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Microarray analysis of mRNA from cumulus cells following *in vivo* or *in vitro* maturation of mouse cumulus–oocyte complexes

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The IVM of mammalian cumulus–oocyte complexes (COCs) yields reduced oocyte developmental competence compared with oocytes matured *in vivo*. Altered cumulus cell function during IVM is implicated as one cause for this difference. We have conducted a microarray analysis of cumulus cell mRNA following IVM or *in vivo* maturation (IVV). Mouse COCs were sourced from ovaries of 21-day-old CBAB6F1 mice 46 h after equine chorionic gonadotrophin (5 IU, i.p.) or from oviducts following treatment with 5 IU eCG (61 h) and 5 IU human chorionic gonadotrophin (13 h). IVM was performed in α-minimal essential medium with 50 mIU FSH for 17 h. Three independent RNA samples were assessed using the Affymetrix Gene Chip Mouse Genome 430 2.0 array. In total, 1593 genes were differentially expressed, with 811 genes upregulated and 782 genes downregulated in IVM compared with IVV cumulus cells; selected genes were validated by real-time reverse transcription–polymerase chain reaction (RT-PCR). Surprisingly, haemoglobin α (*Hba-a1*) was highly expressed in IVV relative to IVM cumulus cells, which was verified by both RT-PCR and western blot analysis. Because haemoglobin regulates O_2 and/or nitric oxide availability, we postulate that it may contribute to regulation of these gases during the ovulatory period *in vivo*. These data will provide a useful resource to determine differences in cumulus cell function that are possibly linked to oocyte competence.

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Cumulus cell microarray

In vitro maturation continues to produce oocytes of poorer competence than those mature *in vivo*, despite potential benefits for clinical infertility treatment and animal breeding. Cumulus cells are important to oocyte health, so we examined differences in the global gene expression of cumulus cells from *in vivo*- and *in vitro*-matured cumulus–oocyte complexes. Important gene expression differences were revealed that reflected differences in cumulus cell function. This included haemoglobin, which is found only within *in vivo*-matured cumulus cells.

Introduction

The ability to mature mammalian oocytes *in vitro* is both a valuable experimental model and a potentially useful clinical procedure. However, current IVM systems deliver suboptimal outcomes in terms of subsequent developmental competence (Leibfried-Rutledge *et al*. 1987; van de Leemput *et al*. 1999; Rizos *et al*. 2002), limiting the use of this tool clinically (Thompson *et al*. 2007). Data collected from mouse models within our own laboratory demonstrate that, compared with *in vivo*-matured (IVV) oocytes, IVM yields poorer embryo development, increased levels of apoptosis in resulting blastocysts and reduced implantation rates and fetal weights (Banwell *et al*. 2007; Albuz *et al*. 2010). An understanding of why oocyte competence is adversely affected by IVM and the differences in biological measurements between IVM and IVV oocytes within the follicular microenvironment should provide the basis for improved developmental outcomes following IVM.

Oocytes mature in the presence of associated cumulus cells and are coupled via the cumulus cell transzonal cytoplasmic projections that form gap junctions at the oocyte surface (Albertini *et al*. 2001). It is through these gap junctions that the cumulus cells are able to provide nutrients and factors to the oocyte. In turn, the oocyte secretes paracrine growth factors (oocyte-secreted factors) that regulate cumulus cell function (Gilchrist *et al*. 2008). This relationship is of paramount importance for oocyte competence (Barrett and Albertini 2010), with removal of cumulus cells before maturation resulting in perturbed cytoplasmic maturation and decreased developmental competence (Vanderhyden and Armstrong 1989; Zhang *et al*. 1995). In turn, the oocyte secretes factors that regulate various cumulus cell functions, including proliferation, expansion, differentiation, metabolism and gene expression (Vanderhyden *et al*. 1992; Matzuk *et al*. 2002; Sugiura *et al*. 2005; Gilchrist *et al*. 2008), which, in turn, impact oocyte developmental competence (Hussein *et al*. 2006).

Because of this close relationship, events affecting the health of the cumulus cells are likely to impact the oocyte and vice versa. Indeed, cumulus cell-specific gene expression is known to be associated with oocyte health (McKenzie *et al*. 2004; Zhang *et al*. 2005; Feuerstein *et al*. 2007; van Montfoort *et al*. 2008; Gebhardt *et al*. 2011; Wathlet *et al*. 2011). The impact of IVM on global expression patterns of cumulus cell genes compared with those associated with IVV COCs may indicate areas of deficient cumulus cell function. Significant differences have been reported in specific gene expression in mouse IVM COCs (Dunning *et al*. 2007), as well as in global gene expression profiles between cumulus cells from IVM and IVV human COCs (Jones *et al*. 2008; Wells and Patrizio 2008) and between cumulus cells from IVM and IVV bovine COCs (Tesfaye *et al*. 2009).

The aim of the present study was to compare the global gene expression profile of murine cumulus cells from COCs matured *in vitro* with that of cumulus cells from COCs matured *in vivo* to identify cumulus cell genes with important contributions to oocyte developmental competence and to further understand the impact of the maturation environment on cumulus cell function. In doing so, we have surprisingly discovered that IVV cumulus cells express mRNA and produce protein for haemoglobin. This finding supports previous suggestions that control of O_2 and nitric oxide (NO) gases is critical in the maturing oocyte environment and further suggests that haemoglobin synthesised within the COC may contribute to this control.

