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# Title: Mode of oocyte maturation affects EGF-like peptide function and oocyte competence

## Running Title: EGF-like peptide signalling during oocyte IVM

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**ABSTRACT**

The function and impact of EGF-like peptide signalling during ovulation and *in vivo* oocyte maturation (IVV) has been recently characterised, however little is currently known about the effect of oocyte *in vitro* maturation (IVM) on this pathway. The aim of this study was to examine expression and functional aspects of three EGF-like peptides (amphiregulin, epiregulin and betacellulin) and their common receptor (EGFR) in cumulus cells during mouse oocyte IVM compared to IVV. Cumulus-oocyte complexes (COCs) were collected from prepubertal mice either 46h post-eCG (IVM) or after eCG plus 0.5-12 h post-hCG (IVV). Time course experiments showed mRNA expression of all three EGF-like peptides and amphiregulin protein in IVM media were significantly lower for the majority of FSH-supplemented IVM compared with IVV. The supplementation of EGF during IVM yielded EGF-like peptide expression levels comparable to IVV and amphiregulin/epiregulin supplemented IVM. However, despite this, EGF activation of the COC EGFR remained significantly lower at 3h and 6h of IVM than *in vivo*, and levels were similar to those observed during FSH-supplemented IVM. The addition of exogenous epiregulin during IVM significantly increased blastocyst rates, and epiregulin and amphiregulin improved blastocyst quality, compared with FSH or EGF. In conclusion, findings from this study suggest that the widely used IVM additives, FSH and EGF, are inadequate propagators of the essential EGF-like peptide signalling cascade. By contrast, the use of epiregulin and/or amphiregulin during IVM leads to improved oocyte developmental competence and therefore may be preferable IVM additives than FSH or EGF.

## INTRODUCTION

Oocyte *in vitro* maturation (IVM) is a reproductive technique that involves the collection of immature oocytes from unstimulated or minimally stimulated ovaries that are then matured *in vitro* in medium containing low doses of gonadotropins, usually follicle stimulating hormone (FSH). The use of IVM in a clinical setting remains poor since, compared to IVF, IVM success rates (embryos and offspring generated per oocyte collected) are lower and miscarriage rates are higher (Banwell and Thompson 2008; Buckett et al. 2008; Child et al. 2002). This is attributed to reduced developmental competence of oocytes after IVM (Gilchrist and Thompson 2007), however the molecular mechanisms underlying this remain unclear. In order to improve IVM outcomes, an understanding of the mechanisms that confer oocyte developmental competence *in vivo* and how they are affected by maturation *in vitro* is imperative.

New insight into the mechanism by which luteinizing hormone (LH) induces resumption of oocyte maturation, cumulus cell matrix expansion and oocyte ovulation in the mammalian ovary have come to light within the last decade. Studies have demonstrated that epidermal growth factor (EGF)-like peptides are important propagators of the LH signal. LH induces fast and transient upregulation in expression of three members of the EGF growth factor family: amphiregulin (AREG); epiregulin (EREG); and betacellulin (BTC) in the mural granulosa cells and cumulus cells (CCs) of several animal species as well as human (Ashkenazi et al. 2005; Ben-Ami et al. 2006; Freimann et al. 2004; Motola et al. 2008; Park et al. 2004; Sekiguchi et al. 2004; Shimada et al. 2006; Zamah et al. 2010). The importance of EGF-like peptides in the propagation of the LH signal *in vivo* was revealed when mice null for *Areg* or *Ereg* exhibited compromised oocyte meiotic resumption, cumulus matrix expansion and ovulation; mice null for one EGF-like peptide generally only exhibit a mild phenotype, which is believed to be due to a compensatory mechanism by the other EGF-like peptides (Hsieh et al. 2007). LH

upregulates expression of EGF-like peptides by acting on mural granulosa cells to induce activation of the p38 mitogen-activated protein kinase (p38MAPK), which in turn leads to sequential upregulation of the three EGF-like peptides (Shimada et al. 2006). The EGF-like peptides are then shed from the cell surface by proteolytic cleavage and bind to the EGF receptor (EGFR; a member of the ERbB family of tyrosine kinases) on both mural granulosa and cumulus cells, in an autocrine and paracrine fashion, respectively (Conti et al. 2006; Shimada et al. 2006). BTC and EREG have also been shown to signal through another member of the ERbB family, ERbB4; however evidence suggests that this receptor does not play a significant role in mediating EGF-like peptide signalling in mural and cumulus granulosa cells (Zamah et al. 2010). Cumulus cells are not directly affected by LH as they express only minute levels of the LH receptor, and hence initially rely on paracrine EGF-like peptide stimulation from the mural granulosa cells before they can autonomously produce these peptides (Conti et al. 2012). Ligand binding in cumulus cells leads to EGFR phosphorylation, for which a key downstream effector is extracellular signal-regulated kinase 1/2 (ERK1/2) (also known as MAPK3/1) and it is clear that ERK1/2 activity is essential for cumulus expansion, resumption of meiosis and ovulation (Downs and Chen 2008; Fan et al. 2009; Hsieh et al. 2007; Shimada et al. 2006; Su et al. 2002). ERK1/2 activation in both granulosa subtypes also induces autonomous production of the EGF-like peptides by a prostaglandin $E_2$ and p38MAPK dependent process (Downs 2010).

