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BRCA1/BARD1 complex interacts with steroidogenic factor 1—A potential mechanism for regulation of aromatase expression by BRCA1

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ABSTRACT

Germline mutations in BRCA1 predispose women to early onset of breast and ovarian cancers. Findings from previous studies support the notion that the tissue- and gender-specific tumor suppression function of BRCA1 is associated with its role in negative regulation of aromatase expression, the rate-limiting step in estrogen biosynthesis. The molecular mechanism of BRCA1 in regulating aromatase promoter activity remains to be elucidated. In this study, we demonstrate that, in an ovarian granulosa cell line KGN, steroidogenic factor 1 (SF-1) is required for aromatase PII promoter basal activity as well as the elevated aromatase expression mediated by BRCA1 knockdown. Furthermore, BRCA1 in KGN cells exists mainly as a heterodimer with BARD1. We provide evidence that the BRCA1/BARD1 complex interacts with SF-1 both *in vivo* and *in vitro*. However, the intrinsic ubiquitin E3 ligase activity of BRCA1/BARD1 does not appear to contribute to ubiquitynation of SF-1. We propose that the interaction between SF-1 and BRCA1/BARD1 may recruit BRCA1/BARD1 complex to the aromatase PII promoter for BRCA1/BARD1-mediate transcriptional repression.

1. Introduction

Mutations of tumor suppressor gene *BRCA1* account for about half of familial breast cancer cases in women and 80% of cases with combined breast and ovarian cancers [1]. The molecular mechanism of the BRCA1-mediated tumor suppression has been the subject of intense research in the past two decades. A wealth of information has established that BRCA1 plays an important role in DNA repair and DNA checkpoint control, which almost certainly contributes to genomic instability and tumorigenesis in BRCA1-associated cancer [2].

BRCA1 has also been implicated in transcriptional regulation. Of particular interest, findings from several transcription-based studies provide possible explanations for the gender- and tissue-specific phenomenon in BRCA1-related tumors [3–7]. For example, BRCA1 has been shown to modulate transcriptional activity of estrogen receptor ERα, which could explain why BRCA1 mutations mainly lead to cancers in estrogen responsive tissues such as breast and ovary [4]. In addition, several laboratories including ours reported that BRCA1 negatively modulates expression of aromatase, a key enzyme in estrogen biosynthesis, in ovarian granulosa cells as well as adipose stromal cells [6–8]. Furthermore, BRCA1 is associated with the proximal promoters of the aromatase gene [7]. Animal studies further show that, in ovarian granulosa cells where aromatase gene expression is responsible for circulating estrogen level in premenopausal individuals, loss of *Brca1* leads to benign tumors on the uterine horn and in the ovaries in a cell non-autonomous manner [5,9]. Interestingly, the female mice with inactive *Brca1* in ovarian granulosa cells also display altered estrus cycle [9]. Recently, a study using clinical samples indicates that local aromatase expression in breast tissue is increased in *BRCA1* mutation carriers [10]. Despite these emerging evidences that link BRCA1 function with aromatase expression, the molecular mechanism by which BRCA1 represses aromatase expression remains to be elucidated.

Aromatase is expressed in a tissue-restricted manner, via activation of multiple tissue-specific promoters. In pre-menopausal women, circulating estrogen level is dictated by aromatase levels in ovarian granulosa cells. Aromatase expression in ovarian granulosa cells is controlled by a proximal promoter, PII. There are two proximal DNA elements in the promoter that are essential for PII activity: a binding site for the orphan nuclear receptor SF-1 and a cAMP-responsive element for cAMP-induced transcription [11]. The SF-1 binding site is required for both basal and cAMP-induced transcription of the aromatase gene. Importantly, SF-1 is thought to be the critical factor that confers ovary-specific transcription activity of the PII promoter. As BRCA1 is associated with the PII promoter and preferentially regulates its tissue-specific transcription activity, we examined in the current work potential physical and functional relationships between SF-1 and BRCA1 in regulation of

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aromatase transcription in an ovarian granulosa cell line KGN. Our findings provide a potential molecular mechanism by which BRCA1 negatively regulates aromatase expression.

2. Materials and methods

2.1. Human cell lines and drug treatment

Human ovarian granulosa cell line KGN is a gift from Dr. Hajime Nawata and has been previously described [12]. KGN cells were grown in DMEM/F-12 Nutrient Mixture (DMEM/F12) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. HEK293T cells were purchased from American Type Culture Collection (Manassas, VA) and maintained in DMEM supplemented with 10% fetal bovine serum, 0.1 mM non-essential amino acids (Invitrogen #11140-050), 1 mM sodium pyruvate (Invitrogen #11360-070), 2 mM L-glutamine (Invitrogen #25030-081), 500 μg/ml geneticin (Invitrogen #10131-035), 100 U/ml of penicillin, and 100 μg/ml of streptomycin. Forskolin was purchased from Sigma and is used at a final concentration of 25 μM.

2.2. Plasmids

The wild-type FLAG-BARD1 expression constructs have been described previously [13]. The hemagglutinin (HA)-tagged ubiquitin expression plasmid, pMT123, was kindly provided by Dirk Bohmann (University of Rochester, USA). To generate the HA-BARD1 expression construct, human BARD1 cDNA sequence was amplified by PCR using pcBb-Flag-BARD1 as the template. The amplified fragment was inserted between the *Eco*RV and *Xho*I sites of pcDNA3-3HA. To construct the expression vectors for SF-1, human SF-1 cDNA sequence (a kind gift from Dr. Larry Jameson, Northwestern University) was amplified by PCR using primers, hSF1-Full N-term-F (ggaattcagatcgactattcgtacg) and hSF1-Full N-term-R (ggggtacccctcaagtctgctt). The amplified fragment was inserted between the *Eco*RI and *Kpn*I sites of pCMV-Myc (Clontech). To generate the Flag-tagged BRCA1-1-771aa, pCR3-Flag-BRCA1 was digested by *Hind*III and *Kpn*I. The corresponding fragment was inserted between the *Hin*dIII and *Kpn*I sites of pcDNA3 (Invitrogen). All subcloned DNA sequences were verified by DNA sequencing. GST-BRCA1 fragments 1–6 were gifts from Dr. Livingston [14] and GST-BARD1 was a gift from Dr. Pan [15].