Materials and methods

Collection of mouse COCs

All experiments were conducted according to the National Health and Medical Research Council of Australia guidelines for the use of animals and following approval from The University of Adelaide and Institute of Medical and Veterinary Science ethics committees. For all IVM experiments, COCs were collected from female hybrid CBAB6F1 mice (21 days old) that had been injected intraperitoneally with 5 IU equine chorionic gonadotrophin (eCG; Folligon; Intervet, Boxmeer, The Netherlands) 46 h before each oocyte collection. The COCs were isolated in HEPES-buffered α-minimal essential medium (αMEM) supplemented with 50 μg mL^{-1} streptomycin sulfate, 75 μg mL^{-1} penicillin G and 5% fetal bovine serum (FBS; Invitrogen, Mulgrave, Vic., Australia) by gently puncturing visible antral follicles present on the ovary surface with a 30-gauge needle. Germinal vesicle-stage oocytes with an intact vestment of cumulus cells were collected and pooled. For IVV COCs, mice were injected intraperitoneally with 5 IU eCG and 5 IU human chorionic gonadotrophin (hCG; Pregnyl; Organon, Sydney, NSW, Australia) 61 and 13 h before recovery, respectively. The IVV COCs were collected from excised oviducts placed into HEPES-buffered αMEM, as described previously (Banwell *et al*. 2007).

In vitro *maturation of COCs*

The COCs were matured (10 per drop) in 100-μL drops of bicarbonate-buffered αMEM supplemented with 50 μg mL^{-1} streptomycin, 75 μg mL^{-1} penicillin G, 5% FBS and 50 mIU mL^{-1} recombinant human (rh) FSH (Puregon; Organon) under oil in 35-mm Falcon 1008 culture dishes (Becton Dickinson Labware, Franklin Lakes, NJ, USA). The COCs were matured for 17 h at 37°C in pre-equilibrated modular incubation chambers (Billups-Rothenburg, Del Mar, XX, USA) filled with 20% O_2, 6% CO_2 dioxide and the balance N_2, as described previously (Banwell *et al*. 2007). Culture dishes were prepared a day ahead and allowed to equilibrate in the modular incubation chambers overnight at 37°C.

Cumulus cell collection for microarray and real-time reverse transcription–polymerase chain reaction

Mature complexes were transferred into 150-μL drops of αMEM HEPES containing 25 U mL^{-1} ovine hyaluronidase (Sigma, St Louis, MO, USA) and all cumulus cells were dissociated by gentle pipetting with a narrow-bore glass pipette. Oocytes were removed and the remaining cumulus cells were collected rapidly in 20 μL medium. The numbers of COCs pooled for cumulus cell collection are described below.

For microarray analysis, COC collection was performed on nine separate occasions to generate 15 pools of cumulus cells from 40–70 IVM COCs (from groups of four or eight mice at each collection) and 15 pools of cumulus cells from 40–70 IVV COCs (from groups of two or four mice per at each collection) for RNA extraction. The COCs from individual mice were combined and randomly allocated to pools at the time of collection. For real-time reverse transcription–polymerase chain reaction (RT-PCR) analysis, a further four COC collections were performed to generate 12 pools of cumulus cells from 50–60 IVM COCs (from six mice at each collection) and nine pools of cumulus cells from 40–60 IVV COCs (from four mice at each collection) for RNA extraction.

Cumulus cell RNA extraction

Total RNA was isolated from the cumulus cell pools using the RNeasy Micro Kit (Qiagen, Doncaster, XX, USA) according to the manufacturer's instructions. DNA that may copurify was removed by the addition of DNase (0.34 Kunitz units μL^{-1}; supplied with the kit). The RNA was eluted in 14 µL RNAse-free water and stored at –80°C. The concentration of the RNA was calculated using a Nano-drop ND-100 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). RNA was extracted from 15 IVM cumulus cell pools and 15 IVV cumulus cell pools for microarray analysis and an additional 12 IVM cumulus cell pools and nine IVV cumulus cell pools for real-time RT-PCR.

Affymetrix microarray

For microarray analysis, from the 15 RNA samples per treatment, equivalent amounts of RNA from five samples were then pooled to provide sufficient RNA for one array and ensuring that each array had no duplication of samples collected on one specific day. This process was repeated three times, generating three independent samples per treatment (n = 3 IVV; n = 3 IVM). Four hundred ng RNA from each of the six samples was sent to the Australian Genome Research Facility (AGRF) in Melbourne (Vic., Australia). The quality and quantity of the RNA was assessed using an Agilent Bioanalyser (Agilent Technologies, Santa Clara, CA, USA). The six RNA samples then underwent Two-Cycle Target Labelling (Affymetrix, Santa Clara, CA, USA) with biotin, followed by hybridisation to Affymetrix GeneChip Mouse Genome 430 2.0 GeneChip arrays (GPL 1261) and scanning. Analysis of RNA integrity, hybridisation and washing were performed by the AGRF facility according to the manufacturer's instructions.

