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1 **Pre-maturation with cAMP modulators in conjunction with EGF-like peptides during IVM**
2 **enhances mouse oocyte developmental competence**

3

4 Running Title: Improving IVM with pre-IVM and EGF peptides

5

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35
36
37 Abbreviations: cAMP, cyclic adenosine monophosphate; EGF, epidermal growth factor; AREG,
38 amphiregulin; EREG, epiregulin; BTC, betacellulin; EGFR, EGF receptor; ERK1/2, extracellular signal-
39 regulated kinase 1/2; FSK, forskolin; IBMX, 3-isobutyl-1-methylxanthine; IVM, *in vitro* maturation; IVV,
40 *in vivo* maturation; FSH, follicle stimulating hormone; LH, luteinizing hormone; eCG, equine chorionic
41 gonadotropin; hCG, human chorionic gonadotropin; ICM, inner cell mass; TE, trophectoderm; BSA,
42 bovine serum albumin; SPOM, simulated physiological oocyte maturation; PKA, protein kinase A;
43 cGMP, cyclic guanine monophosphate; PDE, phosphodiesterase; dbcAMP, dibutyryl cAMP.

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ABSTRACT

Recent studies have independently shown that cAMP modulation prior to IVM and epidermal growth factor (EGF)-like peptide supplementation during IVM improve subsequent oocyte developmental outcomes. This study investigated the effects of an IVM system that incorporates these two concepts. COCs were collected from pre-pubertal mice either 46 h post-eCG (IVM) or post-eCG+post-hCG (*in vivo* maturation; IVV). IVM COCs were treated with the cAMP modulators forskolin and IBMX for 1, 2 or 4 h (pre-IVM phase) prior to IVM. COCs then underwent IVM with the EGF-like peptides amphiregulin or epiregulin, or with the common IVM stimulants FSH or EGF. A pre-IVM phase increased subsequent blastocyst inner cell mass compared to standard IVM, regardless of subsequent IVM treatment ($P<0.05$). However unlike FSH or EGF, amphiregulin or epiregulin significantly increased blastocyst quality (trophectoderm and total cell numbers) and/or yield ($P<0.01$) compared to standard IVM, and were the only treatments that yielded blastocysts comparable with IVV-derived blastocysts. Forskolin acutely up-regulated EGF-like peptide mRNA expression after a 2 h pre-IVM phase ($P<0.001$), however EGF receptor and ERK1/2 activities were not significantly different to control. IVV-like EGF-like peptide mRNA expression during IVM was only maintained by EGF-like peptides and EGF, since expression with FSH was significantly lower than during IVV. However, EGFR and ERK1/2 phosphorylation levels were not significantly different across treatment groups. In conclusion, a pre-IVM phase in conjunction with IVM in the presence of EGF-like peptides endows high oocyte developmental competence as evidenced by increased embryo yield and/or quality relative to FSH and EGF.

1 **INTRODUCTION**

2

3 Oocyte *in vitro* maturation (IVM) is used as a reproductive technique in human and animal assisted
4 reproduction, and for research applications. IVM generates viable metaphase II oocytes that upon
5 fertilisation yield viable embryos and pregnancies (Cross and Brinster 1970). The clinical use of IVM in
6 human assisted reproductive technology has been limited due to its significantly lower success rate
7 compared to conventional *in vitro* fertilization (Gremeau et al. 2012), possibly as oocytes undergo
8 aberrant maturation *in vitro* (Gilchrist 2011). Optimization of IVM protocols remains a substantial
9 challenge and an innovative approach to IVM is required to make it a more successful and,
10 consequently, viable option for infertility treatment.

11

12 *In vivo*, the oocyte is meiotically arrested at the prophase I stage by a moderate concentration of cyclic
13 adenosine 3'5'-monophosphate (cAMP) that is synthesised within the oocyte and by the granulosa and
14 cumulus cells (Conti et al. 2002; Conti et al. 2012; Downs 2010). Somatic cell cAMP is supplied to the
15 oocyte by cumulus cells through gap junctions and acts to inhibit germinal vesicle breakdown via
16 activation of protein kinase A (PKA) (Bornslaeger and Schultz 1985; Dekel et al. 1981; Horner et al.
17 2003; Webb et al. 2002). Granulosa and cumulus cells play an essential role in maintaining adequate
18 cAMP levels within the oocyte by also supplying it with cyclic guanine 3'5'-monophosphate (cGMP) to
19 inhibit oocyte phosphodiesterase (PDE) activity, the enzyme that degrades cAMP (Norris et al. 2009;
20 Tornell et al. 1991). Cyclic AMP has a dual role in regulating oocyte maturation as, paradoxically, it both
21 inhibits and promotes oocyte maturation. Oocyte meiotic resumption and maturation are initiated in
22 response to the ovulatory luteinizing hormone (LH) surge, however, LH exerts its effect by acutely and
23 transiently increasing cAMP concentrations within the mural granulosa cells of the follicle (Dekel et al.
24 1988; Yoshimura et al. 1992). This cAMP pulse, in turn, induces rapid and transient expression of the
25 epidermal growth factor (EGF)-like peptides amphiregulin (AREG), epiregulin (EREG), and betacellulin

1 (BTC), via a p38 MAPK-dependent pathway (Downs 2010). EGF-like peptides act as key mediators of
2 the LH stimulus as they induce oocyte maturation and ovulation, as well as cumulus expansion
3 (Ashkenazi et al. 2005; Park et al. 2004). Hence, during the normal course of oocyte maturation and
4 ovulation *in vivo*, the complex interplay between the ovulatory endocrine signal and the cellular
5 processes within the follicle are mediated to a great extent by cyclic nucleotides and EGF family
6 signalling (reviewed by Conti et al. (2012)).
7
8 After synthesis, EGF-like peptides are proteolytically cleaved from granulosa cells into the follicular
9 fluid, where they act on mural granulosa and cumulus cells to amplify this signal in an autocrine and
10 paracrine manner via activation of the EGF receptor (EGFR); a receptor tyrosine kinase and a member
11 of the ErbB receptor tyrosine kinase family (Oda et al. 2005). EGF-like peptide activation of the EGFR
12 elicits downstream signalling where a key downstream effector is extracellular signal-regulated kinase
13 1/2 (ERK1/2) (Downs 2010; Shimada et al. 2006). ERK1/2 activation is regarded as a central signalling
14 mechanism for the induction of oocyte maturation and ovulation by EGF-like peptide signalling, as
15 ERK1/2 activity is essential for cumulus expansion, resumption of meiosis and ovulation (Fan et al.
16 2009; Shimada et al. 2006). However recent data suggests that EGFR activation also decreases
17 follicular cGMP levels, leading to cAMP degradation and, consequently, oocyte meiotic resumption
18 (Conti et al. 2012; Hsieh et al. 2011; Norris et al. 2010; Tsuji et al. 2012; Vaccari et al. 2009). A new
19 study has now shown that somatic cell EGF-like peptide signalling also regulates translation of a subset
20 of mRNAs within the mouse oocyte during maturation; perturbation of EGF-like peptide-mediated
21 translation impacts fecundity (Chen et al. 2013). A recent study has shown that COCs matured under
22 standard IVM conditions are deficient in EGF-like peptides and EGFR activity, indicating that the oocyte
23 does not receive important regulatory signals (Richani et al. 2013). Unsurprisingly, the addition of
24 exogenous EGF-like peptides, instead of the commonly used additives follicle stimulating hormone

(FSH) and/or epidermal growth factor (EGF), to IVM culture systems, improves oocyte developmental competence (Akaki et al. 2009; Prochazka et al. 2011; Richani et al. 2013).

The intricate process in which an oocyte matures *in vivo* is dissimilar to the way in which it matures *in vitro*. Removal of an immature COC from its antral follicle leads to a rapid drop in COC cAMP (*c.f.* an increase *in vivo*), due to the loss of granulosa-supplied cGMP and cAMP, which leads to activation of the oocyte PDE3, and hence cAMP hydrolysis (Albuz et al. 2010; Norris et al. 2009; Vaccari et al. 2009). The drop in cAMP leads to PKA inactivation within the oocyte which culminates in the resumption of meiosis (Norris et al. 2009; Vaccari et al. 2009). Meiotic resumption due to a loss of the inhibitory cAMP signal (rather than its inductive stimulus via EGF-like peptide signalling) is termed 'spontaneous' oocyte maturation (Wassarman et al. 1976).

