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ARTICLE

Blastocysts from patients with polycystic ovaries exhibit altered transcriptome and secretome


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Abstract Polycystic ovaries (PCO) is a common phenotype of women presenting for infertility treatment. This study investigated whether blastocysts derived from women with PCO have an altered molecular signature which could be a causative factor contributing to reproductive failure. Morphologically similar blastocysts derived from women with PCO and donor oocyte cycles were analysed for transcription and protein secretion. Unsupervised hierarchical clustering demonstrated that the transcriptome profiles of blastocysts derived from PCO patients and control blastocysts were markedly different with complete branch separation. Statistical analysis revealed 829 genes with significantly different expression: 784 decreased (94.6%) and 45 increased (5.4%) in blastocysts derived from women with PCO compared with controls ($P < 0.05$). Functional annotation of these genes revealed predominant gene ontology biological processes including protein metabolism (30%), transcription (22%), signal transduction (15%), biosynthesis (15%) and cell cycle (14%). Proteomic profiling identified 12 biomarkers that displayed significant decrease in expression in blastocysts derived from women with PCO compared with controls ($P < 0.05$). These data indicate molecular alterations in human blastocysts derived from PCO patients, potentially demonstrating for the first time a link between patient aetiology/phenotype and subsequent embryo development, which in part may explain the observed reduction in reproductive capacity. 

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KEYWORDS: blastocyst, gene expression, polycystic ovaries

Introduction

Polycystic ovarian syndrome (PCOS) is a common endocrine and metabolic disorder in women of reproductive age. It is estimated that between 4%–11% of childbearing women are affected by this disorder (Asuncion et al., 2000;

Knochenhauer et al., 1998). The PCOS phenotype is variable and may include polycystic ovaries (PCO), hyperandrogenism, reduced fecundity, hirsutism, obesity and hyperinsulinaemia from insulin resistance (Azziz, 2006). PCOS patients are associated with exaggerated ovarian responsiveness to FSH (Mason et al., 1994), an increased risk of developing

ovarian hyperstimulation syndrome (Swanton et al., 2010), implantation failure (Ludwig et al., 1999) and pregnancy loss (Balen et al., 1993; Sagle et al., 1988).

The aetiology of PCOS remains unclear although the documentation of familial segregation is supportive of a genetic basis for this disorder (Franks et al., 2006). Ongoing association studies focusing on candidate genes involved in ovarian function and metabolism have as yet to find any significant associations (Simoni et al., 2008). Considerable progress has been made in the diagnosis and treatment of PCOS; nevertheless, there are still challenges with understanding the pathophysiology of the disorder, the prediction of response to ovulation stimulation during infertility treatment and the impact of a potentially impaired follicular environment.

Analysis of several ovarian cell types from PCOS patients have been performed in an attempt to document irregularities that may play a role in the physiological and morphological differences between PCOS and normal women. Tissues analysed include ovaries (Diao et al., 2004; Jansen et al., 2004), follicular fluid (Berker et al., 2009), theca cells (Wood et al., 2003), cumulus cells (Zhao et al., 2010) and oocytes (Wood et al., 2007). Analysis of PCOS patients to date indicate perturbations of many of the mechanisms involved in human follicular development including theca cell androgen biosynthesis (Nelson et al., 2001) and cumulus–cell–oocyte communication (Kenigsberg et al., 2009).

Transcriptome analysis of PCOS oocytes has revealed altered expression of mitotic cell cycle and maternal effect genes, with many of these differentially expressed genes containing receptor-binding sites on their promoters for androgens and other activators, suggesting that the altered transcriptome will impact oocyte developmental competence (Wood et al., 2007). The mature pre-ovulatory metaphase-II oocyte is a unique cell required to complete multiple tasks including meiosis, fertilization, early embryonic cleavage divisions and the activation of the embryonic genome (Eichenlaub-Ritter and Peschke, 2002). Consequently, an altered oocyte transcriptome could affect the molecular physiology of the resultant blastocyst. Indeed, alterations of the oocyte proteome have been shown to persist throughout embryonic development (Johnson et al., 2009).

Research has shown that women with ultrasound-confirmed PCO share some of the metabolic characteristics of women with PCOS including subtle endocrine disturbances (Carmina et al., 1997; Kousta et al., 1999). PCO women account for approximately 20–30% of the female population (Farquhar et al., 1994; Polson et al., 1988) and have been documented in up to 34% of women presenting for fertility treatment (Balen et al., 1993).

