EPIDERMAL GROWTH FACTOR-LIKE PEPTIDE SIGNALLING AND OOCYTE IN VITRO MATURATION

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Thesis submitted to the University of Adelaide in total fulfilment of the requirements for the degree of Doctor of Philosophy in Medicine.

February 2014

THE UNIVERSITY of ADELAIDE

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ABSTRACT

A growing body of evidence has recently implicated follicular epidermal growth factor (EGF)-like peptide signalling as essential for the propagation within the ovarian follicle of the LH stimulus that induces oocyte maturation and ovulation. The EGF-like peptides amphiregulin, epiregulin, and betacellulin are produced in mural granulosa and cumulus cells in response to LH, and signal via the EGF receptor (EGFR) in these cells to ultimately induce oocyte maturation, cumulus expansion, and ovulation. Although the function and impact of EGF-like peptide signalling on oocyte maturation *in vivo* has been characterised, little is currently known about the effect of oocyte *in vitro* maturation (IVM) on this important signalling network. This thesis aimed to investigate the regulation of EGF-like peptide signalling in mouse cumulus cells and the effect of various IVM models on this network.

FSH is a universal IVM additive, and EGF is occasionally used in animal IVM. The effect of FSH-stimulated IVM, EGF-stimulated IVM versus *in vivo* maturation (IVV) on cumulus cell EGF-like peptide mRNA and/or protein expression, the activity of EGFR, and its classic downstream effector, ERK1/2, were examined. EGF-like peptide mRNA expression, amphiregulin protein expression, and EGFR phosphorylation were significantly lower using FSH-stimulated IVM than during IVV. EGF stimulated significantly lower EGFR phosphorylation, but not EGF-like peptide mRNA expression. These data demonstrate that this signalling network is perturbed in IVM cumulus cells.

The effect of FSH, EGF, amphiregulin and epiregulin in IVM on subsequent blastocyst development revealed that epiregulin and amphiregulin significantly increased blastocyst yield and/or the proportion of inner cell mass in blastocysts, than FSH or EGF. Examination of the metabolic profiles of IVM cumulus-oocyte complexes (COCs) matured in the presence of these stimulants revealed that EGF-like peptides and EGF induced significantly higher COC glucose metabolism via the hexosamine biosynthesis pathway than FSH, consequently enabling more hyaluronic acid synthesis and protein β-O-linked glycosylation in the cumulus cells. Epiregulin significantly increased intra-oocyte FAD^{++} and the REDOX ratio compared to FSH, and all three EGF-like peptides induced more oocyte mitochondrial activity than EGF or FSH.

Evidence has shown that increasing 3'-5'-cyclic adenosine monophosphate (cAMP) using pharmacological agents significantly increases IVM oocyte developmental competence. This concept, in the form of a pre-IVM culture period with cAMP modulators, was examined in conjunction with IVM in the presence of epiregulin, amphiregulin, EGF or FSH. A pre-IVM phase in conjunction with IVM with EGF-like peptides endowed greater oocyte developmental competence than with FSH or EGF, as evidenced by increased embryo yield and/or quality, which were comparable in embryo development and/or quality rates from IVV oocytes.

This thesis provides the physiological basis for, and evidence that, EGF-like peptides are more appropriate IVM additives than FSH or EGF. EGF-like peptides endow greater oocyte developmental competence than FSH, possibly by regulating important aspects of COC metabolism. Combining this concept with cAMP modulation of IVM COCs may represent a more physiological IVM system than existing IVM approaches, as it yields more blastocysts of higher quality. The knowledge provides new opportunities for the treatment of infertility in women and for the *in vitro* production of embryos and advanced breeding in animals.

DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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February 2014

Dulama Richani

ACKNOWLEDGEMENTS

As I sit down to write these acknowledgements, the one person at the forefront of my mind is my wonderful husband, Adnaan Baraky. I could not have wished for a more supportive partner as I travelled down this road. Adnaan, you have always lifted me up, supported me, consoled me, encouraged me, inspired me, and made me believe I can achieve anything. You have gone on this journey with me and filled every second of it with love and encouragement. I can never thank you enough for what you give me and what you do for me on a daily basis. I love you!

I owe a great deal of thanks to my supervisors Associate Professors Robert Gilchrist and Jeremy Thompson. I very honestly say that I couldn't have asked for better supervisors. Thank you for your mentorship, friendship, and guidance. Thank you for giving me the freedom to pursue my own interests within my project. I know you probably had no idea what I was up to in the lab at times but you had faith in me. Most of all, thank you for teaching me how to be a good researcher and for nurturing my love of research. You have always had your doors open to me and always went above and beyond the call of duty to help me. I am forever grateful!

A special thanks goes to Lesley Ritter for so many things. Lesley, thank you for your support, advice, and guidance throughout my PhD. Thank you for patiently answering the *thousands* (maybe even hundreds of thousands?) of questions I asked you over the years, for your support in the lab, and for our chats.

I would really like to thank my fellow lab members who became my friends and laughed a lot with me: Laura Frank, Mel White, Ryan Rose, Marie Anastasi, Jacky Sudiman, Xiaoqian Wang, Dianne Feil, Annie Whitty, Mel McDowall, and all those I have not mentioned by name. I will miss all our weird and wonderful conversations and our outings to the pub. Laura, you helped me keep my sanity when things weren't going well in the lab and you have been my PhD soul mate. Mel White and Marie, thanks for all the wild times (you know what I'm talking about). Ryan and Jacky, thanks for all the silly times in our PhD room; you made our office fun.

Last, but certainly not least, I would like to thank my highly supportive family who have been a source of encouragement though out my PhD. You all light up my life. I would like

to especially thank Mum and Nick for all that they have done for me and given me. Thank you for babying and spoiling me, even to this day, and for always being there. I love you all!

As I reflect back, I feel very grateful and lucky to have completed my PhD surrounded by such wonderful people. Although it was a rollercoaster ride at times, you are all the reason I loved going to work every day. I am so happy to see how much I have grown as a scientist since the beginning of my PhD studies.

ABBREVIATIONS

AC	adenylate cyclase
ANOVA	analysis of variance
AREG	amphiregulin
ART	assisted reproductive technology
ATP	adenosine triphosphate
BMP15	bone morphogenetic factor 15
BSA	bovine serum albumin
BTC	betacellulin
cAMP	cyclic adenosine monophosphate
CEI	cumulus expansion index
CC	cumulus cell
cGMP	cyclic guanine monophosphate
CNP	C-type natriuretic peptide
COC	cumulus-oocyte complex
DNA	deoxyribonucleic acid
DO	denuded oocyte
eCG	equine chorionic gonadotropin
ECL	enhanced chemiluminescence
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EREG	epiregulin
ERK	extracellular signal-regulated kinase
ErbB	avian erythroblastosis oncogene B
FAD^{++}	flavin adenine dinucleotide
FSH	follicle stimulating hormone
FSK	forskolin
GC	granulosa cell
GDF9	growth differentiation factor 9
GFPT	glutamine:fructose-6-phosphate dehydrogenase
GlcNAc	N-acetylglucosamine
GV	germinal vesicle
GVBD	germinal vesicle breakdown
HBP	hexosamine biosynthesis pathway

hCG	human chorionic gonadotropin
IBMX	3-isobutyl-1-methylxanthine
ICM	inner cell mass
ICSI	intracytoplasmic sperm injection
IVF	*in vitro* fertilization
IVM	*in vitro* maturation
JNK	c-Jun N-terminal kinase
LH	luteinising hormone
MI	metaphase I
MII	metaphase II
mg	milligram(s)
MGC	mural granulosa cell
mL	millilitre(s)
mM	millimolar
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
NAD^+/NADH	nicotinamide adenine dinucleotide
$NADP^+$/NADPH	nicotinamide adenine dinucleotide phosphate
NAD(P)H	combined NADH and NADPH
NS	non siginificant
O-GlcNAcase	β-N-acetylglucosaminidase
OGT	β-*O*-linked glycosylation
P	probability
PBS	phosphate buffered saline
PDE	phosphodiesterase
PDE3	phosphodiesterase subtype 3
PFK	phosphofructokinase
PI	propidium iodide
PI3K	phosphoinositide-3-kinase
PPP	pentose phosphate pathway
REDOX	reduction-oxidation
RIPA	radioimmunoprecipitation
RNA	ribonucleic acid
RT	reverse transcription
SD	significant difference

SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard error of the mean
TCA	tricarboxylic acid
TE	trophectoderm
µL	microliter
µM	micromolar

PUBLICATIONS

Scientific publications generated throughout PhD candidature:

1. **Dulama Richani**, Lesley J. Ritter, Jeremy G. Thompson and Robert B. Gilchrist. 2013. Mode of oocyte maturation affects EGF-like peptide function and oocyte competence. *Molecular Human Reproduction* 19(8):500-509. {Appendix 1}

2. Robert B. Gilchrist and **Dulama Richani**. 2013. Somatic guidance for the oocyte. *Developmental Cell* 27:603-605. {Appendix 4}

3. **Dulama Richani**, Melanie L. Sutton-McDowall, Laura A. Frank, Jeremy G. Thompson and Robert B. Gilchrist. 2014. Effect of epidermal growth factor-like peptides on the metabolism of *in vitro*-matured mouse oocytes and cumulus cells. *Biology of Reproduction* 90(3):49, 1–10. {Appendix 2}

4. **Dulama Richani**, Xiaoqian Wang, Hai-tao Zeng, Johan E.J. Smitz, Robert B. Gilchrist and Jeremy G. Thompson. 2014. Pre-maturation with cAMP modulators in conjunction with EGF-like peptides during IVM enhances mouse oocyte developmental competence. *Molecular Reproduction and Development* 81:422-435. {Appendix 3}

5. Hai-tao Zeng, **Dulama Richani**, Melanie L. Sutton-McDowall, Zi Ren, Johan E.J. Smitz, Yvonne Stokes, Robert B. Gilchrist and Jeremy G. Thompson. 2014. Pre-maturation with cyclic adenosine monophosphate modulators alters cumulus cell and oocyte metabolism and enhances developmental competence of *in vitro* matured mouse oocytes. *Biology of Reproduction* [In Press, BIOLREPROD/2014/118471].

6. Hannah Brown, Marie Anastasi, Laura Frank, Karen Kind, **Dulama Richani**, Rebecca Robker, Darryl Russell, Robert Gilchrist and Jeremy Thompson. 2014. Haemoglobin: A gas-transport molecule that is hormonally regulated in the ovarian follicle. *Human Reproduction* [Submitted 11 June 2014, HUMREP-14-0702].

CONFERENCE PROCEEDINGS

Abstracts (published)

D. Richani, M.L. Sutton-McDowall, L.A. Frank, J.G. Thompson and R.B. Gilchrist (2013) *'Effect of epidermal growth factor-like peptides on the metabolism of in vitro matured mouse oocytes and cumulus cells'*, Society for Reproductive Biology, Sydney, Australia

D. Richani, L.J. Ritter, J.G. Thompson and R.B. Gilchrist (2012) *'Consequences of in vitro maturation of oocytes on cumulus cell EGF-like peptide signalling'*, Society for the Study of Reproduction, Pennsylvania, USA

D. Richani, L.J. Ritter, J.G. Thompson and R.B. Gilchrist (2011) *'Cumulus cell EGF-like peptide and receptor signalling during oocyte in vitro maturation'*, The Society of Reproductive Biology, Cairns, Australia

Meeting abstracts (unpublished)

D. Richani, M.L. Sutton-McDowall, L.A. Frank, J.G. Thompson and R.B. Gilchrist (2013) *'Effect of EGF-like peptides on the metabolism of oocytes and their associated somatic cells'*, Australian Society for Medical Research, Adelaide

D. Richani, L.J. Ritter, J.G. Thompson and R.B. Gilchrist (2012) *'Consequences of in vitro maturation of oocytes on cumulus cell EGF-like peptide signalling'*, Faculty of Health Sciences Postgraduate Research Conference, the University of Adelaide

D. Richani, L.J. Ritter, J.G. Thompson and R.B. Gilchrist (2011) *'The effect of oocyte in vitro maturation on EGFR pathway signalling'*, Australian Society for Medical Research, Adelaide

Conference presentations

Oral presentations

2013 Annual meeting of Society of Reproductive Biology, Sydney, Australia
'Effect of EGF-like peptides on the metabolism of oocytes and their associated cumulus cells'

2013 Annual meeting of Australian Society for Medical Research, Adelaide, Australia
'Effect of EGF-like peptides on the metabolism of oocytes and their associated somatic cells'

2011 Annual meeting of Society of Reproductive Biology, Cairns, Australia
'Cumulus cell EGF-like peptide and receptor signalling during oocyte in vitro maturation'

Poster presentations

2012 Annual meeting of the Society for the Study of Reproduction, Pennsylvania, USA, *Consequences of in vitro maturation of oocytes on cumulus cell EGF-like peptide signalling'*

2012 Faculty of Health Sciences Postgraduate Research Conference, the University of Adelaide, *'Consequences of in vitro maturation of oocytes on cumulus cell EGF-like peptide signalling'*

2011 Annual meeting of Australian Society for Medical Research, Adelaide
'The effect of oocyte in vitro maturation on EGFR pathway signalling'

AWARDS & SCHOLARSHIPS

Competitions

2013 Finalist in the David Healy New Investigator Award, 44th Annual Meeting of the Society for Reproductive Biology, Sydney, Australia

2012 Finalist in the Adelaide Research & Innovation Pty Ltd Award, Faculty of Health Sciences Postgraduate Research Conference, the University of Adelaide

Visit to overseas group

2012 Professor Marco Conti, Department of Obstetrics/Gynaecology & Reproductive Sciences, University of California, San Francisco (UCSF), San Francisco, USA

Scholarships and Grants

Scholarships

2010-2013	Australia Postgraduate Award Research Scholarship
2010-2013	PhD Top-up Scholarship ($15,000)
2013	PhD Top-up Scholarship ($14,222)

Travel grants

2013	Robinson Institute, the University of Adelaide
2013	School of Paediatrics and Reproductive Health, the University of Adelaide
2013	Annual Meeting of Society of Reproductive Biology, Sydney
2012	Faculty of Health Sciences, the University of Adelaide
2012	Robinson Institute, the University of Adelaide
2012	Research Centre for Reproductive Health, the University of Adelaide
2011	Research Centre for Reproductive Health, the University of Adelaide
2011	Annual Meeting of Society of Reproductive Biology, Cairns

CHAPTER ONE

INTRODUCTION

1.1 Oocyte and follicle growth

Oocytes formation and growth occurs via a well-documented process called oogenesis (van den Hurk and Zhao 2005). Oocyte developmental competence is sequentially acquired during oocyte growth. Oogenesis comprises three sub-processes: firstly, the formation of oocytes via mitosis (oocytogenesis); then the initiation of meiosis until oocytes reach the dictyate phase of the prophase I stage (ootidogenesis, meiosis 1); and, following follicular luteinising hormone (LH) stimulation, the resumption of meiosis to the metaphase II (MII) stage where they are then haploid and fertilisable (ootidogenesis, meiosis 2).

Oogenesis is coincident and closely associated with follicular growth, a sub-process called folliculogenesis where densely packed somatic cells form around the developing oocyte (Fig. 1.1) (van den Hurk and Zhao 2005). Follicle growth is gonadotropin-independent until the follicle forms an antrum (and is subsequently referred to as an antral follicle), at which point its growth becomes follicle-stimulating hormone (FSH)-dependent and the oocyte it contains is fully grown. By the time a follicle reaches the preovulatory stage, it contains a fully grown oocyte surrounded by layers of a sub-population of granulosa cells called cumulus cells, mural granulosa cells, a layer of theca cells, and a fluid-filled antrum (Fig. 1.1). Gap-junctions connect the oocyte to surrounding somatic cells throughout oocyte development (Anderson & Albertini 1976; Eppig 1991; Makabe *et al.* 2006), which allow the transfer of regulatory molecules and metabolites between the two cell types to facilitate oocyte and follicle development (Brower & Schultz 1982; Kidder & Vanderhyden 2010; Buccione *et al.* 1990).

1.2 Oocyte maturation and cumulus expansion

Oocyte maturation refers to a series of nuclear and cytoplasmic changes, induced by the ovulatory LH surge, which give rise to an oocyte that is competent for fertilisation. Oocyte nuclear maturation involves changes in chromosome morphology during meiosis. From the primary oocyte stage of oogenesis, mammalian oocytes commence meiosis and are arrested at the prophase I phase until oocyte maturation is induced. The oocyte contains an enlarged nucleus, termed the germinal vesicle (GV), containing decondensed, dispersed, and transcriptionally active chromosomes. In response to pituitary stimuli, the somatic cells of the follicle induce GV breakdown (GVBD) and meiotic resumption to the metaphase II phase, where meiotic maturation is again arrested and is competent for fertilization (Voronina and Wessel 2003).