Many groups have now independently shown that artificial modulation of cAMP levels during IVM significantly improves oocyte developmental competence (Albuz et al. 2010; Luciano et al. 2004; Luciano et al. 1999; Nogueira et al. 2003a; Nogueira et al. 2003b; Nogueira et al. 2006; Shu et al. 2008; Thomas et al. 2004b; Vanhoutte et al. 2009a; Vanhoutte et al. 2009b; Zeng et al. 2013). In broad terms, there are two approaches in this respect: 1) prevention of the drop in cAMP of COCs by conducting IVM with specific or non-specific PDE inhibitors that prevent cAMP degradation, or 2) IVM culture with agents such as forskolin or dibutyryl cAMP (dbcAMP) that lead to a large increase in COC cAMP. We developed a cAMP-mediated IVM system, termed simulated physiological oocyte maturation (SPOM) (Albuz et al. 2010), that uses a 1-2 h pre-IVM phase where, post-oocyte collection, COCs are treated with forskolin and 3-isobutyl-1-methylxanthine (IBMX). This pre-IVM phase attenuates spontaneous oocyte maturation and allows prolongation of cumulus-oocyte gap-junctional communication, leading to a significant improvement in subsequent developmental outcomes including implantation, fetal yield, and fetal weight (Albuz et al. 2010; Zeng et al. 2013). An optimal pre-IVM duration is yet to be

established. There is limited evidence suggesting that the duration of pre-IVM treatment affects oocyte developmental competence, with a longer pre-IVM phase (2 hours) yielding greater embryo quality than a shorter pre-IVM phase (0.5 to 1 hour) (Albuz (2010).

We hypothesised that remodelling an IVM system to incorporate the use of cAMP modulators and exogenous EGF-like peptides would restore in the IVM oocyte key events that occur during *in vivo* maturation, and hence endow greater developmental competence on the oocyte. In this study, we examined the effect, and duration, of COC cAMP modulation prior to IVM, through the use of forskolin and IBMX, on EGF-like peptide signalling since cAMP is an important regulator of EGF-like peptide levels. The effect of exogenous EGF-like peptides to the conventional IVM additives, FSH and EGF, on EGF-like peptide signalling and subsequent embryo development was also examined.

RESULTS

Duration of pre-IVM phase affects oocyte developmental competence

Murine and bovine studies have investigated the effects of 1 h and 2 h pre-IVM periods where COCs were incubated with cAMP modulators or analogues prior to maturation (Albuz et al. 2010; Guixue et al. 2001; Luciano et al. 2004; Zeng et al. 2013), however an optimal pre-IVM period has not been established. Hence, the effects of 0 h, 1 h, and 2 h pre-IVM periods (in the presence of forskolin and IBMX), followed by standard (FSH) IVM, on blastocyst development were compared (Fig. 1). An incremental increase in day 6 total and hatching blastocyst rates was observed with increasing pre-IVM duration; only a 2 h pre-IVM period yielded significantly higher rates than no pre-IVM ($P<0.05$). This indicated that a minimum 2 h pre-maturation is desirable and raised the question of whether a pre-IVM period longer than 2 h would further increase oocyte developmental competence. Hence, pre-IVM periods of 2 h and 4 h were compared in subsequent experiments.

A pre-IVM phase followed by IVM with EREG/AREG yields the most developmentally competent oocytes

The evidence to date suggests that EGF-like peptides are superior to FSH or EGF for IVM of immature oocytes (Akaki et al. 2009; Prochazka et al. 2011; Richani et al. 2013), but they have not been examined in conjunction with cAMP- regulated pre-IVM. Hence, embryo development of COCs matured via IVV, standard IVM, or with a 2 h or 4 h pre-IVM phase (+FSK & IBMX) followed by IVM in the presence of either FSH, EGF, AREG, or EREG was assessed. COCs undergoing 4 h pre-IVM followed by IVM with EREG was the only IVM system that yielded day 6 blastocyst rates equivalent to those matured *in vivo* (IVV; P>0.05), and rates were significantly higher than COCs undergoing standard IVM, 2 h pre-IVM + FSH or EGF, and 4 h pre-IVM + EGF IVM (P<0.01, Fig. 2). Cleavage rate was not significantly different between treatment groups.

COCs undergoing 4 h pre-IVM followed by IVM with EREG or AREG, or 2 h pre-IVM followed by AREG IVM, yielded significantly larger blastocyst size (ICM, TE, and total cell numbers) compared to standard IVM COCs (P<0.01, Fig. 2), and blastocyst size was statistically equivalent to IVV-derived blastocysts (P>0.05). Interestingly, all treatments that contained a pre-IVM phase, whether it be 2 h or 4 h, led to a significant increase in ICM cell number compared to standard IVM (P<0.05). The distribution of embryonic cells was unaltered across all treatments as the percentage of ICM cell was similar across all treatment groups (P>0.05). There was no significant difference in the parameters examined between 2 h and 4 h pre-IVM, except where TE, ICM and total cell numbers were significantly higher with 4 h pre-IVM than 2 h pre-IVM when followed by EREG IVM.

Forskolin acutely up-regulates cumulus cell EGF-like peptide mRNA expression during pre-IVM

The relative abundance of cumulus cell mRNA transcripts from COCs was measured at the end of the 2 h pre-IVM phase in the presence or absence of FSK and IBMX. FSK induced significantly higher mRNA expression of *Btc* (>14-fold), *Areg* and *Ereg* (>570-fold) than IBMX or control (no treatment) after 2 h of pre-IVM ($P<0.001$, Fig. 3A). However, pEGFR and pERK1/2 levels were unaffected by pre-IVM with FSK and IBMX (Fig. 3B). There was no significant difference in mRNA expression levels between the three FSK doses tested (10-100 μM). IBMX-treated COCs had similar *Areg* and *Btc* expression to control ($P>0.05$), and *Ereg* expression was detectable in the presence of IBMX but not with control. No additive effect of FSK and IBMX, above that of FSK alone, was observed on EGF-like peptide expression ($P>0.05$). *Egfr* mRNA expression remained constant across all treatments.

EGF-like peptide expression following pre-IVM is sustained by EGFs but not by FSH

COC EGF-like peptide and *Egfr* mRNA expression was measured over a time course of standard IVM ± 2 h pre-IVM with FSK+IBMX (Fig. 4). *Areg* and *Ereg* expression was significantly lower at 3 h IVM when COCs were previously exposed to FSK+IBMX during the pre-IVM period ($P\leq0.04$). *Btc* and *Egfr* mRNA expression was not significantly different at any time point in the presence or absence of FSK+IBMX in pre-IVM.

To compare our cAMP pre-IVM system to *in vivo* matured and standard IVM oocytes, EGF-like peptide and *Egfr* mRNA expression were measured next in COCs matured via IVV (6 h), standard IVM (6 h), or with a 2 h or 4 h pre-IVM phase (+FSK & IBMX) followed by 6 h IVM in the presence of either FSH, EGF, AREG, or EREG (Fig. 5). A 6 h time point was selected for examination as previous work has shown a marked deficiency in standard IVM COC EGF-like peptide mRNA and protein expression, and EGFR activity, compared with IVV COCs at 6 h of maturation (Richani et al. 2013). COCs exposed to 2 h or 4 h pre-IVM followed by FSH IVM had similar *Ereg,* and lower *Areg* and *Btc,* expression than COCs undergoing standard IVM. COCs exposed to 2 h pre-IVM followed by IVM in the presence of

AREG, EREG, or EGF exhibited significantly higher EGF-like peptide expression than standard IVM COCs, and *Areg* and *Btc* expression levels were equivalent to IVV COCs. COCs exposed to pre-IVM for 4 h exhibited significantly lower *Areg* and *Btc* expression compared to those exposed to pre-IVM for 2 h, and expression was either similar to, or lower, than standard IVM COCs. Collectively these results (Figs. 4 and 5) suggest that, following 2 h pre-IVM, treatment of IVM COCs with EGF family members, but not FSH, can induce *in vivo*-like expression levels of *Areg* and *Btc* and enhance *Ereg* expression.

