Prolonged post-ovulatory pro-inflammatory signaling in the fallopian tube epithelium may be mediated through a BRCA1/DAB2 axis*.

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STATEMENT OF TRANSLATIONAL RELEVANCE

Recent evidence strongly implicates the fallopian tube as the source of ‘ovarian’ high-grade serous carcinoma, the most common and lethal form of epithelial ovarian cancer. In light of this, our work aims to identify molecular alterations in non-malignant 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 high-grade serous carcinoma in women at known risk.

ABSTRACT

Purpose: To assess inflammation-related gene expression in non-malignant fallopian 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 DAB2 on nuclear factor-κB (NFκB)-mediated pro-inflammatory signaling.

Experimental Design: A list of inflammation-related and NFκB-responsive genes was compiled through gene set enrichment and PubMed database search, corresponding probes identified and unpaired t-tests performed to identify differentially expressed genes in previously profiled FTE samples. ES2 and A549 cells were co-transfected with DAB2- or BRCA1-targeting siRNA and an NFκB-responsive luciferase reporter, treated with TNFα and luciferase activity determined. To determine if DAB2 or BRCA1 alters mRNA expression of NFκB target genes,
cells were transfected with siRNA, treated with TNFα and harvested for total RNA extraction and RT-qPCR.

Results: A subset of BRCA1-mutated luteal phase samples previously found to group with adnexal high-grade serous carcinomas 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 post-ovulatory environment. Both BRCA1- and DAB2-targeting siRNA increased TNFα-induced NFκB activity and mRNA expression of NFκB-dependent target gene SOD2 relative to non-targeting siRNA, suggesting that both proteins repress pro-inflammatory signaling.

Conclusions: These data provide evidence of elevated pro-inflammatory 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 NFκB activity, indicating a novel link between BRCA mutation status, ovulation and predisposition to high-grade serous carcinoma.

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 non-malignant 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
(PR) signaling in FTE as a function of BRCA mutation status was not observed (3). This suggests that differential response of BRCA1/2-mutated FTE to factors associated with the post-ovulatory 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 remodelling 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 inflammatory cytokines, including tumor necrosis factor (TNF)-α, reactive oxygen species (ROS) and steroids (5), in addition to pro-inflammatory cytokines secreted by released cumulus cells (6). Thus, an acute pro-inflammatory environment is created following ovulation at the surface of the ovary and within the distal fallopian tube. Pro-inflammatory signaling is primarily mediated by nuclear factor-κB (NFκB), most notably the active subunit RelA/p65. NFκB induces several pro-inflammatory genes, including cytokines (e.g. IL-1, TNFα, 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 pro-inflammatory cytokines by NFκB generates a positive feedback loop, leading to amplification of the inflammatory response. NFκB 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 NFκB may increase the risk for tumor development by induction of genomic instability, through production of potentially mutagenic reactive oxidants, while NFκB-dependent induction of genes involved in cell cycle progression and prevention of apoptosis would promote the survival of damaged cells (7, 9).

Preliminary re-analysis of our previously generated gene expression profiles (2) revealed that 110/630 (17.5%) of differentially expressed genes in BRCA1-mutated luteal phase FTE...
specimens found to cluster with HGSC based on 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 to 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 (Supplemental Figure 1), 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 pro-inflammatory (i.e. activator protein (AP)-1 (15)) signaling. The objective of the present study was therefore to perform 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 NFκB-mediated pro-inflammatory signaling.

**MATERIALS & 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, 6 follicular phase each) and 13 HGSC.
A list of potential inflammation-related and NFxB-responsive genes was compiled using two broad categorical definitions. The first was a gene set enrichment (“V$NFKAPPAB65_01”) identifying 190 genes with promoters matching the RelA consensus binding site (16). The second was an in-house MySQL database containing all publications prior to 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 >50 articles for the keyword “inflammatory” and >2 articles for the keywords “p65/RelA.” The resulting set of genes was combined with the aforementioned gene set enrichment category and a non-redundant list of corresponding probes were identified (n=2510) 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 performed 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 to the remaining FTE samples irrespective of ovarian cycle stage.