Oocyte nuclear maturation is accompanied by structural and metabolic changes to the cytoplasm of the oocyte which include cessation of transcription, up-regulation of protein synthesis, organelle reorganisation, and alterations to cell signalling pathways (Eppig et al. 1994). Cytoplasmic maturation is usually assessed as the mature oocyte's ability to fertilise and develop into a blastocyst as there is currently no accurate measure of cytoplasmic maturity. Oocytes derived from the assisted reproductive technologies *in vitro* maturation and *in vitro* fertilisation can have asynchronous nuclear and cytoplasmic maturation, demonstrated in several species including human, horse, cat, hamster, and macaque (Bell et al. 1997; Goudet et al. 1997; Gross et al. 1996; Kito and Bavister 1997; Merlo et al. 2005; Schramm and Bavister 1994; Sundstrom and Nilsson 1988). Asynchronous oocyte nuclear and cytoplasmic maturation has been associated with poorer oocyte activation, fertilization, and subsequent embryo development (Eppig 1996; Eppig et al. 1994).

A coincident process during oocyte maturation is a phenomenon termed cumulus expansion. Cumulus expansion refers to a process that involves the production of hyaluronic acid by the cumulus cells, which is then secreted into the intracellular spaces and stabilized by accessory proteins (Ball et al. 1982; Mattioli 1994). To date, the fully expanded cumulus oophorus is known to play four important biological roles: it supports oocyte maturation prior to ovulation by providing regulatory metabolites and molecules; it is essential for the commencement of ovulation; it conducts the oocyte into the oviduct during ovulation; and it plays a role in facilitating access of spermatozoa to the oocyte during fertilisation (reviewed by Tanghe et al. (2002)). Cumulus cells communicate with each other and the oocyte via gap-junctions (Eppig 1982; Furger et al. 1996; Moor et al. 1980). Cumulus cells are essential for developmental competence since separation of the oocyte from its cumulus vestment prior to *in vitro* maturation decreases oocyte maturation and subsequent embryo development (Hashimoto et al. 1998). Cumulus cells play a pivotal role in regulating oocyte maturation by: keeping the oocyte meiotically arrested through the supply of cyclic nucleotides (see section 1.3.1); participating in the induction of meiosis through mediation of the LH stimulus (see section 1.3.2); and supporting cytoplasmic maturation through the supply of metabolites (see section 1.4). The oocyte plays a key role in the induction of cumulus expansion. The oocyte secretes soluble growth factors that regulate cumulus expansion; growth differentiation factor-9 (GDF9) and/or bone morphogenetic protein 15 (BMP15) are thought to be the main oocyte secreted factors, with important differences between species as to the relative contributions and interactions between GDF9 and BMP15 (reviewed by Gilchrist et al. (2008)).

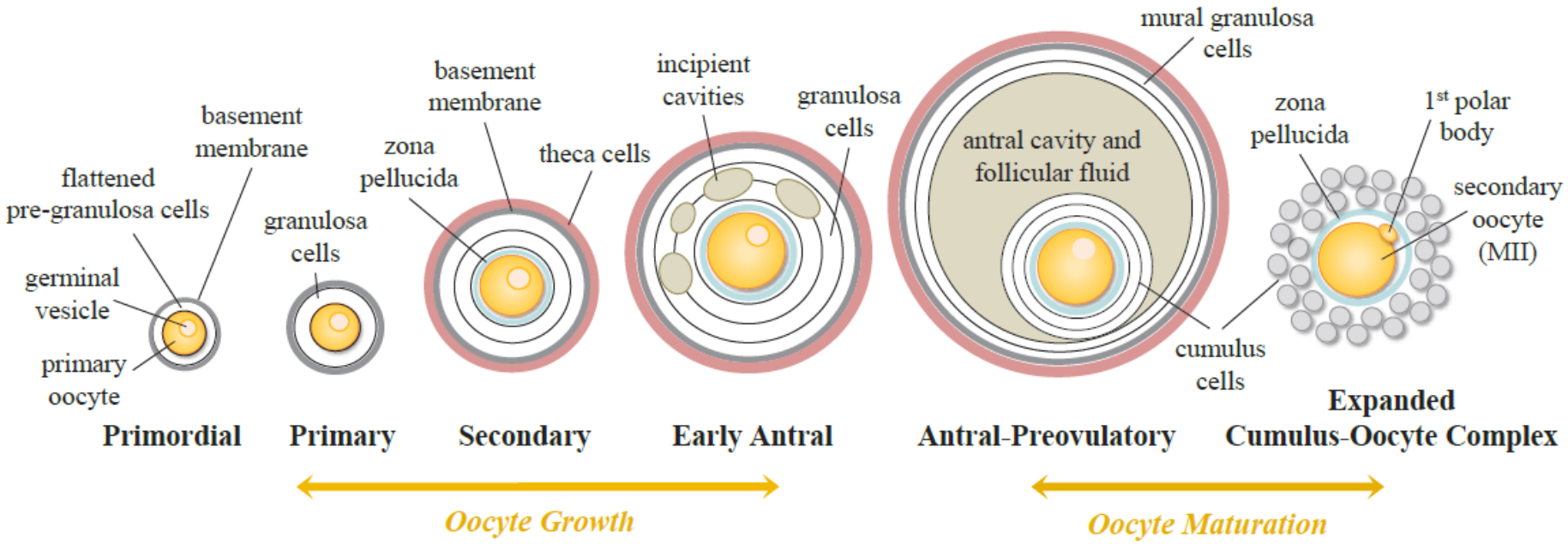

Figure 1.1: Simplified representation of oogenesis and folliculogenesis. Follicle developmental stages are indicated, and the different cell layers at each stage are represented as full lines without cellular divisions. Figure from Collado-Fernandez et al. (2012).

1.3 Mechanisms regulating oocyte maturation and ovulation *in vivo*

Initiation of oocyte meiotic resumption and nuclear maturation is triggered by the mid-cycle LH peak, causing the breakdown of the nuclear envelope, or germinal vesicle (GV), of the oocyte (Heikinheimo and Gibbons 1998). The mechanisms holding the oocyte in meiotic arrest at the meiotic prophase I stage prior to the LH surge are currently emerging. LH signals through LH-chorionic gonadotropin receptors (LHRs) which are abundantly expressed on theca and mural granulosa cells. However, despite being greatly impacted by the LH surge, oocytes and their surrounding cumulus cells express almost no LHRs (Erickson 2000). Therefore, it was unclear how the LH signal at ovulation was transmitted to the oocyte to induce its maturation and ovulation. This puzzle is now progressively being resolved. A series of studies, concentrating predominantly on *in vivo* oocyte maturation in the mouse, have recently revealed two new effectors of the LH surge: cyclic guanine monophosphate (cGMP) (Norris et al. 2010; Norris et al. 2009; Vaccari et al. 2009) and epidermal growth factor (EGF)-like peptides (Fig 1.2) (Ashkenazi et al. 2005; Hsieh et al. 2007; Park et al. 2004; Shimada et al. 2006; Su et al. 2010).

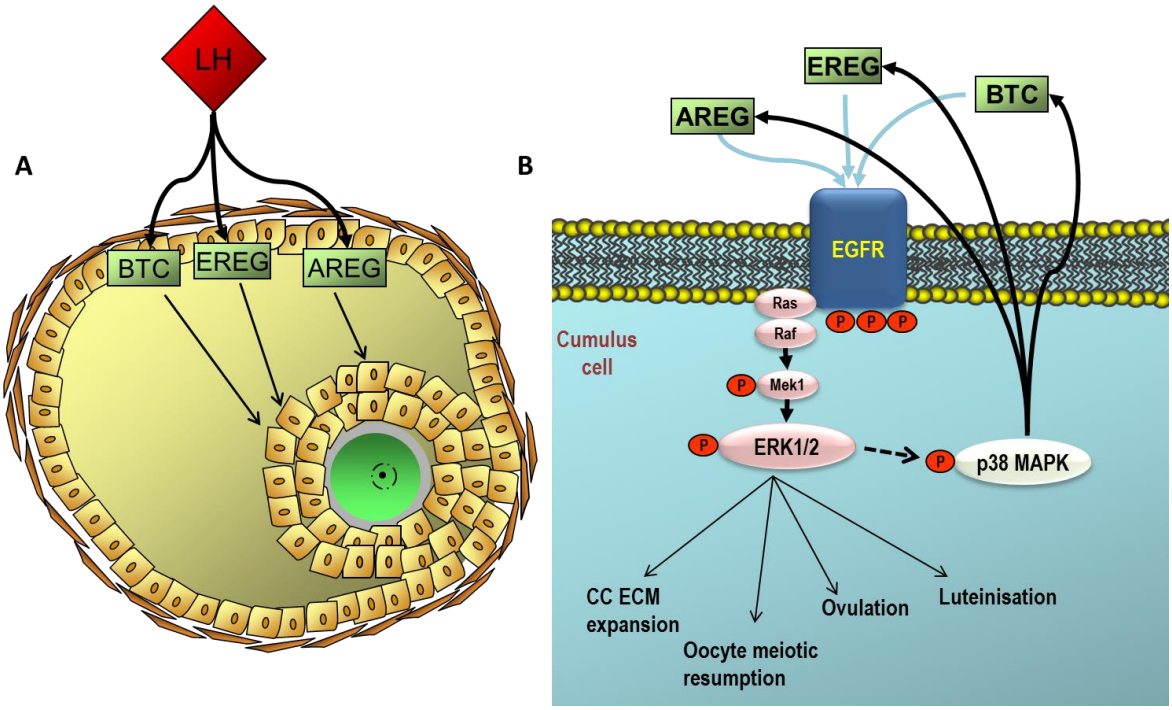

Figure 1.2: **LH induction of follicular EGF-like peptide signalling.** (A) LH induces granulosa cell production of the EGF-like peptides AREG, EREG, and BTC, which are then proteolytically cleaved and released into the follicular fluid, where (B) they bind and phosphorylate the EGF receptor (EGFR) on cumulus cells to activate ERK1/2 pathway signalling. ERK1/2 activation in cumulus cells is required for oocyte meiotic resumption and ovulation, cumulus expansion, and luteinisation. ERK1/2 activation also induces transcriptional events which lead to autocrine up-regulation of EGF-like peptide expression via a p38 MAPK mediated pathway.

1.3.1 cAMP & cGMP second messengers cooperatively regulate meiotic progression

3'-5'-cyclic adenosine monophosphate (cAMP) is a critically important gonadotropin second messenger. It is an important regulator of meiosis that, paradoxically, maintains oocyte meiotic arrest and initiates meiotic resumption (Downs et al. 1989). Cyclic AMP is synthesised in follicular mural and cumulus granulosa cells when gonadotropins (LH or FSH) bind to their respective receptors, leading to activation of guanine nucleotide-binding proteins (G-proteins) that sequentially activate adenylate cyclase (AC) (Downs 2010). AC acts to convert adenosine triphosphate (ATP) to cAMP. Cumulus cells supply cAMP to the oocyte via gap-junctions (Anderson and Albertini 1976), however the oocyte is also capable of endogenously producing cAMP through constitutively active G-protein coupled receptor 3 (Mehlmann 2005; Vaccari et al. 2008) (Fig 1.3). In the oocyte, cAMP exerts its meiotic inhibitory effect through activation of the cAMP-dependent protein kinase A (PKA). PKA activation initiates a cascade of PKA pathway protein phosphorylation that regulates cyclin dependent protein kinases to inhibit germinal vesicle breakdown (Fig. 1.3) (Downs 2010; Jones 2008). The paradox of cAMP in the oocyte is that moderate levels maintain meiotic arrest throughout folliculogenesis, however it also mediates the meiotic inducing effects of LH. The ovulatory LH stimulus induces acute and transient up-regulation in follicular cAMP. This transient pulse in cAMP is believed to produce a meiosis-inducing stimulus, the mechanisms of which remain under investigation (Dekel et al. 1988; Downs and Chen 2008; Gilchrist 2011; Yoshimura et al. 1992).

Recent studies have also elucidated the involvement of cyclic guanine monophosphate (cGMP) in the LH-dependent meiotic maturation of mouse oocytes *in vivo*. Cyclic GMP, synthesised in granulosa and cumulus cells, passes through gap junctions from the cumulus cells into the oocyte where it inhibits the hydrolysis of cAMP by inhibiting phosphodiesterase 3A (PDE3A) activity (Fig. 1.3). PDE3A inhibition allows the maintenance of a moderate concentration of intra-oocyte cAMP which inhibits meiotic resumption (Norris et al. 2009; Vaccari et al. 2009). LH induces meiotic resumption, at least in part, by decreasing granulosa and cumulus cell C-type natriuretic peptide (CNP) levels (the peptides that stimulate production of cGMP), thereby decreasing cGMP and its inhibition of oocyte PDE3A (Kawamura et al. 2011; Zhang et al. 2010).

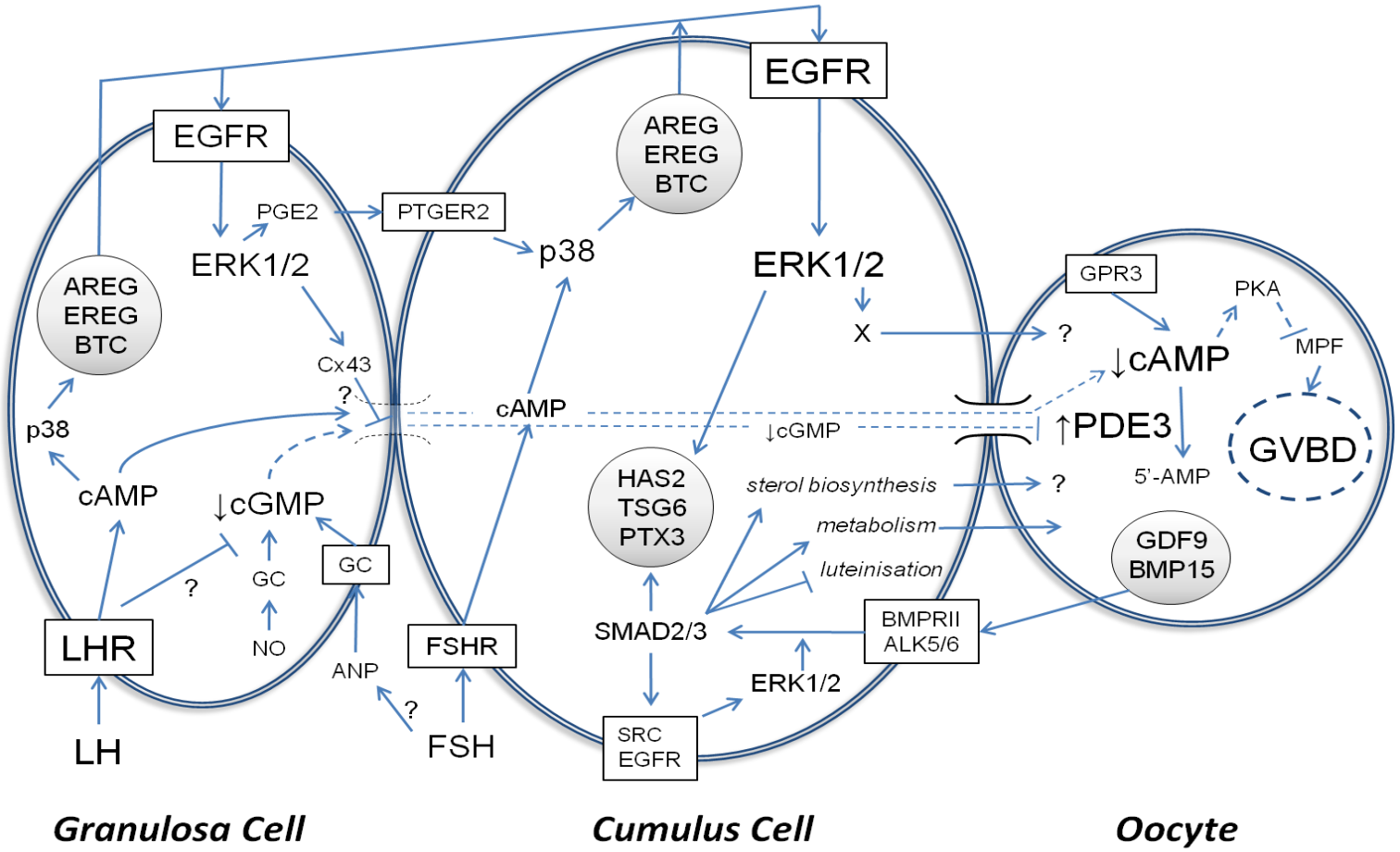

Figure 1.3: Schematic diagram illustrating (1) regulation of oocyte maturation- cAMP and cGMP are supplied from cumulus cells to the oocyte via gap-junctions to regulate nuclear maturation, and (2) LH-induction of EGF-like peptide signalling in granulosa and cumulus cells. Dotted arrows indicate signals of diminishing intensity. 'X' represents a hypothetical meiotic-inducing factor. Figure from Gilchrist (2011).

