Human Cancer Biology

Prolonged Postovulatory Proinflammatory Signaling in the Fallopian Tube Epithelium May Be Mediated through a BRCA1/DAB2 Axis

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Abstract

Purpose: To assess inflammation-related gene expression in nonmalignant fallopian tube epithelium (FTE) from BRCA1/2 mutation carriers and control patients obtained during the luteal and follicular phase, and to determine the impact of BRCA1 and disabled homolog 2 (DAB2) on NF-κB–mediated proinflammatory signaling.

Experimental Design: A list of inflammation-related and NF-κB–responsive genes was compiled through gene set enrichment and PubMed database search, corresponding probes identified, and unpaired t tests conducted to identify differentially expressed genes in previously profiled FTE samples. ES2 and A549 cells were cotransfected with DAB2- or BRCA1–targeting siRNA and an NF-κB–responsive luciferase reporter, treated with TNF-α and luciferase activity determined. To determine whether DAB2 or BRCA1 alters mRNA expression of NF-κB target genes, cells were transfected with siRNA, treated with TNF-α, and harvested for total RNA extraction and quantitative real-time PCR.

Results: A subset of BRCA1–mutated luteal phase samples previously found to group with adnexal high-grade serous carcinomas (HGSCs) differentially expressed 124 inflammation–associated probesets relative to remaining FTE samples. These samples also differentially expressed 264 probes relative to other luteal phase samples exposed to the same postovulatory environment. Both BRCA1- and DAB2–targeting siRNA increased TNF-α–induced NF-κB activity and mRNA expression of NF-κB–dependent target gene SOD2 relative to nontargeting siRNA, suggesting that both proteins repress proinflammatory signaling.

Conclusions: These data provide evidence of elevated proinflammatory signaling in a subset of BRCA1–mutated luteal phase FTE, consistent with an altered response to ovulation-associated cytokines. Furthermore, both BRCA1 and DAB2 affect NF-κB activity, indicating a novel link between BRCA mutation status, ovulation, and predisposition to HGSC. Clin Cancer Res; 18(16): 4334–44. ©2012 AACR.

Introduction

High-grade serous carcinoma (HGSC) is the most common and lethal histologic subtype of ovarian and tubal cancer with up to 20% of cases associated with a germline mutation in BRCA1/2 (1). We previously generated and compared gene expression profiles from laser capture–microdissected nonmalignant fallopian tube epithelium (FTE) from BRCA1/2-mutation carriers (FTEb) and control patients at a baseline risk for adnexal HGSC (FTEn) to identify alterations predisposing to malignant transformation. Notably, FTEb samples collected during the luteal phase exhibited global molecular profiles more closely resembling that of HGSC than FTE collected during the follicular phase or from control patients (2). Despite a marked elevation of progesterone during the luteal phase, an overall difference in progesterone receptor signaling in FTE as a function of BRCA1 mutation status was not observed (3). This suggests that differential response of BRCA1/2–mutated FTE to factors associated with the postovulatory luteal phase other than progesterone underlie its molecular similarity to HGSC.

Of particular relevance, ovulation is recognized as an acute localized inflammatory event. Each ovulatory cycle involves infiltration by leukocytes, production of inflammatory mediators, and extensive tissue remodeling, leading to follicular rupture (4). Ovulation results in the release of an oocyte with its adherent cumulus granulosa cells into the adjacent fallopian tube. At this time, the ovarian surface and fimbria are bathed with follicular fluid containing...
**Translational Relevance**

Recent evidence strongly implicates the fallopian tube as the source of “ovarian” high-grade serous carcinoma (HGSC), the most common and lethal form of epithelial ovarian cancer. In light of this, our work aims to identify molecular alterations in nonmalignant fallopian tube epithelium from BRCA1/2 mutation carriers that may contribute to malignant transformation. Discovery and characterization of these molecular alterations could contribute to a better understanding of the earliest events of serous carcinogenesis, which could lead to improved detection of those patients at greatly enhanced risk, development of prevention strategies, and ultimately significant reductions in ovarian cancer–associated mortality. The data presented in this study further supports the role of ovulation-associated inflammatory signaling in serous carcinogenesis, and could provide targets for prophylaxis to prevent the development of adnexal HGSC in women at known risk.

