Original Research

Altered expression of inflammation-associated genes in oviductal cells following follicular fluid exposure: Implications for ovarian carcinogenesis

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Abstract

Evidence indicates that high-grade serous ovarian carcinoma (HGSOC) may originate from lesions within the distal fallopian tube epithelium (FTE). Our previous studies indicate that fallopian tube epithelial cells from carriers of germline mutations in breast cancer susceptibility genes exhibit a pro-inflammatory gene expression signature during the luteal phase, suggesting that delayed resolution of postovulatory inflammatory signaling may contribute to predisposition to this ovarian cancer histotype. To determine whether exposure of tubal epithelial cells to periovulatory follicular fluid alters expression of inflammation-associated genes, we used an *ex vivo* culture system of bovine oviductal epithelial cells. Oviductal cells grown on collagen IV-coated transwell membranes assumed a cobblestone appearance and immunocytochemistry for FoxJ1 and Pax8 indicated that both ciliated and secretory epithelial cells were maintained in the cultures. Oviductal cells were exposed to human follicular fluid or culture medium for 24 h following which total cellular RNA was extracted at various time points. Expression of genes associated with inflammation was determined by quantitative real-time RT-PCR. Exposure to follicular fluid transiently increased the transcript levels of interleukin 8 (*IL8*) and cyclooxygenase 2 (*PTGS2*), and decreased the expression of mitochondrial superoxide dismutase (*SOD2*), glutathione peroxidase 3 (*GPX3*), disabled homolog 2 (*DAB2*), and glucocorticoid receptor (*NR3C1*). Tumor necrosis factor (*TNF*) and *IL6* levels were also decreased while those of nicotinomide phosphoribosyltransferase (*NAMPT*) were unaffected. This study demonstrates that periovulatory follicular fluid can act directly upon oviductal epithelial cells to alter gene expression that might contribute to early carcinogenic events. Furthermore, these findings illustrate the potential use of bovine oviductal cells to study signaling events implicated in ovarian carcinogenesis.

Keywords: Fallopian tube, ovarian cancer, inflammatory genes, follicular fluid, bovine

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Introduction

Epithelial ovarian cancer encompasses multiple histological subtypes with distinct etiology, gene involvement, and clinical course (as reviewed in Ref. 1). The most common histotype is high-grade serous ovarian carcinoma (HGSOC) for which germline mutations in breast cancer susceptibility genes (*BRCA1/2*) confer substantial increased risk.[2,3] Additional identified risk factors are associated with the number of lifetime ovulatory events. Pregnancy, lactation, oral contraceptive use, early menopause, and late menarche correlate with reduced incidence of HGSOC and are all consistent with fewer lifetime ovulatory events.[4–8]

Recent studies support the idea that HGSOC, which morphologically resembles the fallopian tube epithelium (FTE), originates from within this epithelium rather than from the surface epithelium of the ovary.[9] Putative HGSOC precursor lesions, referred to as tubal intraepithelial carcinomas (TICs), have been identified within the FTE of *BRCA* mutation carriers undergoing risk-reducing surgery. In contrast, precursor ovarian lesions have not been identified.[10,11] Fimbrial TICs have been identified in up to 60% of

unselected pelvic HGSOC cases[12–15] and possess *TP53* mutations identical to those of the corresponding invasive tumors,[13,16,17] supporting the idea that these two entities are clonally related both in *BRCA1*-associated and sporadic populations. We have shown that non-malignant FTE collected during the luteal phase of the menstrual cycle from *BRCA* mutation carriers, but not control patients, molecularly resembles HGSOC cells.[18] Further characterization of these gene expression profiles indicated an increased expression of pro-inflammatory and tumor-promoting NFκB target genes, consistent with diminished glucocorticoid receptor (*NR3C1*) signaling, in luteal phase FTE from *BRCA* mutation carriers relative to control FTE.[19] These findings suggest that FTE from *BRCA* mutation carriers may have a reduced ability to resolve a local pro-inflammatory environment within the distal fallopian tube following ovulation.

As a result of the LH surge and leading up to ovulation, inflammatory cytokines and mediators accumulate within the follicular fluid.[20,21] Upon follicle rupture, the follicular fluid and cumulus–oocyte complex is released into the distal fallopian tube exposing the epithelium to the follicular fluid milieu. Prolonged exposure to an inflammatory environment can lead to increased DNA adduct formation and gene mutations contributing to malignant transformation.[22–24] Thus, repeated exposure of FTE to an inflammatory environment might contribute to initiating events in HGSOC.

