 ug of plasmid DNA by using Lipofectamin 2000 reagent according to the manufacture's instructions (Invitrogen, Carlsbad, CA, U.S.A). The cells were analyzed at 72 h post-transfection with performing puromycin selection.

Total RNA isolation and quantitative real-time RT-PCR

The total RNA was isolated from the cultured cells with using QIAGEN RNA-Mini kits according to the manufacture's instructions (Qiagen, Valencia, CA). All the RNA preparations were treated with DNase I (Roche, Boehringer-Mannhein, Germany). For the RNA analysis, equivalent amounts of total RNA served as the template for cDNA synthesis with using reverse-transcriptase (Invitrogen, Carlsbad, CA, U.S.A). Real-time RT-PCR assays were carried out in a fluorometric thermal cycler (Rotor-Gene 2000, Corbett Research, Australia). For performing the quantitative real-time RT-PCR, SYBR-Green (Qiagen, Valencia, CA) was added to the reaction mixture, and the fluorescence was measured after each cycle. After the native cDNA copy number of each gene was calculated with using the comparative ΔΔCT method for each sample, the relative ratios of the tested gene for *GAPDH* were generated. The date is shown as the mean and standard deviation values of the three measurements per data point.

Western blotting

Whole-cell extracts were prepared from the cell lysates in a buffer that contained 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 1 mM PMSF, 5 mM EDTA and 4 mg/ml of protease inhibitors cocktail (Sigma, MO, USA). The samples were separated on 8% SDS polyacrylamide gels and then blotted onto PVDF membranes. The primary antibodies used were anti-BRCA1 (C-20, Santa Cruz Biotechnology, 1:200) and anti-β-actin (AC-15, Sigma, 1:5,000). After incubation with horseradish peroxidase-coupled secondary antibodies (anti-rabbit, Upstate, 1:1000 or anti-mouse, Calbiochem, 1:1000), the signals were detected by performing chemiluminescence with using ECL (Amersharm, Pharmacia, Piscataway, NJ).

X chromosome-specific cDNA microarrays

One-hundred ninety X-linked clones were provided by KRIBB (Korea Research Institute Bioscience and Biotechnology), and the clone information is listed in Table 1. Those clones were spotted in a X-linked cDNA microarray from Digital Genomics (Digital Genomics, Seoul, Korea). The RNA was prepared by using RiboAmp RNA amplification kits according to the manufacture's instruction (Arcturus, Carlsbad, CA). Each 3 μg of aRNA (antisense RNA) was labeled with either Cy3-UTP or Cy5-UTP (NEN Life Science Products, Boston, MA, U.S.A.) during the reverse transcription (RT) (Invitrogen, Carlsbad, CA, U.S.A). After hybridization, the slide was dried by centrifugation, and then it was scanned on a ScanArray 4000XL (Packard Bioscience, Billerica, MA, U.S.A.). The unified image was quantified with using ImaGene™ version 4.0 (BioDiscovery, Inc., Los Angeles, CA, U.S.A.). Data analysis were performed using the GeneSight™ 3.10 (BioDiscovery, Inc., Los Angeles, CA, U.S.A.).

Table 1. X Chromosomal Gene List of the cDNA Microarray

Gene	locus	Gene	locus	Gene	locus
GRIPAP1	Xp11	P17.3	Xp11.3	ASMTL	Xp22.3
ELK1		DDX3X		MOSPD2	Xp22.31
HADH2	Xp11.2	OATL1	Xp11.3-p11.23	FAM9C	
RBM3		PCTK1		STS	Xp22.32
UTX		TIMP1		CD99	
DT1P1A10		CASK		SLC25A6	
FLJ10613	Xp11.22	MGC39350		PRPS2	Xp22.3-p22.2
TFE3		MID12	Xp11.4	HEPH	Xq11-q12
UREB1		TM4SF2		FLJ12525	Xq12-q13
JARID1C	Xp11.22-p11.21	USP9X		OGT	
SMC1L1		ARAF1	Xp11.4-p11.2	PGK1	Xq13
DKFZp761A052		TCTE1L	Xp21	PIN4	
FLJ21687	Xp11.23	CA5B	Xp21.1	TNRC11	
FTSJ1		ZFX	Xp21.3	DLG3	
GPKOW		CTPS2	Xp22	KIF4A	Xq13.1
HDAC6		SEDL		LOC389866	