Inflammatory cytokines, including TNF-α, reactive oxygen species (ROS), and steroids (5), in addition to proinflammatory cytokines secreted by released cumulus cells (6). Thus, an acute proinflammatory environment is created following ovulation at the surface of the ovary and within the distal fallopian tube. Proinflammatory signaling is primarily mediated by NF-kB, most notably the active subunit RelA/p65. NF-kB induces several proinflammatory genes, including cytokines (e.g., IL-1, TNF-α, and IL-6) and their respective receptors, chemokines (e.g., IL-8) responsible for recruitment of inflammatory leukocytes, cell adhesion molecules, matrix metalloproteinases, and inflammatory enzymes (7, 8). Production of proinflammatory cytokines by NF-kB generates a positive feedback loop, leading to amplification of the inflammatory response. NF-kB is constitutively activated in many human cancers, and has been shown to be the critical link between chronic inflammation and tumor development in some model systems (7). Chronic activation of the inflammatory response by NF-kB may increase the risk for tumor development by induction of genomic instability, through production of potentially mutagenic reactive oxidants, whereas NF-kB–dependent induction of genes involved in cell-cycle progression and prevention of apoptosis would promote the survival of damaged cells (7, 9).

Preliminary reanalysis of our previously generated gene expression profiles (2) revealed that 110 of 630 (17.5%) of differentially expressed genes in BRCA1-mutated luteal phase FTE specimens found to cluster with HGSC on the basis of their global gene expression profiles [referred to as “FTEb(S)”] of known function are involved in inflammation/immune response. A gene of particular interest was tumor suppressor disabled homolog 2 (DAB2). DAB2 expression was decreased in HGSC and luteal compared with follicular phase FTE obtained from both BRCA1/2 mutation carriers and control patients, although the extent of downregulation was greater in carriers. Of note, we observed loss of cytoplasmic DAB2 immunoreactivity specifically in secretory FTE cells, the proposed cell of origin of HGSC, during the luteal phase. This is in stark contrast to intense RelA immunopositivity observed in secretory FTE (Supplementary Fig. S1), suggesting a potential inverse relationship between these two proteins. Several functions likely contribute to the tumor suppressive role of BRCA1 in addition to its role in DNA damage repair, including modulation of steroid hormone (including progesterone, estrogen, and androgen) receptor activity (10–13). DAB2 has similarly been implicated as an important tumor suppressor, and has been shown to regulate both steroid hormone (i.e., androgen receptor (14)) and proinflammatory [i.e., activator protein (AP)-1 (15)] signaling. The objective of the present study was therefore to conduct a comprehensive analysis of inflammation-related gene expression in these previously generated profiles, and to test whether BRCA1 or DAB2 are involved in regulation of NF-kB–mediated proinflammatory signaling.

**Materials and Methods**

**Gene expression analysis**

Gene expression data derived from laser capture–microdissected FTE and HGSC specimens (GEO #GSE10971) were used to evaluate expression levels of inflammation-associated genes. These samples included histologically normal FTE from 12 confirmed BRCA1/2 mutation carriers and 12 control patients (6 luteal and 6 follicular phase each) and 13 HGSC.

A list of potential inflammation–related and NF-kB–responsive genes was compiled using 2 broad categorical definitions. The first was a gene set enrichment (“VSNFKAPPAB65_01”) identifying 190 genes with promoters matching the RelA consensus-binding site (16). The second was an in-house MySQL database containing all publications before April 2010 found in PubMed indexed to the complete set of human and mouse Entrez gene symbols (and synonyms) found in their abstracts. Through a series of SQL joins, keyword searching retrieved a list of all potential gene names mentioned, along with the number of article abstracts in which they were found. Results were restricted to those genes with more than 50 articles for the keyword “inflammatory” and more than 2 articles for the keywords “p65/RelA.” The resulting set of genes was combined with the aforementioned gene set enrichment category and a nonredundant list of corresponding probes was identified (n = 2,510) on the Affymetrix array.

The expression of these probes was assessed in our previously profiled samples. CEL files were imported into GeneSpringGX (v10.0, Agilent), and normalization was conducted using the Robust Multi-Array Average algorithm, followed by the median measurement for each probeset across all samples. An unpaired t-test with a Benjamini and Hochberg false discovery rate of P < 0.05 was used to identify differentially expressed probes in FTEb(S) samples compared with the remaining FTE samples, irrespective of ovarian cycle stage. Regrouping and analysis of the dataset were then conducted to identify differentially expressed...
probes in FTEb(S) samples relative to the remaining luteal phase samples (uncorrected unpaired t-test P < 0.05). The expression of significantly altered probesets among samples was visualized by hierarchical clustering (Pearson-centered correlation distance metric with average linkage rules).

Cell culture
ES2 ovarian cancer cells, MCF7 breast cancer cells, A549 lung cancer cells, and MIA PaCa-2 pancreatic cells were verified by short tandem repeat profiling by The Centre for Applied Genomics Genetic Analysis Facility (Toronto, ON, Canada). All cells were grown in Dulbecco’s modified Eagle’s medium/F-12 supplemented with 10% FBS, 100 U/mL penicillin, 100 μg streptomycin and fungizone (Gibco Cell Culture Products/Invitrogen). Medium containing charcoal-stripped FBS was used during experiments requiring hormone/cytokine treatments. Dexamethasone was dissolved in ethanol and recombinant human TNF-α (R&D Systems) was reconstituted in PBS containing 0.1% bovine serum albumin, and were further diluted with culture medium. Vehicle treatment consisted of an equivalent diluted amount of ethanol.