2.3. Transient transfection

Transient transfection in HEK293T cells was performed with Lipofectamine 2000 (Invitrogen) according to manufacturer's instruction. For each transfection, the total plasmid DNA was adjusted to 8 μg/60-mm dish by adding parental empty vectors.

2.4. Cell extract preparation and immunoblotting

Whole-cell extracts were made with lysis buffer (50 mM Tris, pH 6.8; 2% sodium dodecyl sulfate; 10% glycerol), and protein concentration was determined with the BCA assay (Pierce Chemical Co., Rockford, IL). An equal amount of protein extracts was resolved by SDS–PAGE. Western Blot analysis was carried out using the following antibodies: α-BRCA1 Ab1 (OP92; Calbiochem), α-aromatase (MCA2077; Serotec), α-tubulin (CD06; Calbiochem), α-Flag (F1804; Sigma), α-HA (A190-108A; Bethyl Laboratories, Montgomery, TX), α-SF1 (07-618, Millipore), α-LRH1 (sc-25389; Santa Cruz Biotechnology), α-c-Myc (sc-789; Santa Cruz Biotechnology) and α-BARD1 H-300 (sc-11438; Santa Cruz Biotechnology). Peroxidase-conjugated α-mouse IgG and α-rabbit IgG antibodies (31430, 31460; Pierce) were used as the secondary antibodies for Western blotting. Blots were visualized with the enhanced chemiluminescence method (Pierce).

2.5. RNA isolation and real-time PCR

Drug or siRNA-treated KGN cells were harvested, and total RNA was isolated using TRIzol Reagent (Invitrogen) according to manufacturer's instructions. RNA was reverse-transcribed using the random primers of the ImPrompII kit from Promega. The SYBR Green-based real-time PCR assay was conducted in an AB 7900HT fast real-time PCR system, following manufacturer's procedures (Applied Biosystems). GAPDH was used for normalizing real-time PCR results. The sequences of PCR primers are:

Aromatase-f: 5′ TGGAATTATGAGGGCACATCC 3′
Aromatase-r: 5′ GTCCAATTCCCATGCAGTAGC 3′
hBRCA1Ex20-f: 5′ CCAAAGCGAGCAAGAGAATCC 3′
hBRCA1Ex21-r: 5′ TGAAGGGCCCATAGCAACAG 3′
BARD1-f: 5′ ACACCATTGCATGAAGCTTGC 3′
BARD1-r: 5′ CCAATGCCTTATGCTGGAGC 3′
LRH-1-f: 5′ CGAGAGTTCGTATGTCTGAAATTCTT 3′
LRH-1-r: 5′ GCATTGACTTGTTCCTGGACA C 3′
GAPDH-f: 5′ CCATCAATGACCCCTTCATTG 3′
GAPDH-r: 5′ GACGGTGCCATGGAATTTG 3′

2.6. siRNA knockdown

The siRNA oligos for luciferase (Cat. # D-001100-01-80), control (D-001810-10-20), BRCA1 (Cat. #. D-003461-07), BARD1 (Cat. # D-003873-04), SF1 (Cat. # M-003429-00), and LRH1 (Cat. # M-003430-01) were purchased from Dharmacon. 1x siRNA buffer (diluted from 5x siRNA buffer, Cat. # B-002000-UB-100) was used for mock transfection. For the knockdown experiment, cells at a density of 60% were transfected with Lipofectamine RNAiMAX (Invitrogen 13778-150) and siRNA oligos at a final concentration of 20 nM according to manufacturer's instruction. Cells were harvested for protein and RNA analysis 72 h after transfection.

2.7. Immunoprecipitation

For immunoprecipitation, cells were harvested 24 h after transfection and lysed by incubating at 4 °C for 30 min with 1 ml/60-mm dish high salt lysis buffer (50 mM Tris–HCl pH 8.0, 1% Nonidet P-40, 500 mM NaCl, 20 mM NaF, 1 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). Upon passing through a 21G needle 6 times, cell lysates were clarified by centrifugation at 16,000 × *g* at 4 °C for 15 min. The supernatants (1 ml) were mixed with 15 μl anti-flag-conjugated agarose beads (Cat. # A2220; Sigma) or 7.5 μl anti-c-Myc-conjugated agarose beads (Cat. # sc-40AC; Santa Cruz Biotechnology) at 4 °C for overnight. Proteins bound to the beads were washed three times with high salt lysis buffer and boiled in Laemmli SDS loading buffer for 10 min. The samples were resolved by SDS–PAGE followed by immunoblotting.

