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Oocyte maturation and quality: role of cyclic nucleotides

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Running Head: Regulation of oocyte quality by cAMP/cGMP

Abstract

The cyclic nucleotides, cAMP and cGMP, are the key molecules controlling mammalian oocyte meiosis. Their roles in oocyte biology have been at the forefront of oocyte research for decades and many of the long standing controversies in relation to the regulation of oocyte meiotic maturation are now resolved. It is now clear that the follicle prevents meiotic resumption through the actions of natriuretic peptides and cGMP inhibiting the hydrolysis of intra-oocyte cAMP and that the preovulatory gonadotrophin surge reverses these processes. The gonadotrophin surge also leads to a transient spike in cAMP in the somatic compartment of the follicle; research over the past 2 decades has conclusively demonstrated that this surge in cAMP is important for the subsequent developmental capacity of the oocyte. This is important, as oocyte *in vitro* maturation (IVM) systems practiced clinically do not recapitulate this cAMP surge *in vitro*, possibly accounting for the lower efficiency of IVM compared to clinical IVF. This review focuses in particular on this latter aspect – the role of cAMP/cGMP in the regulation of oocyte quality. We conclude that clinical practice of IVM should reflect this new understanding of the role of cyclic nucleotides, thereby creating a new generation of ART and fertility treatment options.

Introduction

Oocyte maturation and oocyte quality are fundamental to fertility in all mammalian species. This is particularly evident in modern human infertility treatment where oocyte quantity and quality are rate-limiting to the success of nearly all artificial reproductive technologies (ART). We now know a

great deal about the regulation of oocyte maturation. It has long been recognised that one of the most important classes of molecules regulating mammalian oocyte maturation are the cyclic nucleotides; namely, cyclic adenosine 3',5'-monophosphate (cAMP) and cyclic guanosine 3',5'-monophosphate (cGMP). These, in particular cAMP, have been the subject of intensive oocyte research for the past 40 years.

The earliest report on the role of cAMP used a permeable cAMP, dibutyryl-cAMP (dbcAMP), demonstrating oocytes can be maintained in meiotic arrest *in vitro* after removal from their follicular environment (Cho *et al.* 1974). For the ensuing 20 years, cAMP and cGMP and their roles in the regulation of oocyte maturation were and remain intensively researched worldwide. This research focused largely on the regulation of meiosis; the role of the nucleotides in the maintenance of meiotic arrest and the resumption of meiosis, principally using rodents models (reviews from that period; (Dekel 1988, Eppig 1989)). This research area was complicated by the cAMP paradox, whereby high levels of oocyte cAMP maintain oocyte meiotic arrest, but at the time of ovulation, high follicular levels of cAMP induce meiotic resumption (Dekel *et al.* 1988). Understanding the mechanisms regulating oocyte meiotic resumption were further complicated by the controversy over the participation of falling cAMP levels and the simultaneous loss of cumulus-oocyte gap junctional communication (GJC). Many of the controversies of that period are now settled following landmark discoveries, including; the role of phosphodiesterases (PDEs) (Masciarelli *et al.* 2004, Tsafriri *et al.* 1996), the sources and roles of cAMP (Mehlmann *et al.* 2002) and cGMP in the oocyte (Norris *et al.* 2009, Vaccari *et al.* 2009) and the participation of natriuretic peptides (Zhang *et al.* 2010).

Hence today we have a thorough understanding of the participation of cAMP and cGMP in the regulation of mammalian oocyte meiosis. However, it is striking that the large numbers of studies from the 1970s-1990s, principally in mice, were largely limited to investigating oocyte meiosis and

did not follow the oocyte's subsequent capacity to support embryo development, or oocyte developmental competence. Curiously, even today, this central function of oocytes is not typically studied by mouse oocyte developmental biologists, but rather is the subject of major research efforts conducted by domestic animal oocyte biologists. It was first discovered in bovine and porcine oocytes that the spike of follicular cAMP that occurs at ovulation is important for the oocyte's subsequent capacity to support embryo development (Aktas *et al.* 1995, Funahashi *et al.* 1997). This important discovery has led to a whole new area of research over the past 2 decades, which has shifted the focus of cyclic nucleotides away from oocyte meiosis towards oocyte developmental competence or oocyte quality, and how this is applied to improve outcomes in an ART context.

1) Cyclic nucleotides and oocyte maturation *in vivo*

1.1 Cyclic nucleotides maintain meiotic arrest

1.1.1 The follicle maintains meiotic arrest

A basic tenant of oocyte maturation is that oocytes in mid-sized antral and preovulatory follicles are competent to undergo oocyte meiotic maturation but are arrested at the germinal vesicle (GV) stage of meiosis by the follicle environment. Hence, oocytes removed from the follicle and placed *in vitro* will undergo hormone-independent, spontaneous meiotic maturation (Edwards 1965, Pincus and Enzmann 1935). It is the cyclic nucleotides, cAMP and cGMP, of follicular somatic and germ cell origin, that are the principal molecules responsible for maintaining oocyte meiotic arrest (see below).

1.1.2 The follicle endows the oocyte with developmental competence

During folliculogenesis, oocytes undergo changes at both the nuclear and cytoplasmic level that confer the oocyte with developmental competence, defined as the capacity to support

preimplantation embryo development (Gilchrist and Thompson 2007). Among other processes, changes in large-scale oocyte chromatin structure are essential for the onset of developmental competence (reviewed in (Luciano and Lodde 2013)). The proper maintenance of cumulus cell (CC)-oocyte gap-junctional communication (GJC) appears to have a crucial role in chromatin remodeling and the gradual transcription silencing processes that occur in fully grown oocytes, from early antral through to the latter half of antral folliculogenesis (De La Fuente and Eppig 2001, Lodde *et al.* 2008). CC-oocyte GJCs in turn are regulated by cyclic nucleotides, as FSH or a range of cAMP-modulating pharmaceuticals, sustain functional CC-oocyte communication (Atef *et al.* 2005, El-Hayek and Clarke 2015, Luciano *et al.* 2004, Thomas *et al.* 2004a). In addition, treatment with FSH *in vivo* leads to oocyte chromatin condensation and suppress of transcription (De La Fuente and Eppig 2001, Zuccotti *et al.* 1998). The use of bovine cumulus-oocyte complex (COC) culture systems that prolong GJC, sustain oocyte growth and allow early chromatin compaction events are associated with the oocyte acquiring the ability to mature and be fertilized *in vitro* (Luciano *et al.* 2011). However, when GJ functionality is experimentally interrupted, chromatin rapidly condenses and RNA synthesis abruptly ceases. Interestingly, this effect is nullified by preventing cAMP hydrolysis specifically within the oocyte (Luciano *et al.* 2011). Hence, since the preservation of an appropriate cAMP content in the oocyte, even in the absence of functional GJC, is able to prevent the abrupt condensation of chromatin, this suggests the cAMP cascade is the likely regulator of GJ-mediated actions on chromatin remodelling. These findings suggest that cAMP could be involved in the control of the activity of factors that modulate oocyte transcription and large-scale chromatin remodelling in fully grown oocytes during their final phase of development, immediately before the resumption of meiosis.