PCO women undergoing infertility treatment, like PCOS women, have been shown to have an increased risk of ovarian hyperstimulation syndrome when compared with women with normal ovaries (MacDougall et al., 1993; Swanton et al., 2010). They may also have a similar altered follicular environment and compromised oocyte development as observed in studies of PCOS patients. Nonetheless, studies have yet to examine the effects of the PCO ovarian environment in relation to the subsequent developing embryo and ultimately implantation potential.

The aim of this study was to analyse the molecular signature, at both the gene and protein level, of the human blastocyst from women with PCO compared with donor oocyte fertile controls in an attempt to link an altered follicular environment to embryo phenotype and infertility aetiology.

Materials and methods

Subjects

Blastocysts ($n = 48$ in total) were donated for research from couples presenting for infertility treatment at the Colorado Center for Reproductive Medicine between 2001 and 2006. Female partners in the PCO group were ultrasound diagnosed with polycystic ovaries. Blastocysts in the control group originated from female oocyte donors. No male factor infertility was reported for any of these couples. The study was approved by the Institutional Review Board.

Human blastocyst culture

Standard IVF protocols for controlled ovarian stimulation, oocyte retrieval and fertilization were performed. Assessment of fertilization took place 15–18 h after injection. Embryos with two pronuclei were group cultured in microdrops of sequential media at 5% O₂, 6% CO₂ at 37°C (Vitrolife, Englewood CO, USA) with media changeover occurring on day 3 of embryo development. On the morning of day 5, the percentage of blastocyst formation was determined and each blastocyst was assigned a score using the system of Gardner and Schoolcraft (1999). Blastocysts with a distinguishable inner cell mass (>grade 3BB) were identified for either fresh embryo transfer or cryopreservation. Only transferable grade, good quality blastocysts (>grade 3BB) were cryopreserved for patients, using slow freezing techniques as previously described (Veeck et al., 2004). Upon thawing blastocysts were allowed to recover in individual microdrops of G2 culture medium (Vitrolife) for 4 h. In both groups, only fully recovered, good-quality blastocysts with >80% cell survival were selected for transcriptome and secretome analysis.

RNA isolation and amplification

Total RNA was isolated from groups of thawed blastocysts ($n = 6$ per each array) using PicoPure RNA Isolation Kit with modifications (Molecular Devices, Sunnyvale CA, USA). Blastocysts were allowed to expand for 4 h and embryos of similar morphology selected for gene analysis. Samples were lysed and bound to a silica-based filter where they were treated with RNase-free DNase I (Qiagen, Valencia CA, USA), washed several times and eluted in 20 µl. Following isolation, two rounds of linear RNA amplification incorporating a T7 polymerase promoter were performed using the MessageAmp II aRNA Amplification Kit (Ambion, Austin TX, USA). On average, samples had a starting concentration of 165 ng and a final concentration of 200 µg after two rounds of amplification. RNA integrity was evaluated with the 2100 Bioanalyser and RNA 6000 Nano Assay Kit (Agilent, Palo Alto CA, USA) with concentration determined using the NanoDrop spectrophotometer (Thermo Scientific, Waltham MA, USA).

Microarray transcriptome analysis

Samples were fragmented and hybridized to the Codelink Human Whole Genome Bioarray (Applied Microarrays, Tempe AZ, USA) according to the manufacturer's instructions. This platform contains 57,347 transcripts including 45,674 discovery genes. Each microarray was scanned using the GenePix 4000B Scanner (Molecular Devices). Analysis of the microarray data was performed using GeneSpring software (Agilent Technologies, Santa Clara CA, USA). Four arrays were analysed in each patient group. Data was transformed prior to statistical analysis to set measurements less than 0.01 to 0.01 followed by normalization per chip to the 50% centile and normalization per gene to the mean. Unsupervised hierarchical clustering grouped individual microarrays according to similarities in gene expression profiles. Student's *t*-test revealed transcripts that were called 'present' and differentially expressed between the two groups with significance equal to a *P*-value less than 0.05. The list of differentially expressed transcripts was classified according to their gene ontology including both biological process and molecular function (www.ease.com).