Currently, most research and clinical IVM protocols supplement FSH and/or EGF into the culture medium for oocyte maturation (Banwell and Thompson 2008). FSH was shown to upregulate EGF-like peptide production by mural granulosa cells and cumulus cells during IVM (Downs and Chen 2008; Prochazka et al. 2011). A recent human study has shown that amphiregulin mRNA expression at the end of IVM is significantly lower than that seen after *in vivo* oocyte maturation (IVV) in cumulus cells (Guzman et al. 2012). We have also shown in a previous study, using global microarray analysis, that expression of the three EGF-like peptides at the end of maturation is significantly lower in IVM-derived

mouse cumulus cells relative to their IVV counterparts, and apart from haemoglobin α an β peptide sequences, genes for the three peptides were the most differentially expressed (Kind et al. 2012). Several studies have investigated the use of exogenous EGF-like peptides during IVM with findings that amphiregulin, epiregulin, and to a lesser extent betacellulin, effectively stimulate meiotic maturation and cumulus expansion in cumulus-oocyte complexes (COCs) (Park et al. 2004; Prochazka et al. 2011). Furthermore, amphiregulin induced mRNA expression of all three EGF-like peptides in COCs cultured *in vitro* (Shimada et al. 2006). Prochazka *et al.* investigated the effect of EGF-like peptides in IVM on porcine oocyte developmental competence in comparison with gonadotropins and found that IVM with amphiregulin and/or epiregulin improved blastocyst development in comparison with FSH, LH and eCG (Prochazka et al. 2011).

The primary objective of this study was to examine the consequences of FSH- and EGF-supplemented IVM on functional EGF-like peptide signalling in a mouse model. The secondary objective of the study was to examine the effect of EGF-like peptide supplementation during IVM on oocyte developmental competence.

## RESULTS

### Comparison of *in vitro* versus *in vivo derived* cumulus cell EGF-like peptide and receptor mRNA expression

Global microarray analysis from our previous study revealed that the relative number of transcripts for *Areg, Ereg* and *Btc* are greatly reduced in cumulus cells derived from IVM relative to those derived from IVV (Kind et al. 2012). Here we have used quantitative RT-PCR to validate these microarray results and further examine the expression profile of the EGF-like peptides and their receptors. The relative abundance of cumulus cell mRNA transcripts during IVM and IVV was measured over the time course

of oocyte maturation (0-12 h) (Fig. 1). Cumulus cell mRNA expression of the three EGF-like peptides was absent following 46 h of ovarian stimulation with eCG when oocytes are at the germinal vesicle stage. During oocyte maturation, expression of all three peptides was significantly lower for the majority of IVM compared with IVV. *Areg* and *Ereg* expression levels were substantially lower in IVM cumulus cells at 6 h, 9 h and 12 h ($p<0.005$), while *Btc* expression was lower at 9 h and 12 h ($p<0.01$), compared to cumulus cells during maturation *in vivo*. There was notable expression of all three EGF-like peptides at just the 3 h time-point of IVM, at levels statistically comparable to IVV ($p>0.05$). IVV cumulus cell expression of the three peptides was relatively constant from 3 h to 12 h, with the exception of *Ereg* which increased 3.5-fold from 9 to 12 h ($p<0.05$).

There was no difference in *Egfr* mRNA expression between IVM and IVV at any time point measured. It was interesting to note, however, that the expression of *Egfr was* significantly down-regulated after the initial addition of gonadotropins in both maturation scenarios. *ErbB4* mRNA was not detectable until 9 h in both maturation scenarios, and even when detected at 9 h and 12 h, its expression was very low making statistical analysis impractical; thus *ErbB4* mRNA expression was not measured in subsequent experiments. The lack of *ErbB4* expression during the early stages of maturation in this study is consistent with reports in the literature suggesting that EGFR is the predominant receptor mediating EGF-like peptide signalling pathway in follicular mural and cumulus granulosa cells (Zamah et al. 2010).