There have been no studies to date examining the impact of follicular fluid exposure on FTE cells. Recent studies by Levanon *et al*.[25] and Fotheringham *et al*.[26] described an *ex vivo* culture approach to study human FTE cells. In this system, FTE cells are seeded onto collagen IV-coated transwell inserts, enabling the maintenance of secretory and ciliated epithelial cells. A limitation to this approach is the availability of human FTE cells. In most cases only a limited amount of surgical material is released for research purposes since the pathological assessment of the tissues is required to rule out occult carcinoma. In this study, bovine oviductal tissue was used to study the impact of periovulatory follicular fluid on the expression of inflammatory-associated genes by tubal epithelial cells in an *ex vivo* culture model. Bovine oviducts are available from abattoirs and epithelial cells are readily released from these oviducts through mechanical means. Moreover, the bovine genome more closely resembles that of the human as compared to more commonly used rodent animal models.[27]

Materials and methods

Bovine oviductal tissue

Oviducts from Holstein cows were collected at a certified commercial abattoir (Cargill Meat Solutions, Guelph, ON) in accordance with Canadian Food Inspection Agency guidelines. The facility allowed only approved trained laboratory staff admittance and agreed to the use of the tissue for this study. Adherent tissue was removed and luminal epithelial cells were extruded by forcefully running sterile forceps along the outside of the tube. Extruded cells were disaggregated by passing through a 30-gauge needle and washed 3× in 37°C sterile phosphate-buffered saline (PBS; 137 nM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, and 1.76 mM KH_2PO_4, pH 7.4) with centrifugation at 300 *g* for 5 min. Cells were resuspended in DMEM/F-12 Ham's Medium (Invitrogen, Burlington, ON) supplemented with 10% fetal bovine serum (Fisher Scientific, Ottawa, ON), 100 units/mL penicillin, 100 g/mL streptomycin and 0.625 μg amphotericin B (Invitrogen). Cells were plated in 24-well plates or collagen IV-coated 6.5 mm diameter polyester membrane transwell inserts with 0.4 μm pores (Corning, Tewksbury, MA) as described by Fotheringham *et al*.[26] For transwell cultures, 500 μL of culture medium was placed in the lower chamber and 100 μL in the upper chamber with medium changes every two days until confluency was attained. Cultures were maintained in a 37°C humidified incubator at 5% CO_2.

Immunocytochemistry

Cultures were fixed in 4% paraformaldehyde for 20 min, permeabilized with 0.3% Triton X-100 (Sigma, St. Louis, MO), and blocked with 10% normal goat serum (Vector Laboratories, Burlingame, CA) for 1 h.[28] Cells were incubated overnight at 4°C with mouse anti-FoxJ1 (1:5000; Abcam Inc, Cambridge, MA), rabbit anti-Pax8 (1:1000; ProteinTech, Chicago, IL), or mouse anti-vimentin (1:100; Dako, Burlington, ON) antibody as markers of ciliated and secretory epithelial and stromal cells, respectively, or the corresponding non-specific IgG. Cells were then incubated with biotinylated goat anti-rabbit or anti-mouse IgG secondary antibody (1:200; Vector Laboratories) for 1 h at room temperature followed by incubation with streptavidin-peroxidase (1:400; Vector Laboratories) for 30 min. Staining was visualized by incubation in diaminobenzidine (Sigma). Cell images were captured using an Image Micropublisher 4.0 RTV camera with a Leica DIMIL LED inverted microscope with 20× objective, and processed with QCapture Pro 6.0 Software (QImaging, Surrey, BC). Staining was quantified by calculating the area of positive staining relative to total area within five independent randomly selected fields per well (2–5 wells) using northern eclipse version 8 software (Empix Imaging, Mississauga, ON).

Follicular fluid

Human follicular fluid was obtained from 14 patients undergoing oocyte retrieval as part of their *in vitro* fertilization procedure at the Centre for Fertility and Reproductive Health at Mount Sinai Hospital. All patients had provided written consent for the use of this fluid for research purposes and the Mount Sinai Hospital Institutional Research Ethics Board approved the use of this material for this study. All women had received human chorionic gonadotropin (hCG) approximately 36 h prior to oocyte retrieval as part of their stimulation protocol. Follicular fluid was centrifuged at 500 *g* for 10 min at 4°C and the supernatant was stored at −80°C. Cytokines were measured on individual samples using a multiplex assay (MILLIPLEX; Millipore, Billerica, MA) as per manufacturer's protocol.

Cortisol and progesterone were measured by ELISA (Elecsys, Olathe, KS) in the pooled follicular fluid.