Real time RT-PCR

For real-time RT-PCR, first-strand cDNA was synthesised from total RNA using random hexamer primers (Geneworks, Hindmarsh, SA, Australia) and Superscript III reverse transcriptase (Invitrogen Australia, XXX, XX, Australia). Specific gene primers for real-time RT-PCR were designed against published sequences on the NCBI Pubmed database using Primer Express software (PE Applied Biosystems, Foster City, CA, USA) and synthesised by Geneworks. Eight genes that were either upregulated (n = 3) or downregulated (n = 5) according to microarray analysis in cumulus cells following IVM were selected. Primer pairs and sequences for *Bmp4*, *Hba-a1*, *Has2*, *Igfbp5*, *Ptx3*, *Il6*, *Adamts1*, *Amhr*, *RpL19* and *18S* rRNA are listed in Table 1. Real-time RT-PCR was performed in duplicate for each sample on an ABI GeneAmp 5700 sequence detection system (PE Applied Biosystems). In each reaction, 4 µL cDNA (equivalent to 10 ng total RNA), 0.1 µL forward and reverse primers and 10 µL SYBR Green master mix were added, with H_2O added to a final volume of 20 µL, with the exception of *18S* rRNA, in which case 1 µL cDNA (equivalent to 2.5 ng total RNA) was analysed. All primers were used at a concentration of 10 pmol μL^{-1}. The PCR cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 amplification cycles of 95°C for 15 s and 60°C for 1 min. Controls included omission of the cDNA template in otherwise complete reaction mixtures. An ovarian standard was generated by pooling cDNA generated from RNA extracted from ovaries of three random naturally cycling C57BL/6 mice. Relative mRNA expression for each gene of interest was calculated using the standard curves produced from serial dilutions of the whole ovary standard cDNA. The geometric mean of the expression of *18S* rRNA and *Rpl19* was used to normalise samples for the amount of cDNA used per reaction. Results are presented as the relative expression of each gene after normalisation against the mean of *18S* and *Rpl19* expression, as well as a fold change relative to the *in vivo* group. Analysis of the dissociation curves confirmed that a single product was amplified in all reactions. Real-time RT-PCR expression was compared between groups using the Wilcoxon–Mann–Whitney *U*-test. Differences were considered significant at $P < 0.05$.

Microarray data analysis and statistical analysis

For each chip, GCOS 1.4 software (Affymetrix) was used to generate CEL files. The MAS5.0 algorithm in GCOS 1.4 was then used to scale the CEL files globally to a target intensity of 150 for generation of CHP files. The free online program RACE (Remote Analysis Computation for Gene Expression data; http://race.unil.ch/, accessed xx XXX 200x; Psarros *et al*. 2005), which implements various bioconductor packages for quality control and other tasks, was used for data analysis. All six chips were rated as good quality. The GCRMA procedure within RACE was used for normalisation, followed by empirical Bayes statistics for determination of differential expression (Smyth 2004), comparing the IVF group minus the IVV group (IVV = baseline). This enabled fold change estimates (where fold change = 2^M) and, in combination with the variability between replicate arrays, provided confidence levels for differential expression, as well as Benjamini-Hochberg ’95 correction for false discovery rate. Probes were ranked according to B = log odds that the gene is differentially expressed.

Ingenuity pathway analysis

To investigate the biological processes correlated with altered gene expression in cumulus cells from either IVM or IVV oocytes, Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, CA, USA) was used to annotate genes from the dataset that showed M-values ≥1.0. Genes with known gene symbols and their corresponding expression values were uploaded into the software and each gene symbol was mapped to its corresponding gene object in the IPA knowledge base. Networks of genes were generated algorithmically based on their connectivity and assigned a score. The score was used to rank networks according to how relevant they are to the genes in the input dataset, but is not necessarily an indication of the most significant biological differences between the two sources of cumulus cells. Examples of identified networks of interest are presented graphically, indicating the molecular relationships between genes and/or gene products. Genes coloured green represent those upregulated in IVV cells, whereas genes coloured red are upregulated in IVM cells. Uncoloured genes were not identified as being differentially expressed.

Western analysis of haemoglobin α1 within cumulus cells

As a consequence of the results obtained, we investigated further the expression of haemoglobin α (HbA) protein in cumulus cells using western blot analysis. COCs were collected from six mice to generate 100 IVV COCs and from another six mice to generate 100 IVM COCs. Cumulus cells were isolated as described earlier, then pelleted and resuspended in phosphate-buffered saline (PBS). All cumulus cell samples were frozen rapidly in liquid nitrogen and stored at –80°C until use. Thawed cumulus cells were mixed with Laemmli loading buffer containing 100 mM dithiothreitol (DTT; Bio-Rad, Hercules, CA, USA) and proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; 4%–20% Precast Gel; Bio-Rad). Proteins were subsequently electrotransferred onto nitrocellulose membranes (Hybond-ECL; GE Healthcare Life Sciences, Uppsala, Sweden) in 25 mM Tris and 19.2 mM glycine containing 20% methanol. All gels for western blotting included prestained protein molecular weight markers (Bio-Rad) and a protein sample extracted from mouse heart by homogenisation (Precellys; Bertin Technologies, Montigny-le-Bretonneux, France) in RIPA buffer (Sigma) containing Protease Inhibitor Cocktail P8340 (Sigma). The protein concentration of the heart sample was determined using a Bradford assay (Bradford 1976) and 40 μg heart protein was loaded onto each gel. Heart protein extract is the positive control recommended by Santa Cruz Biotechnology (Santa Cruz, CA, USA) for western blot analysis of HbA, with blood suggested as the primary source of haemoglobin proteins in heart extracts (Gelman *et al*. 2010). Membranes were blocked for 2 h at room temperature with 5% (w/v) skim milk, 1× TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.05% Tween 20) and incubated overnight at 4°C with a rabbit monoclonal antibody specific for HbA1 (1 : 1000; NBP1-42065; Novus Biologicals, Littleton, XX, USA). This was followed by incubation with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1 : 5000; Santa Cruz Biotechnology) for 1 h and detection by enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Amersham, Crown Scientific, XXX, XX, Australia). The antibodies were removed with acidic glycine stripping buffer (1% SDS, 25 mM glycine, pH 2) and incubated overnight at 4°C with mouse monoclonal anti-β-actin (1 : 10 000; Sigma). This was followed by incubation with an HRP-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology) and ECL, as described above.