**Comparison of IVM versus IVV derived COCs on AREG protein expression**

To further validate the gene expression profile of EGF-like peptides expression in FSH-driven IVM versus IVV cumulus cells, COC AREG protein was measured in both maturation scenarios over a time course using ELISA (Fig. 2). Consistent with the *Areg* mRNA expression profile, IVM COCs produced and secreted significantly higher concentrations ($p<0.03$) of AREG protein than their IVV counterparts at 3-6 h of maturation. Moreover, AREG was significantly higher in media conditioned by IVV COC at 6-

9 h and 9-12 h maturation ($p<0.03$) compared with IVM (Fig. 2B). EREG was also measured by ELISA however levels were below the limit of detection of the assay in both IVM and IVV COCs and conditioned media.

**Effects of FSH,EGF and EGF-like peptides *in vitro* and hCG *in vivo* on EGF-like peptide and *Egfr* mRNA expression**

As oocytes are most commonly matured *in vitro* with FSH or alternatively with FSH+EGF, we examined the effects of these treatments on cumulus cell EGF-like peptide mRNA expression compared to IVV matured oocytes and IVM matured oocytes with EGF-like peptides (Fig. 3). Cumulus cells were collected 6 h post hCG (IVV) and from IVM COCs matured in the presence of either no treatment (control), FSH, EGF, AREG, EREG or BTC for 6 h. In the absence of any IVM treatment, no EGF-like peptides were detected in cumulus cells. FSH treatment of IVM COCs failed to stimulate expression of all three EGF-like peptides to the level of IVV COCs. By contrast, treatment of IVM COCs with EGF, AREG or EREG significantly ($p<0.05$) increased *Areg, Ereg* and *Btc* expression compared to FSH to levels that were not significantly different to IVV expression levels. BTC-stimulated expression of *Areg* and *Ereg* was significantly ($p<0.05$) lower than AREG-, EREG-, EGF- and IVV-stimulated expression. However, BTC still promoted significantly ($p<0.05$) higher expression levels of *Areg* and *Btc* than FSH ($p<0.05$). No significant differences were observed in *Egfr* expression between IVV cumulus cells and all IVM treatments; however expression was significantly up-regulated in the absence of any IVM treatment (control).

**Effects of FSH and EGF *in vitro* and hCG *in vivo* on activation of EGFR and ERK1/2**

Immunodetection was used to measure EGFR and ERK1/2 phosphorylation in IVM COCs at a number of time points following culture with FSH and/or EGF, or after IVV (Fig. 4). Both FSH- and/or EGF-supplemented IVM COCs contained significantly ($p\leq0.022$) lower levels of phosphorylated EGFR

(pEGFR) than IVV COCs at 3 h and 6 h of maturation. EGF elicited early phosphorylation of EGFR, where pEGFR was significantly ($p \leq 0.033$) higher at 0.5 h in the presence of EGF and EGF+FSH, compared with FSH or IVV. No significant differences in pEGFR were seen at 9 h and 12 h of maturation. Despite the decreased pEGFR during IVM at 3 h and 6 h, there were no significant differences in total ERK1/2 (tERK1/2) (data not shown) or phosphorylated ERK1/2 (pERK1/2) between any of the stimulated IVM treatment groups (FSH/EGF/FSH+EGF) and IVV at any time point. pERK1/2 levels in unstimulated (control) COCs were significantly lower than all other treatment groups at 6 h, and at 9 h (with the exception of EGF), and at 12 h (with the exception of EGF and FSH+EGF).

**Effects of FSH, EGF, AREG and EREG on oocyte developmental competence**

Since mRNA expression of the EGF-like peptides was significantly lower in standard (FSH) IVM cumulus cells compared with their IVV counterparts, IVM COCs were cultured in the presence of exogenous FSH, EGF, AREG and/or EREG and embryo development was compared. Day 6 blastocyst rate was significantly higher for COCs matured with EREG ($p<0.05$), but not AREG or AREG+EREG, compared with those cultured with FSH or EGF (Table II). Furthermore, COCs cultured with EREG showed a trend ($p=0.0589$) for a higher hatching blastocyst rate compared with those cultured with FSH. There were no significant differences in cleavage rate, day 5 blastocyst and hatching blastocyst rates, and day 6 hatching blastocyst rates between any groups. Embryo quality was examined via quantification of the trophectoderm (TE) and inner cell mass (ICM) of day 6 blastocysts. Although there were no significant differences in TE and ICM cell numbers between treatment groups, the proportion of ICM cells per total blastocyst cells was significantly ($p \leq 0.036$) higher in the presence of EREG or AREG, compared with FSH and EGF (Table II). Such a change if cell ratio is indicative of an improvement of blastocyst quality and post-transfer developmental potential (Lane and Gardner 1997).