Regrouping and analysis of the dataset was then performed 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 amongst 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. All cells were grown in DMEM/F-12
supplemented with 10% fetal bovine serum (FBS), 100 units/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. Dex was dissolved in ethanol and recombinant human TNFα (R&D Systems) was reconstituted in PBS containing 0.1% BSA, and were further diluted with culture medium. Vehicle treatment consisted of an equivalent diluted amount of ethanol.

Cell lines were selected based on expression of proteins of interest (GR, DAB2, RelA and BRCA1), ease of transfection and responsiveness to dex and/or TNFα treatment. A549 cells are a commonly used cell line for studies on NFκB signaling.

Western Blot Analysis. Western blot analysis was performed as previously described (17) using monoclonal anti-DAB2 (1:500; BD Transduction Laboratories), monoclonal anti-BRCA1 (1:50, EMD Biosciences), rabbit polyclonal anti-NFκB p65(A) (1:500), monoclonal anti-GR (3D5) (1:50), goat polyclonal anti-actin (1:500), and HRP-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/GR 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 Andrulis (Mount Sinai Hospital, Toronto, ON). All expression constructs were sequence-verified prior to use. siGENOME SMARTpool DAB2-targeting siRNA, BRCA1-targeting siRNA and non-targeting siRNA were purchased from Dharmacon. Western blotting confirmed specific knockdown of DAB2 (70%) and BRCA1 (80%) relative to non-targeting siRNA control (shown in Figure 2).
**Luciferase Assays.** To determine the impact of DAB2 and BRCA1 on endogenous NFκB transcriptional activity, cells were plated at 100,000 cells/well in 24-well plates and transiently transfected with 0.2μg/well pNFκB-luciferase or pTAL-luciferase (negative control; Clontech), 0.1μg/well β-galactosidase, and 50pmol each of DAB2-, BRCA1- or non-targeting siRNA 24h after plating using Lipofectamine LTX (Invitrogen) according to the manufacturer’s protocol. Cells were treated with 10ng/mL TNFα or vehicle 48h following transfection, and harvested in Reporter Lysis Buffer (Promega) 8h after treatment. The impact of DAB2 and BRCA1 on GR activity was determined by co-transfection with 0.2μg/well MMTV luciferase, 0.1μg/well β-galactosidase, and either 50pmol of siRNA (DAB2-, BRCA1- or non-targeting) or varying concentrations of expression constructs (DAB2, BRCA1 or pcDNA3 empty vector). Cells were treated with 10nM dex or vehicle and harvested 24h 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 non-targeting siRNA/empty vector-transfected cells receiving active treatment (TNFα or dex). Statistical analysis was performed using one-way ANOVA followed by Newman-Keuls Multiple Comparison post-hoc test (p<0.05).