1.1.3 cAMP control of meiotic arrest

It has long been known that moderate to high intra-oocyte levels of the second messenger cAMP maintain oocyte meiotic arrest (Cho *et al.* 1974). Cyclic AMP is synthesized from ATP by an active

adenylyl cyclase (AC). In rodent oocytes, AC3 has been reported to be present and functional (Horner *et al.* 2003). GPR3 is a functional receptor found in the oocyte and hence the oocyte can independently synthesize cAMP (Mehlmann *et al.* 2002, Olsiewski and Beers 1983). However, a major source of intra-oocyte cAMP are the somatic cells surrounding the oocyte by virtue of the electrophysiological syncytium between the oocyte, cumulus and granulosa cells. Activation of CC ACs by FSH or forskolin loads the oocyte with many fold-increases in cAMP concentrations (Thomas *et al.* 2002). Sustained levels of intra-oocyte cAMP activates protein kinase A (PKA), which in turn prevents the activation of maturation promoting factor, retaining the oocyte in M-phase. Cyclic nucleotide participation in the control of the meiotic cell cycle is reviewed elsewhere (Conti *et al.* 2012, Downs 2010).

1.1.4 cGMP and phosphodiesterases

The oocyte possesses a potent PDE that must be kept in check to maintain meiotic arrest. The study of oocyte PDEs began several decades ago, where it was found that non-specific PDE inhibitors, such as theophylline (Cho *et al.* 1974) and 3-isobutyl-1-methylxanthine (IBMX) (Dekel and Beers 1978, Magnusson and Hillensjo 1977) maintain meiotic arrest of oocytes *in vitro*. Two studies published in the 1990's reported the presence of a specific family of PDEs within the rodent oocyte, namely PDE3A; identified using in situ hybridization (Reinhardt *et al.* 1995) and sub-type specific PDE inhibitors such as milrinone and cilostamide (Tsafriri *et al.* 1996). Several years later, activity of the oocyte PDE3 was shown to increase prior to both spontaneous and gonadotrophin-induced meiotic resumption (Richard *et al.* 2001). The effect of specific PDE3 inhibitors on maintaining oocyte meiotic arrest *in vitro* has now been demonstrated across many mammalian species; rat (Tsafriri *et al.* 1996), mice (Wiersma *et al.* 1998), cattle (Mayes and Sirard 2002, Thomas *et al.* 2002), monkeys (Jensen *et al.* 2002), human (Nogueira *et al.* 2003a) and swine (Laforest *et al.* 2005). Demonstration of sterility of female mice bearing a PDE3A-null mutation due to the

ovulation of GV-stage oocytes was the final proof of the central role of PDE3A in maintaining oocyte meiotic arrest (Masciarelli *et al.* 2004).

PDEs are organized into eleven families with the differing PDE isoenzymes capable of hydrolysing cAMP or cGMP or both nucleotides. An important finding was that PDE3A, which is the prominent PDE in the oocyte, is a cGMP-inhibited cAMP-hydrolysing enzyme (Maurice and Haslam 1990). It was long known that cGMP is an oocyte maturation inhibitor (Hubbard and Terranova 1982) and that ovarian levels of cGMP decline after LH stimulation (Ratner 1976). Three decades later the significance of this became apparent when two key papers revealed that it is cGMP permeating from the granulosa/CC compartment into the oocyte via gap-junctions that inhibits the oocyte's PDE3A (Norris *et al.* 2009, Vaccari *et al.* 2009). Hence, cGMP from the follicular somatic cells maintains sufficient intra-oocyte cAMP to maintain the oocyte in meiotic arrest (Figure 1).

1.1.5 Contribution of natriuretic peptides to meiotic arrest

The signalling model for the maintenance of meiotic arrest has recently been enhanced by the finding of the important contribution of natriuretic peptides. The natriuretic peptide family is composed of three major types: atrial natriuretic peptide, brain natriuretic peptide and C-type natriuretic peptide (CNP). In an important study, it was shown that granulosa cells secrete CNP and CCs express its receptor, NPR2, which is a member of the guanylyl cyclase receptor family. NPR2 stimulation by CNP increased cGMP intracellular concentrations in both CCs and the oocyte and maintained meiotic arrest (Zhang *et al.* 2010). CNP has since been identified as an oocyte meiotic inhibiting peptide in a range of species in addition to mouse (Zhang *et al.* 2010); swine (Santiquet *et al.* 2014), cattle (Franciosi *et al.* 2014) and rats (Zhang *et al.* 2015). This knowledge now provides us with a logical model whereby the follicular compartment maintains oocyte meiotic arrest in vivo by supplying CNP-induced cGMP from the granulosa/CCs, via gap-junctions, to the oocyte to inhibit PDE3A, thereby maintaining sufficient cAMP to inhibit GV breakdown (GVBD; Figure 1).

1.2 The ovulatory cascade:

1.2.1 LH-induced changes in cyclic nucleotides

The preovulatory surge of LH induces oocyte maturation, but neither oocytes nor CCs express LH receptors, so how does the LH surge lead to oocyte maturation? The exact cellular mechanisms and sequences of events that transduce the LH signal from the mural granulosa cells to the oocyte has been the topic of intense debate for decades. It is not the intention of this review to delve into these debates; fortunately, this is reviewed comprehensively elsewhere (Downs 2010).

LH induces an acute transient spike in cAMP in the somatic compartment of the follicle. This is of the order of an 80- to 200-fold increase in cAMP, depending on the compartment measured (Albuz *et al.* 2010, Mattioli *et al.* 1994, Tsafriri *et al.* 1972, Yoshimura *et al.* 1992). The spike in cAMP appears in general to occur prior to GVBD, with levels falling sharply at around the time of GVBD. There is contradictory evidence as to whether the LH-induced spike in cAMP is transmitted into the oocyte (Norris *et al.* 2009, Yoshimura *et al.* 1992). This preovulatory spike in cAMP has meiotic inducing consequences, as cAMP pulsing of explanted follicles or isolated COCs in vitro induces the resumption of meiosis in the presence of inhibitory factors (Dekel *et al.* 1981, Downs *et al.* 1988, Tsafriri *et al.* 1972). Hence, the acute changes in cAMP concentrations that follow the gonadotropin surge play a significant role in oocyte function, and this is an important point to note for cAMP-mediated oocyte in vitro maturation (IVM) systems (see Section 2 below).

Despite this ovulatory pulse in follicular cAMP levels, activation of the oocyte PDE3A and a consequent fall in intra-oocyte cAMP is clearly a pre-requisite for de-phosphorylation of PKA, activation of maturation-promoting factor (MPF) and meiotic resumption (Figure 2). How is the rapid loss of oocyte cAMP achieved? LH administration leads to a fall in follicular cGMP

(Hubbard 1986) and a loss of gap-junctions (Sela-Abramovich *et al.* 2005). The involvement of cGMP in the process of meiotic resumption was recently strengthened by the work of Shuhaibar *et al*. (Shuhaibar *et al.* 2015). Using follicles from mice expressing a FRET sensor, real-time monitoring of cGMP showed that within one minute of LH exposure, cGMP concentrations start to decrease from the peripheral granulosa cells and by 20 minutes the concentration of cGMP has decreased by more than 20-fold and was uniformly low across the follicle (Shuhaibar *et al.* 2015). Consequently, it is likely that oocyte cAMP concentration decreases because of relief of the inhibitory actions of cGMP on PDE3A in the oocyte (Norris *et al.* 2009, Vaccari *et al.* 2009).

