Effects of BRCA1 Knockdown on CYP19a1/Aromatase and Steroid Receptor Expression in Ovarian and Tubal Cells

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Introduction

Over 90% of ovarian cancer is described as epithelial, but the pathology of this cancer is highly heterogeneous and researchers have been trying to define the cell of origin of the various types of epithelial ovarian cancer (EOC) for decades [1-3]. High numbers of ovulations in a reproductive lifetime have long been thought to increase risk of serous EOC [4-7]. The initial hypothesis that the cells of the ovarian surface epithelium (OSE) transform after repeated ovulations, linked to gonadotropin stimulation [6,8], was extended by the idea that Fallopian tube epithelium might also be the source of primary lesions [9,12], especially in women carrying BRCA1 mutations [BRCA1+]. Prophylactic salpingo-oophorectomy in BRCA1+ women revealed primary lesions in the distal tubal epithelia and particularly the fimbria, rather than on the surface of the ovary. In addition, activation of TP53 expression in patches of the tubal epithelia (the so-called p53 signature) suggested these cells might be the source of serous EOC [7,17-19].

Mutations in human BRCA1 increase the risk of familial breast and epithelial ovarian and Fallopian tube cancers, particularly among younger, premenopausal women [20-22]. BRCA1 plays an active role in DNA repair and is thought to interact with other proteins implicated in cell cycle regulation and DNA repair, such as p53 [23,24] and ATM, the protein mutated in Ataxia Telangectasia [25]. Although somatic BRCA1 mutations are not found in sporadic breast tumors, BRCA1 methylation has been shown to occur and to be associated with reduced gene expression in such cancers. BRCA1 has also been shown to bind to tissue-specific promoter regions in the CYP19a1 gene and can directly control expression of this enzyme in adipose tissue [26]. CYP19a1 expression is also regulated by estrogen [27], whereas stimulation of CYP19a1 expression by phorbol-12-myristate-13-acetate (PMA), ER, or estrogen receptor α (ERα) resulted in increased CYP19a1 expression only in KGN cells and this resulted in a decrease in CYP19a1 expression equivalent to 2 nM siRNA knockdown. We conclude that lowering BRCA1 expression in OSE and tubal epithelium cell lines does not stimulate CYP19a1 expression or change steroid receptor expression significantly. The mechanism by which BRCA1 methylation increases risk of serous EOC remains to be elucidated.

Material and Methods

Cell culture

The MCF-7 mammary carcinoma cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD). This cell line represented an ER-positive breast cancer cell line, used previously to investigate the relationships between BRCA1 and steroid receptor
regulation [29-31]. MCF-7 cells were grown at 37°C in DMEM with 10% (v/v) fetal bovine serum (FBS; Bio International, New Zealand) and 2 nM L-Glutamine (Invitrogen, Auckland, NZ) in a 10% CO2 atmosphere, as described previously [32]. The BRCA1-mutated breast cancer cell line (HCC1937) and a stably-transfected form of HCC1937 expressing BRCA1 were obtained from Professor Georgia Chenevix-Trench at the Queensland Institute of Medical Research (QIMR) and cultured in RPMI-1640 complete media containing 1% Penicillin and Streptomycin solution (Invitrogen) and 10% FBS, in a 5% CO2 atmosphere [33]. HCC1937 contains a frameshift mutation (5382insC) in BRCA1 that produces a premature stop codon near the carboxy terminus, resulting in a non-functional truncated protein [31,34,35].

These cell lines were used to investigate the effects of increased BRCA1 expression on CYP19a1 expression.

The steroidogenic, human granulosa cell-like tumor cell line KGN [36] was obtained from the laboratory of Associate Professor Andrew Shelling, University of Auckland, NZ, and used as a positive control for CYP19a1 expression. KGN cells were cultured in DMEM/F12 medium (Invitrogen) with 10% FBS, 2 nM L-Glutamine and Phenol Red indicator, in a 5% CO2 atmosphere.