Cell lines were selected on the basis of expression of proteins of interest [glucocorticoid receptor, DAB2, RelA, and BRCA1], ease of transfection, and responsiveness to dexamethasone and/or TNF-α treatment. A549 cells are a commonly used cell line for studies on NF-κB signaling.

Western blot analysis
Western blot analysis was conducted as previously described (17) using monoclonal anti-DAB2 (1:50; BD Transduction Laboratories), monoclonal anti-BRCA1 (1:50, EMD Biosciences), rabbit polyclonal anti-NF-kB p65(A) (1:500), monoclonal anti-glucocorticoid receptor (3D5; 1:50), goat polyclonal anti-actin (1:500), and horse-radish peroxidase-labeled secondary antiserum (1:1500; all (3D5; 1:50), goat polyclonal anti-actin (1:500), and horse-radish peroxidase-labeled secondary antiserum (1:1500; all from Santa Cruz Biotechnology). Immunoreactive bands were detected by ECL (Amersham Pharmacia Biotech). Protein expression patterns observed included: ES2 (BRCA1+, DAB2++, GR+, RelA+++) , MIA PaCa-2, and MCF7 (BRCA1 low, DAB2/GD undetectable, RelA++) and A549 (BRCA1+, DAB2++, GR+, RelA++) (not shown).

Expression constructs and siRNA
Human DAB2 cDNA (MEC-1764; American Tissue Type Collection) was cloned into pcDNA3.1/Hygro (Invitrogen). Human BRCA1 cDNA in pcDNA3.1 was obtained from Dr. Irene Andrilis (Mount Sinai Hospital, Toronto, Ontario, Canada). All expression constructs were sequence verified before use. siGENOME SMARTpool DAB2–targeting siRNA, BRCA1–targeting siRNA, and nontargeting siRNA were purchased from Dharmacon. Western blotting confirmed specific knockdown of DAB2 (70%) and BRCA1 (80%) relative to nontargeting siRNA control (shown in Fig. 2).

Luciferase assays
To determine the impact of DAB2 and BRCA1 on endogenous NF-κB transcriptional activity, cells were plated at 100,000 cells/well in 24-well plates and transiently transfected with 0.2 μg/well pNF-κB–luciferase or pTAL–luciferase (negative control; Clontech), 0.1 μg/well β-galactosidase, and 50 pmol each of DAB2–, BRCA1– or nontargeting siRNA 24 hours after plating using Lipofectamine LTX (Invitrogen) according to the manufacturer’s protocol. Cells were treated with 10 ng/mL TNF-α or vehicle 48 hours following transfection, and harvested in Reporter Lysis Buffer (Promega) 8 hours after treatment. The impact of DAB2 and BRCA1 on glucocorticoid receptor activity was determined by cotransfection with 0.2 μg/well mouse mammary tumor virus luciferase, 0.1 μg/well β-galactosidase, and either 50 pmol of siRNA (DAB2–, BRCA1–, or nontargeting) or varying concentrations of expression constructs (DAB2, BRCA1, or pcDNA3 empty vector). Cells were treated with 10 nmol/L dexamethasone or vehicle and harvested 24 hours later. Luciferase activity was measured following addition of Luciferase Assay Substrate (Promega) and was normalized to β-galactosidase activity. Triplicate wells were included for each condition within each experiment. Normalized data are expressed as average fold change (± SEM) relative to nontargeting siRNA/empty vector–transfected cells receiving active treatment (TNF-α or dexamethasone). Statistical analysis was conducted using one-way ANOVA, followed by Newman–Keuls multiple comparison post hoc test (P<0.05).

