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# Modulation of aromatase expression by BRCA1: a possible link to tissue-specific tumor suppression

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**Mutations in *BRCA1* increase risks of familial breast and ovarian cancers, particularly among premenopausal women. While BRCA1 plays an active role in DNA repair, this function alone may not be sufficient to explain why *BRCA1*-associated tumors predominantly occur in estrogen-responsive tissues. Aromatase is the rate-limiting enzyme in estrogen biosynthesis and a key target in breast cancer treatment. Aromatase expression in ovarian granulosa cells dictates levels of circulating estrogen in premenopausal women, and its aberrant overexpression in breast adipose tissues promotes breast cancer growth. Here, we show that BRCA1 modulates aromatase expression in ovarian granulosa cells and primary preadipocytes. The cyclic AMP-dependent expression of aromatase in ovarian granulosa cells is inversely correlated with the protein level of BRCA1. Importantly, transient knockdown of BRCA1 enhances aromatase expression in both ovarian granulosa cells and primary preadipocytes. We propose that BRCA1 deficiency in epithelial and certain nonepithelial cells may result in combined effects of aberrant estrogen biosynthesis and compromised DNA repair capability, which in turn may lead to specific cancers in the breast and ovary.**
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Mutations in *BRCA1* account for about half of the hereditary forms of breast cancer and 80–90% of the combined hereditary breast and ovarian cancers. A large body of evidence has established a pivotal role of BRCA1 in various aspects of cellular response to DNA damage such as DNA repair and checkpoint (Venkitaraman, 2002; Starita and Parvin, 2003). However, loss of BRCA1 function in DNA damage response, a cellular process that is universally important to genetic stability of all cell types in both genders, may be insufficient to explain why *BRCA1* mutations predominantly affect breast and ovarian epithelia, the two major estrogen-responsive tissues in women. The ovary is the main source of circulating estrogen in premenopausal women. Conversion of androgen to estrogen, which is the rate-limiting step in estrogen biosynthesis, is catalysed by aromatase P450 (*CYP19*) in ovarian granulosa cells. Thus, ovary-specific expression of aromatase primarily determines circulating levels of estrogen in premenopausal women. In addition, aromatase expression in peripheral tissues such as adipose tissue also contributes to local estrogen production (Simpson *et al*., 2002). Indeed, over-expression of intratumoral aromatase in mammary adipocytes has been associated with breast cancer development (Sasano and Harada, 1998).

## An inverse correlation of aromatase and BRCA1 expression in ovarian granulosa cells

The release of pituitary gonadotropin follicle-stimulating hormone (FSH) greatly stimulates aromatase expression in mural granulosa cells of preovulatory follicles via a cAMP-dependent pathway (Richards, 1994). Interestingly, murine *Brca1* is highly expressed in ovarian granulosa cells of developing follicles. However, in large antral/preovulatory follicles, *Brca1* expression significantly decreases in mural granulosa cells and becomes restricted to cumulus granulosa cells, which do not contain abundant aromatase as compared to mural ones (see illustration in Figure 1a) (Marquis *et al*., 1995; Phillips *et al*., 1997; Rajan *et al*., 1997). Consistent with the published observation by *in situ* hybridization and immunohistochemistry, we found an inverse correlation of *Brca1* and aromatase mRNA and protein levels in cumulus and mural granulosa cells, using tissue fractions isolated from large antral/preovulatory follicles of mice ovaries (Figure 1a).

Using a human granulosa cell line (KGN) that maintains the steroidogenic function of granulosa cells *in vivo* (Nishi *et al*., 2001), we compared the ovarian expression of human BRCA1 and aromatase in response

