Failure to launch: aberrant cumulus gene expression during oocyte *in vitro* maturation

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Abstract

In vitro maturation (IVM) offers significant benefits for human infertility treatment and animal breeding, but this potential is yet to be fully realised due to reduced oocyte developmental competence in comparison with *in vivo* matured oocytes. Cumulus cells occupy an essential position in determining oocyte developmental competence. Here we have examined the areas of deficient gene expression, as determined within microarrays primarily from cumulus cells of mouse COCs, but also other species, between *in vivo* matured and *in vitro* matured oocytes. By retrospectively analysing the literature, directed by focussing on downregulated genes, we provide an insight as to why the *in vitro* cumulus cells fail to support full oocyte potential and dissect molecular pathways that have important roles in oocyte competence. We conclude that the roles of epidermal growth factor signalling, the expanded extracellular matrix, cumulus cell metabolism and the immune system are critical deficiencies in cumulus cells of IVM COCs.

Reproduction (2017) **153** R109–R120

Introduction

In vitro maturation (IVM) of oocytes is a potentially valuable technology for both clinical infertility treatment and assisted reproductive technologies in animals (Gilchrist & Thompson 2007). IVM has been and remains a valuable research tool in understanding factors that irrefutably influence oocyte developmental competence. Indeed, the great majority of studies investigating the regulation of oocyte competence are performed *in vitro*. Through such studies, it is clear that the cumulus has substantial influence on oocyte biology, including (but certainly not exclusively): the supply of small molecular weight molecules involved in the regulation of meiotic maturation and metabolism of the oocyte; the acquisition of cytoplasmic signalling mechanisms enabling further development by the oocyte; the induction of sperm capacitation enabling penetration of the oocyte. This is clearly observed when developmental competence is compared between cumulus-enclosed and denuded oocytes during IVM (e.g. Younis & Brackett 1991, Hazeleger *et al.* 1995, Zhang *et al.* 1995, Luciano *et al.* 2005), where denudation results in a significant drop in developmental capability. Furthermore, this is not attributed to simply poor fertilisation, as parthenogenic activation rates differ in oocytes denuded of cumulus

cells prior to maturation compared with intact COCs in the presence of FSH (Chian *et al.* 1995).

Despite several decades of research, IVM-derived oocytes of most species have a perceptively poorer developmental outcome than those derived following *in vivo* maturation, even despite recent advances suggesting the gap is closing (Walls *et al.* 2012, 2015). Bi-directional communication between the cumulus vestment and oocyte is critical for oocyte competence and has been the subject of many previous reviews (Gilula *et al.* 1978, Gilchrist *et al.* 2008, Kidder & Vanderhyden 2010). However, this communication is under stress during *in vitro* maturation relative to *in vivo* maturation. We proposed that, compared to *in vivo* derived COCs, the cumulus cells of an *in vitro* matured COC would exhibit significantly different gene expression patterns which may indicate aspects of cumulus cell function that are deficient, therefore leading to reduced oocyte quality. Our analysis was primarily informed by the differential expression between *in vivo* and *in vitro* mouse cumulus cell genes published not only by Kind *et al.* (2013), but also by Tesfaye *et al.* (2009) (bovine CC) and Ouandaogo *et al.* (2012) (human CC). We acknowledge that variations in species and in methodology used for the generation

Figure 1 During IVM, the dynamic niche environment controlled by the ovarian follicle *in vivo* is lost. Global analysis of gene expression differences between *in vivo* and IVM-derived COC demonstrate deficiencies in a range of cellular pathways. Metabolites and endocrine hormones provided through the maternal system are dysregulated, and paracrine factors from follicular somatic cells (granulosa and or theca) are diminished, dysregulating the sensitive progression to oocyte competence.

of both *in vivo* and *in vitro* matured COCs may cause differences in the cumulus cell transcriptome, and that differences in microarray platforms will contribute a significant source of variation in results. Our intent is not to discuss closely the results of these microarrays, but use them as a guide to direct us to the major networks in cumulus cells leading to reduced developmental competence of oocytes *in vitro* (Fig. 1). In doing so, we have focussed on major distinct physiological processes with evident dysregulation because of IVM, and expand to what is known about their role in oocyte maturation.