**Quantitative RT-qPCR.** To determine the impact of DAB2 and BRCA1 on mRNA expression of select NFκB-regulated genes, ES2 cells (100,000 cells/well in 24-well plates) were transfected with 50pmol DAB2-, BRCA1- or non-targeting siRNA. Cells were treated with 10ng/mL TNFα or vehicle 48h following transfection, and harvested 8h later in 250μL TRIzol reagent (Invitrogen). Two wells/condition were pooled to increase yield. RNA was isolated, genomic DNA was removed (DNA-free kit, Ambion), and reverse-transcription performed using Superscript III Reverse Transcriptase and Oligo(dT)20 primers (Invitrogen). Samples were diluted to 1.6ng/μL.
and quantitative PCR was performed as previously described (2). Primer sequences (and relative primer concentrations) used included: *ACTB* forward 5′-GCATTGTTACAGGAAGTCCC-TTG-3′ and reverse 5′-CTATCACCTCCCTGTGTGGA-3′ (900nM forward: 300nM reverse); *DAB2* forward 5′-CTAGCTATTGCAAATGAGGGAAG-3′ and reverse 5′-GGTAATACTACTTTG-AACCCAGGAGCA-3′ (300nM:300nM); *BIRC3* forward 5′-TCTCTGCAAGAAGCTGAAGCTGT-GT-3′ and reverse 5′-TCTCCGCAATTGTTCTTCCACTGGT-3′ (300nM:50nM); *IL8* forward 5′-TTCCAAGCTGGCGTGCTC-3′ and reverse 5′-TGTGTTGCGCAGTGTGGTCC-3′ (300nM:50nM); *GPX3* forward 5′-TGACCGCCTCTTCTGGGAACCC-3′ and reverse 5′-CTGCC-GCCTCATGTAGGACAGGA-3′ (900nM:900nM); *SOD2* forward 5′-AACAACAGGCCTTATTCC-ACTG-3′ and reverse 5′-ACGATCGTGGTTACTTTGGC-3′ (300nM:900nM). All experiments included triplicate wells of each sample for both target and reference gene. The comparative CT method for relative quantitation was performed and normalized to *ACTB* expression. Results are expressed as average fold-change (+/-SEM) relative to TNFα-treated cells transfected with non-targeting 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 NFκB-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 (Supplemental Table 1), as described in Methods. From this list, 124 probesets...
(Supplemental Table 2) 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). 466 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 (Figure 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 post-ovulatory environment (Supplemental Table 3). Due to 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 (Figure 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 TNFα-induced NFκB 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 NFκB transcriptional activity. Cytokine-responsive ES2 ovarian cancer cells were co-transfected with DAB2-targeting, BRCA1-targeting, or non-targeting siRNA and an NFκB-responsive luciferase reporter construct. Cells were subsequently treated with 10ng/mL TNFα or...
vehicle, and harvested 8h later for determination of luciferase activity. As expected, TNFα treatment induced NFκB-dependent luciferase activity in ES2 cells transfected with non-targeting siRNA (Figures 2A, 2C). Importantly, both DAB2- (Figure 2A, p<0.001) and BRCA1-targeting (Figure 2C, p<0.001) siRNA led to enhanced TNFα-dependent induction of NFκB activity relative to control siRNA. Similar results were also observed when these experiments were repeated in A549 lung cancer cells (Figures 2B, 2D), indicating that the impact of DAB2 and BRCA1 on NFκB is not cell line-specific.

We next determined whether the observed differences in NFκB-dependent activity resulted in altered expression of known endogenous target genes, specifically those showing increased expression in FTEb(S) samples. ES2 cells were transfected with either DAB2-, BRCA1-, or non-targeting siRNA, treated with TNFα or vehicle and harvested 8h later for RNA extraction and RT-qPCR. Consistent with our reporter studies, TNFα treatment increased SOD2 mRNA expression in cells transfected with non-targeting siRNA. Expression was further increased in TNFα-treated cells transfected with either DAB2- (Figure 3A, p<0.05) or BRCA1-targeting (Figure 3C, p<0.05) siRNA, suggesting that both proteins inhibit TNFα-induced SOD2 expression. DAB2-targeting siRNA similarly increased TNFα-induced expression of IL8 (Figure 3B, p<0.05), while expression of BIRC3 was not affected by DAB2 status (Supplemental Figure 2A). In contrast to its effects on SOD2, BRCA1-targeting siRNA led to a decreased expression of both IL8 (Figure 3D, p<0.001) and BIRC3 (Supplemental Figure 2B, p<0.05) in TNFα-treated cells. A trend for increased expression of GPX3 in TNFα-treated cells was also observed in cells transfected with DAB2-, but not BRCA1-targeting siRNA (data not shown). Altogether, these findings indicate that DAB2 and BRCA1 have differing effects on the expression of specific NFκB target genes despite a similar impact on overall transcriptional activity.