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Results

Global gene expression profile of in vivo- *versus* in vitro-*derived cumulus cells*

In all, 1593 transcripts with B > 1.4 and M-values ≥1.0 (of ~45 000) were categorised as differentially expressed between cumulus cells derived from IVM and IVV oocytes. Of these, 811 were upregulated and 782 were downregulated in cumulus cells derived from IVM compared with IVV COCs (see Table 2 for selected genes; a list of all 1593 transcripts is available as Supplementary Material to this paper).

Microarray data validation by quantitative real-time PCR

Quantitative real-time PCR analysis was used to validate the expression profile of eight selected transcripts from the microarray experiment. The relative abundance of the mRNA transcripts of *Hba-a1*, *Ptx3, Igfbp5* and *Amhr2* differed significantly between the two cumulus cell groups ($P \leq 0.05$; Fig. 1). The relative abundance of *Igfbp5* and *Amhr2* was significantly higher in cumulus cells from IVM COCs, whereas the relative abundance of *Hba-a1* and *Ptx3* was significantly reduced in cumulus cells from IVM compared with IVV COCs. The abundance of *Bmp4* mRNA in cumulus cells tended to be higher following IVM ($P = 0.08$). No significant differences were observed in the expression of *Has2, Il6* and *Adamts1* between the two cumulus cell sources when analysed by RT-PCR.

Functional annotation of the in vivo *versus* in vitro *cumulus cell gene list using IPA*

Analysis of the microarray data using IPA generated a series of networks and canonical pathways summarising various biological pathways identified in cumulus cells affected by IVM. The top three canonical pathways that represented the greatest degree of difference between the two sources of cumulus cells were aryl hydrocarbon receptor signalling, lipopolysaccharide (LPS) and/or interleukin (IL)-1-mediated signalling and pyruvate metabolism. Several high-level functional categories were identified by IPA analysis, with the top five (and the genes represented within each network) presented in Table 3.

A network of particular interest to us was associated with cardiovascular disease, cell cycle and lipid metabolism (Network 2 in Table 3 and Fig. 2). This network contained a significant number of genes that were downregulated (24 of 35) in cumulus cells from oocytes that were matured *in vitro*. It is of note that this network includes *Il-6* showing a high degree of downregulation in IVM-derived cumulus cells, with a significant number of interactions with other genes within the network that may indicate a significant effect on IL-6 signalling pathways under differing oocyte maturation conditions.

A second network of interest to us was Network 5 (cell death, cell assembly and function, hair and skin development and function; Table 3), which revealed that transcripts for the epidermal growth factor (EGF)-like peptides ampiregulin (*Areg*), betacellulin (*Btc*) and epiregulin (*Ereg*) are greatly reduced in IVM-derived cumulus cells compared with IVV-derived cells. These peptides are important to the propagation of the EGF receptor signalling cascade following the ovulatory LH surge and essential for cumulus expansion and ovulation.

A third network of interest (but not presented in Table 3 because it was ranked ninth) was identified to be associated with connective tissue disorders, genetic disorders, and cellular growth and proliferation (Fig. 3). This network contained several genes that were both up- and downregulated in the microarray of cumulus cells from oocytes that were matured *in vitro* (19 of 35 genes represented were downregulated following IVM). This network is of particular interest because it contains multiple members of the a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family of peptidases with lower expression following IVM, as well as several interactions with various tissue-specific inhibitors of metalloproteinases (TIMPs), which exhibit higher levels of expression under IVM conditions. Also represented in this network is *Igfbp5*, a growth factor-binding protein that is upregulated in cumulus cells following IVM.

Detection of HbA1 protein in cumulus cells

Because of the surprising nature of finding *Hba-a1* mRNA within cumulus cells, western blot analysis was performed to confirm expression of the HbA protein within IVV cumulus cells. A clear band for HbA was present at 14 kDa in cumulus cells from IVV COCs, collected after hCG injection, whereas no evidence of HbA was found within the IVM group. Reprobing the western blot with an antibody to β-actin confirmed that similar amounts of protein were present in both samples. To address possible blood contamination as a source of *Hba-a1* mRNA in our array data, we examined the GCRMA normalised log intensity values of two known erythrocyte markers, namely *Gata1 and Eraf* (Richter *et al*. 2009). For all six arrays, each of these genes had normalised values <2.5, representing negligible levels. In contrast, values from the three IVV COCs arrays for *Hba-a1* were >13, whereas those for the three IVM COCs were <1.5, clearly demonstrating the high degree of differential expression identified by the array and confirming that blood contamination did not contribute to the results observed for haemoglobin expression.