1.3.2 EGF-like peptides are key mediators of the ovulatory LH signal

In vivo studies in several mammalian species have implicated members of the epidermal growth factor (EGF)-like growth factors as critical mediators of the LH signal in the ovarian follicle. LH induces a rapid secondary cascade of members of the EGF-like family of growth factors, predominantly amphiregulin (AREG), epiregulin (EREG) and betacellulin (BTC) in follicular somatic cells (Hsieh et al. 2009; Park et al. 2004). Production of these EGF-like peptides is shown to be induced by LH in mouse and human mural granulose cells (Freimann et al. 2004; Park et al. 2004; Zamah et al. 2010) and also in rat preovulatory follicles (Sekiguchi et al. 2004). Amphiregulin has also been detected in the follicular fluid of women following ovarian hyperstimulation, and amphiregulin levels were shown to positively correlate with oocyte developmental competence (Zamah et al. 2010).

These peptides are initially synthesized as integral membrane precursors in the mural granulose cells. In response to the LH surge (or its analogue human chorionic gonadotropin), increased follicular cAMP activates the p38 mitogen-activated protein kinase (MAPK) signalling pathway, which sequentially up-regulates amphiregulin, epiregulin, and betacellulin production in cumulus and mural granulosa cells within 1-3 hours (Fig.1. 2) (Park et al. 2004). These EGF-like peptides are shed from the cell surface as mature soluble factors by proteolytic cleavage of the ectodomain; the extracellular proteases responsible for release of these growth factors are not well-characterised, however several metalloproteases are believed to be involved (Ashkenazi et al. 2005; Curry and Osteen 2003). Once shed, these EGF-like peptides act as intermediate-range signals by binding in an autocrine and paracrine manner to members of the ErbB family of receptors (a sub-family of the receptor tyrosine kinases) that are found on cumulus and mural granulosa cells (Holbro and Hynes 2004; Yarden and Sliwkowski 2001). Amphiregulin binds specifically to the EGF receptor (EGFR; also known as ErbB1), while epiregulin and betacellulin can bind both EGFR and ErbB4 (Johnson et al. 1993; Riese et al. 1996; Riese et al. 1998). Ligand binding leads to receptor dimerization and autophosphorylation on multiple tyrosine residues which elicit downstream signalling events. EGFR activity is essential for oocyte maturation and cumulus expansion as the effects of LH and growth factors are blocked by tyrosine kinase inhibitors (Ashkenazi et al. 2005; Park et al. 2004). There is strong evidence to suggest that EGFR (in the form of a homodimer) is the central mediator in the propagation of the LH surge from mural granulosa cells, through cumulus cells to the oocyte (Hsieh et al. 2007; Reizel et al. 2010).

Although the literature strongly suggests that EGFR is the predominant receptor involved, the involvement of other members of the ErbB family, particularly ErbB4, cannot be ruled out (Conti 2013; Zamah et al. 2010). Transcripts of all four members of the ErbB family have been detected in preovulatory follicle granulosa and cumulus cells following hCG stimulation, hence it has been suggested that EGFR not only forms homodimers, but also heterodimers with one or more of the other ErbB receptors upon ligand binding (Conti 2013). The physiological role of tyrosine kinase receptors other than EGFR remains to be determined in the ovary.

The classical downstream effector of EGFR signalling in cumulus cells after ligand binding is extracellular signal-regulated kinase 1/2 (ERK1/2) (also commonly known as mitogen-activated protein kinase 3/1, or MAPK3/1); although the mechanisms involved are not yet fully characterised, ERK1/2 phosphorylation is clearly essential for cumulus matrix expansion, resumption of meiosis, and ovulation (Dragovic et al. 2007; Fan et al. 2009; Su et al. 2002). The mechanism by which ERK1/2 elicits oocyte meiotic resumption remains unclear, however it is hypothesised that ERK1/2 achieves this through the production of meiosis-inducing factors in the cumulus cells, and/or via induction of cumulus-cumulus cell gap-junction closure through the phosphorylation of connexin 43, which would inhibit the flux of the meiotic inhibitors cAMP and cGMP to the oocyte (Norris et al. 2008; Norris et al. 2009; Sela-Abramovich et al. 2005). ERK1/2 activation induces a cascade of prostaglandin E2 and p38MAPK induction which in turn up-regulate production of the EGF-like peptides in cumulus and mural granulosa cells (Fig. 1.2) (Ashkenazi et al. 2005; Downs and Chen 2008; Park et al. 2004; Shimada et al. 2006).

Aside from ERK1/2, EGFR signalling can initiate other signal transduction pathways, including the PI3K-AKT, PAK-JNKK-JNK, and Src pathways (Mendoza et al. 2011; Oda et al. 2005; Yarden and Sliwkowski 2001). Although the vast majority of studies into follicular EGFR signalling have focussed on ERK1/2 downstream activity, a new study has shown that amphiregulin activation of cumulus cell EGFR, both *in vitro* and *in vivo*, regulates the translation of a subset of mRNAs within the mouse oocyte via PI3K-AKT-mTOR signalling; perturbation of this signalling cascade decreases fecundity (Chen et al. 2013; Gilchrist and Richani 2013). This new insight suggests that EGF-like peptide signalling downstream effectors other than ERK1/2 likely play a key role in the regulation of oocyte maturation.

The importance of EGF-like peptides in the propagation of the LH signal *in vivo* was elucidated when mice null for *Areg* or *Ereg* exhibited perturbed 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). *Egfr*-null mice are not viable as they die at peri-implantation, gestation, or soon after birth, depending on the strain, making fertility analysis impossible (Sibilia and Wagner 1995; Sukocheva et al. 2006). Double mutant mice null for *Areg* and homozygous for the *Egfr* waved 2 allele (*Egfrr*$^{wa2/wa2}$) were shown to have dramatically impaired oocyte maturation, cumulus expansion, and ovulation (Hsieh et al. 2007). Furthermore, immunodepletion of amphiregulin in human follicular fluid following gonadotropin hyperstimulation abolished the ability of the follicular fluid to stimulate oocyte maturation and cumulus expansion (Zamah et al. 2010). Hence, these studies demonstrate that LH induction of EGF-like peptides and EGFR transactivation are essential for the regulation of oocyte maturation and ovulation.

1.3.3 EGFR signalling controls cGMP function

The induction of oocyte maturation and ovulation by EGF-like peptide signalling is traditionally thought to be due to ERK1/2 activation as ERK1/2 activity is essential for cumulus expansion, resumption of meiosis and ovulation (Fan et al. 2009; Shimada et al. 2006). However recent data suggests that EGFR activation also decreases follicular cGMP levels by supressing CNP production, which leads to cAMP degradation and, consequently, oocyte meiotic resumption (Conti et al. 2012; Norris et al. 2010; Tsuji et al. 2012; Vaccari et al. 2009). This suggests that EGF-like peptide signalling, acting via cGMP and cAMP, plays a pivotal role in the complex signalling network that exists to finely regulate oocyte meiotic progression *in vivo*.

1.4 Metabolism and the cumulus-oocyte complex

1.4.1 Glucose metabolism by the maturing cumulus-oocyte complex

Glucose is the preferred energy substrate for the maturing COC. The oocyte itself has a poor capacity for glucose uptake as it possesses glucose transporters 1, 3, and 8, which have a low affinity for glucose. In addition, the oocyte possesses relatively low phosphofructokinase (PFK) activity, one of the rate-limiting enzymes of glycolysis, and

hence has a low glycolytic rate. As such, the oocyte is heavily dependent on its cumulus oophorus for the metabolism of glucose and the supply of substrates such as pyruvate and lactate (reviewed by Sutton-McDowall et al. (2010)). Along with glucose transporters 1, 3, and 8, cumulus cells also possess the glucose transporter 4, which has a high glucose affinity (Nishimoto et al. 2006; Roberts et al. 2004; Williams et al. 2001). Glucose can be used by the COC for energy (ATP) production, nuclear maturation, production of cumulus matrix substrates, and for cellular homeostasis and signalling. There are four documented pathways by which glucose can be metabolised in the COC: glycolysis, the hexosamine biosynthesis pathway, the pentose phosphate pathway, and the polyol pathway (reviewed by Sutton-McDowall et al. (2010)). COC glucose metabolism has been predominantly studied using IVM as this is the easiest and most accessible means for research.

1.4.2 Glycolysis and energy production

The majority of glucose is metabolised via glycolysis in the COC (Downs and Utecht 1999). The rate of glycolysis has been shown to remain constant throughout maturation (Sutton et al. 2003). Glucose is almost exclusively consumed by the cumulus cells where it is metabolised via glycolysis to produce oxidisable lactate and/or pyruvate (Fig. 1.4). Glycolysis plays a critical role in oocyte energy production since the pyruvate produced can be transported to the oocyte via gap-junctions where it is metabolised via the tricarboxylic acid (TCA) cycle that is coupled with mitochondrial oxidative phosphorylation, the predominant ATP synthesis pathway within the oocyte (Steeves and Gardner 1999). Pyruvate is the preferred energy substrate of the oocyte and remains so until the 8-cell stage in the mouse embryo. Oocyte developmental competence has been shown to positively correlate with the glycolytic rate of the oocyte itself, despite the relatively low activity of this pathway within the oocyte. This was demonstrated in sheep and cow studies, whereby oocytes with lower developmental capacity, such as those matured via IVM or derived from pre-pubertal donors, exhibited decreased glycolytic activity than those with higher developmental capacity, such as those matured *in vivo* or derived from adult donors (O'Brien et al. 1996; Steeves and Gardner 1999).

1.4.3 The hexosamine biosynthesis pathway

The hexosamine biosynthesis pathway (HBP) accounts for the fate of approximately 1-3 % of the glucose consumed by somatic cells (Marshall et al. 1991; Sayeski and Kudlow 1996). However, in the maturing COC, the HBP activity is significantly increased;

approximately 25% of the glucose consumed by the cumulus cells is metabolised via the HBP as it is responsible for generating the hyaluronic acid used in matrix production as the cumulus matrix expands (Fig. 1.4) (Sutton-McDowall et al. 2004). The HBP converts fructose-6-phosphate to glucosamine-6-phosphate by the rate-limiting enzyme glucosamine:fructose-6-phosphate transaminase (GFPT), which is then eventually converted to UDP-N-acetyl glucosamine. In cumulus cells, the majority of UDP-N-acetyl glucosamine is converted to the glycosaminoglycan hyaluronic acid via the enzymatic action of hyaluronic acid synthase 2 (HAS2); hyaluronic acid is the major constituent of the expanded cumulus extracellular matrix (Ball et al. 1982).

An alternative fate of the UDP-N-acetyl glucosamine generated via the HBP is for the β-O-linked glycosylation of intracellular proteins. β-O-linked glycosylation is an inducible and dynamic post-translational regulatory modification of cytosolic and nuclear proteins that can modulate transcriptional and signal transduction events (reviewed by Wells et al. (2003)). β-O-linked glycosylation involves the attachment of a single UDP-N-acetyl glucosamine to protein hydroxyl groups of serine or threonine residues; this form of glycosylation is reversible and regulates protein function in a similar manner to phosphorylation as these two modifications compete for binding sites and turn over at a similar rate to switch proteins on and off (Slawson et al. 2006). These two post-translational modifications often target the same, or adjacent, protein sites in what has been described as a "yin-yang" relationship (Whelan and Hart 2003). Despite its relatively recent characterisation only 3 decades ago (Torres and Hart 1984), β-O-linked glycosylation is now recognised as one of the most common forms of post-translational modifications (Frank et al. 2014). The fuel-sensing role of the HBP appears to be mediated by β-O-linked glycosylation of proteins (Sutton-McDowall et al. 2010). The pathways targeted by this modification within cumulus cells are currently undefined. Only one enzyme to date, O-linked β-N-acetylglucosaminyltransferase (OGT; Fig. 1.4), has been shown to β-O-linked glycosylate proteins (Kreppel et al. 1997; Kreppel and Hart 1999; Lubas et al. 1997; Lubas and Hanover 2000). Moreover, its counterpart, β-N-acetylglucosaminidase (O-GlcNAcase) is the only known enzyme responsible for the de-glycosylation of proteins (Gao et al. 2001; Wells et al. 2002).

1.4.4 The pentose phosphate pathway

Pentose phosphate pathway (PPP) glucose metabolism is thought to account for only a relatively small proportion of the glucose consumed by the COC; PPP activity in whole COCs is yet to be measured, however the majority of PPP acitivity is believed to lie within the oocyte and accounts for >3% of oocyte glucose metabolism (Cetica et al. 2002; Urner and Sakkas 1999). Glucose metabolised via this pathway is used for the production of reduced nicotinaminde adenine dinucleotide phosphate (NADPH), a coenzyme that acts as the main intracellular reducing agent in anabolic reactions in all cell types and acts to protect cells from reactive oxygen species (ROS) toxicity (Xu et al. 2005) (Fig. 1.4). The PPP is also used for phosphoribosyl pyrophosphate synthesis, which is used as the sugar component for de novo nucleic acid synthesis (Banfalvi 2006); consequently, the PPP plays an important role in oocyte meiotic maturation (Downs et al. 1998; Downs et al. 1996; Gutnisky et al. 2013).

1.4.5 The polyol pathway

The polyol pathway is utilized for the conversion of glucose to sorbitol by aldose reductase (AR), which is then converted to fructose by sorbitol dehydrogenase (SDH) (Fig. 1.4). Only a minute amount of glucose is metabolised via this pathway in somatic cells as AR has a low binding affinity to glucose under normal glycaemic conditions (Sutton-McDowall et al. 2010). The relative amount of glucose metabolism via the polyol pathway is yet to be determined in the oocyte.

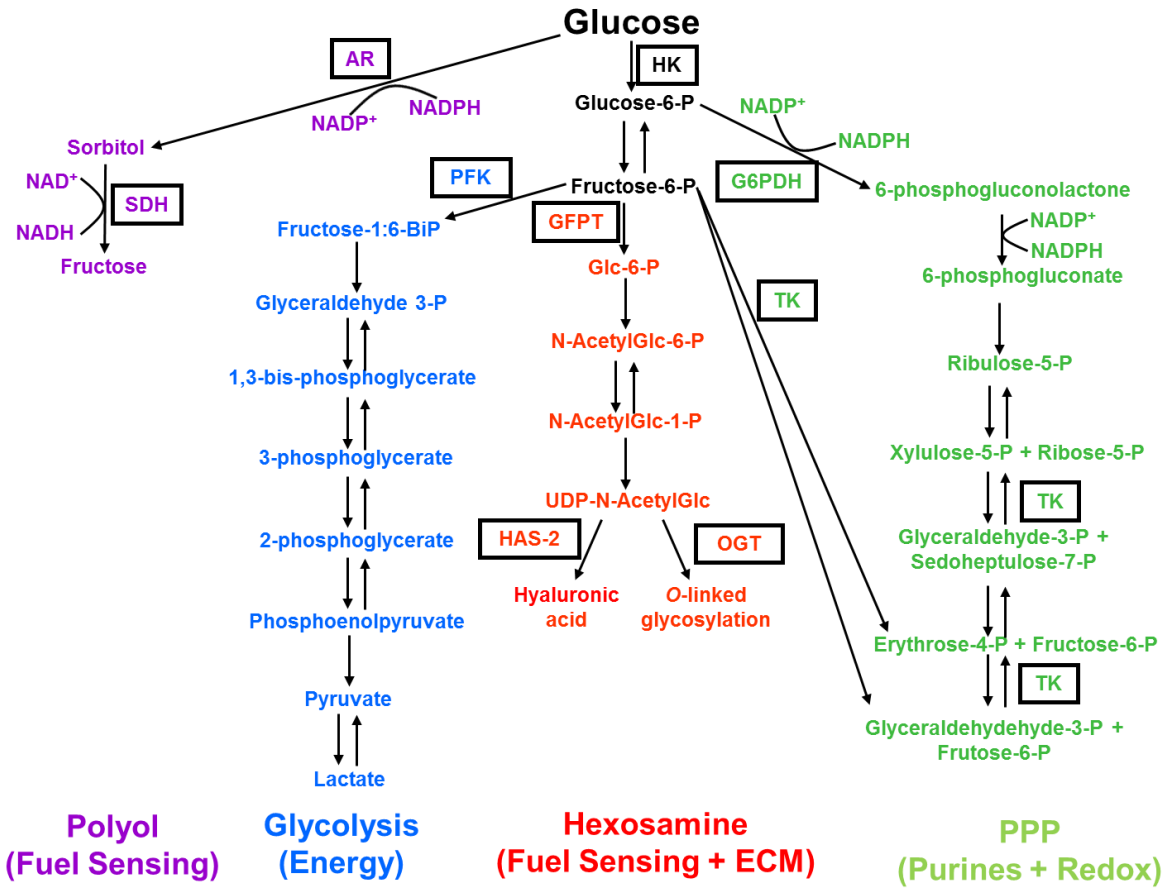

Figure 1.4: Metabolic pathways through which glucose can be utilised within the cumulus-oocyte complex (COC). Pathways known to be active in the COC include the polyol pathway (purple), glycolysis (blue), hexosamine pathway (red) and pentose phosphate pathway (green). Text within boxes indicates rate-limiting or important enzymes. AR, aldose reductase; ECM, extracellular matrix; G6PDH, glucose-6-phosphate dehydrogenase; GFPT, glucosamine:-fructose acetyl transferase; HAS2, hyaluronan synthase 2; HK, hexokinase; OGT, O-linked glycosylation transferase; PFK, phosphofructokinase; SDH, sorbitol dehydrogenase. Figure from Sutton-McDowall et al. (2010).