Since DAB2 levels were found to be further decreased in luteal samples from BRCA mutation carriers in our previous study, we tested the impact of BRCA1-targeting siRNA on the expression of DAB2. TNFα treatment decreased DAB2 mRNA levels in cells transfected with
non-targeting siRNA (p<0.05), while BRCA1-targeting siRNA had no effect (Figure 4). This suggests that the previously observed loss of DAB2 in luteal phase FTE from both mutation carriers and controls (2) may in part be due to the local elevation of TNFα following ovulation, and that BRCA1 does not directly affect DAB2 expression.

DAB2 and BRCA1 enhance dexamethasone-induced GR activity. The pro-inflammatory cascade leading to ovulation also activates a compensatory anti-inflammatory cascade to 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 (GR) to mediate anti-inflammatory signaling and restore tissue homeostasis (4, 8, 18). To determine if DAB2 and BRCA1 impact GR activity, ES2 cells were co-transfected with DAB2-, BRCA1- or non-targeting siRNA and a glucocorticoid-responsive luciferase reporter. Cells were treated with 10nM dexamethasone (dex) or vehicle for 24h prior to determination of luciferase activity. As expected, dex increased GR transactivation activity in cells transfected with non-targeting siRNA (Figure 5A, 5C). In contrast to its effects on NFκB activity, both DAB2- (Figure 5A, p<0.05) and BRCA1-targeting (Figure 5C, p<0.001) siRNA decreased dex-induced GR activity. Consistently, transfection of ES2 cells with increasing amounts of DAB2 resulted in a dose-dependent increase in dex-induced luciferase activity (Figure 5B, p<0.001). Similar results were observed upon transfection with increasing amounts of BRCA1 in MIA PaCa-2 cells (Figure 5D, p<0.001). Finally, enhanced dex-induced GR activity was observed following upregulated DAB2 expression in MIA PaCa-2 cells and either DAB2 or BRCA1 in MCF7 breast cancer cells (Supplemental Figure 3), indicating that both DAB2 and BRCA1 promote GR 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 to 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 non-malignant FTE from BRCA1/2 mutation carriers and control patients, as well as tubal and ovarian HGSC (2). Importantly, BRCA1-mutated samples obtained during the post-ovulatory 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 (OCPs) 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 pre-cancer 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 pro-inflammatory response requiring resolution during the ensuing luteal phase. Our observation of differential inflammatory gene expression in luteal phase FTEb(S) samples suggests that FTE from a proportion of BRCA1 mutation carriers have an altered ability to resolve the local pro-inflammatory 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 NSAIDs (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 et al, 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’) (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-γH2AX in tubal epithelial cells of super-ovulated mice (30). Induction of ovulation was also associated with an increased infiltration by pro-inflammatory macrophages in the oviduct, and exposure of FTE cells in culture to H$_2$O$_2$ or macrophage-conditioned medium recapitulated the DNA damage observed in vivo.

Inflammation associated with menstruation may also promote serous carcinogenesis. Salvador et al 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 vs. 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 et al 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). While 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 is 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 NFκB and GR, 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 to 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), TGFβ signaling (38) and the canonical Wnt pathway (36). Most notably, DAB2 has been shown to inhibit activator protein (AP)-1 signaling (15); this transcriptional complex
activates a subset of inflammatory genes and is a target of transrepression by GR (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- vs. pro-inflammatory signaling, providing further explanation for their tumor suppressive roles in HGSC.

Interestingly, while BRCA1 and DAB2 similarly affect overall NFκB 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 TNFα-dependent expression of NFκB 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 demonstrated in (ovine and human) OSE cells (9), but similar studies have not been performed 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 H$_2$O$_2$-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 to controls (43).