Discussion

The use of IVM both clinically and experimentally faces significant challenges because current protocols result in oocytes that are less developmentally competent than their *in vivo* counterparts (Eppig and Schroeder 1989; van de Leemput *et al*. 1999; Blondin *et al*. 2002; Combelles *et al*. 2002). Because oocytes matured *in vitro* are developmentally compromised compared with IVV oocytes, it seems feasible that the cumulus cell transcriptome would reflect this altered competence. In the present study, we investigated the changes in global gene expression in murine cumulus cells derived from IVM oocytes compared with IVV oocytes and elucidated the biological pathways most affected by *in vitro* culture systems.

Cumulus cell gene expression is of great interest in identifying markers of human oocyte quality and has been the focus of many recent studies (McKenzie *et al*. 2004; Hasegawa *et al*. 2005; Zhang *et al*. 2005; Cillo *et al*. 2007; Feuerstein *et al*. 2007; Hasegawa *et al*. 2007; Gebhardt *et al*. 2011). Similarly, microarray studies have uncovered several genes that are differentially expressed in cumulus cells of oocytes with varying developmental potential (Assou *et al*. 2008; Hamel *et al*. 2008; van Montfoort *et al*. 2008; Hamel *et al*. 2009; Wathlet *et al*. 2011). Significant differences have been reported in the global gene expression profile between cumulus cells from IVM and IVV bovine oocytes (Tesfaye *et al*. 2009), which included upregulation of key genes associated with cumulus expansion and regulation of oocyte maturation in the IVV-derived cumulus cells and upregulation of stress response genes in cumulus cells derived from IVM oocytes.

In the present study, 1593 genes were found to be significantly different between the two groups, showing that the maturation conditions had an acute effect on cumulus cell gene expression, consistent with previous observations (Dunning *et al*. 2007; Jones *et al*. 2008; Tesfaye *et al*. 2009). Several high-level functional categories of interest were identified by IPA analysis. The functions identified of particular interest between cumulus cells from *in vivo*- versus *in vitro*-derived oocytes included cell death (Hussein *et al*. 2005; Zhang *et al*. 2005), cumulus cell growth, proliferation, morphology and function (Ebner *et al*. 2000; Eppig *et al*. 2002; Diaz *et al*. 2007; Gilchrist *et al*. 2008), gene expression (Jones *et al*. 2008; Tesfaye *et al*. 2009), cell signalling, carbohydrate metabolism (Downs *et al*. 2002; Harris *et al*. 2007), amino acid metabolism (Sutton *et al*. 2003; Eppig *et al*. 2005; Harris *et al*. 2005; Curnow *et al*. 2008), lipid metabolism (Crosier *et al*. 2001; Cetica *et al*. 2002; Dunning *et al*. 2010) and embryo development (Thompson 1997).

We have observed several transcripts underexpressed in cumulus cells derived from IVM oocytes compared with those matured *in vivo*. The abundance of Pentraxin 3 (*Ptx3*), Hyaluronan synthase 2 (*Has2*), and *Adamts1* transcripts has been shown previously to be higher in cumulus cells from IVV oocytes (Dunning *et al*. 2007; Tesfaye *et al*. 2009). Pentraxin 3 is produced by cumulus cells and colocalises with hyaluronan in the expanding COC matrix during ovulation and oocyte maturation (Varani *et al*. 2002; Salustri *et al*. 2004). One role for pentraxin 3 is to stabilise and retain hyaluronan molecules in the intercellular spaces of human and mouse COCs (Salustri *et al*. 2004; Garlanda *et al*. 2005). Pentraxin 3 binds to other matrix molecules and may also interact with spermatozoa to promote efficient fertilisation (Varani *et al*. 2002; Salustri *et al*. 2004).

The expression of *Adamts1* as assessed by RT-PCR was variable, and although there was an overall trend for an approximate 80% decrease, the differences did not reach statistical significance. It has been shown that ADAMTS-1 cleaves versican (*Vcan*) in ovulating mouse COCs (Russell *et al*. 2003). In other systems, ADAMTS-1 modulates the activity of growth factors, including fibroblast growth factor, vascular endothelial growth factor and the EGF-like factors (Luque *et al*. 2003; Liu *et al*. 2006; Suga *et al*. 2006; Dunning *et al*. 2007), with the latter two identified in Networks 2 and 5, respectively and downregulated in IVM-derived cumulus. Previously, ADAMTS-1 and its major substrate versican have been shown to be more predominantly products of mural granulosa cells with the protein products translocating and binding to the hyaluronan-rich COC matrix (Russell *et al.* 2003); as such, each is markedly reduced in IVM compared with IVV COCs (Dunning *et al.* 2007). Furthermore, the mRNA abundance of both *Adamts1* (Yung *et al.* 2010) and *Vcan* (Gebhardt *et al.* 2011; Wathlet *et al.* 2011) is positively correlated with oocyte developmental competence, supporting the suggestion that each is involved in oocyte maturation.