Real-time quantitative RT-PCR (RT-qPCR)

Total RNA was isolated using TRIzol (Invitrogen) and genomic DNA was removed using a TURBO-DNA Free kit (Ambion, Austin, TX) according to manufacturer's protocol. Quality and quantity of RNA samples were confirmed using agarose gel electrophoresis and NanoDrop 1000 spectrophotometry (Fisher Scientific). DNAse-treated RNA was reverse transcribed using Superscript III reverse transcriptase (Invitrogen). Primers designed to target specified bovine genes were selected using NCBI Primer BLAST program (Table 1). RT-qPCR was performed using SYBR Green PCR Master Mix (Applied Biosytems, Foster City, CA) on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Amplification of a single product at the expected melting temperature was confirmed. All runs included triplicate wells of each sample for both target and reference genes. CT values were determined with ABI PRISM Sequence Detection software version 2.0 (Applied Biosystems). A relative standard curve method, with β-actin (ACTB) as calibrator, was used to calculate and normalize target gene transcript levels.

Statistical analysis

Data are expressed as mean ± SEM. Comparison of transcript levels determined by RT-qPCR was performed using two-way ANOVA (treatment by time) followed by a Holm-Sidak multiple comparison test. All statistical tests were performed with SigmaStat statistical software (SigmaStat, Systat Software Inc, Chicago, IL) with statistical significance set at $P < 0.05$.

Results

Characterization of bovine oviductal epithelial primary cultures

Epithelial cells mechanically derived from bovine oviducts were grown in primary culture. Cells grown on plastic assumed a fusiform morphology that was maintained at confluence (Figure 1(a)). In contrast, cells grown on collagen IV-coated transwell membranes assumed a cobblestone appearance consistent with a polarized epithelial sheet (Figure 1(b)). Both culture systems allowed for the survival of both ciliated and secretory epithelial cell populations. However, a more robust ciliated population was present in transwell cultures and could be visually differentiated by the presence of rapidly beating cilia.

The presence of ciliated and secretory epithelial cells and stromal cells was assessed by immunocytochemistry for specific cell-type markers. Staining of transwell cultures for FoxJ1, a transcription factor that regulates expression of genes involved in the generation of motile cilia,[29] demonstrated the presence of ciliated cells within the cultures, consistent with the visualization of beating cilia (Figure 2(a)). More than 50% (52.8 ± 2.3%) of the cells stained positive for FoxJ1. Staining for Pax8, a transcription factor and well-established lineage marker for secretory epithelial cells of Mullerian duct origin,[30] demonstrated the presence of secretory cells within the cultures (Figure 2(c)). Approximately 40% (40.2 ± 1.6%) of cells stained positive for Pax8. Vimentin staining, used to visualize stromal cells (Figure 2(e)), indicated that less than 5%

Table 1 Real-time quantitative PCR primer sets

Gene primer	Sequence	Tm (°C)	Amplicon Size (bp)
ACTB forward	5′-GCATTGTTACAGGAAGTCCTTTGC-3′	60–63	108
ACTB reverse	5′-CGATCACCTCCCCTGTGTGGA-3′		
PTGS2 forward	5′-TGCCCAGCACTTCACCCATCA-3′	63	134
PTGS2 reverse	5′-GGCGCAGCTTATGCTGTCTCTCT-3′		
DAB2 forward	5′-GGGCCCCTCCCGGGTCTAG-3′	62	110
DAB2 reverse	5′-CATGGCGCCCGGGACCATTG-3′		
GPX3 forward	5′-CGGCTCTTCCGGGCATCCT-3′	63	100
GPX3 reverse	5′-ACCAGCGTGGCAGTCCGTCT-3′		
NR3C1 forward	5′-GATGCCAAGGGTCTGGAGATG-3′	60	133
NR3C1 reverse	5′-GAGGAACTGGATGGAGGAGAAC-3′		
IL6 forward	5′-TGAGTGTGAAAGCAGCAAGGA-3′	60	101
IL6 reverse	5′-TCGCCTGATTGAACCCAGAT-3′		
IL8 forward	5′-GGCAGCTTTCCTGCTCTCTGCA-3′	60	110
IL8 reverse	5′-GGGTGGAAAGGTGTGGAATGTGTT-3′		
NAMPT forward	5′-TGCGGCAGAAGCCGAGTTCAA-3′	63	87
NAMPT reverse	5′-TTGCTTGTGTTGGGTGGGTACTGTT-3′		
SOD2 forward	5′-TGTTGTCGCGAGCGGCGTG-3′	63	115
SOD2 reverse	5′-TCCAGGGCGCCGTAGTCGTA-3′		
TNF forward	5′-CCGGTAGCCCACGTTGTAGC-3′	63	83
TNF reverse	5′-TGGCCATGAGGGCATTGGCAT-3′		