Epidermal growth factor receptor signalling

Of the seven recognised cognate epidermal growth factor-like ligands (EGF-L), three are known to be expressed in granulosa cells and COCs; amphiregulin (AREG), epiregulin (EREG) and betacellulin (BTC), and all were significantly reduced in IVM COCs compared to *in vivo* (Kind *et al.* 2013). Deficient expression of EGF-L factors in IVM has been confirmed in a number of subsequent studies (Richani *et al.* 2013, 2014b). Additionally, Versican is another factor with demonstrated EGF-like activity in COC (Dunning *et al.* 2015), which was also reduced in IVM compared to *in vivo* COCs as has been previously demonstrated (Dunning *et al.* 2007). The EGF signalling pathway is fundamental for oocyte maturation and this has been established over several decades (Downs *et al.* 1988). More recently, the specific importance of AREG, EREG and BTC was demonstrated (Park *et al.* 2004). The EGF-L factors and Versican are rapidly (within 1 h) induced in granulosa cells responding to the LH-surge and transmit the ovulatory signal to cumulus cells which are not directly LH responsive (Eppig *et al.* 1997). This important intermediary paracrine role played by granulosa cell derived EGF-L factors in activation of oocyte maturation has been confirmed using whole follicle culture (Ashkenazi *et al.* 2005) and genetic dissection (Hsieh *et al.* 2011). Likewise, a role for granulosa-derived Versican in expansion of the COC

matrix has been demonstrated (Dunning *et al.* 2012, 2015). However it is also clear that once maturation of the COC is activated by granulosa derived factors, these same factors are subsequently induced in cumulus cells and hence likely to maintain an autocrine activation of the EGF signalling pathway throughout oocyte maturation, ovulation and fertilisation (Shimada *et al.* 2006b). Expression of these factors in cumulus cells has been shown to associate with oocyte developmental potential (Gebhardt *et al.* 2011, Huang *et al.* 2015), and their deficient expression in IVM systems is therefore likely to be a key contributor to lower quality outcomes. Recently it has come to light that the EGF receptor activation in cumulus cells stimulates PI3K/AKT signalling in the oocyte which controls protein translation and developmental competence (Chen *et al.* 2013, Franciosi *et al.* 2016). The increase in protein translation promotes release of factors such as IL7 from oocytes which act back on cumulus cells (Cakmak *et al.* 2016). Exactly how the cumulus cell response is transferred to the oocyte remains unknown, but the importance of EGF-L induction in granulosa cells and hence the importance of replicating this pathway *in vitro* is clear.

These findings have initiated a trend towards including EGF or EGF-L factors in stimulated IVM systems. A number of studies have undertaken comparison of IVM outcomes using treatment with FSH vs EGF or each of the EGF-L factors as COC maturation stimuli. While more evaluation of dose responses are required to properly compare the activity of each factor in IVM COCs, the consensus findings indicated that when compared at 50 ng/mL, recombinant EREG had the greatest beneficial effects on mouse COC glycolysis (Sugimura *et al.* 2014), oocyte mitochondrial activity (Richani *et al.* 2014a) and embryo developmental potential (Richani *et al.* 2014b), although why EREG was better at promoting these processes is undetermined. Other studies have suggested AREG may play the most important role in oocyte maturation. A non-redundant

contribution of AREG to mouse oocyte maturation *in vivo* was illustrated by null mutation of AREG causing delayed meiotic progression (Hsieh *et al.* 2007) and a significant 40–50% reduction in the proportion of oocytes that could achieve fertilisation (Chen *et al.* 2013). Likewise in human and rhesus monkeys AREG protein was found to be more abundant (median 30 ng/mL, 36 h after hCG) than other EGF-L factors in the follicular fluid of mature follicles (Zamah *et al.* 2010, Peluffo *et al.* 2012). The concentration of AREG in follicular fluid was correlated with oocyte developmental potential (Zamah *et al.* 2010), however, potential juxtacrine signalling within COC by AREG or the other EGF-L is not considered in this approach investigating the free ligand in antral fluid which may indeed be less biologically relevant. A dose–response analysis in porcine COCs showed that AREG at 100 ng/mL significantly increased oocyte maturation to MI (Sugimura *et al.* 2015). Another recent study found AREG mRNA induction in human granulosa cells or in COC correlated with ICSI success parameters including numbers of oocytes retrieved, meiotic progression, fertilisation and embryo quality (Huang *et al.* 2015). These studies together suggest that AREG may be most important and should be tested in primate IVM.