Anti-Müllerian hormone (AMH) is a member of the transforming growth factor (TGF)-β superfamily produced by cumulus and mural granulosa cells. Growing follicles secrete AMH, which binds AMH receptor type 2 and inhibits primordial follicle recruitment and decreases sensitivity to FSH (Durlinger *et al.* 1999, 2001). The higher transcript abundance of *Amhr2* in cumulus cells from IVM mouse oocytes may infer altered hormonal modulation during IVM. Bone morphogenetic proteins (BMPs) are also members of the TGF-β superfamily of growth factors. BMP-4 is expressed strongly in the ovary, most prominently in thecal cells, whereas BMP receptor expression is highest in granulosa cells, suggesting a paracrine role for BMP-4 (Shimasaki *et al.* 1999). In rat ovary, BMP-4 was found to be highest in healthy follicles, but was barely detectable in follicles undergoing atresia (Shimasaki *et al.* 1999). It is therefore surprising that the present microarray analysis detected higher levels of BMP-4 transcript in cumulus cells derived from IVM oocytes and a trend towards increased levels on quantitative PCR analysis. IL-6 is an immunomodulatory cytokine that has been shown previously to be induced in maturing COC (Hernandez-Gonzalez *et al.* 2006; Shimada *et al.* 2007; Liu *et al.* 2009). We found reduced expression of *Il6* mRNA in IVM oocytes when assessed by microarray, but a significant reduction was not confirmed by RT-PCR. Similarly, *Il6* production was found previously to be equal in IVM and IVV COCs (Liu *et al.* 2009).

Insulin-like growth factor-binding proteins (IGFBPs) are involved in the systemic and local regulation of insulin-like growth factor (IGF) activity. In the present study, *Igfbp5* was significantly upregulated in cumulus cells from IVM oocytes. IGFBP5 has a role in the regulation of cell growth, negative regulation of cell migration, apoptosis and proliferation (Beattie *et al.* 2006). IGFBP5 is able to bind to protein and glycosaminoglycan components of extracellular matrices (Beattie *et al.* 2006) and directly to individual extracellular matrix components (Type III and IV collagen, laminin and fibronectin; Jones *et al.* 1993). The expression of *Igfbp5* is associated with follicular atresia in the rat ovary (Onoda *et al.* 1995). The expression profile of follicular fluid IGFBP has been used to better predict bovine oocyte developmental competence (Nicholas *et al.* 2005) and may play a role in follicular selection (Fortune *et al.* 2004). Little is known about the direct role of IGFBP5 in COCs; our finding suggests that it may play a role in controlling the normal IGF responses in cumulus cells during oocyte maturation.

A surprising result from the microarray were the relatively high levels of *Hba-a1* mRNA within ovulated COC cumulus compared with IVM-derived cumulus cells, which we confirmed by western blot analysis. Significantly, non-erythrocyte localisation of haemoglobin is emerging in other tissues (Dassen *et al*. 2008; Richter *et al*. 2009; Grek *et al*. 2011) and we are confident our result does not reflect blood contamination in our preparations, because other known erythrocyte markers, such as *Gata1 and Eraf*, which have been used to test erythrocyte contamination (Richter *et al*. 2009), were expressed at negligible levels and not differentially expressed within the array. Furthermore, Tesfaye *et al*. (2009) found that *Hbb* mRNA within bovine cumulus cells was 3.2-fold higher in IVV versus IVM cells, but this was not discussed in their work. Haemoglobin is a gaseous scavenger molecule, able to sequester NO, as well as O_2. NO is a well-characterised stimulator of soluble guanylate cyclase (Krumenacker *et al*. 2004) and has been implicated in the regulation of meiosis, because production or inhibition of NO activity, especially *in vitro*, has been shown to alter meiotic kinetics (Nakamura *et al*. 2002; Bu *et al*. 2003; Sela-Abramovich *et al*. 2008). High cGMP levels exist in follicles before the LH surge, implicating NO as possible mediator of cGMP levels. A major role of cGMP is to inhibit the specific oocyte phosphodiesterase PDE3 (Vaccari *et al*. 2009). This assists in maintaining high oocyte levels of cAMP, which, in turn, through the action of protein kinase A, prevents the activation of maturation-promoting factor (MPF). Therefore, regulation of NO has been suggested to have a necessary function within the coordination of ovulatory signalling events, although there is little evidence of changes to NO activity during ovulation.

A further potential role for haemoglobin is its classical role of sequestering O_2, although in this case it is within the cells of the follicle, not erythrocytes. Increasing the availability of O_2 would prevent the activation of hypoxic responses, mediated by hypoxia inducible factors (HIFs). Several reports indicate that a relatively low O_2 concentration exists within mammalian antral follicular fluid, which decreases with increasing size (Fischer *et al*. 1992; Van Blerkom *et al*. 1997). Elegant mathematical modelling suggests this must be the case and. indeed. may even be the stimulus for antral formation (Redding *et al*. 2007, 2008). However, previously we have reported that HIF activity is not associated with follicular growth at any stage of mouse ovarian folliculogenesis, but is evident immediately following the ovulatory signal, associated with granulosa cell luteinisation (Tam *et al*. 2010). This leads to an interesting conundrum: COCs within antral follicles should theoretically experience hypoxic conditions, but appear not to. Interestingly, using electron paramagnetic resonance oximetry with a unique oxygen probe injected within denuded oocytes, Higaki *et al*. (2010) recently revealed that oocytes incubated under 35 mmHg O_2 (5%) had an intracellular O_2 concentration of 50 mmHg, supporting the notion that O_2 sequestering may indeed occur within oocytes and cumulus cells.