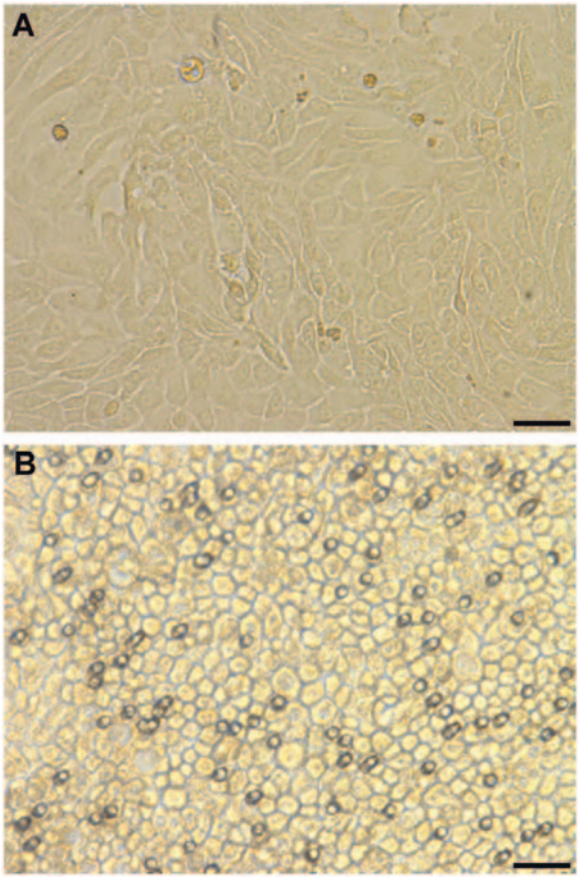

Figure 1 Bovine oviductal epithelial cells assume a cobblestone appearance when grown on collagen IV versus plastic. Cells mechanically released from bovine oviducts were plated directly on plastic culture plates (a) or on collagen IV-coated transwell inserts (b) and grown to confluence. Cells were visualized using light microscopy. Bars = 50 μm. (A color version of this figure is available in the online journal)

(2.0%) of the cultured cells were stromal. These findings indicate that similar to the findings reported for human and monkey fallopian tube cell culture, the transwell assay system supports the culturing of both secretory and epithelial bovine oviductal cells, while imparting a morphologic phenotype resembling an intact epithelial sheet.[26,28]

Impact of follicular fluid exposure on inflammatory gene expression

To address whether the exposure of oviductal epithelial cells to periovulatory follicular fluid alters the expression of genes involved in inflammatory signaling or responses, bovine oviductal cells were treated with follicular fluid obtained from human *in vitro* fertilization patients. These patients underwent a standard hyperstimulation protocol to generate multiple mature follicles, with oocyte retrieval performed approximately 36 h following hCG injection. Follicular fluid was collected from the largest and first follicle aspirated from each patient during oocyte retrieval. Consistent with previous reports, the collected follicular fluid contained varying amounts of cytokines, chemokines, growth factors, and steroids (Table 2). Equal amounts of each sample were pooled for use in further experiments.

To examine the effects of follicular fluid on oviductal cell gene expression, cells grown on transwell inserts were treated by replacing the culture medium in the upper transwell chamber with undiluted follicular fluid. Medium within the upper chamber of parallel control cultures was replaced with culture medium. In both cases, the bottom chamber contained culture medium that was replaced every 48 h. At 24 h after follicular fluid application, the follicular fluid or medium was aspirated from the upper chamber and replaced with fresh serum-free culture medium. Total cellular RNA was extracted from the cultured cells at various times following follicular fluid removal (Figure 3) and assayed for selected inflammation-associated genes by real-time RT-qPCR. Experiments were performed in triplicate within each of five independent bovine samples.

We previously reported that expression of disabled homolog 2 (*DAB2*), an adaptor protein implicated in tumor necrosis factor (*TNF*) signaling, is decreased in luteal phase FTE.[18] Consistent with these findings, exposure of bovine oviductal cells to follicular fluid resulted in decreased expression of *DAB2* that was observed at all time points with the exception of 96 h (Figure 3). Our previous studies also indicated altered expression of interleukin 8 (*IL8*), *IL6*, mitochondrial superoxide dismutase (*SOD2*), cyclooxygenase 2 (*PTGS2*), *NR3C1*, glutathione peroxidase 3 (*GPX3*), nicotinamide phosphoribosyltransferase (*NAMPT*), and *TNF* in luteal phase FTE of *BRCA* mutation carriers but not of control patients,[18,19] suggesting altered expression of these genes by ovulatory events.