In contrast to SOD2, and opposite to that expected based on the pattern of expression observed in FTEb(S) samples, BRCA1 appears to increase TNF$\alpha$-dependent expression of NF$\kappa$B target genes *IL8* and *BIRC3*. Firstly, 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). Secondly, increased expression of *BIRC3* in luteal samples is consistent with previous reports of its induction by genotoxic stress, whereupon it contributes to anti-apoptotic NF$\kappa$B activation (47). The discrepancy between the impact of BRCA1 on NF$\kappa$B luciferase activity and *IL8/BIRC3* expression has several possible explanations. For instance, there are likely important differences between loss of wildtype BRCA1 expression vs. the presence of inherited mutations; furthermore, specific mutations could lead to specific changes of function and/or downstream gene expression. Secondly, we have treated cells with TNF$\alpha$ alone, not the complex milieu of the luteal phase/follicular fluid. Thirdly, 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), while *BIRC3* is a target of GR transactivation (49). An additional possibility is that BRCA1 has promoter-specific effects, consistent with a previous study reporting a co-activator role in TNF$\alpha$- and IL1$\beta$-dependent transcription of NF$\kappa$B 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 NFκB-dependent transcription was not determined.

Despite complex effects on specific targets, the data presented herein supports 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- vs. anti-inflammatory signaling during the post-ovulatory luteal phase, and further, that this balance is determined by the underlying status of key genes such as BRCA1 and DAB2 (Figure 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 GR and inhibition of pro-inflammatory NFκB 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 NFκB 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 anti-apoptotic 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. Pro-inflammatory signalling is recognized to play a role in carcinogenesis for multiple cancers. It is likely that BRCA1 and DAB2 would affect resolution of a pro-inflammatory 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 signalling (53, 54).

In summary, we propose that predisposition of *BRCA1* mutation carriers to HGSC results, in part, from a delayed resolution of pro-inflammatory signaling within the distal fallopian tube following ovulation. While 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.

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**REFERENCES**


**FIGURE LEGENDS**

**Figure 1.** Differential expression of NFκB-regulated and inflammation-related genes in select BRCA1-mutated FTE samples during the luteal phase. A list of NFκB-dependent and/or inflammatory genes was compiled using a combination of gene set enrichment categories and PubMed database searches, corresponding probesets were identified and unpaired t-tests were performed to identify differentially expressed genes in FTEb(S) samples. Panel (A) shows unsupervised hierarchical cluster analysis of all FTE samples (n=24) using 124 probes found to be significantly altered in FTEb(S) relative to the remaining FTE (FDR p<0.05),
while (B) shows the clustering of luteal phase samples (n=12) using the 264 probesets differentiating FTEb(S) and the remaining luteal phase FTE (p<0.05). Two main cluster groups emerged in both analyses (shown at bottom of each panel). The cluster groups are shown at the top, with each line representing one sample. The type of sample is indicated at the bottom of each heatmap (blue, FTEn; red, FTEb), with the stage of the ovarian cycle at the time of surgery indicated below (purple, follicular; yellow, luteal). The distinct FTEb(S) sample subgroup is outlined in yellow in each panel.

Figure 2. DAB2 and BRCA1 inhibit TNFα-induced NFκB-dependent luciferase activity. To determine if DAB2 or BRCA1 directly impact endogenous NFκB transcriptional activity, ES2 ovarian cancer cells were co-transfected 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 NFκB-responsive luciferase reporter, treated with 10ng/mL TNFα or vehicle, and harvested 8h 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 NFκB luciferase activity in individual wells (normalized to β-galactosidase and expressed relative to the average activity in wells transfected with non-targeting siRNA (‘NTsi’) and treated with TNFα), whereas horizontal lines represent average luciferase activity for each condition. Statistically significant differences in average κB 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 3. Impact of DAB2 and BRCA1 on TNFα-induced NFκB target gene expression. ES2 cells were transfected with DAB2-targeting (‘Dsi) (A-B) or BRCA1-targeting (‘Bsi’) (C-D)
siRNA, treated with 10ng/mL TNFα or vehicle and harvested 8h later for total RNA extraction and RT-qPCR for previously identified NFκB-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 IL8 (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 non-targeting siRNA ('NTsi') and treated with TNFα), 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 4. TNFα but not BRCA1 loss decreases DAB2 mRNA expression.** ES2 cells were transfected with BRCA1-targeting ('Bsi') or non-targeting ('NTsi') siRNA, treated with 10ng/mL TNFα or vehicle, and harvested 8h later for total RNA extraction and RT-qPCR for DAB2 (n=4 wells/condition). Circles represent normalized DAB2 mRNA levels in individual wells (relative to NTsi transfected-/TNFα-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).