CNP activation of the NPR2 guanylyl cyclase is a principal source of cGMP in the follicle (Zhang *et al.* 2010), and LH down-regulates CNP transcript expression in mouse granulosa cells and CNP protein in follicular fluid (Kawamura *et al.* 2011). In human, an ovulatory dose of hCG results in a decrease in CNP levels in follicular fluid (Kawamura *et al.* 2011). LH induces a decrease in NPR2 guanylyl cyclase activity within 20 minutes (Robinson *et al.* 2012) that can be explained by dephosphorylation and inactivation of NPR2 in granulosa cells (Egbert *et al.* 2014). Hence, a current model of oocyte meiotic resumption is that LH induces a spike in follicular cAMP and a simultaneous decline in CNP and cGMP, leading to activation of oocyte PDE3A causing a decline in intra-oocyte cAMP sufficient to activate MPF leading to meiotic resumption (Figure 2).

1.2.2 The LH surge and cAMP spike activates the EGF network

The cAMP spike within the mural granulosa cells initiates a signal transduction cascade which activates the EGF receptor-ERK1/2 pathway in order to induce oocyte maturation and ovulation. LH-induced cAMP production rapidly upregulates production of the EGF-like peptides (EGF-p) amphiregulin, epiregulin, and betacellulin to induce EGF receptor-ERK1/2 pathway signaling (Ashkenazi *et al.* 2005, Fan *et al.* 2009, Park *et al.* 2004, Shimada *et al.* 2006). Transcription of EGF-p is induced by cAMP activation of PKA, leading to the activation of the cAMP-response

element (CRE) site in the gene's promoter region via a p38MAPK-CREB dependent process (Fan *et al.* 2009, Richards 2001, Shimada *et al.* 2006). Mature form EGF-p are cleaved from mural granulosa cells and bind to the EGF receptor (ERBB1), expressed on mural granulosa cells (autocrine) as well as on CCs (paracrine) (Hsieh *et al.* 2007, Yamashita *et al.* 2007). Ligand binding in both cell types leads to receptor dimerization and auto-phosphorylation on multiple tyrosine residues, which induce downstream RAS and, ultimately, ERK1/2 activation (Fan and Richards 2010, Yamashita *et al.* 2007). ERK1/2 consequently promotes the production of prostaglandin E2 by upregulating prostaglandin synthase 2 expression. Prostaglandin E2 then acts through the prostaglandin receptor PTGER2, expressed in both granulosa cell types, to induce further production of the EGF-p by activation of the cAMP-PKA-CREB pathway (Shimada *et al.* 2006), thus perpetuating the maturation-inducing stimulus in the LH-unresponsive CCs. Hence, LH-induced upregulation of the cAMP-EGF-p-ERK1/2 signalling axis is involved in CC expansion, decreasing somatic cell cGMP, closure of GJs and possibly a meiotic-inducing stimulus of CC origin (Chen *et al.* 2013, Norris *et al.* 2010, Su *et al.* 2002).

1.2.3 Loss of gap-junctions

In the mammalian ovary, intercellular coupling between oocyte and CCs undergoes dynamic changes during follicle development (Anderson and Albertini 1976) and the patency of GJC between the oocyte and CC compartments decreases in parallel with the meiotic resumption of the oocyte (Eppig 1982, Larsen *et al.* 1986). In both *in vivo* and in IVM, the progressive disruption of GJC occurs concomitantly with the retraction and degeneration of CC transzonal projections (Hyttel *et al.* 1986). Whether this preovulatory loss of CC-oocyte GJC causes meiotic resumption, due to the termination of cAMP transfer from CCs to oocyte, as originally hypothesized (Dekel and Beers 1978), has remained the subject of much debate for decades. Currently there appears to be strong evidence for the hypothesis that diffusion of cGMP from the oocyte to the somatic compartment

through functional GJs during GVBD has a crucial role in the reinitiation of meiosis (Norris *et al.* 2009, Shuhaibar *et al.* 2015, Vaccari *et al.* 2009).

2) Cyclic nucleotides and oocyte quality

2.1 In vitro maturation (IVM)

Advances in our understanding of the role of cyclic nucleotides in oocyte maturation have important practical applications in ART, in particular to oocyte IVM. IVM is an ART whereby COCs are collected at the immature GV stage from unstimulated or FSH-primed ovaries and matured as intact COCs in vitro prior to fertilisation (Edwards 1965). The most significant application of IVM is in the global production of livestock species, in particular cattle. It is also conducted in other domestic species, including pig, sheep, goat, deer, cat, camels and horse, but to a much less extent compared with cattle breeding. Global cattle embryo production by IVM/IVF exceeded 400,000 for the first time in 2013 (Perry 2014), with growth predicted to continue. Nevertheless, this reported figure is likely to be grossly underestimated. In this industry, immature oocytes are harvested from cows usually without exogenous hormone treatment, often on a regular basis (e.g. monthly) even during early pregnancy, which leads to a shortening of the intergenerational interval and genetic enrichment. In this industry, IVM is widely regarded as routine and safe.

IVM has proved less successful in humans and its use and further development as a fertility treatment has been relatively limited compared to classical IVF following hormonal stimulation of ovaries. The principal reason for the poor uptake of human IVM appears to be its lower efficiency at generating pregnancies compared to conventional IVF, and not due to safety concerns (Buckett *et al.* 2007, Kuhtz *et al.* 2014, Spits *et al.* 2015) or other practical aspects of use of the technology. Women with polycystic ovaries (PCO) are excellent candidates for treatment with IVM, because of

their high number of antral follicles that can be aspirated for oocyte retrieval. In addition, these women have a particularly increased risk of ovarian hyperstimulation syndrome, a potentially life-threatening iatrogenic complication of gonadotrophin stimulation, which has never been reported after IVM treatment. Currently, IVM is principally used for women with PCO. However, an important new application of IVM is for fertility preservation for young women who are diagnosed with cancer and face a substantial risk of gonadotoxicity secondary to chemotherapy or radiotherapy. For these women, for whom time is usually pressing, IVM is advantageous as it is possible to harvest oocytes at short notice without prior hormone therapy, and without elevated oestrogen levels which is contraindicated in cases of hormone sensitive tumors (De Vos *et al.* 2014).

In the context of modern milder approaches to ART, the increasing demand from patients for a simpler, cheaper, more patient friendly reproductive technology, the search for improvements in IVM are continuing and improved pregnancy rates have recently been established by a number of centers (Junk and Yeap 2012, Ortega-Hrepich *et al.* 2013, Walls *et al.* 2015a). Nonetheless, the reduced pregnancy rates per started cycle compared with conventional IVF, at least in the majority of centers where IVM is applied, represents a major obstacle that needs to be overcome for widespread uptake of IVM. This lower efficiency manifests at multiple levels: particularly lower metaphase II rates (typically 50-60%), but also lower subsequent embryo development rates (Walls *et al.* 2015b), and at least in the past, higher miscarriage rates. The application of cAMP-modulators to IVM offers great promise to improve IVM pregnancy rates. More than 10 years of animal data using a range of cAMP-modulated IVM systems, together provide compelling evidence that IVM efficiency and pregnancy outcomes can be substantially improved by carefully controlling cAMP levels during IVM.

2.2 Cyclic AMP-mediated IVM Systems

The following section reviews the various oocyte IVM systems/technologies, as pertaining to IVM regulated by the cyclic nucleotides. The modes of actions of some of the pharmacological agents used to manipulate cyclic nucleotides in IVM are listed in Table 1. For ease of discussion, the IVM systems are broadly divided into four IVM approaches (Figure 3), although we recognise that there is overlap between them. Whilst Standard IVM remains the approach used almost universally in human and veterinary clinical practice, the other three approaches can be considered to be at the pre-clinical stage of development, with substantial evidence of benefit accumulated (Table 2). The basic rationale for moving beyond Standard IVM is that oocyte maturation does not naturally occur "spontaneously" *in vivo*, but rather is an induced process that occurs in response to a rapid and transient surge in somatic/COC cAMP (Dekel and Beers 1978).

