Global gene expression profiles of ovarian surface epithelial cells *in vivo*

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Abstract

Epithelial ovarian cancer, the leading cause of death from gynecological malignancy in Western countries, is thought to arise from the ovarian surface epithelium (OSE). It has been postulated that the constant rounds of proliferation and repair following ovulation contributes to neoplastic transformation. However, there is little information on the genes and pathways which are involved in the normal functions of the ovarian epithelium, in particular genes that are hormone responsive and those central to functions such as proliferation and apoptosis during ovulation. We used laser microdissection and cDNA microarrays to profile gene expression specifically in mouse ovarian epithelial cells, first compared with other ovarian cells, and secondly between ovarian epithelium collected at different physiological stages. We identified over 1000 transcripts that were consistently more highly expressed in the ovarian epithelium compared with remaining ovarian cell types, including genes involved in cell growth, transcription, and cell adhesion. At the various physiological stages examined, the highest number of regulated genes was found during the estrous cycle, specifically on the evening of proestrus, coincident with the ovulatory surge of hormones and just prior to ovulation. The expression of several selected genes, identified by the microarray analysis, including *Villin 2*, *Keratin 8*, *Arginine-rich mutated in epithelial tumors*, and *Tumor-associated calcium signal transducer 1*, was validated by independent methods. The identification of genes expressed and regulated in the OSE, and characterization of the pathways involved, will contribute to a more detailed understanding of the ovarian epithelium transcriptome and ultimately lead to a better understanding of the aberrations leading to malignant transformation in the ovarian epithelium.

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Introduction

Epithelial ovarian cancer (EOC) is the leading cause of death from gynecological malignancy in Western women, and is generally thought to arise in the ovarian epithelium either on the surface of the ovary or in inclusion cysts. However, the underlying mechanisms leading to malignant transformation are not known. Gonadotrophins that initiate each ovulation stimulate ovarian surface epithelium (OSE) proliferation and have been implicated in OSE cell transformation (Riman *et al.* 2004). By contrast, progesterone, a component of the oral contraceptive pill, produced at high levels during pregnancy, is thought to be protective against EOC (Risch 1998, Ho 2003), potentially by stimulating apoptosis (Rodriguez *et al.* 1998).

The ovarian epithelium is a single cell layer that covers the entire ovarian surface (Gillett *et al.* 1991), continuous with the peritoneal mesothelium, and derived from the coelomic epithelium. This epithelial layer varies morphologically from simple flattened, to

cuboidal, and to low pseudostratified columnar, in association with the cyclic changes in the ovary (Clement 1987, Clow *et al.* 2002, Gaytan *et al.* 2005). It also has the capacity to remodel the ovarian cortex (Woessner *et al.* 1989, Auersperg *et al.* 1991) and participate in gonadotrophin-induced follicular rupture. Prior to ovulation, OSE proliferation increases at sites adjacent to follicular development (Bjersing & Cajander 1974, Burdette *et al.* 2006) and ovulation-related desquamation of the surface epithelial cells involves programmed cell death or apoptosis (Bjersing & Cajander 1975, Ackerman & Murdoch 1993, Murdoch 1994, 1995). Following ovulation, the wound at the ovarian surface is rapidly repaired by proliferation of the OSE from the perimeter of the ruptured follicle, and ovarian epithelial cells have also been postulated to be involved in the deposition and restructuring of the extracellular matrix of the tunica albuginea (Motta 1980, Osterholzer *et al.* 1985, Auersperg *et al.* 1991, Kruk & Auersperg 1992, Gaytan *et al.* 2005, Burdette *et al.* 2006). The constant rounds of

proliferation and repair following ovulation provide the opportunity for replication errors and dysregulation of the genes involved in the normal processes of proliferation, apoptosis, and DNA repair, and thus may contribute to neoplastic progression in the OSE (Fathalla 1971). However, the specific molecular pathways underlying the development of EOC remain unclear.

A number of studies have documented the expression of known genes in normal OSE (reviewed in Auersperg *et al.* (2001)). Human OSE express cell adhesion-related proteins including integrins, collagens, fibronectin, and vitronectin, and secrete chymotrypsin- and elastase-like peptidases, metalloproteases, and plasminogen activator inhibitor (Carreiras *et al.* 1996, Auersperg *et al.* 1998). Normal OSE also express α - and β -catenin (Davies *et al.* 1998), as well as a number of growth factors, including amphiregulin, and express receptors for epidermal growth factor, ovarian hormones, gonadotrophins, and hepatocyte growth factor/scatter factor (Johnson *et al.* 1991, Gulati & Peluso 1997, Auersperg *et al.* 2001). Although extensive genomic studies of the whole ovary (Espey & Richards 2002, Rinn *et al.* 2004, Zhang *et al.* 2004, Herrera *et al.* 2005) or various components, such as oocytes (Kocabas *et al.* 2006) and granulosa cells at various stages of development (Sasson *et al.* 2004) have been conducted, the limited material and delicate nature of the OSE has been a major barrier to studying OSE genomics using high throughput techniques, such as microarrays. Attempts have been made to address these problems using ovarian epithelial cells maintained in short-term cultures or immortalized cell lines, however Zorn *et al.* (2003) found that the gene expression profiles of cultured and immortalized cell lines are quite different compared with normal ovarian epithelial cell brushings, and thus may not accurately represent the phenotype of their cell of origin.

While there is a growing list of genes that are expressed by the OSE, most of these data have been gathered from ovarian epithelial cells grown in culture, and although some studies have been reported using tissue samples, there is almost no information on the regulation of these gene products under normal physiological circumstances *in vivo*. Since ovulation and cyclical ovulatory hormones have been implicated in the development of EOC, we examined gene expression profiles specifically in the OSE at defined ovulatory stages. We used laser microbeam microdissection and cDNA microarrays to profile gene expression in ovarian epithelial cells compared with the other cell types within the mouse ovary and identified gene sets differentially expressed in the OSE at different stages of the estrous cycle and different stages of development.

Materials and methods

Animals and tissue preparation

BALB/c mice were housed in humidity- and temperature-controlled rooms under a 12 h light:12 h darkness cycle, with food and water provided *ad libitum*. Ovaries were obtained from mice at 27 days of age (immature, $n=4$) and 10–13 weeks of age during the estrous cycle, namely proestrus ($n=4$) and estrus ($n=4$), and between 10 and 14 days of pregnancy (mid-pregnancy, $n=4$).

For cycling mice, vaginal smears were taken daily from the age of 8 weeks and used to monitor progression through the estrous cycle (Nelson *et al.* 1982). Mice exhibiting two consecutive 4- to 5-day cycles were killed on either the evening of proestrus (2200 h) or the morning of estrus (1000 h). All mice were anesthetized with ketamine (100 $\mu\text{g/g}$ body weight) and xylazine (10 $\mu\text{g/g}$ body weight) and then culled by cervical dislocation. Both ovaries were collected, removed from the ovarian bursa, snap frozen, and stored in liquid nitrogen until use. All experiments were approved by the Institutional Animal Care and Ethics Committee.