**DISCUSSION**

This study aimed to examine the consequences of FSH- and EGF-supplemented IVM on EGF-like peptide signalling. Here we have shown that EGF-like peptides are deficient in cumulus cells undergoing standard FSH-stimulated IVM, and that, while the addition of EGF to an IVM system yields EGF-like peptide mRNA expression comparable to IVV levels, it does not improve EGFR or ERK1/2 activation, or improve oocyte developmental competence above that of FSH. This study also examined the effect of amphiregulin and epiregulin supplementation during IVM on oocyte developmental competence, and our results have shown that epiregulin increases blastocyst formation and quality and thereby improves oocyte developmental competence.

LH-induced EGF-like peptide signalling in mural granulosa and cumulus cells is a critical event that occurs in the pre-ovulatory follicles of mammalian species, thus far confirmed in rodent, pig, cow, horse, macaque, and human (Ashkenazi et al. 2005; Ben-Ami et al. 2006; Freimann et al. 2004; Motola et al. 2008; Park et al. 2004; Sekiguchi et al. 2004; Shimada et al. 2006; Zamah et al. 2010). The autocrine and paracrine expression of EGF-like peptides in both these somatic cell types plays a fundamental role in oocyte maturation, cumulus matrix expansion and ovulation (Conti et al. 2012). LH exerts its effect to upregulate expression of EGF-like peptides by acting on mural granulosa cells only. Cumulus cells are not directly stimulated by LH as they do not possess LH receptors, and therefore initially rely on paracrine EGF-like peptide stimulation from the mural granulosa cells before they can autonomously produce these peptides (Conti et al. 2006). Hence, one of the earliest signals the COC receives to resume meiosis and prepare for embryonic development, is from EGF-like peptides produced by mural granulosa cells.

Evidence in the literature exists showing that initial activation of the EGF network by LH is quickly amplified and maintained during at least the first half of oocyte maturation (Ben-Ami et al. 2006; Motola

et al. 2008; Panigone et al. 2008; Shimada et al. 2006). The current study is the first to directly characterise and compare the cumulus cell EGF network throughout *in vivo* versus *in vitro* maturation. FSH is a universal hormonal additive in IVM. Here we show that, although FSH induces mRNA upregulation of the three EGF-like peptides in cumulus cells at the earliest stages of IVM, there is a sharp and significant decline in expression during the remainder of IVM. In contrast, EGF-like peptide mRNA expression by hCG during IVV is amplified and maintained over time. Furthermore, cumulus cell mRNA expression of all three EGF-like peptides and amphiregulin protein expression are significantly lower during the majority of FSH-supplemented IVM than during IVV. It is therefore not surprising that the level of activated EGFR during IVM is also significantly lower at 3 h and 6 h, compared to IVV. Mural granulosa cells are the major epithelial component of the pre-ovulatory follicle and are the source of a significant proportion of the EGF-like peptides produced and secreted in the follicle in response to LH (Eppig 1994; Park et al. 2004; Sekiguchi et al. 2004). The deficiency in EGF-like peptide expression and EGFR activation in FSH-supplemented IVM cumulus cells may be due to the absence of mural granulosa cells, which would otherwise secrete and expose cumulus cells to large amounts of EGF-like peptides, leading to their auto-amplification of this signalling network. Hence, it is possible that a major contributing factor to the poor developmental competence of oocytes derived from standard FSH-driven IVM systems is the continuous exposure of COCs to sub-optimal concentrations of one or all three EGF-like peptides throughout oocyte maturation. This idea is supported by our findings which showed that; (i) EGFR phosphorylation at the early stages of maturation is significantly lower in COCs treated with FSH during IVM than their IVV counterparts; and (ii) the exposure of IVM COCs to a high concentration of epiregulin significantly increases blastocyst rate and, epiregulin and/or amphiregulin improve blastocyst quality, compared with FSH. Amphiregulin and/or epiregulin supplementation during IVM has also been shown by others to induce higher developmental competence in porcine oocytes compared with FSH or LH (Prochazka et al. 2011).