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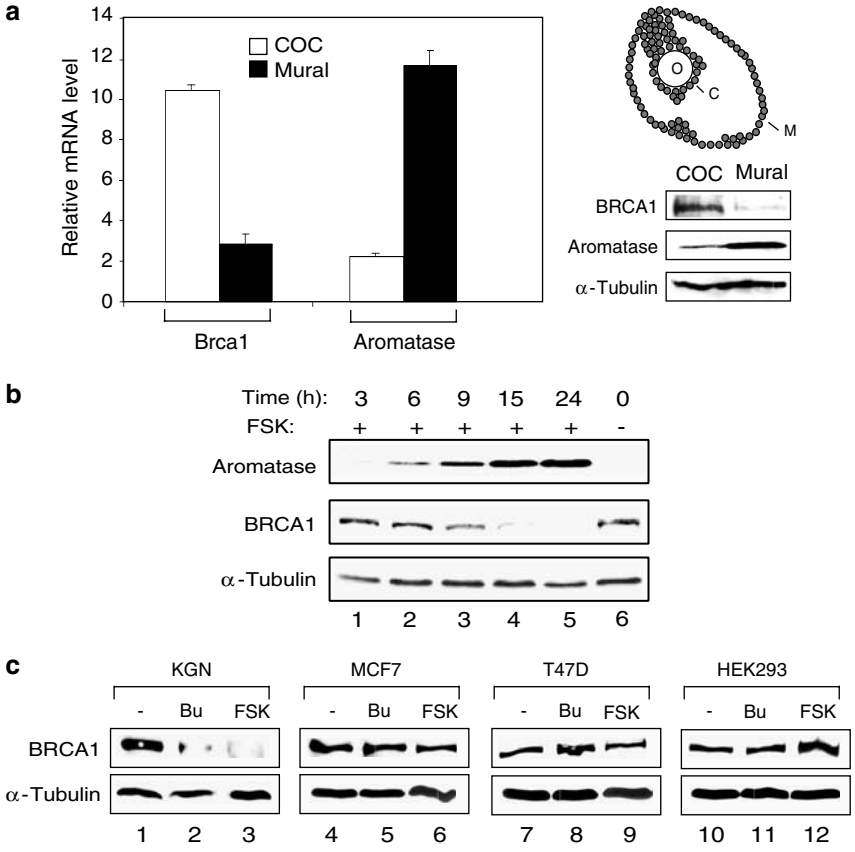


**Figure 1** An inverse correlation between aromatase and BRCA1 expression in ovarian granulosa cells. (**a**) Comparison of murine *Brca1* and aromatase expression in cumulus and mural granulosa cells. Tissue fractions that were enriched with either cumulus granulosa cell–oocyte complex (COC) or mural granulosa cells (Mural) were retrieved from ovaries of 7-week-old female mice by puncturing the large antral/preovulatory follicles with 30-gauge needles and microscopic dissection. Fractionation was validated by several markers, specific for cumulus and mural granulosa cells (data not shown). The mRNA levels of *Brca1* and aromatase were analysed by real-time PCR. Total RNA was isolated using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. RNA was reverse-transcribed using the ImPrompII kit from Promega. SYBR Green-based real time PCR assay was conducted following the manufacturer's procedures (Applied Biosystems for ABI7300). 18s rRNA was used for normalization. Protein extracts from COC and mural granulosa cell-enriched fractions were resolved by SDS–PAGE, and immunoblotted with the following antibodies: α-BRCA1 (Santa Cruz Biotechnology, H-100), α-aromatase (Serotec, MCA-2077), and α-tubulin (Calbiochem, CP06). Also shown is an illustration of oocyte (O), cumulus (C), and mural (M) granulosa cells in an antral follicle. (**b**) The human ovarian granulosa cell line KGN (Nishi *et al*., 2001) was treated with forskolin (FSK; 25 $\mu$M) and cells were harvested at the time intervals indicated on top of the panels. Immunoblotting was performed using specific antibodies against aromatase (Serotec, MCA-2077), BRCA1 (Oncogene, Ab-1/OP-92), and α-tubulin (Calbiochem, CP06). (**c**) $(Bu)_2$cAMP (0.5 mM) or forskolin-induced BRCA1 reduction is specific to ovarian granulosa cells. BRCA1 expression was assessed in KGN, MCF7 (breast carcinoma), T47D (breast carcinoma), and HEK293 cells (embryonic kidney). All immunoblots were developed using an ECL kit from Piece (Cat. # 34080-22) unless specified otherwise