2.2.1 Standard IVM (low cAMP):

Standard IVM refers to the isolation of immature COCs from antral follicles and their subsequent maturation in medium lacking cAMP modulating agents (Figure 3A). This method is based on the original principals of spontaneous oocyte meiotic maturation as described many decades ago (Edwards 1965, Pincus and Enzmann 1935). Standard IVM systems typically contain FSH or other additives such as EGF, EGF-p and/or LH/hCG (Figure 3A). FSH leads to a transient rise in COC cAMP (Li *et al.* 2012) (Figure 3E), but if COC collection and processing is slow (see 2.2.5) or if FSH is omitted from IVM, then cAMP levels fall rapidly causing spontaneous meiotic resumption (Aktas *et al.* 1995, Luciano *et al.* 2004). In some species, FSH has negligible effects on MII rates and oocytes mature spontaneously (e.g. murine, bovine, ovine), whereas in others FSH significantly improves MII rates suggesting an element of meiotic induction (e.g. porcine, human). As cAMP hydrolysis is permitted in this system, intra-oocyte cAMP levels fall (Figure 3E), leading to inactivation of PKA and rapid progression to GVBD (Li *et al.* 2012, Norris *et al.* 2009, Vaccari *et al.* 2009).

2.2.2 Biphasic-IVM (moderate cAMP):

Biphasic IVM systems use a relatively high concentration of a PDE inhibitor to prevent spontaneous GVBD of COCs upon removal from the follicle for an extended period (e.g. 24 hours or more; Figure 3B), thereby preserving the moderate cAMP levels stimulated by FSH (Figure 3E) (Kawashima *et al.* 2008, Nogueira *et al.* 2003b, Nogueira *et al.* 2006). Examples of PDEs used include: Org9935, cilostamide, milrinone, IBMX, and CNP (Table 2). Then in the second IVM phase the inhibitor is washed out allowing cAMP levels to fall and oocyte maturation to proceed (Figure 3B). In general, biphasic IVM systems have been shown to lead to modest improvements in oocyte developmental competence, relative to standard IVM (see Table 2 for references).

2.2.3 Induced IVM (moderate cAMP):

For oocytes that are naturally GV-arrested (e.g. intra-follicular) or artificially GV-arrested (e.g. isolated COCs arrested using a PDE inhibitor), meiosis can be readily induced using natural ligands such as FSH, EGF and EGF-p (Dekel and Beers 1978, Dekel and Sherizly 1985, Downs *et al.* 1988). Oocyte maturation is "induced" as meiotic maturation is inhibited in the absence of such meiotic stimulating ligands, and the actions of these ligands are mediated by CCs as they induce GVBD in intact explanted follicles or in COCs *in vitro*, but importantly not in DOs *in vitro* (Downs *et al.* 1988, Park *et al.* 2004). Hence, Induced IVM systems typically incorporate the simultaneous application of a meiotic inhibitor and a meiotic inducing ligand (Figure 3C) (Thomas *et al.* 2004b). GVBD and progression to MII occurs in the presence of the meiotic inhibitor, at moderate-low levels of COC cAMP (Figure 3E). This system, as pioneered by Downs and Eppig (Downs *et al.* 1988), has been used extensively for decades as a mouse oocyte experimental model to study the cellular and molecular control of meiotic induction (Downs 2010), but curiously has not been examined in detail in oocyte developmental competence studies (see Table 2 for references).

2.2.4 Induced IVM (high cAMP):

The distinguishing feature of this approach to IVM is the inclusion of pharmacological agent(s) that increase COC cAMP or induce the synthesis of large quantities of cAMP in the COC (Figure 3D). This approach was pioneered by Funahashi H. *et al* by treating porcine COCs with dbcAMP leading to improved subsequent blastocyst yield (Funahashi *et al.* 1997). Using dbcAMP has since proved highly successful and is now in widespread use in porcine IVM embryo production systems (Table 2). Other cAMP-elevating agents of note used for this approach include invasive adenylate cyclase (iAC; (Aktas *et al.* 1995, Luciano *et al.* 1999)) and forskolin (Ali and Sirard 2005, Shu *et al.* 2008). Forskolin in particular, leads to rapid and large increases in whole COC and intra-oocyte cAMP (Bernal-Ulloa *et al.* 2016, Thomas *et al.* 2002); to levels that approximate the spike in COC cAMP levels that occurs *in vivo* in response to LH (Figure 3E) (Wang *et al.* 2011). As such, one such approach has been named simulated physiological oocyte maturation (SPOM; (Albuz *et al.* 2010, Gilchrist *et al.* 2015)). The COC cAMP profile in response to these pharmacological agents is usually more acute and notably higher than that achieved by FSH treatment of COCs, as per Standard IVM (Albuz *et al.* 2010). Such Induced IVM systems typically incorporate a pre-IVM (Luciano *et al.* 1999) or biphasic approach (Funahashi *et al.* 1997) (Figure 3D), whereby COCs are exposed to the cAMP-elevating agent for several hours (e.g. 2h; pre-IVM) to up to 22-24h (biphasic). This is then usually followed by an IVM phase lacking pharmacological AC activators, either in the presence (Zeng *et al.* 2013) or absence of FSH (Sugimura *et al.* 2015). A PDE inhibitor such as cilostamide can be included in the IVM phase, as used in SPOM version 1 (Albuz *et al.* 2010), or omitted as per SPOM version 2 ((Gilchrist *et al.* 2015, Li *et al.* 2016, Richani *et al.* 2014b, Zeng *et al.* 2013, Zeng *et al.* 2014)) and in the iAC and dbcAMP approaches (Funahashi *et al.* 1997, Guixue *et al.* 2001, Luciano *et al.* 1999).

It is noteworthy that in all Induced IVM (high cAMP) approaches, oocyte meiotic maturation is induced as a result of the elevated CC cAMP, even in the presence of a PDE inhibitor (Dekel *et al.*

1988, Shu *et al.* 2008) (N.B. this does not or is less likely to occur in Biphasic IVM). This may seem paradoxical, as pharmacological stimulation of cAMP synthesis in CCs increases intra-oocyte cAMP by at least an order of magnitude (Thomas *et al.* 2002), initially preventing GVBD. But in fact this cAMP surge induces CC synthesis of potent meiotic inducing factors (e.g. EGF-p, (Richani *et al.* 2014b); see section 2.3.5), that may recapitulate at least some of the meiotic inducing events that occur during oocyte maturation *in vivo*. It is interesting that in Induced IVM systems, GVBD occurs at a higher intra-oocyte cAMP concentration than in Standard IVM (Wang *et al.* 2011). However, despite the clear improvements across species in oocyte developmental competence using Induced IVM systems (Table 2), this mode of oocyte maturation does not necessarily led to clear improvements in MII rates, which would be useful in species such as human where IVM MII rates are typically low (~50%) (Shu *et al.* 2008, Zeng *et al.* 2013).