2.8. In vitro protein binding assay

GST and GST fusion proteins were purified from *Escherichia coli* using glutathione-agarose beads and retained as 50% slurry according to manufacturer's instruction (Phamacia). SF-1 was translated *in vitro* in the presence of ^{35}S-methionine using TNT quick coupled transcription/translation system (Promega, Inc.). In each binding reaction, 10 μl of reticulocyte lysate containing ^{35}S-SF-1 was mixed with 6 μg of GST or GST fusion proteins in 500 μl binding buffer (150 mM NaCl, 50 mM Tris pH8, 1% NP-40, 20 mM NaF, 1 mM $Na_4P_2O_7$, 1 mM Na3VO4, 1 mM PMSF, 1 μg/ml leupeptin,

1 μg/ml aprotinin, 1 μg/ml pepstatin A). After incubating at 4 °C for overnight, the beads were then washed four times with the same binding buffer and subjected to SDS–PAGE. The gel was dried and exposed to an X-ray film for overnight.

2.9. In vivo ubiquitination assay

HEK 293T cells were first transfected with siRNA oligos. Twenty-four hours after siRNA transfection, cells were split into two 60 mm dishes. Twenty-four hours after cell reseeding, 293T cells were transfected with Myc-SF-1 or Myc-SF-1 plus HA-ubiquitin constructs. Transfected cells were washed once with PBS and dissolved in one volume of 1% SDS, boiled for 10 min, then diluted with nine volumes of dilution buffer (50 mM Tris pH 8.0, 1% Nonidet P-40, 0.5% DOC, 20 mM NaF, 1 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). An equal amount of protein extracts was used for immunoprecipitation. HA-ubiquitin was immunoprecipitated with 15 μl anti-HA affinity matrix (Cat. # 11-815-016-001; Roche) for 12 h at 4 °C. Immunoprecipitated proteins were washed three times with RIPA buffer (150 mM NaCl, 50 mM Tris pH 8, 1% NP-40, 0.5% DOC, 0.1% SDS) and eluted by boiling for 5 min in Laemmli SDS loading buffer.

2.10. In vitro ubiquitination assay

The experiment procedure to obtain the BRCA1-771aa/BARD1 E3 immunocomplex immobilized on anti-Flag-conjugated agarose beads was described [16]. Briefly, cells were harvested 24 h after transfection and lysed by incubating at 4 °C for 30 min with 1 ml/60-mm dish buffer A (15 mM Tris–HCl/pH 7.5, 0.35% Nonidet P-40, 500 mM NaCl, 20 mM NaF, 1 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 150 μg/ml benzamidine). After passing through a 21G needle 6 times, cell lysates were clarified by centrifugation at 16,000 × *g* at 4 °C for 15 min. The supernatants (1 ml) were mixed with 15 μl anti-Flag-conjugated agarose beads (Cat. # A2220; Sigma) at 4 °C overnight. The proteins bound to the beads were washed three times with buffer A, two times with buffer B (50 mM NaCl, 25 mM Tris–HCl pH 7.5, 0.01% Nonidet P-40, 10% glycerol, 1 mM EDTA) and used in the Ub ligation assay. The experiment procedure to obtain the Myc-SF-1 immobilized on