Mode of oocyte maturation does not appear to affect EGFR or ERK1/2 activation

Relative levels of pEGFR and pERK1/2 were measured in COCs matured from IVV (6 h), standard IVM (6 h), or with a 2 h or 4 h pre-IVM phase (+FSK & IBMX) followed by 6 h IVM in the presence of either FSH, EGF, AREG, or EREG (Fig. 6). COCs undergoing IVM in the presence of EGF, regardless of their pre-IVM period, contained significantly lower pEGFR levels than all other treatment groups except 4 h pre-IVM + EREG IVM. No significant differences in pEGFR were observed between all other treatment groups with the exception of 4 h pre-IVM + EREG IVM, which yielded significantly less pEGFR than IVV and 2 h pre-IVM + FSH/AREG IVM. As for the effect of pre-IVM duration, in general there were no significant differences in COC pEGFR between 2 h and 4 h pre-IVM, except in the presence of EREG, where pEGFR was significantly higher in 2 h pre-IVM than 4 h pre-IVM. Relative pERK1/2 levels were similar across all treatment groups ($P>0.05$).

DISCUSSION

Since standard IVM COCs are deficient in, and benefit from, both cAMP and exogenous EGF-like peptide addition (Albuz et al. 2010; Funahashi et al. 1997; Luciano et al. 2004; Prochazka et al. 2011; Richani et al. 2013; Vanhoutte et al. 2009b), we investigated the effects of a novel IVM system that elevates COC cAMP levels prior to IVM, followed by IVM in the presence of exogenous EGF-like

1 peptides; the natural mediators of the ovulatory LH signal. Findings from this study confirm that
2 increasing cAMP during a pre-IVM phase is beneficial to oocyte competence as it increases blastocyst
3 inner cell mass regardless of the IVM treatment. Moreover, a pre-IVM phase in conjunction with IVM in
4 the presence of EGF-like peptides leads to an increase in embryo yield and/or size relative to FSH,
5 EGF, or standard IVM.
6
7 Culture conditions, media composition, growth factors, and hormones have been shown to influence
8 mammalian oocyte *in vitro* maturation and subsequent developmental capacity (Gilchrist 2011; Lane
9 and Gardner 1997). Cyclic AMP is a central molecule controlling oocyte meiotic arrest and resumption,
10 and oocyte developmental competence. IVM COCs undergo some aberrant cellular processes due to
11 the resultant loss of cAMP and its control of meiotic maturation. The literature over the last 30 years,
12 predominantly in animal models, demonstrates that cAMP management during IVM is an effective and
13 logistically feasible means to improve subsequent developmental outcomes (Smitz et al. 2011).
14 Prolonged maintenance of cAMP using PDE inhibitors has been shown to yield modest improvements
15 in oocyte competence across several species including porcine, bovine, murine, and human (Downs et
16 al. 1986; Nogueira et al. 2003a; Nogueira et al. 2003b; Thomas et al. 2004b; Vanhoutte et al. 2009a;
17 Vanhoutte et al. 2009b). Furthermore, up-regulation of COC cAMP levels prior to IVM (either through
18 the use of dibutyryl cAMP or adenylate cyclase activators) seems to be an effective approach, as this
19 has been shown to significantly improve subsequent developmental competence in human, murine,
20 bovine, ovine and porcine IVM oocytes (Albuz et al. 2010; Funahashi et al. 1997; Guixue et al. 2001;
21 Luciano et al. 1999; Rose et al. 2013; Shu et al. 2008; Zeng et al. 2013).
22
23 The optimal pre-IVM duration is currently unknown. The culture of porcine COCs in the presence of
24 dbcAMP for the first half (20 hours) of IVM was shown to greatly increase subsequent embryo yield
25 (Funahashi et al. 1997), and this concept is now widely used for embryo production in this species. In

this study we investigated the effect of 0, 1, and 2 hour pre-IVM phases on subsequent embryo development. An incremental increase in total and hatching blastocyst rates with increasing pre-maturation time was observed. Hence, the duration of pre-IVM appears to affect oocyte developmental competence and this experiment raised the question of whether pre-maturation for longer than 2 hour adds further benefit. Subsequently, we compared the effects of 2 and 4 hour pre-IVM periods; there was a tendency for improved embryo development at 4 hours compared with 2 hours, as reflected by ICM and trophectoderm cell numbers, and blastocyst yield after IVM only in the presence of epiregulin. However, in general we did not see any further benefit with pre-maturation between 2 and 4 hours pre-IVM.

We have recently shown that EGF-like peptide expression and EGFR activity are highly deficient in standard IVM COCs (Richani et al. 2013). Consistent with this, we and others have also shown that IVM supplementation with EGF-like peptides, instead of FSH or EGF, yields oocytes with significantly higher developmental competence (Prochazka et al. 2011; Richani et al. 2013). Furthermore, cAMP is a known stimulator of somatic cell EGF-like peptide expression (Panigone et al. 2008; Shimada et al. 2006). Hence, use of exogenous cAMP modulators seems a logical means to induce EGF-like peptide levels in IVM COCs that are otherwise deficient in these peptides. Therefore, we examined cumulus cell EGF-like peptide and receptor expression at the end of a 2 hour pre-IVM phase where cAMP was elevated using forskolin and maintained using IBMX. As expected, the large increase in cAMP by forskolin vastly up-regulated expression of all three EGF-like peptides compared to control, however this did not translate into increased activity of the EGFR or its main downstream effector ERK1/2. The mechanism by which EGFR and ERK1/2 activation is suppressed in the presence of such high levels of EGF-like peptides remains unclear; however cAMP may play a critical role in regulating the function of EGFR.

1 FSH has been a universal additive to IVM systems for over 20 years due to its recognized positive
2 effects on oocyte developmental competence relative to spontaneous IVM (Eppig et al. 1992; Izadyar et
3 al. 1998). FSH-induced oocyte maturation is now known to induce up-regulation of EGF-like peptide
4 expression in cumulus cells (Park et al. 2004). However, we have recently shown that FSH is a
5 relatively poor stimulator of EGF-like peptide expression in IVM cumulus cells as it induces
6 considerably lower expression levels than EGF-like peptides or hCG *in vivo* (Richani et al. 2013). In this
7 study, we found the increased cumulus cell EGF-like peptide expression, as a result of the cAMP-
8 mediated pre-IVM phase, is not maintained during IVM in the presence of FSH, as expression levels
9 were similar to, or less than, those seen during standard IVM. Conversely, IVM in the presence of
10 exogenous EGF-like peptides or EGF, following a 2 h pre-IVM phase, led to expression of the EGF-like
11 peptides at levels similar to those seen *in vivo,* and notably higher than those of standard IVM.
12 Interestingly, prolonged exposure to high cAMP (4 h pre-IVM phase) prior to IVM appeared to down-
13 regulate EGFR activity compared to a shorter exposure time (2 h pre-IVM phase). This was not due to
14 down-regulated EGFR expression as our data shows no difference in mRNA expression across all
15 treatments. However it could be due to over-activation of the EGFR causing a negative feedback loop,
16 leading to internalization of the receptor (Madshus and Stang 2009; Roepstorff et al. 2008). EGFR
17 activity is normally tightly regulated as this receptor is a well-studied oncogene and its overactivity has
18 been shown to culminate in cancer development (Madshus and Stang 2009; Riese et al. 2007; Yarden
19 and Sliwkowski 2001).
20
21 The use of epiregulin or amphiregulin, instead of FSH or EGF, in an IVM system has been shown to
22 improve subsequent embryo development in the mouse and pig (Prochazka et al. 2011; Richani et al.
23 2013). In the mouse, epiregulin and amphiregulin were shown to increase the proportion of inner cell
24 mass cells, however only epiregulin significantly increased embryo yield (Richani et al. 2013). Here we
25 found that, unlike FSH or EGF, amphiregulin and epiregulin increased blastocyst size (trophectoderm

