REPRODUCTION
REVIEW

Oocyte maturation and quality: role of cyclic nucleotides

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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 pre-ovulatory 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 two 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 practised clinically do not recapitulate this cAMP surge *in vitro*, possibly accounting for the lower efficiency of IVM compared with clinical IVF. This review particularly focuses 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.

Reproduction (2016) **152** R143–R157

Introduction

Oocyte maturation and oocyte quality are fundamental to fertility in all mammalian species. These are evident in modern human infertility treatment in which 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). Since then, cAMP and cGMP and their roles in the regulation of oocyte maturation remain intensively researched worldwide. This research largely focused on the regulation of meiosis and the role of the nucleotides in the maintenance of meiotic arrest and the resumption of meiosis, principally using rodent 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 was 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

ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/REP-15-0606
Online version via www.reproduction-online.org

of phosphodiesterases (PDEs) (Tsafriri *et al.* 1996, Masciarelli *et al.* 2004), 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 to 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 two 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 the context of ART.

Cyclic nucleotides and oocyte maturation *in vivo*

Cyclic nucleotides maintain meiotic arrest

Follicle maintains meiotic arrest

A basic tenant of oocyte maturation is that oocytes in mid-sized antral and pre-ovulatory 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 (Pincus & Enzmann 1935, Edwards 1965). 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.

Follicle endows the oocyte with developmental competence

During folliculogenesis, oocytes undergo changes at both the nuclear and cytoplasmic levels that confer the oocyte with developmental competence, defined as the capacity to support pre-implantation embryo development (Gilchrist & Thompson 2007). Among other processes, changes in large-scale oocyte chromatin structure are essential for the onset of developmental competence (reviewed in Luciano & Lodde 2013). The proper maintenance of cumulus cell (CC)–oocyte gap junctional communication (GJC) appears to have a crucial role in chromatin remodelling and the gradual transcriptional silencing processes that occur in fully grown oocytes, from early antral through to the latter half of antral folliculogenesis (De La Fuente & 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 (Luciano *et al.* 2004, Thomas *et al.* 2004*a*, Atef *et al.* 2005, El-Hayek & Clarke 2015). In addition, treatment with FSH *in vivo* leads to oocyte chromatin condensation and suppression of transcription (Zuccotti *et al.* 1998, De La Fuente & Eppig 2001). The use of bovine cumulus–oocyte complex (COC) culture systems that prolong GJC, sustain oocyte growth and allow early chromatin compaction events is associated with the oocyte acquiring the ability to mature and be fertilised *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). 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 that 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.

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 synthesised 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 synthesise cAMP (Olsiewski & Beers 1983, Mehlmann *et al.* 2002). However, a major source of intra-oocyte cAMP is 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 cAMP, increasing cAMP concentrations in the oocyte manyfold (Thomas *et al.* 2002). Sustained levels of intra-oocyte cAMP activate protein kinase A (PKA), which in turn prevent the activation of maturation-promoting factor, retaining the oocyte in the M-phase. Cyclic nucleotide participation in the control of the meiotic cell cycle is reviewed elsewhere (Downs 2010, Conti *et al.* 2012).

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, when it was found that non-specific PDE inhibitors, such as theophylline (Cho *et al.* 1974) and 3-isobutyl-1-methylxanthine (IBMX) (Magnusson & Hillensjo 1977, Dekel & Beers 1978), maintain meiotic arrest of oocytes *in vitro*. Two studies published in the 1990s reported the presence of a specific family of PDEs within the rodent oocyte, namely PDE3A, identified using *in situ* hybridisation (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 before 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: rats (Tsafriri *et al.* 1996), mice (Wiersma *et al.* 1998), cattle (Mayes & Sirard 2002, Thomas *et al.* 2002), monkeys (Jensen *et al.* 2002), humans (Nogueira *et al.* 2003*a*) 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 organised into 11 families with 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 & Haslam 1990). It was long known that cGMP is an oocyte maturation inhibitor (Hubbard & Terranova 1982) and that ovarian levels of cGMP decline after LH stimulation (Ratner 1976). After three decades, its significance became apparent when two key papers revealed that cGMP permeating from the granulosa/CC compartment into the oocyte via gap junctions 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 (Fig. 1).