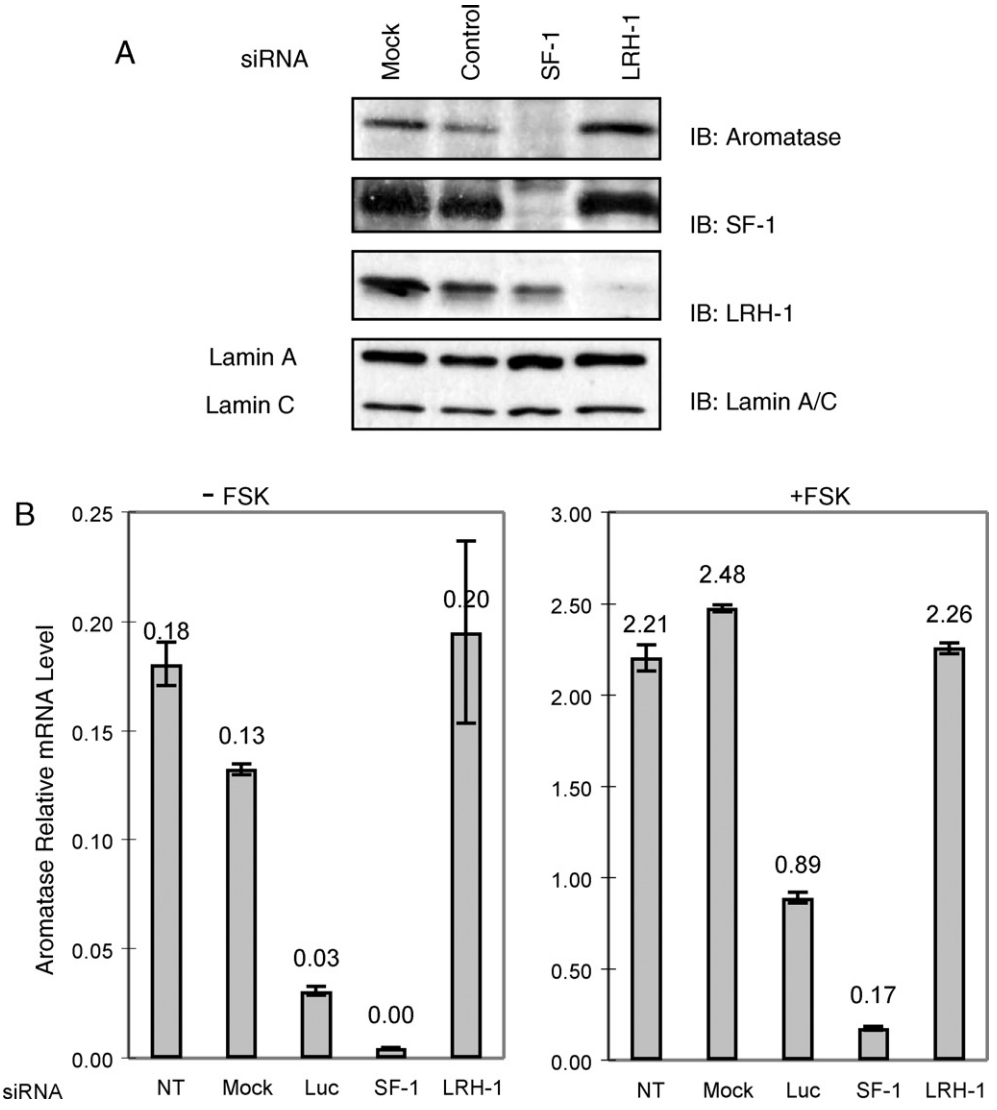

Fig. 1. SF-1 is required for basal and cAMP-induced aromatase PII promoter induction in KGN cells. Successful knockdown of SF-1 and LRH-1 by siRNA as demonstrated by Western blot (A). (B) Knockdown of SF-1, but not LRH-1, abolishes basal (−FSK, left panel) as well as cAMP-induced (+FSK, right panel) aromatase expression, measured by real-time RT-PCR. siRNA against luciferase (Luc) is included as a negative control.

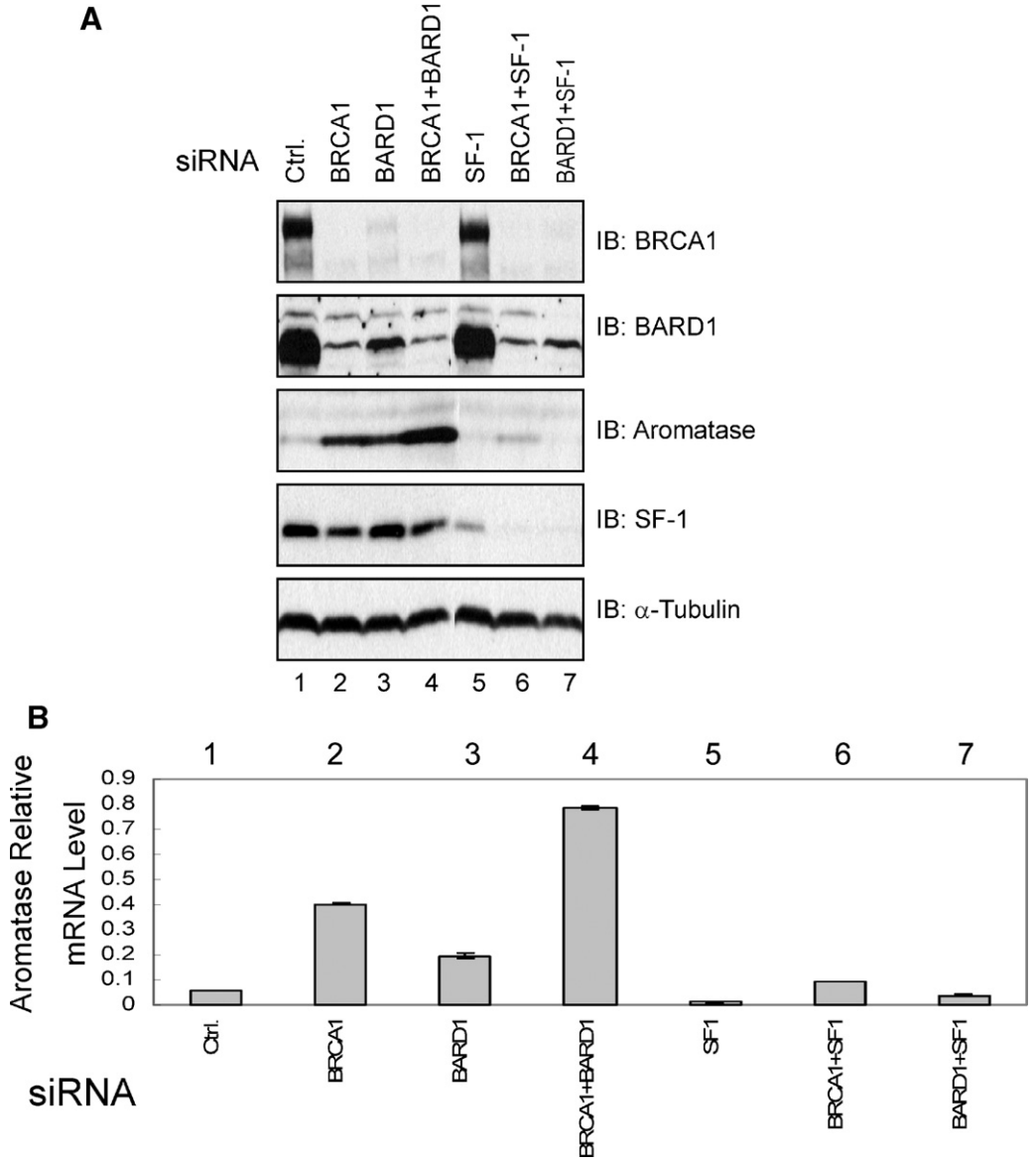

Fig. 2. BRCA1/BARD1-knockdown-mediated aromatase elevation requires SF-1. Knockdown of BRCA1, BARD1 and SF-1 alone or in combination shown by Western blot (A). The effect of knockdown of BRCA1, BARD1 and SF-1 on aromatase expression shown by Western blot (A) or real-time RT-PCR (B).