EGF is rarely used in clinical IVM and is occasionally used in non-human research and veterinary IVM systems, usually in combination with FSH (Banwell and Thompson 2008). EGF induces oocyte meiotic resumption, cumulus expansion, and improves developmental competence in comparison with spontaneous oocyte maturation in several animal species (Rieger et al. 1998). Like FSH, EGF elicits cumulus expansion and oocyte meiotic resumption through activation of ERK1/2 (Conti et al. 2006; De La Fuente et al. 1999; Su et al. 2002); although unlike FSH, it does this by directly binding to and phosphorylating EGFR (Massague and Pandiella 1993). Here, we directly compared the effects of EGF and FSH during IVM, and hCG during IVV, on EGF-like peptide mRNA expression and EGFR signalling. Unlike FSH, EGF exposure during IVM stimulated cumulus cell mRNA expression of all three EGF-like peptides to levels comparable to IVV and amphiregulin- and epiregulin-supplemented IVM. Despite its ability to restore EGF-like peptide expression levels to those seen *in vivo*, EGF did not elicit an improvement in oocyte developmental competence over FSH. This may be attributed to its inability to maintain sufficient EGFR phosphorylation throughout IVM (Fig. 4); pEGFR levels were significantly higher in COCs during EGF-supplemented IVM than IVV after 30 minutes of maturation but were then lower at 3 and 6 hours of maturation, with levels similar to those of FSH matured IVM COCs. Perhaps unexpectedly, there were no detectable differences in ERK1/2 phosphorylation between EGF matured IVM COCs and their IVV counterparts, despite the difference in EGFR activity. This may be due to the phosphorylation of ERK1/2 via other signalling cascades since inhibition of EGFR activity only inhibits ~50% of ERK1/2 phosphorylation in preovulatory follicles (Panigone et al. 2008). Alternatively, the lack of a difference in ERK1/2 phosphorylation, coupled with the improved developmental competence induced by amphiregulin and epiregulin, may suggest that EGF and the EGF-like peptides differentially regulate other EGFR activated pathways. EGFR ligands have distinct binding specificities and affinities, and depending on ligand interaction, EGFR activation can initiate several signal transduction pathways, predominantly the MAPK, AKT and JNK pathways (Oda et al. 2005). Further analysis of alternative

EGFR activated pathways may elucidate a mechanism by which EGF and the EGF-like peptides differentially regulate EGFR signalling.

When the effects of the three EGF-like peptides, FSH and EGF during IVM on EGF-like peptide mRNA expression were examined, EGF, amphiregulin and epiregulin were found to induce comparable expression levels. Betacellulin, however, appears to be a less potent stimulator of EGF-like peptide signalling since it induces significantly lower *Areg* and *Ereg* expression than EGF, amphiregulin or epiregulin. This is likely the reason betacellulin is a poorer stimulator of oocyte meiotic resumption (Park et al. 2004).

We compared the effects of FSH, EGF, amphiregulin and epiregulin during IVM on oocyte developmental competence and found epiregulin to increase day 6 blastocyst yield, and epiregulin or amphiregulin to improve blastocyst quality, over FSH and EGF. It is curious that EGF, epiregulin and amphiregulin yield differences in oocyte developmental competence despite acting through the same receptor, EGFR. As previously mentioned, EGFR ligands exhibit significant differences in intrinsic activity and can produce distinct biological outcomes (Wilson et al. 2009; Wilson et al. 2012), and this may explain, at least in part, the differences in developmental competence observed. For example, Wilson et al. (2012) have shown that amphiregulin possesses greater intrinsic activity than EGF, and that EGF competitively antagonises amphiregulin in human myeloid and breast cells. Different EGFR ligands also phosphorylate the receptor on distinct sets of tyrosine residues which likely alter EGFR signalling (Wilson et al. 2009). For instance, EGF elicits strong phosphorylation of EGFR Tyr 1045, whereas amphiregulin does not (Gilmore et al. 2008). Wilson et al. (2009) hypothesised that differential Tyr 1045 phosphorylation by different ligands leads to differences in the duration of the EGFR signal, with data suggesting that the duration of EGF-induced EGFR signalling is shorter than that of amphiregulin-induced signalling (Wilson et al. 2009).

Although it has yet to be investigated, it has been suggested that amphiregulin and epiregulin accumulate together, and hence may have additive or synergistic effects on maturation *in vivo* (Conti et al. 2006). We investigated oocyte developmental competence outcomes when IVM COCs were matured with both epiregulin and amphiregulin and saw no synergistic or additive effects; rather, the positive effect of epiregulin on blastocyst yield was lost when amphiregulin was also present. Although we did not see an additive or synergistic effect, we cannot exclude the possibility of such effects at other concentrations of amphiregulin and epiregulin.

In conclusion, findings from this study suggest that the common IVM additives, FSH and EGF, are inadequate propagators of the essential EGF-like peptide signalling cascade that occurs in cumulus cells *in vivo*. We have shown that FSH does not promote sufficient expression of EGF-like peptides when compared to levels *in vivo*. Furthermore, both FSH and EGF do not maintain adequate activation of the EGFR. The current study indicates that EGF-like peptides, rather than FSH or EGF, should be added to IVM systems as they were shown by us and others to improve embryo development. Such an approach may represent a more physiological form of IVM as amphiregulin, epiregulin and betacellulin are naturally induced in the somatic cells of the follicle to induce cumulus expansion, oocyte maturation and ovulation.