A transient increase in *IL8* and *PTGS2* was observed 24 h after initiation of follicular fluid exposure, with levels returning to baseline by 12 h after removal of the follicular fluid (Figure 3). In contrast, *SOD2* and *NR3C1* transcript levels showed a delayed response to follicular fluid, with transiently decreased levels observed at 60 and 72 h for *SOD2* and 60, 72, and 96 h for *NR3C1* after initiation of treatment. *GPX3* expression was also transiently decreased by follicular fluid exposure. Surprisingly, follicular fluid exposure did not affect *NAMPT* expression, and decreased both *IL6* and *TNF* transcript levels (Figure 3).

Discussion

This study demonstrates that direct exposure of oviductal epithelial cells to follicular fluid transiently alters expression of several genes associated with inflammation. Exposure of the FTE to pro-inflammatory cytokines as a result of ovulation and exposure to follicular fluid has been postulated to contribute to early events in adnexal HGSOC[1]; however, previous studies have not assessed the impact of periovulatory follicular fluid exposure on gene expression in tubal epithelial cells. We previously showed that approximately 18% of differentially expressed genes within luteal phase FTE from *BRCA* mutation carriers that grouped with HGSOCs have known roles in immune response or inflammation.[19] This finding led us to hypothesize that this expression may represent a persistent response of these cells to follicular fluid exposure.

The preovulatory LH surge acts upon multiple cell types within the mature follicle to stimulate cumulus cell