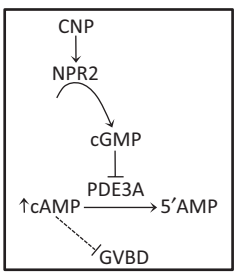

Figure 1 The coordination between CNP, cGMP and cAMP in the control of oocyte meiotic arrest. See text for abbreviations.

Contribution of natriuretic peptides to meiotic arrest

The signalling model for the maintenance of meiotic arrest has recently been enhanced by the discovery of the important roles of the natriuretic peptides. The natriuretic peptide family is composed of three major types: atrial natriuretic peptide, brain natriuretic peptide and C-type natriuretic peptide (CNP). A study showed 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-meiosis-inhibiting peptide in a range of species such as mice (Zhang *et al.* 2010), swine (Santiquet *et al.* 2014), cattle (Franciosi *et al.* 2014) and rats (Zhang *et al.* 2015). The physical characteristics and mode of action of CNP suggest it is likely to be the oocyte maturation inhibitor (OMI) found in follicular fluid, as described several decades ago (Tsafriri *et al.* 1976). 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; Fig. 1).

Ovulatory cascade

LH-induced changes in cyclic nucleotides

The pre-ovulatory 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 have 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 80–200-fold increase in cAMP, depending on the compartment measured (Tsafriri *et al.* 1972, Yoshimura *et al.* 1992, Mattioli *et al.* 1994, Albuz *et al.* 2010). The spike in cAMP appears, in general, to occur before 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 (Yoshimura *et al.* 1992, Norris *et al.* 2009). This pre-ovulatory spike in cAMP is able to induce meiosis, as cAMP pulsing of explanted follicles or isolated COCs *in vitro* induces the resumption of meiosis in the presence of inhibitors (Tsafriri *et al.* 1972, Dekel *et al.* 1981, Downs *et al.* 1988). Hence, the acute changes in cAMP concentrations that follow the gonadotrophin 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 'Cyclic nucleotides and oocyte quality' section).

Despite this ovulatory pulse in follicular cAMP levels, activation of the oocyte PDE3A and a consequent fall in intra-oocyte cAMP are clearly a prerequisite for de-phosphorylation of PKA, and activation of maturation-promoting factor (MPF) and meiotic resumption (Fig. 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 1 min of LH exposure, cGMP concentrations start to decrease from the peripheral granulosa cells and by 20 min the concentration of cGMP 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 the 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 downregulates 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 min (Robinson *et al.* 2012), which can be explained by de-phosphorylation and inactivation of NPR2 in granulosa cells (Egbert *et al.* 2014). Hence, the present 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 that leads to meiotic resumption (Fig. 2).

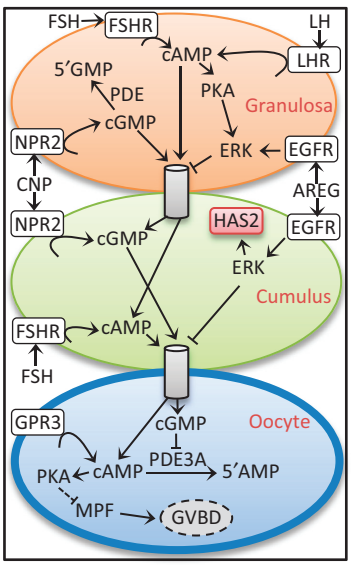

Figure 2 The cyclic nucleotides transmit the ovulatory cascade from the somatic to germ cell compartment of the follicle, instructing the oocyte to resume meiosis in the preparation for ovulation. See text for abbreviations.

LH surge and cAMP spike activate the EGF network

The cAMP spike within the mural granulosa cells initiates a signal transduction cascade that activates the EGF receptor-ERK1/2 pathway to induce oocyte maturation and ovulation. LH-induced cAMP production rapidly upregulates the production of EGF-like peptides (EGF-p) amphiregulin, epiregulin and betacellulin to induce EGF receptor–ERK1/2 pathway signalling (Park *et al.* 2004, Ashkenazi *et al.* 2005, Shimada *et al.* 2006, Fan *et al.* 2009). 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 (Richards 2001, Shimada *et al.* 2006, Fan *et al.* 2009). 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 dimerisation and auto-phosphorylation on multiple tyrosine residues, which induce downstream RAS and, ultimately, ERK1/2 activation (Yamashita *et al.* 2007, Fan & Richards 2010). 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 meiosis-inducing stimulus of CC origin (Su *et al.* 2002, Norris *et al.* 2010, Chen *et al.* 2013).