Validation of microarray transcriptome analysis: quantitative real-time PCR

Microarray results were validated by quantitative real-time polymerase chain reaction (PCR) on the Roche Biosciences LightCycler using the LightCycler FastStart DNA Master^{PLUS} SYBR Green I with 60 ng of amplified RNA template. All reactions were performed in duplicate and standard curves were calculated on 10-fold serial dilutions of input reference RNA. After a 10 min incubation at 95°C, the following thermal cycling profile was performed for 45 cycles of amplification: denaturation at 95°C for 10 s, annealing for 5 s at primer-dependent temperature and extension at 72°C for varying times depending on the size of the amplicon. The final cycle was for 15 s at 65°C and then cooling to 40°C. The following were the genes chosen for validation: *LAMB1* (GenBank NM_002291), *SUGT1* (NM_001130912), *HIST1H1A* (NM_005325) and *KHDRBS1* (NM_006559). The quantification of each gene was calculated relative to the transcription in every sample of the housekeeping gene *ACTB*. Statistical analysis was performed with REST-2005 software using bootstrap randomization techniques. Gene-expression fold differences with *P* < 0.05 were considered significant.

Proteomic analysis

Post-thawing blastocysts were allowed to recover for 4 h in individual microdrops of G2 culture medium (Vitrolife) prior to total RNA isolation. Each microdrop of spent culture media was stored at -80°C until analysis. Metal affinity chromatographic surface ProteinChip arrays (BioRad, Hercules CA, USA) were activated with CuSO₄ (100 mmol/L) prior to individual sample spot incubation for 60 min at room temperature. ProteinChip arrays were then washed three times to remove unbound proteins and other contaminants, followed by a quick rinse in distilled water to remove salts before air-drying. The energy absorbing molecule sinapinic acid (BioRad) was prepared as a saturated solution in 50%

acetonitrile/0.5% trifluoroacetic acid and 1 µl was loaded twice onto each spot surface prior to time-of-flight mass spectrometry (TOF-MS) using the PCS-4000 Series MS (BioRad). Time-of-flight data was collected from an average 1060 laser shots per spot, laser intensity 3000 nJ, capturing peptides and proteins less than 30 kDa. Mass accuracy was calibrated externally with a peptide standard (BioRad). Protein profiles generated following TOF-MS were normalized to the total ion current with peaks selected with a signal-to-noise ratio higher than 6. BioMarker Wizard (BioRad) was employed using the Mann Whitney non-parametric test to calculate significant differences between the groups (*P* < 0.05). The Swiss-Prot database (www.expasy.org) was used to search for preliminary candidate protein identifications.

Results

Of the 45,674 discovery transcripts investigated on the Codelink Whole Genome Bioarray, about 23,000 were identified as being consistently expressed in control and/or PCO blastocysts. Unsupervised hierarchical clustering demonstrated that the transcriptome profiles of blastocysts derived from PCO patients and control blastocysts were markedly different with complete branch separation (Figure 1). In contrast, the transcriptome profiles within each group were noticeably similar indicating greater differences between the groups than within the groups (Figure 1).

Statistical analysis revealed 829 genes with significantly different expression levels; 784 down-regulated (94.6%) and 45 up-regulated (5.4%) in blastocysts derived from women with PCO compared with controls (*P* < 0.05). Functional annotation of the differentially expressed genes revealed gene ontology biological processes including protein metabolism (30%), transcription (22%), signal transduction (15%), biosynthesis (15%) and the cell cycle (14%) (Figure 2A). Gene ontology molecular functions of the differentially expressed genes included nucleic acid binding (32%), hydrolase activity (14%), protein binding (11.5%), transferase activity (11.5%) and transporter activity (11%) (Figure 2B). Pathway annotation of the 94.6% significantly down-regulated genes revealed multiple hits for base excision repair, mitogen activated kinase-like protein signalling, cell cycle, focal adhesion and glutathione metabolism, implicating biological significance.

Candidate genes (*LAMB1*, *SUGT1*, *HIST1H1A* and *KHDRBS1*) were chosen from the list of differentially expressed genes to validate and provide independent confirmation of the microarray data using quantitative real-time PCR relative to the housekeeping gene *ACTB*. *LAMB1* is a component of basement membranes. *SUGT1* is involved in kinetochore function. *HIST1H1A* functions in the compaction of chromatin. *KHDRBS1* is active in post-transcriptional mRNA metabolism. Differential expression was confirmed for each of these down-regulated genes in blastocysts from PCO patients compared with controls (*P* < 0.05) (Figure 3).