## 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.

296 129/SV female mice were used for all experiments. COCs were collected from 21- to 28-day old mice,
297 46 h after intraperitoneal injection of 5 IU of equine chorionic gonadotropin (eCG; Folligon, Intervet,
298 Boxmeer, Holland) to stimulate follicular growth. Human chorionic gonadotropin (hCG, 5 IU; Organon,
299 Sydney, Australia) was administered 46 h post-eCG for IVV experiments to induce oocyte maturation *in*
300 *vivo*. COCs were isolated from preovulatory follicles using a 27-gauge needle and collected using
301 flame-pulled borosilicate Pasteur pipettes in HEPES buffered αMEM (Gibco, Invitrogen, Carlsbad, USA)
302 supplemented with 3 mg/mL bovine serum albumin (BSA).
303
304 **Oocyte *in vitro* maturation**
305 COCs were cultured in bicarbonate buffered αMEM (Gibco, Life Technologies, NY, USA) supplemented
306 with 3 mg/mL BSA (ICPbio, Glenfield, New Zealand) and either: recombinant human FSH (100 mIU/mL;
307 Puregon, Organon, USA), recombinant human EGF (10 ng/mL; R&D Systems, Minneapolis, USA),
308 recombinant mouse AREG (50 ng/mL; R&D Systems), recombinant mouse EREG (50 ng/mL; R&D
309 Systems), or recombinant mouse BTC (50 ng/mL; R&D Systems) at 37ºC and 5% $CO_2$ in air. Doses of
310 EGF family growth factors were based on previous studies using EGF (De La Fuente et al. 1999; Li et
311 al. 2008) and EGF-like peptides (Downs and Chen 2008).
312
313 **RNA Isolation**
314 Following the indicated periods of *in vivo* or *in vitro* maturation, cumulus cells were separated from
315 COCs by mechanical shearing using a P200 pipette and washed with PBS. Total RNA was extracted
316 using the RNeasy Micro Kit (Qiagen, Germantown, USA) according to the manufacturer's instructions.
317 DNA that may have been co-purified was removed by addition of DNase (0.34 Kunitz units/µL supplied
318 with kit). RNA was eluted in 14 µL of RNAse-free water and stored at -80ºC. The final RNA
319 concentrations were determined by absorbance using a NanoDrop ND-1000 spectrophotometer
320 (Thermo Fisher Scientific, Scoresby, Australia).

**RT-qPCR**

An equal amount of total RNA from each sample was reverse transcribed using random hexamers (Life Technologies (Invitrogen) Mulgrave, Australia) and Superscript III reverse transcriptase (Invitrogen). Primers (10 pmol/reaction; Table I) and cDNA were added to 20 µL total reaction volume with SYBR Green (Applied Biosystems, Mulgrave, Australia). PCRs were then performed using a Corbett Rotor-Gene 6000 (Qiagen). A seven point serial dilution standard curve was produced for each transcript from cDNA generated from mural granulosa cells. The relative gene expression values were calculated using the standard curve method and presented relative to a calibrator and normalised to the geometric mean of two housekeeping genes (*Mrpl19* and *Ppia*). To validate primer pairs, amplicons generated from mouse cumulus cell cDNA were run on 2% agarose gels and primer pairs were considered valid when a single product of the correct size was observed and primer efficiency was above 95%.

**ELISA**

AREG and EREG protein levels in COC extract and conditioned media were quantified using a commercially available mouse enzyme-linked immunosorbent assay kits (ELISA; R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. IVM COCs were cultured *in vitro* as described above in the presence of 100 mIU/mL FSH and 3 mg/mL BSA for 3, 6 or 9 h and then transferred into 100 µL culture media without FSH and cultured for a further 3 h. IVV COCs (50 per treatment) were collected at 3, 6 or 9 h post-hCG and cultured in 100 µL culture media without FSH for 3 h. The media and COCs were then collected separately and snap frozen in liquid nitrogen and stored at -80$^0$C. For the ELISA assay, COC samples were resuspended in 40 µL RIPA buffer containing protease inhibitors and freeze-thawed 4X in liquid nitrogen to lyse, and 50 µL of the kit's reagent diluent containing protease inhibitors was added. Eighty microlitres of media or COC lysate were then assayed.