The normal *in vivo* induction of EGF-L factors involves sequential increases in the three factors with overlapping but different kinetics. This may promote a specific temporal pattern of signalling in the COC which is difficult to replicate in IVM and the physiological importance of this sequence for full oocyte developmental potential is unknown. Supplementation of IVM cultures with EGF promotes EGF-L mRNA expression to levels quantitatively similar to *in vivo* COCs, yet levels of EGF receptor phosphorylation at Tyrosine Tyr¹⁰⁶⁸ remain generally lower than *in vivo* and differ in temporal kinetics (Richani *et al.* 2013). Variable responses to the different EGF-L ligands are mediated in part through interaction with distinct receptors or receptor complexes. All seven cognate EGF related factors can bind and activate EGF-receptor (EGFR or ERBB1), but some also bind other members of the ERBB receptor family, such as BTC which can activate all the ERBB members (Dahlhoff *et al.* 2014). Likewise Neuregulin 1 (NRG1) is an EGF-like factor induced in granulosa cells during oocyte maturation *in vivo* (Noma *et al.* 2011), but which binds only ERBB2 and ERBB3, not ERBB1. The rapid induction of *Nrg1* in granulosa cells may initially suppress oocyte maturation and has thus been proposed to be an important granulosa-derived factor helping to synchronise maturational events *in vivo* (Kawashima *et al.* 2014). Because *Nrg1* is not a cumulus gene product it is not found among differentially expressed genes in IVM COC; however, it is deficient due to the absence of granulosa cells and may explain the abnormally rapid meiotic maturation of IVM oocytes (Kawashima *et al.* 2014).

In addition to the different ligand-receptor affinities, variable phosphorylation of intracellular Tyr residues on the EGF receptors is activated by the different ligands (Wilson *et al.* 2012), with important distinctions in internalisation and recycling of the receptors and affecting the signalling duration. Amphiregulin promotes receptor recycling to epithelial cell membranes, while EGF and BTC traffic the receptor to lysosomes for degradation (Roepstorff *et al.* 2009), which will truncate signal duration and blunt the cell's capacity to respond to other EGF-L whether exogenously added or endogenously produced. This outcome is consistent with what has been described for EGFR Tyr¹⁰⁶⁸ phosphorylation in IVM COC, which show a sharp rapid induction, but premature downregulation (Richani *et al.* 2013). Finally, some of the EGF-L ligand's signalling is modulated by heparan sulphate proteoglycans on the cell surface or the local ECM environment. Amphiregulin action is dependent on heparan sulphate (Mahtouk *et al.* 2006), and the expression of many HSPG is altered under IVM conditions (Watson *et al.* 2012). Together all these differences weave a tapestry of complex signal transduction consequences contingent on the mix and timing of ligands and receptors present. The specific combination and timing of EGFR signalling in COC maturation is clearly important, but has yet to be investigated in detail. Likewise, the involvement of HSPG in cumulus cell signalling is just emerging (Watson *et al.* 2012). The collective deficiency in expression of AREG, EREG and BTC and other signalling molecules is clearly suboptimal and likely to result in lower autocrine activation of the EGF pathway in IVM COCs.

An alternative IVM technology that uses a pre-maturation treatment to elevate cAMP before stimulating COC with FSH and/or EGF, improved oocyte competence in cows (Albuz *et al.* 2010) and mice (Zeng *et al.* 2014) and enhances EGF-L peptide expression (Richani *et al.* 2013, 2014b). However, ERK1/2 phosphorylation, which is a downstream consequence of EGF-L activity, was not different (Richani *et al.* 2013, 2014b). The results are suggestive that increased EGF-L production caused by elevated cAMP activated signalling in the pre-maturation phase was responsible for higher oocyte developmental competence (Richani *et al.* 2014b).

Versican is profoundly deficient in IVM COC in mouse (Dunning *et al.* 2007) and its expression in human cumulus cells has been repeatedly found to be associated with successful ART outcomes (Gebhardt *et al.* 2011, Wathlet *et al.* 2011, Ekart *et al.* 2013, Hammond *et al.* 2015). The capacity of Versican to activate EGFR in cumulus cells and promote COC maturation was also recently discovered (Dunning *et al.* 2015). While the ability of Versican to bind and activate EGFR is known (Du *et al.* 2010), its specific downstream signal transduction mechanism is unknown. Importantly, the temporal kinetics of gene

induction mediated by Versican in COC was more similar to the *in vivo* pattern than that achieved by EGF treatment (Dunning *et al.* 2015). Of particular interest, induction of *Ereg* and *Ptgs2* by Versican was more sustained than with EGF (Dunning *et al.* 2015) mimicking more closely the expression profile observed *in vivo* (Shimada *et al.* 2006b). Based on the sustained signalling capacity of Versican, we believe it to be an important component of the periovulatory signalling cascade and that its absence during IVM impacts on oocyte quality. It will be of interest to determine whether Versican has similar signalling capacity in human cumulus cells and whether this is a key missing aspect of EGF pathway activation important for high oocyte developmental potential after IVM.