Proteomic profiling of individual blastocyst spent media also revealed differential signatures in the secretome of blastocysts derived from patients with PCO compared with oocyte donor controls (*P* < 0.05). Control drops of culture media without the presence of a blastocyst were run alongside all spent-media proteomic profiles to confirm

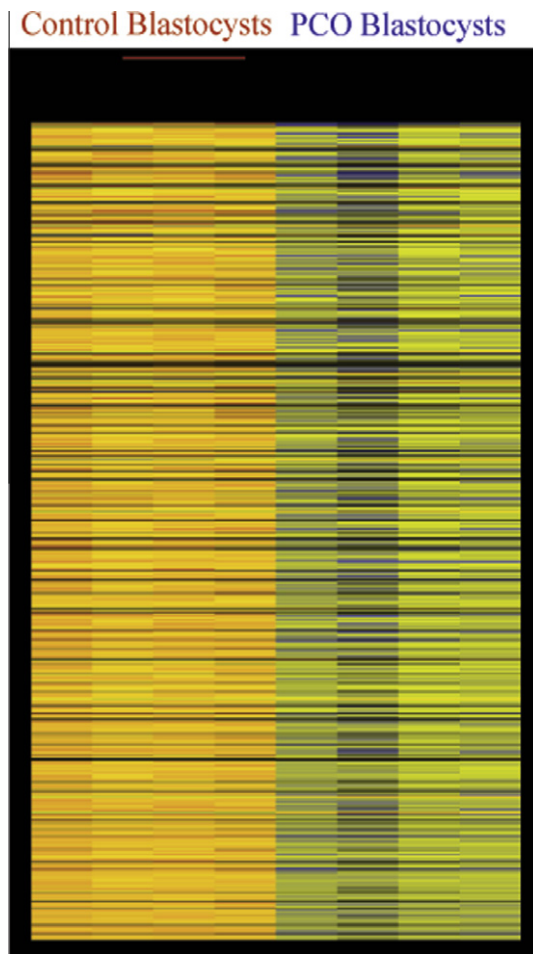


Figure 1 Unsupervised hierarchical clustering of blastocyst microarray samples showing normalized gene expression ratios depicted by colour intensity, with the highest expression corresponding to red and the lowest expression corresponding to blue. Clear separation of the two blastocyst groups was observed.

embryo-derived peaks/biomarkers and all data was normalized to total ion current prior to statistical analysis. A snap-

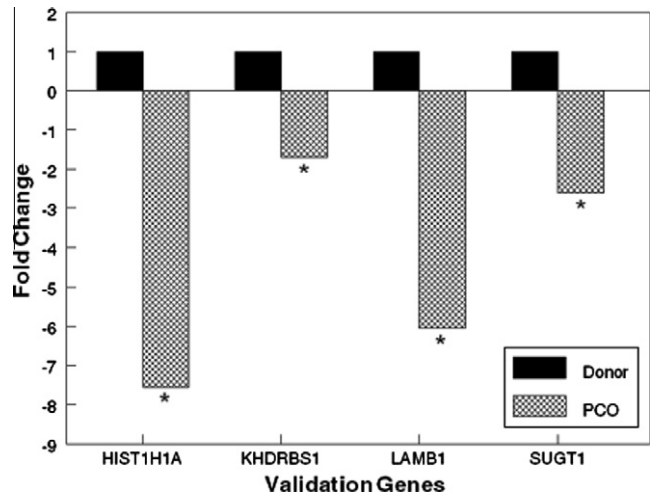


Figure 3 Quantitative real-time PCR validation of microarray data. The expression of candidate genes, *LAMB1*, *SUGT1*, *HIST1H1A* and *KHDRBS1* relative to the housekeeping gene *ACTB*, revealed significant down-regulation in PCO patient blastocysts compared with donor oocyte control blastocysts, in concordance with the microarray data. * $P < 0.05$.

shot (4.8–5.2 kDa) of an individual proteomic profile is shown in **Figure 4**. Black boxes highlight examples of peaks/biomarkers with differential expression ($P < 0.01$). A total of 12 differentially expressed peak/biomarkers across the lower molecular range (< 30 kDa) were identified between blastocysts derived from PCO patients and donor oocyte control blastocysts ($P < 0.05$). Each of these 12 differentially expressed peaks/biomarkers displayed a decrease of expression in the secretome of blastocysts derived from patients with PCO compared with the secretome of blastocysts from donor oocyte controls (**Figure 4**).

