Tumor suppressor BRCA1 inhibits a breast cancer-associated promoter of the aromatase gene (CYP19) in human adipose stromal cells

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BESIDES ITS FUNCTION IN ENERGY STORAGE, adipose tissue is increasingly recognized as an important endocrine organ (27). In particular, it is well established that adipose tissue is a significant extragonadal source of estrogen. Obesity-associated elevation of estrogen production increases risk of breast cancer in postmenopausal women. Aromatase (CYP19), which converts androgen to estrogen, is a key enzyme in estrogen biosynthesis. In normal adipose tissue, transcription of the aromatase gene is initiated from a relatively weak adipose-specific promoter (I.4). However, in breast cancer, a switch of promoter utilization from I.4 to a strong ovary-specific promoter, PII, leads to increased aromatase expression and, hence, elevated estrogen production. Here, we report an intriguing relationship between the breast cancer susceptibility gene BRCA1 and aromatase expression in human adipose stromal cells (ASCs). Upon stimulation by phorbol ester or dexamethasone, increased aromatase expression in ASCs was accompanied by significant reduction of the BRCA1 level. In addition, adipogenesis-induced aromatase expression was also inversely correlated with BRCA1 abundance. Downregulation of BRCA1 expression in response to various stimuli was through distinct transcription or posttranscriptional mechanisms. Importantly, siRNA-mediated knockdown of BRCA1 led to specific activation of the breast cancer-associated PII promoter. Therefore, in addition to its well-characterized activities in breast epithelial cells, a role of BRCA1 in modulation of estrogen biosynthesis in ASCs may also contribute to its tissue-specific tumor suppressor function. Tumor suppression; estrogen biosynthesis

Clinical target in breast cancer treatment. Aromatase inhibitors, such as letrozole, have become standard adjuvant treatment for postmenopausal breast cancer (14). The abundance of aromatase in ovaries and extragonadal tissues dictate estrogen levels in circulation and in situ, respectively (34, 35). Expression of aromatase is controlled by tissue-specific promoters and alternative splicing between a tissue-specific, noncoding exon 1 and the common coding exon 2 (Fig. 1A) (1). For example, aromatase in ovarian granulosa cells is expressed via the action of a proximal, gonad-specific promoter (PII) in response to pituitary gonadotropins, whereas aromatase expression in normal adipose tissue is driven by an adipose-specific promoter (I.4) that is located >70 kb upstream of the initiation codon. Interestingly, switch of promoter utilization from the relatively weak I.4 to the strong PII accounts for the elevated intratumoral aromatase expression and, hence, enhanced in situ estrogen biosynthesis in breast tumors (30, 34).

BRCA1 mutation carriers have ~80% lifetime risk of developing breast cancer (18). For obvious reasons, the vast majority of BRCA1 studies have been conducted in breast epithelial/carcinoma cells. Intense research has implicated BRCA1 in multiple essential nuclear functions, including DNA repair, DNA damage checkpoint, gene regulation, and centrosome duplication (24, 31, 37). In particular, loss of BRCA1 function in DNA repair is thought to be the major cause for BRCA1-associated cancer development. However, because BRCA1 is expressed in a broad spectrum of tissues and cell types (20, 23), it is not clear how loss of BRCA1 function in DNA repair would lead to tissue- and gender-specific cancers.

Recent studies (4, 15) suggest that BRCA1 might also play an important biological function in nonepithelial cells. Tissue-specific knockout of Brcal in mouse ovarian granulosa cells induces tumors in the ovaries and uterine horns (4). In addition, small interfering RNA (siRNA)-mediated reduction of BRCA1 expression in human ovarian granulosa cells stimulates aromatase expression (15). Given the importance of ovarian granulosa cells in estrogen production in premenopausal women, these findings point to a possible cell nonautonomous function of BRCA1 and offer a reasonable explanation for the tissue specificity of BRCA1-associated cancers. In light of the importance of adipose tissue in extragonadal estrogen production, we extended our previous work by examining a functional relationship between BRCA1 and aromatase in ASCs. Our work revealed an inverse correlation between the BRCA1 and aromatase; estrogen biosynthesis; cancer development; tissue-specific TSE
matase expression when ASCs were exposed to various stimuli, including adipogenic agents. Furthermore, our results indicate that BRCA1 represses aromatase expression by inhibiting the transcriptional activity of the breast cancer-associated PII promoter of the aromatase gene.