1.4.6 Oocyte mitochondrial activity and oxidative phosphorylation

Like most other cell types, the oocyte relies on mitochondria as the primary source of energy production as it is the site for TCA cycle metabolism and oxidative phosphorylation. Evidence suggests that mitochondria are determinants of oocyte developmental competence. Mitochondrial parameters shown to influence competence include oocyte mitochondrial content and membrane potential, as well as oocyte ATP levels. Mitochondrial DNA levels are highly variable, even between sister oocytes (Barritt et al. 2002; Reynier et al. 2001; Tamassia et al. 2004). Several human and animal studies have linked low oocyte mitochondrial DNA copy number with poor meiotic spindle formation, fertilisation, and developmental outcomes (El Shourbagy et al. 2006; May-Panloup et al. 2005; Reynier et al. 2001; Santos et al. 2006; Wai et al. 2010; Zeng et al. 2007; Zeng et al. 2009), whilst others found no correlation between oocyte mitochondrial DNA copy number and developmental outcomes (Barritt et al. 2002; Tamassia et al. 2004); hence it remains unclear whether mitochondrial content affects oocyte developmental competence. Increased oocyte ATP levels of both *in* vivo and *in vitro* matured oocytes have been strongly associated with improved developmental outcomes, including fertilisation, spindle formation, and blastocyst development, in several species including human (Stojkovic et al. 2001; Tamassia et al. 2004; Thouas et al. 2006; Thouas et al. 2004; Van Blerkom et al. 1995; Zeng et al. 2007; Zeng et al. 2009). An exception to this has been porcine oocyte developmental competence, which was not linked to oocyte ATP content (Brad et al. 2003; Brevini et al. 2005); this indicates that a correlation between ATP content and oocyte competence may be species-specific. Mitochondrial membrane potential is used as an indicator for mitochondrial activity, and is therefore thought to affect ATP levels and oocyte developmental competence (Diaz et al. 1999; Richter et al. 1996). Decreased oocyte mitochondrial membrane potential is associated with aberrant spindle formation, chaotic mosaicism, and decreased developmental potential of human oocytes or pre-implantation embryos (Wilding et al. 2003); furthermore, mitochondrial membrane potential in MII oocytes is negatively correlated with maternal age (Wilding et al. 2001).

1.4.7 Cellular reduction-oxidation (REDOX) state

The intracellular reduction-oxidation (REDOX) state refers to a complex interaction of the relative concentrations of reduced and oxidised forms of a number of molecules including flavins, pyridine nucleotides, ubiquitones, peroxides, and thiosulphides. It is difficult to

measure the REDOX state with accuracy; it is usually measured by the direct or indirect measurement of REDOX molecules such as NAD^{+}/ NADH, $NADP^{+}$/NADPH and FAD^{++}/FADH. The pyridine nucleotides NADH and NAD(P)H, and the thioltripeptide glutathione are the major reducing equivalents in somatic cells. The ratio of FAD^{++}: NAD(P)H autofluorescence has been used as an indicator REDOX ratio in COCs (Skala and Ramanujam 2010; Sutton-McDowall et al. 2012). NADH (nicotinamide adenine dinucleotide) acts as a reducing agent in a number of metabolic pathways in both the cytoplasm (glycolysis and β-oxidation) and the mitochondria (tricarb oxylic acid (or Krebs) cycle and oxidative phosphorylation) (Fig. 1.5). In contrast, NADPH (nicotinamide adenine dinucleotide phosphate) is found in smaller quantities within the cytoplasm and mitochondria and acts as a reducing agent in the PPP, tricarboxylic acid cycle, and for glutathione reduction (Fig. 1.5). The autofluorescence of NADH and NADPH is indistinguishable, and as such are measured in combination. FAD^{++} (reduced flavin adenine dinucleotide) is localised exclusively within mitochondria and is involved in the electron transport chain. An oocyte REDOX ratio can be determined by FAD^{++}:NAD(P)H, whereby increasing ratios are indicative of increased oxidative metabolic activity within cells (Skala and Ramanujam 2010).

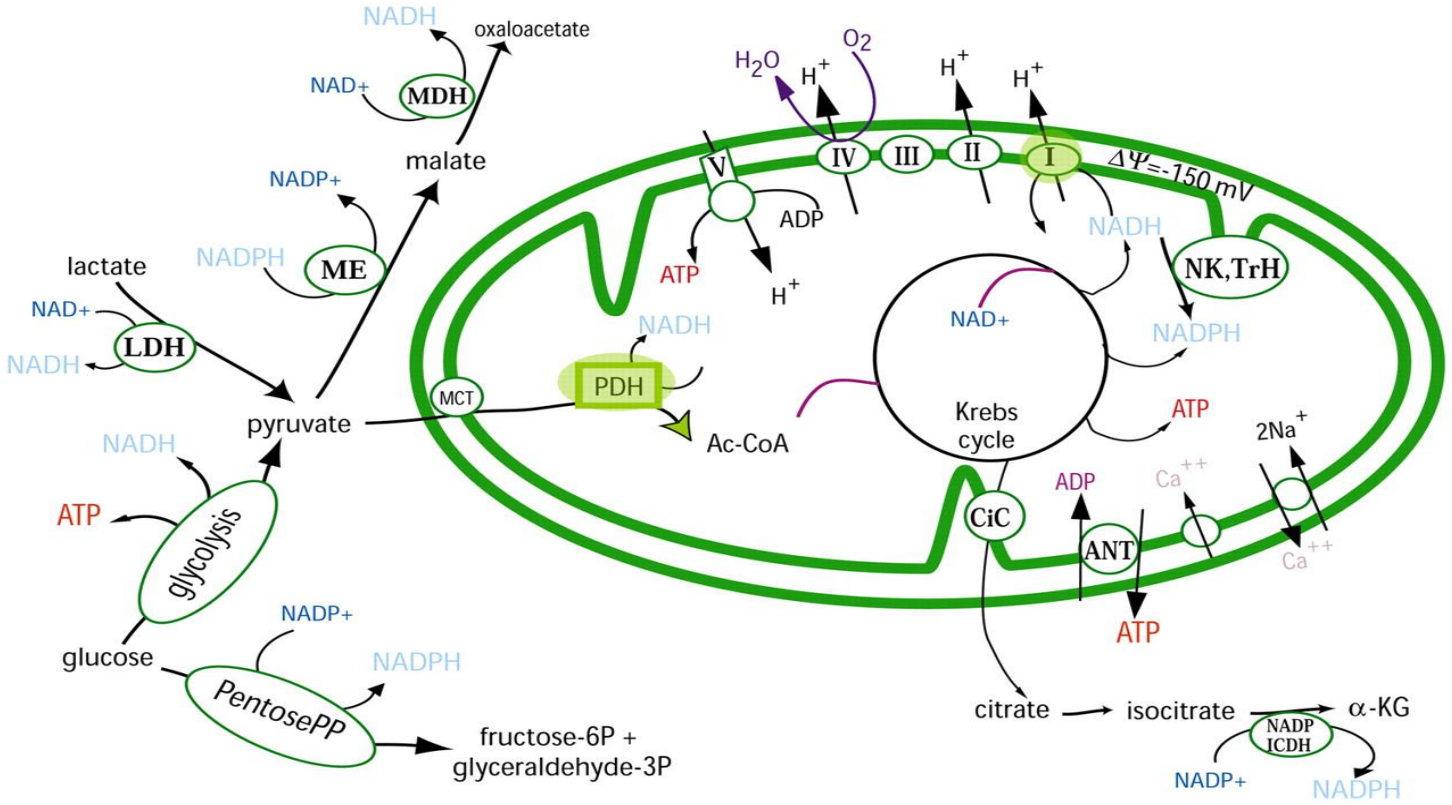

Figure 1.5: NAD(P)H and FAD^{++} autofluorescence originate from both mitochondrial and cytosolic compartments in mouse oocytes. Schematic representation of the metabolic pathways producing NADH and NADPH in the cytosol and the mitochondria of a eukaryotic cell. The possible sources of FAD^{++} fluorescence in the mouse oocyte are highlighted in green and are the pyruvate dehydrogenase (PDH) complex and the complex I of the mitochondrial electron transport chain. α-KG, alpha-ketoglutarate; Ac-CoA, acetyl coenzyme A; ANT, adenine nucleotide translocase; CiC, citrate carrier; LDH, lactate dehydrogenase; ME, malic enzyme; MDH, malate dehydrogenase; MCT, mono carboxylate transporter; $NAD(P)^{+}$, oxidised nicotinamide adenine dinucleotide (phosphate); NADH, reduced nicotinamide adenine dinucleotide, NADP-ICDH, NADP-dependent isocitrate dehydrogenase; NK, NAD kinase; PentosePP, pentose phosphate pathway, TrH, transhydrogenase. Figure from Dumollard et al. (2007).

1.5 Increasing demand and use of assisted reproductive technologies

Australia's overall fertility rate has been declining over the last 35 years, in part due to a growing trend for delayed childbearing. In 2008, the median age of all first mothers was 30.8, indicating that many women are now attempting to fall pregnant over the age of 35 (Laws and Sullivan 2009). This is the approximate age when oocyte quantity and quality begin to sharply decline, and this is concerning since oocyte quality is a key limiting factor in female fertility. To exacerbate this situation, the impact of damaging lifestyle choices such as poor nutrition, obesity, smoking and diseases such as diabetes also adversely affect fertility (Sharma et al. 2013). The increasing infertility rate and the declining birth rate is resulting in more people turning to assisted reproductive technologies (ARTs) such as *in vitro* fertilization (IVF) to achieve pregnancy; IVF cycle numbers have increased by over 10% in the last decade, with ~3.3% of babies born in Australia now derived from IVF (Wang et al. 2009).

1.6 Drawbacks to the use of IVF

ARTs such as IVF are dependent on gonadotropic hormone hyperstimulation of the ovary to generate large numbers of mature oocytes. Human ovaries contain approximately 400,000 oocytes at puberty that have varying potential to mature, ovulate and fertilize. Of these oocytes, 99.9% never ovulate. Treatment of patients with gonadotropins is used in IVF to prevent some of these oocytes from undergoing atresia. Although ovarian hyperstimulation is commonly and successfully used, it is associated with a number of significant drawbacks. These include health risks caused by moderate to severe ovarian hyperstimulation syndrome, a condition that occurs in ~5% of treated women; ~1% of treated women develop potentially fatal complications (Lowe et al. 2005). Women most vulnerable to developing this syndrome are those who suffer from polycystic ovaries, which is 23% of the Australian female population (Lowe et al. 2005). Other side effects of the use of gonadotropins include embryo aneuploidy (Baart et al. 2007), perturbed genomic imprinting (Market-Velker et al. 2010), as well as potential increased risk of the patient developing ovarian, breast, and endometrial cancers (Brinton 2007; Pappo et al. 2008), and increased incidence of stroke (Demirol et al. 2007). The use of gonadotropins in IVF also has a large financial impact on the Australian Government's Pharmaceutical Benefits Scheme which subsidises about $100 million per annum on hormones alone (Griffiths et al. 2010). Patients undergoing IVF also carry a large financial burden. Therefore, a safe and reliable alternative to traditional IVF treatment that reduces or eliminates the need for the

administration of gonadotropins and allows better access to patients who would not otherwise be treated is highly desirable.

1.7 Oocyte *in vitro* maturation (IVM): an ART with much potential

A reproductive technology that has always had potential to be an adjunct ART to clinical IVF, is *in vitro* maturation (IVM). IVM involves the artificial removal of immature cumulus-oocyte complexes (COCs) from antral follicles of unstimulated or minimally stimulated ovaries that are then cultured to maturation and fertilized *in vitro* in simple oocyte maturation media that contain small doses of gonadotropins, usually follicle stimulating hormone. COCs are cultured until they have meiotically matured to the metaphase II stage; maturation time is species dependent.

The concept of IVM was first demonstrated by Pincus and Enzmann (1935) when they reported that germinal-vesicle stage rabbit oocytes can spontaneously mature when removed from the follicle. Edwards *et al*. later reported this to be the case in oocytes from humans as well as in several animal species (1965a; 1965b; 1969). The first attempts at maturing human oocytes from small antral follicles *in vitro* was first reported by Cha et al. (1991), who successfully matured and fertilised oocytes from unstimulated follicles, five of which were then implanted into a woman suffering from premature ovarian failure who subsequently delivered triplet girls. To date, ~400 IVM births have been reported in the literature but recent estimates place the actual figure at one to two thousand babies globally (Suikkari 2008). An accurate estimate of the prevalence of IVM, however, is difficult to achieve as there is no global registry for IVM pregnancies and births, and the majority of modern fertility clinics do not report results in the published literature (Gilchrist et al. 2011). IVM obstetrics outcomes, such as multiple-pregnancy rates, mode of delivery, cord pH, and pregnancy complications, are comparable with those obtained via IVF or intra-cytoplasmic sperm injection (ICSI). IVM postnatal outcomes, including gestational age, growth restriction, Apgar scores, birth weights, and sex ratios, are also comparable to IVF and ICSI, and indeed natural conception. Information on IVM post-natal outcomes is limited since this technology is relatively new thus the children are still young, and the number of IVM children reported in the literature is insufficient to calculate absolute risks for specific health abnormalities (Gilchrist et al. 2011; Suikkari 2008). Despite this, studies thus far have not indicated that IVM increases obstetric or prenatal complications above IVF or ICSI (Buckett et al. 2007; Jurema and Nogueira 2006; Shu-Chi et al. 2006; Suikkari

and Soderstrom-Anttila 2007). Furthermore, no long-term health concerns have arisen in animal IVM.