Table 1. Continued

Gene	locus	Gene	locus	Gene	locus
JM4		PIGA	Xp22.1	LOC51248	
PIM2		SAT		NONO	Xq13.1
PLP2		ACATE2		PJA1	
PQBP1		EIF1AX	Xp22.13	RPS4X	
RBM10		PRDX4		IGBP1	Xq13.1-q13.3
SUV39H1	Xp11.23	RAB9A	Xp22.2	MGC874	Xq13.2
TIMM17B		CXorf15		COX7B	
TMEM29		RBBP7	Xp22.22	DKFZp564K142	Xq13.3
UBE1		REPS2		SH3BGRL	
USP11		SYAP1		RNF12	Xq13-q21
WDR13		EIF2S3		ATP6AP2	Xq21
WDRX1		PDHA1	Xp22.2-p22.1	BRODL	Xq21.1
UBQLN2	Xp11.23-p11.1	PHKA2		CHM	Xq21.2
EBP		OFD1	Xp22.2-p22.3	PHKA1	Xq21-q13
SLC35A2	Xp11.23-11.22	GYG2	Xp22.3	PRPS1	Xq21-q27
UXT		MSL3L1		GLA	Xq22
HNRPH2		RAP2C	Xq25	DKC1	
KU008456	Xq22	STAG2		DNASE1L1	
MORF4L2		APLN	Xq25-26.3	DXS9879E	
TM4SF6		PDCD8	Xq25-q26	F8A	
CSTF2		UPF3B		FAM11A	
RPL36A	Xq22.1	OCRL	Xq25-q26.1	FAM3A	
TCEAL1		COVA1	Xq25-q26.2	FLNA	
FLJ12969	Xq22.1-q22.3	THOC2	Xq25-q26.3	GDI1	
LOC139231	Xq22.2	ELF4	Xq26	HCBP6	
NGFRAP1		MAGEC1		HCFC1	
DSIPI		GPC4		IDH3G	
MGC44287	Xq22.3	HPRT1	Xq26.1	IDS	
PSMD10		RBMX2		IKBKG	
RNF128		HTATSF1	Xq26.1-q27.2	IRAK1	
NXF3	Xq22-23	MST4	Xq26.2	LOC91966	Xq28
RPL39		DKFZP564B147		MAGEA3	
MCTS1	Xq22-q24	LOC159090		MAGEA4	
PGRMC1		LOC159091		MECP2	
CUL4B		LOC389890	Xq26.3	MGC15827	
FLJ22965		MGC27005		MPCP1	
KIAA1701	Xq23	MOSPD1		MPP1	
MDS031		VGLL1		MTMR1	
NXT2		ZNF75		NSDHL	
ZBTB33		FMR1	Xq27.3	SLC10A3	
DKFZp686L20145		ABCD1		SLC6A8	
IL13RA1	Xq24	ARD1		SSR4	
LAMP2		ARHGAP4		TRAG3	
NDUFA1		ATP6AP1	Xq28	UBL4	
UBE2A	Xq24-q25	BCAP31		VBP1	
SLC25A5	Xq24-q26	BGN		ZNF275	
C1GALT2	Xq25	CETN2			
HDGF		CXorf12			