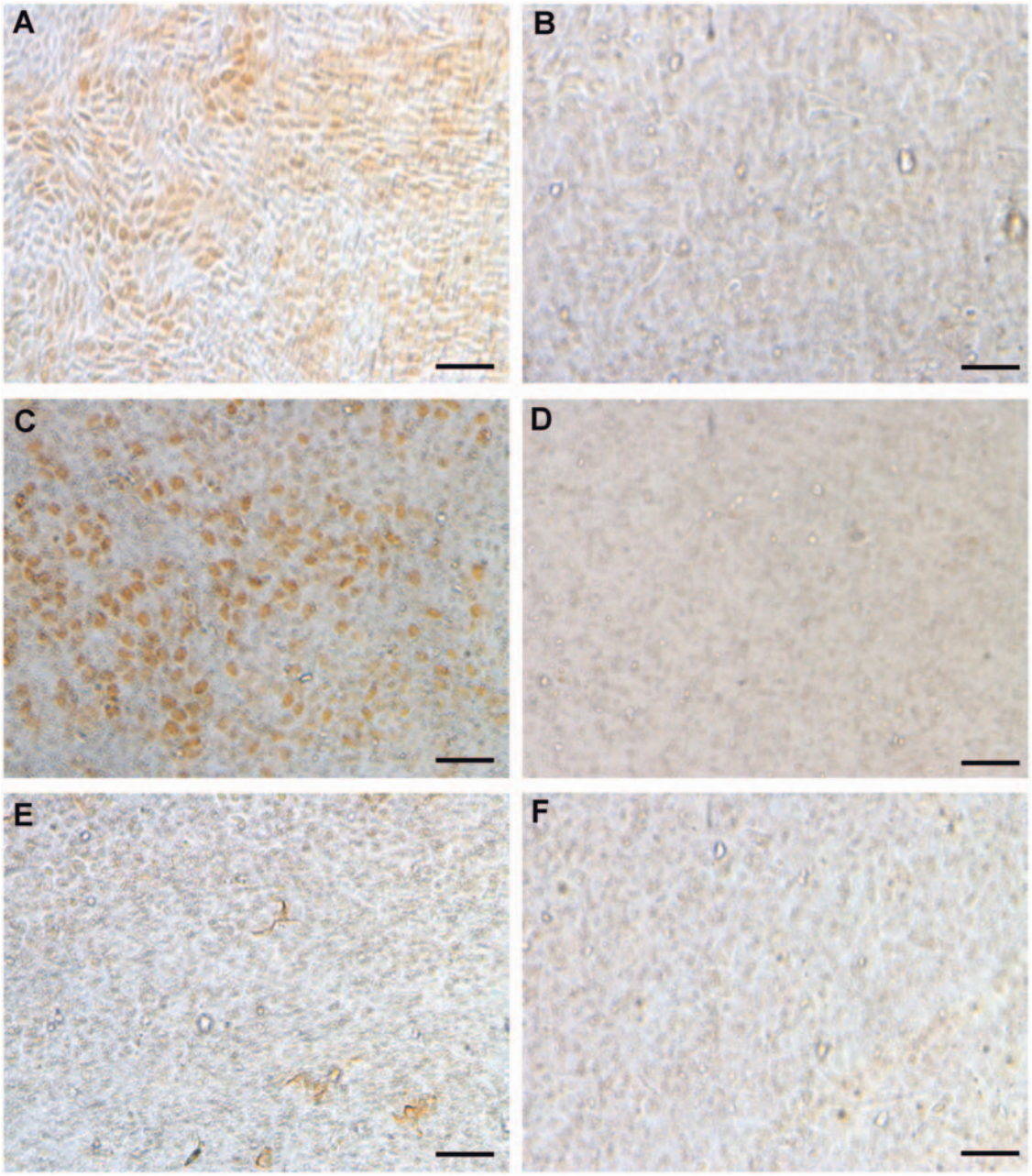

Figure 2 Immunocytochemical evidence of ciliated and secretory epithelial cells grown on collagen IV-coated transwell inserts. Cultures grown to confluence were fixed and stained for FoxJ1 (a), Pax8 (c) or vimentin (e) indicating ciliated epithelial, secretory epithelial, and stromal cells, respectively. Staining was visualized with diaminobenzidine (brown staining). Panels b, d, and f show staining obtained with non-specific IgG substituted for primary antibody. Bars = 50 μm

expansion, follicular wall breakdown, oocyte maturation and release, progesterone production, and luteinization of granulosa and thecal cells.[31,32] These remarkable changes involve coordination of multiple signaling pathways, including pro-inflammatory signaling, growth factor signaling, matrix remodeling, and angiogenesis. As a result, the follicular fluid, which enters the fallopian tube along with the cumulus-oocyte-complex, accumulates multiple signaling molecules, steroids, and reactive oxygen species.[20,21] Cortisol, which generally suppresses anti-inflammatory signaling, is also present in periovulatory follicular fluid, along with high concentrations of progesterone that can also bind glucocorticoid receptors to suppress pro-inflammatory signaling.[33] Thus, ovulation appears to create a limited and localized pro-inflammatory event that is quickly resolved. This is supported by the transient nature of the changes we observed in oviductal cells following exposure to follicular fluid.

The genes selected for examination in this study were altered in luteal phase FTE microdissected from *BRCA* mutation carriers relative to luteal phase FTE from control patients.[18,19] IL8 is a major mediator of the pro-inflammatory response and macrophage-induced angiogenesis.[34] Within tissues, IL8 acts to attract monocytes,

Table 2 Quantification of substances in periovulatory follicular fluid

Substance	Mean (SEM) pg/mL	Number of samples with detectable levels
Cytokines		
IP-10	870.01 (150.51)	14
sCD40L	214.22 (66.19)	14
G-CSF	124.79 (14.34)	14
Eotaxin	94.77 (8.95)	14
GM-CSF	30.85 (3.36)	14
TNFα	5.91 (0.53)	14
IFNγ	1.72 (0.44)	14
IL17	0.46 (0.06)	14
IFNα2	17.087 (3.68)	13
Flt-3 ligand	41.54 (9.32)	12
IL10	16.22 (5.75)	12
IL1ra	73.35 (45.15)	10
IL3	18.53(3.78)	7
IL7	14.7 (3.52)	6
IL15	4.83 (1.19)	6
IL1α	23.434 (8.41)	5
IL12(p70)	1.66 (0.23)	5
IL6	32.82(9.58)	4
IL12(p40)	5.58(2.13)	4
IL9	2.86 (1.06)	4
sIL2Rα	87.51 (21.45)	3
IL5	3.85 (1.39)	3
Chemokines		
MDC	726.18 (43.07)	14
GRO	469.64 (46.22)	14
IL8	281.53 (44.20)	14
MIP-1β	51.41 (5.27)	14
MIP-1α	20.07 (2.59)	13
MCP-1	446.96 (201.56)	8
Fractalkine	26.89 (7.75)	6
Growth factors		
VEGF	2303.59 (514.9)	14
FGF-2	84.02(16.69)	14
EGF	32.94 (6.85)	13
TGFα	5.12 (1.24)	5
Steroid hormones		
Cortisol	543.00 nmol/L	Pooled
Progesterone	62,080 nmol/L	Pooled

neutrophils and T-cells, thereby accentuating local pro-inflammatory signaling.[35]