1 and total cell numbers), and consistent with our previous study, only epiregulin significantly increased
2 day 6 blastocyst rates compared to standard IVM, with rates comparable to *in vivo* matured oocytes.
3 Increased blastocyst yield and size have been associated with improved developmental outcomes
4 (Lane and Gardner 1997). Hence, it appears that increasing cAMP prior to IVM via a pre-IVM phase is
5 beneficial as it increases blastocyst inner cell mass regardless of the IVM conditions. However, a pre-
6 IVM phase in conjunction with IVM in the presence of EGF-like peptides, particularly epiregulin in the
7 mouse, endows even greater oocyte developmental competence as it leads to increased embryo yield
8 and/or quality relative to FSH and EGF. A comparison of the effect of these treatments on post-
9 implantation fetal development is required, however the increased embryo yield and/or quality observed
10 in this study is promising as IVM treatments that lead to small increases in embryo yield and, in
11 particular, in inner cell mass size, have been shown to significantly increase murine fetal development
12 and survival following IVM (Albuz et al. 2010; Sudiman et al. 2014).
13
14 We examined the effect of EGF as it is a common additive to animal research and veterinary IVM
15 systems (Banwell and Thompson 2008), and is a member of the same family of proteins as EGF-like
16 peptides and signals via EGFR to induce oocyte maturation and cumulus expansion *in vitro* (De La
17 Fuente et al. 1999; Downs 1989; Downs et al. 1988; Rieger et al. 1998). Despite its ability to induce
18 comparable EGF-like peptide expression levels to those induced *in vivo*, and by epiregulin and
19 amphiregulin *in vitro*, EGF did not elicit the same level of EGFR activity or endow the same oocyte
20 developmental competence as these EGF-like peptides. EGF has previously been shown to yield
21 significantly lower blastocyst yield and quality than epiregulin and amphiregulin in an IVM system
22 without a pre-IVM phase (Richani et al. 2013). This suggests that differing EGFR ligands produce
23 differences in IVM oocyte developmental competence despite acting through the same receptor. EGFR
24 ligands are known to have distinct binding specificities and affinities, and depending on ligand
25 interaction, EGFR activation can initiate several signal transduction pathways including the PI3K-AKT,

Src, and PAK-JNKK-JNK pathways (Mendoza et al. 2011; Oda et al. 2005; Yarden and Sliwkowski 2001). Our results here and elsewhere suggest that EGF is not an optimal IVM additive, and that EGF-like peptides are more appropriate.

Mechanisms that have been associated with increased oocyte developmental competence resulting from cAMP modulation and EGF-like peptide supplementation include oocyte metabolic alterations, prolonged COC gap-junctional communication, altered chromatin remodelling, and increased EGF-like peptide expression. The increased oocyte developmental competence derived from pre-maturation with cAMP modulators observed in this study is possibly the result of prolonged oocyte-cumulus gap-junctional communication that enables increased exchange of regulatory molecules and metabolites; gap-junctional communication is abruptly terminated during standard IVM (Thomas et al. 2004a; Thomas et al. 2004b) but is prolonged by a pre-IVM phase utilizing forskolin and IBMX (Albuz et al. 2010). Premature gap-junction termination during standard IVM leads to rapid chromatin condensation and RNA transcription cessation, however maintenance of this communication using PDE inhibitors has been shown to alter chromatin remodelling and transcription, which is related to increased oocyte developmental competence (Dieci et al. 2013; Lodde et al. 2013; Luciano et al. 2011). Increased cAMP levels and EGF-like peptides have also been associated with metabolic alterations (Richani et al. 2014). It has recently been shown that forskolin and IBMX in a pre-IVM phase significantly increase oocyte mitochondrial activity and ATP levels, both of which are markers of increased oocyte developmental competence (Zeng et al. 2013). Furthermore, EGF-like peptides have been shown to increase IVM oocyte mitochondrial activity and alter COC glucose metabolism compared to FSH and EGF (Richani et al. 2014). The current study examined the effect of increased cAMP and exogenous EGF-like peptides during IVM on EGF-like peptide expression and signalling. We hypothesised that the increased oocyte developmental competence acquired via increased cAMP and exogenous EGF-like peptides during IVM would be linked to EGF-like peptide expression levels as well as EGFR and

1 ERK1/2 activation. However, EGFR and ERK1/2 activities were not reflective of EGF-like peptide
2 levels, and these factors were not reflective of embryo developmental outcomes. Hence, the level of
3 EGFR signalling via ERK1/2 may not be a limiting factor in oocyte developmental competence, however
4 we cannot exclude the possibility that differential EGFR phosphorylation via alternative tyrosine
5 residues than the one measured in this study (Tyr 1068) may be occurring. Furthermore, investigation
6 into the differential effects of EGF-like peptides, EGF, and FSH on alternative signalling pathways to
7 ERK/12 is required since a new study by Chen et al. (2013) has shown that granulosa and cumulus cell
8 amphiregulin signalling regulates the translation of a subset of mRNAs within the mouse oocyte via the
9 P(I)3K-AKT-mTOR signalling pathway. Further investigation is required into the mechanisms by which
10 increased cAMP, in combination with exogenous EGF-like peptides, increased oocyte developmental
11 competence. This may include differential activation of downstream signalling pathways, metabolic
12 alterations and/or improvements attributable to prolonged COC gap-junctional communication.
13
14 In conclusion, the current study demonstrates an improved IVM model that incorporates the use of
15 cAMP modulators in a pre-maturation phase and exogenous EGF-like peptides during maturation to
16 yield oocytes with higher developmental competence than those matured via standard IVM, and are
17 comparable to those matured *in vivo*. The improvement to IVM oocyte developmental competence
18 through pre-maturation with cAMP modulators has strong precedence in the literature, however this
19 study suggests that combining this concept with oocyte maturation in the presence of exogenous EGF-
20 like peptides, rather than the presently used IVM additives FSH or EGF, further increases oocyte
21 developmental competence. Such an approach may represent a more physiological IVM system as it
22 ameliorates key deficiencies in current systems and likely mimics key *in vivo* signalling events more
23 closely.
24
25

MATERIALS AND METHODS

Unless otherwise specified, all chemicals were obtained from Sigma Aldrich (St Louis, USA).

COC collection

Mice were maintained in accordance with the Australian Code of Practice for Care and Use of Animals for Scientific Purposes and with the approval of the Adelaide University Animal Ethics Committee. 129/Sv female mice were used for all experiments. Ovaries were collected from 21- to 26-day old mice, 46 h after intraperitoneal injection of 5 IU of equine chorionic gonadotropin (eCG; Folligon, Intervet, Boxmeer, Holland) in HEPES-buffered alpha minimum essential medium (αMEM; Gibco, Life Technologies, NY, USA) supplemented with 3 mg/mL bovine serum albumin (BSA). In one treatment group, human chorionic gonadotropin (hCG, 5 IU; Organon, Sydney, Australia) was administered 46 h post-eCG to induce oocyte maturation *in vivo* (IVV). Ovaries were harvested at indicated times post-hCG and COCs were collected.

COC *in vitro* culture

Pre-IVM

COCs were freed from preovulatory follicles using a 27-gauge needle into HEPES-buffered αMEM (Gibco) with 3 mg/mL BSA ± 50 μM forskolin (the dose used unless otherwise stated; FSK) and 50 μM IBMX, and collected using flame-pulled borosilicate Pasteur pipettes. COCs were then immediately transferred into bicarbonate-buffered αMEM (Gibco) with the same additives and placed at 37ºC with 5% CO_2 in air for the remainder of the pre-IVM period. Timing of the pre-IVM period (1, 2 or 4 h) was commenced once all COCs were freed from their follicle.