anti-c-Myc-conjugated agarose beads (Cat. #sc-40AC; Santa Cruz Biotechnology) is essentially the same as described above for the BRCA1 complex with the following modifications. The cell lysate was sonicated at 50% amplitude, 0.5 s on/off for 8 s. Before buffer A wash, Myc-SF-1 protein bound to the anti-c-Myc-conjugated agarose beads was washed once with TE-sarcosyl buffer (50 mM Tris–HCl pH 8.0, 2 mM EDTA, 0.2% sacosyl), TSEI buffer (150 mM NaCl, 20 mM Tris–HCl pH 8.0, 2 mM EDTA, 0.1% SDS, 1% Triton X-100), TSEII buffer (500 mM NaCl, 20 mM Tris–HCl pH 8.0, 2 mM EDTA, 0.1% SDS, 1% Triton X-100) and TSEIII buffer (250 mM LiCl, 10 mM Tris–HCl pH 8.0, 1 mM EDTA, 1% sodium deoxycholate, 1% Nonidet P-40).

The BRCA1-771aa/BARD1 E3 immunocomplex immobilized on anti-Flag-conjugated agarose beads and Myc-SF-1 protein immobilized on anti-c-Myc-conjugated agarose beads were added to a Ub ligation reaction mixture (30 μl) that contained 50 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 2 mM NaF, 10 nM okadaic acid, 2 mM ATP, and 0.6 mM DTT as well as 40 ng of E1 (Cat. # E-304; Boston Biochem), 0.3 μg of E2 (Cat. # E2-627; Boston Biochem), and 1 μg of ubiquitin (Cat. # U-100H; Boston Biochem). The mixture was incubated for 120 min at 37 °C with shaking, and the reaction was terminated by boiling in Laemmli SDS loading buffer with a final concentration of 0.1 M DTT.

3. Results

3.1. SF-1 is essential for aromatase promoter activity in ovarian granulosa cell line KGN

A number of studies implicate SF-1 in directing aromatase transcription from promoter II in gonadal tissues [11,17,18]. However, liver receptor homologue-1 (LRH-1), another member of the nuclear receptor 5A (NR5A) family to which SF-1 belongs, has also been shown to regulate PII promoter [19–23]. While the role of LRH-1 in PII-driven aromatase expression in breast preadipocytes is well established, there are controversial reports regarding the relative contributions of SF-1 and LRH-1 to gonadal PII activity [18,20,21]. We therefore set out to examine the roles of SF-1 and LRH-1 in PII transcription control in an ovarian granulosa cell line, KGN.

Expression of SF-1 or LRH-1 can be specifically knocked down using gene-specific siRNA (Fig. 1A). As shown in Fig. 1B, both basal (without forskolin) and cAMP-induced (with forskolin) aromatase expression in KGN was abolished by SF-1 knockdown. In contrast, LRH-1 knockdown did not impair aromatase mRNA level at all. As PII is the major promoter for aromatase expression in KGN cells [6,24] (Hu, data not shown), this data strongly suggest that, SF-1 plays a major role in regulating PII activity in ovarian granulosa cells.

3.2. SF-1 is required for elevated aromatase expression upon BRCA1/BARD1-knockdown

Our previous studies demonstrate an inverse correlation between BRCA1 level and ovarian granulosa aromatase expression [6]. Furthermore, knockdown of BRCA1 as well as its partner, BARD1, leads to elevation of basal aromatase expression from Promoters PII and PI.3 in KGN cells [6]. To explore the relationship between SF-1 and BRCA1/BARD1 in regulating aromatase PII promoter, SF-1 knockdown was conducted in combination with that of BRCA1/BARD1. As shown in Fig. 2A, knockdown of BRCA1 or BARD1 decreased both BRCA1 and BARD1 protein levels, due to the well-established co-stability of the two proteins in the BRCA1/BARD1 complex. The SF-1 protein levels were not affected by BRCA1/BARD1 knockdown (lanes 2–4; Fig. 2A). Reciprocally, knockdown of SF-1 did not change the BRCA1 or BARD1 protein level (lane 5). Consistent with our previous report, knockdown of BRCA1 and BARD1 led to elevation of aromatase mRNA level (Fig. 2B, columns 1–4). A greater degree of aromatase gene activation was achieved upon simultaneous knockdown of BRCA1 and BARD1 (Fig. 2B, column 4). However, the effect of BRCA1/BARD1 knockdown was abolished when SF-1 was depleted from KGN cells (Fig. 2B, columns 6 and 7). This data suggest that BRCA1/BARD1-knockdown-induced aromatase transcription still depends on the presence of SF-1 protein.

3.3. BRCA1/BARD1 Interacts with SF-1 both in vivo and in vitro

To determine whether BRCA1/BARD1 interacts with SF-1 *in vivo*, Flag-tagged BARD1 or BRCA1 and myc-tagged SF-1 were co-expressed in HEK 293T cells and the potential interaction was examined by coimmunoprecipitation. As shown in Fig. 3A, when Flag-BARD1 was immunoprecipitated using an anti-Flag antibody, myc-SF-1 was present in the Flag-BARD1 immunoprecipitates. However, when Flag-BRCA1 was over-expressed, myc-SF-1 could not be detected in the Flag-BRCA1 immunocomplex (data not shown). Conversely, when myc-SF-1 was immunoprecipitated using an anti-Myc antibody, both Flag-BRCA1 and endogenous BARD1 could be detected in the precipitates (Fig. 3B). These data suggest that SF-1 might interact with BRCA1/BARD1 complex via directly contacting BARD1. To further confirm the physical interaction between SF-1 and BRCA1/BARD1, we conducted GST pulldown using various GST-BRCA1/BARD1 fusion proteins and *in vitro* translated ^{35}S labeled SF-1. Fig. 3C shows that ^{35}S labeled SF-

