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Bidirectional communication between cumulus cells and the oocyte: old hands and new players?

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Abstract

Cumulus cell – oocyte communication is an essential feature of mammalian reproduction. Established mechanisms involve the bidirectional transfer of ions and small molecules through gap-junctions that fundamentally regulate the process of oocyte maturation. Also well-established is the paracrine signalling from the oocyte to the cumulus, which regulates much of the flow of ions and molecules to the oocyte and orchestrates many of the associated local signalling events around ovulation, which is the key to establishing oocyte competence to sustain early embryo development. Less well characterised and new potential players include exosomal transfer of non-coding RNAs from cumulus to oocytes and the recent observations of the presence of haemoglobin in oocytes and cumulus cells. The impact of these new communication pathways is either poorly defined or even unknown. Finally, signalling between the two cell types most likely continues after ovulation and even fertilisation, however, this too is largely undefined, but may play roles in substrate transport, sperm chemotaxis and "trapping" and potentially signalling to the rest of the reproductive tract.

Introduction

The importance of the communication between the oocyte and its surrounding nurse-cells, the cumulus cells, is profound to the nuclear regulation of meiosis and the subsequent developmental capacity of the oocyte. This communication is bi-directional and involves regulation of nutrient transfer and signalling between the two very different cell types. Once thought of as a passive recipient of cumulus cell activity, the oocyte is now appreciated as the driver of this relationship [1, 2]. Furthermore, most of these functions are dynamic, as they alter both during follicular growth and following the ovulatory signal. Much progress has occurred in our understanding of this bi-directional communication and impact on oocyte maturation over the last two decades (for recent reviews, see also [3-6]), yet in doing so, this has revealed both common and species-specific mechanisms [7]. Better characterised players include meiotic and gap-junction regulation by cyclic nucleotides and the role of oocyte-secreted factors in regulating cumulus cell function. Here we discuss these well characterised mechanisms described in the literature, plus emerging additional players in this communication axis. These emerging players demonstrate the breadth and complexity of this communication, some having undefined roles that require further investigation to their significance.

Gap junctional communication

Gap junctions are multi-domain, trans-membrane protein structures, comprising of six connexin (Cx) proteins to form a connexon, which may (but not always) be tethered to zona occludin proteins (the membrane proteins forming tight-junctions). The connexin family of proteins has as many as 21 members, but the two relevant to gap junctional communication between the oocyte and cumulus cells are Cx43 and Cx37 [8]. At least in the mouse, the structure of gap junctions between the oocyte and cumulus exclusively involves a hexamer of Cx37 on the coronal process membrane and the oolemma, whereas between cumulus cells themselves, the structure is exclusively made of Cx43 [8]. This appears not to hold for other species, as in the pig, Cx43 is the most prominent, and Cx37 has yet to be isolated [9]. However, the composition of connexons forming gap junctions of other tissues can vary in both the composition on the opposing membranes and within the connexon itself.

Gap junctions are normally associated with the transfer of hydrophilic molecules, with a molecular weight less than 1 kDa [8]. Elegant experiments using radioisotopes with mouse COCs demonstrated an array of molecules were able to traverse gap-junctions from cumulus cells to the oocyte, especially nucleotides, amino acids and simple carbohydrates [10, 11], in addition to ions and other small molecular weight molecules. Of particular significance to embryo development following maturation and fertilization is the transfer of cumulus-sourced, reduced glutathione to the pig oocyte by gap-junctions [12, 13]. In contrast, most proteins, large nucleic acids and complex carbohydrates are thought not to be transferred, although this is now being questioned (see below).

Gap junction communication between cumulus cells and the oocyte occurs through the aptly named trans-zonal processes (TZP), which are specialised extensions of corona radiata cells [14]. These extend through the zona and form the junction with the oolemma [15]. Cumulus-oocyte complex (COC) communication through these junctions has been well described. Evidence of the bi-directional transfer of small molecular weight molecules through junctions has been demonstrated by 1) transfer of labelled molecules and/or fluorescence probes from the cumulus-cell to the oocyte (e.g. radioisotopes and calcein, [10, 11, 16]; 2) transfer of molecules/and or probes blocked by gap-junction inhibitors (e.g. carbenoxolone [17, 18]; and 3) injected probes into the oocyte passing to the coronal cells of the cumulus oophorus (e.g. FITC-dextran, lucifer yellow [18-20].