MATERIALS AND METHODS

Cell culture. Primary human adipose stromal cells (ASCs) were isolated from a 58-yr-old female patient (body mass index 24.1) undergoing elective surgical procedures at the University of Virginia, using methods previously published and approved by the University of Virginia’s Human Investigation Committee (16). The patient has no history of diabetes but is presently on Premarin (estrogen replacement) because of prior hysterectomy nearly three decades ago. The cells were cultured in DMEM-F-12 medium with 10% FBS and antibiotic-antimycotic solution, using previously described methods (16). Cells were treated with the phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA; Sigma) or dexamethasone (Dex; Sigma) at the indicated concentrations and for the indicated time periods. For the mRNA stability assay, cells were treated with 5 μg/ml actinomycin D (Acros Organics) with or without TPA (2 nM) for the time intervals as indicated in Fig. 4.

RNA isolation and real-time PCR. Drug or siRNA-treated ASCs were harvested, and total RNA was isolated using TRIzol Reagent (Invitrogen) according to the manufacturer’s instructions. RNA was reverse-transcribed using the random primers of the ImPrompII kit from Promega. To examine the abundance of the alternative first exons of the aromatase gene were used to perform real-time PCR. Values are means ± SD of triplicates of 1 representative experiment. P values of Student’s t-test were calculated using the Sigma Plot, and the ranges are designated in the following manner: *P < 0.05; **P < 0.01.

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siRNA knockdown. Double transient transfection of the luciferase (control) or BRCA1-specific siRNA oligonucleotides was conducted using Lipofectamine 2000 and, subsequently, Oligofectamine (Invitrogen). The luciferase-(catalog no. D-001100-01-80) and BRCA1-specific siRNA oligonucleotides (BRCA1–2 catalog no. D-003461-
03; BRCA1–4 catalog no. D-003461-07) were purchased from Dharmacron. 1× Buffer was used for the mock transfection. The knockdown experiment was performed as previously described (15). Briefly, cells at a density of 60% were first transfected with Lipofectamine 2000 and siRNA oligos at a final concentration of 100 nM. Twenty-four hours after the first transfection, cells were transfected again with Oligofectamine and siRNA at a final concentration of 200 nM. Cells were harvested for protein and RNA analysis 72 h after the first transfection.

Immunoblotting. Whole cell lysates were prepared in the SDS sample buffer along with a cocktail of protease inhibitors. Protein concentrations were quantified by the BCA protein assay reagent (Pierce), and an equal amount of total protein was resolved by 5 (for BRCA1) or 10% (for α-tubulin and lamin A/C) SDS-PAGE. The proteins were detected by an anti-BRCA1 (Ab1 from Calbiochem), anti-α-tubulin (Calbiochem), or anti-lamin (Santa Cruz biotechnology) antibody, respectively, using SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Adipogenesis. ASCs were allowed to grow to become fully confluent. They were then exposed to the adipogenic medium [50:50 DMEM/Ham’s F-12 medium (Invitrogen), 10% FBS (Invitrogen), 1 μM dexamethasone (Sigma), 10 μM insulin (Sigma), 200 μM indomethacin (Sigma), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma), and 1% antibiotic-antimycotic solution (Invitrogen)]. Adipogenic medium was changed every 2 days until most of the cells started producing fat globules, and cells were harvested for protein and RNA analysis on day 12.

RESULTS

Activation of aromatase expression in ASCs. ASCs, also known as human adipose-derived adherent stromal cells and adipose-derived stem cells, were isolated from subcutaneous adipose tissue as previously described (16). It has been documented previously that ASCs display multilineage developmental plasticity in vitro and in vivo (8, 11, 13, 29, 40). To determine the steroidogenic potential of these cells, early-passage ASCs were treated with various chemicals that are known to stimulate aromatase expression in adipose tissue (21, 33). As shown in Fig. 1B, treatment with the phorbol ester (TPA) or Dex significantly induced aromatase gene expression. On the other hand, treatment of cells with forskolin (FSK) had little effect on aromatase expression (Fig. 1B, lane 2), which is consistent with the previously reported observation that growth factors present in regular serum blunts the effect of FSK on aromatase expression (21).

Aromatase expression is mediated by multiple tissue-specific promoters that span over a 93-kb upstream region of the coding exons of the aromatase gene. Whereas all aromatase-expressing tissues share the same coding exons, a tissue-specific noncoding exon 1 transcribed by a distinct promoter is alternatively spliced to the commonly transcribed coding exons (see diagram in Fig. 1A) (1). We used exon 1-specific real-time PCR primers to determine the aromatase promoters involved in the TPA- or Dex-stimulated gene activation. TPA treatment predominantly activated the promoter activity of I.3 (adipose and ovary; Fig. 1C, lane 4) and PII (ovary; Fig. 1C, lane 8), whereas Dex exclusively stimulated transcription from promoter I.4 (skin and adipose; Fig. 1D, lane 6). Neither drug stimulated transcription from the placenta (I.1) or endothelium-specific promoter (I.7). Thus, with regard to the promoter utilization of the aromatase gene, the primary human ASCs responded to TPA and Dex in a promoter- and stimuli-specific manner.