IVM

1 Following the pre-IVM period, COCs were washed thoroughly with bicarbonate-buffered αMEM
2 supplemented with 3 mg/mL BSA to remove pre-IVM treatments. COCs were then cultured in
3 bicarbonate-buffered αMEM supplemented with 3 mg/mL BSA and either: recombinant human FSH (50
4 mIU/mL; Puregon, Organon, Oss, The Netherlands), recombinant human EGF (10 ng/mL; R&D
5 Systems, Minneapolis, USA), recombinant mouse AREG (50 ng/mL; R&D Systems), or recombinant
6 mouse EREG (50 ng/mL; R&D Systems) at 37ºC with 5% CO_2 in air. Doses of EGF family growth
7 factors are based on previous studies (De La Fuente et al. 1999; Downs and Chen 2008; Li et al.
8 2008). "Standard IVM" conditions were considered IVM in the presence of 50 mIU/mL FSH and no pre-
9 IVM period.
10
11 **RNA Isolation**
12 Following the indicated periods of IVV or IVM, 50 COCs, or their cumulus cells, were collected and
13 washed once with PBS. Total RNA was extracted using the RNeasy Micro Kit (Qiagen, Germantown,
14 USA) according to the manufacturer's instructions. DNA that may have been co-purified was removed
15 by the addition of DNase (0.34 Kunitz/µL supplied with kit). RNA was eluted in 14 µL of RNase-free
16 water and stored at -80ºC. Final RNA concentrations were determined by absorbance using a
17 NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Scoresby, Australia).
18
19 **RT-qPCR**
20 An equal amount of total RNA from each sample was reverse transcribed using random hexamers
21 (Invitrogen, Carlsbad, USA) and Superscript III reverse transcriptase (Invitrogen). Primers (10
22 pmol/reaction; Table 1) and cDNA were then added to 20 µL total reaction volume with SYBR Green
23 (Applied Biosystems, Mulgrave, Australia). PCRs were performed using a Corbett Rotor-Gene 6000
24 (Qiagen). Thermal cycling conditions were set as follows: denaturing for 10 min at 95°C, then 40 cycles
25 of annealing and extension at 95°C for 15 sec and 60°C for 60 sec, followed by dissociation at 95°C,

1 60°C, and 95°C for 15 sec each. A seven point serial dilution standard curve was produced for each
2 transcript from cDNA generated from mural granulosa cells collected 3 h post-hCG. The relative gene
3 expression values were calculated using the standard curve method and presented relative to a
4 calibrator and normalised to the geometric mean of two housekeeping genes (*Mrpl19* and *Ppia*). To
5 validate primer pairs, amplicons generated from mouse cumulus cell cDNA were run on 2% agarose
6 gels and primer pairs were considered valid when a single product of the correct size was observed and
7 primer efficiency was above 90%.
8
9 **Protein Immunodetection**
10 Immunodetection of phosphorylated EGFR (pEGFR), phosphorylated ERK1/2 (pERK1/2), total ERK1/2
11 (tERK1/2) and β-actin was performed as described by Richani et al. (2013). Briefly, 50 COCs per
12 treatment group were collected and suspended in RIPA buffer (10 mM Tris, 150 mM NaCl, 1 mM
13 EDTA, 1 % Triton X-100) containing phosphatase and protease inhibitor cocktails (Roche, Penzberg,
14 Germany), snap frozen in liquid nitrogen, and stored at -80ºC. Samples were loaded onto a 7.5% SDS–
15 polyacrylamide gel for electrophoresis and proteins were transferred to Hybond-ECL membranes (GE
16 Healthcare, Waukesha, USA). Membranes were cut in half horizontally; the upper half was incubated
17 with the primary antibody anti-pEGFR (Cell Signalling Technology, Beverley, USA, cat. no. 3777)
18 diluted 1:1000, and the lower half was incubated with anti-pERK1/2 (Sigma, cat. no. M8159) diluted
19 1:10,000 at 4°C overnight, followed by incubation with goat anti-rabbit IgG peroxidase-conjugated
20 (diluted 1:200,000, Santa Cruz Biotechnology Inc, Santa Cruz, USA, cat. no. SC-2004) and donkey
21 anti-mouse peroxidase-conjugated (diluted 1:400,000, Santa Cruz, cat. no. SC-2314) secondary
22 antibodies, respectively. Binding was detected using the ECL Advance kit and exposure to Hyperfilm
23 (GE Healthcare). The lower half membrane was then stripped using an acidic glycine stripping buffer
24 (1% SDS, 25mM glycine, pH 2.0) and was incubated with anti-ERK1/2 (Sigma, cat. no. M5670) diluted
25 1:10,000 at 4°C overnight, followed by incubation with goat anti-rabbit IgG peroxidase-conjugated

1 (diluted 1:400,000). This membrane was then stripped again and incubated with anti-β-actin peroxidise
2 conjugated antibody (diluted 1:20 million, Sigma, cat. no. A3854). Band intensities were measured
3 using Image J software (NIH; Bethesda, USA). Band intensities for pEGFR and pERK1/2 were
4 normalised to β-Actin and tERK1/2 band intensities, respectively, and standardised relative to post-hCG
5 values. Data is represented as the mean of four replicate experiments.
6
7 ***In vitro* embryo production and differential staining**
8 As described above, standard IVM COCs were matured for 18 h, and IVM COC with a pre-IVM phase
9 (2 h or 4 h) were matured for 20 h following the pre-IVM phase (albeit, for Fig. 1, COCs exposed to a
10 pre-IVM phase were cultured for 18 h only), however 1 mg/mL fetuin was also added to the COC
11 handling and IVM culture media to prevent zona pellucida hardening. IVV COCs were collected 16 h
12 post-hCG from eCG primed mice. The extended IVM maturation period of 20 h used for COCs
13 subjected to a pre-IVM phase with cAMP modulators was based on our previous experience examining
14 the kinetics of meiosis under these conditions; although extended maturation time generally causes
15 decreased developmental competence of standard IVM oocytes due to oocyte aging, we observe an
16 increase in competence using this model (Albuz et al. 2010; Shu et al. 2008; Thomas et al. 2004b;
17 Zeng et al. 2013). CBA x C57BL6 FI hybrid male mice (6- to 8- weeks old) were used as sperm donors
18 for *in vitro* fertilization; COCs were fertilised with capacitated epididymal sperm for 4 h at 37ºC at 6%
19 CO_2 in air in Research VitroFert media (Cook Medical, Eight Miles Plain, QLD, Australia). COCs were
20 then transferred into Research VitroWash media (Cook Medical) and cumulus cells were removed by
21 mechanical shearing with a P200 pipette. Presumptive zygotes were then washed and incubated in
22 Research VitroCleave media (Cook Medical) at 37ºC with 6% CO_2, 5% O_2 and balance of nitrogen.
23 Blinded assessment of embryo development at days 2, 5 and 6 post-insemination was performed using
24 the scoring system reported by Gardner et al. (2004).
25

Day 6 blastocyst trophectoderm (TE) and inner cell mass (ICM) cell numbers were counted using differential staining as described by Hardy et al. (1989). Briefly, blastocysts were incubated in 0.5% pronase at 37°C to remove the zona pellucida. Blastocysts were then placed in protein-free wash medium and then in 10% 2,4,6-trinitrobenzene sulfonic acid for 10 min at 4°C. They were then incubated with anti-2,4-8 dinitrophenol (1:10) for 10 min at 37°C, followed by complement (1:1; 2 µg/ml propidium iodine:guinea pig serum) for a further 5 min. Blastocysts were incubated in 25 µg/ml Hoechst 33342 (bisbenzimide) in ethanol at 4°C overnight, and were then washed in 100% ethanol and transferred into 5 µl drops of 100% glycerol on microscope slides and covered with a cover slip. An epifluorescent microscope (Nikon, TE 2000-E; excitation 340-380 nm, emission 440-480 nm) was used to visualize differential staining of blastomeres. The ICM cells (stained blue) and TE cells (stained pink) of the embryo were counted.

Statistical Analyses

Statistical analyses were conducted using SigmaPlot 11.0 software (Systat Software, San Jose, USA). Statistical significance was assessed by one-way ANOVA followed by Student-Newman-Keuls multiple-comparison post-hoc tests to identify individual differences between means for PCR, immunodetection, and blastocyst differential staining data. In the case data were not normally distributed, statistical significance was assessed by non-parametric one-way ANOVA by ranks. Two sample *t*-tests were used where only two sample means were compared. All values are presented with their corresponding standard error of the mean (SEM). For embryo culture, statistical significance was assessed using Chi-Squared testing. Probabilities of $P \leq 0.05$ were considered statistically significant.

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1 **CONFLICT OF INTEREST**

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4

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1 **TABLE LEGEND**

2 **Table 1:** Sequences of primers used for RT-qPCR.