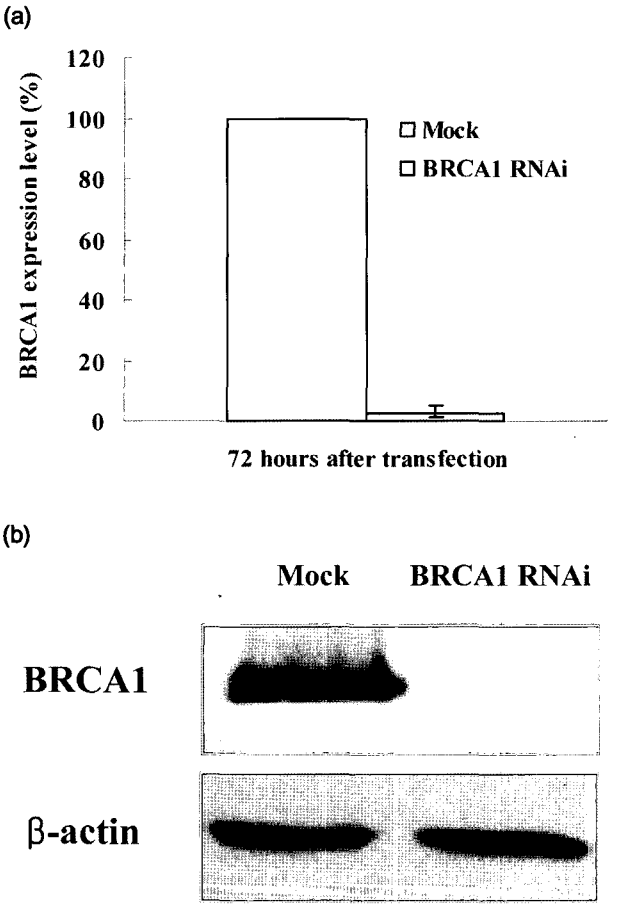

Fig. 1. BRCA1-RNAi can reduce the expression level of BRCA1 in MCF7 cells. (a) MCF7 cells were transfected with BRCA1-RNAi or mock. Cells were treated with pSuper. retro.puro-BRCA1 for 72 h. Quantitative real-time RT-PCR analysis of the BRCA1 mRNA by comparing the BRCA1 knockdown cells with the mock-transfected MCF7 cells. (b) Total cells lysates of BRCA1-RNAi and mock-transfected cells. The BRCA1 protein was separated by western blotting analysis; expression levels were normalized for loading by probing for β-actin.

Results

BRCA1 knockdown by the RNAi method

We used RNAi for examining the gene expression change according to BRCA1 knockdown in the MCF7 cells. The RNAi was produced by employing the pSuper.retro.puro-vector system, which was indicated in the material and methods section (Fabbro *et al.*, 2004). Successful use of this technology depends on effective mRNA degradation and the protein turnover. Bae *et al* reported the knockdown of BRCA1 by using the RNAi method in MCF7 cells (Bae *et al.*, 2004). We used the same vector with identical sequences for the BRCA1 knockdown.

We assessed the silencing effect BRCA1-RNAi on its target mRNA by RT-PCR and Western blotting. Real-time RT-PCR data (Fig. 1a.) showed that BRCA1 mRNA level was markedly downregulated in MCF7 cells transfected with BRCA1-RNAi for 72 h. The Y axis indicates the expression level of BRCA1 mRNA that was normalized by using GAPDH. The data are presented as the mean ± SE of the individual values from three separate experiments. The knockdown level of the BRCA1 protein was also confirmed by western blot analysis (Fig. 1b). BRCA1 protein was not detected at

72 hrs after transfection. The 97% BRCA1 knockdown was shown at both the transcriptional and translational levels.

X-linked gene expression patterns by cDNA microarray

We performed the cDNA microarray to identify the expression pattern of the X-linked genes according to BRCA1 knockdown. Our X-linked cDNA microarray contained 190 X-linked genes; the lists of genes are shown in Table 1. The total RNA was extracted from both BRCA1-RNAi and mock-transfected MCF7 cells. The probe was generated via RT-PCR from the BRCA1-RNAi samples and the mock-transfected samples, respectively. We first compared the X-linked gene expression by performing BRCA1 knockdown. To verify the different incorporation rates of the Cy-3 and Cy-5 dye, we swapped the fluorescent dye (Fig. 2a). The schematic diagram for the change of the expression levels of the X-linked genes was according to the X chromosomal physical map (Fig. 2b). The overall differentially expressed genes between the BRCA1-RNAi and the mock-transfected MCF7 cells were described according to the X chromosome loci (Fig. 2b). The Y axis represents the expression level ($\log_2$ ratio>1, $\log_2$ ratio>-1) in the BRCA1-RNAi transfected MCF7 cells. Similar results were shown by the three repeated experiments. A total of seven percent of the 190 X-linked genes (14/190) were differentially expressed, and 28% were at Xp and 72% were at Xq. Two genes (2/190, 1%), RBM10 and VBP1, were up-regulated, and 12 genes (12/190, 6.3%), UBE1, OFD1, GYG2, NONO, TAF9L, TSPAN6, MCTS1, MTMR1, FAM3A, GDI1, CXorf40 and SLC10A4, were down-regulated by over 2-fold in the BRCA1-RNAi transfected MCF7 cells.