Gap-junctions and cumulus-oocyte signalling

The major regulatory signalling role of gap junctions is the passage of cyclic nucleotides into the oocyte from the cumulus cells which play a critical role in the regulation of meiosis [17], and therefore are impacted by the events surrounding ovulation (reviewed by [21]). Cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) play critical inter-related roles in firstly preventing spontaneous meiotic activation from the germinal vesicle stage (prophase 1) prior to the ovulatory signal and then enabling re-induction of meiosis following the ovulatory signal. It has been long recognised that oocyte intracellular levels of

cAMP regulates re-entry into meiosis, whereby cAMP levels promotes protein kinase A (PKA)-dependent phosphorylation of cyclin-dependent kinase 1 (CDK1), thereby inhibiting activity of Meiosis Promoting Factor (MPF) (Figure 1). Removal of the COC from the follicle initiates spontaneous meiotic resumption, in response to rapidly falling cAMP levels in the cumulus and oocyte [22], as a consequence of rapid degradation by phosphodiesterase (PDE) activity (in the mouse oocyte, specifically PDE3). The source of intra-oocyte cAMP is from a constitutive G-coupled receptor with adenylate cyclase activity and cumulus cell-derived cAMP also plays a role via transfer through gap-junctions, especially following gonadotrophin stimulation [23]. The reciprocity of this is the loss of gap junctional communication in response to falling cAMP levels [22, 24]. More complex is the ovulation signal-induced reduction in intra-oocyte cAMP. Primarily modelled from studies in the mouse, cAMP levels are maintained by the inhibition of the intra-oocyte PDE activity by cGMP [25] which in turn, is generated from a cumulus cell-specific guanylate cyclase (Natriuretic Peptide Receptor 2, NPR2) activity [26]. Regulation of NPR2 guanylate cyclase activity is via natriuretic peptide signalling [26], most likely under the influence of oestradiol via FSH [27]. Closure of gap junctions during in vivo maturation is also associated with activity of Epidermal Growth Factor Receptor (EGFR) kinase activity [28], induced by the production of epidermal growth factor-like peptides, ampiregulin, epiregulin and betacellulin from the mural granulosa cells by LH-induction of ovulation [29].

The discrepancy in oocyte developmental competence between ovulated and *in vitro* matured COCs has led to attempts to recapitulate some of the events that are hallmarks of COC communication signalling during ovulation. In particular, temporal cAMP manipulation by the use of analogues or modifiers of PDEs and/or adenylate cyclase activity in mouse and cattle COCs [30-32], which are known to delay or inhibit germinal vesicle break down (GVBD), have yielded improvements in developmental competence. Such strategies in conjunction with the stimulation of EGFR signalling and application of oocyte-secreted factors (see below) have impacts on both prolonging gap-junction communication and oocyte developmental competence.

Contribution of Oocyte Secreted Factors (OSF) to the COC and oocyte developmental competence

Oocytes fundamentally depend on cumulus cells to perform many of the functions oocytes require to support preimplantation embryo development. Cumulus cells are differentiated granulosa cells and the oocyte must actively prevent the default pathway of granulosa cell differentiation which is towards the mural granulosa cell phenotype [33]. Oocytes dictate cumulus cell differentiation and function for their own purposes via the local secretion of potent growth factors, commonly referred to as oocyte-secreted factors (OSFs). These consist of, as a minimum, growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15), and likely others including BMP6 and some of the fibroblast growth factors. Some of the important processes that OSFs appear to regulate include the regulation of granulosa/cumulus proliferation and the rate of follicle growth [34, 35], promotion of cumulus cell glycolysis required for oocyte metabolism [36], acquisition of cumulus cells EGF family signalling capability required for the COC to recognise to ovulatory cascade [37, 38], and the control of cumulus cell mucification and expansion needed for ovulation [39, 40].