Together these findings on the role of EGF-L factors in activation of COC maturation and the evident importance of local EGF-L production in COC for acquisition of full oocyte developmental potential suggest that the lower EGF-L production in IVM is expected to be one aspect that has a negative influence on developmental potential. The most plausible explanation at present is that a vicious cycle arises whereby key mural granulosa cell derived contributors to COC maturation are absent *in vitro* resulting in deficient expression of critical factors (e.g. EGF-L, Versican, or HSPG), which further diminish the activation of key signalling pathways in the IVM COC.

Extracellular matrix

The *in vivo* action of the ovulatory LH-surge also stimulates production of an extensive extracellular matrix in the COC. Again this requires granulosa cells which produce a variety of factors that impinge on cumulus cells resulting in the induction of ECM gene expression while some granulosa cell secreted proteins (e.g. versican and *Adams1*) are directly incorporated into the COC matrix (Russell & Salustri 2006, Russell & Robker 2007). The resultant phenomenon, known as cumulus expansion, occurs concomitantly with oocyte meiotic maturation and is required for ovulation (Russell & Robker 2007). Cumulus expansion has been described in nearly all mammalian species investigated to date, yet historically its role was thought to have been restricted to its functionality during oocyte pick-up by the infundibulum (Odor & Blandau 1973, Lam *et al.* 2000) and during fertilisation in the oviduct (Salustri *et al.* 2004). In recent years studies have shown that formation of a compositionally complete and functional cumulus matrix is associated with oocyte quality (McKenzie *et al.* 2004, Cillo *et al.* 2007, Yung *et al.* 2010, Gebhardt *et al.* 2011, Wathlet *et al.* 2011, Ekart *et al.* 2013), ovulation rate (Varani *et al.* 2002, Fulop *et al.* 2003, Ochsner *et al.* 2003, Mittaz *et al.* 2004, Brown *et al.* 2006), cumulus cell signalling (Dunning *et al.* 2015), adhesive and invasive capacity of the COC (Akison *et al.* 2012) and

molecular filtration (Dunning *et al.* 2012). Importantly, the cumulus matrix formed during *in vitro* maturation is compositionally and functionally altered and may account for the poorer quality of oocytes produced using this technology (Dunning *et al.* 2007, 2012, Kind *et al.* 2013).

In vivo, the EGF-L peptides secreted by granulosa cells, as described above, act on cumulus cells to induce genes such as prostaglandin endoperoxide synthase 2 (*Ptgs2*) involved in cumulus matrix gene expression and hyaluronan synthase (*Has2*), tumour necrosis alpha induced protein 6 (*Tnfaip6*), pentraxin 3 (*Ptx3*). These matrix components interact, along with versican from the granulosa cells and inter-alpha trypsin inhibitor heavy chain from circulation, to assemble the COC matrix (Russell & Salustri 2006). Another granulosa product, *Adams1* is progesterone-dependently induced in granulosa cells following the LH-surge and found in abundance in the expanded COC (Russell *et al.* 2003a,b). *In vitro* it is clear that expression of cumulus matrix genes is truncated or temporally altered (Jones *et al.* 2008, Wells & Patrizio 2008, Tesfaye *et al.* 2009, Kind *et al.* 2013, Dunning *et al.* 2015) and this is associated with the poor oocyte developmental competence. Cumulus expansion also requires calpain-mediated migration of cumulus cells along the newly synthesised cumulus matrix (Kawashima *et al.* 2012). Whether cumulus cell motility during IVM is altered and functionally impacts oocyte quality is yet to be determined.

We have shown that following IVM the expanded mouse COC is completely void of the cumulus matrix proteins ADAMTS1 and Versican despite their abundance in the *in vivo* matured COC (Dunning *et al.* 2007). The absence of these proteins and the reduced abundance of other cumulus matrix components results in a compositionally deficient and functionally altered matrix. We have shown that the *in vivo* matured matrix is able to restrict diffusion of metabolites to the oocyte such as glucose and sequester locally produced signalling molecules including prostaglandin E2 (Dunning *et al.* 2012). This molecular filtration property of the cumulus matrix is however deficient following IVM (Dunning *et al.* 2012). The ability of the cumulus matrix to regulate metabolite supply while retaining cumulus cell synthesised signalling factors is likely to affect oocyte quality. It is logical that the loss of the ECM capacity to retain and concentrate locally produced factors contributes to poor IVM quality since the gene products involved in prostaglandin synthesis, oocyte secreted growth factors and glucose metabolism are associated with oocyte quality, and supplementation of IVM media with the products of these pathways improves oocyte quality (Hussein *et al.* 2005). Thus, the reduced oocyte quality following IVM may be due to dysregulated control of oocyte exposure to metabolites and signalling molecules. Whether similar deficiencies in cumulus matrix filtration occurs following IVM of human COCs

has not been determined, nor whether repletion of the mouse cumulus matrix through exogenous addition of proteins such as Versican restores this function.

