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ORIGINAL ARTICLE *Embryology*

Simulated physiological oocyte maturation (SPOM): a novel *in vitro* maturation system that substantially improves embryo yield and pregnancy outcomes

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BACKGROUND: Oocyte *in vitro* maturation (IVM) reduces the need for gonadotrophin-induced ovarian hyperstimulation and its associated health risks but the unacceptably low conception/pregnancy rates have limited its clinical uptake. We report the development of a novel *in vitro* simulated physiological oocyte maturation (SPOM) system.

METHODS AND RESULTS: Bovine or mouse cumulus–oocyte complexes (COCs) were treated with cAMP modulators for the first 1–2 h *in vitro* (pre-IVM), increasing COC cAMP levels ~100-fold. To maintain oocyte cAMP levels and prevent precocious oocyte maturation, COCs were treated during IVM with an oocyte-specific phosphodiesterase inhibitor and simultaneously induced to mature with FSH. Using SPOM, the pre-IVM and IVM treatments synergized to increase bovine COC gap-junctional communication and slow meiotic progression (both $P < 0.05$ versus control), extending the normal IVM interval by 6 h in bovine and 4 h in mouse. FSH was required to complete maturation and this required epidermal growth factor signalling. These effects on COC had profound consequences for oocyte developmental potential. In serum-free conditions, SPOM increased bovine blastocyst yield (69 versus 27%) and improved blastocyst quality (184 versus 132 blastomeres; both $P < 0.05$ versus standard IVM). In mice, SPOM increased (all $P < 0.05$) blastocyst rate (86 versus 55%; SPOM versus control), implantation rate (53 versus 28%), fetal yield (26 versus 8%) and fetal weight (0.9 versus 0.5 g) to levels matching those of *in vivo* matured oocytes (conventional IVF).

CONCLUSIONS: SPOM is a new approach to IVM, mimicing some characteristics of oocyte maturation *in vivo* and substantially improving oocyte developmental outcomes. Adaption of SPOM for clinical application should have significant implications for infertility management and bring important benefits to patients.

Key words: oocyte maturation / *in vitro* maturation / cyclic AMP / phosphodiesterase / embryo

Introduction

In Western societies today up to 4% of all children born are conceived using assisted reproduction technologies (ARTs), with IVF now the clinically preferred method of treatment of infertility (Andersen *et al.*, 2009). Treatment using IVF is largely dependent on treating women for 1–3 weeks, often with high doses of exogenous gonadotrophin hormones to generate sufficient numbers of mature oocytes. However, hormonal stimulation of the ovary is associated with a number of significant drawbacks, including potentially serious health risks caused by severe ovarian hyperstimulation syndrome (Whelan and Vlahos, 2000), embryo aneuploidy (Baart *et al.*, 2007) and perturbed genomic imprinting (Market-Velker *et al.*, 2010), substantial patient discomfort and psychological impact, and a major financial burden to patients and/or national health care providers. The high cost of gonadotrophins alone restricts access to IVF, particularly in less developed countries.

A reproductive technology which has always shown great potential to alleviate these drawbacks of IVF is oocyte *in vitro* maturation (IVM). The major benefit of IVM over IVF is that it eliminates or drastically

reduces the need for hormonal ovarian hyperstimulation (Edwards, 2007). IVM involves harvesting immature oocytes from ovarian antral follicles, followed by culture to generate mature oocytes (Edwards, 1965), after which fertilization and embryo transfer can provide a viable pregnancy (Schroeder and Eppig, 1984). Oocyte IVM is a strategy used to generate mature oocytes for a range of applications including human infertility treatment, embryo production for livestock artificial breeding programmes, and cloning, stem cell and transgenic technologies. It is also an important research tool in reproductive and developmental biology.

Despite the clear advantages to the patient of IVM over stimulated IVF, it is noteworthy that IVM is not widely practiced in infertility treatment, even though its potential was first recognized more than 40 years ago (Edwards, 1965) and is a widespread, routine practice in cattle breeding. There are two explanations for the restricted clinical practice of human IVM; firstly, IVM has a substantially lower success rate than IVF, and secondly, there are ongoing concerns about the safety of IVM. Comprehensive, long-term follow-up studies of IVM offspring are required, although examination of small cohorts of IVM children (Shu-Chi *et al*., 2006; Soderstrom-Anttila *et al*., 2006; Buckett *et al*., 2007), detailed long-term examination of mouse IVM offspring (Eppig *et al*., 2009) and the precedent of hundreds of thousands of cattle IVM offspring (Thibier, 2006), provide an optimistic outlook in terms of the safety of IVM.

However, it is abundantly clear from the literature that current processes for IVM are at best approximately half as efficient as IVF, as measured by such parameters as live birth rate, implantation rate and blastocyst rates. A recent study in mice by Eppig *et al*. (2009) illustrates this point, reporting a live birth rate of 52% using IVF and 21% from IVM. Importantly, this discrepancy in reproductive efficiency between IVM and IVF has existed for decades and is universal across mammalian species. For example, embryo development potential is notably lower using IVM than IVF in sheep [blastocyst rate; 35 versus 75%, respectively (Thompson *et al*., 1995)], cattle (Leibfried-Rutledge *et al*., 1987; Rizos *et al*., 2002), mice (Nogueira *et al*., 2003b; Vanhoutte *et al*., 2009b) and in women [implantation rate; 9.5 versus 17%, respectively (Child *et al*., 2002)]. Moreover in women, even once a pregnancy is established using IVM, the miscarriage rate is significantly higher than in gonadotrophin-stimulated IVF (Buckett *et al*., 2008), ranging from 20 to 57% (Mikkelsen and Lindenberg, 2001; Cha *et al*., 2000, 2005). As the lower efficiency of IVM is the major impediment to its clinical application, a notable increase in IVM efficiency would have significant consequences for the management and treatment of human infertility (Edwards, 2007).

All ART clinics offering clinical IVM (human and veterinary) use a simple, standard system of IVM called spontaneous oocyte maturation. Using spontaneous IVM, oocytes irrevocably reinitiate meiosis when removed from the inhibitory environment of the follicle and are placed in culture (hereafter termed 'standard IVM'). Cumulus-oocyte complexes (COCs) are usually cultured for 24–48 h in medium designed for somatic cell culture, consisting of a complex nutrient mix with supplemented serum or albumin and specific hormones, including FSH (Sutton *et al*., 2003). In the ART clinic, laboratory aspects of the IVM technique have changed little in the past 20 years (Cha *et al*., 1991; Trounson *et al*., 1994a).

There have been some major advances in animal oocyte biology reported in recent years, which have not yet been applied to *in vitro* embryo production in any species. A critically important signalling molecule is the gonadotrophin second messenger, cyclic AMP (cAMP), which is synthesized in the oocyte by constitutively active G-protein coupled receptors (Mehlmann *et al*., 2002) and is also supplied to the oocyte by adjacent cumulus cells through gap junctions (Anderson and Albertini, 1976). High levels of oocyte cAMP keep the oocyte meiotically arrested. Importantly, the somatic cells also supply cyclic GMP (cGMP) to the oocyte (Tornell *et al*., 1991) which inhibits phosphodiesterase (PDE) activity, the enzyme that degrades cAMP. *In vivo*, the ovulatory gonadotrophin surge causes (i) a drop in follicular and oocyte cGMP levels (Norris *et al*., 2009; Vaccari *et al*., 2009) and (ii) induces a secondary cascade of epidermal growth factor (EGF)-like proteins in the somatic cells of the follicle that are required for oocyte maturation (Park, *et al*., 2004; Ashkenazi *et al*., 2005). Together these processes induce oocyte maturation as cAMP-mediated meiotic arrest is lost. This intricate process is in stark contrast to standard IVM, which occurs in the absence of the complete gonadotrophin/EGF cascade. As a result, standard IVM is likely to occur in the absence of both these critical maternal signals from cumulus cells required to coordinate major oocyte meiotic and cytoplasmic events, and components that are required for complete developmental competence of the oocyte (Gilchrist and Thompson, 2007).