Loss of gap junctions

In the mammalian ovary, intercellular coupling between oocyte and CCs undergoes dynamic changes during follicle development (Anderson & 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 IVM, the progressive disruption of GJC occurs concomitantly with the retraction and degeneration of CC transzonal projections

(Hyttel *et al.* 1986). Whether this pre-ovulatory loss of CC–oocyte GJC causes meiotic resumption due to the termination of cAMP transfer from CCs to oocyte, as originally hypothesised (Dekel & Beers 1978), has remained the subject of much debate for decades. There is strong evidence supporting the hypothesis that diffusion of cGMP from the oocyte to the somatic compartment through functional GJs during GVBD has a crucial role in the re-initiation of meiosis (Norris *et al.* 2009, Vaccari *et al.* 2009, Shuhaibar *et al.* 2015).

Cyclic nucleotides and oocyte quality

In vitro *maturation (IVM)*

Understanding the role of cyclic nucleotides in oocyte maturation has important practical applications in ART, particularly in oocyte IVM. IVM is an ART in which COCs are collected at the immature GV stage from unstimulated or FSH-primed ovaries and matured as intact COCs *in vitro* before fertilisation (Edwards 1965). The most significant application of IVM is in the global production of livestock species, especially cattle. It is also conducted in other domestic species, including pigs, sheep, goats, deer, cats, camels and horse, but to a much less extent compared with cattle breeding. Global cattle embryo production by IVM/IVF exceeded 400,000 in 2013 (Perry 2014), with growth predicted to continue. Nevertheless, this figure is likely a gross underestimation. Based on this method, immature oocytes are harvested from cows usually without exogenous hormone treatment, often on a regular basis (e.g. monthly) even during early pregnancy. Thus, it leads to 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. Its use and further development as a fertility treatment have been relatively limited compared with 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 with 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 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. IVM is principally used for women with PCO. However, an important new application of IVM is also used for fertility preservation in young women who are diagnosed with cancer and who 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 the cases of hormone-sensitive tumours (De Vos *et al.* 2014).

In the context of modern milder approaches to ART and 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 centres (Junk & Yeap 2012, Ortega-Hrepich *et al.* 2013, Walls *et al.* 2015*a*). Nonetheless, the reduced pregnancy rates per cycle compared with conventional IVF 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.* 2015*b*), and in some centers, higher miscarriage rates. The use of cAMP modulators in human IVM offers great promise to improve pregnancy rates. Animal data obtained from more than 10 years of research using various cAMP-modulated IVM systems provide evidence that IVM efficiency and pregnancy outcomes can be improved by controlling cAMP levels during IVM.

Cyclic AMP-mediated IVM Systems

This section reviews the various oocyte IVM systems/technologies pertaining to IVM regulated by the

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
Org9935, cilostamide, milrinone	PDE3-specific inhibitors	Target the PDE in the oocyte (PDE3A) to prevent intra-oocyte cAMP hydrolysis
Rolipram	PDE4-specific inhibitor	Target the PDE in CC and MGC (PDE4) to prevent cAMP hydrolysis
Dipyridamole	PDE8-specific inhibitor	Inhibits PDE8 in CCs (bovine) to prevent cAMP hydrolysis
CNP	NPR2 agonist	Stimulates CC and MGC cGMP synthesis thereby antagonising PDE
Sildenafil	PDE5 and PDE6 inhibitor	Target CC and MGC PDEs to prevent cGMP hydrolysis
Forskolin, iAC	AC activators	Elevate CC/oocyte cAMP
dbcAMP, 8-bromo-cAMP	cAMP analogous	Elevate CC/oocyte cAMP

AC, adenylate cyclase; CC, cumulus cell; CNP, C-type natriuretic peptide; dbcAMP, dibutyryl cAMP; IBMX, 3-isobutyl-1-methylxanthine; MGC, mural granulosa cell; NPR2, natriuretic peptide receptor 2; PDE, phosphodiesterase.

cyclic nucleotides. The modes of actions of some pharmacological agents used to manipulate cyclic nucleotides in IVM are listed in Table 1. IVM systems are broadly divided into four approaches (Fig. 3), although some may overlap. Although standard IVM is used in human and veterinary clinical practice, the other three approaches can be considered at the preclinical stage of development, which has shown