Laser microdissection, RNA isolation, and amplification

One ovary chosen randomly from each mouse was embedded in Tissue-Tek OCT medium (Sakura Finetek, Torrance, CA, USA) and 10 μm sections were mounted on RNase- and UV-treated PALM MembraneSlides (PALM Microlaser Technologies AG, Bernried, Germany), frozen on dry ice, and stored at -80°C until use. Prior to microdissection, the slides were fixed in 70% ethanol for 2 min, stained with Mayers hematoxylin (Sigma–Aldrich) for 1.5 min, rinsed with deionized water, and sequentially dehydrated in graded alcohols (70, 95, and 100% ethanol). The OSE were then laser microdissected from ~ 20 ovarian sections per mouse using the PALM Robot-Microbeam system (PALM Microlaser Technologies) under $200\times$ magnification. In addition to collecting the OSE, the remaining ovarian tissue (including follicles, stroma, corpora lutea, and blood vessels) was also collected and placed separately in lysis buffer containing 0.7% β -mercaptoethanol. Samples were vortexed for 5 s, centrifuged, incubated for 10 min at 55°C , snap frozen on dry ice, and stored at -80°C .

Total RNA was extracted from each mouse ovarian epithelial cell and residual ovarian tissue sample using the Stratagene Absolutely RNA Nanoprep or Microprep kit (Stratagene, La Jolla, CA, USA) respectively, following the manufacturer's instructions. RNA was eluted in 15 (Nanoprep kit) or 30 μl (Microprep kit) elution buffer and stored at -80°C . RNA quality/integrity was

measured using the RNA 6000 Nano LabChip kit in combination with the Agilent 2100 Bioanalyzer. For each mouse, 10 μ l OSE and $\sim$ 500 ng residual ovarian tissue total RNA were amplified using the MessageAmp aRNA kit (Ambion, Austin, TX, USA), following the manufacturer's instructions. Two rounds of amplification were performed on each sample.

cDNA microarrays

Microarray slides were obtained from the Australian Genome Research Facility (Melbourne, Australia) and consisted of $\sim$ 15 000 expressed sequence tags from the National Institute of Ageing 15K mouse clone library. More details about this clone set, as well as further gene annotation and informatics, are available from <http://lgsun.grc.nia.nih.gov/cDNA/15k.html>. Printed on all microarray slides was a range of positive, negative, and calibration controls, including the Amersham Lucidea scorecard (Amersham Biosciences). All arrays were hybridized with 3 μ g Cy3- and Cy5-labeled cDNA generated from 3 μ g double-amplified RNA using a modified protocol from <http://brownlab.stanford.edu/protocols.html>, as described by Graham *et al.* (2005).

Microarray comparisons

Thirty-six microarray hybridizations were performed using amplified RNA prepared from the OSE and residual ovarian tissue collected from 16 mice (4 replicate mice per group). Two main comparisons were performed. The first comparison examined OSE-specific

gene expression, consisting of a direct comparison between OSE and residual ovarian tissue from immature (IM), proestrus evening (PE), and mid-pregnant (P) mice (12 slides, Fig. 1B). The second comparison examined developmental and estrous stage-specific gene expression profiles, consisting of a direct comparison between OSE from IM, PE, estrus morning (EM), and mid-pregnant mice (24 slides, Fig. 1C). Each group comparison compared RNA from four independent biological replicates, with two hybridizations labeled with each fluorescent dye to account for dye effects.

Microarray normalization and analysis

Hybridized arrays were scanned using a GenePix 4000B scanner and the images analyzed with GenePix PRO 6.0 software (Molecular Devices Corp., Sunnyvale, CA), using the median-fixed circles for foreground and the morphological opening method to calculate the local background for each spot. Spots not found by the image analysis software or not significantly different to local background (background plus twice the s.d. of the background) in either channel were defined as low quality and weighted 0.1 out of 1. The results were analyzed in the statistical computing environment R (www.r-project.org) through the Bioconductor Project (Dudoit *et al.* 2003), using the *marray* and *limma* packages (Gentleman *et al.* 2004). The expression values were calculated as the log ratio of dye-normalized red (Cy5) and green (Cy3) channel signals. All microarrays were normalized using the weighted robust splines normalization method. The empirical Bayes linear modeling approach was then used to rank genes in order for differential expression (Smyth 2004, see

Figure 1 (A) Fluctuating hormone levels during the estrous cycle in rodents. The cycle can be divided into four main stages: diestrus, proestrus, estrus, and metestrus. Black bars indicate the dark period, with each phase commencing at midnight. The dotted line represents when ovulation begins, after the surge of hormones on the evening of proestrus. Adapted from Smith *et al.* (1975). (B and C) Microarray design. (B) OSE obtained from immature (IM), proestrus evening (PE), and pregnant (P) mice ($n=4$ /stage) were compared directly with the residual ovarian (RO) tissue, making a total of 12 hybridizations (4 hybridizations/group). (C) OSE from IM, PE, estrus morning (EM), and P mice ($n=4$ /stage) were directly compared, making a total of 24 hybridizations (4 hybridizations/group). Direction of the arrow head represents the sample labeled with Cy5.

Supplementary information for the details in the online version of the Journal of Endocrinology at <http://jme.endocrinology-journals.org/content/vol40/issue6/>). Briefly, data were divided into each physiological condition (four arrays per group) and an average ratio (*M*-value) for each gene was calculated, by fitting a simple linear model for each gene. Genes were only included if they were weighted as high quality in 75% of the arrays (i.e., in three out of four arrays per group) and not defined as a control. Next, empirical Bayes statistics were calculated for differential expression, using moderated *t*- and *B*-statistics (Smyth 2004). For these experiments, genes displaying $B > 0$ (log posterior odds) and at least a 1.2-fold change were considered differentially expressed. For comparison, analysis of normalized data was also conducted using Serial Analysis of Microarray (SAM) software (Tusher *et al.* 2001), freely available from the TM4 package (<http://www.tm4.org/>; Saeed *et al.* 2003).

Gene identification and function (ontology) was assigned based on the SOURCE database (<http://source.stanford.edu>). The Kyoto Encyclopedia of Genes and Genomes pathway database (Ogata *et al.* 1999, Dennis *et al.* 2003) was used to identify whether any genes were involved in any known cellular pathways and the Gostat program (<http://gostat.wehi.edu.au/>) was used to identify whether any gene ontologies were statistically over-represented in particular gene profiles (Beissbarth & Speed 2004).

Real-time quantitative RT-PCR

Expression levels of *arginine-rich mutated in epithelial tumors* (*Armet*) and *tumor-associated calcium signal transducer* (*Tacstd1*) were analyzed by real-time quantitative RT-PCR (RT-qPCR). For each RNA sample, cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen), following the manufacturer's instructions. RT-qPCR was performed using Platinum SYBR Green Master Mix (Invitrogen) and the Rotor-Gene 3000 (Corbett Research, Sydney, NSW, Australia). For each gene, RT-qPCR reactions were conducted using RNA that was used for the microarray analysis. All quantifications were normalized to 18S RNA. Primer sequences used were as follows: *Armet* forward, 5'-CACCAGCCACTATTGAAGAAGAA-3'; *Armet* reverse, 5'-TCCAATGTAGTAGCACAACCG-3'; *Tacstd1* forward, 5'-CCGAAGAACCGACAAGGACAC-3'; *Tacstd1* reverse, 5'-AGTAGGTCCTCACGCGCTCG-3'; 18S RNA forward, 5'-GTAACCCGTTGAACCCCAT-3'; and 18S RNA reverse, 5'-CCATCCAATCGGTAGTAGCG-3'.