to cAMP stimulation. The aromatase protein level increased steadily during the first 24 h post-treatment with forskolin (FSK), an activator of adenylyl cyclase (compare lanes 1–5 with 6 in Figure 1b). The cAMP-stimulated expression of aromatase was accompanied by a gradual reduction of BRCA1 protein in the human granulosa cells (Figure 1b), consistent with *in vivo* observations with murine Brca1 (Phillips *et al*., 1997; Turner *et al*., 2002) (Figure 1a). Treatment with a cAMP analog, $(Bu)_2$cAMP, caused similar inversely correlated changes of BRCA1 and aromatase levels (data not shown). The decrease in the BRCA1 protein level is unlikely to be due to a general chemical-triggered cell cycle arrest, as the same forskolin or $(Bu)_2$cAMP treatment in several other cell lines that do not express aromatase (MCF7, T47D, and HEK293) did not lead to significant changes in BRCA1 expression (Figure 1c) or any detectable aromatase expression (data not shown).

## Reduction of BRCA1 levels increases aromatase expression in the ovarian granulosa cell line and primary adipocytes

To directly test a causal relationship between aromatase and BRCA1 expression, several small interfering RNA (siRNA) oligonucleotides that target various regions of *BRCA1* were used to transiently reduce its expression in the KGN granulosa cell line (compare lanes 1 and 2 with 3–6; Figure 2a). Real-time RT-PCR analysis showed

