Decreased progesterone receptor isoform expression in luteal phase fallopian tube epithelium and high-grade serous carcinoma

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Abstract

We previously reported that *BRCA1/2*-mutated fallopian tube epithelium (FTE) collected during the luteal phase exhibits gene expression profiles more closely resembling that of high-grade serous carcinoma (HGSC) specimens than FTE collected during the follicular phase or from control patients. Since the luteal phase is characterised by high levels of progesterone, we determined whether the expression of progesterone receptor (PR) and PR-responsive genes was altered in FTE obtained from *BRCA* mutation carriers during the luteal phase of the menstrual cycle. RT-qPCR confirmed a decreased expression of *PR* mRNA in FTE during the luteal phase relative to follicular phase, in both *BRCA1/2* mutation carriers and control patients. Immunohistochemistry using isoform-specific antibodies confirmed a low level of both PR-A and PR-B in HGSC and a lower level of staining in FTE samples obtained during the luteal phase compared with the follicular phase. No significant difference in PR-A or PR-B staining was found based on patient *BRCA* mutation status. Analysis of our previously reported gene expression profiles based upon known PR-A- and PR-B-specific target genes did not partition samples by *BRCA* mutation status, indicating that overall FTE PR response is not altered in *BRCA* mutation carriers. HGSC samples grouped separately from other samples, consistent with the observed loss of PR expression. These findings indicate no overall difference in PR signalling in FTE as a function of *BRCA* mutation status. Thus, the molecular similarity of *BRCA1/2*-mutated luteal phase FTE and HGSC likely results from an altered response to luteal phase factors other than progesterone.

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Introduction

Epithelial ovarian cancers, which constitute the 5th leading cause of cancer-related death in North American women, are thought to arise through two basic pathways. While most histological subtypes likely arise from the ovarian surface epithelium (OSE), accumulating evidence provides strong support that high-grade serous adnexal cancer, commonly attributed to an ovarian origin, arises from the distal fallopian tube epithelium (FTE; Colgan *et al*. 2001, Piek *et al*. 2001, Finch *et al*. 2006, Medeiros *et al*. 2006, Crum *et al*. 2007, Kindelberger *et al*. 2007,

Folkins *et al*. 2008, Shaw *et al*. 2009). We have previously demonstrated that non-malignant FTE collected during the luteal phase from breast cancer susceptibility gene 1/2 (*BRCA1/2*) mutation carriers exhibits gene expression profiles more closely resembling that of ovarian and fallopian tube high-grade serous carcinoma (HGSC) than FTE from control patients (Tone *et al*. 2008). This finding suggests that the FTE of *BRCA* mutation carriers (FTEb) responds differently to the luteal phase milieu, which may contribute to its predisposition to malignant transformation. Since elevated progesterone secretion is a hallmark of the luteal phase, the altered gene expression in these samples may reflect a differential response to progesterone.

Progesterone classically signals by binding to intracellular progesterone receptors (PR), leading to receptor dimerisation and binding to specific hormone response elements located in the promoter region of target genes to regulate transcription through interaction with co-regulatory proteins (Mulac-Jericevic & Conneely 2004, Shao *et al*. 2006, Gellersen *et al*. 2009). Progesterone can also lead to rapid activation of a number of genes through protein–protein interactions involving cytoplasmic PR with components of the Src/mitogen activated protein kinase, phosphoinositide 3-kinase/Akt and janus kinase/signal transducer and activator of transcription signalling pathways (Leonhardt *et al*. 2003, Boonyaratanakornkit *et al*. 2008, Gellersen *et al*. 2009). The canonical PR is expressed as two isoforms, PR-A and PR-B, which are encoded by a single gene but arise through alternative use of two promoters (Boonyaratanakornkit *et al*. 2008). PR-A and PR-B are co-expressed at roughly equivalent levels in most target tissues (Shao *et al*. 2006). PR-A is truncated at the amino-terminal domain and has been shown to exhibit differential functional properties compared with the full-length PR-B isoform, including regulation of distinct genes (Jacobsen *et al*. 2005) and differential effects on target tissues in knockout mice (Mulac-Jericevic & Conneely 2004, Shao *et al*. 2006). Interestingly, PR-A has been shown to act as a transdominant inhibitor of PR-B (Vegeto *et al*. 1993, Mote *et al*. 2002), and the relative expression of these two isoforms has been shown to vary in reproductive tissues as a result of development and hormonal status and carcinogenesis (Mulac-Jericevic & Conneely 2004). Phosphorylation of PR in the absence of ligand, through the activation of cytokine or growth factor signalling pathways such as epidermal growth factor, can activate PR-dependent gene expression. In breast epithelial cells, ligand-independent signalling is primarily through the PR-A isoform (Jacobsen *et al*. 2005).

The genes regulated by ligand-independent signalling are largely distinct from those regulated by ligand-dependent PR activation (Jacobsen *et al*. 2005). Differential expression of PR isoforms in FTEb could thus underlie the altered gene expression profiles, particularly during the luteal phase when circulating progesterone levels are elevated.

The purpose of this study was to determine whether PR isoform expression is altered in FTEb relative to FTE from normal control patients (FTEn), particularly during the luteal phase. Real-time quantitative RT-PCR (RT-qPCR) and immunohistochemistry using isoform-specific antibodies were performed on patients' samples of FTE and HGSC. To our knowledge, this is the first comparative study of PR expression in non-malignant FTE in the follicular and luteal phases of the ovarian cycle in *BRCA1/2* mutation carriers and control patients.

Materials and methods

Study samples

Gene expression data derived from laser capture microdissected (LCM) snap-frozen FTE and HGSC specimens as part of our original profiling study (Tone *et al*. 2008) were used to evaluate expression levels of PR and PR-responsive genes. Specific clinical characteristics associated with these samples were previously presented (Tone *et al*. 2008). Briefly, these samples included histologically normal fallopian tubes from 12 confirmed *BRCA1/2* mutation carriers and 12 control patients undergoing salpingo-oophorectomy for reasons other than family history or adnexal malignancy, and 13 HGSC diagnosed as being of tubal or ovarian origin. Of the 12 *BRCA* patients, ten had confirmed mutations in *BRCA1* and two had confirmed mutations in *BRCA2*. Non-malignant samples were stratified according to the phase of the menstrual cycle, with six samples collected during the luteal phase and six collected during the follicular phase in each group. RNA extracted from the same representative LCM samples used in our original study (histologically normal FTE from six *BRCA1* mutation carriers and controls from both the luteal and follicular phases and six HGSC samples) was used to compare PR expression levels by RT-qPCR.