There are several advantages to the use of clinical IVM. Since the use of gonadotropins on the mother is eliminated or dramatically decreased, IVM removes the risk of ovarian hyperstimulation syndrome and reduces the risk of other adverse side-effects to patients, and simplifies and considerably reduces the cost of treatment. Furthermore, IVM can provide genuine treatment opportunities for preserving the fertility of cancer patients (whose fertility is invariably destroyed by chemotherapy and radiotherapy) where ovarian hyperstimulation is usually not an option for patients with oestrogen-sensitive cancers (Smitz et al. 2011). In domestic animal breeding, IVM has become routine practice and an important platform technology for artificial breeding, cloning and transgenic animal production, especially in cattle breeding where close to 500,000 live offspring were produced in 2011 (Stroud 2012). IVM has become popular in advanced cattle breeding since it yields a significant reduction in the costs of commercial embryo production and transfer, and a reduction in the generation interval (Brackett et al. 1982; Hoshi 2003). However, IVM is not widely used to treat human infertility since, compared to conventional IVF using *in vivo* matured oocytes, IVM success rates (embryos and offspring generated per oocyte collected) are approximately 30-50% lower, and miscarriage rates are higher (Buckett et al. 2008; Child et al. 2002; Gremeau et al. 2012). However, no randomised, controlled human trials comparing IVM to IVF currently exist. A retrospective case-controlled study in polycystic ovarian syndrome (PCOS) patients by Child et al. (2002) reported implantation rates of 17% versus 9.5% for IVF and IVM, respectively. More recently, Gremeau et al. (2012) also reported implantation rates of 19.4% vs. 12.9%, clinical pregnancy rates of 50.5% vs. 19.6%, and live birth rates of 44.3% vs. 16.5% in IVF vs. IVM groups, respectively, in a PCOS retrospective case-controlled study. The discrepancy in efficiency between IVM and IVF is also seen across mammalian species including murine (Albuz et al. 2010; Eppig et al. 2009; Nogueira et al. 2003b; Vanhoutte et al. 2009b), ovine (Thompson et al. 1995), and bovine (Leibfried-Rutledge et al. 1987; Rizos et al. 2002) (Table 1.1).

Table 1.1: A comparison of *in vivo* versus IVM oocyte maturation on developmental outcomes.

		Mode of oocyte maturation		
Species	Outcomes	*In vivo*	IVM	References
Human	Implantation	17 / 19%	9.5 / 13%	Childs et al. 2002/Gremeau et al. 2012
Human	Miscarriage	13%	25%	Buckett et al. 2008
Murine	Live birth	52 / 22%	21/ 8%	Eppig et al. 2009/Albuz et al. 2010
Bovine	Blastocysts	58%	39%	Rizos et al. 2002
Ovine	Blastocysts	74%	35%	Thompson et al. 1995

The key contributing factor to the low success rates of IVM is poorer oocyte quality. This is attributed to the fact that, for IVM: 1) the COCs are generally collected from mid-sized antral follicles that have not completed oocyte capacitation (the phase of oogenesis when the oocyte acquires the cytoplasmic machinery necessary to fully support embryo development) and hence are not fully developmentally competent; and 2) mechanically removing the COC from the follicle results in loss of the natural meiotic inhibiting environment, leading to "spontaneous" meiotic maturation of the oocyte *in vitro* (Gilchrist and Thompson 2007; Wassarman et al. 1976). Spontaneous meiotic maturation refers to the resumption of meiosis in the absence of the inducing gonadotropin/epidermal growth factor (EGF)-like peptide cascade, hence oocytes that resume meiosis spontaneously during IVM are unlikely to have undergone the elaborate paracrine and endocrine molecular signalling events that usually induce maturation *in vivo*. Gilchrist and Thompson (2007) have proposed that spontaneous resumption of oocyte meiosis is aberrant and as such leads to compromised oocyte developmental competence. Attempts have been made to enhance oocyte developmental competence during IVM, however in order to achieve this we must elucidate key factors contributing to oocyte developmental competence during maturation *in vivo* and develop new IVM conditions to mimic this (Gilchrist and Thompson 2007).

1.8 IVM additives: FSH and EGF

FSH is synthesized and secreted by gonadotropes of the anterior pituitary gland. FSH regulates growth and development, pubertal maturation, and, in the female, is indispensable for folliculogenesis; it acts in synergy with luteinizing hormone (LH) to regulate such processes (Jeppesen et al. 2012). Although LH is the gonadotropin primarily responsible for the resumption of oocyte maturation *in vivo*, FSH is a potent stimulator of oocyte maturation *in vitro* and stimulates cumulus cell mucification to yield expanded COCs *in vitro* that appear similar to *in vivo* matured COCs (Lindner et al. 1974). As such, FSH became the IVM additive of choice since cumulus cells possess only minute levels of LH receptors, but express the FSH receptor abundantly (Jeppesen et al. 2012; Peng et al. 1991). FSH is also widely used due to its recognized positive effects on oocyte developmental competence relative to spontaneous IVM (Eppig 1992; Izadyar et al. 1998).

Epidermal growth factor (EGF) is the first discovered member of the EGF-family of proteins, members of which have highly similar structural and functional characteristics (Dreux et al. 2006). EGF is naturally found in many cell types where it is involved in cell proliferation, differentiation, and survival (Herbst 2004). EGF binds and signals through EGFR (Herbst 2004; Oda et al. 2005). FSH stimulation of the granulosa cells of the follicle were shown to trigger EGF synthesis, and exogenous EGF was found to mimic FSH-stimulated DNA synthesis (Roy and Greenwald 1990; Roy and Greenwald 1991a; Roy and Greenwald 1991b). Furthermore, EGF induces oocyte meiotic resumption, cumulus expansion, and improves developmental competence in comparison with spontaneous oocyte maturation in several animal species (Das et al. 1992; Dekel and Sherizly 1985; Goud et al. 1998; Prochazka et al. 2000; Rieger et al. 1998). This suggested that gonadotropin-induced events in the follicle may have been mediated by EGF, however this remained controversial for many years since little to no EGF was detected in follicular fluid (Hsu et al. 1987; Reeka et al. 1998; Westergaard and Andersen 1989). A seminal study by Park et al. (2004) demonstrated that the ovulatory LH signal was not mediated by EGF, but rather by other members of the EGF family, namely amphiregulin, epiregulin, and betacellulin (see section 1.3.2). To date, the application of amphiregulin, epiregulin, and betacellulin in IVM has not been thoroughly investigated (see section 1.10). EGF is not commonly used in clinical IVM (human or veterinary) and is only occasionally used in research IVM, usually in combination with FSH (Banwell and Thompson 2008). Like FSH, EGF elicits its effects 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).

1.9 Emerging concepts and technologies to improve IVM oocyte developmental competence

Some recent advances that improve the developmental competence of IVM oocytes have been made. These include the use of follicular fluid meiosis-activating sterol (FF-MAS) (Smitz et al. 2007), oocyte-secreted factors (particularly GDF9 and BMP15) (Hussein et al. 2006; Yeo et al. 2008), and factors that activate protein kinase C (PKC) including FSH and phorbol myristate acetate (Ali and Sirard 2005). However, one of the most effective approaches to date has been the attenuation of spontaneous meiotic maturation using cAMP modulating agents such as phosphodiesterase (PDE) inhibitors and adenylate cyclase activators (Luciano et al. 2004; Nogueira et al. 2003b; Shu et al. 2008; Thomas et al. 2004).

1.9.1 COC cAMP modulation improves the developmental competence of IVM oocytes

As the oocyte grows and acquires developmental competence prior to ovulation, moderate levels of oocyte cAMP keep the oocyte meiotically arrested. However, when COCs are removed from the follicle for IVM, COC cAMP levels acutely drop and, as such, gap-junctions close, the inhibitory effect of cGMP and cAMP on meiotic maturation is lost, and the oocyte undergoes aberrant meiotic maturation in the absence of the stimulatory follicular stimuli which is asynchronous with its cytoplasmic maturation (Gilchrist 2011).

One means of delaying spontaneous meiotic resumption involves the addition of kinase or protein synthesis inhibitors to IVM media. Although reversible meiotic inhibition can be achieved using such inhibitors, these studies conclusively demonstrate that they do not enhance oocyte quality during IVM as they commonly result in significantly poorer embryo yield (Anderiesz et al. 2000; Fulka et al. 1991; Grupen et al. 2006; Kubelka et al. 2000; Lonergan et al. 2000; Lonergan et al. 1997; Mermillod et al. 2000).

To date, controlling maturation of IVM oocytes through cAMP modulation has proven to be the most promising approach as it improves developmental competence; however this concept is yet to be adopted into routine clinical human IVM. The literature over the last

30 years, predominantly in animal models, demonstrates that cAMP management in IVM oocytes and cumulus cells is an effective concept to improve subsequent developmental outcomes (Smitz et al. 2011). Prolonged maintenance of cAMP using PDE inhibitors during the first half or for all of IVM has yielded modest improvements in murine, bovine, and human IVM oocyte developmental outcomes (Downs et al. 1986; Nogueira et al. 2003a; Nogueira et al. 2003b; Thomas et al. 2004; Vanhoutte et al. 2009a; Vanhoutte et al. 2009b). A more effective approach has been the up-regulation of COC cAMP levels prior to IVM or for the first half of IVM (either through the use of dibutyryl cAMP or adenylate cyclase activators) as this has been shown to significantly improve subsequent developmental competence in human, murine, bovine, and porcine IVM oocytes (Albuz et al. 2010; Funahashi et al. 1997; Luciano et al. 1999; Shu et al. 2008; Zeng et al. 2013).

1.9.2 Simulated physiological oocyte maturation

Our group has developed a novel IVM system that yields higher blastocyst and fetal yields than standard IVM by altering COC cAMP levels during culture (Albuz et al. 2010). This system, called simulated physiological oocyte maturation (SPOM), increases and maintains oocyte cAMP via the addition of cAMP modulators to IVM media to prolong the exchange of positive regulatory molecules and metabolites between the cumulus cells and oocyte, which delays oocyte maturation and improves subsequent oocyte quality (Fig. 1.6). The fundamental aspect of SPOM lies in a short pre-IVM phase (1-2 hours) whereby COCs are collected in media containing the cAMP modulators forskolin (an adenylate cyclise activator that induces cAMP synthesis), and 3-isobutyl-1-methylxanthine (IBMX; a non-specific PDE inhibitor that prevents cAMP degradation). These agents cause a >100-fold spike in oocyte-cumulus cAMP levels, mimicking the spike that occurs *in vivo* after the LH surge (Dekel et al. 1988; Yoshimura et al. 1992).

This is in contrast to standard IVM, where COC cAMP levels rapidly decline after the COC is removed from the follicle (Albuz et al. 2010). This critically important pre-IVM phase delays meiotic maturation and increases cumulus-oocyte gap-junctional communication. The IVM phase during SPOM involves culturing of oocytes in media containing the PDE inhibitor, cilostamide, which acts to prevent oocyte meiotic maturation *in vitro*. Cilostamide is a type-3 specific PDE inhibitor which targets oocyte cAMP only as PDE3 is oocyte-specific in the mouse. The IVM phase also requires a high concentration of FSH (100 mIU/mL in mouse) in order to override cAMP-mediated arrest and drive

meiotic induction. This indicates that oocyte maturation during SPOM is hormone-dependent and not spontaneous as in standard IVM.

The requirement of EGFR signalling during SPOM has been briefly investigated. Meiotic resumption during SPOM occurs by a mechanism that is dependent on EGFR signalling since inhibiting EGFR activity inhibits SPOM (Albuz et al. 2010). This indicates that SPOM mimics some of the key molecular signals that take place during oocyte maturation *in vivo*, leading to improved developmental outcomes.

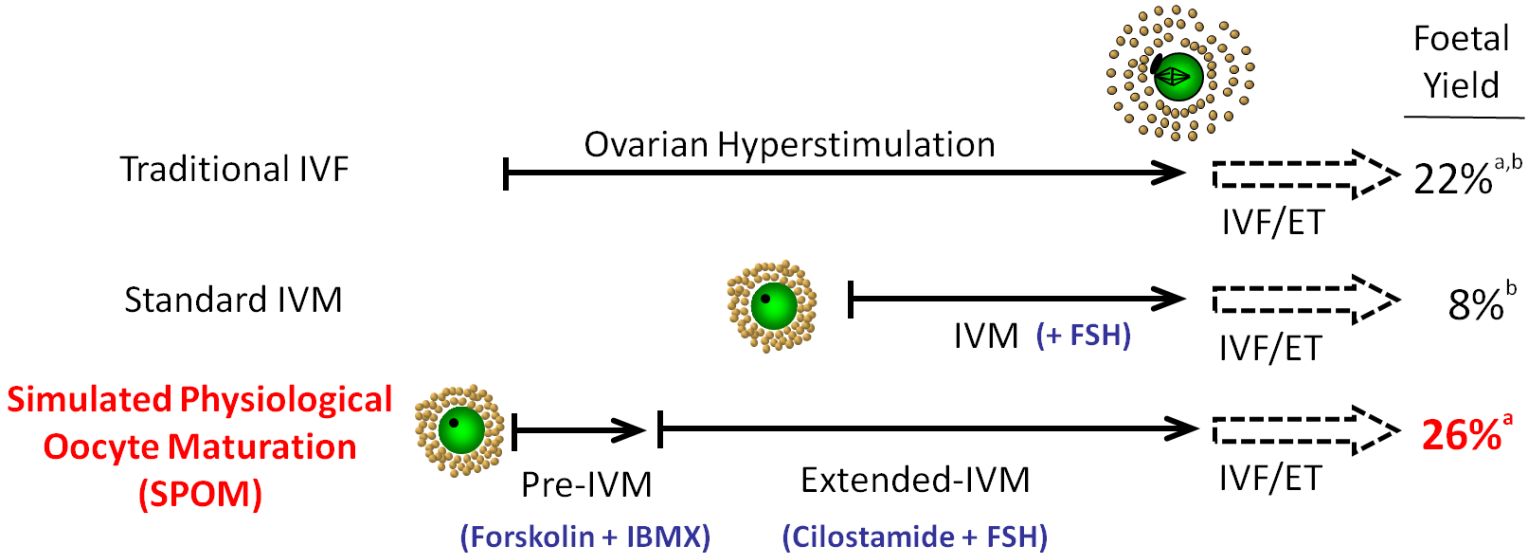

Figure 1.6: Model illustrating the methodology and mouse foetal yield of SPOM relative to traditional IVF and standard IVM. SPOM is characterised by a short pre-IVM phase where COC are collected in media containing cAMP modulating agents (forskolin & IBMX) that cause a spike in cAMP. The extended IVM phase involves FSH induced oocyte maturation in the presence of an oocyte-specific PDE inhibitor (cilostamide). SPOM generates mouse fertilization, blastocyst and implantation rates and foetal yields similar to those achieved through conventional IVF. Figure adapted from Albuz et al. (2010).

1.10 EGF-like peptide signalling and IVM

Currently, standard research and clinical IVM protocols supplement FSH into the culture medium for oocyte maturation (Banwell and Thompson 2008). FSH was shown to up-regulate 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). A study from our group has also shown, 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 and β peptide sequences, genes for the three peptides were the most differentially expressed (Kind et al. 2012). Hence, these studies suggest that EGF-like peptide signalling may be perturbed during IVM.

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/or cumulus expansion in COCs (Ben-Ami et al. 2011; Park et al. 2004; Prochazka et al. 2011). Furthermore, amphiregulin induces 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). A human study by Ben-Ami et al. (2011) investigated the effects of amphiregulin + epiregulin supplementation during "rescue IVM" (see below); this study reported a significant increase in oocyte maturation rate relative to spontaneous maturation, but no significant differences in fertilization and cleavage rates, or embryo morphology. Results from this study, however, should be interpreted with caution since there were several limitations to the study including the use of germinal vesicle stage oocytes that had not responded to gonadotropin hyperstimulation during IVF treatment (termed "Rescue IVM"); the partial denuding of oocytes prior to IVM; and the use of media designed to meet the nutritional needs of a growing embryo, and not a maturing oocyte.

1.11 Synopsis

It is clear from existing literature that normal oocyte maturation is a complex process that involves communication between the oocyte and the follicular cells with which it shares its environment. Recent breakthroughs in our understanding of oocyte maturation *in vivo* have highlighted the critical role EGF-like peptide signalling plays in mediating the ovulatory LH signal to induce the maturation and ovulation of a developmentally competent oocyte.

Although the function and impact of EGF-like peptide signalling *in vivo* has been characterised, little is currently known of the effect of oocyte IVM on this pathway. I hypothesise that EGF-like peptide expression and signalling in cumulus cells is perturbed during standard IVM, and supplementation of exogenous EGF-like peptides during IVM ameliorates this deficiency and increases IVM oocyte developmental competence. To test this hypothesis, the effect of IVM on cumulus cell EGF-like peptide mRNA and protein expression, the activity of EGFR, and its main downstream effector, ERK1/2, will be examined. Furthermore, the effect of exogenous EGF-like peptide supplementation on IVM oocyte developmental competence will be examined via their effect on blastocyst yield and quality.

Next, I will seek to characterise and compare the metabolic profiles of IVM COCs matured in the presence of the EGF-like peptides versus the traditional IVM additives FSH or EGF. I hypothesise that these ligands will exert differential effects on COC metabolism which will relate to oocyte developmental competence. To test this hypothesis, I aim to examine glucose metabolism via the two major glucose metabolic pathways in the maturing COC, glycolysis and the HBP, and examine oocyte REDOX state and mitochondrial activity. Alterations to these metabolic parameters have been associated with altered oocyte developmental competence.