An inverse correlation between aromatase and BRCA1 expression in ASCs. Our recent work (15) demonstrates that FSK-stimulated aromatase expression in human ovarian granulosa cells is accompanied by a significant decrease in the BRCA1 protein level. To determine whether a similar correlation exists in ASCs, we measured the mRNA as well as protein levels of BRCA1 in ASCs following the TPA or Dex treatment. As shown in Fig. 2A, aromatase mRNA expression peaked at the 12-h time point following the TPA treatment (Fig. 2A, lane 5). Concomitantly, the BRCA1 mRNA level was significantly diminished within the first 12 h following drug treatment (Fig. 2B, lanes 1–5) but rebounded at the 24-h time point when aromatase expression declined (compare lane 5 with lane 6 in Figs. 2A and 2B). BRCA1 protein underwent a biphasic change similar to the transcript (Fig. 2C). The relatively low sensitivity of a commercially available anti-aromatase antibody precluded us from detecting the changes of aromatase protein in ASCs. A similar inverse correlation between the aromatase and BRCA1 mRNA expression was observed in the Dex-treated ASCs (Fig. 3), although the magnitude and duration of the Dex-induced BRCA1 reduction were not as substantial as seen in the TPA-treated cells (compare Figs. 2B and 3B). Taken together, our data strongly suggest that activation of aromatase expression in response to distinct
stimuli is coupled with a transient depletion of endogenous BRCA1 in ASCs.

The TPA- or Dex-induced decrease of the BRCA1 mRNA level could be due to decreased transcription and/or increased mRNA degradation. To distinguish these two possibilities, ASCs were treated simultaneously with TPA and actinomycin D, which inhibits transcription. BRCA1 mRNA levels at multiple time points were measured by quantitative PCR. As shown in Fig. 4, in the absence of active transcription, BRCA1 mRNA was decayed at the same rate with and without TPA (compare closed triangles and open squares). This result indicates that TPA does not affect the half-life of the BRCA1 transcript. Rather, reduced transcription of the BRCA1 gene most likely accounts for the transient depletion of BRCA1 mRNA.

Given that ASCs can be induced to differentiate into mature adipocytes (16, 40), we sought to assess the impact of adipogenesis on BRCA1 expression. After prolonged incubation of ASCs in the adipogenic medium, expression of peroxisome proliferation-activated receptor-γ and CCAAT/enhancer-binding protein-β, two well-established markers for adipogenesis (12, 19), was significantly elevated (Fig. 5A). In addition, maturation of adipocytes was also indicated by the elevated level of visfatin (Fig. 5A), an adipokine enriched in mature adipocytes in visceral fat (10). Adipogenesis resulted in an increase in aromatase expression (Fig. 5B). In contrast to the observation made in the TPA- or Dex-treated ASCs (Figs. 2 and 3), relatively little change in BRCA1 mRNA abundance was observed during adipogenesis (Fig. 5B, left). However, BRCA1 protein level was reduced in mature adipocytes, indicating the involvement of a regulatory mechanism at the translation and/or posttranslational level. Therefore, although BRCA1 expression can be modulated at different levels in response to distinct external cues, changes of the BRCA1 protein levels are in consistent contrast with those of aromatase expression.

Reduction of BRCA1 in ASCs leads to activation of the ovary-specific aromatase promoter. To explore a functional relationship between BRCA1 and aromatase expression, we reduced BRCA1 expression in ASCs using two different BRCA1-specific siRNA oligonucleotides (Fig. 6A, inset). Consistent with our previous finding (15), partial depletion of BRCA1 in ASCs resulted in elevated aromatase expression (Fig. 6A). The quantitative difference of the aromatase expression in the two knockdown samples (Fig. 6A, lanes 3 and 4) may be due to different kinetics of BRCA1 reduction by the two siRNA oligos over the course of the transient transfection. To extend the initial observation, we sought to interrogate the impact of BRCA1 siRNA knockdown on the transcriptional activity of individual aromatase promoters. Using promoter-specific primer sets, we found that BRCA1 reduction led to significant increases of the PII-specific aromatase transcripts (Fig. 6D). The I.3-specific transcripts were also moderately elevated in the BRCA1 knockdown cells (Fig. 6C). In contrast, BRCA1 knockdown had minimal effects on the I.1, I.4, and I.7 promoters (Fig. 6, B, E, and F, respectively). Given the importance of PII in conferring elevated aromatase expression and estrogen production in breast tumors, these results strongly suggest that BRCA1-mediated downregulation of the PII promoter in normal adipose tissue may suppress and/or delay the development of breast cancer.