We constructed two tissue microarrays using formalin-fixed paraffin-embedded samples from the University Health Network Ovarian Tissue Bank, as described in our previous publication (Tone *et al*. 2008). The fallopian tube array included one 1.5 mm^2 core from 11 luteal and 16 follicular phase non-malignant specimens from confirmed *BRCA1/2*

mutation carriers, and 11 luteal and 12 follicular phase specimens from normal control patients. This array included the FTEn and FTEb cases that were used for the original gene expression analysis, as well as additional samples from the same groups. The second array included two distinct 0.6 mm^2 cores from 51 cases of HGSC naive to chemotherapy, all but two of which were independent from those subjected to gene expression profiling. Clinical features of all carcinoma specimens included in this second tissue array are shown in Supplementary Table 1, see section on supplementary data given at the end of this article. The use of all tissues in this study was approved by the University Health Network Research Ethics Board, and all patients provided informed consent.

RT-qPCR for *PR* mRNA

Real time RT-qPCR was performed as described previously (Tone *et al*. 2008) with the following modifications. Primer sequences used included PR: forward 5′-GAACAGCGGATGAA-AGAATCATC-3′ reverse 5′-AGGAACTCTTCTTGGCTAACTTGAAG-3′; ACTB: forward 5′-GCATTGTTACAGGAAGTC-CCTTG-3′ and reverse 5′-CTATCACCTCCCC-TGTGTG-GA-3′. The PR primer pair used recognises a specific sequence within the 1500 bp region immediately upstream of the poly(A) tail and therefore amplifies both PR-A- and PR-B-specific transcripts. All experiments included triplicate wells of each sample for both target and reference genes. The comparative C_T method for relative quantitation was performed and normalised to ACTB expression. A one-way ANOVA followed by the Newman–Keuls multiple comparison test was used to compare groups. Statistical significance was determined as $P < 0.05$.

PR isoform-specific immunohistochemistry

Immunohistochemistry was performed using standard procedures as described previously (Tone *et al*. 2008) with the following modifications. PR-A protein was detected using mouse monoclonal anti-PR antibody (PGR-312 (16), Novocastra Laboratories, Newcastle, UK; Mote *et al*. 2001) at a dilution of 1 in 200. PR-B was detected using anti-PR (B-form) antibody (PGR-B (SAN27), Novocastra Laboratories) at a dilution of 1 in 200. The ScanScope CS slide scanner (Aperio Technologies, Inc., Vista, CA, USA) was used to capture digitised images at 40× magnification. The images were then analysed core by core using the Positive Pixel Count Algorithm included with Image-Scope software (Aperio Technologies, Inc., version 10.0). This algorithm counts the number of pixels

within three user-defined intensity ranges (weak, positive and strong). Pixels that are stained but do not meet the positive colour specification are counted as negative-stained pixels; this is used to determine the overall percentage of positive-stained pixels. A pseudo-colour mark-up image is generated as an algorithm result to confirm the accurate measurement of pixels within each intensity range (examples shown in Supplementary Figure 1, see section on supplementary data given at the end of this article). To control for potential variability during immunohistochemistry, duplicate 5 μm sections of the fallopian tube array were included on each slide. Since the HGSC array consisted of duplicate distinct cores, the two values for each sample were averaged. Statistical analysis of mean percentage of strong staining by sample group was conducted using ANOVA followed by the Newman–Keuls multiple comparison test. The distribution of cases into quartiles was analysed using Pearson's χ^2 test. Comparisons were considered statistically significant if $P < 0.05$.

Gene expression analysis

Unsupervised hierarchical clustering was performed on our original gene expression data based upon corresponding probe sets of potential PR isoform-specific genes identified by Jacobsen *et al*. (2005). CEL files (Gene Expression Omnibus Series accession #GSE10971) were imported into GeneSpringGX (version 10.0, Agilent, Santa Clara, CA, USA). Normalisation was performed, first using the robust multi-array average (RMA) algorithm and then by the median measurement for each probe set across all samples. A two-way hierarchical cluster analysis with average linkage using a Pearson centered similarity distance metric was then performed.

Classification accuracy of subsets of PR isoform-specific genes was assessed using the supervised, shrunken centroid class prediction method (prediction analysis of microarrays (PAM); Tibshirani *et al*. 2002) found in the pamr (v1.44) package of R (v2.10.0, R Foundation for Statistical Computing, Vienna, Austria). Each gene set was tested against a predefined categorical binary variable relevant to a subset of patient expression data from Tone *et al*. (2008). Expression data for each set of patients under consideration were normalised in GeneSpringGX independently using the RMA algorithm as well as to the median expression value of each gene across all samples. All prediction analyses were performed with exported log-transformed values in *R*. In order to use all the probes under consideration in this prediction

method, a fixed threshold of 0 for the shrinkage factor was used. Model building and prediction was done using a leave-one-out cross-validation, with the number of cross validation folds equal to the smallest number of samples in one of the classes under consideration.

Results

Decreased expression of *PR* mRNA in luteal phase FTE and HGSC specimens

Analysis of previously obtained gene expression profiles of microdissected non-malignant FTE and HGSC specimens (Tone *et al.* 2008) revealed a decreased expression of *PR* mRNA in FTE samples obtained during the luteal phase relative to follicular phase in both *BRCA* mutation carriers ($P < 0.001$) and normal controls ($P < 0.01$; Fig. 1A). No differences in *PR* mRNA expression were observed in samples obtained from mutation carriers versus normal controls during either phase of the ovarian cycle. A subset of four *BRCA1*-mutated luteal specimens, which we have previously highlighted because they grouped closely with HGSC based on global gene expression (denoted as FTEb(S); Tone *et al.* 2008), showed similar expression of PR compared with other samples obtained during the luteal phase. Finally, HGSC specimens exhibited decreased expression of PR, similar to that observed in the luteal phase FTE.

To confirm these findings, RT-qPCR was performed using RNA from the same representative LCM samples from each group used for RT-qPCR in our previous study. Similar to the Affymetrix profiling data, RT-qPCR revealed a decreased expression of *PR* mRNA in the luteal versus follicular samples in both mutation carriers ($P < 0.05$) and controls ($P < 0.05$). In addition, no differences in PR expression were observed between samples obtained from *BRCA1/2* mutation carriers versus normal controls overall or within the same phase of the cycle (Fig. 1B). Importantly, *PR* mRNA was barely detectable in HGSC specimens, with expression levels > 100-fold lower than those observed in FTEn follicular samples ($P < 0.01$).