Immunohistochemistry

Ovaries were embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA, USA) and 10 µm serial

sections were cut on a cryotome (Microm HM 505E) using disposable blades, at -25°C . The sections were mounted on Superfrost Plus slides and stored at -20°C until use. The sections were fixed in either cold acetone for 10 min (TROMA-1) or cold 3.7% formaldehyde in PBS for 30 min (*Ezrin*). Endogenous peroxidase was blocked by incubation in 0.003% hydrogen peroxide solution for 10 min and then rinsed with distilled water. To block for endogenous biotin, the sections were treated with the Dako Biotin Blocking system (DakoCytomation, Carpinteria, CA, USA), according to the manufacturer's instructions. After a brief rinse with $1\times$ PBS, the sections were incubated for 30 min with normal goat serum and diluted 1:1 in PBS. Excess normal goat serum was removed before incubation with the primary antibody.

The TROMA-1 rat anti-mouse monoclonal antibody that recognizes *Keratin 8* (Brulet *et al.* 1980) was obtained from the Developmental Studies Hybridoma Bank (The University of Iowa, Department of Biological Sciences, Iowa City, IA, USA). The sections were incubated with 4.5 ng/µl TROMA-1 antibody diluted in PBT (PBS with 0.5% (v/v) Triton X-100 (Amresco, Solon, OH, USA)) at 37°C for 1 h. *Villin 2*, also known as *Ezrin*, was detected using rabbit anti-human *Ezrin* polyclonal antibody (Upstate, supplied by Auspep Pty Ltd, Parkville, Australia), which cross-reacts with mouse *Villin 2/Ezrin*. The sections were incubated with 5 ng/µl *Villin 2/Ezrin* antibody diluted in PBT at room temperature for 1 h.

After incubation with the primary antibody, the sections were rinsed with PBT, and PBS and the appropriate biotinylated secondary antibody was added. After rinsing, the sections were then incubated with streptavidin-biotinylated peroxidase according to the manufacturer's instructions (Zymed Laboratories, Inc., San Francisco, CA, USA) and then rinsed, as described above. Bound antibody was visualized using diaminobenzidine (DAB; DakoCytomation) and the reaction was stopped in distilled water. The sections were counterstained with Harris hematoxylin, differentiated with 1% acid alcohol, and allowed to air-dry before mounting with histolene and normount. To control for non-specific staining, adjacent sections were stained as above, except the primary antibody was replaced with PBT.

Results

RNA quality and microarray design

In laboratory mice, the estrous cycle generally lasts for 4–6 days. It can be divided into four major phases (diestrus, proestrus, estrus, and metestrus), with each stage distinguished by changes in structures and function of the sex organs. Generally, proestrus and estrus constitute the follicular phase of the cycle,

whereas metestrus and diestrus constitute the luteal phase of the ovarian cycle (Fig. 1A). To investigate global gene expression changes in the OSE under various hormonal conditions (Fig. 1A), we used laser microdissection to isolate pure populations of ovarian epithelial cells (Fig. 2) from ovaries collected from mice at four distinct physiological stages: immaturity, proestrus, estrus, and mid-pregnancy. Individual ovarian epithelial cell samples were arrayed rather than using pooled samples, to reduce identifying genes with high biological variability in their expression levels (Seltmann *et al.* 2005). RNA from the laser microdissected material was amplified twice to obtain sufficient material. Previous studies have shown that RNA amplification does not significantly change the expression profile (Wang *et al.* 2000, Baugh *et al.* 2001, Feldman *et al.* 2002). To further confirm this, we compared hybridization data between total RNA and

amplified RNA obtained from two cell lines to verify that the amplification process was not biasing our microarray results. Analysis of these cDNA microarrays resulted in correlation coefficients greater than 0.8 for first round amplification and greater than 0.7 between total RNA and double amplified RNA (data not shown).

Genes highly expressed in the OSE compared with other ovarian cell types

To determine gene expression profiles that were specific to the OSE compared with other cell types within the ovary, epithelial cells were laser microdissected from 12 mouse ovaries (Fig. 2) and RNA extracted was directly analyzed by comparative hybridization to cDNA microarrays (Fig. 1B) containing 14 948 transcripts, representing 5742 known genes. Using

Figure 2 Laser microdissection of the epithelial cells from the ovarian surface using the Robot-Microbeam system. (A) Ten μm ovarian sections were mounted on PALM MembraneSlides and stained with hematoxylin. Inset represents area in (B). (B) The laser was used to cut and separate the OSE from the underlying ovarian cortex. (C) Ovarian section with underlying cells removed, separating the OSE from the other ovarian cells. Original magnification, (A and C) $200\times$ and (B) $400\times$.

linear models, 3075 transcripts were identified as differentially expressed ($B > 0$) between the OSE and the residual ovarian tissue (Supplementary Table 1 in the online version of the Journal of Molecular Endocrinology at <http://jme.endocrinology-journals.org/content/vol40/issue6/>), irrespective of the mouse's physiological condition, i.e., in all 12 microarrays.

We applied a further statistical method, SAM (Tusher *et al.* 2001), to confirm the accuracy of the linear model approach to identify differentially expressed genes. Applying SAM to the same normalized data set identified the same top two transcripts as in the linear model analysis and ranked the remaining transcripts in a similar order (Table 1), with a median FDR of 0.000% and a 90th percentile FDR of 0.046%. Only 116 (4%) transcripts were identified as being differentially expressed in the SAM analysis and were not by the linear model analysis (data not included). These results indicate that both methods of analysis are able to identify very similar sets of differentially

expressed genes and further confirm the reliability of the linear model approach.

Out of the 3075 differentially expressed transcripts, identified by linear models, the majority of transcripts (1964/3075, 64%) were more highly expressed in the residual ovarian tissue compared with the OSE (Supplementary Table 1), a result that was consistent with the high level of physiological activity and diverse number of cell types present within the ovary. Of these, a number of genes have already been previously identified expressed in various cell types of the ovary, including *inhibin-α* (Drummond *et al.* 1996), *disintegrin-like and metalloprotease with thrombospondin type 1 motif, 1* (Robker *et al.* 2000), and *forkhead box O1* (Shi & LaPolt 2003), which have all been shown to be expressed in ovarian granulosa cells. Over 1000 transcripts (1111/3075, 36%) were more highly expressed in the OSE (Supplementary Table 1) compared with the residual ovarian tissue in all groups examined. Among the 1111 transcripts, the majority

Table 1 Results of the top 25 transcripts/genes identified as differently expressed between the ovarian surface epithelium (OSE) and residual ovarian tissue (RO) using empirical Bayes linear modeling and serial analysis of microarrays (SAM) analysis.