Discussion

This is the first study to identify differences in the molecular signature between morphologically similar human blastocysts derived from PCO patients and donor oocyte fertile

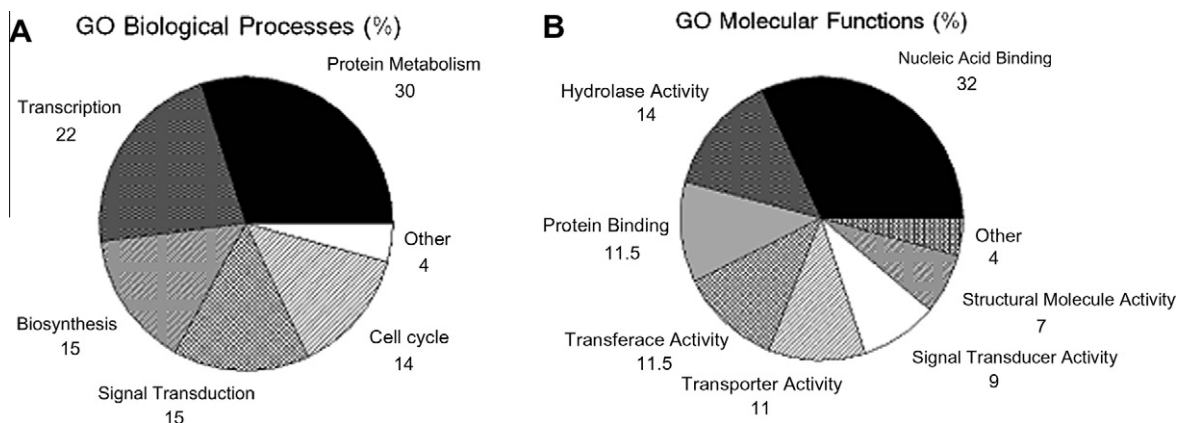


Figure 2 Differentially expressed genes were annotated to identify (A) gene ontology (GO) biological processes and (B) GO molecular functions.