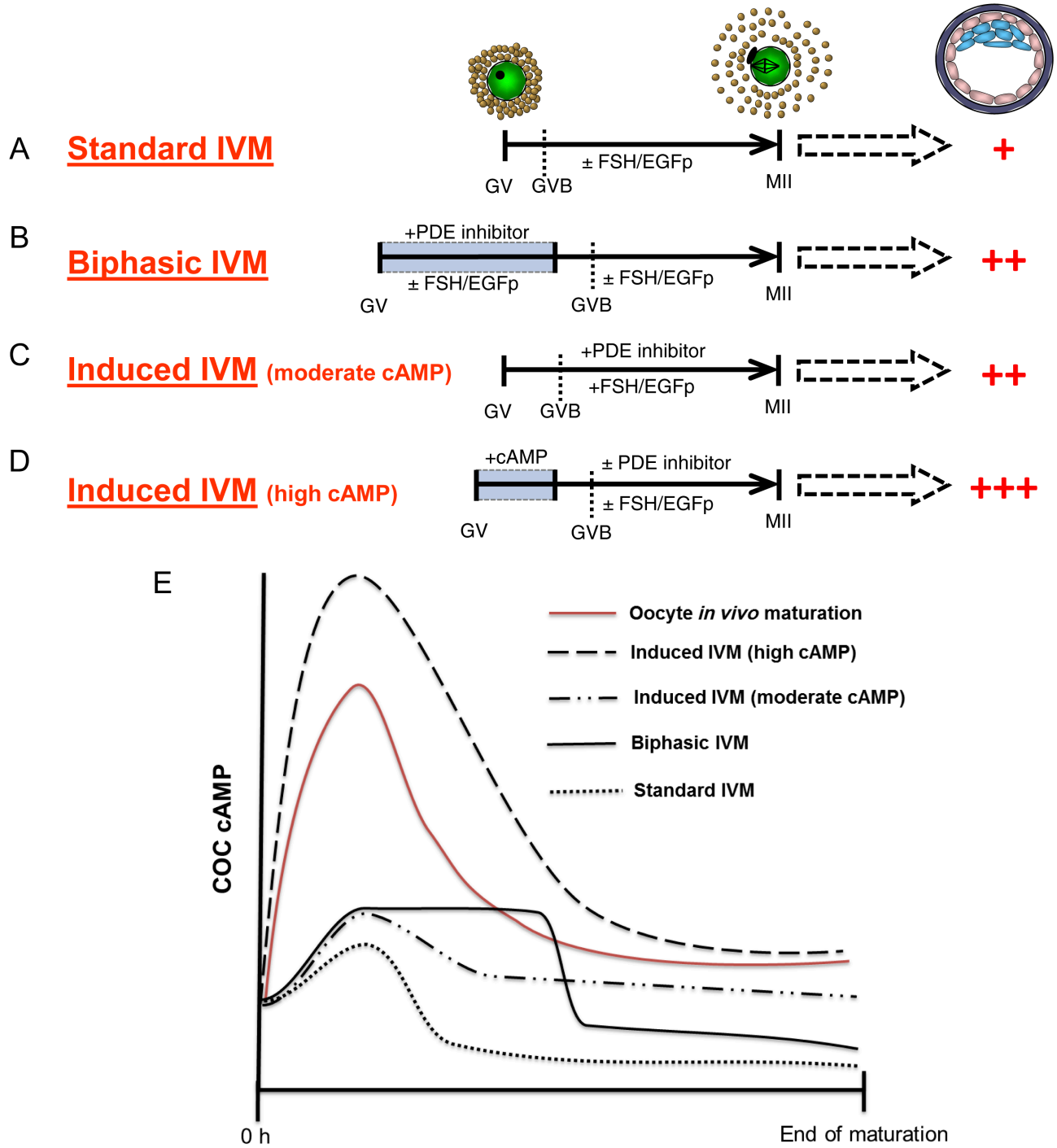

Figure 3 Differing approaches to cAMP-mediated IVM. Schematic comparison of standard IVM containing FSH but no cAMP-modulating agents (A) to various cAMP-mediated IVM systems, including (B) biphasic IVM using a PDE inhibitor for the first phase followed by washout and PDE inhibitor free in the second 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 with standard IVM and in oocytes matured *in vivo*. In the absence of FSH in standard IVM, COC cAMP levels decrease rather than increase (modified from (Thompson & Gilchrist 2013)). EGF-p, epidermal growth factor-like peptides; GV, germinal vesicle; GVB, germinal vesicle breakdown; MII, metaphase II; PDE, phosphodiesterase.