Human ovarian surface epithelial cell lines hOSE 17-1 and hOSE 11-12 were obtained from the laboratory of Dr Kai-Fai Lee (University of Hong Kong) and originated from primary cultures, grown from scraping the surface of a non-neoplastic ovary and immortalized using the human papilloma virus (HPV) viral oncogenes E6 and E7 [37]. The similarly immortalized human Fallopian tube epithelial cell line OE-E6/E7 was also obtained from the laboratory of Dr Kai-Fai Lee [38-43]. The hOSE and OE-E6/E7 lines were chosen to represent potential precursor cells for EOC [10,44,45]. The ciliated OE-E6/E7 cells were cultured in DMEM/F12 medium (Invitrogen) with 10% FBS and 2 nM L-Glutamine in 5% CO2. hOSE 17-1 and hOSE 11-12 cell lines were cultured in RPMI medium 1640 (Invitrogen), supplemented with 10% FBS, 1% penicillin and streptomycin (Invitrogen) plus 2 nM L-Glutamine in 5% CO2. Media for OE-E6/E7 and hOSE cells did not contain Phenol Red.

Stimulation of aromatase/CYP19a1 expression with forskolin and phorbol methyl acetate

MCF-7, KGN and hOSE 17-1 (2.5 X 10^5 and 5.0 X 10^5 cells per well) were seeded into 6-well culture dishes and cultured for 24 hours as described above. Cells were cultured in medium without FBS for a further 24 hours, before treatment with 25 µM forskolin (Sigma) in FBS supplemented medium for subsequent 24 hours. Cells were harvested and RNA extracted for qRT-PCR analysis. hOSE 17-1 cells were also treated with the phorbol ester phorbol-12-myristate-13-acetate (PMA: Sigma Aldrich) at a range of concentrations and incubation times. A stock solution of 1.5nM was prepared using dimethyl sulfoxide (DMSO – Sigma Aldrich), which was then diluted in complete RPMI-1640 medium containing 0.25% DMSO [46]. hOSE 17-1 cells (1 x 10^5 cells/ml) were treated with 1-100 nM PMA for 1h, 6h, 12h, and 24h at 37°C and relative expression of BRCA1 and CYP19a1 analyzed by qRT-PCR. All treatments were repeated in triplicate.

siRNA transfection and BRCA1 knockdown

Small interfering RNAs (siRNA) were designed by Thermo Scientific Dharmacon (ON-TARGETplus siRNA BRCA1 [J-003461-11] and ON-TARGETplus Non-targeting pool [D-001810-10]). siRNA reverse transfection of all cell lines was performed using Lipofectamine RNAiMAX (Invitrogen) as outlined in the manufacturer’s instructions. Optimized assays used 2nM siRNA in 6-well plates with 5 X 10^4 cells per well, compared with earlier experiments using 100 nM siRNA [31]. RNA was collected after 24 hours, protein after 48 and 72 hours. Three experiments using duplicate replication were completed for each cell line.

RNA isolation

Total RNA was isolated using TRIzol reagent (Invitrogen) and a total RNA Purification Kit (Norgen Biotek, Innovative Sciences, Dunedin, NZ) according to manufacturer’s instructions. The RNA was eluted in Elution Buffer (Norgen Biotek). The RNA concentration was measured at OD260 by spectrophotometer (NanoDrop, Wilmington, DE, USA) and RNA purity was assessed by measurement of the OD260/OD280 ratio. RNA degradation was assessed by electrophoresis of 400-500 ng of each sample through a 1% non-denaturing agarose gel (data not shown). Samples showing signs of RNA degradation were not assessed for mRNA expression by qRT-PCR.

Quantitative RT-PCR

First-strand cDNA was synthesized from 500 ng total RNA using Superscript III (Invitrogen) with random hexamers and oligo(dT20) primers. The SYBR Green-based (Platinum SYBR Green qPCR SuperMix-UDG with ROX, Invitrogen) real-time PCR was performed on an ABI 7300 Real-Time PCR System (Applied Biosystems, Auckland, NZ). Delta Ct relative quantification and multiple reference gene normalization (GAPDH, HPRT and ACTB) were calculated using qBase (qBasePLUS, Biogazelle, Ghent University, Belgium). Primers for breast cancer 1, early onset (BRCA1) were designed between Exons 12 and 13: Forward: (5’ AACGACCTCTAATGGTTGAGGG 3’); Reverse: (5’ ATGACCAAGTCCACCAGCAGG 3’) [47]. Other mRNA-specific primer sequences were obtained from the RTPrimers database (medgen.ugent.be/rtprimers/index.php) and included estrogen receptor 1 (ESR1); ID: 1945; estrogen receptor 2 (ESR2); ID: 1954; progesterone receptor (PGR); ID: 2395; androgen receptor (AR); ID: 1506; glyceraldehyde 3-phosphate dehydrogenase (GAPDH; ID: 3); β-actin (ACTB) (ID: 2219) and hypoxanthine phosphoribosyltransferase (HPRT; ID: 5).