**EGFR and ERK1/2 Immunodetection**

Following the indicated periods of *in vivo* or *in vitro* maturation, whole COCs were collected as described above and suspended in RIPA buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100) containing phosphatase (Roche, Penzberg, Germany) and protease inhibitor cocktails, snap frozen in liquid nitrogen and stored at -80ºC. Samples were mixed with loading buffer containing 100 mM dithiothreitol, heated at $100^0$C for 5 mins, and loaded onto a 7.5% SDS–polyacrylamide gel for electrophoresis. Proteins were transferred to Hybond-ECL membranes (GE Healthcare, Waukesha, USA). Membranes were then blocked with 2% blocking reagent (supplied in an ECL Advance kit; GE Healthcare) diluted in Tris-buffered saline containing 0.1% (v/v) Tween 20. Each membrane was cut in half horizontally and the upper half was incubated with the primary antibody anti-phospho-EGFR (pEGFR; Cell Signalling Technology, Beverley, USA, cat. no. 3777) diluted 1:1000, and the lower half was incubated with anti-phospho-ERK1/2 (pERK1/2; Sigma, cat. no. M8159) diluted 1:10,000 at 4°C overnight, followed by incubation with goat anti-rabbit IgG peroxidase-conjugated (diluted 1:200,000, Santa Cruz Biotechnology Inc, Santa Cruz, USA, cat. no. SC-2004) and donkey anti-mouse peroxidase-conjugated (diluted 1:400,000, Santa Cruz, cat. no. SC-2314) secondary antibodies, respectively. Binding was detected using the ECL Advance kit and exposure to Hyperfilm (GE Healthcare). The lower half membrane was then stripped using an acidic glycine stripping buffer (1% SDS, 25mM glycine, pH 2.0) and was incubated with anti-ERK1/2 (tERK1/2, Sigma, cat. no. M5670) diluted 1:10,000 at 4°C overnight, followed by incubation with goat anti-rabbit IgG peroxidase-conjugated (diluted 1:400,000). This membrane was then stripped again and incubated with anti-β-Actin peroxidise conjugated antibody (diluted 1:20 million, Sigma, cat. no. A3854). Band intensities were measured using Image J software (NIH; Bethesda, USA). Band intensities for pEGFR, pERK1/2 and tERK1/2 were normalised to β-Actin band intensities and standardised relative to post-hCG values

(except at 3 h maturation where they were standardised to FSH&EGF values). Data is represented as the mean of at least three replicate experiments.

**Oocyte Developmental Competence**

To examine the effects of EGF-like peptides on oocyte developmental competence, COCs underwent IVM with various treatments, followed by IVF and *in vitro* embryo development to day 6. Immature COCs were collected from preovulatory follicles 46 h post-eCG into HEPES buffered αMEM. IVM was performed in bicarbonate buffered αMEM supplemented with 3 mg/ml BSA, 1 mg/ml fetuin and FSH or EGF peptides (as described above). Following 17 h of IVM, COCs were placed in Research VitroFert fertilization media (Cook Medical, William A. Cook Australia Pty Ltd., QLD, Australia, cat. no. K-RVFE-50) with capacitated CBA x C57BL6 F1 epididymal sperm for 4 h at 37ºC at 6% $CO_2$, 5% $O_2$ and balance of nitrogen. COCs were then transferred into Research VitroWash media (Cook Medical, cat. no. K-RVWA-50) and cumulus cells were removed by mechanical shearing with a P200 pipette. Presumptive zygotes were then washed and incubated in Research VitroCleave media (Cook Medical, cat. no. K-RVCL-50) at 37ºC with 6% $CO_2$, 5% $O_2$ and balance of nitrogen. Embryo development was assessed at days 2, 5 and 6 post-insemination using the scoring system reported by Gardner *et al.* (Gardner et al. 2004).

Day 6 blastocyst trophectoderm (TE) and inner cell mass (ICM) cell numbers were quantified by differential staining using a published protocol (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 placed in 10% 2,4,6-trinitrobenzene sulfonic acid for 10 mins at 4°C. They were then incubated with anti-2,4-8 dinitrophenol (1:10) for 10 mins at 37°C, followed by complement (1:1; 2 µg/ml propidium iodine:guinea pig serum) for a further 10 mins. Blastocysts were then 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. Stains were visualised using an epifluorescent microscope excitation 340-380 nm, emission 440-480 nm). 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. For PCR and Western blot data, statistical significance was assessed by ANOVA followed by Tukey's multiple-comparison post-hoc tests to identify individual differences between means. Where data were not normally distributed, statistical significance was assessed by non-parametric Kruskal-Wallis one-way ANOVA by ranks. T-tests were used to analyse ELISA data. 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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**AUTHORS' ROLES**

R.B.G. conceived the study and secured funding. All authors contributed to the design of the study. D.R. performed all experiments with guidance from L.J.R. D.R. carried out statistical analyses and prepared the figures. D.R. analysed the data with input from all authors. D.R. wrote the manuscript with review by all authors.