We hypothesized that establishing an IVM system that induces oocyte maturation would recapitulate *in vitro* some of the inter- and intra-cellular signals that operate in the COC *in vivo* and thereby improve the quality and developmental competence of the resulting mature oocyte. We have tested this hypothesis in the two most commonly employed mammalian models of IVM: bovine IVM, currently widely used in veterinary clinical practice; and mouse, the species in which many of the recent advances in basic oocyte biology have been made. All experiments in both models, from oocyte collection through to blastocyst production, were conducted using entirely serum-free procedures. It is known that serum-free conditions impede oocyte and embryo development, however defined culture procedures are required for clinical applications. We herein report the development of a novel *ex vivo* simulated physiological oocyte maturation (SPOM) system that more than doubles embryo and fetal yield compared with standard IVM systems, to levels that match gonadotrophin-stimulated IVF.

Materials and Methods

Source of oocytes

Bovine COCs were obtained from ovaries of cows of unknown reproductive status collected at a local abattoir. COCs were aspirated within 2–3 h of collection of ovaries (held at 35–37°C) using an 18-gauge needle and a 10-ml syringe from ~3 to 8 mm follicles [mid-sized antral follicles—bovine ovulatory follicles are 12–14 mm (Sirois and Fortune, 1988)] of mixed growth and atresia status. Following standard procedures, murine COCs were collected from juvenile (21–26 days old) 129/Sv inbred mice. In broad terms of developmental competence, IVM/IVF-generated embryos from inbred mouse strains lie between outbreds and the robust F1 strains. Immature COCs were collected 46 h after administration of 5 IU of equine chorionic gonadotrophin (eCG; Folligon, Intervet, The Netherlands). For collection of *in vivo* matured control COC, mice were primed with eCG, then 46 h later with 5 IU hCG

(Pregnyl, Organon, Oss, The Netherlands), and ovulated COCs were collected 14 h later from oviducts (conventional IVF).

Oocyte collection (pre-IVM phase)

We designed oocyte collection (pre-IVM) conditions that more realistically replicated typical clinical IVM conditions than those used in research laboratories. For example, in human and bovine clinical IVM, the aspirated follicular fluid containing the immature COC is rapidly diluted or removed completely, and the COC is typically left for 0.5–2 h in either buffered culture medium or simple phosphate-buffered saline prior to IVM. This differs notably from typical research conditions, where COCs are collected and retained in pure follicular fluid (e.g. bovine) or in PDE inhibitor-containing medium [e.g. hypoxanthine, 3-isobutyl-1-methylxanthine (IBMX); mouse]. To mimic conditions used clinically, within 5 min of aspiration, bovine COCs were removed from aspirated follicular fluid and transferred into an in-house prepared HEPES-buffered collection medium containing 50 μg/ml gentamycin (Sigma) and 0.2 mg/ml fatty acid-free bovine serum albumin (BSA; ICPbio Ltd, Auckland, NZ), but lacking cAMP modulators. Removal of bovine COCs immediately from follicular fluid is not normal practice in our laboratory, and so for comparison, a further cohort of collected COCs remained in follicular fluid for ~0.5–2 h, as we know this can positively influence developmental competence by transiently maintaining cAMP levels (unpublished data).

Puncture of murine ovaries and collection of COCs was performed in HEPES-buffered alpha minimal essential medium (αMEM; Invitrogen, Carlsbad, USA) supplemented with 3 mg/ml BSA and 1 mg/ml fetuin (Sigma). Depending on individual experimental design, COCs were exposed during pre-IVM to the adenylate cyclase activator, forskolin (FSK, Sigma: bovine, 100 μM; murine, 50 μM), which potently increases whole COC and intraoocyte cAMP levels (Thomas *et al*., 2002). COCs were also treated during pre-IVM with or without the PDE inhibitor IBMX (Sigma) (bovine, 500 μM; murine, 50 μM). This is a non-specific PDE inhibitor acting on oocyte and cumulus cell PDEs. Millimolar stock concentrations of the cAMP modulators were stored at −20°C dissolved in anhydrous dimethylsulphoxide (DMSO, Sigma) solutions and were diluted fresh for each experiment. COCs were maintained in pre-IVM treatments under atmospheric conditions at 38.5°C (bovine) or 37°C (murine) for 2 h (all experiments in Figs 2–5) or 1 h (all experiments in Figs 6–8). At the end of the pre-IVM phase, COCs were washed twice in their respective IVM treatments, before transfer to IVM drops.

Oocyte IVM (IVM phase)

Bovine COCs were matured in Bovine VitroMat (IVF Vet Solutions, Adelaide, Australia) containing 4 mg/ml BSA (ICPbio Ltd), 50 μg/ml gentamycin (Sigma), and unless indicated otherwise, 100mTU/ml recombinant human FSH (Puregon, Organon). Depending on the experiment, bovine COCs were cultured throughout IVM with or without the type 3-specific PDE inhibitor, cilostamide (20 μM; Biomol, Plymouth Meeting, PA, USA). As cilostamide is five times more potent than milrinone (Tsafriri *et al*., 1996), 20 μM cilostamide is equivalent to the 100 μM milrinone we have previously used (Thomas *et al*., 2004b). Owing to the compartmentalization of PDE subtypes in the COC (Tsafriri *et al*., 1996), PDE3 inhibitors act on the oocyte and do not affect cumulus cell cAMP degradation (Thomas *et al*., 2002). In one experiment (Fig. 4C), COCs were cultured in the presence of cilostamide, either without FSH or with FSH and an increasing dose (0–1 μM) of the EGF receptor kinase inhibitor, AG1478 (Calbiochem, La Jolla, CA, USA). Cilostamide and AG1478 were added from millimolar stock solutions stored at −20°C dissolved in DMSO, and DMSO carrier controls were added to experiments (data not shown). Thirty COCs were cultured in pre-equilibrated 300 μl drops overlaid with mineral oil (Sigma) and incubated at 38.5°C with 6% CO_2 in humidified air for the time intervals indicated.

Following the pre-IVM treatments, murine COCs were matured in bicarbonate-buffered αMEM medium supplemented with FSH and antibiotic (as described above) and 3 mg/ml BSA and 1 mg/ml fetuin, to prevent zona hardening under serum-free conditions (Schroeder *et al*., 1990). COCs were matured with or without 0.1 μM cilostamide as indicated: this low concentration is based on preliminary dose–response experiments conducted to determine a non-inhibiting concentration in the presence of FSH (data not shown).Thirty to forty COCs were matured in pre-equilibrated 500 μl drops with mineral oil and incubated at 37°C with 5% CO_2 in humidified air for 18 or 22 h prior to IVF.

Assessment of COC

cAMP contents of COCs or oocytes were measured using a radioimmunoassay described and validated previously (Reddoch *et al*., 1986; Thomas *et al*., 2002). Measurements were performed on groups of 6–10 bovine COCs or 30 murine COCs. For measurement of intraoocyte cAMP levels (Fig. 2b), bovine COCs were cultured intact for 24 h, then denuded of cumulus cells by pipetting and cAMP measurements were made on groups of 21–24 denuded oocytes (4 replicates). Cumulus cell-oocyte gap junctional communication was measured in individual COCs (10–12 COCs/replicate, 4 replicate experiments) by quantitative fluorescence microscopy of calcein transfer to the oocyte as described and validated previously (Thomas *et al*., 2004a, b). Gap junctional communication was measured either after 2 h of pre-IVM or after a further 3 h of IVM with or without cilostamide. Oocyte meiotic status was assessed on paraformaldehyde fixed and permeabilized denuded oocytes using 4′,6-diamidino-2-phenylindole (Sigma) and fluorescence microscopy. Meiosis experiments were replicated 3–4 times with 40–45 oocytes/treatment for each replicate.