Extracellular matrix proteins can also function as signalling molecules. As described above, recombinant Versican induces cumulus-specific gene expression during IVM (Dunning *et al.* 2015). Other ECM proteins such as heparan sulphate proteoglycans can also either directly transduce an intracellular signal (e.g. Syndecans) or modulate signalling by EGF, FGF or TGF β family ligands.

That the cumulus matrix is important for oocyte quality is clearly supported by several studies associating human cumulus ECM gene expression with oocyte developmental potential. The expression of Versican (VCAN) has been positively correlated with oocyte developmental potential in four independent studies including a positive association with live birth (Gebhardt *et al.* 2011, Wathlet *et al.* 2011, Ekart *et al.* 2013, Hammond *et al.* 2015). Similar positive associations have been shown for *Ptgs2*, *Adamts1* and *Has2* (McKenzie *et al.* 2004, Cillo *et al.* 2007, Yung *et al.* 2010, Xu *et al.* 2015). These retrospective results have yet to be confirmed using randomised controlled trials; however, the independent findings at different sites of consistent associations between cumulus matrix gene expression and oocyte developmental outcomes provide strong evidence that formation of an appropriate cumulus matrix is essential for human oocyte quality. Whether human cumulus expression of matrix genes is similarly reduced during IVM as occurring in the rodent is yet to be determined and should be investigated.

Metabolism

The importance of the cumulus cell supply of nutrients and substrates to the oocyte to achieve developmental competence has been increasingly acknowledged over the last two decades (Dumesic *et al.* 2015). Metabolism of cumulus cells is an important contributor to the bi-directional communication between the oocyte and cumulus vestment (Eppig 1991, Albertini *et al.* 2001, Matzuk *et al.* 2002). The environment to which a COC is exposed to during maturation, both *in vivo* and *in vitro*, largely impacts its metabolism and developmental competence (Krisher 2013, Dumesic *et al.* 2015). For example, maternal hyperglycaemia and hyperlipidaemia compromise COC health, embryo development and pregnancy outcomes (Chang *et al.* 2005, Leroy *et al.* 2008, Robker 2008, Purcell & Moley 2011, Van Hoesck *et al.* 2011). It is well established that the oocyte predominantly utilises oxidative phosphorylation, whereas the cumulus vestment has a high rate of glycolytic activity (Thompson *et al.* 2007). The primary substrate of cumulus cells is glucose and is metabolised via numerous pathways to provide energy, substrates for ECM, nucleic acid synthesis and stress/fuel sensing

(reviewed by Sutton-McDowall *et al.* 2010). Therefore the discovery that several genes in the glucose metabolic pathway have reduced expression during IVM suggests glycolytic deficiency is likely to negatively impact on oocyte developmental potential. These included *Slc2a1* (facilitated glucose transporter 1), lactate dehydrogenase (*Ldhd*), enolase (*Eno2*) and hexokinase (*Hk2*) expression and indicate a reduced glucose uptake and metabolism (Kind *et al.* 2013). Cumulus cell glucose metabolism is regulated by oocyte-secreted factors, which influence the expression of such genes (Sugiura & Eppig 2005, Sugiura *et al.* 2005, 2007), and the array results are in line with a reduction in OSF expression during IVM (Mester *et al.* 2015). Furthermore, glucose (and glutamine)-dependent hyaluronic acid matrix production is most likely inhibited by reductions in glutamine-fructose-6-phosphate transaminase (*Gfpt1*) and hyaluronic acid synthase 2 (*Has2*) expression within *in vitro* matured cumulus cells (Caixeta *et al.* 2013). This is despite many IVM systems utilising follicle stimulating hormone, a potent stimulator of cumulus glucose metabolism and nuclear maturation within COCs (Downs *et al.* 1996, Sutton-McDowall *et al.* 2004).

Fatty acid metabolism via beta-oxidation is a potent source of cellular energy. The importance of beta-oxidation during COC maturation is becoming increasingly recognised. *In vivo*, the LH-surge results in a significant increase in beta-oxidation-related genes in COCs and functional assays demonstrate a significant increase in beta-oxidation during *in vitro* maturation (Dunning *et al.* 2010). Further, pharmacological inhibition of beta-oxidation impairs oocyte developmental competence, while promotion with L-carnitine during IVM improves subsequent embryo development in several species (Dunning *et al.* 2010, 2014, Dunning & Robker 2012). Thus it appears that supply of a fatty acid milieu and necessary co-factors such as L-carnitine that replicates the *in vivo* environment is likely to result in improved oocyte maturation conditions *in vitro*.