Quantitative real-time PCR
To determine the impact of DAB2 and BRCA1 on mRNA expression of selected NF-κB–regulated genes, ES2 cells (100,000 cells/well in 24-well plates) were transfected with 50 pmol DAB2–, BRCA1– or nontargeting siRNA. Cells were treated with 10 ng/mL TNF-α or vehicle 48 hours following transfection, and harvested 8 hours later in 250 μL TRIzol reagent (Invitrogen). Two wells per condition were pooled to increase yield. RNA was isolated, genomic DNA was removed (DNA-free kit, Ambion), and reverse transcription conducted using Superscript III Reverse Transcriptase and Oligo(dT)20 primers (Invitrogen). Samples were diluted to 1.6 ng/μl and quantitative PCR was conducted as previously described (2). Primer sequences and (relative primer concentrations) used included: ACTB forward 5’-CTATCACCTCCCCCTGTGTTGGA-3’ and reverse 5’-AACAACAGGCCTTATTCC-3’ (900 nmol/L forward: 300 nmol/L reverse); DAB2 forward 5’-CTACGCTATTGAAATGGAAGCC-3’ and reverse 5’-GGATTACTACTTTGACCCAGAGGAG-3’ (300 nmol/L: 900 nmol/L); BIRC3 forward 5’-CTCTGTGAAAGCCTGAACGTG-3’ and reverse 5’-CTCCTGGAATTGTTCTTG-3’ (300 nmol/L: 900 nmol/L; IL8 forward 5’-TTCCAAGCTGGCCGTGGCTCGTCC-3’ and reverse 5’-TTCCGAGCTGGCGTGGCTCC-3’ (300 nmol/L: 50 nmol/L); GPX3 forward 5’-TGACGCCCTCTTCTGGGAACCC-3’ and reverse 5’-CTGCCGCTTGTGGTTGGACAGGA-3’ (900 nmol/L: 1,900 nmol/L); and SOD2 forward 5’-AAACAGCGCTATCCCACTCG-3’ and reverse 5’-ACCATCTGGTATTCCTTTGGC-3’ (300 nmol/L: 900 nmol/L). All experiments included triplicate wells of each sample for both target and reference gene. The comparative CT method for relative quantitation was conducted and normalized to ACTB expression. Results are
expressed as average fold change (± SEM) relative to TNF-α-treated cells transfected with nontargeting siRNA (n = 4 experiments). A one-way ANOVA followed by Newman–Keuls multiple comparison test (P < 0.05) was conducted for statistical comparisons.

Results

Differential expression of NF-κB–regulated and inflammation-related genes

In our initial study (2), FTE from 12 BRCA carriers and 12 control women were stratified by whether they were in the follicular phase or luteal phase (n = 6 for each genotype and cycle phase). When analyzed as a group, the 6 luteal phase BRCA mutation carrier specimens partitioned more closely with HGSC and distant from the follicular phase and the control luteal phase samples. Upon closer examination considering the individual profiles, 4 of the BRCA luteal phase samples partitioned closely with the HGSC specimens [FTEb(S)]. To characterize inflammatory gene expression in profiled FTE samples, we compiled a list of inflammatory process–related genes (Supplementary Table S1), as described in Materials and Methods. From this list, 124 probesets (Supplementary Table S2) were found to be significantly altered in FTEb(S) relative to the remaining FTE obtained during both the luteal and follicular phase (Benjamini and Hochberg false discovery rate P < 0.05). Four hundred and sixty-six probesets were significant without a multiple testing correction (P < 0.05, data not shown).

Hierarchical clustering was used to visualize how well expression of these probes categorized patient samples. As expected, FTEb(S) samples grouped together and were more closely related to other luteal samples. In contrast, no clear
separation of samples based solely on BRCA1/2 mutation status was observed (Fig. 1A). Regrouping and analysis of the dataset using only luteal phase samples found 264 differentially expressed inflammatory probesets (uncorrected t test P < 0.05) in the FTEb(S) relative to the remaining luteal phase samples exposed to a similar postovulatory environment (Supplementary Table S3). Because of the smaller numbers of samples in this comparison, an FDR-corrected t test revealed no significant changes (P < 0.05). Clustering of patient samples using this list separated samples into two distinct groups not directly related to mutation status; however, as expected, the unique pattern of gene expression in BRCA1-mutated FTEb(S) samples was evident (Fig. 1B). Notable genes with greatly increased expression in FTEb(S) samples in both comparisons included chemokines such as interleukin (IL)-8, detoxifying enzymes such as glutathione peroxidase 3 (GPX3), and superoxide dismutase 2 (SOD2), and survival genes such as baculoviral IAP repeat-containing 3 (BIRC3). Altogether, these data provide compelling evidence of altered inflammatory signaling in a subset of BRCA1-mutated luteal phase samples.

**DAB2 and BRCA1 directly impact TNF-α–induced NF-κB transcriptional activity**

These findings, combined with the inverse pattern of DAB2 and RelA protein expression within secretory FTE cells during the luteal phase, led us to investigate whether DAB2 or BRCA1 impact NF-κB transcriptional activity. Cytokine-responsive ES2 ovarian cancer cells were cotransfected with either DAB2-targeting ("Dsi") (A, knockdown of DAB2 protein shown in inset) or BRCA1-targeting ("Bsi"; C, knockdown of BRCA1 protein shown in inset) siRNA and an NF-κB–responsive luciferase reporter, treated with 10 ng/mL TNF-α or vehicle, and harvested 8 hours later for luciferase activity determination (n = 12 wells/condition). Similar experiments were repeated with DAB2- (B) or BRCA1-targeting (D) siRNA in A549 lung cancer cells (n = 3 wells/condition). Circles in each panel indicate the NF-κB luciferase activity in individual wells (normalized to β-galactosidase and expressed relative to the average activity in wells transfected with nontargeting siRNA ("NTsi") and treated with TNF-α), whereas horizontal lines represent average luciferase activity for each condition. Statistically significant differences in average xB luciferase activity were determined by one-way ANOVA followed by the Newman–Keuls multiple comparison test. Groups with different letters are statistically different from one another (P < 0.05).