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

IVM system	cAMP modulator	Species	Effect on oocyte developmental competence*	References
Biphasic IVM (moderate cAMP)	Org 9935	Human	None	Nogueira *et al.* (2006)
		Murine	Improved	Nogueira *et al.* (2003*b*)
	Cilostamide	Human	None	Vanhoutte *et al.* (2007)
		Human	Improved	Vanhoutte *et al.* (2009*a*,*b*)
		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.* (2004*b*)
		Porcine	None	Grupen *et al.* (2006)
	Rolipram	Bovine	Improved	Thomas *et al.* (2004*b*)
	Dipyridamole	Bovine	Decreased	Sasseville *et al.* (2009)
	Hypoxanthine	Murine	None	Downs *et al.* (1986)
Induced IVM (high cAMP)	dbcAMP	Porcine	Improved	Funahashi *et al.* (1997), Somfai *et al.* (2003), Bagg *et al.* (2006), Kim *et al.* (2008), Akaki *et al.* (2009), Nascimento *et al.* (2010), Sugimura *et al.* (2015)
			None	Park and Yu (2013), Appeltant *et al.* (2015)
	iAC	Bovine	Improved	Luciano *et al.* (1999, 2004), Guixue *et al.* (2001)
		Bovine	None	Aktas *et al.* (1995)
	Forskolin	Human	Improved	Shu *et al.* (2008)
		Murine	Improved	Albuz *et al.* (2010), Zeng *et al.* (2013, 2014), Richani *et al.* (2014*b*)
		Bovine	Improved	Ali and Sirard (2005), Albuz *et al.* (2010), Li *et al.* (2016)
		Bovine	None/Decreased	Ulloa *et al.* (2014), Guimaraes *et al.* (2015), Bernal-Ulloa *et al.* (2016)
		Ovine	Improved	Rose *et al.* (2013)
		Ovine	None	Buell *et al.* (2015)

*Relative to standard IVM control. As assessed by embryo development typically to the blastocyst stage.
CNP, C-type natriuretic peptide; dbcAMP, dibutyryl cAMP; iAC, invasive adenylate cyclase; IBMX, 3-isobutyl-1-methylxanthine.

substantial benefit (Table 2). The rationale for moving beyond standard IVM is that oocyte maturation does not 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 & Beers 1978).

Standard IVM (low cAMP)

Standard IVM refers to the isolation of immature COCs from antral follicles and their subsequent maturation in medium without cAMP-modulating agents (Fig. 3A). This method is based on the principles of spontaneous oocyte meiotic maturation described previously (Edwards 1965, Pincus & Enzmann 1935). Standard IVM systems typically contain FSH or other additives such as EGF, EGF-p and/or LH/hCG (Fig. 3A). FSH leads to a transient rise in COC cAMP (Li *et al.* 2012) (Fig. 3E). However, if COC collection and processing is slow (see "The oocyte collection phase" section) or if FSH is omitted from IVM, then cAMP levels fall rapidly, resulting in spontaneous meiotic resumption (Aktas *et al.* 1995, Luciano *et al.* 2004). In some species, FSH has negligible effects on MII rates and hence oocytes mature spontaneously (e.g. murine, bovine and ovine), whereas in others, FSH significantly improves MII rates, suggesting meiotic induction (e.g. porcine and human). As cAMP hydrolysis is permitted in this system, intra-oocyte cAMP levels decrease (Fig. 3E), leading to inactivation of PKA and rapid progression to GVBD (Norris *et al.* 2009, Vaccari *et al.* 2009, Li *et al.* 2012).

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 h or more; Fig. 3B), thereby preserving the moderate cAMP levels stimulated by FSH (Fig. 3E) (Nogueira *et al.* 2003*b*, Nogueira *et al.* 2006, Kawashima *et al.* 2008). Examples of PDE inhibitors used include: Org9935, cilostamide, milrinone, IBMX and CNP (Table 2). In the second phase, the inhibitor is washed out, decreasing cAMP levels and enabling oocyte maturation (Fig. 3B). In general, biphasic IVM systems lead to modest improvements in oocyte developmental competence, relative to standard IVM (see Table 2 for references).