IVF, embryo culture and transfer

Oocyte quality was assessed by examining the capacity of the oocyte post-IVM to support preimplantation embryo development and near-term fetal development. In all experiments, pre-IVM, IVM, IVF and embryo culture were undertaken using defined, serum-free conditions. standard procedures were employed for bovine and murine IVF, embryo culture and assessment, embryo transfer and fetal assessment. Bovine IVF and embryo culture were undertaken as previously described (Hussein *et al*., 2006). In brief, following IVM for either 24 or 30 h, COCs were washed twice in Bovine VitroWash (IVF Vet Solutions) and inseminated in Bovine VitroFert (IVF Vet Solutions) with 1×10^6 frozen–thawed bull sperm. After 24 h, cumulus cells were removed by gentle pipetting and putative zygotes were cultured in 20 μl drops of Bovine VitroCleave (IVF Vet Solutions) under mineral oil at 38.5°C in 7% O_2, 6% CO_2 and balance N_2 for 5 days. On Day 5, embryos were transferred in groups of 5–10 to 20 μl drops of Bovine VitroBlast (IVF Vet Solutions) under oil and cultured to Day 8. Blinded embryo assessment was performed on Day 8 and blastocyst quality was assessed using standard differential staining procedures (Hussein *et al*., 2006). Bovine IVM/embryo experiments were replicated 4 times with 45 oocytes/treatment for each replicate.

Murine IVF and embryo culture were performed as previously described (Yeo *et al*., 2008), except media used were the Vitro Research Media series (generously donated by Cook Australia). Blastocyst differential staining was undertaken using standard methods (Gardner *et al*., 2000). Murine IVM/embryo experiments were replicated 3–4 times with 42–210 oocytes/treatment for each replicate (see figure captions for numbers in individual experiments). For embryo transfer experiments, Swiss recipient female mice were mated with vasectomized males and anaesthetized with

2% Avertin on Day 3.5 of pseudopregnancy, as described (Yeo et al., 2008). Six blastocysts from a treatment were randomly transferred to a uterine horn. A total of 192 embryos across the three treatments were transferred to 16 recipients. Pregnancy outcomes were assessed following euthanasia 3 days prior to term, on Day 18.5 of pregnancy. This enabled the collation of implantation rates, fetal survival, fetal and placental weights and fetal crown to rump lengths. All mouse experiments were approved by local animal ethics committees and conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Statistical analysis

Statistical analyses were conducted using Prism 5.00 GraphPad for Windows (GraphPad Software, San Diego, CA, USA). Treatment effects were assessed by one-way or two-way analysis of variance followed by either Dunnett's or Bonferroni's multiple-comparison *post-hoc* tests to identify individual differences between means. All values are presented as means with their corresponding SEM. Implantation rate and fetal yield were analysed using Fisher's exact test. Statistical significance was set at $P < 0.05$.

Results

We first examined the effect of maturing mouse oocytes *in vitro* versus *in vivo* on COC cAMP levels (Fig. 1). It is well known that removal of the COC from the follicle prior to IVM causes a precipitous fall in COC cAMP levels (Vivarelli *et al.*, 1983), which we also observed here (Fig. 1B). In contrast to IVM and similar to pig COC maturation *in vivo* (Mattioli *et al.*, 1994), mouse oocyte meiotic maturation *in vivo* is associated with a substantial spike in COC cAMP levels (Fig. 1A). Hence, we conducted a series of experiments with both bovine and mouse COCs to establish a pre-IVM, oocyte collection phase which (i) prevents rapid COC cAMP degradation that occurs at collection during standard IVM, and (ii) induces a substantial rise in cAMP that resembles that which occurs naturally during *in vivo* oocyte maturation.

Interactions between pre-IVM and IVM treatments

As expected (Vivarelli *et al.*, 1983; Luciano *et al.*, 2004), and consistent with the mouse results shown in Fig. 1, bovine COC cAMP levels dropped rapidly within 2 h of removal from the follicle (Fig. 2A). Treatment of bovine COCs at collection with either FSK or IBMX prevented this fall in COC cAMP levels, whereas combined FSK + IBMX treatment substantially increased COC cAMP levels by 10-fold (Fig. 2A). In the IVM phase, COCs were then treated with cilostamide, an oocyte-specific (type 3) PDE inhibitor, which therefore permits cAMP hydrolysis in the cumulus cell compartment but prevents it in the oocyte (Thomas *et al.*, 2002). Figure 2B illustrates the interacting effects of pre-IVM and IVM cAMP modulators on bovine intraoocyte cAMP levels at the end of IVM (24 h). Intraoocyte cAMP levels only remained notably elevated at the end of IVM when the cAMP modulators were included in both the pre-IVM and IVM phases. IVM treatments alone (cilostamide + FSH) had no effect on oocyte cAMP levels, whereas FSK + IBMX in pre-IVM followed by standard IVM (+FSH) led to a moderate 2-fold increase ($P < 0.05$). Hence, while FSK + IBMX in the pre-IVM phase substantially increased COC cAMP levels, the inclusion of the PDE3 inhibitor

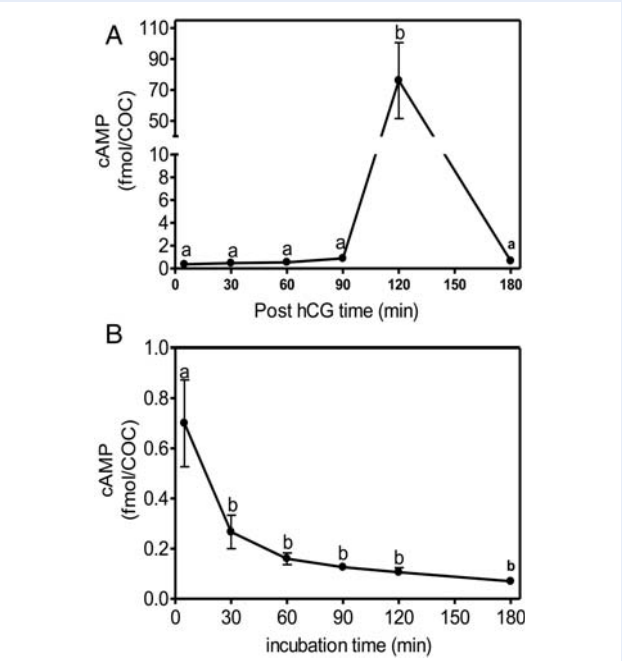

Figure 1 cAMP concentrations in murine COC during *in vivo* maturation (**A**) and during standard IVM (**B**). Mice were primed in both groups with 5 IU equine chorionic gonadotrophin (eCG). To generate *in vivo* maturing COC, mice were injected with 5 I U hCG 46 h after eCG, and groups of COC were collected at 30-min intervals after hCG administration. For standard IVM, 46 h after eCG treatment COCs were collected using typical IVM collection conditions (i.e. no FSH). Data points represent mean cAMP/COC ± SEM of three replicates. Each measurement was conducted on 30 COCs. Means within a graph with different letters are significantly different ($P < 0.05$).

cilostamide in the IVM phase retarded the decline in intraoocyte cAMP levels, that normally occurs during standard IVM (Fig. 2). This had multiple profound consequences for COC function.