![Figure 2](image-url)

**Figure 2.** DAB2 and BRCA1 inhibit TNF-α–induced NF-κB–dependent luciferase activity. To determine whether DAB2 or BRCA1 directly impact endogenous NF-κB transcriptional activity, ES2 ovarian cancer cells were cotransfected with either DAB2-targeting ("Dsi") (A, knockdown of DAB2 protein shown in inset) or BRCA1-targeting ("Bsi"; C, knockdown of BRCA1 protein shown in inset) siRNA and an NF-κB–responsive luciferase reporter, treated with 10 ng/mL TNF-α or vehicle, and harvested 8 hours later for luciferase activity determination (n = 12 wells/condition). Similar experiments were repeated with DAB2- (B) or BRCA1-targeting (D) siRNA in A549 lung cancer cells (n = 3 wells/condition). Circles in each panel indicate the NF-κB luciferase activity in individual wells (normalized to β-galactosidase and expressed relative to the average activity in wells transfected with nontargeting siRNA ("NTsi") and treated with TNF-α), whereas horizontal lines represent average luciferase activity for each condition. Statistically significant differences in average xB luciferase activity were determined by one-way ANOVA followed by the Newman–Keuls multiple comparison test. Groups with different letters are statistically different from one another (P < 0.05).
siRNA. Expression was further increased in TNF-α-treated cells transfected with either DAB2- (Fig. 3A, \( p < 0.05 \)) or BRCA1-targeting ("Bsi"; C and D) siRNA, suggesting that both proteins inhibit TNF-α-induced SOD2 expression. DAB2-targeting siRNA similarly increased TNF-α-induced expression of IL8 (Fig. 3B, \( p < 0.05 \)), whereas expression of BIRC3 was not affected by DAB2 status (Supplementary Fig. S2A). In contrast to its effects on SOD2, BRCA1-targeting siRNA led to a decreased expression of both IL8 (Fig. 3D, \( P < 0.001 \)) and IL-8 (B and D) mRNA expression is shown. Circles in each panel represent expression levels in individual samples (normalized to β-actin and expressed relative to cells transfected with nontargeting siRNA ("NTsi") and treated with TNF-α), whereas horizontal lines represent average relative mRNA expression for each experimental condition. Groups with different letters are statistically different from one another, as determined by one-way ANOVA followed by the Newman–Keuls multiple comparison test (\( P < 0.05 \)).

Figure 3. Impact of DAB2 and BRCA1 on TNF-α-induced NF-κB target gene expression. ES2 cells were transfected with DAB2-targeting ("Dsi"; A and B) or BRCA1-targeting ("Bsi"; C and D) siRNA, treated with 10 ng/mL TNF-α or vehicle and harvested 8 hours later for total RNA extraction and qRT-PCR for previously identified NF-κB-induced genes with increased expression in FTEb(S) samples (\( n = 4 \) wells/condition). The impact of DAB2/BRCA1 status on SOD2 (A and C) and IL-8 (B and D) mRNA expression is shown. Circles in each panel represent expression levels in individual samples (normalized to β-actin and expressed relative to cells transfected with nontargeting siRNA ("NTsi") and treated with TNF-α), whereas horizontal lines represent average relative mRNA expression for each experimental condition. Groups with different letters are statistically different from one another, as determined by one-way ANOVA followed by the Newman–Keuls multiple comparison test (\( P < 0.05 \)).

DAB2 and BRCA1 enhance dexamethasone-induced glucocorticoid receptor activity

The proinflammatory cascade leading to ovulation also activates a compensatory anti-inflammatory cascade to