GenBank accession	Gene symbol	Function	Linear model		SAM analysis		More highly expressed in:
			Rank	<i>B</i> -value ^a	Rank	Score(d) ^b	
BG062958	Rtn4	Membrane	1	20.77	1	25.19	OSE
CK334553	Hspa5	Response to stress	2	19.93	2	23.32	RO
BG063925	Mt2	Cell growth and/or maintenance	3	18.89	4	20.65	OSE
C79809	Acadl	Lipid metabolism	4	18.88	3	21.44	RO
BG065235	Metap2	Protein metabolism	5	18.03	5	19.96	RO
BG087402	Hnrpu	Unknown	6	17.58	7	18.84	RO
BG086280	Msh6	Cell growth and/or maintenance	7	17.17	6	18.98	RO
BG086479	Sms	Unknown	8	16.53	8	18.11	RO
BG085110	Crip1	Ion binding	9	16.40	11	16.73	OSE
BG077536	Esam1	Cell adhesion	10	16.22	9	17.36	OSE
BG086474	Igfbp5	Cell growth and/or maintenance	11	15.95	12	16.69	OSE
BG088477	Sdha	Oxidoreductase activity	12	15.94	13	16.29	RO
BG079899	1200009K13Rik	Unknown	13	15.80	14	16.03	RO
BG078689	Atp5a1	Cell growth and/or maintenance	14	15.48	15	15.61	RO
BG084796	D6Wsu176e	Unknown	15	15.38	19	15.39	RO
BG073755	9330182L06Rik	Unknown	16	15.03	18	15.41	OSE
BG078109	Tcp1	Protein metabolism	17	15.02	16	15.58	RO
BG087336	Vdac2	Cell growth and/or maintenance	18	15.02	20	15.06	RO
BG069709	Unknown	Unknown	19	15.01	17	15.52	RO
BG078424	Cct4	Protein metabolism	20	14.96	21	14.90	RO
BG070449	Nudt4	Ion binding	21	14.59	23	14.45	RO
BG081456	4921520L01Rik	Unknown	22	14.47	24	14.31	RO
BG063603	Uchl5	Protein metabolism	23	14.44	22	14.62	RO
BG078531	Wdhd1	DNA binding	24	14.32	10	16.82	RO
BG086342	Tdg	Cell growth and/or maintenance	25	14.15	25	14.27	RO

^a $B > 0$ indicates the log odds that a gene is differentially expressed.

^bScore(d), change in gene expression relative to the s.d. of repeated measurements for that gene.

represented unknown genes, with only 25% (276/1111) corresponding to genes with known functions, including *Keratin 8*, *18*, and *19 Villin 2* (*Ezrin*), and *Tacstd1/EpCAM*. Functional groups were assigned using the GOstat (Beissbarth & Speed 2004) and over-representation of several processes was identified, including genes involved in defense response ($P < 0.0001$, Fisher's exact test), immune response ($P < 0.001$), cell proliferation ($P < 0.0001$), and organ development ($P < 0.0002$). We also analyzed known cellular pathways (Ogata *et al.* 1999) and found the pathways with the highest number of genes from this gene set included those involved in the regulation of the actin cytoskeleton, focal adhesion, and MAPK and Wnt signaling pathways (Fig. 3).

Validation of OSE-specific gene expression

To validate the microarray results, two genes differentially expressed between the OSE and the residual ovarian tissue, *Keratin 8* and *Villin 2*, were assessed by immunohistochemical analysis using independent ovarian tissues. Immunohistochemistry confirmed the microarray data for *Keratin 8* (fold change (FC) = 3.36, $B = 5$) and *Villin 2* (FC = 3.68, $B = 9.88$) with both proteins shown to be highly expressed in the OSE when compared with the residual ovarian tissue (Fig. 4).

Figure 4 Validation of differential expression of *Keratin 8* and *Villin 2* by immunohistochemical staining. Cryostat sections (e.g. 4 μ m) were stained for (A) *Keratin 8* in an ovary from a pregnant mouse and (C) for *Villin 2* in an ovary removed on the evening of proestrus. (B and D) No primary antibody negative controls. Staining was visualized using biotin-avidin amplification and horseradish peroxidase/diaminobenzidine and counterstained with hematoxylin. Original magnification, 600 $\times$.

Figure 3 OSE genes involved in the regulation of the actin cytoskeleton, focal adhesion, cell cycle, apoptosis, MAPK signaling, and Wnt signaling pathways. Analysis of all 1111 genes identified as highly expressed in mouse OSE compared with RO, irrespective of the estrous developmental stage, using the KEGG pathway analysis program (Ogata *et al.* 1999) found multiple genes (as listed) involved in the actin cytoskeleton, focal adhesion, cell cycle, apoptosis, MAPK signaling, and Wnt signaling pathways.

Profiling transcriptional changes of the OSE at different physiological stages

Currently, there are few data available on the hormonal regulation of gene expression specifically in the OSE *in vivo*. To explore gene expression profiles under both low and high endogenous hormone stimulation, epithelial cells were laser microdissected from ovaries obtained from 1) IM mice (low hormone levels), 2) cycling mice on PE (high hormone levels just prior to ovulation), 3) cycling mice on EM (low hormone levels just after ovulation), and 4) mid-pregnant mice (P, high hormone levels). To define patterns of gene expression specifically in the OSE, we designed our experiment to include all six possible comparisons between each of the four physiological stages (Fig. 1C). The numbers of differentially expressed genes identified in each of the six comparisons is represented in the inset in Fig. 5.

Genes associated with ovarian growth and development

Results from all microarrays were also combined to identify subsets of genes that were specifically associated with each physiological stage or had restricted patterns of expression (Fig. 5). We found 13 transcripts to be consistently up-regulated in the OSE from IM mice compared with the epithelial cells collected from cycling mice (PE and EM, Fig. 5). Five were genes with known

Figure 5 Number of genes identified as being differentially expressed in the OSE at each physiological stage. OSE were isolated from immature (IM), proestrus evening (PE), estrus morning (EM), and mid-pregnant (P) mice and amplified RNA from each stage were compared with all other physiological stages by cDNA microarrays. The number of differentially expressed genes/ESTs identified (up- and down-regulated) in the OSE between each of the six cDNA microarray comparisons (Fig. 1C) are indicated in the inset. Results from these microarrays were also combined to identify subsets of genes that were specifically associated with each physiological stage or had restricted patterns of expression. The numbers in the diagram represent the number of genes differentially expressed at each physiological stage compared with the other stages.

functions (Table 2), including *tsc22 domain family member 1* (*Tsc22d1*) (transcription) and *Nucleoporin like 1* (*Nupl1*) (transport), and nine were genes/ESTs with unknown functions. Out of these 13 transcripts, one gene, *microorchidia 1* (*Morc1*), was also more highly expressed in the OSE compared with the remaining ovary.

Genes associated with the estrous cycle

The highest number of differentially expressed genes was found in epithelial cells collected on PE directly prior to ovulation (Fig. 5, inset), probably reflecting the high level of metabolic activity or hormone regulation

in the OSE in response to the increase in ovulatory hormones and gonadotrophins (Fig. 1A).

When the OSE from PE mice were compared with the three other stages, 161 genes/ESTs were found to be specifically induced in the OSE just prior to ovulation (PE), whereas 35 genes/ESTs were down-regulated (Fig. 5). A subset of genes up- and down-regulated in epithelial cells collected on the evening of proestrus is represented in Table 3, and the full data set is supplied in Supplementary Table 2 in the online version of the Journal of Molecular Endocrinology at <http://jme.endocrinology-journals.org/content/vol40/issue6/>. Out of the 161 transcripts more highly expressed in

Table 2 Genes differently expressed in the ovarian surface epithelium (OSE) from immature (IM) mice compared with estrous cycling mice, proestrus evening and estrus morning-OSE (PE and EM-OSE). Values represent the fold change (FC) and *B*-value calculated for each gene/EST from four microarrays using linear modeling.