Discussion

The genotypes of hereditary breast cancers are reflected in the different gene expression profiles (Rosen *et al.*, 2003). However, the expression pattern of the X-linked

Table 2. List of the X-linked genes that were Differentially Expressed by 2-fold or Greater

Assession	Gene symbol	Gene name	Cytogenetic band	Pair mean ratio
A K025854	RBM10	RNA binding motif protein 10	Xp11.23	1.3578
NM_003372	VBP1	von Hippel-Lindau binding protein 1	Xq28	1.6021
NM_003334	UBE1	ubiquitin-activating enzyme E1	Xp11.23	-1.6372
NM_003611	OFD1	oral-facial-digital syndrome 1	Xp22.2-p22.3	-1.4995
NM_003918	GYG2	glycogenin 2	Xp22.3	-3.5103
BC010049	NONO	non-POU domain containing, octamer-binding	Xq13.1	-1.0469
NM_01575	TAF9L	TAF9-like RNA polymerase II, TATA box binding protein	Xq13.1-q21.1	-1.4446
NM_003270	TSPAN6	tetraspanin 6	Xq22	-1.1116
NM_014060	MCTS1	malignant Tcell amplified sequence 1	Xq22-q24	-1.3775
AF057354	MTMR1	myotubularin related protein 1	Xq28	-1.0619
AK024601	FAM3A	family with sequence similarity 3, member A	Xq28	-1.2583
AL833629	GDI1	GDP dissociation inhibitor 1	Xq28	-1.4218
AY074889	CXorf40	chromosome X open reading frame 40	Xq28	-1.1926
NM_019848	SLC10A3	solute carrier family 1 (sodium/bile acid cotransporter family), member 3	Xq28	-1.7118

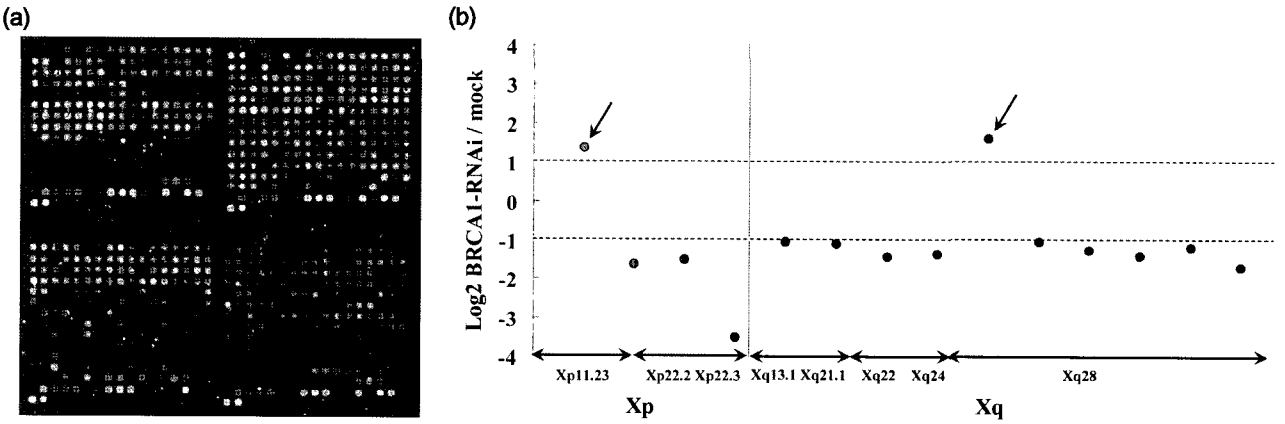

Fig. 2. The differential gene expression was monitored by X chromosome-specific cDNA arrays by performing hybridization with the BRCA1-RNAi and mock-transfected MCF7 cells. (a) Two dye-swapped microarray experiments were performed for comparing the BRCA1-RNAi to the mock-transfected MCF7. A similar result was shown by the three repeated experiments. (b) The 14 circles indicate the differentially expressed genes with the X chromosomal location. Each circle represents a single gene. The X axis displays the loci of the X chromosomal genes. The up-regulated two genes, RBM10 (Xp11.23) and VBP1 (Xq28), are indicated by the arrows.

genes is incompletely understood in BRCA1-related breast cancer. This study is the first data concerning the expression pattern of the X-linked genes after BRCA1 knockdown with using RNAi by performing DNA microarray experiments. After the BRCA1 knockdown, we experimented with the cDNA microarray to identify the expression pattern of the X-linked genes.

A new function for BRCA1 was reported in the heterochromatin formation, especially in the inactive X chromosome (Ganesan *et al.*, 2002). However, it remains unclear whether BRCA1 modulates the expression of the X-linked genes to a global (affecting the entire X chromosome) or specific degree. Welcsh *et al.* have reported the upregulation of the XIST RNA levels, and this was followed by the BRCA1 expression (Welcsh *et al.*, 2002). However, we found that the XIST expression was not changed by BRCA1 knockdown (data not shown), even though the XIST expression was low in the MCF7 cells. Sudbral *et al.* employed X chromosome-specific cDNA arrays to identify the genes that escaped from the X-inactivation (Sudbrak *et al.*, 2001). We also employed X chromosome-specific cDNA arrays that spotted 190 X chromosome genes to identify the differentially expressed X-linked genes according to the BRCA1 knockdown (Jeong *et al.*, 2004). However, almost all the X-linked genes were not changed by BRCA1 knockdown in the MCF7 cells.