Firstly, FSK + IBMX in the pre-IVM phase significantly increased bovine oocyte-cumulus cell gap-junctional communication (Fig. 3). In the absence of this pre-IVM treatment, oocyte-cumulus gap-junction communication was rapidly lost during early IVM, although this loss was partially attenuated by the inclusion of the PDE3 inhibitor cilostamide, as we have previously reported (Thomas *et al.*, 2004a). Effects of the cAMP modulators in pre-IVM persisted into the IVM phase and appeared to be unaffected by the presence of cilostamide during IVM (Fig. 3). Secondly, treatments that enhanced oocyte-cumulus gap-junction communication simultaneously slowed the resumption and completion of oocyte meiotic maturation (Fig. 4A and B and data not shown), consistent with our previous work (Thomas, *et al.* 2004a, b). Seventy percent of oocytes had not resumed meiosis after 9 h (2 h control pre-IVM + 7 h IVM) when treated with cilostamide during IVM, compared with 30% in controls (Fig. 4A). Notably, presence of FSK + IBMX during pre-IVM resulted in >90% of oocytes remaining meiotically arrested at the germinal vesicle stage (GV) after 9 h of IVM, irrespective of the presence or absence of cilostamide ($P < 0.05$, Fig. 4A). Consequently, the completion of oocyte maturation was significantly ($P < 0.05$) delayed by 4–6 h when the cAMP

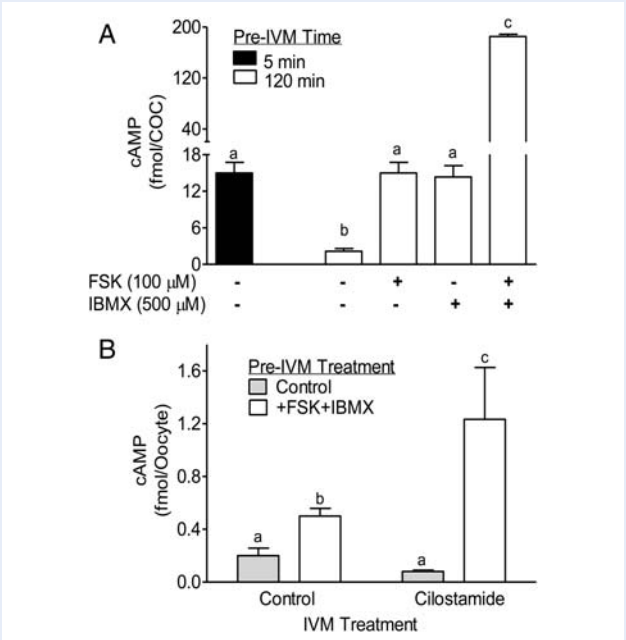

Figure 2 Effect of cAMP modulators during pre-IVM (**A**) and/or during IVM (**B**) on bovine COC/oocyte cAMP levels. (A) cAMP levels were measured in intact whole bovine COCs either immediately after follicle aspiration (5 min) or after 120 min in pre-IVM plus/minus IBMX (PDE inhibitor) and/or FSK (adenylate cyclase activator). Each data observation was made on 6–10 pooled COCs and the experiment was replicated three times. (B) Intraoocyte cAMP levels after 24 h of IVM. A 2 × 2 factorial experiment was conducted where bovine COCs were treated in pre-IVM (120 min) with or without IBMX + FSK and in IVM with or without cilostamide (type 3-specific PDE inhibitor, 20 μM) in the presence of FSH. COCs were denuded of cumulus cells after 24 h of IVM and oocytes immediately snap frozen. cAMP measurements were made on pools of 24 denuded oocytes and the experiment was replicated four times. Data are mean ± SEM. Means within a graph with different letters are significantly different ($P < 0.05$).

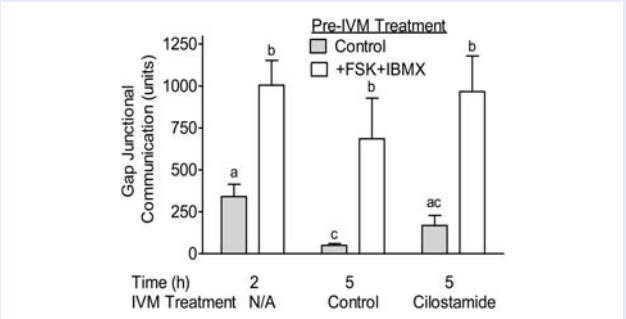

Figure 3 Effect of cAMP modulators during pre-IVM and/or during IVM on bovine cumulus-oocyte gap-junctional communication. Intact bovine COCs were treated in pre-IVM (120 min) with or without IBMX + FSK and in IVM with or without cilostamide (20 μM) in the presence of FSH. Levels of cumulus cell to oocyte gap junctional communication were quantified at the end of pre-IVM (2 h) and after an additional 5 h of IVM, using a fluorometric assay (units = relative fluorescence intensity). Ten to twelve COCs were assessed for each treatment in each of four experimental replicates. Data are mean ± SEM. Means with no common superscripts are significantly different ($P < 0.05$).

modulators were included in both the pre-IVM and IVM phases (Fig. 4B). While these agents were effective alone in either pre-IVM or IVM at delaying resumption of meiosis, the combination of pre-IVM and IVM treatments was required to achieve a notable delay beyond 20 h in the completion of oocyte maturation (Fig. 4B).

A further innovative feature of the novel IVM system described here, and one that distinguishes it from standard IVM and IVM systems that arrest meiosis, is that FSH induced oocyte maturation by overriding the meiosis-inhibiting effects of the elevated cAMP (Fig. 4C). To achieve this, a relatively high dose of FSH (100 mIU/ml) was required (data not shown). Important recent advances in the field of ovarian biology have revealed that oocyte maturation *in vivo* requires EGF signalling in the somatic cell compartment of the follicle (Park *et al.*, 2004; Ashkenazi *et al.*, 2005; Shimada *et al.*, 2006; Downs and Chen, 2008). When bovine oocytes underwent pre-IVM with FSK + IBMX and were matured with cilostamide in the absence of FSH, <40% of the oocytes completed meiosis (metaphase II stage) by 24 h, compared with >80% in the presence of FSH (Fig. 4C). Furthermore, FSH-induction of oocyte maturation required secondary EGF receptor-mediated signalling within the cumulus cells, similar to oocyte maturation *in vivo*, as evidenced by the ability of the EGF receptor tyrosine kinase inhibitor AG1478, at a concentration of 1 μM, to completely prevent the FSH-induced oocyte maturation (Fig. 4C).

This IVM system, hereafter referred to as 'SPOM', entails: (i) a pre-IVM phase of 1–2 h that substantially elevates COC/oocyte cAMP, (ii) an IVM phase containing a type-3 PDE inhibitor with (iii) simultaneous hormonal-induced oocyte maturation and (iv) an extended IVM interval.

SPOM substantially improves bovine embryo yield

To determine the efficacy of the above modifications in pre-IVM and IVM conditions to enhance oocyte developmental competence post IVF, the efficiency of embryo production by the new SPOM system was compared with that of the methods currently most widely used in clinical bovine practice, termed standard Clinical (Bovine) IVM. Oocyte quality post-IVM was assessed using entirely serum-free systems of IVM, IVF and embryo production. To simulate human and veterinary clinical oocyte collection conditions, COC were rapidly removed from follicular fluid after follicle puncture and placed in simple buffered collection media, as typically occurs during clinical bovine and human IVM. Removing COCs from follicular fluid at collection compromised subsequent oocyte developmental competence, significantly ($P < 0.05$) reducing blastocyst rates (blastocysts/2 cells) from the industry standard of 40% (Lonergan, 2007) when collected in follicular fluid to just 26% when collected in simple buffered medium. 'standard clinical IVM' refers to this process, as collection and processing of human oocytes in pure follicular fluid is not clinically practical.