			Microarray comparison			
GenBank or NIA accession	Gene symbol	Function	IM versus PE		IM versus EM	
			FC	<i>B</i> -value	FC	<i>B</i> -value
BG070077	Ptger4	Receptor	2.34	0.73	2.28	0.51
BG084610 ^a	Morc1	Spermatogenesis	1.24	0.04	1.23	0.08
BG081592 ^b	Tsc22d1	Transcription	1.46	1.49	1.43	1.39
BG078397	Nupl1	Transport	1.43	0.79	1.40	0.38
BG080810	Oas1e		1.79	5.34	1.58	2.08
BG079664	C79267		1.80	2.90	1.66	1.69
BG079819			1.82	2.38	1.67	0.69
H3002f10			1.61	4.07	1.47	1.35
H3050F07			1.83	6.60	1.58	2.60
H3063B07			1.60	3.66	1.44	0.44
H3068A09			1.38	6.80	1.27	2.53
H3072B02			2.08	6.41	1.57	0.76
H3088C08			1.19	0.27	1.19	0.45

^aGene highly expressed in the OSE compared with the remaining ovarian tissue.
^bTranscript highly expressed in OSE from mid-pregnant mice.

Table 3 A selection of genes differently expressed in ovarian surface epithelium (OSE) from proestrus evening (PE) compared with immature (IM), estrus morning (EM) and pregnant-OSE (P-OSE). Genes are grouped into broad functional groups based on results obtained from SOURCE. Values represent the fold change (FC) and *B*-value calculated for each gene/EST from 4 microarrays using linear modelling.

GenBank or NIA accession	Gene symbol	Function	Microarray comparison					
			PE versus IM		PE versus EM		PE versus P	
			FC	<i>B</i> -value	FC	<i>B</i> -value	FC	<i>B</i> -value
BG086439	Anxa2	Angiogenesis	2.04	0.52	2.18	1.59	2.48	3.35
BG076263	Hdh	Anti-apoptosis	1.33	2.13	1.35	2.76	1.40	4.01
BG080700	Tnfrsf12a	Apoptosis	1.72	0.76	1.72	0.79	1.84	2.06
BG086102 ^a	Cspg2	Cell adhesion	1.43	0.36	1.45	0.66	1.54	2.41
BG088014 ^{a,b}	Ptprf	Cell adhesion	2.13	4.52	1.71	0.33	2.70	0.44
AW544560	Calml	Cell cycle	1.92	0.20	2.24	2.64	2.08	1.41
AW551029 ^a	Ccnb1	Cell cycle	2.10	3.55	1.84	1.08	2.39	5.76
BG077186	Hspa8	Cell cycle	2.44	3.32	2.77	5.15	2.67	5.92
BG080846	Jun	Cell cycle	1.88	1.33	1.93	1.87	2.00	2.42
BG065443 ^b	Krt8	Cytoskeleton	2.66	0.23	2.82	0.80	3.00	1.33
BG064528 ^b	Myo1d	Cytoskeleton	1.45	1.94	1.38	0.34	1.39	0.52
BG070089 ^b	Tacstd1	Defense response	2.51	7.69	1.72	0.60	2.15	4.91
BG070519 ^b	Cd55	Immune response	1.99	4.55	2.12	5.76	1.76	1.97
BG087169	Pdia6	Ion binding	2.24	0.37	1.82	1.79	2.62	2.39
BG087831 ^a	Kcnd2	Ion transport	1.86	2.45	1.75	1.31	1.90	2.86
BG078816 ^a	Hmgcr	Lipid metabolism	1.46	0.33	1.63	3.29	1.47	0.51
BG072984	Cltc	Membrane	1.65	1.86	1.54	0.19	1.67	2.18
BG070646	Edem1	Membrane	1.56	4.21	1.62	5.01	1.64	0.26
BG072052	Gpiap1	Membrane	1.82	1.81	1.71	0.67	1.98	3.54
BG083639	Mpdu1	Membrane	2.53	6.24	2.67	6.78	2.79	7.82
BG086188 ^a	Ptprg	Membrane	2.17	7.75	1.95	5.20	2.41	9.87
BG063996	Surf4	Membrane	1.89	0.00	2.22	2.50	2.23	2.54
AU045526	Tmem33	Membrane	1.53	5.34	1.74	9.38	1.42	2.68
BG072651 ^{a,b}	Bnc1	Nucleic acid binding	2.33	8.17	1.63	0.74	2.15	6.66
BG079902	Elavl1	Nucleic acid binding	1.76	1.15	1.92	2.85	1.90	2.65
BG077642	Wbp4	Nucleic acid binding	1.19	0.31	1.25	2.97	1.23	2.28
BG069834	Zc3h8	Nucleic acid binding	1.42	1.14	1.58	4.24	1.46	1.76
BG068801	Ube4b	Protein binding	1.34	2.02	1.26	0.03	1.39	4.12
BG064466	Ahsa1	Protein folding	1.89	5.93	1.84	5.52	2.04	8.03
BG077365 ^a	Canx	Protein folding	1.82	4.41	1.74	3.38	1.75	3.59
BG065035	Cct7	Protein folding	2.01	0.56	2.08	1.74	2.17	1.64
AW551675	Dnaja1	Protein folding	2.56	7.36	2.52	7.46	2.62	8.14
BG065483	Dnajb1	Protein folding	1.84	6.00	1.58	2.17	1.82	5.76
BG064128	Fkbp4	Protein folding	1.97	4.00	2.04	4.72	2.38	7.60
BG064500	Hsp110	Protein folding	3.51	7.01	4.04	9.05	3.61	7.71
BG064772	Hsp90aa1	Protein folding	2.66	5.77	2.60	8.29	3.07	7.88
BG078337	Hsp90b1	Protein folding	1.81	0.02	2.24	3.60	2.39	4.89
BG076621	Hspa5	Protein folding	3.16	4.44	3.25	8.18	3.75	6.50
BG073420	Hspa9	Protein folding	1.70	0.11	2.01	3.30	2.15	4.61
BG070071 ^b	Hspb1	Protein folding	3.07	3.00	2.79	1.90	2.78	1.76
BG069463	Hspb8	Protein folding	2.95	11.30	2.02	4.48	2.79	10.42
BG086364	Serpinh1	Protein folding	2.48	7.08	2.01	3.28	2.45	6.88
BG086389	St13	Protein folding	1.62	7.45	1.45	3.74	1.68	8.56
BG081885	Vdp	Protein transport	1.52	2.05	1.87	7.49	1.61	3.85
BG064110	Rnf128	Proteolysis and peptidolysis	2.81	4.96	2.08	0.40	2.50	3.33
BG066107	Acvr2a	Receptor	1.37	2.99	1.38	3.34	1.28	0.46
BG069864	Cry1	Receptor	2.40	7.14	3.62	13.15	2.64	8.78
AA408993	Il13ra1	Receptor	1.40	3.04	1.31	0.49	1.28	0.04
BG077951	Kdelr2	Receptor	1.52	0.79	1.51	0.51	1.56	1.39
BG086503	Kitl	Receptor	2.00	4.69	1.81	2.60	2.20	6.62
BG063992 ^a	Ldlr	Receptor	2.25	5.78	2.85	9.81	2.06	4.16
BG079564	Lif	Receptor	1.66	1.02	1.62	0.74	1.64	0.96
BG073869 ^b	Lsr	Receptor	2.30	2.53	2.03	0.69	2.46	3.56
BG078514 ^b	Ncoa4	Receptor	3.20	9.50	3.03	8.55	3.03	8.59