2.2.5 The oocyte collection phase:

COC collection conditions and the ensuing first hour are paramount to IVM success. A large part of the developmental competence that the oocyte has acquired in the follicle can be lost in this first hour. It is important that during this period; 1) the oocyte receives key nutrient support (Frank *et al.* 2013), and 2) that activation of the oocyte's potent PDE is prevented. Therefore, cAMP-mediated IVM systems require a PDE inhibitor in the oocyte collection medium, otherwise upon isolation of the COC, the loss of follicular cGMP will lead to rapid activation of the oocyte's PDE, loss of cAMP (Aktas *et al.* 1995, Albuz *et al.* 2010, Luciano *et al.* 2004), de-activation of PKA, loss of CC-oocyte gap junctions, cessation of oocyte transcription and irreversible resumption of meiosis (Li *et al.* 2012, Luciano *et al.* 2011). It is noteworthy that in the enormous body of IVM literature using porcine and bovine abattoir-sourced oocytes, these COCs are invariably collected and processed in undiluted or high concentration follicular fluid, which serves the two purposes of COC nutrient provision and PDE inhibition. Therefore, the clinical practice of performing IVM oocyte pick-ups with saline, PBS or simple holding medium is likely to be particularly detrimental to

oocyte quality. We have recently demonstrated that the inclusion of IBMX in human oocyte collection medium supports subsequent oocyte maturation and healthy embryo development (Spits *et al.* 2015).

2.2.6 Cyclic AMP-mediated IVM and oocyte developmental competence:

The number of publications accumulated over the past decade now provides compelling evidence that cAMP-mediated IVM systems can lead to notably improved oocyte quality, compared to Standard IVM, as measured by enhanced subsequent pre-implantation embryo development and quality (see table 2 for citation list). Hence these novel approaches to IVM are now highly attractive for clinical and commercial applications, to bridge the efficiency gap between IVM and IVF. The different cAMP-mediated IVM systems yield differing outcomes. In general terms, biphasic IVM and Induced IVM (low cAMP) approaches led to only modest improvements in blastocyst yield, whereas Induced IVM systems producing high COC cAMP levels generally lead to larger improvements in oocyte quality (Figure 3, Table 2). Induced IVM systems lead to apparently healthy pregnancies and offspring (Akaki *et al.* 2009, Albuz *et al.* 2010, Bernal-Ulloa *et al.* 2016). There remain challenges however in working with these systems (Gilchrist *et al.* 2015). Firstly, manipulating oocyte cAMP has major effects on oocyte meiotic kinetics, and hence timing to MII should be assessed carefully under local laboratory conditions (see 2.3.4). Secondly, IVM systems, where CC and oocyte functions are acutely altered, such as SPOM version 1, can be difficult to work with practically and strict attention to protocol is needed (Gilchrist *et al.* 2015). This led us to develop a more user-friendly SPOM version 2 (Zeng *et al.* 2013, Zeng *et al.* 2014). These issues highlight that, to realise the full potential of these novel approaches to IVM, further refinement of practical aspects of IVM protocols is warranted.

2.3 Impact of cAMP-mediated IVM on CCs and the oocyte

441 Since there is a clear beneficial effect of cAMP-mediated IVM systems on oocyte developmental
442 competence (Table 2), the effect of cAMP-mediated IVM on cellular and molecular aspects of CC
443 and oocyte function are of interest as a means to: 1) provide insights into basic mechanisms
444 regulating oocyte quality, and 2) offer opportunities to further improve IVM efficiency.
445
446 ***2.3.1 CC microarray analysis***
447 Recent microarray analysis of CCs after 6 hour exposure to cAMP-elevating agents (forskolin +
448 IBMX + dipyridamole; see Table 1) was undertaken to elucidate cAMP-induced gene networks
449 (Khan *et al.* 2015). These culture conditions were previously demonstrated to give higher yield of
450 embryo development (Ali and Sirard 2005). The study demonstrated that cAMP significantly and
451 specifically modulated gene expression dynamics including genes involved in cell metabolism, cell
452 communication, signal transduction, steroidogenesis, cell survival and extracellular matrix
453 formation (Khan *et al.* 2015). Genes involved in cell metabolism such as *GFPT2* (glutamine-
454 fructose-6-phosphate transaminase 2) and *HK2* (hexokinase 2) were significantly up-regulated by
455 the cAMP-elevating agents, as well as genes involved in carbohydrate uptake (*SLC2A1*, solute
456 carrier family 2 - facilitated glucose transporter, members 1 and 3, respectively) and steroidogenesis
457 (*STAR*, steroidogenic acute regulatory protein). Interestingly, down-regulation of EGF pathway
458 genes (*AREG*, amphiregulin and *HAS2*, hyaluronan synthase 2), which are involved in cumulus
459 expansion were observed ((Khan *et al.* 2015); see end of section 2.3.5 for temporal effects).
460 Decreased phosphorylation of ERK1/2 supports a possible negative regulatory role of PKA in this
461 process. These findings imply that treatment of COCs with cAMP-elevating agents up-regulate
462 genes in cell metabolism, carbohydrate uptake and steroidogenesis and down-regulate genes of the
463 EGF pathway (Khan *et al.* 2015).
464
465 ***2.3.2 CC-oocyte gap-junctional communication***

A central objective of most modern IVM systems is to preserve CC-oocyte communication as it is viewed as critical to generating a healthy mature oocyte capable of sustaining embryo development (Gilchrist 2011). Under standard IVM conditions, the drop in COC cAMP concentration that occurs soon after removal of the COC from the antral follicle (Aktas *et al.* 1995, Albuz *et al.* 2010) is accompanied by initiation of closure of CC-oocyte GJs (Thomas *et al.* 2004a). This loss of GJC is attenuated to some extent by the inclusion of FSH in standard IVM media (Atef *et al.* 2005). FSH stimulates expression of genes encoding connexins including *Gja1* (El-Hayek and Clarke 2015), possibly via a PKA-regulated mechanism (Yun *et al.* 2012). Inhibiting COC cAMP hydrolysis, in either the CC or oocyte compartment using selective PDE inhibitors, further attenuates the loss of GJC and is usually associated with a delay in GVBD (Luciano *et al.* 2004, Thomas *et al.* 2004a). By contrast, using cAMP elevating agents in IVM, such as forskolin or dbcAMP, not only prevent GJC loss but maintain full patency for extended periods of IVM (Albuz *et al.* 2010, Li *et al.* 2016, Shu *et al.* 2008, Thomas *et al.* 2004a). Using cAMP-mediated approaches to preserve CC-oocyte GJCs in IVM is nearly always associated with an improvement in subsequent oocyte developmental competence (see Table 2 for citations). Furthermore, blocking GJs negates any benefits of cAMP-mediated IVM in terms of subsequent embryo development (Atef *et al.* 2005). The molecular mechanisms underlying the improvement in oocyte quality are less clear; GJ-mediated effects on oocyte metabolism may be important (see 2.3.6), as well as effects on oocyte transcription. As outlined in section 2.1.2, addition of cAMP modulators to IVM prevents premature chromatin condensation and permits continued oocyte RNA synthesis, and it has been hypothesized that this occurs via a GJ-mediated mechanism (De La Fuente and Eppig 2001, Luciano *et al.* 2011).