Compared with standard clinical IVM, SPOM increased embryo cleavage rates when the IVM period was extended to 30 h (Fig. 5A). SPOM increased blastocyst rates at both IVM time points, however the exceptional yield of 69% blastocysts was only achieved with

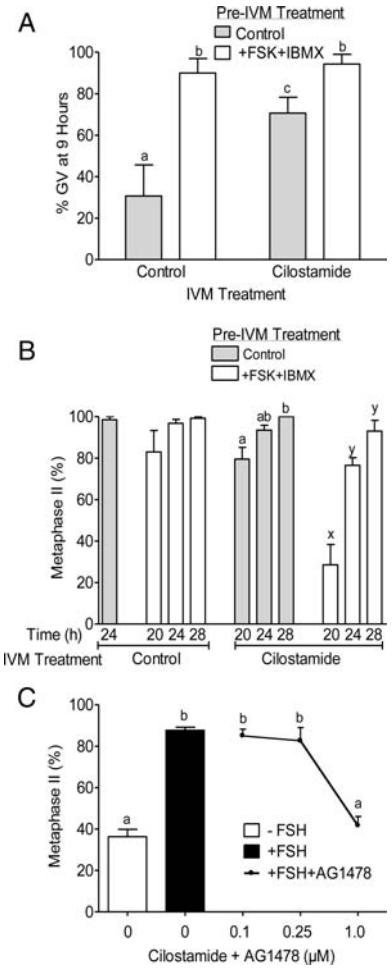

Figure 4 Interacting effects of pre-IVM and IVM conditions on the induction of bovine oocyte meiotic maturation. (**A**) Effect of pre-IVM (with or without IBMX + FSK) and IVM (with or without cilostamide) on oocyte meiotic arrest (GV) after 9 h. Oocytes ($n = 45$) were assessed/treatment in each of four replicate experiments. Means with different superscripts are significantly different ($P < 0.05$). (**B**) The time required to undertake meiosis to metaphase II is affected by pre-IVM and IVM treatments. Forty-five oocytes were assessed/treatment in each of four replicate experiments. Means with no common superscripts are significantly different ($P < 0.05$). (**C**) Dose effect of the EGF receptor inhibitor AG1478 on FSH-induced oocyte maturation. Bovine COC underwent *in vitro* SPOM (pre-IVM with IBMX + FSK and IVM with cilostamide plus/minus FSH). FSH induction of oocyte maturation was prevented by 1 μM of AG1478. Forty oocytes were assessed/treatment in each of three replicate experiments. Data are mean ± SEM. Means with different superscripts are significantly different ($P < 0.05$).

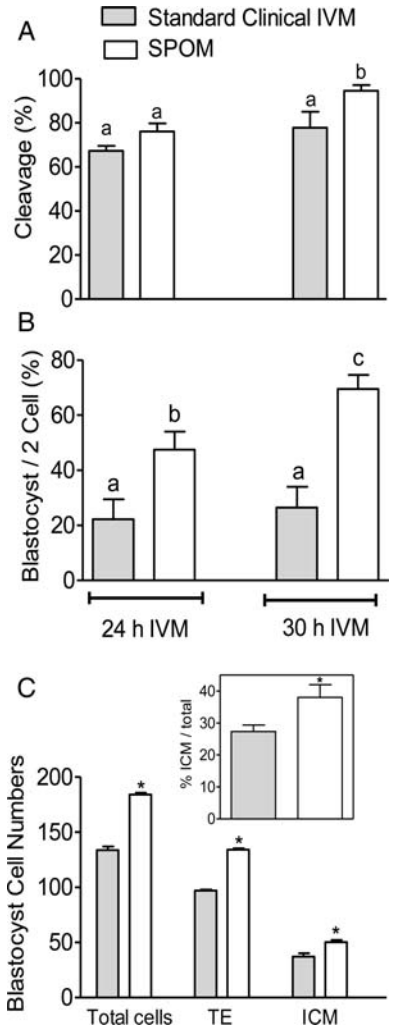

Figure 5 The effect of maturing bovine oocytes *in vitro* using standard clinical IVM or SPOM, for either 24 or 30 h, on oocyte developmental capacity and blastocyst quality. Oocyte developmental capacity was assessed after IVF and embryo development by cleavage rate (**A**, Day 2) and blastocyst rate (**B**, Day 8). standard clinical IVM = control pre-IVM and standard IVM with FSH. SPOM = - pre-IVM with FSK + IBMX and IVM with cilostamide + FSH. (A and B) Columns represent mean ± SEM of 4 replicates with 45 oocytes/treatment for each replicate. Means within a graph with different letters are significantly different ($P < 0.05$). (**C**) Effect of standard Clinical IVM and SPOM on bovine blastocyst quality. Following 30 h of IVM, COCs were fertilized and embryos cultured until Day 8 and then blastocyst quality was quantified by total cell counts and cell allocation to trophoectoderm (TE) or ICM. Columns represent mean ± SEM of 20 blastocysts from 4 replicate experiments. *Significantly different to standard clinical IVM ($P < 0.05$).

delayed insemination (Fig. 5B). These blastocysts were also of higher quality than those generated by standard clinical IVM, as evidenced by significantly ($P < 0.05$) increased total blastomeres, including more trophectoderm and inner cell mass (ICM) cells, and a higher ratio of ICM to total cells (Fig. 5C). SPOM generated bovine blastocysts contained on average 184 blastomeres which is comparable to the 195 cells in *in vivo* generated blastocysts (de la Fuente and King, 1997).

SPOM increases embryo yield and quality in the mouse IVM model

Similar approaches to those used in bovine studies (above) were employed to evaluate the efficacy of the pre-IVM and IVM modifications to enhance oocyte quality in the mouse. Preliminary studies showed

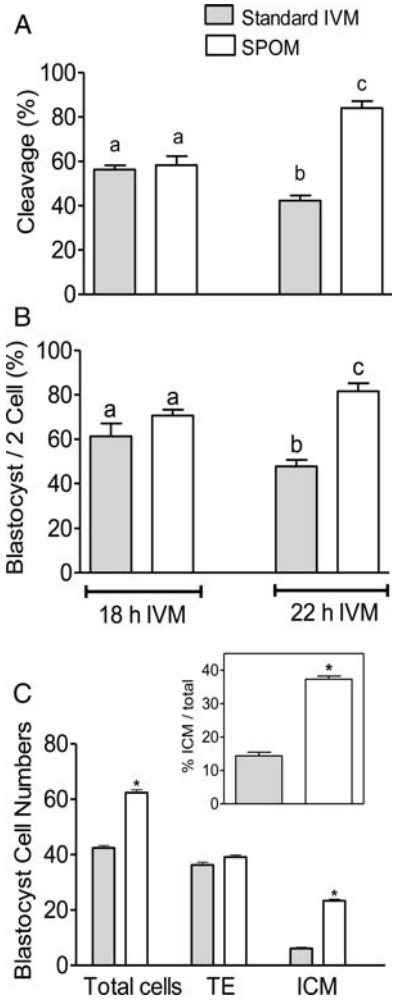

Figure 6 The effect of maturing murine oocytes *in vitro* using standard IVM or SPOM, for either 18 or 22 h, on oocyte developmental capacity and blastocyst quality. Oocyte developmental capacity was assessed after IVF and embryo development by cleavage rate (**A**, Day 2) and blastocyst rate (**B**, Day 5). standard IVM = control pre-IVM and standard IVM with FSH. SPOM = pre-IVM with FSK + IBMX and IVM with cilostamide + FSH. Columns represent means ± SEM of 3 replicates with 45 oocytes/treatment for each replicate. Means within a graph with different letters are significantly different ($P < 0.05$). (**C**) Effect of standard IVM and SPOM on murine blastocyst quality. Following 22 h of IVM, COCs were fertilized and embryos cultured until Day 5 and then blastocyst quality was quantified by total cell counts and cell allocation to TE or ICM. Columns represent mean ± SEM of 20 blastocysts from 3 replicate experiments. *Significantly different to standard IVM ($P < 0.05$).