GDF9 and BMP15 are members of the TGFβ superfamily of growth factors, although they have a number of unusual features that makes them notable from the rest of the family. Firstly, in females their expression is largely restricted to the oocyte where they are co-expressed throughout most of folliculogenesis. Secondly, there are major between-species differences in the expression and activity of the proteins and hence in their respective roles in differing species. For example, human GDF9 is produced in a latent form [41], which may also be the case in most other mono-ovular mammals, where balanced expression of BMP15 and GDF9 may be associated with activation of GDF9 [42, 43], whereas a predominance of active GDF9 leads to a poly-ovular phenotype [41, 44]. Thirdly, the proteins lack the fourth cysteine residue that is usual in the TGFβ superfamily, such that GDF9 and BMP15 do not form dimers stabilized by a disulphide bond. This is consistent with the notion that GDF9 and BMP15 exhibit remarkable interactions with each other, as evidenced by genetic [44, 45], biochemical [46, 47] and functional studies [47-49]. Such important interactions between GDF9 and BMP15 are likely to be mediated by the formation and function of

cumulin, a heterodimer of the two growth factors [43]. Unlike either homodimer, the heterodimer cumulin is a potent activator of both intracellular SMAD pathways (SMAD2/3 and SMAD1/5/8), probably accounting for its potent bioactivity. Nevertheless, despite its production under laboratory conditions, the identification of naturally-occurring cumulin has yet to be established in any species.

Consistent with their key role in controlling cumulus cell functions, OSFs have important roles in regulating oocyte quality, or the oocyte's capacity to support embryo development [3]. This concept has principally been demonstrated using oocyte *in vitro* maturation (IVM) experiments, where OSF expression appears to be perturbed [50]. Hence, exogenous supplementation of IVM with OSFs notably improves oocyte quality as assessed by post-fertilization embryo development potential [51]. This can be readily achieved using "native" OSFs secreted by denuded oocytes, as now demonstrated in a broad range of species [52-57]. OSFs can have profound effects on the developmental program of the oocyte, notably improving subsequent advanced stages of mouse fetal development [56]. Recombinant GDF9 and BMP15 are also effective at enhancing oocyte quality when used as IVM additives, but curiously only when used in their pro-forms [51, 57-60], as mature domain GDF9 and BMP15 are ineffective [56]. Consistent with this, pro-cumulin but not mature cumulin, potently enhances mouse and pig oocyte developmental competence [43]. Hence there are major practical opportunities to improve the efficiency of assisted reproductive technologies in domestic species and in humans through the application of GDF9, BMP15 and cumulin.

Emerging candidates for cumulus-oocyte communication

Haemoglobin?

Recently we have reported that haemoglobin protein (at least HBAA1) is found in mouse cumulus cells and oocytes and human cumulus cells contain considerable amounts of mRNA for *HBA1* and *HBB* [61, 62]. Both were identified in follicular cells recovered from large, peri-ovulatory follicles. The function of haemoglobin in the COC has yet to be identified, but we have evidence from mouse experimental data that mRNA levels are hormonally regulated during the ovulatory

period [61]. There are several potential functions and the most likely involve O_2 and/or NO gas binding, as both these are known to rapidly alter over this period [63-65]. Our evidence that cumulus-derived haemoglobin is transferred to the oocyte from cumulus cells stems from observations that following *in vitro* maturation (IVM), oocytes lack haemoglobin protein. However, co-incubation of two different forms of haemoglobin (oxidised or reduced) during IVM increases intra-oocyte haemoglobin levels, similar to those seen within *in vivo* derived oocytes [61]. The mechanism by which this occurs has yet to be established.

Exosomal communication?