Decreased PR-A and PR-B immunostaining in luteal phase FTE and HGSC specimens

In light of previous studies showing selective loss of either PR-A or PR-B in ovarian (Akahira *et al.* 2000, 2002) and breast (Graham *et al.* 1995, Ariga *et al.* 2001, Mote *et al.* 2002, Jacobsen *et al.* 2005) cancers, immunohistochemistry was performed on tissue microarrays using PR-A- or PR-B-specific antibodies.
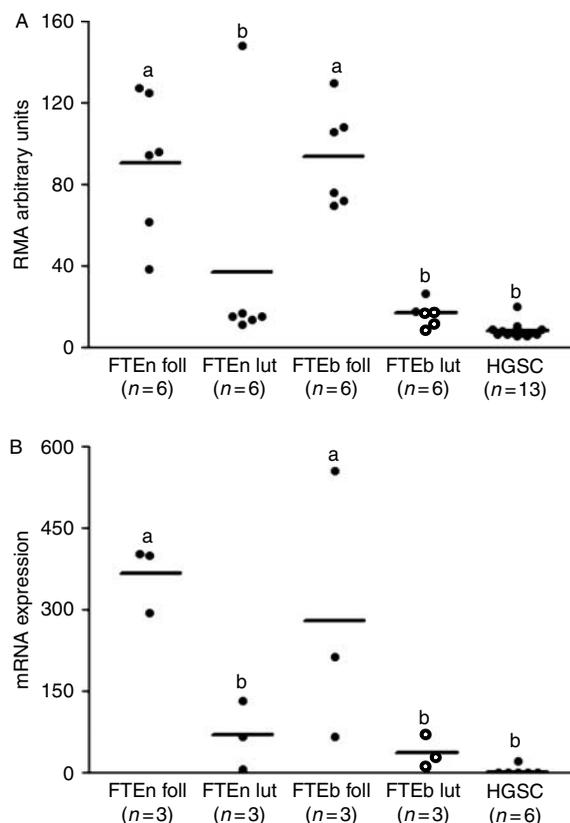

Figure 1 Decreased expression of *PR* mRNA in luteal phase FTE and HGSC specimens. Panel A shows the expression of PR in previously obtained gene expression profiles of non-malignant FTE and HGSC, expressed as values obtained following normalisation by the robust multi-array average (RMA) algorithm. The expression of *PR* mRNA (relative to actin) in representative samples as determined by RT-qPCR is shown in panel B. Circles in each panel indicate the individual sample values, whereas horizontal lines represent average mRNA expression for each group of samples (FTEn foll, follicular phase samples from normal controls; FTEn lut, luteal phase samples from normal controls; FTEb foll, follicular phase samples from *BRCA1/2* mutation carriers; FTEb lut, luteal phase samples from *BRCA1/2* mutation carriers). FTEb(S) samples are indicated by open circles. Statistically significant differences in average mRNA expression were determined by one-way ANOVA followed by the Newman–Keuls multiple comparison test. Sample groups with different letters are statistically different from one another ($P < 0.05$).

Immunostaining for PR-A protein was predominately nuclear, with differences in staining observed due to stage of the ovarian cycle and not *BRCA* mutation status, consistent with our total *PR* mRNA results. A decreased percentage of FTE cells showing strong PR-A immunopositivity was observed in previously profiled luteal phase FTE relative to follicular phase samples in both normal controls and *BRCA1/2* mutation carriers ($P < 0.001$ for both). No differences in percentage of strong PR-A staining were observed in FTEn versus FTEb samples obtained within either the

luteal or the follicular phase (Fig. 2A). Of particular note, expression of PR-A was similar in FTEb(S) and the remaining FTEb luteal phase cases not previously found to group with HGSC. These same differential expression patterns were also observed when PR-A immunostaining was evaluated in additional FTE cases not subjected to gene expression profiling in our previous study. In addition, PR-A protein was barely detectable in an independent set of 51 HGSC specimens (Fig. 2B).

The percentages of strong PR-A staining for all FTE cases were ranked by quartiles and compared between sample groups. A significant difference in distribution was observed between the luteal phase and follicular phase samples independent of *BRCA* status, with a greater proportion of luteal phase samples partitioning in the lower quartiles (Fig. 2C, $P < 0.001$). While a slightly higher proportion of FTEb luteal versus FTEn luteal cases were within the lowest quartile, this distribution pattern was not statistically significant ($P = 0.372$). Representative PR-A staining in follicular, luteal and HGSC specimens is shown in Fig. 2D–F respectively.

Similar results were obtained for PR-B immunohistochemistry. PR-B staining was decreased in nonmalignant FTE during the luteal phase compared with that during the follicular phase in both normal controls

and *BRCA* mutation carriers ($P < 0.001$ for both), in samples obtained from both previously profiled (Fig. 3A) and additional cases (Fig. 3B). No differences in PR-B staining were observed in FTEn versus FTEb overall or within the same stage of the ovarian cycle, and PR-B was barely detectable in HGSC samples. A significant difference in quartile distribution of percentages of strong PR-B staining was observed between the luteal and follicular phase samples independent of *BRCA* status, with a greater proportion of luteal phase samples partitioning in the lower quartiles (Fig. 3C, $P < 0.001$). The distribution of cases was strikingly similar in normal controls compared with mutation carriers. Representative staining for follicular, luteal and HGSC specimens is shown in Fig. 3D–F. Altogether, these data indicate that both PR-A and PR-B protein levels vary according to the stage of the ovarian cycle, and not *BRCA* mutation status. Similar results were obtained when images were analysed using an alternative image analysis program (Visiomorph software, Visiopharm, Hoersholm, Denmark; data not shown).

The analyses presented in this study largely reflect nuclear PR levels. Cytoplasmic staining was observed for PR-A and PR-B; however, the staining was less intense and present in fewer cells than nuclear staining. A difference due to the cycle stage was observed in
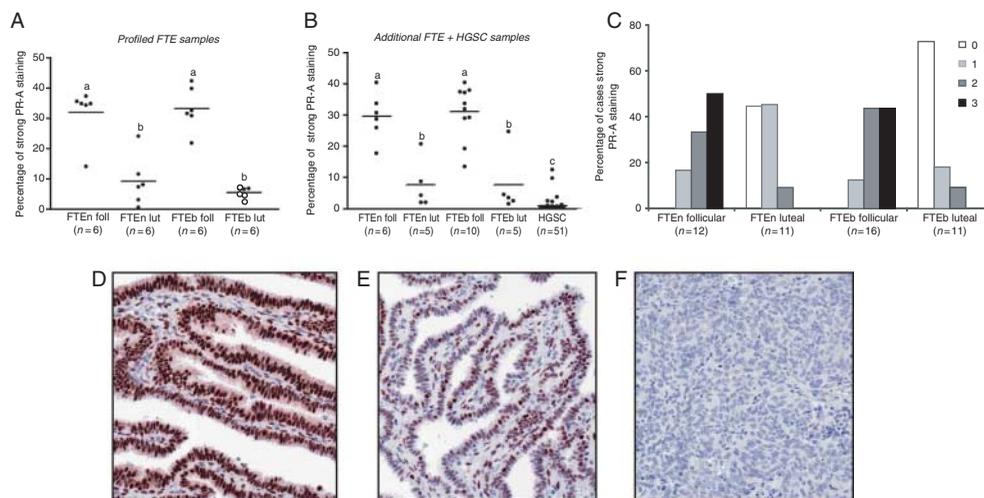