Induced IVM (moderate cAMP)

In 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 & Beers 1978, Dekel & Sherizly 1985,

Downs *et al.* 1988). Oocyte maturation is 'induced' as meiotic maturation is inhibited in the absence of such meiosis-stimulating ligands. The actions of these ligands are mediated by CCs, as they induce GVBD in intact explanted follicles or in COCs *in vitro*, but 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 meiosis-inducing ligand (Fig. 3C) (Thomas *et al.* 2004*b*). GVBD and progression to MII occur in the presence of the meiotic inhibitor at moderate–low levels of COC cAMP (Fig. 3E). This system, 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). However, it has not been examined in oocyte developmental competence studies (see Table 2 for references).

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 (Fig. 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). Use of dbcAMP has proved highly successful and is now widely used 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 & Sirard 2005, Shu *et al.* 2008). Forskolin, in particular, leads to rapid and large increases in whole COC and intra-oocyte cAMP (Thomas *et al.* 2002, Bernal-Ulloa *et al.* 2016) to levels that approximate the spike in COC cAMP levels that occur *in vivo* in response to LH (Fig. 3E) (Wang *et al.* 2011). One such approach is 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) (Fig. 3D), in which COCs are exposed to the cAMP-elevating agent for several hours (e.g. 2 h; (pre-IVM) to up to 22–24h (biphasic)). This is 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 (Zeng *et al.* 2013, Richani *et al.* 2014*b*, Zeng *et al.* 2014, Gilchrist *et al.* 2015, Li *et al.* 2016), and in the iAC and dbcAMP approaches (Funahashi *et al.* 1997, Luciano *et al.* 1999, Guixue *et al.* 2001).

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) (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. In fact, this cAMP surge induces CC synthesis of potent meiosis-inducing factors (e.g. EGF-p (Richani *et al.* 2014*b*); see 'CC EGF signalling' section), which may recapitulate at least some of the meiosis-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). Despite the improvements across species in oocyte developmental competence using induced IVM systems (Table 2), this mode of oocyte maturation does not typically improve 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).

Oocyte collection phase

COC collection conditions and the ensuing first hour are paramount to IVM success. This is because a large part of the developmental competence acquired by the oocyte in the follicle can be lost in the first hour. During this period, the oocyte should receive key nutrient support (Frank *et al.* 2013) and activation of the oocyte's potent PDE should be 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, Luciano *et al.* 2004, Albuz *et al.* 2010), de-activation of PKA, loss of CC–oocyte gap junctions, cessation of oocyte transcription and irreversible resumption of meiosis (Luciano *et al.* 2011, Li *et al.* 2012). It is noteworthy that in the IVM literature using porcine and bovine abattoir-sourced oocytes, these COCs are invariably collected and processed in undiluted or high concentration follicular fluid, which provides the COC with nutrient and inhibits the oocyte's PDE. Therefore, the clinical practice of performing IVM oocyte pickups with saline, PBS or simple holding medium is likely to be 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).

Cyclic AMP-mediated IVM and oocyte developmental competence

Publications accumulated over the past decade provide evidence that cAMP-mediated IVM systems can lead to notably improved oocyte quality, compared with 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. Different cAMP-mediated IVM systems yield differing outcomes. Biphasic IVM and induced IVM (low cAMP) approaches typically lead to modest improvements in blastocyst yield. However, induced IVM systems producing high COC cAMP levels generally lead to larger improvements in oocyte quality (Fig. 3 and 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 are challenges, however, in working with these systems (Gilchrist *et al.* 2015). First, manipulating oocyte cAMP has major effects on oocyte meiotic kinetics, and hence timing to MII should be assessed carefully under local laboratory conditions (see "Kinetics of meiosis" section). Secondly, IVM systems, where CC and oocyte functions are acutely altered such as SPOM version 1, can be difficult to work with; therefore, strict attention to protocol is needed (Gilchrist *et al.* 2015). This led to the development of a more user-friendly SPOM version 2 (Zeng *et al.* 2013, 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.