A potentially new communication pathway is exosome transmission from the cumulus to the oocyte. It is now well established that exosomes are produced by the somatic follicular cells and can be found in abundance in follicular fluid of several species including bovine [66], equine [67] and human [68]. Whether these particles in solution can traverse the zona pellucida to influence the oocyte is unknown. However, the immediate surrounding coronal cell processes that traverse the zona pellucida and the perivitteline space, suggest direct transmission of proteins, RNA species and larger molecular weight molecules is a possibility. It has also been proposed that RNA transcripts may be directly trafficked to the oocyte via the trans-zonal projections of corona radiata cells. Two studies to date, both conducted in cattle COCs, have presented some evidence for this by either PCR amplification of a long non-coding RNA in isolated zona pelucidae [69] or detecting an exogenous synthetic transcript transfected into cumulus cells, transferred into the oocyte [70]. This evidence remains equivocal, and if or how RNA passes from cumulus cell to oocyte (exosomal, through gap junctions or other) is not yet known, however the prospect of active and specific transfer of RNA species from cumulus to oocyte is enticing.

Non-coding RNAs within the follicular fluid and cellular compartments?

Non-coding RNAs are a rapidly growing family of transcripts with the unifying characteristic that they do not encode an open reading frame that can be translated into a protein. MicroRNAs (miRNA) are small RNAs of around 22 nucleotides in length which can regulate gene functions by either modulating mRNA transcript

stability or the translation of specific transcripts through interaction based on sequence homology. MicroRNAs can be found in follicular fluid [67] as well as in circulation [71] and are frequently a part of the cargo carried by exosomes or other cell secreted particles. These exosomes can be taken up by and influence the function of granulosa [66] and cumulus cells [72]. A number of findings have suggested that miRNA from somatic cells participate in intrafollicular signalling and influence oocyte developmental potential. It was shown that bovine cumulus cells and oocytes reciprocally affect the abundance of miRNA in cellular compartment [73], and oocyte developmental stage also influences the microsomal population in follicular fluid [66]. The gene expression program required for cumulus expansion, oocyte maturation and ovulation can be triggered in cumulus cells in culture by treatment with purified exosomes [72]. Perhaps importantly, the corona radiata cells immediately in contact with the human oocyte have been shown to express a different miRNA population than the more distant cumulus cells [74] and gene ontology analysis linked these differentially expressed miRNA to glycolysis and amino acid metabolism processes, suggesting the miRNA regulate the nutritional exchange between oocytes and corona radiate cells. Age-associated changes in human miRNA profile have also been suggested to influence cumulus gene expression required for oocyte quality [75]. Several additional mechanisms for regulation of oocyte maturation by miRNA have been proposed, including miR-378 suppression of aromatase activity in porcine cumulus cells. Polycystic ovary syndrome in humans has been shown in several studies to be associated with altered miRNA profile potentially altering NOTCH [76], or Wnt- and MAPK- [77] pathway signalling in cumulus cells.

Long non-coding RNAs (lncRNA)?

Long non-coding RNAs (lncRNA) are defined as non-coding transcripts of ~200bp or longer. This classification are among the most rapidly growing family of transcripts and the most diverse actions including regulation of gene expression through interactions with enhancer or promoter sequences, antisense transcripts which block mRNA translation, or through interacting with specific proteins. While only recently identified as associated with developmental potential in the human COC [78], the number of lncRNA shown to be important in this process is rapidly growing. Three

lncRNA were associated with bovine embryo quality and also identified in the cumulus cell transzonal projections [69]. Another lncRNA *AK124742* is an antisense sequence to the *PSMD6* (26S proteasome non-ATPase regulatory subunit 6) gene and both have been reported to be correlated with embryo quality [79]. In each case it remains to be determined how these RNA transcripts in cumulus cells influence oocyte developmental potential.

Structure, scaffolding and selection: safe passage at ovulation and beyond

Our understanding of cumulus-oocyte communication is focussed on the period of follicular oocyte growth and peri-ovulatory meiotic events. Nevertheless, oocytes of most mammalian species undergo fertilisation in the presence of cumulus cells, or at least the corona radiata [80]. Is it possible that there are signalling events occurring between cumulus and oocyte during this time?