Figure 2 Decreased PR-A immunostaining in luteal phase FTE and HGSC specimens. Immunohistochemistry was performed on tissue microarrays using a PR-A-specific antibody, and the number of weak-positive, medium-positive and strong-positive pixels in each sample was quantified using the Positive Pixel Count Algorithm. The percentage of strong PR-A immunostaining in tissue cores obtained from previously profiled FTE samples (A) and additional FTE and HGSC specimens (B) is shown. Circles in A and B denote the percentage of strong PR-A immunostaining in individual samples, with FTEb(S) samples indicated by open circles in A. Horizontal lines represent average percentage of strong PR-A staining for each group of samples, and groups with different letters are statistically different from one another (as determined by one-way ANOVA followed by the Newman–Keuls multiple comparison test; $P < 0.05$). Panel C shows the distribution of samples following ranking of PR-A immunostaining data by quartiles (0, < 1st quartile; 1, 1–2nd quartiles; 2, 2–3rd quartiles; 3, > 3rd quartile). The distribution of cases into quartiles was analysed using Pearson's χ^2 test, with comparisons considered statistically significant if $P < 0.05$. Representative PR-A immunostaining in follicular FTE (D), luteal FTE (E) and HGSC (F) samples is shown (magnification $20 \times$).

cytoplasmic PR-A staining, with an absence of staining in the luteal phase samples regardless of *BRCA* mutation status. In contrast, cytoplasmic PR-B staining did not differ with either cycle stage or mutation status (Supplementary Figure 2, see section on supplementary data given at the end of this article).

Because PR-A has been found to affect signalling by PR-B, the relative levels of these two isoforms could be important to the resultant progesterone signalling. The ratio of the total percentage of cells positive for PR-A versus PR-B (PR-A:PR-B ratio) in the same selected area was calculated for each case and compared between FTE groups. No statistically significant differences in average PR-A:PR-B ratio were observed in the luteal phase compared with follicular phase samples, or normal controls versus *BRCA* mutation carriers (Fig. 4A). Interestingly, individual PR-A:PR-B values were tightly grouped in the follicular phase samples, whereas a greater range of ratios was observed in the luteal phase samples. This could potentially reflect different times of the luteal phase when samples were collected, as progesterone secretion is variable during this phase, reaching peak levels at 5 days post-ovulation. In contrast, very little progesterone is secreted throughout the duration of the follicular phase. Ranking of PR-A:PR-B ratio data by quartiles revealed an overall difference in distribution among the four FTE groups ($P = 0.006$). A greater

proportion of luteal phase compared with follicular phase samples partitioned in the lowest quartile ($P = 0.003$; Fig. 4B). In contrast, there was no statistically significant difference in the distribution pattern due to *BRCA* mutation status overall ($P = 0.129$) or in *BRCA* mutation carriers versus normal controls during the luteal phase ($P = 0.09$). These data suggest a subtle shift in the PR-A:PR-B staining ratio during the luteal phase, potentially reflective of a slightly lower level of PR-A relative to PR-B during this phase.

Gene expression analysis of profiled FTE based on proposed *PR-A* and *PR-B* target genes

To determine whether the expression of PR-dependent genes separates FTE samples into meaningful sub-groups, unsupervised clustering of all individual samples was performed using genes previously shown to be altered downstream of PR activation by Jacobsen *et al.* (2005). Genes with altered expression specifically due to PR-A or PR-B activation, both dependent and independent of ligand, were identified in oestrogen receptor (ER)-positive, PR-negative T47D breast cancer cell lines engineered to express PR-A or PR-B under the control of an inducible promoter (Jacobsen *et al.* 2005). Specifically, 37 distinct genes were found to be altered in PR-A+ (relative to PR null)
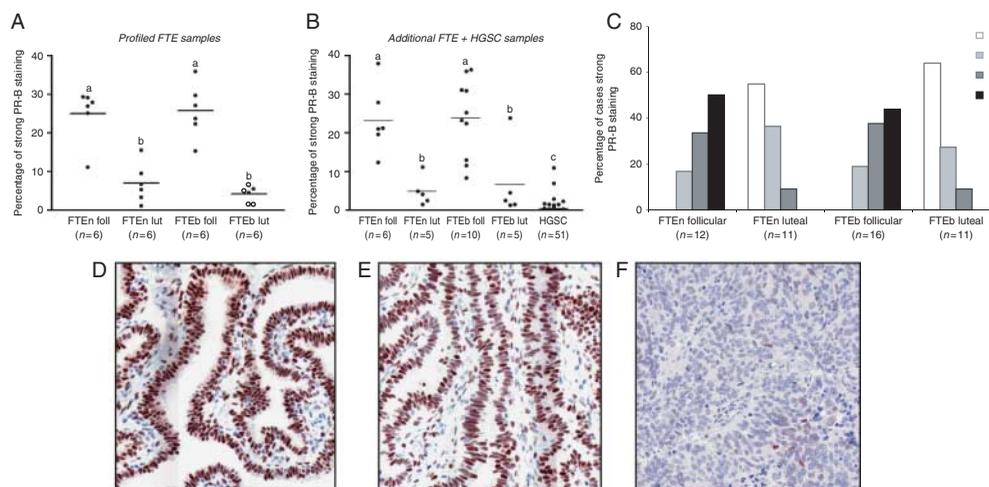