An emerging interest is the negative impact on oocyte competence of 2-carbon glucose metabolic products, such as glyoxal and methylglyoxal (reactive carbonyl species, RCS) (Tatone *et al.* 2010, 2011). RCS are derived from glycolysis and the polyol pathway, plus other sources, and are damaging in that they promote lipid peroxidation (Tatone *et al.* 2010, 2011). Reduction in 2-carbon derivative detoxification enzymes, such as carbonyl reductase (NADPH) 3 (*Cbr3*) within *in vitro* matured cumulus cells is evidence that 2-carbon toxicity may occur (Tatone *et al.* 2010, 2011), and this area requires more attention.

We have recently reported that haemoglobin A1 and B expression (*Hba-a1* and *Hbb*, respectively) greatly differed in mouse cumulus cells derived from *in vivo* or *in vitro* maturation, where they were at nearly undetectable levels following *in vitro* maturation (Kind *et al.* 2013). Indeed, *Hba-a1* was the most

differentially expressed gene from the microarray analysis. This was validated by a substantial loss of HBA protein following *in vitro* maturation (Kind *et al.* 2013, Brown *et al.* 2015). Significantly, this difference in gene expression is supported for both *HBA1* and *HBB* within *in vivo* vs *in vitro* matured human cumulus cells (Ouandaogo *et al.* 2012) (Supplementary Table within publication) and for *HBB* in bovine cumulus cells (Tesfaye *et al.* 2009). Our analysis of the temporal gene expression pattern following the ovulatory luteinising hormone (LH) surge demonstrated these two genes were hormonally regulated, with the peak expression level occurring 12-h post hCG. Haptoglobin, required for iron-recycling from haem degradation, also followed this pattern of expression (Brown *et al.* 2015). Other gene products involved in haem synthesis were also detected, but not hormonally regulated.

The function of haemoglobin within the follicular cells of antral follicles has yet to be determined, but as its presence is hormonally regulated and extends to the oocyte itself (Brown *et al.* 2015), we have proposed it plays a role during the ovulatory period (Thompson *et al.* 2015), perhaps involving O₂ and/or NO binding and sequestering. A further potential role is as an antioxidant (Thompson *et al.* 2015). Work is continuing to investigate these options by developing purposefully designed molecular tools to measure haemoglobin-gas interactions.

In addition to haemoglobins and haptoglobin, there are several well-characterised O₂-sensitive genes that are upregulated within mouse *in vivo* matured cumulus cells relative to *in vitro* matured cumulus cells. These include: endothelin 2 (*Edn2*); enolase 2 (*Eno2*); BCL2/adenovirus E1B interacting protein 1, NIP3 (*Bnip3*); solute carrier family 2 member 1 (*Slc2a1*); lactate dehydrogenase D (*Ldhd*); hexokinase 2 (*Hk2*); N-myc downstream regulated gene 1 (*Ndrg1*), NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4-like 2 (*Ndufa4l2*) and vascular endothelial growth factor A (*Vegfa*) (Kind *et al.* 2013). Such a pattern of expression points to involvement of the hypoxia inducible factor (HIF) family of transcription factors in regulating cumulus cell function, especially in low levels of dissolved O₂. HIF transcription factors are now understood to at least partly mediate gonadotrophin signalling in the ovarian follicle, especially granulosa cells signalling following the luteinising hormone surge (reviewed by Thompson *et al.* 2015). We have experimentally demonstrated that maturation of COCs *in vitro* under low O₂ concentrations (2% and 5%) involves HIF-induced transcription activity within cumulus cells. Similar upregulation of O₂-sensitive genes in bovine cumulus cells is observed following maturation under 5% O₂ relative to 20% O₂ (Bermejo-Alvarez *et al.* 2010). Does this suggest that *in vitro* maturation should be performed at low pO₂ (specifically 5%)? Several publications support the concept that IVM under low pO₂ conditions improves oocyte maturation, including