Figure 4. TNF-α but not BRCA1 loss decreases DAB2 mRNA expression. ES2 cells were transfected with BRCA1-targeting ("Bsi") or nontargeting ("NTsi") siRNA, treated with 10 ng/mL TNF-α or vehicle, and harvested 8 hours later for total RNA extraction and qRT-PCR for DAB2 (\( n = 4 \) wells/condition). Circles represent normalized DAB2 mRNA levels in individual wells (relative to NTsi transfected-/TNF-α-treated wells), with horizontal lines indicating the average DAB2 level within each group. Statistically significant differences in average DAB2 expression were determined by one-way ANOVA followed by the Newman–Keuls multiple comparison test (\( P < 0.05 \)).
limit tissue damage and promote luteinization. This is achieved in part by cytokine-induced upregulation of 11β-hydroxysteroid dehydrogenase type 1, which catalyzes the conversion of cortisol from its inactive precursor cortisone, in granulosa and OSE cells at the site of follicular rupture. This results in elevation of cortisol in follicular fluid, which acts upon the intracellular glucocorticoid receptor to mediate anti-inflammatory signaling and restore tissue homeostasis (4, 8, 18). To determine whether DAB2 and BRCA1 impact glucocorticoid receptor activity, ES2 cells were cotransfected with DAB2-, BRCA1-, or nontargeting siRNA and a glucocorticoid-responsive luciferase reporter. Cells were treated with 10 nmol/L dexamethasone or vehicle for 24 hours before determination of luciferase activity. As expected, dexamethasone increased glucocorticoid receptor transactivation activity in cells transfected with nontargeting siRNA (Fig. 5A and C). In contrast to its effects on NF-kB activity, both DAB2- (Fig. 5A, P < 0.05) and BRCA1-targeting (Fig. 5C, P < 0.001) siRNA decreased dexamethasone-induced glucocorticoid receptor activity. Consistently, transfection of ES2 cells with increasing amounts of DAB2 resulted in a dose-dependent increase in dexamethasone-induced luciferase activity (Fig. 5B, P < 0.001). Similar results were observed upon transfection with increasing amounts of BRCA1 in MIA PaCa-2 cells (Fig. 5D, P < 0.001). Finally, enhanced dexamethasone-induced glucocorticoid receptor activity was observed following upregulated DAB2 expression in MIA PaCa-2 cells and either DAB2 or BRCA1 in MC7 breast cancer cells (Supplementary Fig. S3), indicating that both DAB2 and BRCA1 promote glucocorticoid receptor transactivation activity in multiple cancer cell lines.

Discussion

The cell of origin for pelvic HGSC is now thought to lie within the FTE, regardless of whether the cancer is diagnosed as ovarian, peritoneal, or tubal (19). Women with a germline mutation in BRCA1 or BRCA2 have up to a 60% and 30% chance of developing HGSC, respectively, compared with a 1.6% chance in the general female population (20, 21); however, the underlying mechanisms contributing to an increased propensity for malignant transformation in mutation carriers are incompletely understood.

To identify alterations that may predispose to HGSC development, we previously molecularly characterized nonmalignant FTE from BRCA1/2 mutation carriers and control patients, as well as tubal and ovarian HGSC (2). Importantly, BRCA1-mutated samples obtained during the
postovulatory luteal phase showed global gene expression profiles closely resembling HGSC, suggesting that the luteal phase milieu may contribute to serous carcinogenesis. In contrast, BRCA1/2-mutated samples obtained during the follicular phase, and both follicular and luteal phase samples from normal controls, clustered separately from HGSC. The separation of luteal and follicular phase FTE was similarly observed in an independent set of cases (22), further highlighting the potential role of the luteal phase milieu.

In addition to BRCA1/2, epidemiologic studies have identified additional modifiers of ovarian cancer risk, mostly associated with reproductive history. Several studies have reported a substantial protective effect of oral contraceptives (OCP) in both the general population and BRCA1/2 mutation carriers, with greater risk reduction observed with increasing duration of use (23, 24). Parity and breastfeeding have also consistently been associated with risk reduction in both populations, with greater protection provided by an increased number of full-term pregnancies and longer duration of total lactation time (23, 24). Consistent with suppressive effects of OCP use, pregnancy, and breastfeeding on ovulation, a population-based case-control study found a positive association between estimated lifetime ovulatory (log)years and ovarian cancer risk, specifically among premenopausal women (25). Particular relevance of incessant ovulation in HGSC is also highlighted by two recent studies of BRCA mutation carriers reporting an inverse correlation between presence of a tubal precancer lesion and either parity (26) or duration of OCP use (27).

Exposure of adjacent distal FTE cells to locally elevated inflammatory mediators following ovulation would activate an intrinsic proinflammatory response requiring resolution during the ensuing luteal phase. Our observation of differential inflammatory gene expression in luteal phase FTE(β) samples suggests that FTE from a proportion of BRCA1 mutation carriers have an altered ability to resolve the local proinflammatory environment associated with ovulation, providing a novel explanation for the positive association of number of lifetime ovulations with ovarian/tubal cancer risk, as well as the protective effect of nonsteroidal anti-inflammatory drugs (28). These findings are consistent with other groups proposing an important role for inflammation in HGSC development. For instance, exposure of distal FTE cells to ovulation-associated inflammatory cytokines/ROS has been proposed to contribute to histologic HGSC precursor development. As summarized by Karst and colleagues, repetitive genotoxic stress associated with ovulation could lead to DNA damage and mutation of p53, resulting in clonal expansion of morphologically benign secretory FTE cells with strong immunopositivity for p53 and DNA damage marker γ-H2AX (“p53 signature”; ref. 29). Progression of a p53 signature to a tubal intraepithelial carcinoma (and eventually invasive HGSC) would occur following the acquisition of further genetic aberrations. This idea is supported by a recent study showing increased phospho-γ-H2AX in tubal epithelial cells of superovulated mice (30). Induction of ovulation was also associated with an increased infiltration by proinflamma-

ory macrophages in the oviduct, and exposure of FTE cells in culture to H2O2 or macrophage-conditioned medium recapitulated the DNA damage observed in vivo.