Figure 3 Decreased PR-B immunostaining in luteal phase FTE and HGSC specimens. Immunohistochemistry was performed using a PR-B-specific antibody and quantified using the Positive Pixel Count Algorithm. The percentage of cells with strong PR-B immunostaining in previously profiled FTE cases (A) and independent FTE and HGSC (B) is shown. Circles in A and B denote the percentage of cells with strong PR-B immunopositivity in individual samples, with FTEb(S) samples indicated by open circles. Horizontal lines represent average percentage of strong PR-B staining by group, and groups with different letters are statistically different from one another (as determined by one-way ANOVA followed by the Newman–Keuls multiple comparison test; $P < 0.05$). Panel C shows the distribution of samples following ranking of PR-B immunostaining data by quartiles (0, <1st quartile; 1, 1–2nd quartiles; 2, 2–3rd quartiles; 3, >3rd quartile). The distribution of cases into quartiles was analysed using Pearson's χ^2 test, with comparisons considered statistically significant if $P < 0.05$. Representative PR-B immunostaining in follicular FTE (D), luteal FTE (E) and HGSC (F) samples is shown (magnification 20×).
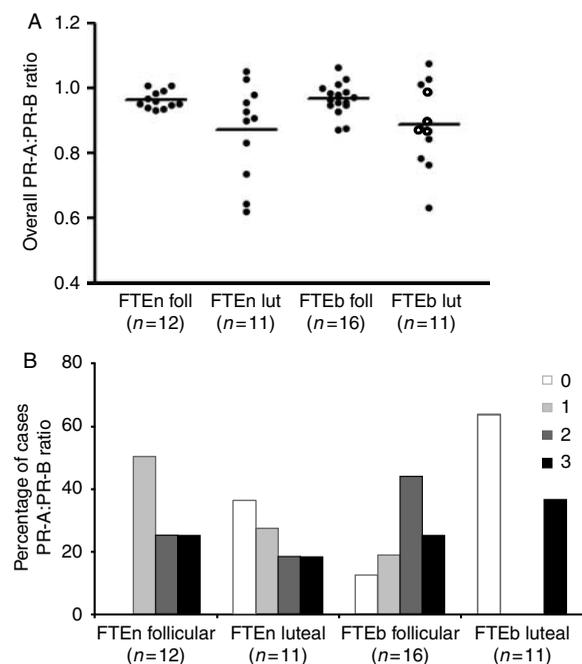
Figure 4 Overall ratio of PR-A to PR-B immunostaining in non-malignant FTE specimens. The ratio of the total percentage of cells positive for PR-A versus PR-B (PR-A:PR-B ratio) in the same selected area was calculated for each FTE case (taking into account weak-, medium- and strong-positive pixels)* and compared between sample groups (shown in panel A). Circles denote the calculated PR-A:PR-B ratio for individual samples, with FTEb(S) samples indicated by open circles. Horizontal lines represent average PR-A:PR-B by group. Panel B shows the distribution of samples following ranking of all PR-A:PR-B data by quartiles (0, <1st quartile; 1, 1–2nd quartiles; 2, 2–3rd quartiles; 3, >3rd quartile). The distribution of cases into quartiles was analysed using Pearson's χ^2 test, with comparisons considered statistically significant if $P < 0.05$. The average overall levels of PR-A and PR-B staining by FTE group ($\pm$s.e.m.) were as follows: FTEn follicular, PR-A $86.1 \pm 1.8\%$ and PR-B $89.1 \pm 1.7\%$; FTEn luteal, PR-A $63.1 \pm 5.9\%$ and PR-B $70.8 \pm 4.1\%$; FTEb follicular, PR-A $83.7 \pm 1.8\%$ and PR-B $86.4 \pm 1.6\%$; FTEb luteal, PR-A $60.8 \pm 5.2\%$ and PR-B $67.6 \pm 3.9\%$.

T47D cells after 6 h, and 54 were found to be specifically altered in PR-B-expressing cells at the same time point (see Supplementary Tables 2 and 3, see section on supplementary data given at the end of this article for PR-A- and PR-B-dependent genes extracted from Jacobsen *et al.* that are included in the current analysis). Probe sets representing each of these genes were identified in the Affymetrix 2.0 Plus arrays (a total of 94 for PR-A-dependent genes and 118 for PR-B-dependent genes), and each list of probe sets was used separately to cluster our original profiling data.

In this study, unsupervised hierarchical clustering of FTE and HGSC based on probe sets corresponding to all (both ligand-dependent and -independent) PR-A-dependent genes resulted in two main cluster groups

(I + II), each of which further divided into two subgroups (a + b; Fig. 5). A clear separation of non-malignant samples based on *BRCA1/2* mutation status or cycle stage was not observed based on the expression of these genes. While group Ia consisted of mostly follicular samples and one luteal sample with the highest PR-A staining, group Ib consisted of a mix of luteal and follicular phase samples from both mutation carriers and normal controls. Notably, FTEb(S) samples all partitioned together (within group IIa) separate from the remaining FTEb luteal samples (group I). However, the presence of FTEn luteal samples in both of these cluster groups indicates that *BRCA* mutation status does not affect the expression of PR-A-responsive genes. Importantly, 12 of the 13 HGSC specimens partitioned separately from non-malignant FTE; since the HGSCs all express low levels of PR, this pattern is consistent with the PR dependence of this cassette of genes.

Similar results were obtained when unsupervised cluster analysis was performed using probe sets corresponding to genes specifically altered down-stream of PR-B activation (Fig. 6). There were two main cluster groups, with no clear separation of non-malignant samples by *BRCA* mutation status or cycle stage. Group Ia consisted of a mix of FTEn and FTEb samples from both the follicular and luteal phases. FTEb(S) samples partitioned together within group Ib separate from the majority of HGSC in group II.

To extend our analysis of PR-dependent gene expression in FTE and HGSC, we tested the ability of these probe sets to predict a given sample category using a shrunken centroid classification methodology (Tibshirani *et al.* 2002; results shown in Table 1). A threshold of 0 for the shrinkage factor was used in order to include all probe sets in the prediction, and all results are based on a leave-one-out cross-validation analysis. Similar to the cluster analysis presented above, PR-A- and PR-B dependent genes were evaluated separately, and PR-A target genes were further subdivided into those found to be altered dependent ('PR-A ligand-dependent') and independent of the presence of ligand ('PR-A ligand-independent') in T47D cells. We chose to include the latter set of genes in our analysis (despite being altered in the absence of progesterone), since the ligand dependency of a particular gene may be influenced by cell or tissue type. As exposure to progesterone leads to down-regulation of both PR isoforms (Lange *et al.* 2000), gene expression could also be altered by decreased levels of PR-A irrespective of the absence or presence of ligand. Furthermore, if ligand-independent genes were found to be predictive of *BRCA* mutation status,
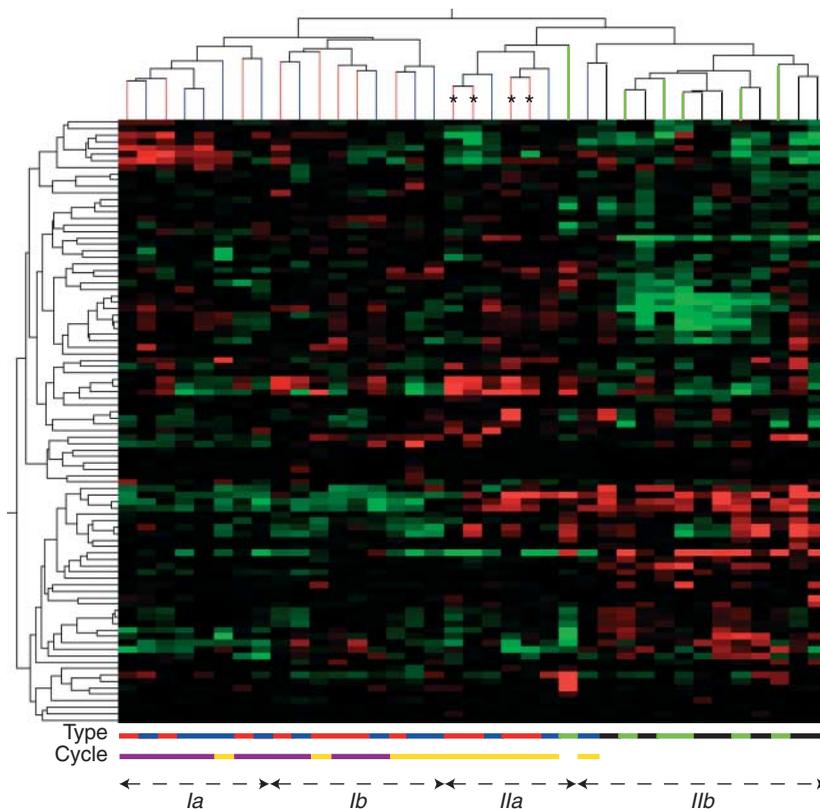
Figure 5 Clustering of profiled non-malignant FTE and HGSC samples using PR-A-dependent probe sets. Unsupervised hierarchical clustering was performed on our original profiling data, using a cassette of probe sets corresponding to genes previously found to be specifically altered following PR-A activation. Two main cluster groups emerged (shown at the bottom). The cluster tree is shown at the top, with each line representing one sample. The type of sample is indicated at the bottom of the heatmap (blue, FTEn; red, FTEb; green, tubal HGSC; black, ovarian HGSC), with the stage of the ovarian cycle for each FTE sample indicated below (purple, follicular; yellow, luteal). *Denotes each FTEb(S) sample.