2.3.4 Kinetics of meiosis

A founding principal of cAMP-mediated IVM systems is to prevent spontaneous GVBD of oocytes upon removal from antral follicles (Gilchrist and Thompson 2007), and as such the kinetics of the meiotic cell cycle are notably different in these cAMP-mediated oocyte maturation systems (Figure

3). GVBD occurs most rapidly under spontaneous IVM (e.g. mouse, ~1 hour; bovine, ~ 6 hours) where there is uncontrolled loss of intra-oocyte cAMP leading to the activation of MPF. Using bi-phasic IVM systems, GVBD is prevented for as long as a cAMP modulator is present; typically 22-48h (Nogueira *et al.* 2003b, Nogueira *et al.* 2006). In Induced IVM systems, GVBD is typically delayed (but not inhibited) by several hours, for example; from 1-3 h in the mouse, or from 6-7 h to 10-12 h in bovine (Albuz *et al.* 2010, Thomas *et al.* 2004a)). Whether time to MII is delayed depends on the type, dose and combination of cAMP modulators used, but commonly MII is either not delayed (Kim *et al.* 2008) or delayed by only several hours (Albuz *et al.* 2010, Rose *et al.* 2013, Thomas *et al.* 2004b). However, as GVBD is always delayed, this means that usually the GVBD to MII interval is commonly shortened using Induced or Biphasic IVM systems, relative to Standard IVM (Kim *et al.* 2008, Thomas *et al.* 2004b). There is strong evidence that this occurs because these IVM systems generate potent meiotic-inducing factors, likely of CC origin requiring EGFR signalling [see next; (Albuz *et al.* 2010, Dekel *et al.* 1988, Downs and Chen 2008)]. The net effect of this rapid progression through meiosis is a reduction in meiotic asynchrony, as originally identified by Funahashi *et al* (1997); i.e. a reduction in the range of times at which a cohort of oocytes reach MII. Hence, a significant benefit of cAMP-mediated IVM systems is likely to be a reduction in *in vitro* ageing of IVM oocytes, which may account for their improved subsequent developmental competence.

2.3.5 CC EGF signalling

The effect of cAMP elevation in CCs in induced IVM systems (e.g. SPOM; Fig 3D) is mediated, at least in part, by EGF receptor activity. The EGF receptor inhibitor AG1478 blocks GVBD in COCs pulsed with dbcAMP to stimulate maturation (Downs and Chen 2008). The same effect is also observed in SPOM COCs exposed to AG1478 (Albuz *et al.* 2010). Moreover, recent genetic dissection of the effect of elevated cAMP in vitro implicates PKA and ERK1/2 pathways, which are interconnected with EGF receptor signaling, as key downstream signalling regulators of cAMP in

vitro (Khan *et al.* 2015). The increased developmental competence of cAMP-mediated IVM oocytes may in part be attributable to the impact of cAMP on EGF pathway signaling in CCs. Standard IVM conditions (including with FSH) exhibit perturbed CC expression of EGF-p relative to those matured in vivo (Richani *et al.* 2013), leading to alterations in COC glucose metabolism and decreased oocyte mitochondrial activity (Richani *et al.* 2014a). Cyclic AMP elevation using forskolin leads to a large, but very transient, increased expression of CC amphiregulin, epiregulin, and betacellulin, compared to unstimulated or IBMX-treated COCs (Richani *et al.* 2014b). However, the large increase in EGF-like peptide expression does not translate into increased activation of the EGF receptor or its downstream target ERK1/2 (Richani *et al.* 2014b), suggesting that the cAMP-induced spike in EGF-like peptides may impact alternate downstream EGF receptor targets (Chen *et al.* 2013), or may alter temporal EGFR signaling through negative feedback. The latter hypothesis is supported by evidence showing increased EGF-p expression after 2 hours of forskolin exposure in the mouse, but not at 4h (mouse) or 6h (cow) (Khan *et al.* 2015, Richani *et al.* 2014b).

2.3.6 COC metabolism and oocyte antioxidant defence

There appears to be an effect of cAMP-mediated IVM on CC and oocyte metabolism, consistent with the established relationship between oocyte developmental competency and oocyte metabolism (Thompson *et al.* 2007). The basic pattern of metabolism during development and maturation of the oocyte is demonstrated as a dynamic process with the consumption of oxygen and the utilisation of nutrients present in culture media; mainly glucose, pyruvate and lactate (Leese 2015). Induced-IVM (high cAMP; SPOMv2; Figure 3D) systems that enhance oocyte quality, lead to increased lactate production by COCs over the course of IVM, suggesting stimulation of CC glycolysis (Zeng *et al.* 2014). Significantly, treating COCs during pre-IVM with forskolin plus IBMX leads to intra-oocyte GSH accumulation in a pre-IVM duration-dependent manner, which is ablated when GJs are blocked (Li *et al.* 2016, Zeng *et al.* 2014). This cAMP-mediated increase in GSH is associated with

lower levels of H_2O_2, suggesting that a key benefit of cAMP-mediated IVM may be an improvement in the oocyte's antioxidant defenses requiring GSH supplied by CCs (Li *et al.* 2016). As increased GSH levels are highly correlated with oocyte developmental competence (de Matos *et al.* 1995) this may at least partly explain why pre-maturation with these agents improves oocyte competence. Cyclic AMP-modulated pre-IVM treatments also increase COC oxygen consumption and oocyte oxidative metabolism, associated with an increase in the oocyte redox ratio and a higher ATP:ADP ratio (Zeng *et al.* 2013, Zeng *et al.* 2014). Therefore, activation of cAMP signalling pathways during oocyte maturation not only impacts on oocyte metabolism but also on oocyte antioxidant defense, in a GJ-dependent manner (Li *et al.* 2016).

Conclusions

The scientific and medical community now have at hand a vast body of literature on the role of cyclic nucleotides in mammalian oocyte function. Their role in the regulation of oocyte meiosis is now clear and more recently, we have acquired substantial experimental evidence that the delicate balance of cyclic nucleotides between the somatic and germ cell compartments plays a key role in oocyte developmental competence. Current research is directed to understanding the possible mechanisms by which cyclic nucleotides improve oocyte quality (Figure 4). Nonetheless, it is clear that application of cAMP modulators in IVM can present practical challenges, for example, on the timing of meiosis, and may present regulatory issues. The challenge therefore to the medical and veterinary disciplines is to capitalise on these new scientific and technological advances to improve the efficacy of IVM, for the benefit of infertile and cancer patients and for domestic animal breeding.

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574

575 **Conflict of Interest**

576

577 The research programs of RBG, MDV, JS and JGT have received commercial funding for the
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Figure Legends:

Figure 1: The coordination between CNP, cGMP and cAMP in the control of oocyte meiotic arrest. See text for abbreviations.

Figure 2: The cyclic nucleotides transmit the ovulatory cascade from the somatic to germ cell compartment of the follicle, instructing the oocyte to resume meiosis in preparation for ovulation. See text for abbreviations.

Figure 3: Differing approaches to cAMP-mediated IVM.

Schematic comparison of Standard IVM containing FSH but no cAMP modulating agents (**A**), to various cAMP-mediated IVM systems, including; (**B**) Biphasic IVM using a PDE inhibitor for the first phase followed by wash-out and PDE inhibitor free in the second phase, (**C**) Induced IVM producing moderate cAMP levels, where oocytes are matured in the simultaneous presence of PDE inhibitor and an inducing ligand, and (**D**) Induced IVM where exogenous cAMP or AC activators produce high levels of COC cAMP. (**E)** Schematic illustration of actual and predicted COC cAMP levels in the differing IVM systems containing FSH, compared to Standard IVM and in oocytes matured *in vivo.* In the absence of FSH in Standard IVM, COC cAMP levels fall rather than rise (modified from (Thompson and Gilchrist 2013)). GV, germinal vesicle; GVB, germinal vesicle breakdown; MII, metaphase II; EGF-p, epidermal growth factor-like peptides; PDE, phosphodiesterase.