A growing body of evidence supports an important role for cumulus cells well after oocyte maturation is complete, to ensure the oocyte is successfully expelled from the ovary at ovulation and that fertilisation is achieved. In models known to alter mouse cumulus expansion (*Ptx3*, *Adamts1*), ovulation and fertilisation are lowered, or completely ablated [81, 82]. Furthermore, it has been proposed that these cumulus cells and their matrix act as a scaffold to protect the oocyte as it is propelled into the oviduct [83]. In addition to this role, a cumulus matrix lacking critical components including the proteoglycan, versican, completely alters the passage of small molecules including glucose and lipids, dramatically altering the environment that the oocyte is maturing in [84, 85]. While it remains unclear whether there is direct disruption of genuine signalling mechanisms between the cumulus cells and the oocyte in this situation, it is clear that external signals arriving at the oocyte are altered. Studies from the diabetes field support this possibility, with a single day exposure to a diabetic mouse reproductive tract, when the cumulus cells are still largely present, is enough to program a plethora of negative effects in the developing embryo [86]. Whether the role of the cumulus cells in mediating, or preventing this effect is active or passive remains unknown. New evidence has emerged that even oocyte protein translation following the ovulatory signal is, for at least some maternally-derived mRNAs, enhanced by the presence of cumulus cells [87].

The cumulus cells and their matrix are also important in facilitating the capture by the fallopian tube [88, 89], and for mediating the interaction with sperm at fertilisation. Cumulus cells have been reported to provide chemoattractants in the oviduct to attract sperm [90], and also a gradient of chemotaxis within the cumulus complex in human (reviewed in [91]). It has been observed that following fertilisation of human oocytes, the intimate contact between the cumulus cells and the oocyte is lost, as a result of the withdrawal of cytoplasmic processes [4], while anecdotally, we have observed that cumulus cell removal from the zona of bovine *in vitro* fertilised oocytes is easier if the oocyte is fertilised than when not, supporting the possibility of communication post-fertilisation. With the cumulus cells reported to be lost early after fertilisation in many species, including bovine [92], it remains unclear whether this in an *in vivo* phenomenon, or an artefact of the *in vitro* system. Cumulus cells have also been proposed to play roles in sperm trapping and selection in many species, including hamster, rat and cow, although many of these experiments are performed entirely in vitro (reviewed in [93]).

Fertilisation is associated with an increased oxidative redox state within the oocyte [94, 95]. We have tantalising evidence that this extends to the cumulus cells as well, at least during *in vitro* fertilisation in cattle. Further effort is required to validate these observations. Furthermore, assessment if such changes occur *in situ* is paramount. In addition to the well-established intra-oocyte calcium oscillations at fertilisation [96], zinc ions have recently been described to efflux dramatically following sperm penetration [97]. These zinc ‘sparks’, conserved in rodents and primates at least, decrease intracellular zinc, a process necessary for meiotic cell-cycle progression. Like much of the data obtained in the field of ionic events during fertilization, these experiments were performed on oocytes stripped of their cumulus cells, thereby preventing assessment of cumulus cell behaviour under such non-physiological conditions.

Concluding remarks

The communication between cumulus cells and oocytes is fundamental to fertility. However, the relationship between these two highly specialised cell types has

provided challenges in elucidating the nature of this communication. Isolation of either cell type has repeatedly been shown to alter the function of the isolated cells, rendering a poor insight into the legacy of the bi-directional communication. Increasingly sophisticated tools that can distinguish the degree of heterogeneity between individual cumulus cells when associated with an oocyte will do much to determine the unique nature of this cellular association. There is no doubt more to determine about this essential function for mammalian reproduction.

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Figure 1: Schematic representation of the bi-directional communication between the cumulus cells and the oocyte.

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669 Figure 1.

670 Diagrammatic representation of the major signalling pathways involved with meiotic
671 maintenance by cyclic nucleotides. Abbreviations: AC = adenylate cyclase; AMP =
672 adenosine monophosphate; ATP = adenosine triphosphate; cAMP = cyclic AMP;
673 CNP = C-type naturetic peptide; cGMP = cyclic GMP; GC = guanylate cyclase; GMP
674 = guanosine monophosphate; Gs = G-protein; GPR3 = G-protein coupled receptor
675 type 3; GTP = guanosine triphosphate; GV = germinal vesicle; PDE =
676 phosphodiesterase; PKA = protein kinase A.

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