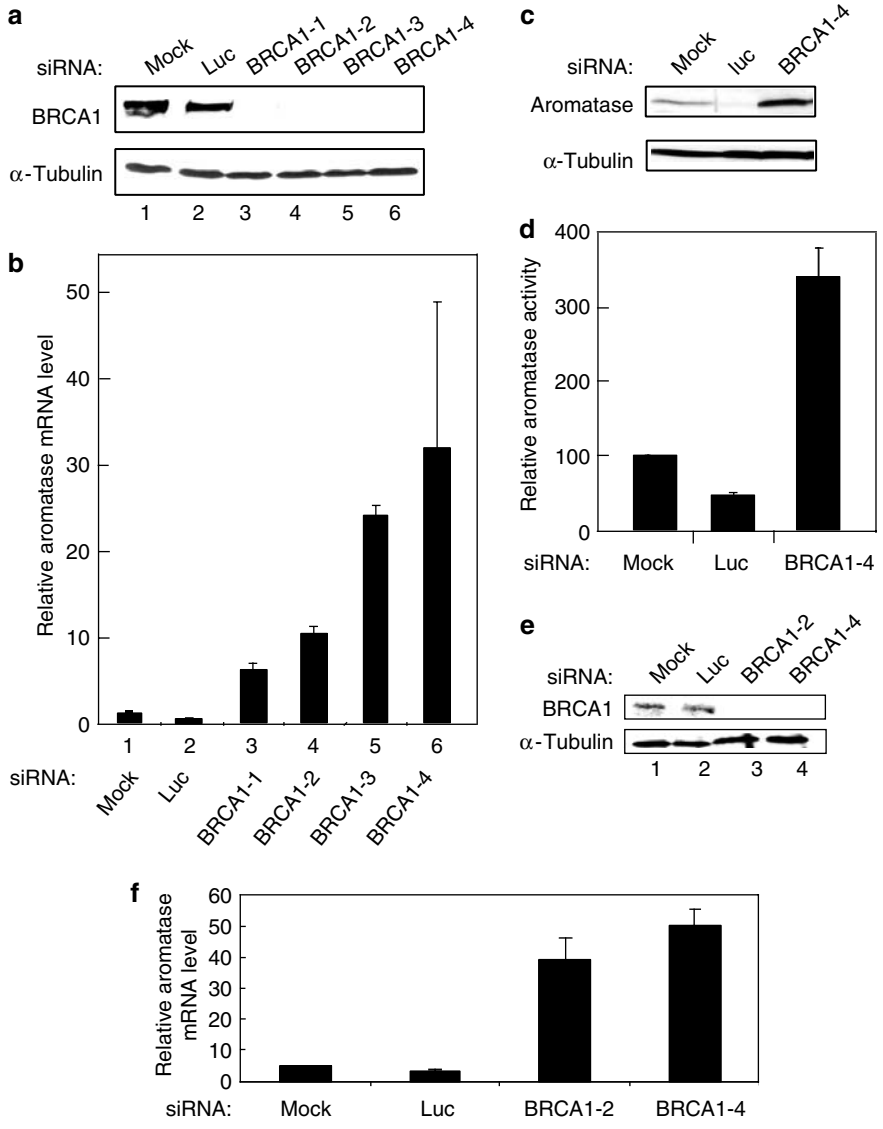


**Figure 2** Reduction of BRCA1 protein levels in ovarian granulosa cells and primary preadipocytes leads to elevated aromatase expression. (**a**) Immunoblotting for BRCA1 and α-tubulin in the control and BRCA1 knockdown granulosa cells. Various siRNAs purchased from Dharmacon (luciferase: D-001100-01-80, BRCA1-1:D-003461-02, BRCA1-2:D-003461-03, BRCA1-3:D-003461-06, BRCA1-4:D-003461-07) were introduced into KGN cells by two consecutive transient transfections at 200 nmol per well of a six-well dish, using Oligofectamine (Invitrogen). (**b**) mRNA levels of aromatase were measured by real-time RT–PCR method. β-Actin was used for normalizing the real-time PCR results. The sequences of the specific PCR primers will be provided upon request. (**c**) Lysates from the control and BRCA1 knockdown KGN cells were immunoblotted with the α-aromatase antibody and detected with ECL-Plus (Amersham). (**d**) Aromatase activity was measured in KGN cells by the tritiated water release assay, as described previously (Yue and Brodie, 1997). (**e**) Human adipose-derived adherent stromal cells (hADASC) were isolated and cultured as described previously (Katz *et al*., 2005). siRNA-mediated reduction of BRCA1 protein in preadipocytes was carried out essentially in the same way as that for KGN cells, except that Lipofectamine 2000 was used in the first transfection. (**f**) Real-time RT–PCR results indicate increased aromatase mRNA in the BRCA1-reduced preadipocytes

that the BRCA1-targeted siRNAs gave rise to a substantial elevation of the aromatase mRNA level as compared to the mock or luciferase siRNA (compare bar 1 and 2 with 3–6; Figure 2b). Furthermore, aromatase expression was not affected by a number of additional unrelated siRNA (data not shown). Consistent with the quantitative RT–PCR result, reduction of BRCA1 level increased the aromatase protein level (Figure 2c) as well as its catalytic activity in converting androgen to estrogen (Figure 2d). Under the forskolin-treated condition, BRCA1 siRNA only resulted in a modest increase over the cAMP-stimulated aromatase expression (data not shown). This was most likely due to the fact that, even in the absence of BRCA1 siRNA, the level of BRCA1 in the forskolin-treated cells was greatly reduced by an intrinsic cAMP-dependent process (Figure 1b and c).

In addition to ovarian granulosa cells, aromatase is also expressed in several peripheral tissues including adipose tissue (Simpson and Davis, 2001). Notably, in

many postmenopausal breast cancer tissues, adipocytes that surround the carcinoma tissues promote cancer growth by increasing the intratumoral production of aromatase and, hence, estrogen (Sasano and Harada, 1998). To assess the effect of BRCA1 on aromatase expression in adipose tissue, primary preadipocytes were transfected with either the control or BRCA1-specific siRNAs. As shown in Figure 2e and f, transient reduction of BRCA1 in preadipocytes resulted in substantial increases of aromatase mRNA expression. BRCA1 knockdown in several cell lines that normally do not express aromatase (e.g. MCF10A, T47D, and HEK293) did not result in the activation of the aromatase gene (data not shown), most likely due to the absence of tissue-specific transcription activators required for aromatase expression (Simpson *et al*., 2002). Thus, the BRCA1 effect on aromatase expression was only observed in those cell types that have the inherent steroidogenic capability, such as ovarian granulosa cells and preadipocytes.