Impact of cAMP-mediated IVM on CCs and the oocyte

As there is a clear beneficial effect of cAMP-mediated IVM systems on oocyte developmental competence (Table 2), the effect of cAMP-mediated IVM on cellular and molecular aspects of CC and oocyte function is of interest as a means to (1) provide insights into basic mechanisms regulating oocyte quality and (2) offer opportunities to further improve IVM efficiency.

CC microarray analysis

A microarray analysis of CCs after 6-h exposure to cAMP-elevating agents (forskolin + IBMX + dipyridamole; see Table 1) was performed to elucidate cAMP-induced gene networks (Khan *et al.* 2015). These culture conditions were demonstrated previously to enhance embryo development (Ali & Sirard 2005). The analysis demonstrated that cAMP significantly and specifically modulated gene expression dynamics including genes involved in cell metabolism, cell communication, signal transduction, steroidogenesis, cell survival and extracellular matrix formation (Khan *et al.* 2015). Genes involved in cell metabolism such as *GFPT2* (glutamine-fructose-6-phosphate transaminase 2) and *HK2* (hexokinase 2) were significantly upregulated by the cAMP-elevating agents, as well as genes involved in carbohydrate uptake (*SLC2A1*, solute carrier family 2-facilitated glucose transporter, members 1 and 3 respectively) and steroidogenesis (*STAR*, steroidogenic acute regulatory protein). Interestingly, downregulation of EGF pathway genes (*AREG*, amphiregulin and *HAS2*, hyaluronan synthase 2), which are involved in cumulus expansion, was observed ((Khan *et al.* 2015); see the end of "CC EGF signalling" section for temporal effects). Decreased phosphorylation of ERK1/2 supports a possible negative regulatory role of PKA in this process. These findings imply that treatment of COCs with cAMP-elevating agents upregulates genes in cell metabolism, carbohydrate uptake and steroidogenesis, and downregulates genes of the EGF pathway (Khan *et al.* 2015).

CC–oocyte gap junctional communication

The central objective of the most recent IVM systems is to preserve CC–oocyte communication, as it is 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 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.* 2004*a*). 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 & Clarke 2015), possibly via a PKA-regulated mechanism (Yun *et al.* 2012). Inhibiting COC cAMP hydrolysis, in either the CC or the 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.* 2004*a*). By contrast, using cAMP-elevating agents in IVM, such as forskolin or dbcAMP, not only prevents GJC loss but also maintains full patency for extended periods of IVM (Thomas *et al.* 2004*a*, Shu *et al.* 2008, Albuz *et al.* 2010, Li *et al.* 2016). Using cAMP-mediated approaches to preserve CC–oocyte GJCs in IVM is usually associated with an improvement in 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 not clear; GJ-mediated effects on oocyte metabolism may be important (see "COC metabolism and oocyte antioxidant defence" section), as well as effects on oocyte transcription. As outlined in

the section on 'Follicle endows the oocyte with developmental competence', addition of cAMP modulators to IVM prevents premature chromatin condensation and permits continued oocyte RNA synthesis. It has been hypothesised that this occurs via a GJ-mediated mechanism (De La Fuente & Eppig 2001, Luciano *et al.* 2011).

Kinetics of meiosis

A founding principle of cAMP-mediated IVM systems is to prevent spontaneous GVBD of oocytes upon removal from antral follicles (Gilchrist & Thompson 2007) and as such the kinetics of the meiotic cell cycle are notably different in these cAMP-mediated oocyte maturation systems (Fig. 3). GVBD occurs most rapidly under spontaneous IVM (e.g. mouse, ~1 h; bovine, ~6 h), where there is uncontrolled loss of intra-oocyte cAMP leading to the activation of MPF. Using biphasic IVM systems, GVBD is prevented as long as the cAMP modulator is present – typically, 22–48 h (Nogueira *et al.* 2003*b*, Nogueira *et al.* 2006). In induced IVM systems, GVBD is typically delayed (but not inhibited) by several hours; for example, from 1 to 3 h in mouse or from 6–7 h to 10–12 h in bovine (Thomas *et al.* 2004*a*, Albuz *et al.* 2010). Delay in time to MII depends on the type, dose and combination of cAMP modulators, but MII is either not delayed (Kim *et al.* 2008) or delayed by only several hours (Thomas *et al.* 2004*b*, Albuz *et al.* 2010, Rose *et al.* 2013). However, as GVBD is always delayed, the GVBD to MII interval is commonly shortened using induced or biphasic IVM systems, relative to standard IVM (Thomas *et al.* 2004*b*, Kim *et al.* 2008). There is strong evidence to suggest that this occurs because these IVM systems generate potent meiosis-inducing factors, likely of CC origin requiring EGFR signalling (see section on 'CC EGF signalling' (Dekel *et al.* 1988, Downs & Chen 2008, Albuz *et al.* 2010)). 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 time range 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 developmental competence.