DISCUSSION

Germ line mutations in BRCA1 specifically lead to increased risks of familial breast and ovarian cancers (24). The past decade has witnessed intense investigation of multiple functions of BRCA1 in breast cancer cells. It is well-documented that BRCA1 functions in DNA repair and damage checkpoint and estrogen production in breast tumors, these results strongly suggest that BRCA1-mediated downregulation of the PII promoter in normal adipose tissue may suppress and/or delay the development of breast cancer.

Fig. 3. Inverse correlation between BRCA1 and aromatase expression in Dex-treated ASCs. Cells were treated with 250 nM Dex for the indicated periods of time. Aromatase RNA (A), BRCA1 mRNA (B), and protein (C) were analyzed in the same manner as shown in Fig. 2, except that Lamin A/C was used as the loading control for the immunoblot. *P < 0.5; **P < 0.05; ***P < 0.005.

Fig. 4. Impact of TPA treatment on the BRCA1 mRNA stability. ASCs were treated with actinomycin D (ActD; 5 μg/ml) for various periods of time, with or without TPA (2 nM). The cultures were harvested and RNA isolated for real-time RT-PCR analysis. Values are means ± SE of triplicates of 1 representative experiment. β-Actin was used as the internal control.
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Fig. 5. Expression of aromatase and BRCA1 in mature adipocytes. ASCs were subjected to adipogenesis, and RNA was isolated from undifferentiated and differentiated cells 12 days after adipogenic treatment. A: adipogenesis was confirmed by 3 adipogenic markers, peroxisome proliferator-activated receptor-γ (PPARγ), visfatin, and CCAAT/enhancer-binding protein-β (CEBPβ). Results are averages of triplicates that are normalized against the mRNA levels of GAPDH. B: real-time PCR was performed to detect the abundance of aromatase and BRCA1 transcript levels (left). In addition, BRCA1 protein levels in ASCs and mature adipocytes were assessed by immunoblotting (right). **P < 0.05.

Epithelial cells (31, 39). However, given that BRCA1 expression is not restricted to breast/ovarian epithelia, it remains a conundrum as to why loss of the BRCA1 function in DNA repair would specifically increase cancer risks in the breast and ovary, two major estrogen-responsive tissues in women. Recent work from others and us (4, 15) on BRCA1 functions in ovarian granulosa cells highlights the merit of exploring the tissue-specific tumor suppressor function of BRCA1 in a broader tissue and cell context. The results from the present study extend the previous findings by uncovering an intriguing inverse correlation between BRCA1 and aromatase expression in ASCs in response to different external cues. Together with the published findings in ovarian granulosa cells (15), our data in ASCs suggest a common theme in the coordinated regulation of aromatase and BRCA1 expression in both gonadal and extragonadal estrogen-producing cells. Furthermore, our work indicates that BRCA1 suppresses the transcriptional activity of the breast cancer-associated aromatase promoter in normal ASCs, thus lending further support to the notion that BRCA1-mediated modulation of estrogen biosynthesis contributes to its tissue-specific tumor suppressor function.

Our recent study (15) indicates that the effect of BRCA1 on aromatase expression in ovarian granulosa cells is due primarily to its action on promoter I.3 and PII. However, the lack of activity of the other tissue-specific promoters of the aromatase gene in ovarian granulosa cells precluded us from assessing a potential effect of BRCA1 on these inactive promoters. The ASCs used in the present work are capable of expressing aromatase from I.3, I.4, and PII promoters in response to different stimuli. Although adipose-specific I.4 was transcriptionally competent, it was not affected by the BRCA1 knock-down. In contrast, I.3 and PII were activated by the reduction of BRCA1 in a manner similar to that seen in ovarian granulosa cells. This result clearly demonstrates a preferential effect of BRCA1 on a subset of the aromatase promoters.