Studies by several groups have independently shown that cAMP modulation prior to IVM improves subsequent oocyte developmental outcomes. I hypothesise that an IVM system that incorporates this concept with an IVM system that utilises EGF-like peptides during the maturation stage would endow greater developmental competence on the oocyte than if the two concepts were applied independently. To test this hypothesis, a pre-IVM phase, whereby cAMP is acutely up-regulated using forskolin and IBMX, combined with an IVM phase that utilises EGF-like peptides versus FSH or EGF, will be examined. The effect of this novel IVM system on oocyte developmental competence, as measured by blastocyst

yield and quality, will be examined. Furthermore, its effect on cumulus cell EGF-like peptide signalling, as measured by EGF-like peptide mRNA and expression and EGFR and ERK1/2 activity, will be examined.

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CHAPTER TWO

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

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Published in Molecular Human Reproduction (Appendix 1)

Statement of Authorship

Title of Paper	Mode of oocyte maturation affects EGF-like peptide function and oocyte competence
Publication Status	⦿ Published, ○ Accepted for Publication, ○ Submitted for Publication, ○ Publication style
Publication Details	Molecular Human Reproduction 19(8):500-509

Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

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2.1 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.

2.2 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 up-regulation 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 up-regulates 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 up-regulation 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 up-regulate 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.

2.3 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. COCs were collected from 21- to 28-day old mice, 46 h after intraperitoneal injection of 5 IU of equine chorionic gonadotropin (eCG; Folligon, Intervet, Boxmeer, Holland) to stimulate follicular growth. Human chorionic gonadotropin (hCG, 5 IU; Organon, Sydney, Australia) was administered 46 h post-eCG for IVV experiments to induce oocyte maturation *in vivo*. COCs were isolated from preovulatory follicles using a 27-gauge needle and collected using flame-pulled borosilicate Pasteur pipettes in HEPES buffered αMEM (Gibco, Invitrogen, Carlsbad, USA) supplemented with 3 mg/mL bovine serum albumin (BSA).

Oocyte *in vitro* maturation

COCs were cultured in bicarbonate buffered αMEM (Gibco, Life Technologies, NY, USA) supplemented with 3 mg/mL BSA (ICPbio, Glenfield, New Zealand) and either: recombinant human FSH (100 mIU/mL; Puregon, Organon, USA), recombinant human EGF (10 ng/mL; R&D Systems, Minneapolis, USA), recombinant mouse AREG (50 ng/mL; R&D Systems), recombinant mouse EREG (50 ng/mL; R&D Systems), or recombinant mouse BTC (50 ng/mL; R&D Systems) at 37ºC and 5% CO_2 in air. Doses of EGF family growth factors were based on previous studies using EGF (De La Fuente et al. 1999; Li et al. 2008) and EGF-like peptides (Downs and Chen 2008).

RNA Isolation

Following the indicated periods of *in vivo* or *in vitro* maturation, cumulus cells were separated from COCs by mechanical shearing using a P200 pipette and washed with PBS. Total RNA was extracted using the RNeasy Micro Kit (Qiagen, Germantown, USA) according to the manufacturer's instructions. DNA that may have been co-purified was removed by addition of DNase (0.34 Kunitz units/µL supplied with kit). RNA was eluted in 14 µL of RNAse-free water and stored at -80ºC. The final RNA concentrations were

determined by absorbance using a NanoDrop ND-1000 spectrophotometer (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 2.1) 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 100^0C 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.

2.4 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. 2.1). 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. 2.2). 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. 2.2B). 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. 2.3). 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. 2.4). 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 2.2). 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 2.2). Such a change if cell ratio is indicative of an improvement of blastocyst quality and post-transfer developmental potential (Lane and Gardner 1997).

2.5 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 up-regulate 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 up-regulation 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. 2.4); 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.

2.6 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.

2.7 ACKNOWLEDGEMENTS

We would like to thank Deanne Feil and Xiaoqian Wang for their assistance and advice with the embryo production experiments. We would also like to thank David Kennaway and Darren Miller for sharing their knowledge and advice on ELISAs.

2.8 FUNDING

This work was supported by National Health and Medical Research Council [grant number APP1007551] and Fellowships APP1023210 and APP627007; and an Australian Postgraduate Award to D.R.

Table 2.1: Sequences of PCR primers used for RT-qPCR

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.

Table 2.2: Embryo development of IVM oocytes cultured in medium supplemented with FSH, EGF and EGF-like peptides.

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]

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

Figure 2.1: Effect of maturation *in vitro* (IVM) compared to *in vivo* (IVV) on cumulus cell EGF-like peptide and receptor mRNA expression. Cumulus cells were harvested from IVM cumulus-oocyte complexes cultured with FSH (grey bars) and from IVV cumulus-oocyte complexes matured *in vivo* with hCG (black bars). mRNA expression was measured using quantitative RT-PCR and normalized to the geometric mean of the *Mrpl19* and *Ppia*. Bars not sharing a common letter are significantly different; IVM^{a-d}, IVV^{x-z} ($p \leq 0.05$). (*) indicate a significant difference ($p \leq 0.05$). (#) indicates below limit of detection. Data is from 3 replicate experiments.

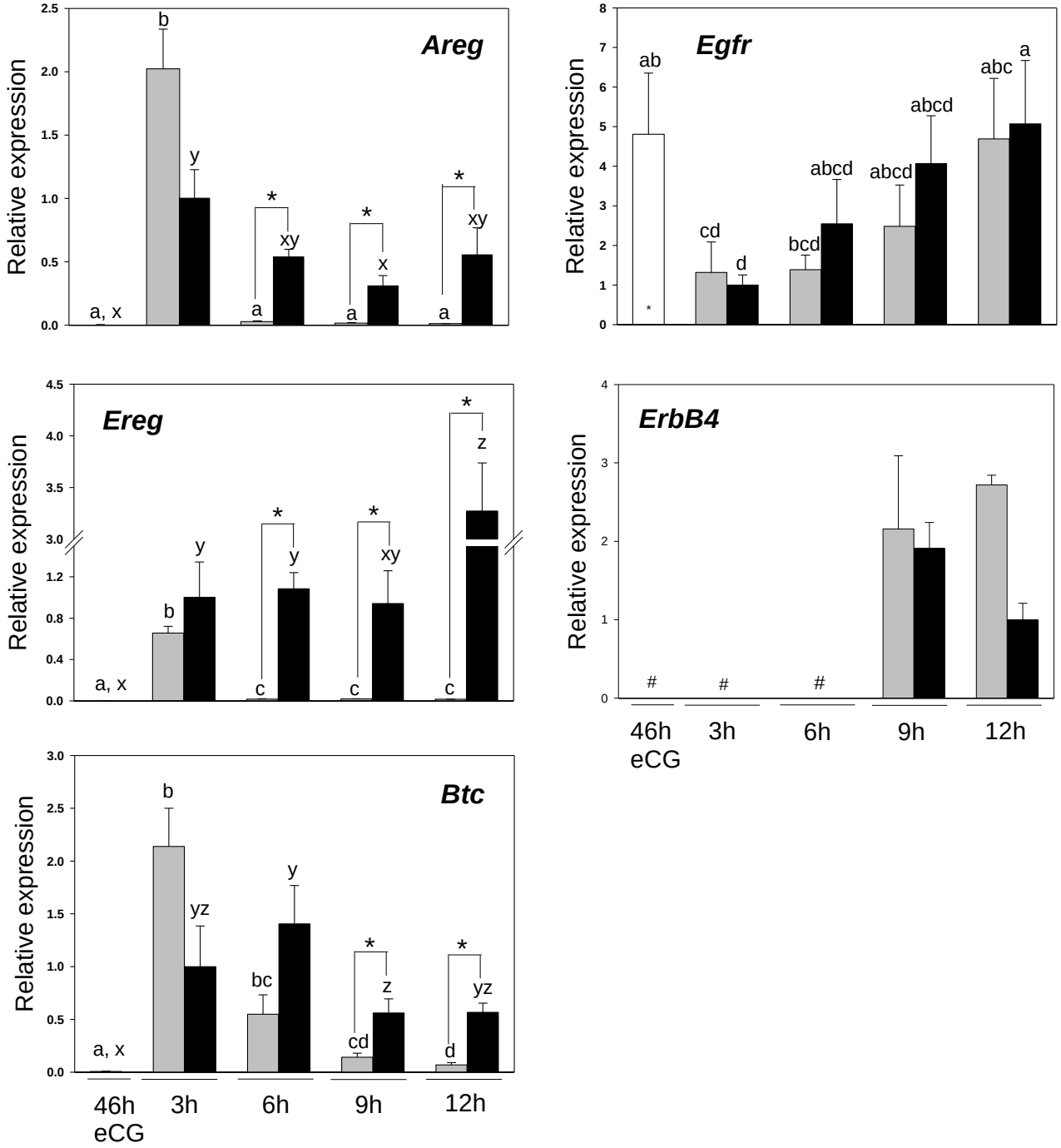

Areg
Egfr
Ereg
ErbB4
Btc
Relative expression
46h eCG
3h
6h
9h
12h

Figure 2.2: Effect of maturation *in vitro* (IVM) compared to *in vivo* (IVV) on amphiregulin production. IVM cumulus-oocyte complexes were cultured *in vitro* with FSH and IVV cumulus-oocyte complexes were matured with hCG. Both cumulus-oocyte complex types were collected after 3 h, 6 h or 9 h of maturation and placed in 100 µL bicarbonate buffered medium with 3 mg/ml BSA and cultured *in vitro* for a further 3 h. Amphiregulin (AREG) protein was measured in the cumulus-oocyte complexes (A) and their conditioned media (B) by ELISA and quantified using the standard curve method. (*) indicate a significant difference ($p<0.03$). (#) indicates below limit of detection. Data is from 4 replicate experiments.

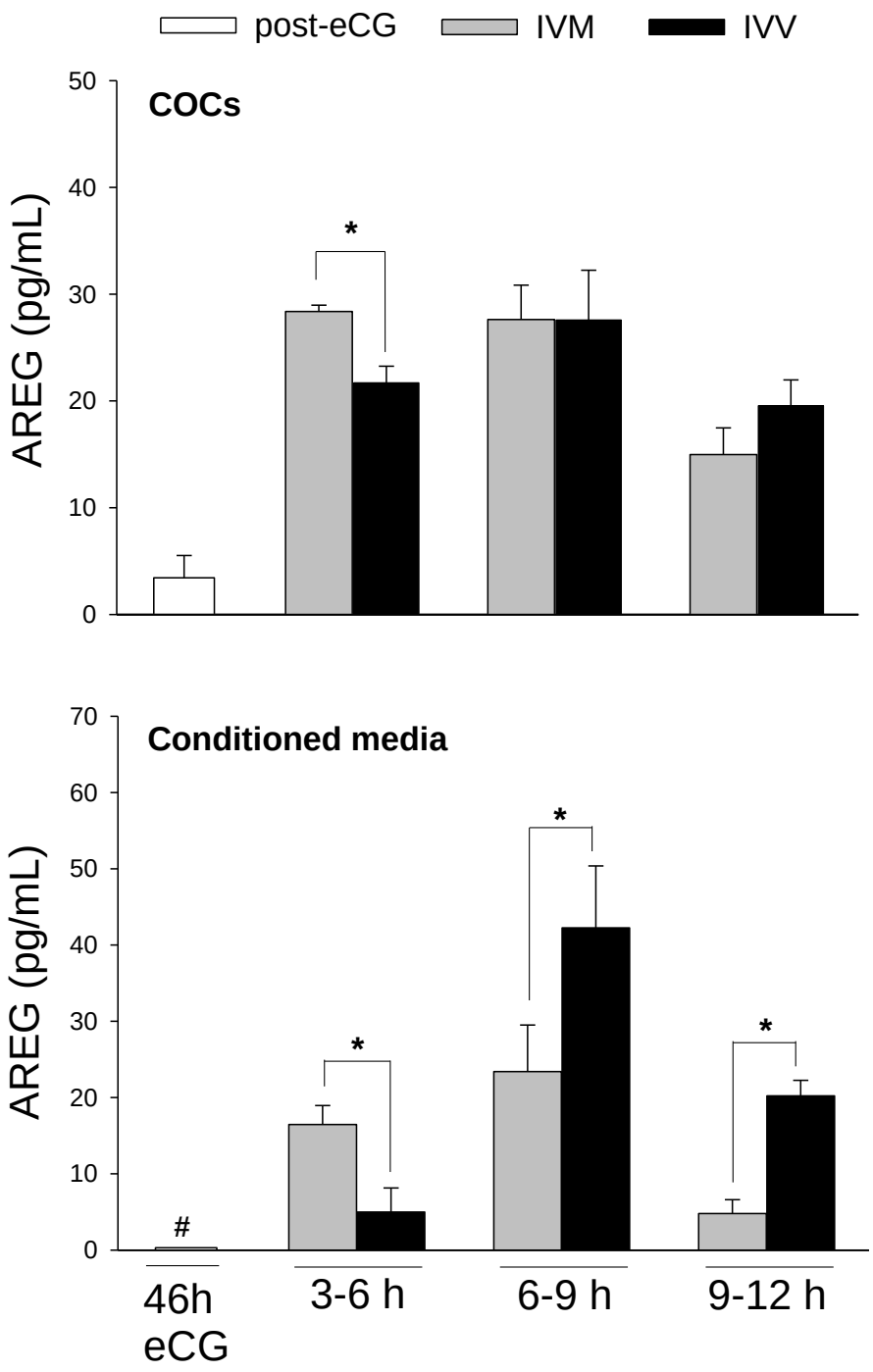

post-eCG
IVM
IVV
COCs
AREG (pg/mL)
50
40
30
20
10
0
*
Conditioned media
AREG (pg/mL)
70
60
50
40
30
20
10
0
#
*
*
*
46h eCG
3-6 h
6-9 h
9-12 h

Figure 2.3: Effects of FSH, EGF and EGF-like peptides *in vitro* and hCG *in vivo* on cumulus cell EGF-like peptide and *Egfr* mRNA expression. Cumulus cells were harvested at 6 h from IVM cumulus-oocyte complexes cultured in the absence (control) or presence of FSH, EGF, AREG, EREG or BTC, or from IVV cumulus-oocyte complexes matured with hCG for 6. Bars not sharing a common letter are significantly different ($p \leq 0.05$). N.D, not detectable. Data is from 6 replicate experiments.