BRCA1 forms a heterodimeric complex with the BRCA1-associated RING domain (BARD1) protein, which is implicated in BRCA1-mediated biological functions (Baer and Ludwig, 2002). Interestingly, siRNA-mediated knockdown of BARD1 in granulosa cells resulted in a significant reduction of the BARD1 as well as BRCA1 protein levels (compare lane 1 with 2–4, Figure 3a). This is reminiscent of the interdependent stability observed for the BRCA1/BARD1 homologues from other species (Joukov *et al*., 2001; McCarthy *et al*., 2003). Importantly, the BARD1-targeted siRNAs led to increases of aromatase mRNA that were comparable to the effects of the BRCA1 siRNAs (Figure 3b). Thus, BARD1 may facilitate the BRCA1-mediated repression of aromatase transcription by stabilizing BRCA1 and/or forming a functional repressive complex with BRCA1.

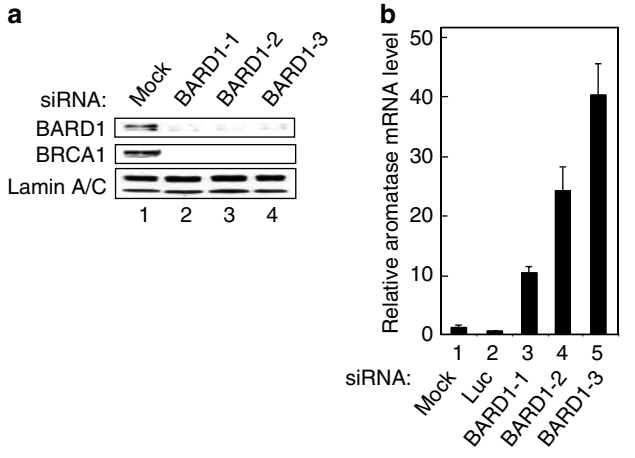


**Figure 3** BARD1 knockdown results in increased aromatase expression. (**a**) BARD1-specific siRNA reduced the protein levels of both BARD1 and BRCA1. Nuclear fractions were analysed for BARD1 (Bethyl, A300-263A) and BRCA1 (Oncogene, Ab-1/OP-92) levels, with lamin A/C (Covance, MMS-107P) as the loading control. (**b**) Real-time PCR indicates elevated total mRNA levels of aromatase in the BARD1 knockdown cells. Transient transfection and real-time PCR were conducted in the same manner as in Figure 2. The following siRNA oligonucleotides were purchased from Dharmacon: BARD1-1 (D-003873-01), BARD1-2 (D-003873-03), and BARD1-3 (D-003873-04)

## A gene- and promoter-specific effect of BRCA1

To further ascertain the specificity of the siRNA knockdown effect, we compared the mRNA levels of several other genes in the control and BRCA1-reduced KGN granulosa cells. These include inhibin $\beta$A, inhibin