3

4 **FIGURE LEGEND**

5 **Figure 1: Effect of 0-2 h pre-IVM followed by standard (FSH) IVM on embryo development.** COCs
6 were subjected to 0 h (standard IVM), 1 h or 2 h pre-IVM culture in the presence of forskolin and IBMX,
7 followed by standard IVM in the presence of FSH for 18 h. Cleavage and days 5 and 6 blastocyst rates
8 were assessed (n≥200 COCs per group over 6 replicate experiments). Bars within each group that are
9 not sharing a common letter are significantly different (P<0.05). Blasts, blastocysts; H/Blasts, hatching
10 blastocysts; D, day.

11

12 **Figure 2: Effect of 2 h and 4 h pre-IVM followed by IVM in the presence of EGF-like peptides or**
13 **EGF on embryo development.** COCs were either matured *in vivo* (IVV) for 16 h, under standard IVM
14 conditions (no pre-IVM + FSH IVM) for 18 h, or were subjected to 2 h or 4 h pre-IVM culture in the
15 presence of forskolin and IBMX, followed by IVM in the presence of FSH, EGF, AREG or EREG for 20
16 h. Cleavage and day 6 blastocyst rates were assessed (n≥120 COCs per group over 5 replicate
17 experiments). Day 6 blastocyst quality was assessed by quantification of the number of inner cell mass
18 (ICM), trophectoderm (TE), and total cells, and the proportion of ICM cells (n=24-27 per group). Bars
19 not sharing a common letter are significantly different (P<0.05). Blasts, blastocysts; D, day.

20

21

22 **Figure 3: Effect of 2 h pre-IVM on EGF-like peptide and EGFR mRNA expression, and EGFR and**
23 **ERK1/2 phosphorylation.** COCs underwent 2 h pre-IVM culture in the presence of control (no
24 treatment), or forskolin and/or IBMX at indicated doses. (A) Cumulus cell were separated from oocytes
25 and their mRNA expression after 2 h pre-IVM was quantified using RT-qPCR and normalised to the

1 geometric mean of the *Ppia* and *Mrpl19* housekeeper genes (n=3). (B) The relative levels of whole
2 COC phosphorylated EGFR (pEGFR) and phosphorylated ERK1/2 (pERK1/2) were measured after 2 h
3 pre-IVM via Western blotting (n=4); a representative blot is shown and the quantified densitometric
4 values below it. Bars not sharing a common letter are significantly different (P<0.05); NS, not
5 significant; N.D., below limit of detection.

6

7 **Figure 4: Effect of cAMP modulators during pre-IVM on EGF-like peptide and EGFR mRNA**
8 **expression during IVM.** COCs either underwent standard IVM with FSH (-pre-IVM), or were
9 maintained for 2 h in the presence of forskolin and IBMX (+pre-IVM) followed by IVM with FSH, for 3 h,
10 6 h, or 12 h . COC mRNA expression was quantified using RT-qPCR and normalised to the geometric
11 mean of the *Ppia* and *Mrpl19* housekeeper genes. (*) denotes a significant difference (P<0.05). Data is
12 from 3 replicate experiments.

13

14 **Figure 5: Effect of 2 h and 4 h pre-IVM followed by IVM in the presence of EGF-like peptides,**
15 **FSH, or EGF on EGF-Like peptide expression.** COCs were either matured *in vivo* (IVV) for 6 h, under
16 standard IVM conditions (no pre-IVM + FSH IVM) for 6 h, or were subjected to 2 h or 4 h pre-IVM
17 culture in the presence of forskolin and IBMX, followed by IVM in the presence of FSH, EGF, AREG or
18 EREG for 6 h. COC mRNA expression was quantified using RT-qPCR and normalised to the geometric
19 mean of the *Ppia* and *Mrpl19* housekeeper genes. Bars not sharing a common letter are significantly
20 different (P<0.05). Data is from 6 replicate experiments.

21

22 **Figure 6: Effect of 2 h and 4 h pre-IVM followed by IVM in the presence of EGF-like peptides,**
23 **FSH, or EGF on EGFR and ERK1/2 activation.** COCs were either matured *in vivo* (IVV) for 6 h, under
24 standard IVM conditions (no pre-IVM + FSH IVM) for 6 h, or were subjected to 2 h or 4 h pre-IVM
25 culture in the presence of forskolin and IBMX, followed by IVM in the presence of FSH, EGF, AREG or

EREG for 6 h. The relative levels of COC phosphorylated EGFR (pEGFR) and phosphorylated ERK1/2 (pERK1/2) were measured. A representative blot is shown and the quantified densitometric values below it. Densitometric measurements of pEGFR were normalised to β-actin and pERK1/2 were normalised to total ERK1/2 and standardised to IVV in each individual blot. Bars not sharing a common letter are significantly different ($P<0.05$). Data is from 4 replicate experiments.

1 Table 1:
2

Gene	GenBank accession no.	Forward primer	Reverse primer	PCR size (bp)
Areg	NM_009704.3	TTGGTGAACGGTGTGGAGAA	CGAGGATGATGGCAGAGACA	111
Ereg	NM_007950.2	AGACGCTCCCTGCCTCTTG	TTCTCCTGGGATGCATGATG	104
Btc	NM_007568.4	TGCCCTGCCCCTCACA	TACCACACAGTGGAGAATTGCAA	115
Egfr	BC023729.1	TCTGGAAACCGAAATTTGTGCTA	ACGGCCTTGCAGTCTTTCTC	116
Ppia	NM_008907.1	TGGCAAATGCTGGACCAA	CCTTCTTTCACCTTCCCAAAGA	106
Mrpl19	NM_026490	GAAAGGTGCTTCCGATTCCA	TGATCGCTTGATGCAAATCC	116

All primers are given in the 5' to 3' orientation.

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4

1 Figure 1

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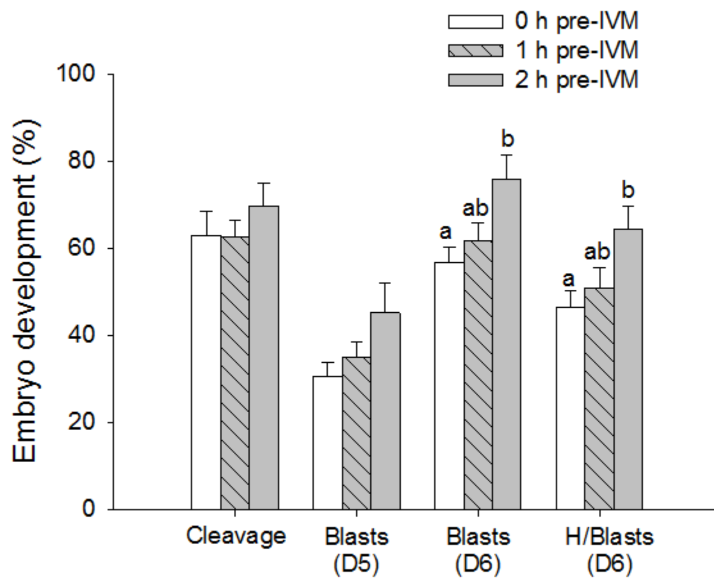