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523

524

525 Table I: Sequences of PCR primers used for RT-qPCR

526

| 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 |
| *ErbB4* | NM_010154.1 | AGAAACTGAATGTCTTTCGGACTGT | TGTGACGAGGTTGGAGAAAACA | 107 |
| *Mrpl19* | NM_026490 | GAAAGGTGCTTCCGATTCCA | TGATCGCTTGATGCAAATCC | 116 |
| *Ppia* | NM_008907.1 | TGGCAAATGCTGGACCAA | CCTTCTTTCACCTTCCCAAAGA | 106 |

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

527

528

529 Table II: Embryo development of IVM oocytes cultured in medium supplemented with FSH, EGF and

530 EGF-like peptides

531

| IVM Treatment | Number of COCs | Cleaving embryos (%) | Day 5 blastocysts/ cleaving embryos (%) | Day 6 blastocysts/ cleaving embryos (%) | Day 6 hatching blastocysts/ cleaving embryo (%) | Day 6 blastocyst inner cell mass/total cells (%) |
|---|---|---|---|---|---|---|
| FSH | 195 | 67 | 29 | 49[a] | 34 | 11.2 ± 1.2 [a] |
| EGF | 230 | 66 | 26 | 48[a] | 37 | 12.9 ± 1.1 [ac] |
| AREG | 200 | 67 | 31 | 54[ab] | 35 | 17.5 ± 1.4 [b] |
| EREG | 249 | 67 | 34 | 60[b] | 45 | 18.0 ± 2.3 [b] |
| AREG+EREG | 217 | 67 | 30 | 53[ab] | 42 | 17.0 ± 1.8 [bc] |

532 Values not sharing a common letter within columns are significantly different ($p \leq 0.05$). Data is from five replicate experiments.

533

534 **FIGURE LEGENDS**

535

536 **Figure 1: Effect of maturation *in vitro* (IVM) compared to *in vivo* (IVV) on cumulus cell EGF-like**
537 **peptide and receptor mRNA expression.** Cumulus cells were harvested from IVM cumulus-oocyte
538 complexes cultured with FSH (grey bars) and from IVV cumulus-oocyte complexes matured *in vivo* with
539 hCG (black bars). mRNA expression was measured using quantitative RT-PCR and normalized to the
540 geometric mean of the *Mrpl19* and *Ppia*. Bars not sharing a common letter are significantly different;
541 $IVM^{a-d}$, $IVV^{x-z}$ ($p \leq 0.05$). (*) indicate a significant difference ($p \leq 0.05$). (#) indicates below limit of
542 detection. Data is from 3 replicate experiments.

543

544 **Figure 2: Effect of maturation *in vitro* (IVM) compared to *in vivo* (IVV) on amphiregulin**
545 **production.** IVM cumulus-oocyte complexes were cultured *in vitro* with FSH and IVV cumulus-oocyte
546 complexes were matured with hCG. Both cumulus-oocyte complex types were collected after 3 h, 6 h or
547 9 h of maturation and placed in 100 µL bicarbonate buffered medium with 3 mg/ml BSA and cultured *in*
548 *vitro* for a further 3 h. Amphiregulin (AREG) protein was measured in the cumulus-oocyte complexes
549 (A) and their conditioned media (B) by ELISA and quantified using the standard curve method. (*)
550 indicate a significant difference ($p<0.03$). (#) indicates below limit of detection. Data is from 4 replicate
551 experiments.

552

553 **Figure 3: Effects of FSH, EGF and EGF-like peptides *in vitro* and hCG *in vivo* on cumulus cell**
554 **EGF-like peptide and *Egfr* mRNA expression.** Cumulus cells were harvested at 6 h from IVM
555 cumulus-oocyte complexes cultured in the absence (control) or presence of FSH, EGF, AREG, EREG
556 or BTC, or from IVV cumulus-oocyte complexes matured with hCG for 6. Bars not sharing a common
557 letter are significantly different ($p \leq 0.05$). N.D, not detectable. Data is from 6 replicate experiments.

558

559 **Figure 4: Immunodetection of cumulus-oocyte complex EGFR and ERK1/2 phosphorylation in**
560 **response to FSH and EGF *in vitro* and hCG *in vivo.*** Cumulus-oocyte complexes were cultured via
561 IVM in the absence (control) or presence of FSH, EGF or FSH+EGF, or matured via IVV with hCG for
562 0.5 h, 3 h, 6 h, 9 h, or 12 h. pEGFR and pERK1/2 levels were measured using Western blots. For each
563 time point, a representative blot of at least 3 replicate experiments is shown and the quantified
564 densitometric values below it. Densitometric measurements of pEGFR were normalised to β-actin and
565 pERK1/2 were normalised to tERK1/2 in each individual blot. The blot bands shown are in order of the
566 following treatments: p-hCG, control, FSH, EGF and FSH+EGF, respectively, and are representatives
567 of pEGFR and pERK1/2. pEGFR, phosphorylated EGFR; pERK1/2, phosphorylated ERK1/2; tERK1/2,
568 total ERK1/2.
569