Western blotting

Cells (5.0 X 10^5 cells per well) were cultured for 48 hours before protein extraction. Duplicate wells of each sample were trypsinized, counted and the lysate prepared according to cell number (10,000 cells/microliter). Whole-cell protein extracts were prepared using cell lysis buffer (10mM Tris HCl pH 8.0, 150mM NaCl, 1mM EDTA, 1%NP40, 0.1%SDS) including 1 X Complete Protease Inhibitor (Roche Diagnostics, Auckland, NZ). Human placental tissue was obtained with written, informed consent from the mother, after ethical approval from The New Zealand Health and Disability Multi-Region Ethics Committee (MREC/07/05/06). Samples (50 mg) were extracted using the same lysis buffer, as a control for CYP19a1-aromatase expression.

The whole-cell protein extracts were resolved on Tris-acetate 3-8% polyacrylamide gradient gels (NuPAGE System, Invitrogen) run in the NuPAGE Tris-Acetate SDS Running Buffer and transferred onto a Polyvinylidene Fluoride membrane (BioTrace PVDF, Pall Corporation Life Sciences, Auckland, NZ).
The blot was blocked in 9% skim milk with 0.1% TBS-Tween 20. The top half of the blot (above 100 kDa) was then incubated in a mouse anti-BRCA1 antibody (Calbiochem MS110 Anti-BRCA1 [Ab-1] Mouse mAb, 1:100 in 1% skim milk in 0.1% TBS-Tween 20) overnight. Cutting the blot in half avoided antibody sequestering by the smaller Δ11 transcript of BRCA1 (Chanel Smart, University of Queensland, Brisbane, Australia; personal communication). The lower half was incubated with a rabbit anti-ERα antibody (Santa Cruz Biotechnologies, 1:1000 dilution) under the same conditions. Blots were also incubated with a mouse anti-human cytochrome P450 aromatase antibody (Serotec MCA 2077S) at 1:250 dilution, under the same conditions. Membranes were then incubated with horseradish peroxidase–coupled secondary antibodies and developed using enhanced chemiluminescence (ECL; Amersham Biosciences, GE Healthcare Life Sciences, Sydney, Australia). Blots were reprobed with an anti-ß-actin antibody (clone AC-15, Sigma MAb A1978; 1:10,000 dilution) as a loading control.

Cell proliferation assay

Cells were transfected in 96-well plates under the conditions described above and subjected to BRCA1 knockdown using 2 nM siRNA. The detection reagent, consisting of SybrGreen 1 (SybrGreen Nucleic Acid Stain I, Invitrogen) in lysis buffer (20mM Tris HCl pH 8.0, 2.5mM EDTA, 0.1% Triton X-100) was dispersed into the assay plates after 24, 48 and 72 hours. The plates were left for 24 hours at 4°C after which fluorescence was measured on a plate reader. Cell proliferation was measured in triplicate in all cell lines.

Statistical analysis

A two-tailed Student T-test was used to determine significant changes in gene expression. Values of p<0.05 were considered significant.

Results

Gene expression characteristics of cell lines

Serial dilution of purified RT-PCR products showed that all primers could detect as few as 1000 copies of the target mRNA. No significant differences in the expression of genes used for normalization (GAPDH, HPRT and ACTB) were demonstrated across treatments, for all cell samples in all experiments (data not shown).

MCF-7: Very low CYP19α1 expression was detected in the MCF-7 cell line. Two independent primer sets were used to detect CYP19α1 expression in both MCF-7 cells and human placental cDNA extracts and equivalent amounts of cDNA were detected with each primer set (data not shown). MCF-7 cells expressed BRCA1 (Ct values of 21-24), ESR1, PGR and AR, but ESR2 expression was low to undetectable.

OE-E6/E7: OE-E6/E7 cells did not express CYP19α1, ESR1, AR or PGR, but very low levels of ESR2 expression (Ct values of 30-33) were detected. Ct values of 20-23 were observed for BRCA1 expression.

hOSE 17-1 and 11-12: CYP19α1 mRNA expression was undetectable in hOSE 11-12, but expressed at very low levels in hOSE 17-1 cells. Both cell lines were ESR1, ESR2 and PGR negative. AR mRNA expression was very low to undetectable in hOSE 17-1 and undetectable in hOSE 11-12 cell lines.