human IVM (Hashimoto *et al.* 2000a, Iwamoto *et al.* 2005, Preis *et al.* 2007, Walls *et al.* 2015). Nevertheless, long-term follow-up studies are virtually absent, with the study of Banwell *et al.* (2007) being the exception. This study reported improved embryo development from low O₂ IVM, but post-transfer outcomes were impacted dependent on O₂ concentration with, surprisingly, 5% O₂ IVM derived fetuses with poorer developmental indices relative to 2% or 20%. A feature of this latter study is that several O₂ concentrations for IVM were assessed. In contrast, virtually all other studies investigate 5% vs 'air' O₂ concentrations. The historical origin for 5% O₂ gas applied to gamete and embryo culture derives from work performed several decades ago showing it was optimal for mouse embryo culture (Quinn & Harlow 1978). Whether it has relevance or not to IVM in any species, in particular human IVM, has not been properly assessed. Furthermore, if haemoglobin does have a role to sequester gases, does this impact the optimal IVM gas composition when haemoglobin is present during IVM conditions, as suggested by Brown *et al.* (2015)? An often touted reason for the use of 5% O₂ atmosphere during IVM is the reduction in reactive oxygen species (ROS) production, therefore safeguarding the integrity of chromatin conformation and prevention of lipid and protein oxidation within the maturing oocyte from ROS-induced damage (Hashimoto *et al.* 2000a,b, Salavati *et al.* 2012). Therefore it would appear that a lower than atmospheric O₂ concentration is preferable, but which concentration is optimal will depend on numerous factors, such as species, composition of medium and the incubation environment as a whole.

Reduced glutathione (GSH) is an essential peptide determining oocyte competence (Sutovsky & Schatten 1997, Furnus *et al.* 1998, de Matos & Furnus 2000, Bing *et al.* 2002). Additionally glutathione peroxidase-1 (GPX1) expression in cumulus cells was also recently shown to be associated with human oocyte competence (Ceko *et al.* 2015). Metabolism in the cumulus cells is directly linked to GSH levels in the oocyte (Xie *et al.* 2016). Increasing intra-oocyte GSH levels by the addition of component amino acids, especially cysteine, into *in vitro* maturation medium formulations is a common strategy to improve embryo production (de Matos & Furnus 2000). Several studies demonstrate cumulus cells are a source of reduced GSH for the oocyte, especially while gap junctions between oocyte and cumulus cells are patent (Curnow *et al.* 2008, 2010, Furnus *et al.* 2008). Glutathione-S-transferase (*Gsta*) is a key enzyme in the process of reducing oxidised glutathione, thus regenerating GSH levels. It is therefore pertinent that microarrays (e.g. Kind *et al.* 2013, Salhab *et al.* 2013) revealed a reduced level of *Gsta* transcription within the cumulus of *in vitro* matured COCs.

With the exception of cholesterol metabolism and steroid synthesis, little is known of lipid synthesis and metabolism within cumulus cells. Much of the focus has

been directed towards the oocyte itself (Thompson *et al.* 2014). Recent insights reveal that compared to lipid within the oocyte, cumulus cell lipid composition is most vulnerable to exogenous factors such as oxidants, suggesting the cumulus lipid content is 'protecting' oocyte cytoplasmic and membrane lipids (Aardema *et al.* 2013, Lolicato *et al.* 2015). Microarray data from human and mouse comparing cumulus cell transcriptome between *in vivo* and *in vitro* maturation highlight differences in some lipid synthesis and metabolism genes (such as *Acs11*, *Acs14*, *Alox5ap*, *Lpgat1*, and *Fabp4as*) (Ouandaogo *et al.* 2012, Kind *et al.* 2013). However, studies on how this impacts lipid composition have not followed-up and is a deficiency in our understanding.

The immune system

Hypotheses surrounding the similarities between the ovulatory cascade and an immune-regulated event have been proposed over several decades (Espey 1980). While latter work suggested an inflammatory-like state was initiated following the LH-surge (Richards *et al.* 2002), more recent insights also implicated the involvement an innate, immune-like process (Richards *et al.* 2008). Research has proposed that an endocrine-immune-cytokine network exists within the pre-ovulatory follicle and involving not only inflammatory molecules but also innate immune molecules within the pattern-recognition receptor family.

While several gene expression studies explore the presence and abundance of a number of 'immune-cell pathway genes', little functional work has been conducted. Microarray data on cumulus cells during *in vivo* maturation and expansion of the cumulus oocyte complex identified a number of genes as upregulated over this period (*Adam8*, *Cd28*, *Pdcd1*, *Cd34*, *Cd52*, *Cd81*, *Cd97*, *Emr1*, *Cd97a*, *Cd147*, *Cd166* (*Alcam*), *Cxcr4*, *Il6*, *Nurp1*, *Ptx3*, *Runx1*, *Saa3*) (Hernandez-Gonzalez *et al.* 2006), with most never described within the ovary. It is significant that our comparison of COCs derived *in vivo* and *in vitro* identified a number of 'immune-cell pathway genes' that were dramatically lower in the *in vitro* derived COCs (Kind *et al.* 2013).