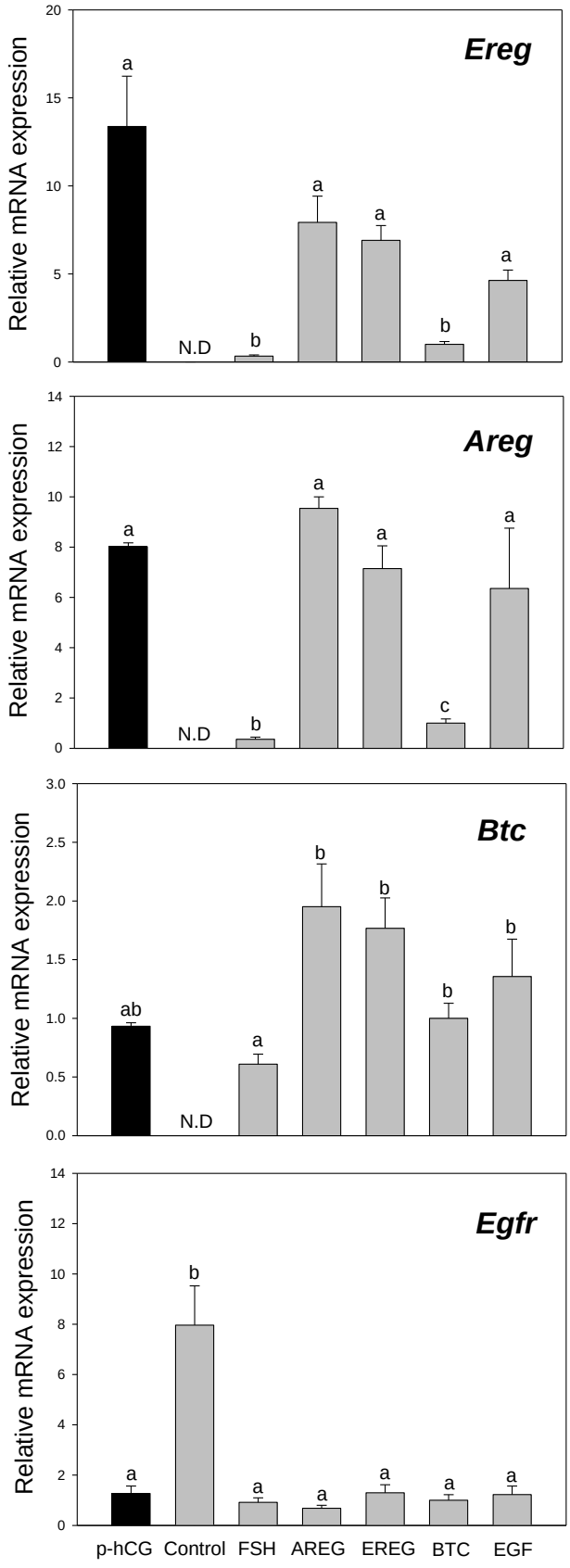
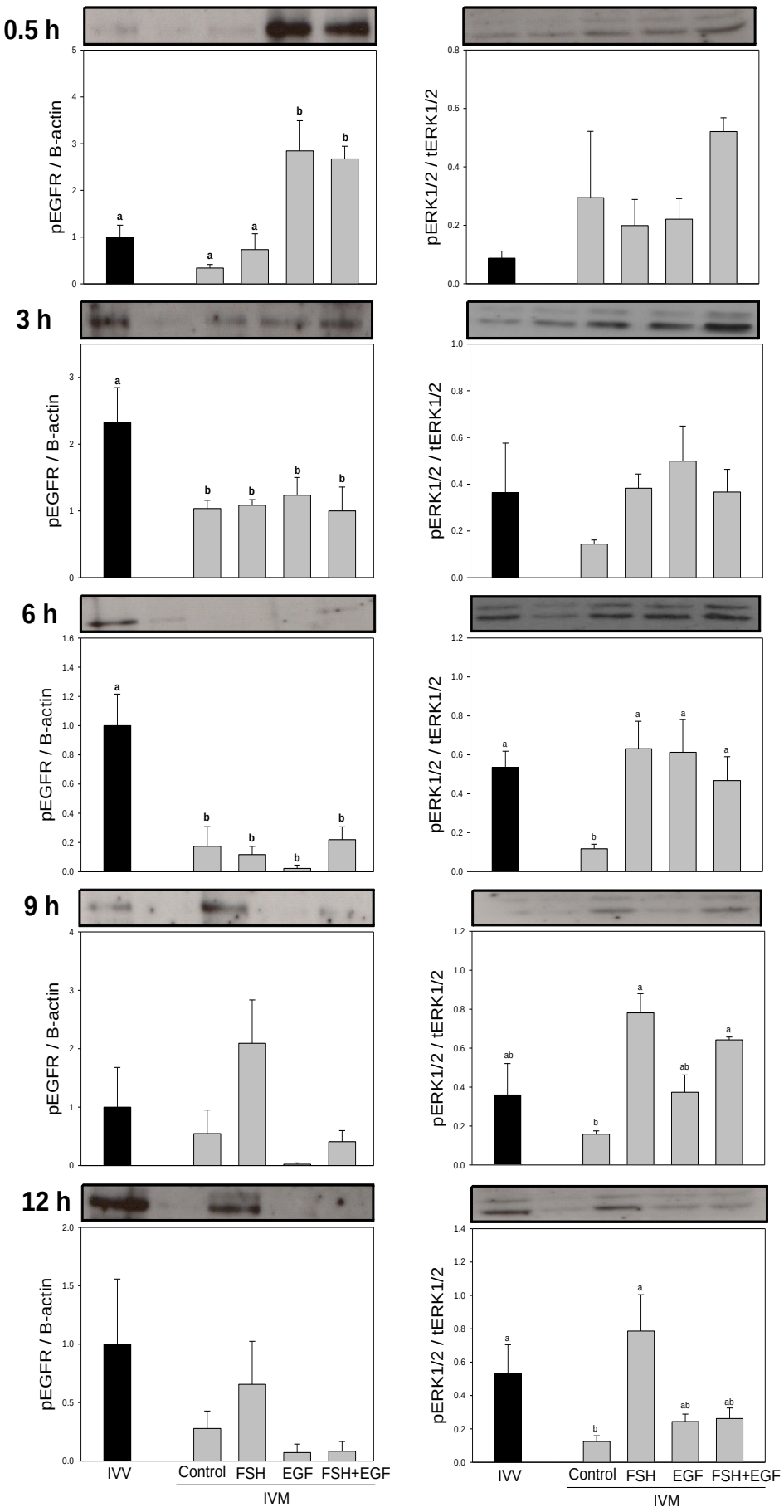

Ereg
Areg
Btc
Egfr
Relative mRNA expression
N.D
p-hCG
Control
FSH
AREG
EREG
BTC
EGF

Figure 2.4: Immunodetection of cumulus-oocyte complex EGFR and ERK1/2 phosphorylation in response to FSH and EGF *in vitro* and hCG *in vivo*. Cumulus-oocyte complexes were cultured via IVM in the absence (control) or presence of FSH, EGF or FSH+EGF, or matured via IVV with hCG for 0.5 h, 3 h, 6 h, 9 h, or 12 h. pEGFR and pERK1/2 levels were measured using Western blots. For each time point, a representative blot of at least 3 replicate experiments is shown and the quantified densitometric values below it. Densitometric measurements of pEGFR were normalised to β-actin and pERK1/2 were normalised to tERK1/2 in each individual blot. The blot bands shown are in order of the following treatments: p-hCG, control, FSH, EGF and FSH+EGF, respectively, and are representatives of pEGFR and pERK1/2. pEGFR, phosphorylated EGFR; pERK1/2, phosphorylated ERK1/2; tERK1/2, total ERK1/2.


pEGFR
pERK1/2
0.5 h
3 h
6 h
9 h
12 h
pEGFR / B-actin
pERK1/2 / tERK1/2
IVV
Control
FSH
EGF
FSH+EGF
IVM

2.9 REFERENCES

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CHAPTER THREE

Effect of epidermal growth factor-like peptides on the metabolism of *in vitro* matured mouse oocytes and cumulus cells

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Published in Biology of Reproduction (Appendix 2)

Statement of Authorship

Title of Paper	Effect of epidermal growth factor-like peptides on the metabolism of in vitro matured mouse oocytes and cumulus cells
Publication Status	○ Published, ◉ Accepted for Publication, ○ Submitted for Publication, ○ Publication style
Publication Details	Published on 22 January 2014 as DOI:10.1095/biolreprod.113.115311 in Biology of Reproduction.

Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

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Statement of Authorship

Title of Paper	Effect of epidermal growth factor-like peptides on the metabolism of in vitro matured mouse oocytes and cumulus cells
Publication Status	○ Published, ◉ Accepted for Publication, ○ Submitted for Publication, ○ Publication style
Publication Details	Published on 22 January 2014 as DOI:10.1095/biolreprod.113.115311 in Biology of Reproduction.

Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

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Richani, D., Sutton-McDowall, M.L., Frank, L.A., Gilchrist, R.B. & Thompson, J.G. (2014) Effect of epidermal growth factor-like peptides on the metabolism of in vitro matured mouse oocytes and cumulus cells.
Biology of Reproduction, v. 90(3):49, pp. 1-10

CHAPTER FOUR

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

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Published in Molecular Reproduction and Development (Appendix 3)

Statement of Authorship

Title of Paper	Pre-maturation with cAMP modulators in conjunction with EGF-like peptides during IVM enhances mouse oocyte developmental competence
Publication Status	○ Published, ◉ Accepted for Publication, ○ Submitted for Publication, ○ Publication style
Publication Details	Accepted 28 January 2014. Molecular Reproduction and Development. Manuscript ID. MRD-13-0300.R1

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Statement of Authorship

Title of Paper	Pre-maturation with cAMP modulators in conjunction with EGF-like peptides during IVM enhances mouse oocyte developmental competence
Publication Status	○ Published, ◉ Accepted for Publication, ○ Submitted for Publication, ○ Unpublished and Unsubmitted work
Publication Details	Accepted 28 January 2014. Molecular Reproduction and Development. Manuscript ID. MRD-13-^300.R1

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4.1 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.

4.2 INTRODUCTION

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

In vivo, the oocyte is meiotically arrested at the prophase I stage by a moderate concentration of cyclic adenosine 3’5’-monophosphate (cAMP) that is synthesised within the oocyte and by the granulosa and cumulus cells (Conti et al. 2002; Conti et al. 2012; Downs 2010). Somatic cell cAMP is supplied to the oocyte by cumulus cells through gap junctions and acts to inhibit germinal vesicle breakdown via activation of protein kinase A (PKA) (Bornslaeger and Schultz 1985; Dekel et al. 1981; Horner et al. 2003; Webb et al. 2002). Granulosa and cumulus cells play an essential role in maintaining adequate cAMP levels within the oocyte by also supplying it with cyclic guanine 3’5’-monophosphate (cGMP) to inhibit oocyte phosphodiesterase (PDE) activity, the enzyme that degrades cAMP (Norris et al. 2009; Tornell et al. 1991). Cyclic AMP has a dual role in regulating oocyte maturation as, paradoxically, it both inhibits and promotes oocyte maturation. Oocyte meiotic resumption and maturation are initiated in response to the ovulatory luteinizing hormone (LH) surge, however, LH exerts its effect by acutely and transiently increasing cAMP concentrations within the mural granulosa cells of the follicle (Dekel et al. 1988; Yoshimura et al. 1992). This cAMP pulse, in turn, induces rapid and transient expression of the epidermal growth factor (EGF)-like peptides amphiregulin (AREG), epiregulin (EREG), and betacellulin (BTC), via a p38 MAPK-dependent pathway (Downs 2010). EGF-like peptides act as key mediators of the LH stimulus as they induce oocyte maturation and ovulation, as well as cumulus expansion (Ashkenazi et al. 2005; Park et al. 2004). Hence, during the normal course of oocyte maturation and ovulation *in vivo*, the complex interplay between the ovulatory endocrine signal and the cellular processes within the follicle are mediated to a great extent by cyclic nucleotides and EGF family signalling (reviewed by Conti et al. (2012)).

After synthesis, EGF-like peptides are proteolytically cleaved from granulosa cells into the follicular fluid, where they act on mural granulosa and cumulus cells to amplify this signal in an autocrine and paracrine manner via activation of the EGF receptor (EGFR); a receptor tyrosine kinase and a member of the ErbB receptor tyrosine kinase family (Oda et al. 2005). EGF-like peptide activation of the EGFR elicits downstream signalling where a key downstream effector is extracellular signal-regulated kinase 1/2 (ERK1/2) (Downs 2010; Shimada et al. 2006). ERK1/2 activation is regarded as a central signalling mechanism for the induction of oocyte maturation and ovulation by EGF-like peptide signalling, as ERK1/2 activity is essential for cumulus expansion, resumption of meiosis and ovulation (Fan et al. 2009; Shimada et al. 2006). However recent data suggests that EGFR activation also decreases follicular cGMP levels, leading to cAMP degradation and, consequently, oocyte meiotic resumption (Conti et al. 2012; Hsieh et al. 2011; Norris et al. 2010; Tsuji et al. 2012; Vaccari et al. 2009). A new study has now shown that somatic cell EGF-like peptide signalling also regulates translation of a subset of mRNAs within the mouse oocyte during maturation; perturbation of EGF-like peptide-mediated translation impacts fecundity (Chen et al. 2013). A recent study has shown that COCs matured under standard IVM conditions are deficient in EGF-like peptides and EGFR activity, indicating that the oocyte does not receive important regulatory signals (Richani et al. 2013). Unsurprisingly, the addition of 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.

4.3 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

Following the pre-IVM period, COCs were washed thoroughly with bicarbonate-buffered αMEM supplemented with 3 mg/mL BSA to remove pre-IVM treatments. COCs were then cultured in bicarbonate-buffered αMEM supplemented with 3 mg/mL BSA and either: recombinant human FSH (50 mIU/mL; Puregon, Organon, Oss, The Netherlands), recombinant human EGF (10 ng/mL; R&D Systems, Minneapolis, USA), recombinant mouse AREG (50 ng/mL; R&D Systems), or recombinant mouse EREG (50 ng/mL; R&D Systems) at 37ºC with 5% CO_2 in air. Doses of EGF family growth factors are based on

previous studies (De La Fuente et al. 1999; Downs and Chen 2008; Li et al. 2008). "Standard IVM" conditions were considered IVM in the presence of 50 mIU/mL FSH and no pre-IVM period.

RNA Isolation

Following the indicated periods of IVV or IVM, 50 COCs, or their cumulus cells, were collected and washed once with PBS. Total RNA was extracted using the RNeasy Micro Kit (Qiagen, Germantown, USA) according to the manufacturer's instructions. DNA that may have been co-purified was removed by the addition of DNase (0.34 Kunitz/µL supplied with kit). RNA was eluted in 14 µL of RNase-free water and stored at -80ºC. Final RNA concentrations were determined by absorbance using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Scoresby, Australia).

RT-qPCR

An equal amount of total RNA from each sample was reverse transcribed using random hexamers (Invitrogen, Carlsbad, USA) and Superscript III reverse transcriptase (Invitrogen). Primers (10 pmol/reaction; Table 4.1) and cDNA were then added to 20 µL total reaction volume with SYBR Green (Applied Biosystems, Mulgrave, Australia). PCRs were performed using a Corbett Rotor-Gene 6000 (Qiagen). Thermal cycling conditions were set as follows: denaturing for 10 min at 95°C, then 40 cycles of annealing and extension at 95°C for 15 sec and 60°C for 60 sec, followed by dissociation at 95°C, 60°C, and 95°C for 15 sec each. A seven point serial dilution standard curve was produced for each transcript from cDNA generated from mural granulosa cells collected 3 h post-hCG. 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 90%.

Protein Immunodetection

Immunodetection of phosphorylated EGFR (pEGFR), phosphorylated ERK1/2 (pERK1/2), total ERK1/2 (tERK1/2) and β-actin was performed as described by Richani et al. (2013). Briefly, 50 COCs per treatment group were collected and suspended in RIPA buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100) containing phosphatase and

protease inhibitor cocktails (Roche, Penzberg, Germany), snap frozen in liquid nitrogen, and stored at -80ºC. Samples were loaded onto a 7.5% SDS–polyacrylamide gel for electrophoresis and proteins were transferred to Hybond-ECL membranes (GE Healthcare, Waukesha, USA). Membranes were cut in half horizontally; the upper half was incubated with the primary antibody anti-pEGFR (Cell Signalling Technology, Beverley, USA, cat. no. 3777) diluted 1:1000, and the lower half was incubated with anti-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 (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 and pERK1/2 were normalised to β-Actin and tERK1/2 band intensities, respectively, and standardised relative to post-hCG values. Data is represented as the mean of four replicate experiments.

In vitro embryo production and differential staining

As described above, standard IVM COCs were matured for 18 h, and IVM COC with a pre-IVM phase (2 h or 4 h) were matured for 20 h following the pre-IVM phase (albeit, for Fig. 4.1, COCs exposed to a pre-IVM phase were cultured for 18 h only), however 1 mg/mL fetuin was also added to the COC handling and IVM culture media to prevent zona pellucida hardening. IVV COCs were collected 16 h post-hCG from eCG primed mice. The extended IVM maturation period of 20 h used for COCs subjected to a pre-IVM phase with cAMP modulators was based on our previous experience examining the kinetics of meiosis under these conditions; although extended maturation time generally causes decreased developmental competence of standard IVM oocytes due to oocyte aging, we observe an increase in competence using this model (Albuz et al. 2010; Shu et al. 2008; Thomas et al. 2004b; Zeng et al. 2013). CBA x C57BL6 FI hybrid male mice (6- to 8-weeks old) were used as sperm donors for *in vitro* fertilization; COCs were fertilised with capacitated epididymal sperm for 4 h at 37ºC at 6% CO_2 in air in Research VitroFert media

(Cook Medical, Eight Miles Plain, QLD, Australia). COCs were then transferred into Research VitroWash media (Cook Medical) 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) at 37ºC with 6% CO_2, 5% O_2 and balance of nitrogen. Blinded assessment of embryo development at days 2, 5 and 6 post-insemination was performed using the scoring system reported by Gardner et al. (2004).

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.

4.4 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. 4.1). 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. 4.2). 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. 4.2), 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. 4.3A). However, pEGFR and pERK1/2 levels were unaffected by pre-IVM with FSK and IBMX (Fig. 4.3B). 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. 4.4). *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≤0.04). *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. 4.5). 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. 4.4 and 4.5) 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. 4.6). 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 ($P>0.05$).