(continued)

Table 3 Continued

GenBank or NIA accession	Gene symbol	Function	Microarray comparison					
			PE versus IM		PE versus EM		PE versus P	
			FC	B-value	FC	B-value	FC	B-value
BG064672	Procr	Receptor	2.31	2.98	1.99	0.68	2.07	1.33
BG076991 ^a	Socs3	Receptor	1.58	0.13	1.89	3.70	1.82	3.18
BG070950	Zdhhc3	Receptor	1.63	0.51	1.67	0.73	1.73	1.97
BG064837 ^a	Stip1	Response to stress	2.05	2.35	2.08	2.47	2.36	4.56
BG065464	Cabin1	Signal transduction	1.88	1.21	1.76	0.16	2.11	3.25
BG078838 ^b	Grb7	Signal transduction	1.64	9.18	1.47	5.26	1.61	8.57
BG069378	Kcnh3	Signal transduction	1.59	1.44	1.61	1.76	1.71	3.32
BG086893	Pacsin2	Signal transduction	1.56	1.43	1.67	3.15	1.73	4.03
BG079375 ^b	Stard5	Steroid biosynthesis	1.83	2.98	1.82	3.12	1.88	3.74
BG084009 ^a	Cldn3	Tight junction	1.59	0.57	1.58	0.36	1.59	0.54
BG072020	Arntl	Transcription	1.49	1.15	1.68	4.33	1.77	5.84
BG084311 ^{a,b}	Esr1	Transcription	1.43	3.87	1.54	7.05	1.41	3.85
BG069739	Hmgcs1	Transcription	2.53	2.72	2.79	4.11	2.32	1.55
BG084377 ^b	Stat3	Transcription	1.67	4.22	1.51	1.41	1.62	3.41
BG084172	Xbp1	Transcription	2.09	3.53	2.05	3.22	2.37	5.78
BG085346	Acaa2	Transferase activity	2.08	3.92	1.95	3.07	2.31	6.19
BG079850	Got1	Transferase activity	1.80	1.04	2.15	4.30	1.94	2.48
BG071867	Lpgat1	Transferase activity	1.57	1.29	1.57	1.58	1.57	1.57
BG071111	Map4k3	Transferase activity	2.00	3.66	1.84	1.99	1.75	0.97
BG086330 ^a	Mgst1	Transferase activity	2.58	4.65	2.29	2.86	2.74	5.54
BG078650	Nme2	Transferase activity	2.96	2.58	3.07	2.99	3.65	4.94
BG064642 ^a	Pcyt1a	Transferase activity	2.17	11.47	1.84	7.49	2.12	11.03
BG074044	Atp2a2	Transport	1.53	0.73	1.73	1.23	2.17	5.87
BG078309	Slc38a4	Transport	1.46	5.11	1.38	3.21	1.41	3.97
BG069742	Cacybp	Ubiquitin cycle	2.38	0.29	2.63	3.57	2.71	4.29
BG076945	Armet		3.36	4.02	4.49	7.16	4.48	7.13

^aTranscript previously reported to be induced by ovulatory hormones or play a role in ovarian biology.^bTranscript more highly expressed in OSE compared with the remaining ovary.

PE-OSE, 105 were genes with known function (65%), 8 were genes with unknown function (5%), and 48 were ESTs (30%). We identified over-representation of several processes using GStat (Beissbarth & Speed 2004), including those involved in protein binding ($P < 0.0001$, Fisher's exact test) and protein folding ($P < 0.02$). Four genes (*calmodulin 1* (*Calm1*), *cyclin B1* (*Ccnb1*), *jun oncogene* (*Jun*), and *heat shock protein 8* (*Hspb8*)) were found to be involved in the cell cycle and two genes (*tumour necrosis factor receptor super family, member 12* (*Tnfrsf12a*) and *huntington disease gene homolog* (*Hdh*)) were found to be involved in apoptosis. We identified 10 genes, (10/105 with known function, 9%) that have previously been shown to have induced expression in response to ovulatory levels of LH, human chorionic gonadotrophin, or follicle-stimulating hormone, in various ovarian cell types *in vitro* (Table 3). In addition to providing support for data obtained by microarray analysis, this suggests that the remaining up-regulated transcripts may represent novel hormone-regulated genes. Out of the 161 transcripts, 21 genes/ESTs were also more highly expressed in the OSE compared with the remaining ovarian tissue.

On the morning of estrus, after ovulation has occurred and hormones levels have dropped (Fig. 1A), 22 genes/ESTs were found to be specifically more highly expressed in EM-OSE compared with the three other stages (Fig. 5; Table 4). This included 11 ESTs (50%), 10 genes with known function (45%) including *nucleobindin 1* (*Nucb1*), *oviductal glycoprotein 1* (*Ovgp1*), and *suppressor of zeste 12 homolog* (*Drosophila*) (*Suz12*), and 1 gene, *PDZ and LIM domain 2* (*Pdlim2*), with unknown function. An over-representation of genes involved in amino- and exopeptidase activity ($P < 0.06$, Fisher's exact test (Beissbarth & Speed 2004)) was identified. Out of the 22 transcripts, only *H3047C02* was also more highly expressed in the OSE compared with the remaining ovarian tissue. No genes were identified to be down-regulated in the EM-OSE compared with the other three stages.

Several transcripts were also identified to be highly expressed in ovarian epithelial cells collected during the estrous cycle compared with cells from IM mice (i.e., PE and EM-OSE compared with IM-OSE, Table 5). These genes are likely to be induced in ovarian epithelial cells as the mouse reaches maturity, and are perhaps not involved in pre-pubertal ovarian growth and development. They

Table 4 Genes differently expressed in ovarian surface epithelium (OSE) from estrus morning (EM) compared with immature (IM), proestrus evening (PE), and pregnant-OSE (P-OSE), as measured by cDNA microarray analysis. Genes are grouped into broad functional groups based on results obtained from SOURCE. Values represent the fold change (FC) and *B*-value calculated for each gene/EST from four microarrays using linear modeling.

GenBank or NIA accession	Gene symbol	Function	Microarray comparison					
			EM versus IM		EM versus PE		EM versus P	
			FC	<i>B</i> -value	FC	<i>B</i> -value	FC	<i>B</i> -value
BG072750	Tgfb1	Cell adhesion	1.35	5.60	1.97	3.46	2.13	4.51
BG067101	Nucb1	DNA binding	1.51	3.23	1.58	1.43	1.87	5.65
BG086606	Txndc12	Electron transport	1.66	1.58	1.45	3.85	1.36	1.49
BG078684	Pex19	Membrane	1.65	0.20	1.44	3.99	1.36	2.15
BG087081	Tmem59	Membrane	1.56	0.80	1.79	3.82	1.52	0.07
BG080500 ^a	Ovvp1	Metabolism	1.59	1.01	1.59	2.37	1.61	2.95
BG086139	Abhd4	Proteolysis	1.49	0.88	2.38	6.61	2.08	4.12
BG065235	Metap2	Proteolysis	1.69	0.64	1.31	0.01	1.30	0.12
BG063378	Suz12	Transcription	1.77	1.08	1.24	2.52	1.26	3.19
BG088209	Tgif	Transcription	1.63	0.40	1.81	6.99	1.71	5.27
AW554179	BC037112		1.73	0.59	1.26	0.54	1.36	3.68
BG077310	Pdlim2		1.50	3.32	1.93	6.53	1.57	1.42
BG065640			1.53	7.21	1.51	5.07	1.72	9.18
H3047C02 ^b			3.25	3.33	2.57	0.75	3.97	5.48
H3082D04			1.89	3.32	1.88	3.13	1.99	4.27
H3055E08			2.40	2.60	2.01	0.01	2.26	1.72
BG076724			1.68	0.45	2.39	16.94	1.41	2.27
BG067247			2.06	2.55	1.88	0.80	2.09	2.87
BG085369			5.56	8.56	4.15	6.15	6.85	10.59
H3048C02			2.43	4.82	1.84	0.43	2.17	3.16
H3055E03			1.86	1.39	1.87	1.42	1.99	2.52
H3048E03			1.19	0.64	1.24	2.40	1.21	1.39