this could suggest a potential role for crosstalk between PR and cytokine signalling pathways that may be locally elevated following ovulation (Denner *et al.* 1990, Pierson-Mullany & Lange 2004). In contrast to PR-A, only two PR-B-specific genes were reported to be altered independent of progesterone in T47D cells (Jacobsen *et al*. 2005), so a separate analysis was not performed for PR-B. Consistent with unsupervised clustering, PR-dependent genes were predictive of whether a particular sample was non-malignant FTE or HGSC (as indicated by a high sample prediction accuracy for each list using dataset A, total $n = 37$ samples). In contrast, none of the PR-dependent subsets (particularly probe sets corresponding to PR-A ligand-independent genes) were successful at predicting whether a particular non-malignant sample was obtained from a *BRCA1/2* mutation carrier or control patient (as indicated by a low sample prediction accuracy for dataset B, total $n = 24$ samples), indicating that the overall PR response is not altered due to *BRCA* mutation status. As expected, all lists were

predictive of the stage of the ovarian cycle of a given FTE sample (dataset C, total $n = 24$ samples), providing further support of the PR dependence of the genes identified by Jacobsen *et al*. (2005). Finally, certain subsets of PR-dependent genes (such as those activated by PR-A overall and PR-B) were relatively successful at predicting whether a particular luteal phase sample belonged to the FTEb(S) subgroup (dataset D, total $n = 12$ samples).

Discussion

The data presented in this study demonstrate that PR expression in the FTE is altered as a function of the ovarian cycle. *PR* mRNA and protein were decreased during the luteal phase, and this represented a decreased expression of both PR-A and PR-B isoforms in both previously profiled and independent FTE samples. A major question addressed in this study was whether PR levels are affected by *BRCA* mutation status, and whether this could be PR isoform specific.
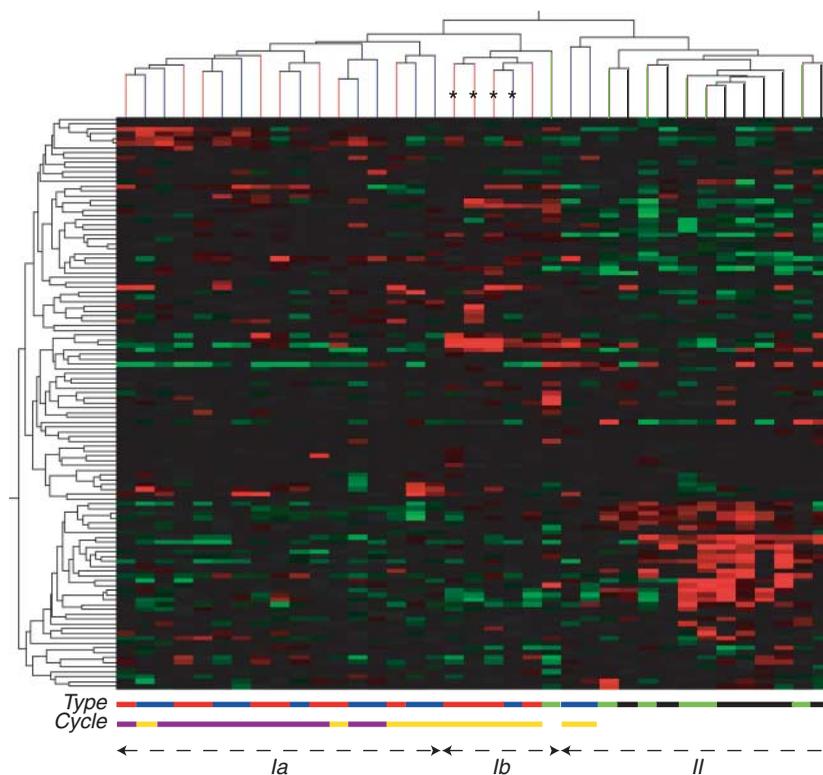
Figure 6 Clustering of profiled non-malignant FTE and HGSC samples using PR-B-dependent probe sets. Unsupervised hierarchical clustering of FTE and HGSC using probe sets corresponding to genes specifically altered downstream of PR-B activation revealed two main sample groupings (indicated at bottom). Sample type is indicated at the bottom (blue, FTEn; red, FTEb; green, tubal HGSC; black, ovarian HGSC), with the stage of the ovarian cycle for each FTE sample indicated below (purple, follicular; yellow, luteal). *Denotes each FTEb(S) sample.

Our results indicate that *BRCA* mutation status does not overtly impact PR-A and PR-B expression in the FTE. Unsupervised clustering and sample prediction analyses using genes reported as PR-A- and PR-B-specific targets further showed that *BRCA*-mutated samples did not exhibit an altered PR response.