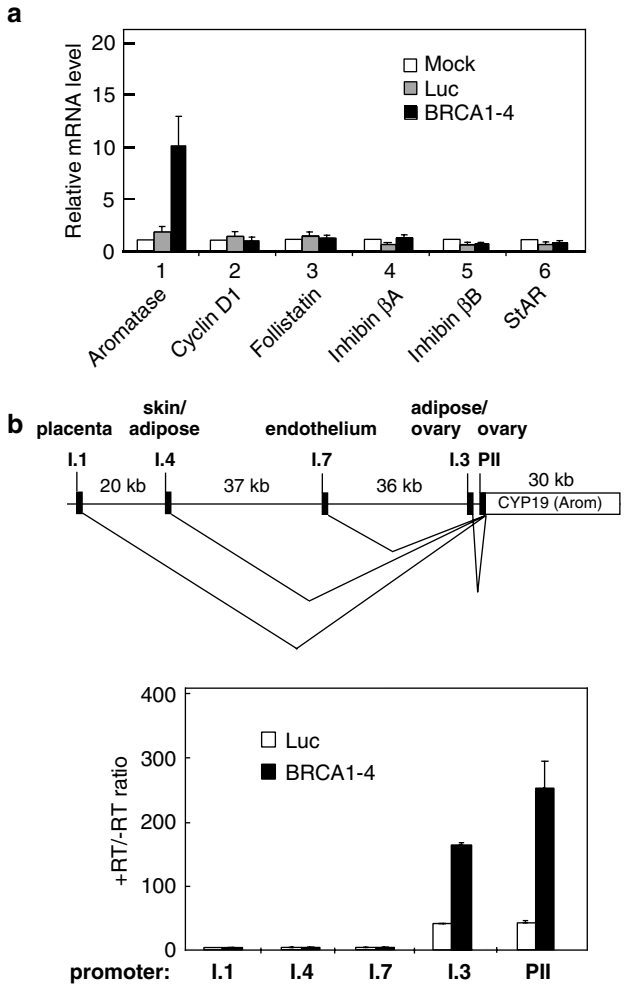


**Figure 4** Gene and promoter-specific effect of BRCA1. (**a**) KGN cells were either mock transfected or with various siRNAs. RNA was isolated and the mRNA levels for different genes were analysed by real-time PCR. The relative mRNA level of the mock sample in each group was set at 1. $\beta$-Actin was used for normalization. (**b**) The same RNA from (**a**) was used to measure the presence of the first exons that are specific to individual tissue-specific aromatase promoters. To eliminate false positive signals due to the possible contamination of genomic DNA in the RNA samples, parallel PCR reactions were carried out using both reverse transcriptase-treated (+RT) and untreated (−RT) samples. The ratio of +RT over −RT was used for measuring the relative promoter activity (1 means no detectable transcripts). The diagram on the top indicates the genomic locations of several tissue-specific promoters of the aromatase gene (*CYP19*) and the tissue-specific alternative splicing patterns of the aromatase transcripts. The solid bars in the diagram designate the promoter-specific first exons. The approximate distances between the promoters are also indicated

$\beta$B, cyclin D1, follistatin, and steroidogenic acute regulatory protein (StAR), most of which are stimulated by gonadotropins at various stages of follicular maturation (Richards, 1994). Real-time PCR analysis revealed that, in contrast to aromatase, none of the other genes examined were significantly affected by the BRCA1 siRNA knockdown (Figure 4a). Thus, it is unlikely that BRCA1 represses an upstream step that is common to the activation of these genes.

Expression of aromatase in various tissues is largely conferred by transcription initiation from tissue-specific promoters as well as alternative splicing that involves a promoter-specific, noncoding exon I (Bulun *et al*., 2003) (Figure 4b). The elevated aromatase expression upon BRCA1 knockdown in the granulosa cells could be due to super-activation of the ovary-specific promoter(s) and/or activation of a different tissue-specific promoter that is otherwise dormant in ovarian granulosa cells. To distinguish these two possibilities, we determined the abundance of the first exons that are specific to various tissue-specific promoters (Figure 4b), including I.1 (placenta), I.4 (skin and adipose), I.7 (endothelium), I.3 (adipose and ovary), and PII (ovary). In order to eliminate spurious signals due to contaminated genomic DNA in the RNA samples, we calculated ratios of real-time PCR values from RNA samples treated with reverse transcriptase (RT) over those without. As shown in Figure 4b, BRCA1 knockdown increased the PII and I.3-specific transcripts. In contrast, no significant amounts of the other promoter-specific transcripts (I.1, I.4, or I.7) were detected in either the control or BRCA1-knockdown cells. This finding indicates that super-activation of the ovarian promoters is the primary cause for the elevated aromatase expression in the BRCA1-depleted granulosa cells.