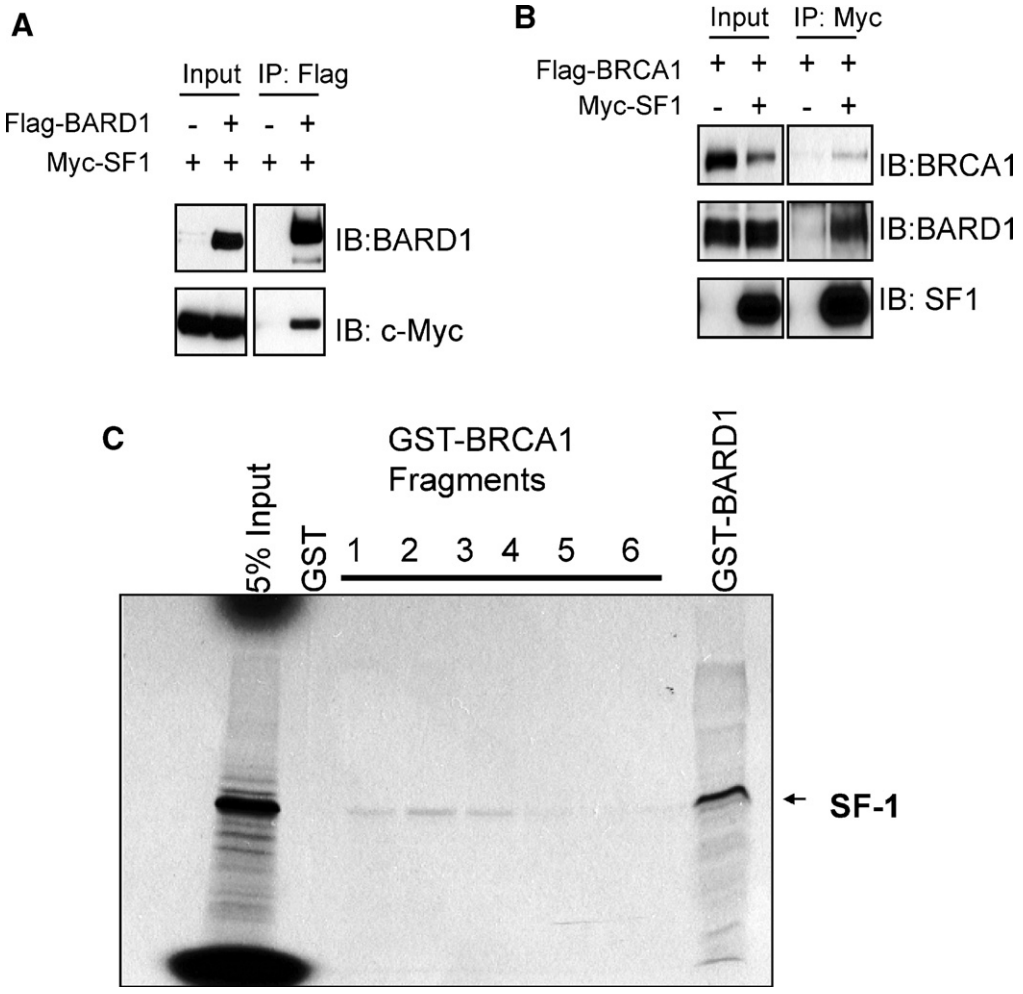

Fig. 3. Interaction of BRCA1/BARD1 with SF-1. (A) myc-SF1 is coimmunoprecipitated with Flag-BARD1. (B) BRCA1 and BARD1 are coimmunoprecipitated with myc-SF1. (C) *In vitro* translated SF1 binds to GST-BARD1, but not to GST-BRCA1 fragments in GST-pulldown assay.

1 interacts with GST-BARD1 much more efficiently than any of the six GST-BRCA1 fragments that encompass the entire BRCA1 coding sequence.

It is important to mention that the interaction between SF-1 and BRCA1/BARD1 can be reliably detected in HEK293T cells, but not in KGN cells, where the endogenous BRCA1/BARD1/SF1 protein levels are much lower. Although it cannot be excluded that the interaction reported in this paper could be an artifact of protein over-expression, it is also likely that the transient interaction between BRCA1/BARD1 and SF-1 could only be detected when there is sufficient amount of complex in the experimental context. Alternatively, the interaction between BRCA1/BARD1 and SF-1 might be intrinsically much weaker in KGN cells (where aromatase promoter basal activity is much higher), due to other unknown regulatory proteins in KGN cells that keep the PII promoter on by preventing efficient repression of BRCA1/BARD1.

3.4. SF-1 is not ubiquitynated by BRCA1/BARD1 in vivo or in vitro

The BRCA1/BARD1 complex possesses an intrinsic ubiquitin E3 ligase activity and has been shown to confer auto-ubiquitynation as well as ubiquitynation of several substrates [15,25,26]. Ubiquitynation of SF-1 has also been reported in the literature [27,28]. We therefore tested whether SF-1 could be a potential substrate for BRCA1/BARD1 E3 ligase activity, using both *in vivo* and *in vitro* ubiquitynation assays.

When myc-SF-1 was co-expressed with HA-tagged ubiquitin in HEK293T cells, ubiquitynated SF-1 was readily detected by anti-HA immunoprecipitation (lane 6 in Fig. 4A). Simultaneous knockdown of BRCA1 and BARD1 did not reduce the intensity of ubiquitynated SF-1 in HEK293T cells (compare lanes 6 and 8).