Follicular fluid treatment caused a sustained decrease in *DAB2* transcript levels consistent with our previous finding of decreased expression of *DAB2* in luteal phase human FTE.[18] *DAB2* is an adaptor protein with multiple activities. It competes for sons of sevenless homolog 1 (SOS) binding to growth factor receptor bound protein 2 (GRB2) to disrupt growth factor signaling[36] and inhibits Wnt signaling,[37] which has been implicated in tumor cell metastasis.[38] *DAB2* is decreased in ovarian cancers and may function as a tumor suppressor.[39] *DAB2* potentiates glucocorticoid receptor signaling, suggesting possible decreased anti-inflammatory signaling by glucocorticoid receptor.[19] We observed a delayed and slight decrease in *NR3C1* transcript levels following treatment with follicular fluid that could reflect negative feedback on expression by activation of glucocorticoid receptor by ligands present in follicular fluid.

SOD2 is a mitochondrial protein that binds and converts superoxides to H_2O_2 as an initial step of the detoxifying process.[40] In the present study, we found that follicular fluid exposure transiently decreased *SOD2* transcript levels whereas *SOD2* levels were increased in luteal phase FTE of *BRCA* mutation carriers relative to FTE from control patients.[18,19] Unless efficiently removed by scavenging enzymes, such as catalase and *GPXs*, excess H_2O_2 generated by *SOD2* can form highly reactive hydroxyl radicals, leading to DNA adduct formation.[41] We found that *GPX3* transcript levels were transiently decreased by follicular fluid in oviductal cells. Thus, it would be of interest to determine the impact of follicular fluid on this and other enzymes involved in H_2O_2 elimination as a function of *BRCA1* levels.

A transient increase in *PTGS2* expression induced by follicular fluid exposure was observed. *PTGS2* is the induced form of cyclooxygenase (prostaglandin endoperoxide synthase) that catalyzes the conversion of arachidonic acid to prostaglandin H2 (PGH2), the rate-limiting step in prostinoid biosynthesis.[42] The specific prostaglandin produced (principally PGE_2, PGI_2, PGD_2, or $PGF_{2\alpha}$) is determined by the tissue-specific synthases/isomerases expressed and act as local mediators to affect tissue homeostasis.[43] *PTGS2* is induced during inflammation and inhibitors have been investigated for use in chemoprevention.[44–46]

Surprisingly, follicular fluid exposure decreased transcript levels of *IL6* and *TNF* and had no impact on *NAMPT*. *NAMPT* is involved in the biosynthesis of nicotinamide adenine dinucleotide (NAD), a co-enzyme involved with a broad range of cellular functions, and acts as a pro-inflammatory cytokine to increase *IL6* and *IL8* expression.[47] *NAMPT*, *IL6* and *TNF* transcript levels were increased in luteal phase FTE from *BRCA* mutation carriers relative to that from control patients, but not in luteal phase FTE compared to follicular phase FTE; thus, it is possible that *BRCA1* levels influence the expression of these cytokines through effects on glucocorticoid receptor signaling.[19]

In summary, this study highlights the potential use of bovine oviductal epithelial cell primary cultures for the study of signaling mechanisms that may contribute to early events associated with adnexal carcinogenesis. Similar to what has been reported for the use of the transwell culture system for human and monkey FTE *ex vivo* culture,[25,26,28] we demonstrate the maintenance of both secretory and ciliated epithelial cells in these bovine cultures. Bovine oviducts are more widely available and generate higher numbers of epithelial cells than human fallopian tube surgical samples. While it is possible to retrieve follicular fluid from bovine ovarian follicles, we elected to use follicular fluid collected from patients undergoing oocyte retrieval as part of their *in vitro* fertilization procedure. Since these patients received hCG 36 h prior to retrieval, the follicular fluid collected is periovulatory