KGN: KGN cells expressed low, but measurable, CYP19α1 mRNA, but neither ESR1 nor ESR2 mRNAs was observed. KGN cells were PGR and AR positive.

Effects of BRCA1 knockdown on mRNA concentrations

BRCA1 expression: An 80-90% reduction in BRCA1 expression was achieved routinely in MCF-7, KGN, hOSE 17-1 and hOSE 11-12 cells with this siRNA concentration. A lower knockdown of BRCA1 mRNA (>60%) was observed in OE-E6/E7 cells, 24 hours after transfection, using 2 nM siRNA (Figure 1a).

In MCF-7 cells, BRCA knockdown with 100 nM siRNA did not increase CYP19α1 mRNA at 24 hours (Figure 1b). The higher siRNA concentration also did not significantly change ESR1 or ESR2 mRNA concentrations in MCF-7 cells (data not shown).

BRCA1 knockdown with 2 nM siRNA had no measurable effects on the expression of either ESR1 or 2, PGR and AR mRNAs, where these genes were expressed in MCF-7, OE-E6/E7, hOSE 17-1 or hOSE 11-12 cell lines (data not shown).

Gene expression in the BRCA1-mutated cell line HCC1937

Expression of CYP19α1 was analyzed in the HCC1937 cell lines [33]. There was an approximately three-fold increase in the expression of BRCA1 in the BRCA1-stably transfected form of HCC1937 (HCC-...
BRCA1) compared with HCC1937 (Figure 2a). There was no apparent change in the expression of CYP19a1 (Figure 2b). These results demonstrate that the regulation of CYP19a1 by BRCA1 is also absent in HCC1937.

The relative expression of ESR1 was also analyzed in the HCC1937 cell lines. Although HCC1937 has been classified as an ER-negative cell line [31], expression of ESR1 mRNA was higher in HCC1937 compared with HCC-BRCA1 (Figure 2c). HCC1937 had similar levels of mRNA expression to the ER-positive MCF-7 cell line (Figure 2c).

Effects of forskolin and PMA treatment on CYP19a1 expression

Treatment of KGN cells with 25 µM forskolin resulted in an increase in CYP19a1 mRNA expression of 80-fold (P<0.02), with a concomitant decrease in BRCA1 expression of 80-95% (Figure 3). In cell lines with low to undetectable basal CYP19a1 expression (MCF-7 and hOSE 17-1 cells) no significant increase in CYP19a1 mRNA expression was observed in response to 25 µM forskolin treatment (data not shown).

PMA enhanced the expression of CYP19a1 in hOSE 17-1 cells around four-fold at 10 nM (Figure 4a). There was no reduction in BRCA1 expression in response to the induction of CYP19a1 in hOSE 17-1 cells (Figure 4b).
**Effects of BRCA1 knockdown on CYP19a1 and ERalpha protein expression**

Reduction of BRCA1 protein, 48 and 72 hours after knockdown with 2 nM siRNA, was confirmed by immunoblotting, in MCF-7, OE-E6/E7, hOSE 17-1 and hOSE 11-12 cells (Figure 5a).

Aromatase: No measurable CYP19a1 protein was detected in OE-E6/E7 or hOSE 17-1 cells by immunoblotting and extremely low concentrations were observed in MCF-7 cells (Figure 5b). An extract of human placental protein gave a band at approximately 55 KDa. [48]. Other immunopositive bands were noted in MCF-7 and hOSE 17-1 cells (Figure 5b). CYP19a1 expression was measured in KGN cells, after BRCA1 knockdown and after forskolin treatment (Figure 5c). No change in CYP19a1 expression was noted 24-hours or 48-hours after BRCA1 knockdown with 2 nM siRNA. Stimulation of KGN cells with 25µM forskolin resulted in a large increase in CYP19a1 expression, at both 24-hours and 48-hours after treatment (Figure 5c).