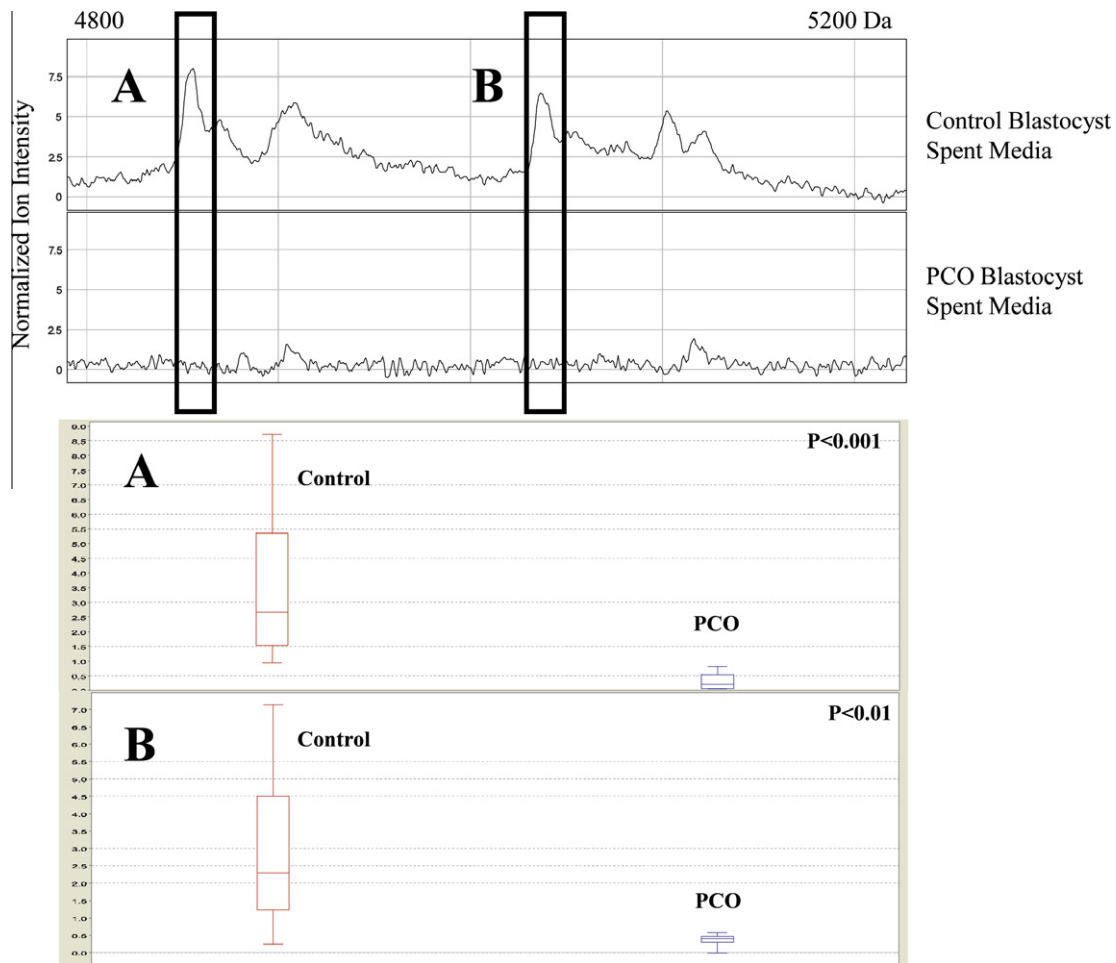


Figure 4 Snapshot (4800–5200 Da) of the proteomic profile from the secretome of (A) donor oocyte control blastocyst and (B) PCO-derived blastocyst. Black boxes = differentially expressed peaks/biomarkers identified following statistical analysis ($P < 0.01$).

controls. Results revealed predominantly decreased gene and protein expression in PCO blastocysts that could potentially be contributing to any observed reduction in reproductive capacity. Future transcriptome and secretome analysis of blastocysts from other infertility groups will assist in confirming the specificity for this patient population group, as well as reveal potential differences associated with other infertility factors. Gene annotation revealed numerous biological processes, potentially affected by the aetiology of PCO including protein metabolism and transcription, as well as molecular functions including nucleic acid binding and hydrolase activity. In addition, this study demonstrated a link between patient aetiology and molecular signatures of the resultant embryo that may elucidate the underlying mechanisms contributing to infertility.

Candidate key genes were chosen to independently confirm the microarray transcriptome using quantitative real-time PCR with each gene demonstrating down-regulation in PCO blastocysts. Diminished function of these candidate genes could contribute to the overall viability of the developing embryo. *LAMB1*, which encodes the beta chain isoform laminin beta 1, is a component of all basement membranes. Specifically, laminin 1 is a major laminin found in embryonic basement membranes during the preimplantation stage. Mutant *LAMB1* embryos lack basement mem-

branes and do not survive beyond embryonic day 5.5 (Miner et al., 2004). *SUGT1* encodes a protein involved in kinetochore function and is required for the G1/S and G2/M mitotic cell cycle transitions. *HIST1H1A* is a member of the histone 1 family, the basic nuclear proteins responsible for nucleosome structure. Histone 1 interacts with DNA between nucleosomes and functions in the compaction of chromatin. *KHDRBS1* encodes a protein involved in post-transcriptional mRNA metabolism and has been implicated in translational regulation of maternal mRNA (Paronetto et al., 2008).

In contrast to the microarray analysis performed on PCOS oocytes that showed 80% of the differentially expressed genes to be up-regulated (Wood et al., 2007), the present study revealed predominantly down-regulated transcripts (94.6%) and decreased protein expression (12 biomarkers) in blastocysts from PCO patients. Wood et al. (2007) postulated from their results that the maternal zygotic transition could be disrupted due to an increased concentration of oocyte mRNA. This disruption may result in inaccurate activation of the embryonic genome, resulting in a compromised blastocyst and thus decreased expression at both the gene and protein level, which is what was observed in the current study's cohort of blastocysts from PCO patients.