The finding that *PR* mRNA is lowest during the luteal phase (in both carriers and controls) is consistent with previous studies of normal human FTE throughout the ovarian cycle, which have shown little or no PR expression during this phase as determined by semi-quantitative RT-PCR (Briton-Jones *et al*. 2005). Similar results have also been obtained for total PR protein, and the sharp decline in PR was found to coincide with the luteal elevation of serum progesterone concentration in these studies (Verhage *et al*. 1980, Pino *et al*. 1984, Helm *et al*. 1987). This is consistent with studies demonstrating the poly-ubiquitination and subsequent degradation of PR protein upon exposure to progesterone (Lange *et al*. 2000). Thus, the decline in PR expression in the luteal phase samples observed in this study likely represents downregulation of PR.

Our results further demonstrate a strong down-regulation of PR expression in HGSC. This is consistent with several studies that have reported decreased expression of PR in ovarian and fallopian tube cancers. Lau *et al*. (1999) have demonstrated a marked reduction in *PR* mRNA in ovarian cancer cell lines compared with primary cultures of normal OSE using semi-quantitative RT-PCR, and others have found low PR protein expression in both BRCA1-associated and sporadic serous ovarian cancers by immunohistochemistry (Aghmesheh *et al*. 2005). However, recent studies have strongly suggested that the FTE is the source of HGSC (Colgan *et al*. 2001, Piek *et al*. 2001, Finch *et al*. 2006, Medeiros *et al*. 2006, Crum *et al*. 2007, Kindelberger *et al*. 2007, Folkins *et al*. 2008, Shaw *et al*. 2009), thus the OSE may not be the appropriate comparator. A single previous study has reported slightly decreased PR protein expression in fallopian tube carcinomas relative to areas of benign FTE in patients with or without a *BRCA* mutation (Cass *et al*. 2005). Our study extends these observations to both tubal and ovarian HGSC samples and to FTE samples obtained from

Table 1 Accuracy for predicting sample categories based on sets of PR-dependent genes using the shrunken centroid classification methodology of Tibshirani *et al.* with a threshold of 0 (all probes used for prediction)

Dataset	Prediction[a]	PR-A prediction[b] accuracy	PR-A LD prediction[b] accuracy	PR-A LI prediction[b] accuracy	PR-B prediction[b] accuracy
[c]A. Non-malignant FTE	Non-malignant	24/24 (100%)	24/24 (100%)	24/24 (100%)	24/24 (100%)
versus HGSC	Cancer	12/13 (92.3%)	11/13 (84.6%)	12/13 (92.3%)	12/13 (92.3%)
[d]B. FTEn	FTEn	6/12 (50%)	8/12 (66.7%)	3/12 (25%)	4/12 (33.3%)
versus FTEb	FTEb	7/12 (58.3%)	8/12 (66.7%)	4/12 (33.3%)	5/12 (41.7%)
[d]C. Luteal	Luteal	9/12 (75%)	9/12 (75%)	9/12 (75%)	10/12 (83.3%)
versus follicular	Follicular	11/12 (91.7%)	11/12 (91.7%)	10/12 (83.3%)	11/12 (91.7%)
[e]D. FTEb(S)	FTEb(S)	3/4 (75%)	3/4 (75%)	2/4 (50%)	3/4 (75%)
versus other luteal	Other luteal	7/8 (87.5%)	6/8 (75%)	7/8 (87.5%)	8/8 (100%)

LD, ligand dependent, LI, ligand independent.
[a]Indicates the sample type attempting to be predicted based on the expression of a given set of PR-dependent genes.
[b]Indicates the proportion of samples in a given category that are predicted correctly based on the expression of a given set of PR-dependent genes. A high prediction accuracy suggests that a given set of PR-dependent genes is successful at differentiating between the two sample types included in the analysis.
[c]Expression data from all non-malignant FTE ($n=24$) and HGSC ($n=13$) samples included in prediction analysis.
[d]Expression data from all non-malignant FTE ($n=24$) samples included in prediction analysis following independent normalisation.
[e]Expression data from all non-malignant FTE obtained during the luteal phase ($n=12$) included in prediction analysis following independent normalisation.

women without adnexal cancer. Our studies further demonstrate a strong downregulation of PR expression in HGSC samples, such that PR expression is nearly absent in malignant cells. Interestingly, previous studies have demonstrated loss of heterozygosity (LOH) at the PR locus (11q22) in up to 50% of analysed ovarian carcinomas of varying histotypes (Gabra *et al.* 1995, Launonen *et al.* 1998), providing a potential explanation for PR loss in HGSC specimens. In one such study, 6/15 (40%) of informative serous carcinoma specimens exhibited LOH; furthermore, LOH at 11q22 was significantly associated with low tumour PR content in the full cohort of 38 ovarian carcinomas studied (Gabra *et al.* 1995). Whether LOH occurs in HGSC precursor lesions in the FTE remains to be determined.

Expression of PR has been reported to be a favourable prognostic indicator in ovarian cancer (Lee *et al.* 2005), but this finding may be misleading due to the inclusion of multiple histotypes in the analysis. Close examination of the reported data indicates that 64% of endometrioid carcinomas were considered positive for PR (>10% of cells stained), compared with only 25% of serous carcinomas of unspecified grade. Patients with endometrioid carcinomas typically present at an earlier stage and have a greatly enhanced survival (Gien *et al.* 2008), which could underlie the apparent impact of PR expression on prognosis in this study. The tissue microarray used in this study to assess PR expression in HGSC also contained other major histotypes of epithelial ovarian cancer (see Supplementary Table 1, see section on

supplementary data given at the end of this article for the clinical characteristics and immunostaining results of individual carcinoma cases). Consistent with the data presented in the previous study, 11/14 (79%) and 12/12 (100%) of evaluable endometrioid cases, compared with 10/51 (20%) and 13/51 (25%) of HGSC cases, showed >10% positivity for PR-A and PR-B respectively (Supplementary Figure 3, see section on supplementary data given at the end of this article). Also consistent was our finding that none of the clear cell carcinomas and only one of six mucinous carcinomas examined showed positive PR-A or PR-B staining, suggesting a potential role for PR loss in these histotypes.

The relative levels of PR-A and PR-B influence the response of target cells to progesterone. For instance, breast cancer cells with predominant expression of PR-A have shown an exaggerated proliferative response to luteal phase levels of progesterone compared with cells with predominant expression of PR-B (Leo & Lin 2008). While PR-A and PR-B are expressed at roughly equivalent levels in normal human breast epithelium throughout the cycle, several studies have found a predominance of PR-A in a high proportion of invasive breast tumours (Graham *et al.* 1995, Ariga *et al.* 2001, Mote *et al.* 2002) and in ductal carcinomas *in situ* (Mote *et al.* 2002). A specific lack of expression of the PR-B isoform has also been observed in normal breast epithelium obtained from *BRCA* mutation carriers compared with control patients, resulting in predominant expression of PR-A in 40% of cases (Mote *et al.* 2004).