**Figure 5. DAB2 and BRCA1 enhance dexamethasone-induced GR transactivation activity.** To determine the impact of DAB2 and BRCA1 on endogenous GR transactivation activity, ES2 cells were co-transfected with either DAB2-targeting ('Dsi') (A, n=3 wells/condition) or BRCA1-targeting ('Bsi') (C, n=6 wells/condition) siRNA and a glucocorticoid-responsive luciferase reporter (mouse mammary tumor virus, MMTV). Cells were treated with 10nM of the synthetic glucocorticoid dexamethasone (dex) or vehicle and harvested 24h later for luciferase activity determination. The dose-dependent effect of DAB2 and BRCA1 were determined by
transfecting ES2 or MIA PaCa-2 cells with increasing amounts of DAB2 (‘D’) (B) or BRCA1 (‘B’) (D) respectively (n=9 wells/condition). Circles in panels A and C indicate the MMTV luciferase activity (normalized to β-galactosidase) in individual wells, expressed relative to cells transfected with non-targeting siRNA (‘NTsi”) and treated with dex. Normalized individual values in the remaining panels are expressed relative to dex-treated cells transfected with pcDNA3 empty vector. Statistically significant differences in average MMTV 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 6. Proposed model for high-grade serous carcinogenesis which incorporates a central role for restoring homeostasis following ovulation. In this model, the risk of malignant transformation of distal (secretory) FTE is increased by an altered balance of pro- vs. anti-inflammatory signaling during the post-ovulatory luteal phase, as determined by DAB2 and BRCA1 expression or mutation status. In FTE cells with sufficient DAB2 and/or BRCA1 (top), these proteins enhance glucocorticoid-induced GR transactivation activity and inhibit cytokine-induced NFκB activity shortly following ovulation. This shifts the balance in favor of anti-inflammatory signaling, leading to a timely resolution of the local inflammatory milieu that could promote mutagenesis. In contrast, FTE cells with deficient DAB2 and/or BRCA1 (bottom) would be unable to adequately mount a GR-mediated anti-inflammatory response or inhibit NFκB-dependent signaling following ovulation. This would result in a sustained pro-inflammatory environment within the distal fallopian tube following each ovulatory event, contributing to an increased propensity for malignant transformation over a woman’s reproductive lifespan.
Figure 1. Differential expression of NFκB-regulated and inflammation-related genes in select BRCA1-mutated FTE samples during the luteal phase.
Figure 2. DAB2 and BRCA1 inhibit TNFα-induced NFκB-dependent luciferase activity.

A.

B.

C.

D.
Figure 3. Impact of DAB2 and BRCA1 on TNFα-induced NFκB target gene expression.

A. C.

B. D.

Research.
Figure 4. TNFα but not BRCA1 loss decreases DAB2 mRNA expression.
Figure 5. DAB2 and BRCA1 enhance dexamethasone-induced GR transactivation activity.

A. MMTV luciferase activity (ES2) (FC rel to NTsi dex)

B. MMTV luciferase activity (ES2) (FC rel to pcDNA3 dex)

C. MMTV luciferase activity (ES2) (FC rel to NTsi dex)

D. MMTV luciferase activity (PaCa-2) (FC rel to pcDNA3 dex)
Figure 6. Proposed model for high-grade serous carcinogenesis which incorporates a central role for restoring homeostasis following ovulation.
Prolonged post-ovulatory pro-inflammatory signaling in the fallopian tube epithelium may be mediated through a BRCA1/DAB2 axis

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