Figure 4: Possible mechanisms by which high levels of COC cAMP during *in vivo* oocyte maturation or by Induced-IVM improve oocyte quality. GJC, gap-junctional communication; GSH, glutathione; ROS, reactive oxygen species; EGF-p, epidermal growth factor-like peptides; GVBD, germinal vesicle breakdown.

Table 1: Pharmacological agents used in IVM to manipulate cyclic nucleotides.

Agent	Mode of Action	Remarks
Hypoxanthine, IBMX	PDE inhibitors: broad spectrum	Act on CC and oocyte PDEs to prevent cAMP hydrolysis
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Abbreviations: IBMX, 3-isobutyl-1-methylxanthine; PDE, phosphodiesterase; CC, cumulus cell; MGC, mural granulosa cell; CNP, C-type natriuretic peptide; NPR2, natriuretic peptide receptor 2; AC, adenylate cyclase; dbcAMP, dibutyryl cAMP.

Table 2: Effect of cAMP-mediated IVM on subsequent oocyte developmental competence

IVM system	cAMP modulator	Species	Effect on oocyte developmental competence*	References
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	Cilostamide	Human	None	(Vanhoutte *et al.* 2007)
		Human	Improved	(Vanhoutte *et al.* 2009a, Vanhoutte *et al.* 2009b)
		Bovine	Improved	(Luciano *et al.* 2011)
		Porcine	Improved	(Dieci *et al.* 2013)
	IBMX	Bovine	Improved	(Lodde *et al.* 2013)
		Porcine	Improved	(Kawashima *et al.* 2008)
	CNP	Bovine	Improved	(Franciosi et al., 2014)
Induced IVM (moderate cAMP)	Milrinone	Bovine	Improved	(Thomas *et al.* 2004b)
		Porcine	None	(Grupen *et al.* 2006)
	Rolipram	Bovine	Improved	(Thomas *et al.* 2004b)
	Dipyridamole	Bovine	Decreased	(Sasseville *et al.* 2009)
	Hypoxanthine	Murine	None	(Downs *et al.* 1986)
Induced IVM (high cAMP)	dbcAMP	Porcine	Improved	(Akaki *et al.* 2009, Bagg *et al.* 2006, Funahashi *et al.* 1997, Kim *et al.* 2008, Nascimento *et al.* 2010, Somfai *et al.* 2003, Sugimura *et al.* 2015)
			None	(Appeltant *et al.* 2015, Park and Yu 2013)
	iAC	Bovine	Improved	(Guixue *et al.* 2001, Luciano *et al.* 2004, Luciano *et al.* 1999)
		Bovine	None	(Aktas *et al.* 1995)
	Forskolin	Human	Improved	(Shu *et al.* 2008)
		Murine	Improved	(Albuz *et al.* 2010, Richani *et al.* 2014b, Zeng *et al.* 2013, Zeng *et al.* 2014)
		Bovine	Improved	(Albuz *et al.* 2010, Ali and Sirard 2005, Li *et al.* 2016)
		Bovine	None/Decreased	(Bernal-Ulloa *et al.* 2016, Guimaraes *et al.* 2015, Ulloa *et al.* 2014)
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		Ovine	None	(Buell *et al.* 2015)

* Relative to standard IVM control. As assessed by embryo development typically to the blastocyst stage.

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Figure 1

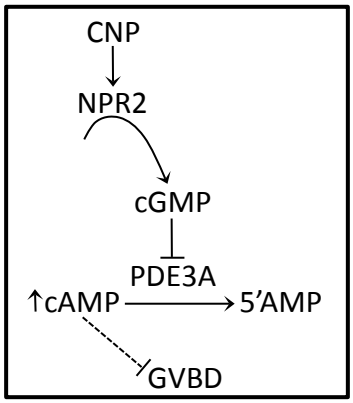
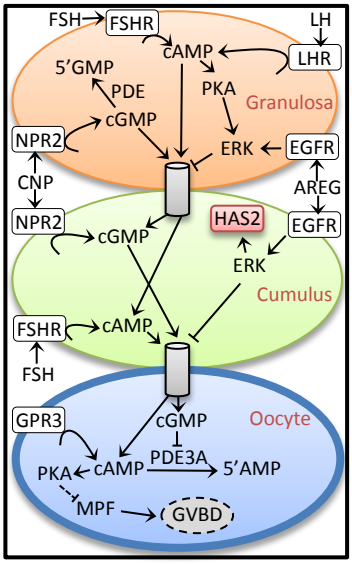


Figure 2


FSH
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LH
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5'GMP
PDE
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NPR2
HAS2
EGFR
cGMP
ERK
Cumulus
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PDE3A
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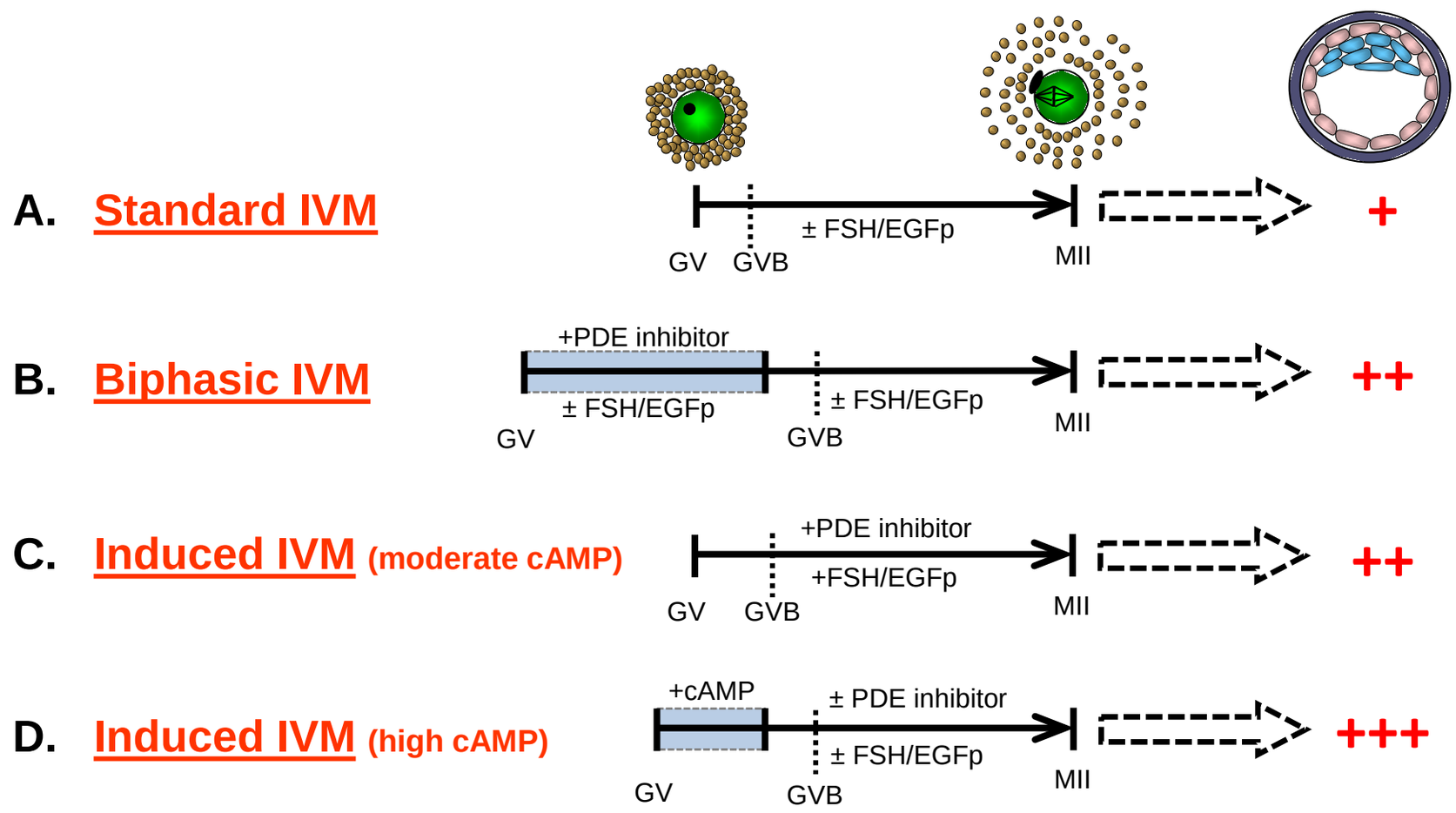
cAMP
5'AMP
MPF
GVBD