A wealth of evidence (30, 35) strongly suggests that elevated synthesis of estrogen by intratumor adipose tissue contributes to the growth of postmenopausal breast cancer. In particular, aberrant activation of the PII promoter of the aromatase gene largely accounts for the increased aromatase expression and, hence, estrogen production in tumor-bearing breast adipose tissue. Given the importance of the PII utilization in breast cancer development, we find it intriguing that partial depletion of BRCA1 has a significant impact on the PII promoter activity in both ovary- and adipose tissue-derived cells. It is tempting to speculate that BRCA1 may use a common underlying mechanism to repress the promoter activity of PII in both cellular settings. It is also conceivable that reduced expression and/or activity of BRCA1 due to a cancer-predisposing mutation or epigenetic changes at the BRCA1 locus may superactivate or reactivate the ovary-specific promoter of the aromatase gene in gonadal and extragonadal estrogen-producing cells. This may in turn result in abnormal estrogen biosynthesis and promote cancer development in estrogen-responsive tissues, including breast and ovary.

The exact underlying mechanisms by which BRCA1 mRNA and/or protein levels are downregulated in response to TPA and Dex remain unclear. However, previous studies of BRCA1 in tumor samples and established carcinoma cell lines have implicated multiple mechanisms in regulation of BRCA1 expression. For example, hypermethylation of the BRCA1 promoter region occurs in 10–15% of sporadic breast and ovarian cancer cases (3, 9), thus accounting for some breast cancer cases with reduced BRCA1 expression. In addition, emerging evidence suggests that BRCA1 abundance can be regulated via several other mechanisms, such as mRNA stability (25), translation (32, 36), and protein stability (5, 6). Indeed, the inverse correlation of BRCA1 and aromatase expression in ASCs suggests a coordination of the two transcriptional events in response to various external stimuli. For example, it is possible that TPA-stimulated PKC might lead to the inhibition of one or more transcription activators at the BRCA1 promoter. Alternatively, the same signal transduction pathway could enhance the inhibitory activity of a transcription repressor. Compared with TPA, the Dex-mediated effect on BRCA1 expression is less robust and more transient. This may be due to an indirect effect of Dex on the promoter activity of the BRCA1 gene. Because siRNA-mediated BRCA1 knockdown does not stimulate the adipose-specific I.4 promoter, Dex-triggered BRCA1 reduction may not contribute, in a significant manner, to the activation of the I.4 promoter following the Dex treatment.

Our result shows that adipogenesis results in reduction in BRCA1 protein, but not mRNA levels. It is conceivable that adipogenesis inhibits BRCA1 translation and/or promotes protein degradation, two mechanisms that have been previously implicated in regulation of BRCA1 protein abundance in different settings (5, 6, 32, 36). In particular, it has recently been shown that proteasome-mediated degradation of BRCA1 is regulated in a cell cycle-dependent manner (6). Given that the adipogenic medium contains Dex, the lack of reduction in BRCA1 mRNA during adipogenesis contrasts with the obser-
vation made in the acutely Dex-treated ASCs. It is conceivable that the quiescent ASCs due to contact inhibition at the initial stage of adipogenesis may respond differently to Dex than the subconfluent cells used in the transient experiment. Alternatively, other components of the adipogenic medium may antagonize the effect of Dex on BRCA1. Finally, Dex may not be able to sustain long-term inhibitory effect on BRCA1 mRNA expression, as evidenced by the transient nature of BRCA1 mRNA reduction in Dex treatment (Fig. 3B).

Our study indicates that aromatase levels are higher in mature adipocytes than in ASCs. However, it has been reported (2, 22) that aromatase is expressed predominantly in undifferentiated preadipocytes, not in mature adipocytes. The basis for the discrepancy between our findings and the previous observation is not clear. It is possible that ASCs with multilineage plasticity, as used in the present study, may represent a different developmental stage from that of the preadipocytes in earlier studies (2, 22). Alternatively, the different results may reflect physiological/pathological differences in the adipose tissues from various donors. It will be of importance to explore the impact of the age, adiposity, and hormone status of the patients as well as the tissue/cell isolation methods and number of passage in vitro on regulation of BRCA1 and aromatase expression.

Besides the highly penetrant mutations in BRCA1, other genetic and nongenetic factors can significantly influence the onset of BRCA1-associated cancers. For example, reproductive factors related to estrogen exposure, such as breastfeeding, parity, and contraceptive use, modify risk in BRCA1 mutation carriers (7, 24). Furthermore, prophylactic oophorectomy, which removes the major source of circulating estrogen in premenopausal women, reduces risk of breast cancer in BRCA1 mutation carriers by 75% and has therefore become a standard cancer-preventive procedure for BRCA1 carriers (17, 28). In addition to the hormonal factors, physical activity and lack of obesity in adolescence can significantly delay breast cancer onset for this group of cancer-predisposed women (18). Investigation of regulation of BRCA1 expression and functions in both epithelial and nonepithelial cells within the tumor microenvironment may shed light on the molecular basis for BRCA1-associated tumorigenesis.

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REFERENCES

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