^aTranscript previously reported to play a role in ovarian biology.

^bTranscript more highly expressed in OSE compared with the remaining ovary.

included genes involved in metabolism, cell growth, and response to stress. Among these eight transcripts, three were genes/ESTs with unknown function and five represented genes with known functions, including

insulin-like growth factor-binding protein 4, *complement component 3 (C3)*, *claudin 10 (Cldn10)*, *adipose differentiation related protein (Adfp)*, and *cystatin B (Cstb)*. Both *Igfbp4* and *Cstb* have previously been found to be expressed in human

Table 5 Genes more highly expressed in the ovarian surface epithelium (OSE) on both proestrus evening and estrus morning-OSE (PE and EM-OSE) compared with immature and pregnant (IM and P) mice, as measured by cDNA microarray analysis. Genes are grouped into broad functional groups based on results obtained from SOURCE. Values represent the fold change (FC) and *B*-value calculated for each gene/EST from four microarrays using linear modeling.

GenBank or NIA accession	Gene symbol	Function	Microarray comparison							
			PE versus IM		EM versus IM		PE versus P		EM versus P	
			FC	<i>B</i> -value	FC	<i>B</i> -value	FC	<i>B</i> -value	FC	<i>B</i> -value
BG084827	<i>Igfbp4</i>	Cell growth	2.34	0.99	2.95	3.79	2.91	3.76	3.67	6.39
BG087429	<i>C3</i>	Complement activation	1.91	0.28	1.98	0.92				
AU018835 ^a	<i>Cldn10</i>	Tight junction	3.08	6.15	2.07	0.64				
BG075211	<i>Adfp</i>	Protein binding	1.48	2.22	1.37	0.04	1.49	2.42	1.38	0.23
BG078674 ^b	<i>Cstb</i>	Proteolysis	1.56	3.04	1.79	6.49	1.52	2.27	1.74	5.99
BG081020	<i>Prr13</i>		1.81	0.56	2.00	2.35				
H3111C05			1.58	2.26	1.49	0.78				
BG076724			1.21	0.98	1.21	1.12	1.29	4.60	1.29	4.61

^aTranscript more highly expressed in OSE compared with the remaining ovary.

^bTranscript previously reported to play a role in ovarian biology.

(Kalli *et al.* 2004) and monkey (Oksjoki *et al.* 2001) ovarian epithelial cells respectively, and like *Cldn10* have dysregulated expression in human EOC (Donninger *et al.* 2004).

Validation of the OSE genes regulated during the estrous cycle

Two genes that were consistently more highly expressed on PE were selected to validate our microarray data using an independent method. We used RT-qPCR to measure the expression of *Armet* and *Tacstd1*. RT-qPCR results confirmed the microarray data (Fig. 6), with expression of *Tacstd1* and *Armet* being higher in PE-OSE when compared with all other stages (Fig. 6). By microarray analysis, *Tacstd1* was induced to a similar level (1.7- to 2.5-fold, Fig. 6) in PE-OSE compared with all other stages (IM, EM, and pregnancy). RT-qPCR revealed induction to a similar level (two- to five-fold) in PE-OSE compared with the other stages (Fig. 6), although the induction of *Tacstd1* in PE compared with IM-OSE was more marked (eight-fold, Fig. 6) when measured by RT-qPCR. Likewise, *Armet* was induced in PE-OSE compared with OSE from IM, EM, and pregnant mice by microarray analysis (3.4 to 4.5-fold, Fig. 6), and RT-qPCR analysis also found higher expression of *Armet* in PE compared with the other stages (approx. two-fold, Fig. 6). Overall, these data illustrate similar changes of gene expression using RT-qPCR providing technical validation of our microarray data.

Genes associated with pregnancy

We identified 17 transcripts (Fig. 5) that were highly expressed in the OSE from mid-pregnant mice compared with OSE from cycling mice (PE and EM). Of these, the vast majority were ESTs (15/17, 83%) and only two were known genes, *Tsc22d1* and *elongation factor RNA polymerase LL, 2 (Ell2)*, both associated with transcriptional processes (Table 6). Interestingly, no genes were found to be consistently and significantly differentially expressed

between the OSE from mid-pregnant and IM mice (Fig. 5, P versus IM).

Discussion

Results from this study provide, for the first time, global expression profiles of pure preparations of normal ovarian epithelial cells collected from the intact animal, revealing complex gene expression profiles that are activated or repressed in response to specific physiological conditions. Furthermore, we have demonstrated the expression of genes and ESTs not previously described in the OSE, and identified diverse cellular pathways that are active in the OSE through the estrous cycle.

Using gene expression profiling of microdissected tissue, we showed first that there were significant differences in gene expression between the OSE and other cell types within the ovary. These results are not surprising considering the numerous cell types that are present in the ovary and the high level of physiological activity but indicate that the OSE are involved in many cellular processes. Many of the differentially regulated genes identified are well characterized, and several have been previously identified to be involved in ovarian epithelial cell biology, including *Villin 2 (Ezrin)* (Moilanen *et al.* 2003), *Keratins 8, 18, and 19* (Auersperg *et al.* 2001), and *ESR1* (Sar & Welsch 1999, Saunders *et al.* 2000).

The marked difference in gene expression between the OSE and the residual ovarian tissue has significant implications considering that several microarray studies have examined EOC gene expression compared with the whole ovary. These results provide at least some insight into why EOC gene expression profiles are not consistent between studies, particularly between studies where EOC specimens are compared with whole ovarian tissue rather than the OSE (Zorn *et al.* 2003).

Figure 6 Validation of gene expression levels in the OSE using RT-qPCR. The relative expression of (A) *Tacstd1* and (B) *Armet* in the OSE from IM, PE, EM, and P mice, as measured using RT-qPCR. Inset, expression levels of *Tacstd1* and *Armet* in PE-OSE compared with IM, EM, and P-OSE by microarray analysis. FC, fold change.

Table 6 Genes differentially expressed in ovarian surface epithelium (OSE) from pregnant mice compared with proestrus evening (PE) and estrus morning (EM) OSE as measured by cDNA microarray analysis. Genes are grouped into broad functional groups based on results obtained from SOURCE. Transcripts with two accession numbers indicate the presence of duplicate spots on the microarray.