Figure 3


A. Standard IVM
± FSH/EGFp
GV
GVB
MII
+
B. Biphasic IVM
+PDE inhibitor
± FSH/EGFp
± FSH/EGFp
GV
GVB
MII
++
C. Induced IVM (moderate cAMP)
+PDE inhibitor
+FSH/EGFp
GV
GVB
MII
++
D. Induced IVM (high cAMP)
+cAMP
± PDE inhibitor
± FSH/EGFp
GV
GVB
MII
+++

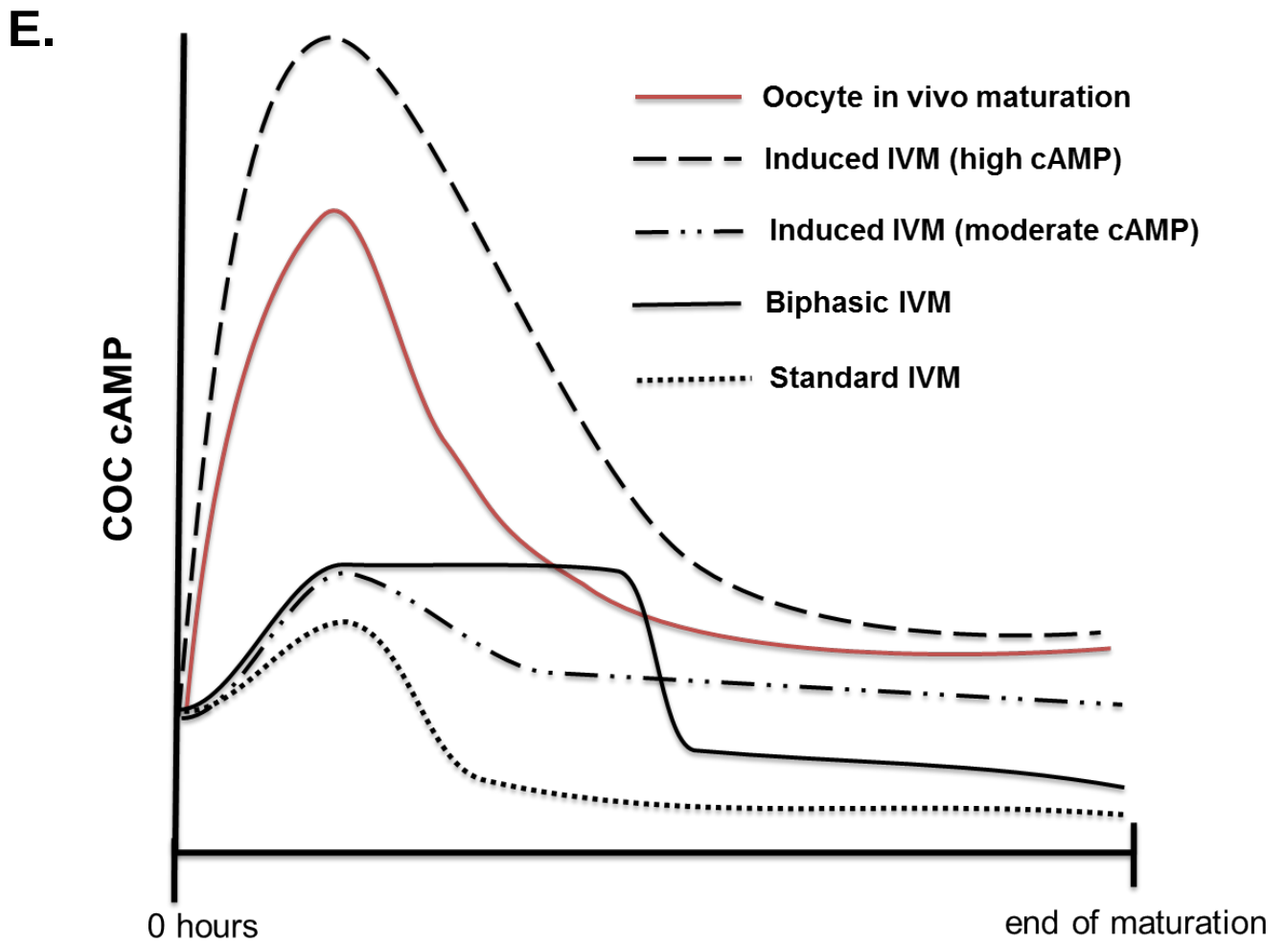
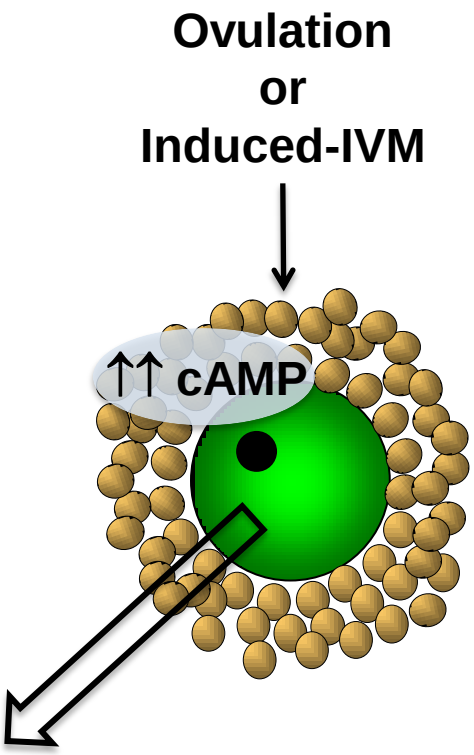

E.
Oocyte in vivo maturation
Induced IVM (high cAMP)
Induced IVM (moderate cAMP)
Biphasic IVM
Standard IVM
COC cAMP
0 hours
end of maturation

Figure 4



- preservation of GJC
- ↑ glycolysis, ↑ oxidative metabolism
- ↑ GSH → ↓ ROS
- activation of EGF-p cascade
- ordered cessation of RNA synthesis
- delayed GVBD
- ↓ meiotic asynchrony

↑ Embryo Development

↑ Fetal Viability

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