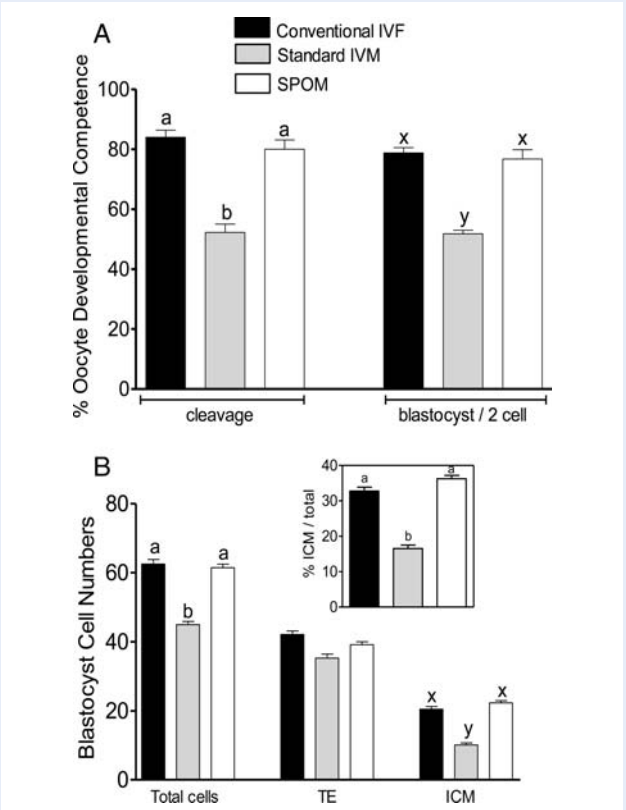

Figure 7 Developmental competence of murine oocytes matured either *in vivo* (conventional IVF), by SPOM or by standard IVM. Conventional IVF = mice were hyperstimulated with eCG and hCG and COC were collected from oviducts 14 h after hCG administration. standard IVM = control pre-IVM and standard IVM with FSH and fertilization at 18 h. SPOM = pre-IVM with FSK + IBMX and IVM with cilostamide + FSH and fertilized at 22 h. Mature COC (42–210/treatment/replicate, 4 replicates) were fertilized and embryos cultured until Day 5 (**A**) and then blastocyst quality (30 blastocysts from 4 replicates) was quantified by total cell counts and cell allocation to TE or ICM (**B**). Columns represent mean ± SEM. Means within a graph for each end-point assessed with different letters are significantly different ($P < 0.05$).

that, consistent with the bovine results in Fig 4B, SPOM extended the interval required for mouse oocytes to reach metaphase II (data not shown). Compared with standard IVM, the complete SPOM system in mouse increased cleavage and blastocyst rates (Fig. 6A and B), but only when insemination was delayed from 18 to 22 h. SPOM also improved embryo quality, reflected in an increased total number of cells compared with standard IVM, which was made up largely of an increased ICM size rather than by trophectoderm cells (Fig. 6C).

Blastocysts produced by SPOM contained on average 62 blastomeres which is comparable to the 72 cells from *in vivo* grown blastocysts (de la Fuente and King, 1997). We next compared the efficacy of the optimized SPOM system to standard IVM and to oocytes matured *in vivo* (conventional IVF using ovarian hyperstimulation). As expected, standard IVM yielded significantly lower cleavage and blastocyst rates (Fig. 7A) and blastocyst quality (Fig. 7B), compared with conventional IVF. Notably, the higher efficiency and quality of embryos generated by conventional IVF were matched in all outcomes assessed when oocytes were matured *in vitro* using SPOM (Fig. 7).

SPOM matches conventional IVF in mouse pregnancy outcomes

The mode of oocyte maturation had profound long-term developmental consequences for embryo and fetal survival and for normal

fetal and placental development (Fig. 8). Murine blastocyst transfer on Day 4.5 to pseudo-pregnant recipient mothers followed by euthanasia 3 days prior to term allowed us to assess in detail multiple developmental parameters. Embryo implantation rate and fetal yield (fetus per embryo transferred) were relatively low (48 and 22%, respectively) in the positive control group (conventional IVF; Fig. 8A and B), as expected, given the serum-free IVM and embryo production conditions and the use of an inbred (129/Sv) mouse strain. Consistent with mouse (Nogueira *et al.*, 2003b; Eppig *et al.*, 2009) and human (Child *et al.*, 2002; Buckett *et al.*, 2008) published results, developmental outcomes were substantially lower when oocytes were matured by standard IVM compared with conventional IVF. In contrast, SPOM approximately doubled the implantation rate (53 versus 28%, $P < 0.01$) and led to a 3-fold increase in fetal yield (26 versus 8%, $P < 0.05$) compared with standard IVM, both to levels that matched those achieved using conventional IVF ($P > 0.05$; Fig. 8A and B).

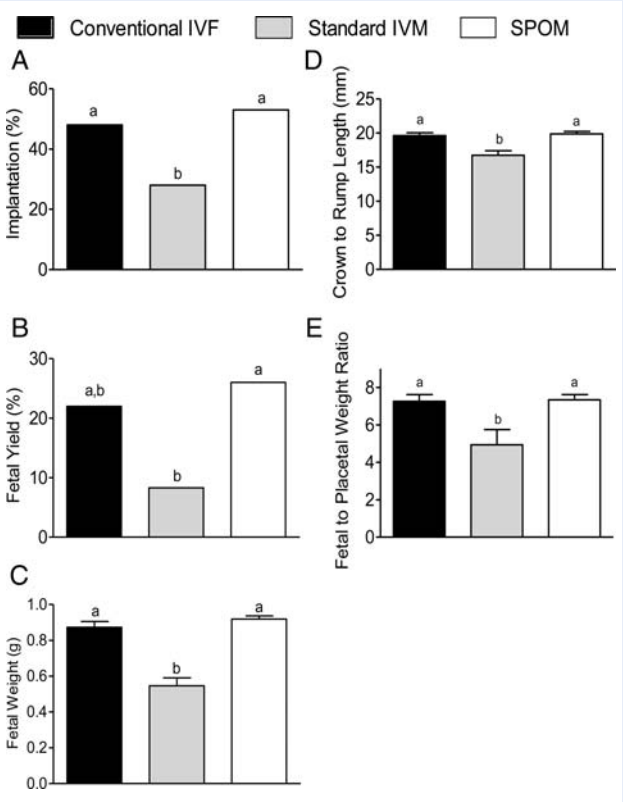

Figure 8 Effect of mode of oocyte maturation on murine pregnancy outcomes and fetal parameters. Day 4.5 blastocysts developed from COCs matured *in vivo* (conventional IVF) or by SPOM or standard IVM, were transferred to pseudo-pregnant recipients and outcomes analysed on Day 18.5 of pregnancy. Conventional IVF = mice were hyperstimulated with eCG and hCG and COCs were collected from oviducts 14 h after hCG administration. standard IVM = control pre-IVM and control IVM with FSH and fertilization at 18 h. SPOM = pre-IVM with FSK + IBMX and IVM with cilostamide + FSH and fertilized at 22 h. (**A**) Implantation rate = implantation sites/embryos transferred; (**B**) fetal yield = day 18.5 fetuses/embryos transferred; (**C**) fetal weight; (**D**) fetal crown to rump length; (**E**) ratio of fetal to placental weight. Columns represent mean ± SEM of 5–16 (range) fetuses from 4 replicates. Means within a graph with different letters are significantly different ($P < 0.05$).

Apart from improving reproductive efficiency, SPOM also normalized fetal development. Fetal weight and length were reduced (Fig. 8C and D) as a consequence of maturing oocytes by standard IVM compared with conventional IVF. However, maturing oocytes *in vitro* using SPOM increased fetal weight and length ($P < 0.05$), normalizing fetal development to conventional IVF sizes. Mode of oocyte maturation had no effect on placental weight (data not shown), however the fetal-to-placental weight ratio was significantly lower when oocytes were matured by standard IVM compared with either SPOM or conventional IVF (Fig. 8E).

Discussion

This study describes a new IVM methodology that substantially improves on existing IVM techniques to generate pregnancy outcomes equivalent to conventional IVF but without the need for hormone treatment. The SPOM system entails a number of components that combine to generate high embryo and fetal yields following embryo transfer. Critical to success of the approach is a pre-IVM phase that generates a rapid increase in COC cAMP levels. Secondly, the system utilizes an extended IVM phase containing sufficient FSH to drive meiotic induction in the presence of a type-3 PDE inhibitor (Fig. 9). The high levels of cAMP in the oocyte and the induced nature of oocyte maturation mimics some of the key, newly characterized molecular signals that occur during oocyte maturation *in vivo*. The SPOM system utilizes specific cAMP modulating agents, previously described in terms of their effects in isolation on oocyte meiosis. However, this study systematically examines the interactions of these individual agents into a new oocyte IVM system specifically designed for embryo production. Technical and conceptual elements were first developed using bovine COCs. Mouse studies were conducted to provide three key additional lines of evidence that were not provided from the bovine data: (i) advanced fetal development and safety data, (ii) direct comparison of SPOM to conventional IVF and (iii) demonstration that the concept of SPOM can be adapted to another mammalian species despite appreciably different oocyte biochemistry.