![Immunoblot of CYP19a1 protein expression](image)

**Figure 5:** BRCA1 protein expression, 48 h after transfection of MCF-7, OE-E6/E7 and hOSE 17-1 and 11-12 cells with 2 nM BRCA1 siRNA oligonucleotide. C is the control transfection; KD is the siRNA knockdown. (A) BRCA1 and (B) CYP19a1 protein expression, measured by immunoblotting, with β-actin as the loading control. Human placental cDNA (P) was used as a positive control for CYP19a1 expression. Approximately 5 µg (P1) and 10 µg (P2) of lysate protein were added to the last two lanes of B. (C) Immunoblot of CYP19a1 protein expression after BRCA1 knockdown and after forskolin treatment in KGN cells. C-24: control expression 24 hours after transfection; KD-24: knockdown expression after 24 hours; C-48: control expression 48 hours after transfection; KD-48: knockdown expression after 48 hours.

**Discussion**

Very low or no basal CYP19a1 mRNA expression was observed in all the cell lines under study, apart from the granulosa cell-derived KGN cells. Low basal CYP19a1 expression or estrogen synthesis has previously been reported in MCF-7 cells [46,49-52], as well as in Fallopian tube epithelia [53-59]. Schoen et al. demonstrated CYP19a1 expression by immunoblot and immunohistochemistry, in the ciliated cells of the bovine oviduct, as well as in primary bovine oviductal cell cultures [59]. CYP19a1 expression has also been demonstrated, using immunohistochemistry, in some but not all hOSE cell lines [46,60], primarily those that were ER gttgggpositive, although the hOSE lines in the current study were not tested [60]. Lower CYP19a1 mRNA expression was observed in ovarian cancers and cysts, than in primary hOSE cells in the same study [60].

None of the cell lines tested, including KGN, responded to BRCA1 knockdown with 2 nM siRNA by increasing CYP19a1 mRNA expression. Furthermore in our hands MCF-7 cells did not show an increase in CYP19a1 expression after knockdown with 100 nM siRNA [61,62]. Although lower siRNA concentrations were used, they did result in a 60-90% drop in BRCA1 mRNA expression in all cell lines. We are also confident that this result was not the result of a problem with the qRT-PCR assay, since we achieved similar results with two different primer sets for CYP19a1 in placental extracts, as well as the KGN cells (data not shown). Cell density was a critical determinant of CYP19a1 expression in adipose stromal cells in culture [63]. In these experiments, wells were seeded with 500,000 cells in all experiments. However, the cell density after 24 hours in culture was not always the same between cell lines, with MCF-7 cells reaching around 80% confluence and OE-E6/E7, hOSE and KGN cells reaching 90-100% confluence.

Previous research has shown that augmenting the expression of CYP19a1 leads to a reduction in BRCA1 expression [28,64]. These studies demonstrated that inducing CYP19a1 expression in SK-BR-3 and breast adipose fibroblasts suppressed the expression of BRCA1. Similar treatments have been used with hOSE cell lines to augment CYP19a1 expression [46]; however the response of BRCA1 was not measured in that report. The KGN cell line responded to forskolin treatment with an 80-fold increase in CYP19a1 mRNA expression. We originally hypothesized that the precursor cell of EOC would demonstrate a relationship between BRCA1 and CYP19a1 expression, but this was only observed in the KGN cells. None of the hypothesized precursor cell lines for EOC responded to BRCA1 knockdown by increasing expression of CYP19a1. The reported relationship between these two genes [26,65,66] may only be observable in cell lines that express moderate amounts of CYP19a1. It is also possible that such a relationship involves methylation of either or both genes, since hypermethylation of the BRCA1 promoter appears to play a role in sporadic breast carcinogenesis [67-70].

Both OSE and tubal epithelia may be exposed to cumulus granulosa cells secreting high concentrations of estrogen and, in BRCA1+ women, these tissues will be sensitive to cytotoxic agents, due to deficiency in DNA repair mechanisms caused by the lack of effective BRCA1 [71,72]. Conditional BRCA1 knockdown in murine granulosa cells leads to ovarian and uterine serous cystadenoma [73-75]. A role for granulosa and/or cumulus cells released at ovulation, in the etiology of epithelial ovarian cancer, has not yet been thoroughly investigated.

In conclusion, no evidence for a relationship between BRCA1 and CYP19a1 expression was found in OSE or tubal cells, where steroidogenic pathways are not strongly expressed. A study of methylation of the BRCA1 promoter in these cells and of the interaction of granulosa cells with OSE and tubal epithelia, using a coculture system [76], may be warranted.
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