We also used an *in vitro* ubiquitynation assay to further verify the *in vivo* finding. In this case, we used an immuno-

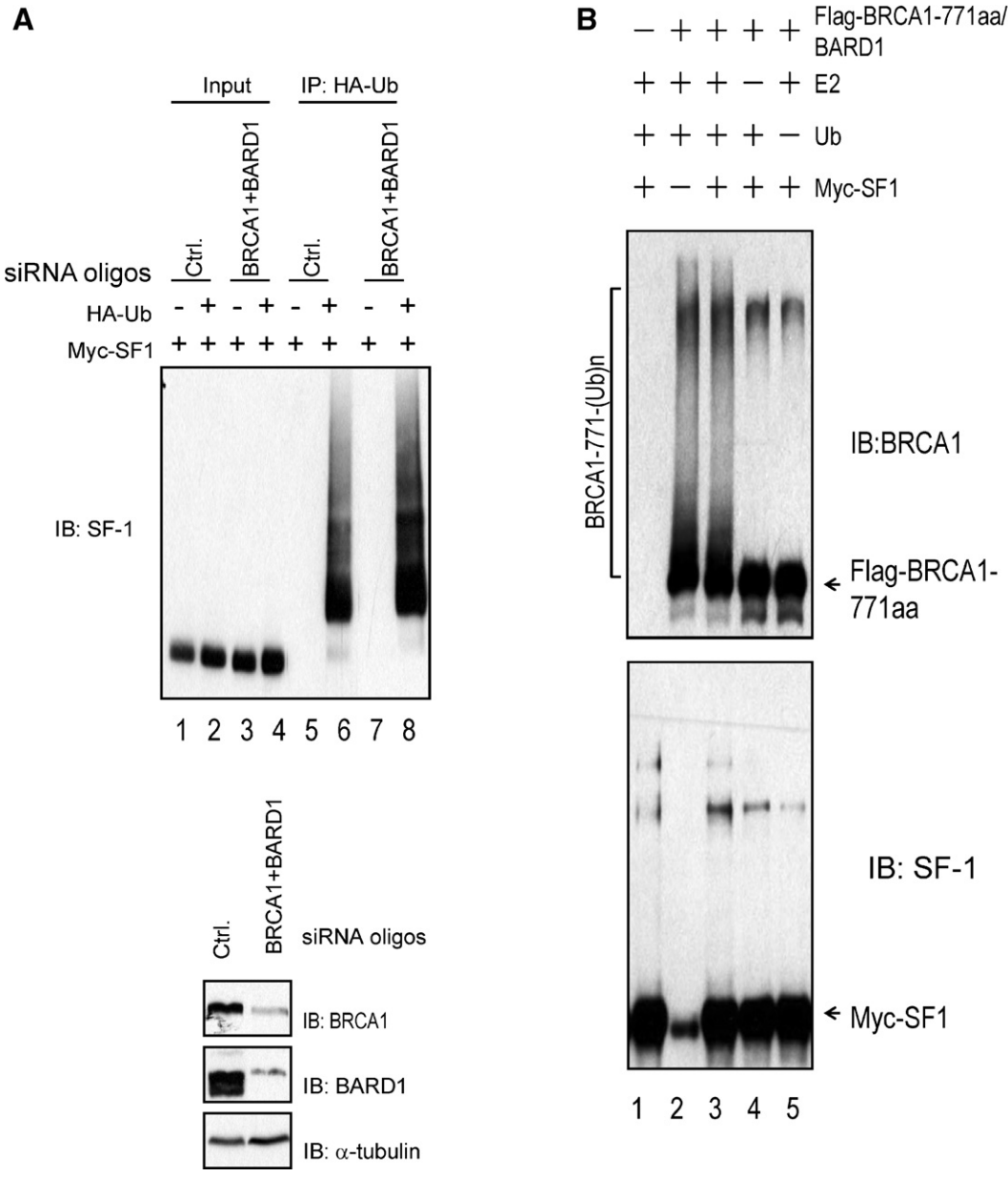

Fig. 4. SF-1 is not ubiquitynated by BRCA1/BARD1 complex in HEK293T cells. (A) *In vivo* ubiquitynation assay. The ubiquitynated SF-1 is detected by immunoprecipitation of HA-ubiquitin followed by SF-1 Western blot. Knockdown of BRCA1/BARD1 has no effect on SF-1 ubiquitynation status in HEK293T cells. (B) *In vitro* ubiquitynation assay. BRCA1(1-771aa)/BARD1 are immunoprecipitated from HEK293T cells and display auto-ubiquitynation (upper panel) in an E2-dependent manner. The same BRCA1(1-771aa)/BARD1 precipitates do not ubiquitynate myc-SF1 precipitated from HEK293T cells in a separate reaction (lower panel).

complex consisting of a BRCA1 fragment (1-771aa) and HA-BARD1, both co-expressed in HEK293T cells. Auto-ubiquitynation of the BRCA1(1-771aa)/BARD1 complex was readily detected in the *in vitro* system as previously reported [25,29] (Fig. 4B upper panel, lanes 2 and 3). However, there was no indication of SF-1 ubiquitynation in the same *in vitro* reactions (Fig. 4B lower panel). These data suggest that SF-1 is not a substrate of the BRCA1/BARD1 E3 ubiquitin ligase.