Inflammation associated with menstruation may also promote serous carcinogenesis. Salvador and colleagues proposed that regular exposure of the fallopian tube to menstrual cytokines/infection through retrograde flow from the endometrium contributes to tubal carcinogenesis; elevated levels of cytokines in fluid from hydrosalpinx versus normal follicular fluid, and the frequent co-occurrence of chronic salpingitis with ovarian cancer are cited as support for this idea (31). A recent review by Vercellini and colleagues proposed that the distal tube would have uniquely prolonged exposure to bloody peritoneal fluid and catalytic iron that has collected in the Douglas pouch as a result of retrograde menstruation; tumorigenesis would then be promoted by iron-induced oxidative stress (32). Although a causative link between retrograde menstruation and ovarian carcinoma is strongly suggested in specific subtypes (endometrioid and clear cell) thought to arise from atypical ovarian endometriosis (33), our data are more consistent with a causative role of inflammation associated with the ovulatory process in HGSC development.

In addition to providing evidence that altered ovulation-associated inflammatory signaling in a subset of FTE samples may contribute to serous carcinoma development; the present study suggests a novel underlying mechanism for this phenomenon by demonstrating an impact of both BRCA1 and DAB2 on the transcriptional activity of NF-κB and glucocorticoid receptor, the main mediators of pro- and anti-inflammatory signaling, respectively. The importance of BRCA1 in HGSC has long been appreciated. Several functions of BRCA1 likely contribute to its tumor suppressive role, including but not limited to maintenance of genomic integrity through facilitation of DNA repair, regulation of cell-cycle progression, and modulation of steroid hormone (including progesterone, estrogen, and androgen) receptor activity (10–13). DAB2 has similarly been implicated as an important tumor suppressor by us and others. It was originally identified in a screen for transcripts downregulated in ovarian cancer cell lines compared with normal OSE (34), and subsequent studies have observed decreased DAB2 in breast, prostate, pancreatic, esophageal, and squamous cell carcinoma (35–37). DAB2 has been implicated in various cellular processes, including proliferation, differentiation, polarity, endocytosis, adhesion, and migration. It has also been shown to regulate several important signaling pathways, including androgen receptor signaling (14), TGF-β signaling (38), and the canonical Wnt pathway (36). Most notably, DAB2 has been shown to inhibit AP-1 signaling (15); this transcriptional complex activates a subset of inflammatory genes and is a target of transrepression by glucocorticoid receptor (39). DAB2 primarily functions as an adaptor protein in these pathways, resulting in alteration of the phosphorylation status of key molecules, disruption of protein complexes, and altered subunit expression. Our results suggest an additional function for BRCA1 and DAB2 in balancing anti-

versus pro-inflammatory signaling, providing further explanation for their tumor suppressive roles in HGSC.

Interestingly, although BRCA1 and DAB2 similarly affect overall NF-kB transcriptional activity, the impact on expression of specific target genes appears to be more complex. Consistent with our luciferase data, both DAB2 and BRCA1 inhibit TNF-α-dependent expression of NF-kB target gene SOD2. This suggests that both proteins may act in concert to regulate the response to elevated (sublethal) ROS levels adjacent to the ovulatory site in vivo. Superoxide anions, released in large amounts by invading inflammatory leukocytes, are dismutated by enzymes (such as SOD2) into H₂O₂. H₂O₂ is further decomposed into molecular oxygen and water by "H₂O₂-detoxifying enzymes" (such as catalase and thioredoxin) to prevent the formation of highly reactive hydroxyl radicals by excess H₂O₂. Both of these steps are crucial, as hydroxyl radicals can readily generate DNA adducts, principally 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG), if not prevented by antioxidant enzymes (40, 41). In contrast to SOD2, FTEb(S) samples show decreased expression of thioredoxin and catalase relative to the remaining luteal phase samples (not shown). Increased expression of SOD2 in the absence of catalase and thioredoxin could lead to accumulation of potentially mutagenic 8-oxo-dG adducts, in addition to preventing induction of p53-dependent apoptosis in damaged cells (41). The connection between ovulation and 8-oxo-dG adducts has been shown in (ovine and human) OSE cells (9), but similar studies have not been conducted in FTE cells. 8-Oxo-dG adducts could result in DNA double-strand breaks and increased risk for mutations/malignant transformation (40) if repair pathways are not activated in the ensuing luteal phase. Interestingly, BRCA1 has previously been reported to increase the expression of several H₂O₂-detoxifying enzymes, including thioredoxin, and to regulate antioxidant response element-driven transcription (42). In addition, a recent study reported an increased level of 8-oxo-dG in leukocyte DNA obtained from BRCA1 mutation carriers (with or without symptoms of breast or ovarian cancer) compared with controls (43).