In this study, we found a decreased expression of both PR-A and PR-B in HGSC relative to its probable cell type of origin. Because expression of both isoforms in HGSC was barely detectable by immuno-histochemistry, consistent with our RT-qPCR data, ratios of PR-A to PR-B were not calculated. Akahira *et al*. (2000) have demonstrated a lower expression of PR-A compared with PR-B for all epithelial ovarian cancer histotypes using both RT-PCR and immuno-histochemistry; relative isoform levels did not vary with grade or stage of tumour. In a follow-up study, this group reported a progressive downregulation of PR-A, but not of PR-B, from normal OSE through benign, borderline and malignant serous ovarian tumours (Akahira *et al*. 2002). In normal OSE and serous adenomas, both isoforms were equally expressed, whereas PR-B predominated in borderline and malignant tumours. However, the grade of the serous carcinomas was not indicated, and while low-grade serous carcinomas are thought to be derived from borderline tumours (Singer *et al*. 2002, Shih & Kurman 2004, May *et al*. 2010), this is not thought to be the case for HGSC. In light of recent studies outlining the progression to HGSC from normal FTE (Crum *et al*. 2007, Lee *et al*. 2007, Folkins *et al*. 2008), studies of relative PR isoform expression in FTE exhibiting focal accumulation of p53 (p53 signatures), tubal intra-epithelial carcinomas and their proposed intermediaries should be performed to determine whether differential isoform expression may play a role at any stage during HGSC development.

A study comparing steroid receptor expression in familial and sporadic serous carcinomas reported no statistical difference in either PR-A or PR-B expression (Aghmesheh *et al*. 2005). Initial studies investigating the effect of *BRCA* mutation status on PR expression in non-malignant cells did not distinguish by isoform, but rather focused on determining total PR protein levels. In these studies, no differential expression was observed in non-malignant FTE and OSE from mutation carriers and controls (Piek *et al*. 2001, 2003). PR protein was expressed abundantly in both ciliated and secretory FTE cells, and expression in morphologically normal and dysplastic areas from mutation carriers was similar (Piek *et al*. 2001). This is consistent with our findings of no differences in either PR-A or PR-B expression as a function of *BRCA* mutation status. In studies of *BRCA*-associated OSE, the only difference observed was an increased expression of PR protein in inclusion cyst epithelium compared with OSE, which the authors attributed to an increased exposure to ovarian stroma-derived hormones including oestrogen (Piek *et al*. 2003).

Interestingly, PR expression was higher in inclusion cyst epithelium from mutation carriers compared with controls, potentially consistent with the repression of ER transactivation activity by wild-type BRCA1 (Zheng *et al*. 2001, Fan *et al*. 2002). BRCA1 has also been reported to function as a PR transcriptional repressor (Ma *et al*. 2006); thus we had expected to see a separation of FTEb and FTEn cases based on established PR-responsive genes. However, the response to progesterone was not affected by mutation status, as indicated by a lack of separation of FTEn and FTEb samples by unsupervised clustering based on PR-A- or PR-B-dependent genes. Furthermore, a low proportion of FTEn and FTEb samples were placed in the correct category when attempting to predict whether a particular FTE sample was obtained from a *BRCA1/2* mutation carrier or a normal control using the same subsets of PR-dependent genes. If altered PR signalling is in fact an important contributor to the initiation of HGSC, we would expect to observe a clear separation of FTEn and FTEb samples by unsupervised clustering and a higher accuracy rate in sample prediction analysis given the greatly enhanced risk of HGSC in *BRCA1/2* mutation carriers. In contrast to FTEn versus FTEb, PR-dependent genes could successfully differentiate malignant versus non-malignant samples, utilising both unsupervised clustering and sample prediction analysis. This is not unexpected, as HGSC samples showed a universal loss of PR expression compared with non-malignant FTE. It is important to note that the gene expression analysis presented in this study is based on target genes identified in breast cancer cells and does not reflect genes affected in a tissue-specific manner. A more definitive analysis therefore awaits the identification of PR target genes more specific to FTE cells.

Several studies suggest that HGSC arises from the secretory rather than the ciliated FTE (Talamo *et al*. 1982, Crum *et al*. 2007, Lee *et al*. 2007). One possibility, therefore, for the partitioning of luteal phase samples with HGSC may be an enrichment of secretory cells in the FTE during the luteal phase. However, studies indicate that the secretory cell type is not overrepresented during this phase. The relative proportions of secretory to ciliated cells in different segments of the human fallopian tube throughout the ovarian cycle were previously studied by Crow *et al*. (1994). They reported an increase in the proportion of ciliated cells (hence a decrease in the proportion of secretory cells) along the length of the tube, with the highest proportion of ciliated cells observed in the fimbriae (the location of the overwhelming majority of HGSC precursor lesions). Importantly, they reported

only very minor differences in the percentages of secretory versus ciliated cells in the luteal versus follicular phase. For example, only 4% more secretory cells were observed in the fimbriated end in the luteal phase compared with the follicular phase. This slight difference would have a negligible impact on global gene expression. We found that luteal samples, in general, grouped separately from follicular phase samples by unsupervised clustering, which is consistent with the increased presence of progesterone during the luteal phase. Our finding of a high accuracy rate with PR ligand-independent gene expression in differentiating the luteal and follicular phase samples could reflect the changes in PR expression as well as indicate that signalling pathways resulting in PR phosphorylation differ with the ovarian cycle.

Our previous work provides evidence suggesting that a differential response by FTE from *BRCA* mutation carriers to the luteal phase milieu contributes to an increased propensity for malignant transformation. FTE from *BRCA* mutation carriers collected during the luteal phase molecularly resembled HGSC, rather than FTE collected during the follicular phase or from normal control patients. This was particularly apparent for four FTEb luteal samples, referred to as FTEb(S), which grouped with HGSC at the global gene expression level (Tone *et al*. 2008). In this study, we investigated the potential for altered PR expression and signalling to contribute to this differential gene expression pattern. Our results indicate that there is no overt difference in PR expression as a function of *BRCA* mutation status. Unsupervised cluster analysis further indicated that a differential response to progesterone based on *BRCA* mutation status also does not likely underlie the molecular similarity of FTEb(S) and HGSC samples, as FTEb(S) samples were found to group separately from the majority of HGSC according to the expression of PR-dependent genes. As we learn more about the specific role of PR-A and PR-B in the fallopian tube, a refined set of genes will likely emerge. It therefore remains possible that a differential clustering pattern could result with a tissue-specific gene list. However, based on the information that is presently available, our data indicate that differential response to factors associated with the luteal phase other than progesterone plays an important role in determining HGSC risk.

Supplementary data

This is linked to the online version of the paper at http://dx. doi.org/10.1530/ERC-10-0235.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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