In conclusion, the present study determined that global cumulus cell gene expression was significantly altered by oocyte maturation conditions. Our findings support the growing understanding that maturation conditions have a significant effect on oocyte–somatic cell signalling, extracellular matrix composition and oocyte developmental competence. The present findings facilitate identification of pathways associated with mouse COC maturation and oocyte developmental competence. One new finding that is of particular interest to us is the potential role of haemoglobin during COC maturation, but this requires further analysis before any role can be further investigated. A thorough understanding of the most significantly affected genes and pathways paves the way for improvements to IVM systems and the future clinical use of oocyte IVM.

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Fig. 1. Quantitative real-time reverse transcription–polymerase chain reaction results for eight transcripts (*Bmp4*, *Hbaa1*, *Has2*, *Igfbp5*, *Ptx3*, *Il-6*, *Adamts1* and *Amhr2*) in cumulus cells derived from *in vivo*- or *in vitro*-matured oocytes. For all genes, values are presented as fold induction from *in vivo*, after normalisation against the internal control genes *Rpl19* and *18S* rRNA. Data are the mean ± s.e.m. *$P \leq 0.05$ compared with *in vivo*-matured oocytes.

Fig. 2. Significant gene ontology network associated with cardiovascular disease, cell cycle and lipid metabolism. Significantly ranked functional network obtained from comparisons of differential expression in cumulus cells associated with differing maturation conditions. Genes are represented as nodes and the biological relationship between two nodes is represented as an edge (line). The red colour intensity of the node indicates the degree of upregulation, whereas the green colour intensity of the nodes indicates the degree of downregulation, in *in vitro*-matured cumulus cells. Uncoloured nodes were not identified as differentially expressed in the present study. A solid line indicates a direct interaction, a dashed line indicates an indirect

interaction, a line without an arrowhead indicates binding only, a line finishing with a vertical line indicates inhibition, a line with an arrowhead indicates 'acts on'.

Fig. 3. Significant gene ontology network associated with connective tissue disorders, genetic disorders, and cellular growth and proliferation. Significantly ranked functional network obtained from comparisons of differential expression in cumulus cells associated with differing maturation conditions. Genes are represented as nodes and the biological relationship between two nodes is represented as an edge (line). The red colour intensity of the node indicates the degree of upregulation, whereas the green colour intensity of the nodes indicates the degree of downregulation, in *in vitro*-matured cumulus cells. Uncoloured nodes were not identified as differentially expressed in the present study. A solid line indicates a direct interaction, a dashed line indicates an indirect interaction, a line without an arrowhead indicates binding only, a line finishing with a vertical line indicates inhibition, a line with an arrowhead indicates 'acts on'.

Fig. 4. Western blot analysis of cumulus cells collected from 100 *in vivo*-matured (IVV) cumulus–oocyte complexes (COCs) or 100 *in vitro*-matured (IVM) COCs probed with an antibody to (*a*) haemoglobin A1 (HbA1) or (*b*) β-actin (ACTB). Protein from mouse heart (40 μg) was included as a positive control for HbA1.

Table 1. Real time reverse transcription–polymerase chain reaction primer sequences

Gene	Amplicon size (bp)	Sequence (5′–3′)	Accession no.
Bmp4	105	Forward: GAGCCAACACTGTGAGGATTGC Reverse: GGATGCTGCTGAGGTTGAAGAG	BC013459
HBAa1	89	Forward: TGGTGCTGAATATGGAGCTGAA Reverse: GGCTTACATCAAAGTGAGGGAAGT	NM_008218
Has2	162	Forward: AAGACCCTATGGTTGGAGGTGTT Reverse: CATTCCCAGAGGACCGCTTAT	NM_008216
Igfbp5	107	Forward: GGGTTTGCCTCAACGAAAAG Reverse: GGAGTAGGTCTCTTCAGCCATCTC	NM_010518
Ptx3	109	Forward: GGACAACGAAATAGACAATGGACTT Reverse: CGAGTTCTCCAGCATGATGAAC	NM_008987
Il-6	129	Forward: AAGTCGGAGGCTTAATTACACATGT Reverse: TCTGGGAAATCGTGGAAATGAGAAAAGAGTTGT	NM_031168
Adamts1	61	Forward: TTGCAAGCCGCCTTCAC Reverse: CATCGTGCGGCATGTTAAAC	NM_009621
Amhr2	101	Forward: GAGATCCTGAGCCGCTGTTC Reverse: TCACAGGCACTGGGATTGC	NM_144547
18s rRNA	91	Forward: AGAAACGGCTACCACATCCAA Reverse: CCTGTATTGTTATTTTTCGTCACTACCT	AF176811
RpL19	103	Forward: CATGCCAAATGGACCAATGTC Reverse: TGCTCAGGTTCCATGCTCATTA	NM_014763

Table 2. List of selected genes differentially regulated, as detected by microarray analysis, in cumulus cells derived from *in vitro*- compared with *in vivo*-matured cumulus–oocyte complexes

Genes with a positive fold change (fold change = 2^{M}) were found to be higher in cumulus cells derived from *in vitro*-matured (IVM) oocytes, whereas genes with a negative fold change were higher in cumulus cells derived from *in vivo*-matured (IVV) oocytes. BMP, bone morphogenetic protein; TGF-β, transforming growth factor-β

Gene name	Accession no.	M-value	*P*-value	Gene function (biological process)
Phosphodiesterase 7B (*Pde7b*)	NM_013875	7.5	0.00016	cAMP phosphodiesterase activity
Mitogen-activated protein kinase 10 (*Mapk10*)	NM_009158	6.6	0.00015	ATP binding, kinase activity