In contrast to SOD2, and opposite to that expected on the basis of the pattern of expression observed in FTEb(S) samples, BRCA1 appears to increase TNF-α-dependent expression of NF-kB target genes IL8 and BIRC3. First, elevated expression of IL8 (a chemokine responsible for recruitment of neutrophils to inflammatory sites) in luteal phase FTE is consistent with previous observations of increased IL-8 immunopositivity in distal FTE during the peri-ovulatory period (44), and infiltration of the fallopian tube (including the epithelium) by neutrophils and macrophages (45, 46). Second, increased expression of BIRC3 in luteal samples is consistent with previous reports of its induction by genotoxic stress, whereupon it contributes to antiapoptotic NF-kB activation (47). The discrepancy between the impact of BRCA1 on NF-kB luciferase activity and IL8/BIRC3 expression has several possible explanations. For instance, there are likely important differences between loss of wild-type BRCA1 expression versus the presence of inherited mutations; furthermore, specific mutations could lead to specific changes of function and/or downstream gene expression. Second, we have treated cells with TNF-α alone, not the complex milieu of the luteal phase/follicular fluid. Third, we have altered the expression of one gene at a time and assayed for target gene expression, which would not recapitulate complex patterns of gene expression in vivo, including the expression of potential genes with redundant and/or opposing functions. For example, AP-1 is also known to regulate IL8 expression (48), whereas BIRC3 is a target of glucocorticoid receptor transactivation (49). An additional possibility is that BRCA1 has promoter-specific effects, consistent with a previous study reporting a coactivator role in TNF-α- and IL-1β-dependent transcription of...
NF-kB target genes Fas and interferon-β (50). This was achieved in part through interaction of endogenous BRCA1 and RelA proteins; however, the effect of BRCA1 on global NF-kB-dependent transcription was not determined.

Despite complex effects on specific targets, the data presented herein support the generation of a refined model of serous carcinogenesis, in which malignant transformation of secretory cells of the distal FTE is promoted by an altered balance of pro- versus anti-inflammatory signaling during the postovulatory luteal phase, and furthermore, that this balance is determined by the underlying status of key genes such as BRCA1 and DAB2 (Fig. 6). We propose that homeostasis would be restored shortly following ovulation in FTE cells with sufficient levels of wild-type BRCA1 and/or DAB2, through induction of anti-inflammatory glucocorticoid receptor and inhibition of proinflammatory NF-kB by an as yet unidentified mechanism. In contrast, FTE cells with insufficient levels of BRCA1 and/or DAB2 would be unable to mount an adequate anti-inflammatory response following each ovulatory event. Incessant activation of the inflammatory response by NF-kB during successive luteal phases could therefore eventually lead to genomic instability and increased tumor risk, through continued production of mutagenic ROS and induction of cell cycle and antiapoptotic genes. Importantly, unlike previous theories suggesting a role for ovulation-associated inflammation in HGSC, the current proposed model emphasizes the differential response to this environment as the predisposing factor, rather than the nature of the environment itself. Proinflammatory signaling is recognized to play a role in carcinogenesis for multiple cancers. It is likely that BRCA1 and DAB2 would affect resolution of a proinflammatory environment in multiple tissues other than the fallopian tube. In our study, we observed results with breast and pancreatic cell lines similar to ovarian cancer cells, supporting the idea that the impact of DAB2 and BRCA1 is not cell line specific. We therefore propose that such a mechanism could contribute to early events of pancreatic and breast carcinogenesis, two cancers known to have loss of expression or mutations in DAB2 and BRCA1 (51, 52) and a predisposing role for inflammatory signaling (53, 54).

In summary, we propose that predisposition of BRCA1 mutation carriers to HGSC results, in part, from a delayed resolution of proinflammatory signaling within the distal fallopian tube following ovulation. Although the precise mechanisms whereby loss or compromised activity of BRCA1 leads to this delay remain unknown, our data suggest a unique role for DAB2. These findings have important implications for the definition of early events in high-grade serous carcinogenesis and identification of effective markers of early disease onset or increased risk. Moreover, this model provides a framework for the ultimate development of preventative strategies, as an alternative to surgery, for women with a predisposition to this cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: A.A. Tone, P. Shaw, T.J. Brown
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References


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