### A nonautonomous function of BRCA1 in ovarian granulosa cells and preadipocytes may contribute to tissue-specific tumor suppression

Recent clinical studies indicate that carriers of *BRCA1* mutations have a marked decrease in cancer risk at menopause as compared to the premenopausal age group (Antoniou *et al*., 2003). Furthermore, prophylactic oophorectomy reduces the risk of breast cancer in carriers of *BRCA1* mutations (Kauff *et al*., 2002; Rebbeck *et al*., 2002). These observations suggest that, as it has long been associated with sporadic breast cancer, circulating estrogen in premenopausal women may also contribute to the development of *BRCA1*-associated tumors. The current study provides evidence for a role of BRCA1 in modulating aromatase expression in ovarian granulosa cells and preadipocytes, two cell types that are important for the production of circulating and local estrogen, respectively. Elevated estrogen levels alone, due to increased aromatase expression may not be sufficient to trigger tumorigenesis in normal epithelial cells, as transgenic mice that overexpress aromatase in the mammary gland develop hyperplasia but not malignant tumors (Tekmal *et al*., 1999). However, by affecting the endocrine/paracrine actions of estrogen, *BRCA1* deficiency in the estrogen-producing cells may promote tumorigenesis in those estrogen-responsive epithelial cells that already display loss of heterozygosity (LOH) for *BRCA1*. Alternatively, accelerated proliferation in breast and ovarian epithelia due to the increased estrogen level could lead to a higher frequency of LOH for *BRCA1* in *BRCA1*-mutation carriers. Thus, a combined loss of the DNA repair function in epithelial cells and the modulation of estrogen biosynthesis in granulosa cells/adipocytes may confer tissue-specific tumor development in the breast and ovary. The model for BRCA1 functions in multiple cell types could also explain the lack of somatic mutations of *BRCA1* in sporadic breast tumors, as it may be an extremely rare event to lose the wild-type alleles of *BRCA1* in multiple cell types in noncarriers of *BRCA1* mutations.

A prediction from our study is that carriers of *BRCA1* mutations would exhibit elevated local and/or circulating levels of estrogen as compared to noncarriers. Correlative studies of estrogen levels and breast cancer risk in premenopausal women have been complicated by the large fluctuation of the plasma levels of estrogen during the menstrual cycle (Key and Verkasalo, 1999), and a direct comparison of the estrogen levels in *BRCA1*-mutation carriers with those in noncarriers is lacking in the current literature. However, it is notable that carriers of *BRCA1* mutations tend to have higher breast density, a surrogate of estrogen level (Huo *et al*., 2002). It is also possible that a paucity of BRCA1 in granulosa cells and/or adipocytes may increase breast cancer risk via cumulative exposure of breast epithelium to a mildly elevated basal level of estrogen, rather than via a significant alteration of the menstrual cycle in carriers of *BRCA1* mutations.

The exact mechanism of BRCA1-mediated repression of aromatase expression remains to be elucidated. Multiple positive and negative site-specific transcription factors have been reported to bind to and regulate transcription from the I.3 and PII promoters. These include steroidogenic factor 1 (SF-1), CREB transcription factor family, Snail/Slug proteins, and several orphan nuclear receptors (Zhou and Chen, 1999; Chen *et al*., 2001; Richards, 2001; Simpson *et al*., 2002). In addition, Jun proteins, which interact with BRCA1 (Hu and Li, 2002), bind to the PII promoter of the aromatase gene and repress its transcriptional activity in KGN cells (Ghosh *et al*., 2005). Thus, it is conceivable that BRCA1 may be recruited to the I.3/PII promoters via its interaction with one or more site-specific transcriptional repressors.

While the current manuscript was in preparation, Chodankar *et al*. reported that ovarian granulosa cell-specific knockout of *Brca1* in mice resulted in the development of cystic tumors in the ovaries and uterine horns (Chodankar *et al*., 2005). Strikingly, the tumors retained the wild-type *Brca1* alleles. This finding led to the notion that *Brca1* in mouse ovarian granulosa cells might indirectly suppress tumorigenesis in the neighboring estrogen-responsive tissues via a granulosa cell-

secreted effector (Chodankar *et al*., 2005). Altered steroidogenic capacity due to elevated aromatase gene expression in the granulosa cells could explain the tumor phenotype observed in the granulosa cell-specific *Brca1* knockout mice. Taken together, both the *in vivo* work in mice and our tissue culture study support a cell nonautonomous function of BRCA1 in suppression of breast- and ovary-specific tumor development, which may be in addition to its established role as a classical tumor suppressor in DNA repair and maintenance of genetic stability.

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