GenBank or NIA accession	Gene symbol	Function	Microarray comparison			
			P versus PE		P versus EM	
			FC	B-value	FC	B-value
BG085211	ElI2	Transcription	1.24	0.10	1.27	1.04
BG081592 ^a	Tsc22d1	Transcription	1.42	0.92	1.39	0.62
BG081142, BG087624	4930562C15Rik		2.30	1.71	2.40	2.33
BG086900	BC034090		1.66	0.94	1.97	4.10
H3134a10			1.99	1.58	1.89	0.81
H3051G01			1.99	2.98	1.72	0.34
H3068A09			1.31	4.44	1.21	0.15
H3018h12			1.55	5.34	1.34	0.54
H3088C08			1.20	0.77	1.20	0.95
BG074026			2.74	0.94	3.37	3.15
BG074023			2.72	1.05	3.30	3.15
BG074024			2.50	0.02	3.07	2.29
BG074022			2.45	0.60	3.03	3.13
BG074021			2.39	0.87	2.84	3.01
BG086541			2.40	0.65	2.46	1.06
H3106C09			1.44	0.78	1.46	1.32
H3133A01			1.54	3.56	1.44	1.54

FC, fold change.

^aTranscript highly expressed in immature OSE.

In addition to profiling gene expression changes between the OSE and the residual ovary, we also examined microarray profiles of the OSE under specific hormonal conditions. Interestingly, no genes were identified to be differentially expressed between the OSE from mid-pregnant and IM mice. This was an unexpected result, given the very different hormonal milieu associated with these phases, immaturity being associated with low systemic hormone levels and pregnancy with high hormone levels. The relative similarity in transcripts between the OSE from IM and mid-pregnant mice may reflect the similar 'dormant' nature of the epithelium during these anovulatory phases.

Several hypotheses have been proposed to explain altered risk of ovarian cancer related to reproductive factors (Fathalla 1971, Risch 1998). The first argues that repeated cycles of ovulation-induced proliferation, trauma, and repair of the OSE at the site of ovulation contributes to ovarian cancer development (Fathalla 1971), and that pregnancies and oral contraceptives protect by suppressing ovulation (Casagrande *et al.* 1979). A second hypothesis is that circulating levels of gonadotrophins increase the risk of malignancy, and that pregnancies and oral contraceptives protect by suppressing secretion of these hormones (Zheng *et al.* 2000, Ho 2003, Riman *et al.* 2004). These two hypotheses are not mutually exclusive and features of our data are consistent with both.

The most extensive differential expression was observed on the evening of proestrus directly prior to ovulation when hormone levels are high and OSE

proliferation is stimulated. This subset included genes involved in the cell cycle and apoptosis, although the processes most over-represented in proestrus were protein folding/binding and transcription factors, indicative of the rapid changes that are occurring in these cells in response to ovulation. We identified several genes that were specifically induced in the OSE on the evening of proestrus, which have been implicated in the early events of ovarian tumorigenesis, including two integral membrane proteins, *claudin 3* (*Cldn3*) and *Tacstd1* (Donninger *et al.* 2004, Heinzelmänn-Schwarz *et al.* 2004). *Cldn3* has previously been shown to be regulated in response to ovulatory hormones, which is consistent with our expression array data (Rimon *et al.* 2004).

Cldn3 has been found to be one of the most highly expressed genes in EOC, identified by several microarray and SAGE studies (Hough *et al.* 2000, Rangel *et al.* 2003, Adib *et al.* 2004, Heinzelmänn-Schwarz *et al.* 2004, Lu *et al.* 2004, Santin *et al.* 2004). *Cldn3* is expressed at low levels in the normal human OSE and epithelial cells lining inclusion cysts, its expression is increased in ovarian adenocarcinomas compared with benign and low malignant potential (LMP) tumors (Heinzelmänn-Schwarz *et al.* 2004, Zhu *et al.* 2006), and it has been shown to be expressed in all EOC subtypes (Heinzelmänn-Schwarz *et al.* 2004, Lu *et al.* 2004, Zhu *et al.* 2006). Recently, small interfering RNA (siRNA) analysis has shown *CLDN3* expression to increase invasion and survival of ovarian tumor cells (Agarwal *et al.* 2005).

Similarly, immunohistochemical analysis has revealed that *Tacstd 1* (commonly known as epithelial cell adhesion/activating molecule, EpCAM/CD326)) is also expressed in all EOC subtypes (Hough *et al.* 2000, Connor *et al.* 2004, Drapkin *et al.* 2004, Heinzelmann-Schwarz *et al.* 2004). While it is expressed at low levels in the normal OSE, its expression increases significantly in ovarian epithelial inclusion cysts and further in ovarian adenocarcinomas compared with benign and LMP tumors (Drapkin *et al.* 2004, Heinzelmann-Schwarz *et al.* 2004). Recently, Cheng *et al.* (2005) demonstrated the expression of *Tacstd1* and *Cldn3* in the serous-like tumors that developed from inoculation of nude mice with transformed mouse ovarian epithelial cells transfected with various members of the homeo box (HOX) family (HOX genes normally regulate müllerian duct differentiation, but are not expressed in normal mouse OSE (Cheng *et al.* 2005)). Although their exact roles in tumorigenesis are still being investigated (Agarwal *et al.* 2005, Baeuerle & Gires 2007), results indicate that both *Cldn3* and *Tacstd1* are widely expressed in ovarian cancer and represent promising targets for detection, diagnosis, and therapy.

Related to the hormonal hypothesis of ovarian cancer development is the proposal that progesterone is protective against ovarian cancer with increased progesterone synthesis during pregnancy, and progestins contained in formulations of oral contraceptives, conferring protection to the ovarian epithelium (Risch 1998, Rodriguez *et al.* 1998, Schildkraut *et al.* 2002). Various mechanisms have been proposed to explain the progestin-mediated reduction in ovarian cancer risk including induction of apoptosis (Murdoch 1995), promotion of repair of ovulation-induced genomic damage (Murdoch & Martinchick 2004), and inhibition of proliferation by progestin-mediated induction of alternative expression of transforming growth factor- β (TGF- β) isoforms (Ho 2003).

Although very few genes were differentially expressed in the OSE during pregnancy compared with the OSE from cycling mice in our study, the expression of one gene, *Tsc22d1* (also known as TGF- β -stimulated clone 22 homolog) is of potential interest in the light of these proposed effects of pregnancy on ovarian epithelial cells. *Tsc22d1* expression has been shown to be stimulated by both TGF- β (Shibanuma *et al.* 1992, Jay *et al.* 1996) and progesterone (Kester *et al.* 1997), and *Tsc22d1* has features consistent with tumor suppression in prostate cancer, salivary gland cancer, and leukemia; however, further investigation is required to determine whether *Tsc22d1* has a role in reduction of ovarian cancer risk.

Overall, we have identified genes that were consistently more highly expressed in ovarian epithelial cells compared with the other ovarian cells, illustrating their specific role in ovarian physiology. We also identified

many known and unknown genes that were differentially expressed only during the estrous cycle, indicating stage-specific roles. Our findings show clear evidence of the highly complex and tightly regulated processes affected by the fluctuations in hormone levels that occur through the estrous cycle and with pregnancy. Analysis of these gene profiles may also help to further elucidate some of these pathways and also help to identify genes that are involved in ovarian epithelial tumorigenesis.

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