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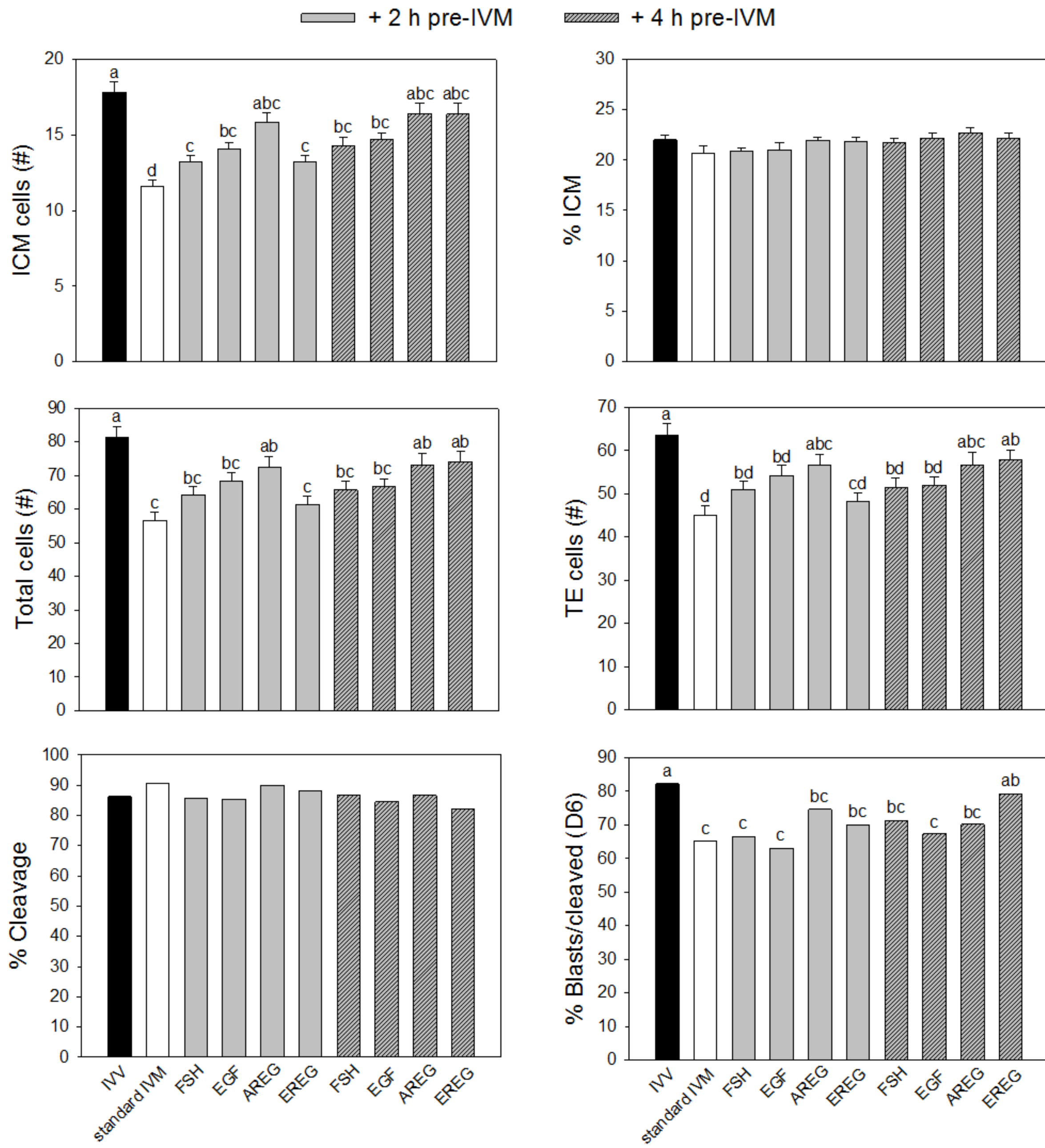
1 Figure 2

2



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1 Figure 3
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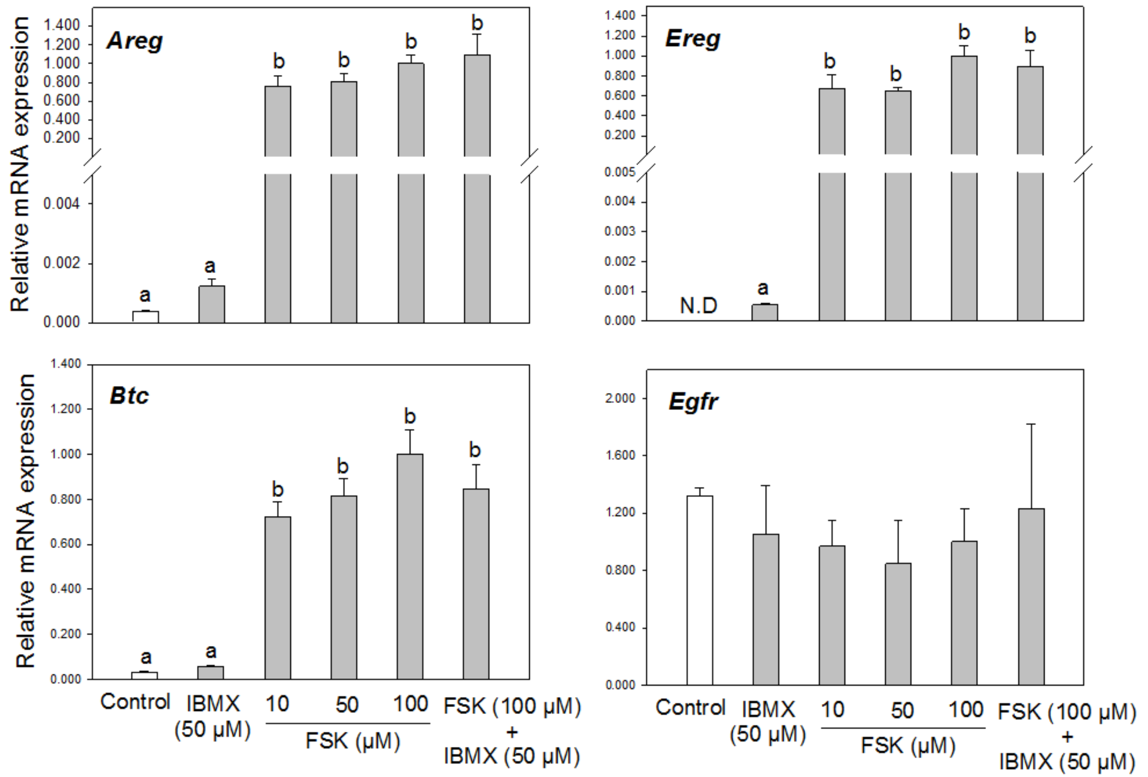

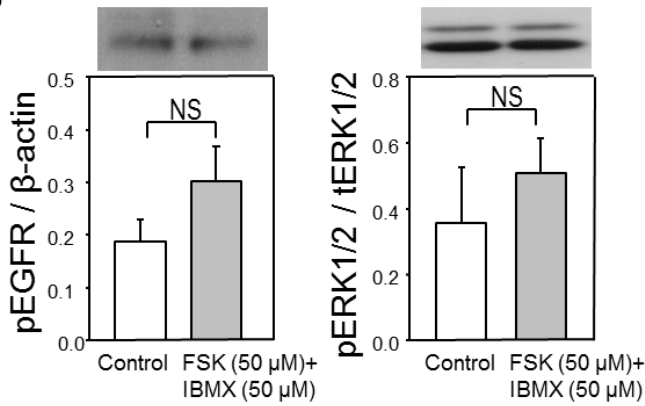

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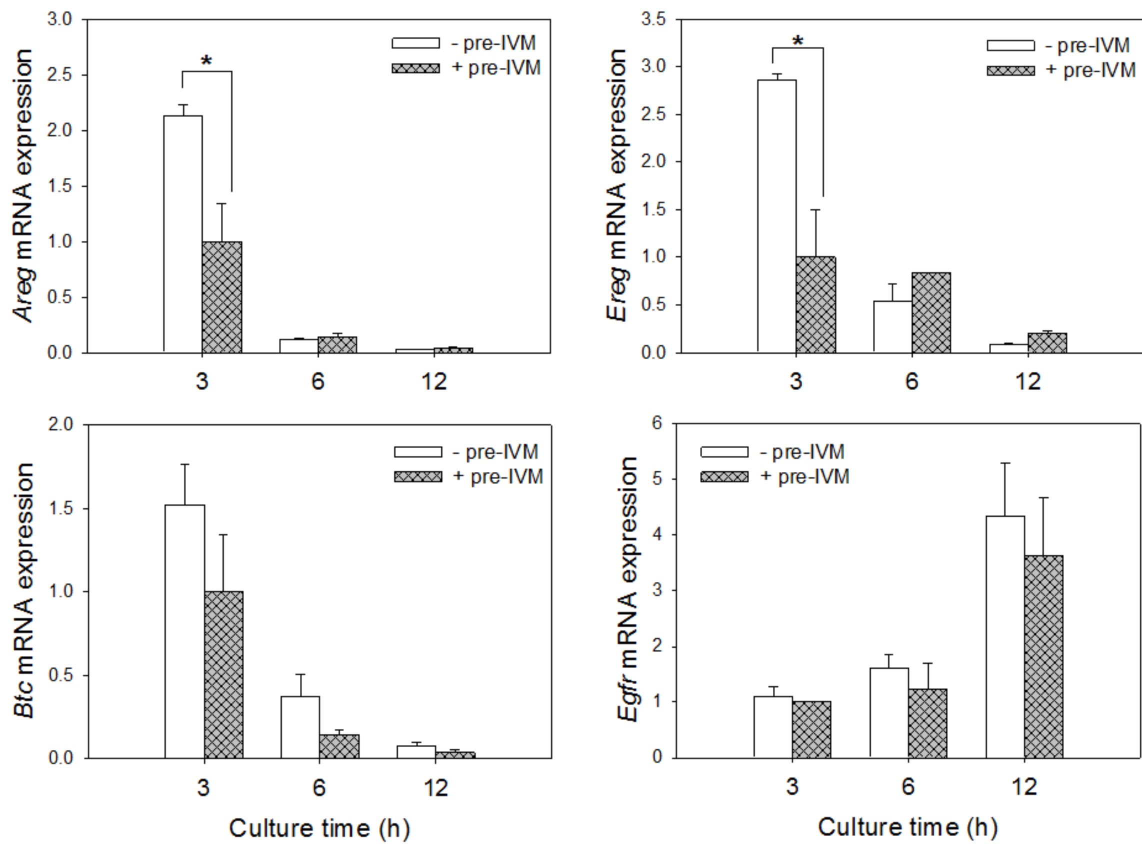
1 Figure 4
2