Clinical practice of IVM uses a system of 'spontaneous IVM', usually containing serum. Using this approach, COCs are aspirated from unstimulated or mildly stimulated ovaries and rapidly removed from the meiotic-inhibiting influence of the follicle and the follicular fluid. Regardless of *in vitro* gonadotrophin treatment, oocytes mature spontaneously *in vitro*, hence undergoing meiotic resumption in the absence of the usual elaborate cascade of endocrine and paracrine molecular signals that induce maturation *in vivo* (Park *et al.*, 2004; Ashkenazi *et al.*, 2005; Shimada *et al.*, 2006; Norris, *et al.*, 2009; Vaccari *et al.*, 2009). As such, the maturation of oocytes by standard IVM techniques is an artefact that compromises subsequent oocyte developmental competence. standard spontaneous oocyte IVM can be regarded as a form of 'precocious' oocyte maturation, because the oocyte would otherwise still be developing *in vivo* acquiring developmental competence (Fair *et al.*, 1995). Spontaneous maturation causes a premature breakdown of oocyte-cumulus cell gap junctions (Thomas *et al.*, 2004a), leading to loss of beneficial cumulus cell metabolites,

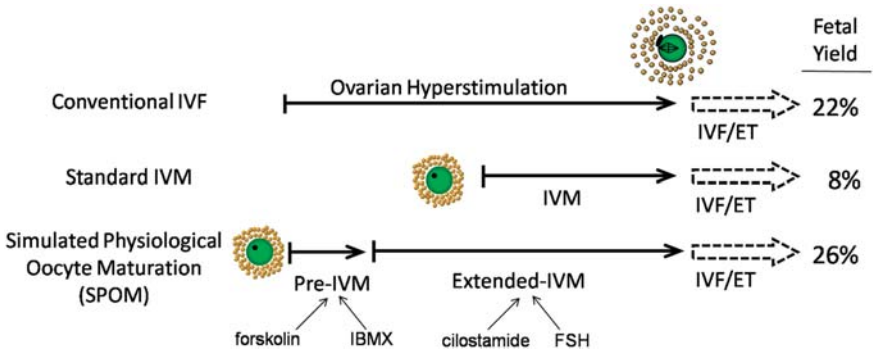

Figure 9 Model illustrating the methodology of SPOM and the fetal yields in mice relative to standard IVM and conventional IVF. SPOM differs from standard IVM with a short pre-IVM phase (COC collection) where COCs are exposed to agents that cause a rapid increase in cAMP. The SPOM system also requires an extended IVM phase where oocytes are induced to mature with FSH in the presence of cilostamide, an oocyte-specific PDE inhibitor. SPOM generates fertilization, blastocyst and implantation rates and fetal yield comparable to hormone-driven conventional IVF.

such as nucleotides and nutrients (Gilchrist and Thompson, 2007). In light of this we have taken the approach of delaying or temporarily preventing spontaneous IVM, in an effort to improve the quality of the developing ooplasm by prolonging interaction with the surrounding cumulus cells *in vitro*.

Experimental approaches to improve the developmental competence of IVM oocytes by regulating spontaneous IVM have largely focused on a 'biphasic IVM' strategy (Downs *et al*., 1986). Using this approach, oocyte meiotic resumption is initially blocked *in vitro* using high concentrations of cAMP analogues or kinase or PDE inhibitors for ~24 h, which are then washed out, allowing the oocyte to undergo spontaneous IVM (reviewed in Gilchrist and Thompson (2007). Biphasic IVM has proven beneficial in porcine IVM (Funahashi *et al*., 1997) where it is now routinely employed, however, it has been less successful in most other species. In mouse (Downs *et al*., 1986; Nogueira *et al*., 2003b; Vanhoutte *et al*., 2009b), cattle (Aktas *et al*., 1995a) and humans (Nogueira *et al*., 2003a, 2006; Shu *et al*., 2008; Vanhoutte *et al*., 2009a, b), biphasic IVM leads to either little or no improvement in embryo yields above standard IVM. Despite major prolonged effort, there remains no established IVM technology that matches conventional IVF for pregnancy outcomes. The SPOM system described here differs notably from biphasic and standard IVM techniques in a number of important ways.

The most important innovation is the inclusion of a pre-IVM phase of 1–2 h including cAMP modulating agents (FSK + IBMX) that generate a substantial (>100-fold) spike in COC cAMP levels within minutes of collection. In mouse meiosis studies, COCs are commonly collected in medium containing IBMX. Collecting COC in IBMX or in pure follicular fluid protects the rapid loss of cAMP that otherwise occurs in standard clinical IVM, but does not notably increase COC cAMP levels (Vivarelli *et al*. 1983; Aktas *et al*., 1995b). Collecting bovine COC in the presence of invasive adenylate cyclase has previously been reported (Aktas *et al*., 1995b; Luciano, *et al*., 1999; Guixue *et al*., 2001), and in the current study the addition of FSK to pre-IVM was necessary to generate the rapid large increase in cAMP that resembles the *in vivo* increase in COC cAMP levels and to achieve high embryo yields. These pre-IVM treatments were designed to target the cumulus cell compartment in particular. We and others have previously shown that an effective way to rapidly increase oocyte cAMP levels during early IVM is to load the oocyte, via gap junctions, with cAMP generated in the cumulus cells (Anderson and Albertini, 1976; Thomas *et al*., 2002). These pre-IVM treatments had long-acting and significant effects on subsequent COC functions and developmental programming of the oocyte. Consistent with our previous approaches (Thomas *et al*., 2004a, b), the cAMP modulators increased the level, and extended the interval of, oocyte-cumulus cell gap junctional communication. This met our objectives of preventing precocious resumption of meiosis while simultaneously promoting oocyte-cumulus cell exchange, elevating intraoocyte cAMP levels at the end of IVM thereby delaying the completion of meiotic maturation.

A second important feature of this new approach to IVM is that oocytes are continuously exposed throughout maturation to a type-3 specific PDE inhibitor. The important discovery that PDE subtypes are compartmentalized in the ovarian follicle (Tsafriri *et al*., 1996) has enabled the exploitation of subtype-specific PDE inhibitors to target either cumulus cells or oocyte PDEs (Gilchrist and Thompson, 2007). In our SPOM system, the non-specific PDE inhibitor IBMX is used in the pre-IVM phase to inhibit both cumulus cell and oocyte PDEs, whereas in the IVM phase the PDE3-specific inhibitor cilostamide is used to regulate the oocyte PDE only. PDE3 inhibitors have been examined in experimental IVM systems, but nearly always using a biphasic IVM approach (Nogueira *et al*., 2003a,b, 2006; Shu *et al*., 2008; Vanhoutte *et al*., 2009a, b). Importantly, the current approach differs as the PDE3 inhibitor is not washed out at any stage of IVM, as occurs in biphasic IVM. Hence, non-inhibiting, low to moderate concentrations of the PDE3 inhibitor are required to allow the oocyte to mature. We have previously shown that attenuating the pace of meiotic maturation by continuously exposing oocytes to a PDE3 inhibitor during IVM improves subsequent oocyte developmental potential (Thomas *et al*., 2004b). Consistent with this, in the current study, the full benefits in terms of blastocyst production were only observed when the IVM interval was extended by 4 h in the mouse and by 6 h in cattle (Figs 5–6). Under these conditions, presumably the oocyte developmental programme benefits in some manner from a more appropriate G2 to M-phase cell cycle

resumption and the extended period of gap junctional communication between the oocyte and cumulus cells, when compared with standard IVM.