4. Discussion and conclusions

BRCA1 has been implicated in multiple cellular functions such as DNA repair, cell cycle checkpoint control, transcriptional regulation, chromatin remodeling, ubiquitynation, and centrosome maintenance. Although DNA repair and maintenance of genome stability play a central role in tumor suppression function of BRCA1, genome instability alone is likely insufficient to explain the tissue- and gender-specificity in BRCA1-associated tumor spectrum. In an effort to address the tissue-specific phenomenon, we and others showed previously that BRCA1 negatively regulates expression of aromatase. Interestingly, a recent clinical study reported that the aromatase mRNA level is indeed higher in breast tissue of cancer-free BRCA1 mutation carriers [10], supporting findings from the tissue culture studies. In the current study, we extended the previous work to address a physical and functional connection between BRCA1 and SF-1, a key transcription factor that regulates aromatase gene expression.

In the current study, we establish that the BRCA1/BARD1 complex interacts with SF-1. However, our data do not provide evidence that SF-1 is a direct substrate of the BRCA1/BARD1 E3 ligase activity. It is conceivable that the physical interaction between SF-1 and BRCA1/BARD1 helps recruit the BRCA1/BARD1 complex to the aromatase PII promoter to exert the repression on PII promoter. It remains to be elucidated as to how BRCA1/BARD1 represses aromatase promoter activity once they are recruited to the promoter. It has been reported BRCA1/BARD1-mediated ubiquitynation does not usually lead to degradation of substrate [16]. Furthermore, when tethered to a synthetic transcription promoter by the GAL4 DNA binding domain, BRCA1/BARD1 could ubiquitynate the RNA Pol II machinery and silence the promoter activity [26]. We suggest that a similar mechanism could be used by BRCA1/BARD1 to repress aromatase PII promoter. In this case, BRCA1/BARD1 may not be stably associated with the PII promoter. Rather they may dissociate from the promoter shortly after the transcription machinery is ubiquitynated and inactivated. Of note, since SF-1 is not expressed in breast tissue, the proposed mechanism does not directly apply to the regulation of PII promoter in breast cancer tissues. On the other hand, BRCA1-associated breast cancer tends to be early onset and in pre-menopausal women, it is conceivable that PII regulation in ovary could contribute to tumorigenesis in breast.

Our protein binding assays suggest that SF-1 may interact with the BRCA1/BARD1 complex via its direct interaction with BARD1. Our earlier work [30] also showed that BRCA1 C-terminus directly interacts with transcription factor Jun proteins, which are present at the PII proximal region [24,31]. It is therefore possible that the BRCA1/BARD1 complex may utilize two PII-binding site-specific transcription factors for promoter recruitment: BRCA1 C-terminus could contact Jun at the CRE site, while BARD1 interacts with SF-1 at its corresponding recognition site (Fig. 5). The two contact points may work concertedly to ensure the recruitment of BRCA1/BARD1 complex to the PII promoter. This model would predict the presence of BRCA1/BARD1 complex at the PII promoter. Chromatin immunoprecipitation (ChIP) assay using BRCA1 antibodies has not yielded convincing results (Lu, data not shown). This could be due to the poor quality of available BRCA1 antibodies, compounded by the

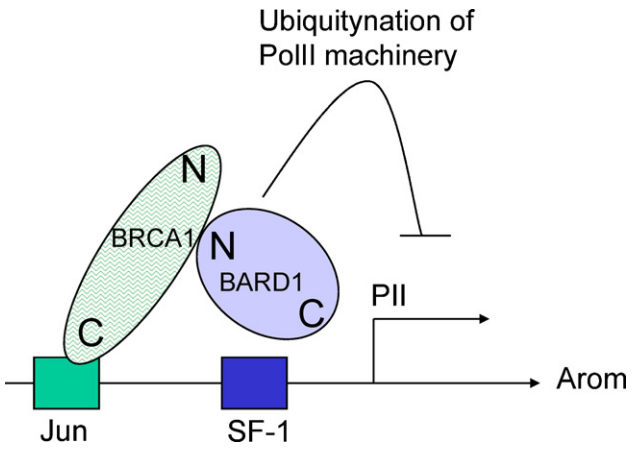

Fig. 5. A model on how BRCA1/BARD1 down-regulates aromatase PII promoter.

fact that BRCA1 is not a sequence-specific DNA binding protein and may be transiently associated with promoters via protein–protein interactions. To circumvent potential antibody deficiency, we used a lentiviral expression system to express full-length BRCA1 with Flag-tagged to its N-terminus. The exogenous BRCA1 is robustly overexpressed by at least 20-fold, compared with the endogenous BRCA1 level, as judged by Western blot. Surprisingly, the Flag monoclonal antibody could only detect a very weak band in Flag-BRCA1 overexpressed extract (Lu, data not shown). It is not clear what causes the discrepancy between Western blots using BRCA1 or Flag antibodies. We reported earlier that BRCA1 N-terminus is heavily ubiquitynated on its lysines [32]. Since Flag epitope contains several lysine amino acids that could be ubiquitynated, the modified Flag-tag may no longer be recognized by the Flag antibody. These technical difficulties make it less feasible at present to examine BRCA1 recruitment to the PII promoter using ChIP analysis. More in-depth experiments will be required in future to test this model.

Our data suggest that both BRCA1 and BARD1 are involved in modulation of aromatase expression. It is known that the BRCA1/BARD1 heterodimer confers much more potent ubiquitin E3 ligase activity than each protein separately. Several BRCA1 cancer mutations located in the BRCA1 RING domain abolish the E3 ligase activity, further suggesting that the E3 ligase contributes to tumor development. In this regard, it is surprising that mice embryonic stem cells carrying a point mutation in BRCA1 (I26A) that abolishes ubiquitin E3 activity display no apparent deficiency in homology-directed DNA double-strand break repair [33]. We speculate that mutations in BRCA1/BARD1 ubiquitin E3 activity might not be directly involved in DNA repair, rather, they could contribute to breast cancer development through loss of control in steroidogenesis.

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