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1 Figure 5
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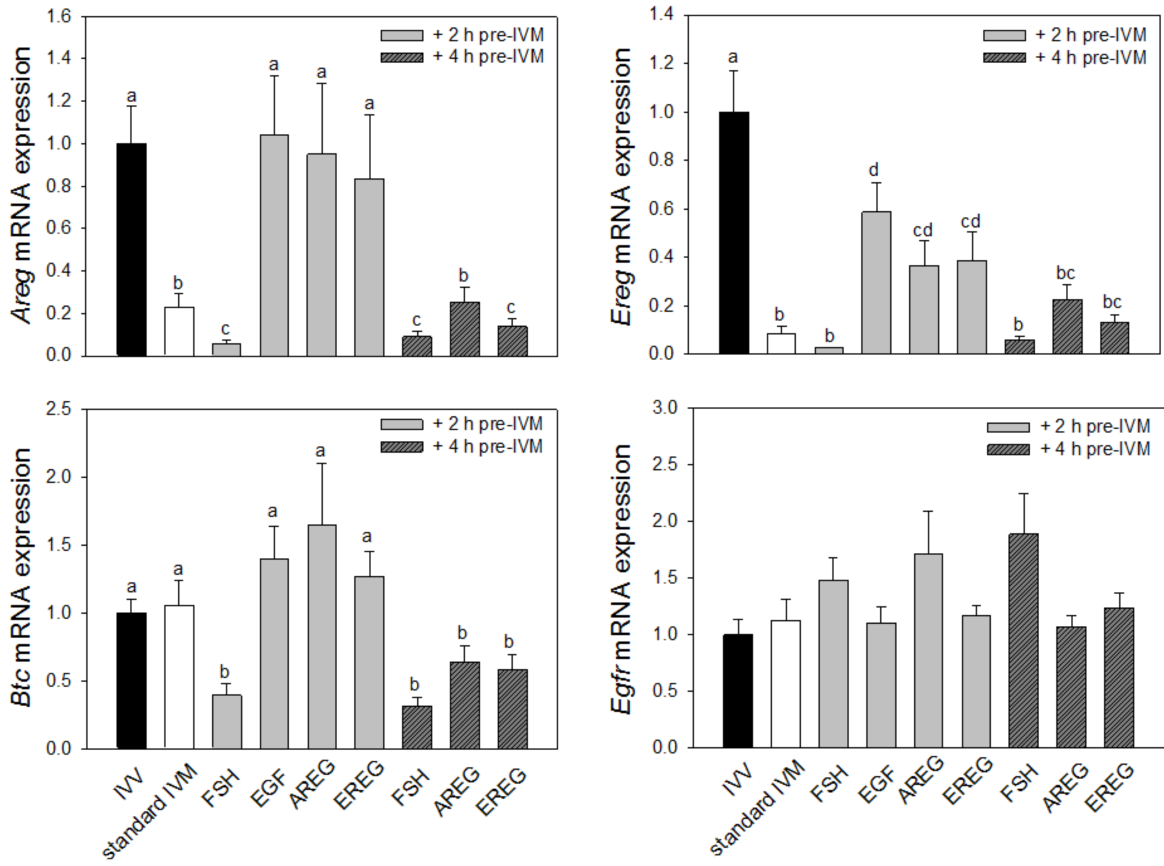

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1 Figure 6
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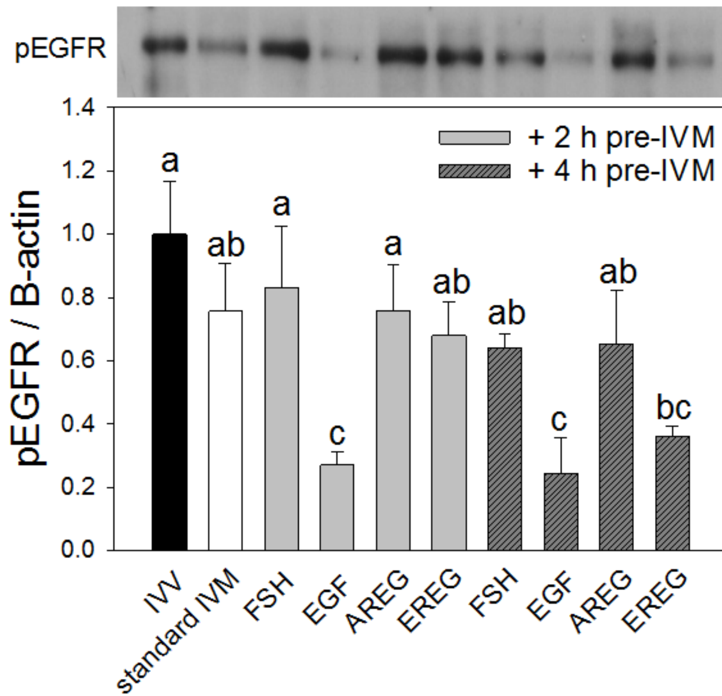

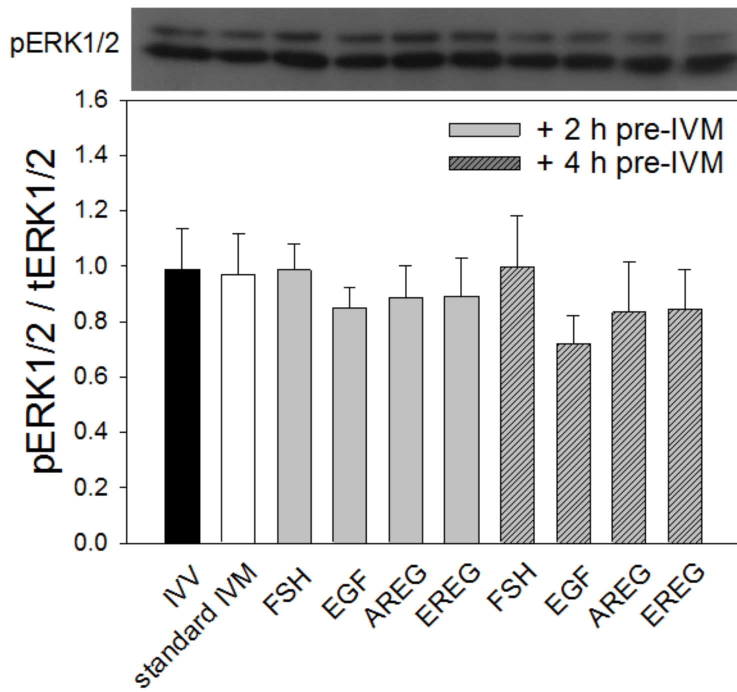

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