Perhaps the most significant advance described in the current study is that the pre-IVM and IVM treatments combine to induce oocyte maturation *in vitro*. Using SPOM, oocytes do not spontaneously mature as occurs using standard IVM. To achieve meiotic maturation, a relatively high dose of FSH (100 mIU/ml) was required to override the meiotic-inhibiting effects of the PDE3 inhibitor. In the absence of FSH or with low-dose FSH, few oocytes matured. Hence, the IVM phase of our new system requires a balance between the mildly inhibiting effects of the PDE3 inhibitor and the inducing effects of FSH. This feature shares some similarities with the well established induced rodent model which has been used for decades to study rodent oocyte meiosis but curiously, to the best of our knowledge, has rarely if ever been used to generate embryos. In the rodent induced oocyte maturation model, oocytes are stimulated to mature by FSH or EGF to override a meiotic arresting agent, most commonly hypoxanthine (Downs *et al*., 1988). Importantly, our SPOM system differs from this rodent model as our pre-IVM phase dramatically increases COC cAMP levels which potentiate the effects of the PDE3 inhibitor during IVM.

In vivo, the ovulatory gonadotrophin surge overrides the meiotic-inhibiting effects of the pre-ovulatory follicle to induce oocyte maturation. Important recent studies have demonstrated that gonadotrophins cause a secondary cascade of follicular EGF-like peptides which act on the cumulus cell EGF receptor to induce maturation requiring an ERK1/2-dependent mechanism (Park *et al*., 2004; Ashkenazi *et al*., 2005; Shimada *et al*., 2006; Downs and Chen, 2008; Fan *et al*., 2009). Consequently, inhibition of signalling through the EGF receptor using AG1478 blocks induced oocyte maturation *in vivo* (Park, *et al*., 2004; Ashkenazi *et al*., 2005). In the current study, FSH was required for meiotic maturation and the EGF receptor kinase inhibitor AG1478 blocked FSH-induced oocyte maturation. Hence, in the SPOM system, FSH is inducing meiotic resumption by a mechanism that requires the EGF receptor, which is likely to involve FSH/cAMP-induced expression of EGF-like peptides by cumulus cells (Downs and Chen, 2008). Hence, SPOM recapitulates some of the molecular signalling events in cumulus cells that occur during oocyte maturation *in vivo*. This enables us to hypothesize that activation of this signalling cascade is associated with the increased developmental potential of SPOM oocytes and is absent or defective in oocytes undergoing standard IVM, contributing to their impaired quality. Alternatively, it is possible that SPOM is able to improve the developmental competence of oocytes from a specific cohort of the heterogeneous population of follicles in bovine ovaries (e.g. atretic or small antral follicles). Further studies are required to test these hypotheses.

IVM is not routinely employed for the treatment of human infertility in any country, despite substantial efforts over the past 20 years to adapt the technique to humans (Trounson *et al*., 1994b; Cha *et al*., 2005; Soderstrom-Anttila *et al*., 2005). While approximately 35 000–50 000 children are conceived each year worldwide from IVF/ICSI (Collins, 2002), it is estimated just 1300 IVM babies have been born over the past 30 years (Suikkari, 2008). IVM is not a routine clinical procedure because of the notably lower success rates of IVM compared with IVF. The most accurate measure of a clinical ART success rate is implantation rate, as pregnancy rates and live birth rates are compounded by the number of embryos transferred. For the past decade, IVM implantation rates have averaged ~12% and varied from 5.5 to 15.7% (Chian *et al*., 2000; Cha *et al*., 2005). In stark contrast, the implantation rate in a conventional IVF cycle is 25–40% in women between 20 and 35 years. It is important to note that, in addition, the miscarriage rate is also significantly higher from IVM compared with IVF (Buckett *et al*., 2008). This considerably lower success rate of IVM compared with IVF is not restricted to the human but is consistent across mammalian species, including in mice (Nogueira, *et al*., 2003b; Eppig *et al*., 2009; Vanhoutte *et al*., 2009b), cattle (Leibfried-Rutledge *et al*., 1987; Rizos *et al*., 2002) and sheep (Thompson *et al*., 1995).

To gauge the value of a new ART technique, markers of IVM/IVF efficiency, such as implantation and blastocyst rates must be carefully interpreted in a species-specific and research versus clinical context. In cattle artificial breeding programmes, standard IVM using serum typically generates 25–35% blastocyst yields with reported rates rarely exceeding 40% (Lonergan, 2007) and these rates are economically marginal. Nevertheless, ~133 000 viable cattle IVM offspring were produced in 2005 alone (Thibier, 2006). Our new SPOM system improves bovine blastocyst rates to 69% in a completely serum-free system which is a major increase in efficiency on current methodologies. In these experiments, we used a standard clinical IVM control (COC collection and processing in simple medium) and a standard research IVM control (collection in follicular fluid). COC collection conditions in the cattle and human clinics are comparable, as in both cases COCs are always rapidly removed from follicular fluid and placed in PDE inhibitor-free media. This causes a rapid loss of COC cAMP resulting in precocious spontaneous oocyte maturation. In contrast, in cattle research IVM laboratories, COCs are collected and processed for up to 2 h usually in pure follicular fluid, which typically yields blastocyst rates of 40% using standard IVM (Hussein *et al*., 2006). Collecting in follicular fluid retards COC cAMP degradation and does not enhance cAMP levels (data not shown), but this is impractical for human and veterinary clinical applications.

In contrast to cattle, blastocyst rates have less significance as an indicator of developmental competence in the mouse model, where rates are widely variable depending on the choice of mouse strain and the oocyte/embryo culture conditions, and especially influenced by the use of serum. As oocytes from hybrids of inbred strains (F1 mice) are more robust, in the current study we used relatively sensitive mouse oocyte/embryo conditions by choosing an inbred mouse strain (129/Sv) and serum-free culture conditions throughout. Consistent with other serum-free standard IVM systems (Preis *et al*., 2007), this yielded moderate control blastocyst rates (~50%) and poor post-transfer outcomes. Despite these adverse conditions, SPOM improved fertilization and blastocyst rates compared with standard IVM, and substantially increased implantation rate and fetal yield. Developmental outcomes were improved to such an extent using the SPOM methodology that they matched outcomes from oocytes matured *in vivo* using conventional IVF.

This study has demonstrated a substantive advance in IVM using the two most commonly employed experimental models of mammalian oocyte biology, but which have disparate oocyte biochemistries. Knowledge gained in relation to the differing SPOM systems in these two differing models should enable translation of the methodology to human clinical IVM. A seminal finding of this study is that the first

1–2 h after oocyte collection have profound long-term consequences on oocyte developmental programming. Current methodologies in clinical IVM pay no particular attention to oocyte collection conditions, especially oocyte cAMP, and the pre-IVM techniques used in the current study can be adapted to the human oocyte. Given that currently the primary impediment to the uptake of IVM is its lower success rate compared with conventional IVF, and that SPOM achieves pregnancy outcomes equivalent to IVF, application of this new technique would dramatically increase the use of IVM for the treatment of infertility. The single most important benefit of IVM is that it spares patients the need for hormone treatment, thereby reducing adverse side effects for women and bringing substantial financial savings to patients and health care providers. Wider application of IVM will also provide access to treatment for patients who react particularly adversely to hormones, such as women with polycystic ovaries, and provide treatment options where there are currently few in less affluent sectors especially in developing countries (Jurema and Nogueira, 2006).

Authors' roles

R.B.G. and J.G.T. conceived the study and secured funding. R.B.G., F.K.A., J.G.T. and M.S. designed the experiments. F.K.A. performed all experiments with some contributions from M.S., M.L. and D.T.A. F.K.A. analysed the data and prepared figures with input from R.B.G. and M.S. R.B.G. wrote the manuscript with input from F.K.A. and review by all authors.

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