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 & Chen 2008). The same effect is also observed in SPOM COCs exposed to AG1478 (Albuz *et al.* 2010). Moreover, genetic

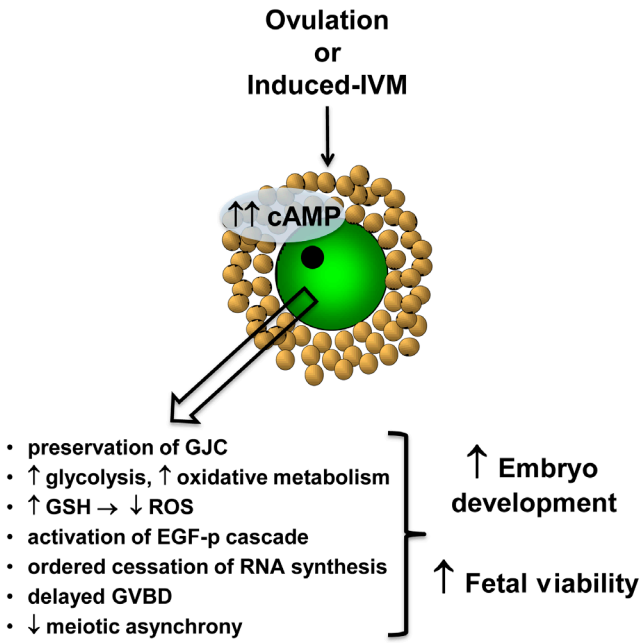

Figure 4 Possible mechanisms by which high levels of COC cAMP during *in vivo* oocyte maturation or by induced IVM improve oocyte quality. EGF-p, epidermal growth factor-like peptides; GJC, gap junctional communication; GSH, glutathione; GVBD, germinal vesicle breakdown; ROS, reactive oxygen species.

expression analysis of the effect of elevated cAMP *in vitro* implicates PKA and ERK1/2 pathways, which are interconnected with EGF receptor signalling, 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 signalling 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.* 2014*a*). Cyclic AMP elevation using forskolin leads to a large, but very transient, increased expression of CC amphiregulin, epiregulin and betacellulin compared with unstimulated or IBMX-treated COCs (Richani *et al.* 2014*b*). However, the increase in expression of EGF-like peptides does not translate into increased activation of the EGF receptor or its downstream target ERK1/2 (Richani *et al.* 2014*b*), 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 signalling 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 4 h (mouse) or 6 h (cow) (Richani *et al.* 2014*b*, Khan *et al.* 2015).

COC metabolism and oocyte antioxidant defence

cAMP-mediated IVM has an effect 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; Fig. 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). Importantly, 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 (Zeng *et al.* 2014, Li *et al.* 2016). This cAMP-mediated increase in GSH is associated with lower levels of H_2O_2, suggesting that a key benefit of cAMP-mediated IVM is an improvement in the oocyte's antioxidant defences 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, 2014). Therefore, activation of cAMP signalling pathways during oocyte maturation affects not only oocyte metabolism but also oocyte antioxidant defence, in a GJ-dependent manner (Li *et al.* 2016).

Conclusions

There is 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. 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 mechanisms by which cyclic nucleotides improve oocyte quality (Fig. 4). Nonetheless, application of cAMP modulators in IVM can present practical challenges, for example, on the timing of meiosis, and may present regulatory body 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.

Declaration of interest

The research programmes of R B G, M D V, J S and J G T have received commercial funding for the development of IVM technologies from Cook Medical.

Funding

This work was supported by grants and fellowships from the National Health and Medical Research Council of Australia (1007551, 627007, 1008137 and 1023210) and by the Agency for Innovation through Science and Technology Flanders (IWT) grant number 110680 and the Fund for Scientific Research Flanders (FWO) grant number GO34313.

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Received 21 December 2015
First decision 20 January 2016
Revised manuscript received 6 July 2016
Accepted 15 July 2016