Perhaps the most widely explored immune modulator in the COC is Interleukin-6 (IL6). IL6 is significantly upregulated during the ovulatory cascade *in vivo* (Hernandez-Gonzalez *et al.* 2006), and known to be regulated during the follicular phase and present in high levels in the periovulatory follicular fluid in humans (Baskind *et al.* 2014). Liu *et al.* (2009) explored a functional role for IL6 in the mouse COC during IVM. They reported a short-term induction of *Il6* mRNA in culture that was upregulated by AREG, FSH and PGE2 (4h hCG, Liu *et al.* 2009), although longer-term regulation *in vitro* was not explored. The induction was blocked both by indomethacin and RU486, suggesting IL6 is a downstream target of PGE2, and that its

expression is modulated by progesterone receptor (Pgr). With a number of these key components dysregulated during IVM (*Ptgs1*, *Ptges*, *Ptger4*, *Star*, *Cyp11a1*) (Kind *et al.* 2013), it is perhaps no surprise that there are alterations in IL6 signalling as a consequence of *in vitro* maturation. Interestingly, addition of IL6 to IVM was able to promote cumulus expansion in mouse COCs, albeit at concentrations much higher than detectable in follicular fluid; 100–1000 ng/mL (Liu *et al.* 2009) vs less than 1–100 pg/mL detected in human follicular fluid) (Buscher *et al.* 1999, Altun *et al.* 2011, Baskind *et al.* 2014).

A limited amount of data in human suggest that the COC may contain CD4+ T cells, which produce both IL4 and LIF (Piccinni *et al.* 2001), while a number of chemo-attractants for immune cells including monocytes, dendritic cells and natural killer cells have also been found (Cxcl14, Kind *et al.* 2013). In addition to this, a number of genes best described as immune cell markers have been found in the COC *in vivo*, and dysregulated *in vitro*. *Cd83*, a marker of fully matured dendritic cells was decreased following *in vitro* maturation, while *Cd14*, a co-receptor for toll-like receptors (TLR), which recognise pathogen-associated molecular patterns (PAMPs), as well as endogenous damage associated molecular patterns (DAMPs) was also decreased *in vitro*, but present and regulated by LH (hCG) *in vivo* (Shimada *et al.* 2006a). Indeed fragmented hyaluronan and Versican (Kim *et al.* 2009, Hu *et al.* 2015) are strong DAMP signals. We propose that the production of inflammatory cytokines and/or immune like behaviour of cumulus cells could represent a final checkpoint prior to ovulation, or at fertilisation, when tissue damage releases DAMP signals that impinge on TLRs and thus are absent when oocytes undergo IVM. As such, IVM followed by intra-cytoplasmic sperm injection (ICSI), where the COC is not exposed to sperm or seminal plasma, may represent a further insult. Future experiments comparing *in vivo* fertilisation, IVF and ICSI may provide valuable insight into the role of immune factors during oocyte maturation and at fertilisation.

Conclusion

IVM remains at the fringe of acceptance both clinically and for animal breeding, as it remains less efficient relative to other embryo production strategies. It occupies several niche applications, but is far from widely accepted. Our analysis of cumulus cell transcriptome reveals many different molecular attributes that separate the functionality of cumulus cells from *in vivo* and *in vitro* matured COCs, providing a view of the molecular mechanisms behind the wide discrepancy in capacity to generate developmentally competent and mature oocytes. Consistent evidence showing deficient EGF-L signalling, ECM composition and function, metabolism and immune gene expression in IVM as

well as demonstrating the importance of these pathways in oocyte maturation lead us to conclude that attention given to understanding these molecular mechanisms and to restoring them in IVM will lead to improved IVM efficacy. It is likely an unattainable goal to achieve parity in the transcriptome of these two sources of oocytes, although management of cAMP levels, use of EGF-L peptides, inclusion of specific growth factors, such as cytokines, oocyte secreted factors, and other growth factors (including Versican), providing the correct oxygenation and substrate composition in the medium, will contribute to normalising cumulus cell function and oocyte developmental competence.

Declaration of interest

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

Funding

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

Acknowledgements

J G T, H M B and M S-M are supported by the Australian Research Council Centre of Excellence for Nanoscale Biophotonics. D L R, J G T and R B G are recipients of National Health and Medical Research Council Fellowships (IDs 1110562, 1077694, 1023210).

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Received 4 August 2016

First decision 12 October 2016

Revised manuscript received 8 November 2016

Accepted 21 November 2016