Specific pathway annotation of the significantly down-regulated genes revealed multiple hits for base excision repair, the cell cycle and glutathione metabolism, implicating their biological significance in the PCO blastocyst. Base excision repair is a cellular mechanism that repairs damaged DNA during the mitotic cell cycle. *PARP1* and *HMBG1* are integral components of base excision repair with both genes down-regulated in PCO blastocysts. Initial *Parp1* knockout studies showed reduced fertility (Menisser-de Murcia et al., 2001) with increased early embryonic death (Henrie et al., 2003) and problems with epigenetic remodelling of chromatin that perpetuated through subsequent cell divisions (Imamura et al., 2004). However, a more recent extensive examination of *Parp1* knockouts failed to report pup litter size differences between controls and knockouts (Yang et al., 2009). *HMBG1*, a regulator of multiple cell functions including DNA repair, typically increases in expression following blastocyst formation and has been correlated with improvements in embryo development (Shen et al., 2006). Inhibition of *HMBG1* expression results in cell death (Cui et al., 2008). Important DNA repair pathways could be compromised in PCO blastocysts, contributing to reduced developmental capacity.

YWHAB, *BUB3* and *MAD2L1*, all of which were down-regulated in PCO blastocysts, are components of the cell cycle. The *YWHAB*-encoded protein is known to mediate signal transduction by binding to phosphoserine-containing proteins, suggesting a role in linking mitogenic signalling with the cell cycle. *BUB3* is a conserved component of the mitotic spindle assembly complex. Mutant embryos fail to survive past 7 days, developing mitotic errors from days 4.5 to 6.5 post conception in the form of micronuclei, chromatin bridging, lagging chromosomes and irregular nuclear morphology (Babu et al., 2003; Kalitsis et al., 2000). Like *BUB3*, *MAD2L1* is a component of the mitotic spindle assembly checkpoint. Specifically, *MAD2L1* prevents the onset of anaphase until all the chromosomes are properly aligned at the metaphase plate. A correlation has been observed between up-regulation of *MAD2L1* and chromosomal stability (Abal et al., 2007). The present data suggest potential disruption of the mitotic cell cycle with the down-regulation of several key cell-cycle genes in PCO blastocysts.

ANPEP and *GSTA4* were identified as members of the glutathione metabolism pathway and were down-regulated in PCO blastocysts. *ANPEP* works in succession with *GSTA4* to metabolize glutathione. *GSTA4* encodes a glutathione S-transferase belonging to the alpha class and functions in the detoxification of lipid peroxidation products alleviating oxidative stress. Oxidative stress can exert deleterious effects during embryo development including early arrest prior to embryonic genome activation and the apoptotic cascade in the blastocyst (Dumollard et al., 2009).

Identification of the differentially expressed peaks/biomarkers observed in the secretome of blastocysts from PCO patients is likely to contribute to the comprehension of any underlying pathophysiology related to embryo phenotype. Preliminary identification has recognized a potential candidate for the 4.8 kDa peak/biomarker to be corticotrophin-releasing hormone (CRH). CRH is a secreted peptide that was included in the transcriptome list with several biological functions including expression in the hypothalamus in response to stress and perhaps a trigger for parturition.

In addition, CRH may play a crucial role during implantation with data indicating involvement in controlled trophoblast invasion as well as prevention of rejection by inducing apoptosis of activated maternal T lymphocytes (Kalantaridou et al., 2007).

This study demonstrates, for the first time, a link between patient aetiology and subsequent molecular signature of the resultant embryo, which may in part explain any reduction in reproductive capacity. Significantly altered transcript and protein expression was observed between morphologically similar blastocysts derived from PCO patients and donor oocyte fertile controls, potentially affecting numerous biological processes and pathways. Further studies investigating these altered genes and proteins may assist in understanding the underlying mechanisms involved in PCO. It would appear that the altered follicular environment in a PCO ovary has an effect on the subsequent developmental capacity of the resulting embryo. Ongoing focus on the molecular signature of gametes and embryos may assist in elucidating the underlying mechanisms of PCO and other indicators of human infertility.

Acknowledgements

This work was partly funded by an EMD Serono Medical Research grant. The authors would like to thank Dr Deb Minjarez for her clinical evaluation and Greg Ochs for his contribution to the writing of this manuscript.

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Declaration: The authors report no financial or commercial conflicts of interest.

Received 15 January 2010; refereed 11 May 2010; accepted 12 May 2010.