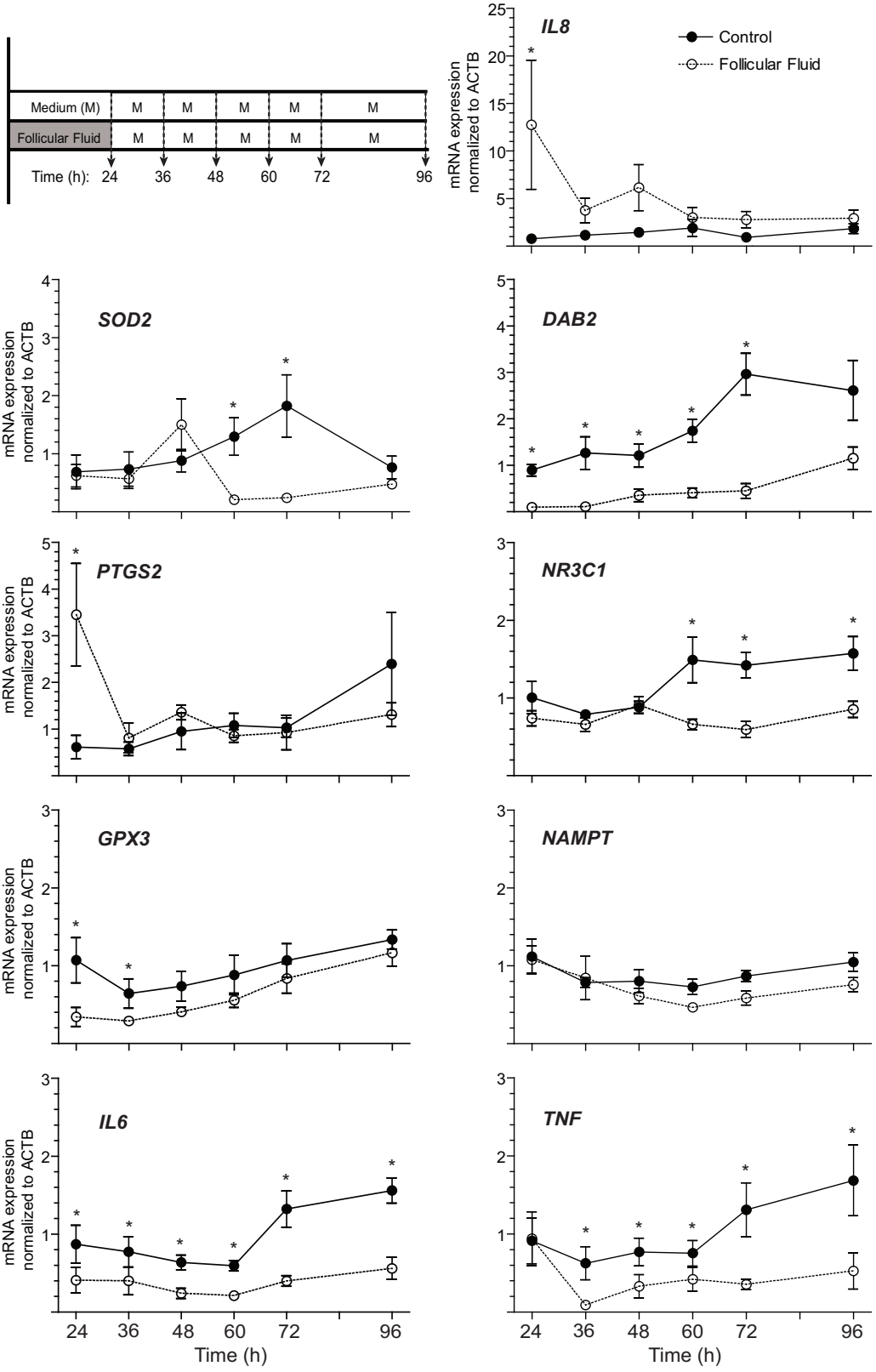

Figure 3 Exposure of bovine oviductal cells to follicular fluid alters expression of genes associated with inflammation. Cells grown on collagen IV-coated transwells were exposed to follicular fluid or culture medium (control) for 24 h. Total cellular RNA was extracted at various times after removal of follicular fluid and assayed for gene transcripts by real-time RT-qPCR. The top left panel shows the experimental design. Data are shown as mean ± SEM of five independent bovine samples. *Indicates a significant difference as compared to cultures treated with culture medium (control) as determined by two-way ANOVA followed by a Holm-Sidak multiple comparison test

and closely reiterates that which would interact with oviductal cells *in vivo* at the time of ovulation. Our finding that oviductal cells respond to follicular fluid exposure with altered expression of inflammation-associated genes provide support for the model that repeated exposure to these fluids may contribute to carcinogenesis. Further studies are underway to determine the impact of altered *BRCA* levels on these gene expression patterns.

Author contributions: AL performed all experimental procedures and statistical analysis and prepared the first draft of the manuscript. AK assisted in all experimental procedures and contributed to the writing of the manuscript. ESJ retrieved all oviducts used in the study and extracted oviductal epithelial cells. AT contributed to the conception and design of the study, ethics approval, and analysis of the follicular fluid cytokine levels. CV contributed to the design of the study and analysis of the follicular fluid cytokine levels. EG collected follicular fluid from IVF patients at time of oocyte retrieval, research ethics approval for collection of the fluid. WAK contributed to the design of the study. TJB, senior responsible author, contributed to the conception and design of the study, ethics approval and writing the manuscript. All authors provided editorial comments on the manuscript.

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