Insulin-like growth factor binding protein 5 (*Igfbp5*)	NM_010518	6.5	0.0003	Growth factor binding
Bone morphogenetic protein 4 (*Bmp4*)	BC013459	5.7	0.0012	BMP receptor binding, growth factor activity
Growth arrest specific 6 (*Gas6*)		5.0	0.0015	Calcium ion binding, metal ion binding
Anti-Müllerian hormone type 2 receptor (*Amhr2*)	NM_144547	4.8	0.00042	Hormone binding, TGF-β activity
A disintegrin-like and metallopeptidase (*Adamts1*)	NM_009621	–4.1	0.00048	Heparin binding
LH/choriogonadotrophin receptor (*Lhcgr*)	NM_013582	–4.9	0.00064	ATPase binding, LH receptor activity
Pentraxin-related gene (*Ptx3*)	NM_008987	–5.2	0.00059	Inflammatory response
Hyaluronan synthase 2 (*Has2*)	NM_008216	–5.3	0.0008	Hyaluronan synthase activity
Interleukin-6 (*Il-6*)	NM_031168	–7.8	0.000067	Cytokine activity, growth factor activity
Betacellulin (*Btc*)	NM_007568	–7.2	0.0026	Growth factor activity
Amphiregulin (*Areg*)	NM_009704	–7.7	0.000068	Growth factor activity, cytokine activity
Epiregulin (*Ereg*)	NM_007950	–10.2	0.000021	Growth factor activity
Haemoglobin β, 2 (*Hbb,b2*)	NM_0033234	–10.9	0.000016	Gas transport
Haemoglobin α, 1 (*Hba,a1*)	NM_008218	–11.9	6.59E-07	Gas transport

Table 3. Genes comprising networks ranked number 1–5 following ingenuity pathway analysis

Each network is denoted with a functional description. Genes are either up- or downregulated from *in vitro*-matured (IVM) compared with *in vivo*-matured (IVV) cumulus cells

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Network rank	Description	IVM cumulus gene expression	Genes
1	Cancer, cell morphology, organ development	Upregulated	*Bok*, *Ca12*, *Cabc1*, *Casp7*,*Casp8*, *Ccnd2*, *Fam130a1*, *Hip1*, *Ift57*, *Ift88*, *Invs*, *Jup*, *Kcnh2*, *Mtch1*, *Nlrp10*, *Pbx3*, *Pinx1*, *Smarca2*, *Wdr6*
		Downregulated	*Axud1*, *Glul*, *Gzmb*, *Has2*, *Kctd11*, *Nr3c1*, *Runx1*, *Stk24*, *Terf1*, *Tinf2*, *Xaf1*
2	Cardiovascular system development and function, cell movement, organismal development	Upregulated	*Acvrl1*, *C5orf13*, *Capns*, *Hsd17b1*, *Htatip2*, *Itga6*, *Itga9*, *Itgb3*, *Ptges*, *Tspan4*, *Tspan8*
		Downregulated	*Cd43*, *Cd47*, *Cxcl14*, *Dmn*, *Efnb2*, *Emp2*, *Lilrb4*, *Plaur*, *Ppap2b*, *Ptger4*, *Ptgs1*, *Slc3a2*, *Slc7a8*, *Slc7a11*, *St6 gal1*, *Stc1*, *Teln1*, *Vegfa*
3	Lipid metabolism, molecular transport, small molecule biochemistry	Upregulated	*Apoc1*, *Blvra*, *Fzd2*, *Fzd6*, *Ly6e*, *Msh2*, *Nphs2*, *Smo*, *Sqrdl*, *Tmem176a*, *Tmem176b*, *Zadh1*
		Downregulated	*Alox5ap*, *Atf3*, *Cebpb*, *Fabp4*, *Fetub*, *Gab2*, *Gas 1*, *Hba2*, *Hbb*, *Hmga1*, *Ifitm3*, *Mgp*, *Pfkl*, *Pvr*, *Snw1*, *Sprr1a*, *Vldlr*
4	Cellular movement, haematological system development and function, immune response	Upregulated	*5430435 g22rik*, *Bgn*, *C1r*, *Cbr1*, *Dhrs4*, *Nutf2*, *Pycard*
		Downregulated	*Cbr3*, *Cfh*, *Cited4*, *Dox58*, *Dmbt1*, *Dusp5*, *E2f7*, *Fam46a*, *Gla*, *Irf7*, *Lgals7*, *Nfkbiz*, *Nup62*, *Nup98*, *Ptx3*, *Rsad2*, *Slc7a1*, *Tap1*, *Tap2*, *Tnfsf9*, *Traip*
5	Cell death, cell assembly and function, hair and skin development and function	Upregulated	*Arhgdig*, *Cd24*, *Dusp6*, *Gas6*, *Hspb1*, *Krt8*, *Krt18*, *Mapk10*, *Mras*, *Rassf2*, *Sorbs1*, *Stmn1*
		Downregulated	*Alcam*, *Areg*, *Arhgap26*, *Btc*, *Cblb*, *Ereg*, *Mapkapk3*, *Rasa2*, *Rassf1*, *Rgs1*, *Rgs2*, *Rgs13*, *Sos2*, *Tpd52 L1*, *Tra*, *Trib1*