Our data is the first to report the X-linked gene expression according to BRCA1 knockdown. We suggest that BRCA1 may not involve the X-linked gene expression, at least the MCF7 cancer cells. We supposed that both the X chromosomes of the MCF7 human breast carcinoma cell line may have active (Sirchia *et al.*, 2005). Further investigations are needed to determine whether BRCA1 is related to Xi through such epigenetic mechanisms as chromatin modification.

Acknowledgments

This study was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (01-PJ10-PG6-01GN13-0002).

References

Easton, D., Ford, D., and Peto, J. (1993). Inherited susceptibility to breast cancer. *Cancer Surv.* 18, 95-113.

Hedenfalk, I., Ringner, M., Ben-Dor, A., Yakhini, Z., Chen, Y., Chebil, G., Ach, R., Loman, N., Olsson, H., Meltzer, P., Borg, A., and Trent, J. (2003). Molecular classification of familial non-BRCA1/BRCA2 breast cancer. *Proc. Natl. Acad. Sci. USA* 100, 2532-2537.

Venkitaraman, A. R. (2002). Cancer susceptibility and the functions of BRCA1 and BRCA2. *Cel.* 108, 171-182.

Narod, S.A. and Foulkes, W.D. (2004). BRCA1 and BRCA2: 1994 and beyond. *Nat. Rev. Cancer* 4, 665-676.

Welcsh, P.L., Lee, M.K., Gonzalez-Hernandez, R.M., Black, D.J., Mahadevappa, M., Swisher, E. M., Warrington, J. A., and King, M. C. (2002). BRCA1 transcriptionally regulates genes involved in breast tumorigenesis. *Proc. Natl. Acad. Sci. U S A* 99, 7560-7565.

Rosen, E.M., Fan, S., Pestell, R.G., and Goldberg, I.D. (2003). BRCA1 in hormone-responsive cancers. *Trends Endocrinol. Metab.* 14, 378-385.

Ganesan, S., Silver, D.P., Drapkin, R., Greenberg, R., Feunteun, J., and Livingston, D. M. (2004). Association of BRCA1 with the inactive X chromosome and XIST RNA. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 359, 123-128.

Ganesan, S., Silver, D.P., Greenberg, R.A., Avni, D., Drapkin, R., Miron, A., Mok, S.C., Randrianarison, V., Brodie, S., Salstrom, J., Rasmussen, T.P., Klimke, A., Marrese, C., Marahrens, Y., Deng, C.X., Feunteun, J., and Livingston, D.M. (2002). BRCA1 supports XIST RNA concentration on the inactive X chromosome. *Cell* 111, 393-405.

Sirchia, S.M., Ramoscelli, L., Grati, F.R., Barbera, F., Coradini, D., Rossella, F., Porta, G., Lesma, E., Ruggeri, A., Radice, P., Simoni, G., and Miozzo, M. (2005). Loss of the inactive X chromosome and replication of the active X in BRCA1-defective and wild-type breast cancer cells. *Cancer Res.* 65, 2139-2146.

Fabbro, M., Savage, K., Hobson, K., Deans, A.J., Powell, S.N., McArthur, G.A., and Khanna, K.K. (2004). BRCA1-BARD1 complexes are required for p53Ser-15 phosphorylation and a G1/S arrest following ionizing radiation-induced DNA damage. *J. Biol. Chem.* 279, 31251-31258.

Bae, I., Fan, S., Meng, Q., Rih, J.K., Kim, H.J., Kang, H.J., Xu, J., Goldberg, I.D., Jaiswal, A.K., and Rosen, E.M. (2004). BRCA1 induces antioxidant gene expression and resistance to oxidative stress. *Cancer Res.* 64, 7893-7909.

Sudbrak, R., Wieczorek, G., Nuber, U.A., Mann, W., Kirchner, R., Erdogan, F., Brown, C. J., Wohrle, D., Sterk, P., Kalscheuer, V.M., Berger, W., Lehrach, H., and Ropers, H.H. (2001). X chromosome-specific cDNA arrays: identification of genes that escape from X-inactivation and other applications. *Hum. Mol. Genet.* 10, 77-83.