4.5 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 peptides; the natural mediators of the ovulatory LH signal. Findings from this study confirm that increasing cAMP during a pre-IVM phase is beneficial to oocyte competence as it increases blastocyst inner cell mass regardless of the IVM treatment. Moreover, a pre-IVM phase in conjunction with IVM in the presence of EGF-like peptides leads to an increase in embryo yield and/or size relative to FSH, EGF, or standard IVM.

Culture conditions, media composition, growth factors, and hormones have been shown to influence mammalian oocyte *in vitro* maturation and subsequent developmental capacity (Gilchrist 2011; Lane and Gardner 1997). Cyclic AMP is a central molecule controlling oocyte meiotic arrest and resumption, and oocyte developmental competence. IVM COCs undergo some aberrant cellular processes due to the resultant loss of cAMP and its control of meiotic maturation. The literature over the last 30 years, predominantly in animal models, demonstrates that cAMP management during IVM is an effective and logistically feasible means to improve subsequent developmental outcomes (Smitz et al. 2011). Prolonged maintenance of cAMP using PDE inhibitors has been shown to yield modest improvements in oocyte competence across several species including porcine, bovine, murine, and human (Downs et al. 1986; Nogueira et al. 2003a; Nogueira et al. 2003b; Thomas et al. 2004b; Vanhoutte et al. 2009a; Vanhoutte et al. 2009b). Furthermore, up-regulation of COC cAMP levels prior to IVM (either through the use of dibutyryl cAMP or adenylate cyclase activators) seems to be an effective approach, as this has been shown to significantly improve subsequent developmental competence in human, murine, bovine, ovine and porcine IVM oocytes (Albuz et al. 2010; Funahashi et al. 1997; Guixue et al. 2001; Luciano et al. 1999; Rose et al. 2013; Shu et al. 2008; Zeng et al. 2013).

The optimal pre-IVM duration is currently unknown. The culture of porcine COCs in the presence of dbcAMP for the first half (20 hours) of IVM was shown to greatly increase subsequent embryo yield (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.

FSH has been a universal additive to IVM systems for over 20 years due to its recognized positive effects on oocyte developmental competence relative to spontaneous IVM (Eppig et al. 1992; Izadyar et al. 1998). FSH-induced oocyte maturation is now known to induce up-regulation of EGF-like peptide expression in cumulus cells (Park et al. 2004). However, we have recently shown that FSH is a relatively poor stimulator of EGF-like peptide expression in IVM cumulus cells as it induces considerably lower expression levels than EGF-like peptides or hCG *in vivo* (Richani et al. 2013). In this study, we found the increased cumulus cell EGF-like peptide expression, as a result of the cAMP-mediated pre-IVM phase, is not maintained during IVM in the presence of FSH, as expression levels

were similar to, or less than, those seen during standard IVM. Conversely, IVM in the presence of exogenous EGF-like peptides or EGF, following a 2 h pre-IVM phase, led to expression of the EGF-like peptides at levels similar to those seen *in vivo,* and notably higher than those of standard IVM. Interestingly, prolonged exposure to high cAMP (4 h pre-IVM phase) prior to IVM appeared to down-regulate EGFR activity compared to a shorter exposure time (2 h pre-IVM phase). This was not due to down-regulated EGFR expression as our data shows no difference in mRNA expression across all treatments. However it could be due to over-activation of the EGFR causing a negative feedback loop, leading to internalization of the receptor (Madshus and Stang 2009; Roepstorff et al. 2008). EGFR activity is normally tightly regulated as this receptor is a well-studied oncogene and its overactivity has been shown to culminate in cancer development (Madshus and Stang 2009; Riese et al. 2007; Yarden and Sliwkowski 2001).

The use of epiregulin or amphiregulin, instead of FSH or EGF, in an IVM system has been shown to improve subsequent embryo development in the mouse and pig (Prochazka et al. 2011; Richani et al. 2013). In the mouse, epiregulin and amphiregulin were shown to increase the proportion of inner cell mass cells, however only epiregulin significantly increased embryo yield (Richani et al. 2013). Here we found that, unlike FSH or EGF, amphiregulin and epiregulin increased blastocyst size (trophectoderm and total cell numbers), and consistent with our previous study, only epiregulin significantly increased day 6 blastocyst rates compared to standard IVM, with rates comparable to *in vivo* matured oocytes. Increased blastocyst yield and size have been associated with improved developmental outcomes (Lane and Gardner 1997). Hence, it appears that increasing cAMP prior to IVM via a pre-IVM phase is beneficial as it increases blastocyst inner cell mass regardless of the IVM conditions. However, a pre-IVM phase in conjunction with IVM in the presence of EGF-like peptides, particularly epiregulin in the mouse, endows even greater oocyte developmental competence as it leads to increased embryo yield and/or quality relative to FSH and EGF. A comparison of the effect of these treatments on post-implantation fetal development is required, however the increased embryo yield and/or quality observed in this study is promising as IVM treatments that lead to small increases in embryo yield and, in particular, in inner cell mass size, have been shown to significantly increase murine fetal development and survival following IVM (Albuz et al. 2010; Sudiman et al. 2014).

We examined the effect of EGF as it is a common additive to animal research and veterinary IVM systems (Banwell and Thompson 2008), and is a member of the same family of proteins as EGF-like peptides and signals via EGFR to induce oocyte maturation and cumulus expansion *in vitro* (De La Fuente et al. 1999; Downs 1989; Downs et al. 1988; Rieger et al. 1998). Despite its ability to induce comparable EGF-like peptide expression levels to those induced *in vivo*, and by epiregulin and amphiregulin *in vitro*, EGF did not elicit the same level of EGFR activity or endow the same oocyte developmental competence as these EGF-like peptides. EGF has previously been shown to yield significantly lower blastocyst yield and quality than epiregulin and amphiregulin in an IVM system without a pre-IVM phase (Richani et al. 2013). This suggests that differing EGFR ligands produce differences in IVM oocyte developmental competence despite acting through the same receptor. EGFR ligands are known to have distinct binding specificities and affinities, and depending on ligand 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 ERK1/2 activation. However, EGFR and ERK1/2 activities were not reflective of EGF-like peptide levels, and these factors were not reflective of embryo developmental outcomes. Hence, the level of EGFR signalling via ERK1/2 may not be a limiting factor in oocyte developmental competence, however we cannot exclude the possibility that differential EGFR phosphorylation via alternative tyrosine residues than the one measured in this study (Tyr 1068) may be occurring. Furthermore, investigation into the differential effects of EGF-like peptides, EGF, and FSH on alternative signalling pathways to ERK/12 is required since a new study by Chen et al. (2013) has shown that granulosa and cumulus cell amphiregulin signalling regulates the translation of a subset of mRNAs within the mouse oocyte via the P(I)3K-AKT-mTOR signalling pathway. Further investigation is required into the mechanisms by which increased cAMP, in combination with exogenous EGF-like peptides, increased oocyte developmental competence. This may include differential activation of downstream signalling pathways, metabolic alterations and/or improvements attributable to prolonged COC gap-junctional communication.

In conclusion, the current study demonstrates an improved IVM model that incorporates the use of cAMP modulators in a pre-maturation phase and exogenous EGF-like peptides during maturation to yield oocytes with higher developmental competence than those matured via standard IVM, and are comparable to those matured *in vivo*. The improvement to IVM oocyte developmental competence through pre-maturation with cAMP modulators has strong precedence in the literature, however this study suggests that combining this concept with oocyte maturation in the presence of exogenous EGF-like peptides, rather than the presently used IVM additives FSH or EGF, further increases oocyte developmental competence. Such an approach may represent a more physiological IVM system as it ameliorates key deficiencies in current systems and likely mimics key *in vivo* signalling events more closely.

4.6 ACKNOWLEDGEMENTS

Many thanks to Ms. Lesley Ritter and Dr. Satoshi Sugimura for their generous advice and guidance.

4.7 CONFLICT OF INTEREST

Part of this work was supported by Cook Medical Pty Ltd. (see funding statement), and R.B.G and J.G.T have consultancy agreements with Cook Medical.

4.8 FUNDING

This work was supported by the National Health and Medical Research Council through Project Grant [APP1007551] awarded to RBG and JES and Senior Research Fellowships [1023210, 627007] awarded to JGT and RBG; by grants from Cook Medical; and an Australian Postgraduate Award to DR.

Table 4.1: Sequences of PCR primers used for RT-qPCR.

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.

Figure 4.1: Effect of 0-2 h pre-IVM followed by standard (FSH) IVM on embryo development. COCs were subjected to 0 h (standard IVM), 1 h or 2 h pre-IVM culture in the presence of forskolin and IBMX, followed by standard IVM in the presence of FSH for 18 h. Cleavage and days 5 and 6 blastocyst rates were assessed (n≥200 COCs per group over 6 replicate experiments). Bars within each group that are not sharing a common letter are significantly different (P<0.05). Blasts, blastocysts; H/Blasts, hatching blastocysts; D, day.

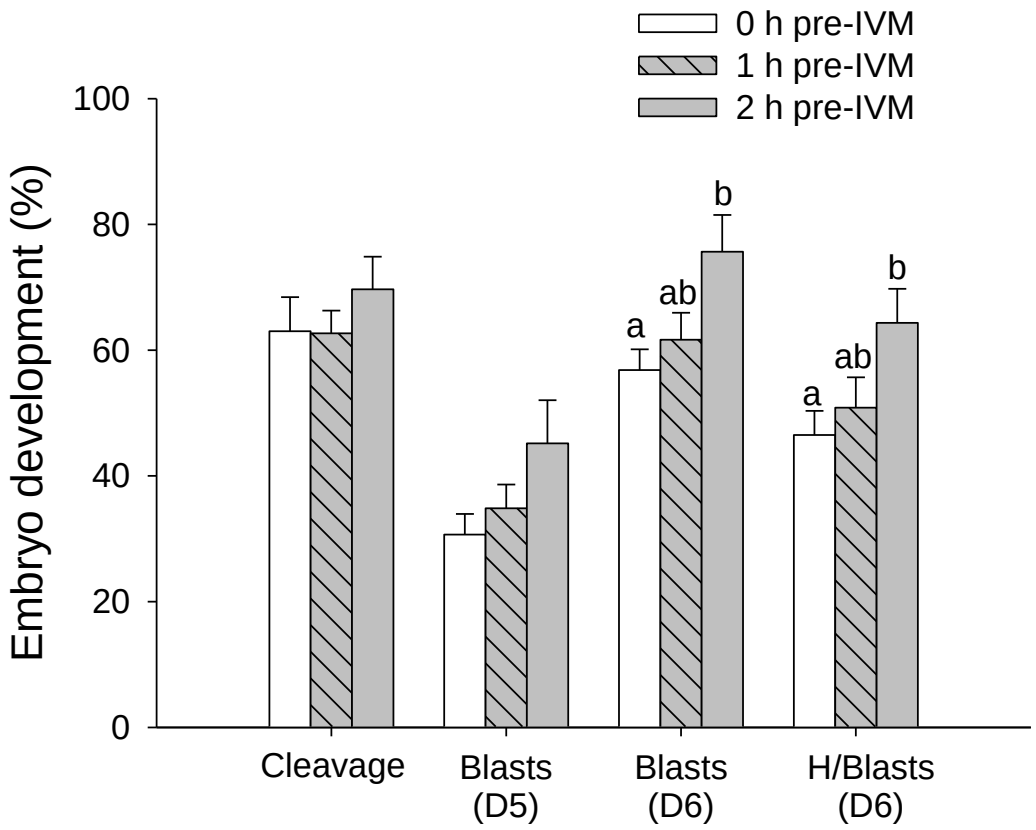

0 h pre-IVM
1 h pre-IVM
2 h pre-IVM
Embryo development (%)
100
80
60
40
20
0
a
ab
b
a
ab
b
Cleavage
Blasts (D5)
Blasts (D6)
H/Blasts (D6)

Figure 4.2: Effect of 2 h and 4 h pre-IVM followed by IVM in the presence of EGF-like peptides or EGF on embryo development. COCs were either matured *in vivo* (IVV) for 16 h, under standard IVM conditions (no pre-IVM + FSH IVM) for 18 h, or were subjected to 2 h or 4 h pre-IVM culture in the presence of forskolin and IBMX, followed by IVM in the presence of FSH, EGF, AREG or EREG for 20 h. Cleavage and day 6 blastocyst rates were assessed (n≥120 COCs per group over 5 replicate experiments). Day 6 blastocyst quality was assessed by quantification of the number of inner cell mass (ICM), trophectoderm (TE), and total cells, and the proportion of ICM cells (n=24-27 per group). Bars not sharing a common letter are significantly different (P<0.05). Blasts, blastocysts; D, day.

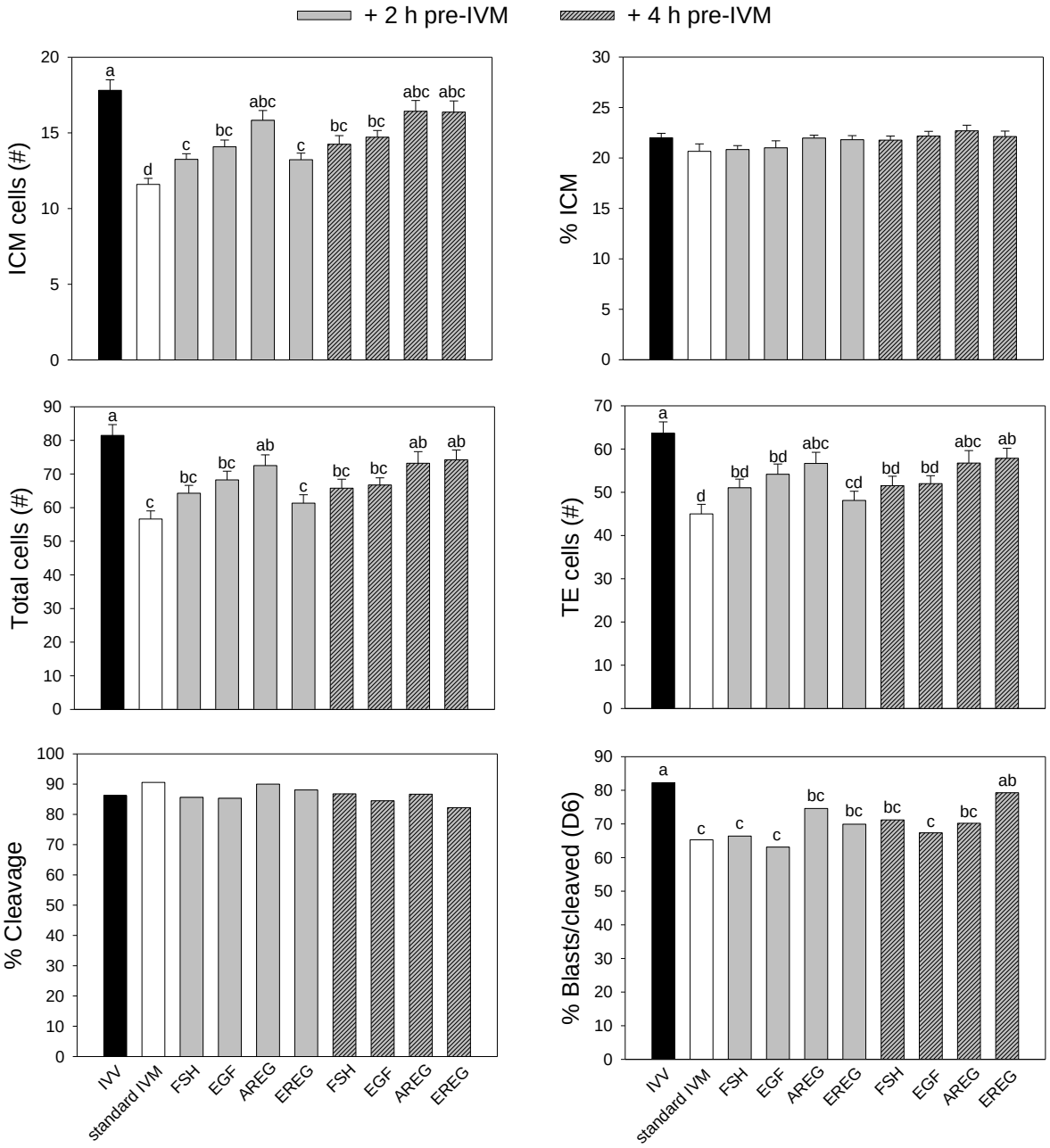
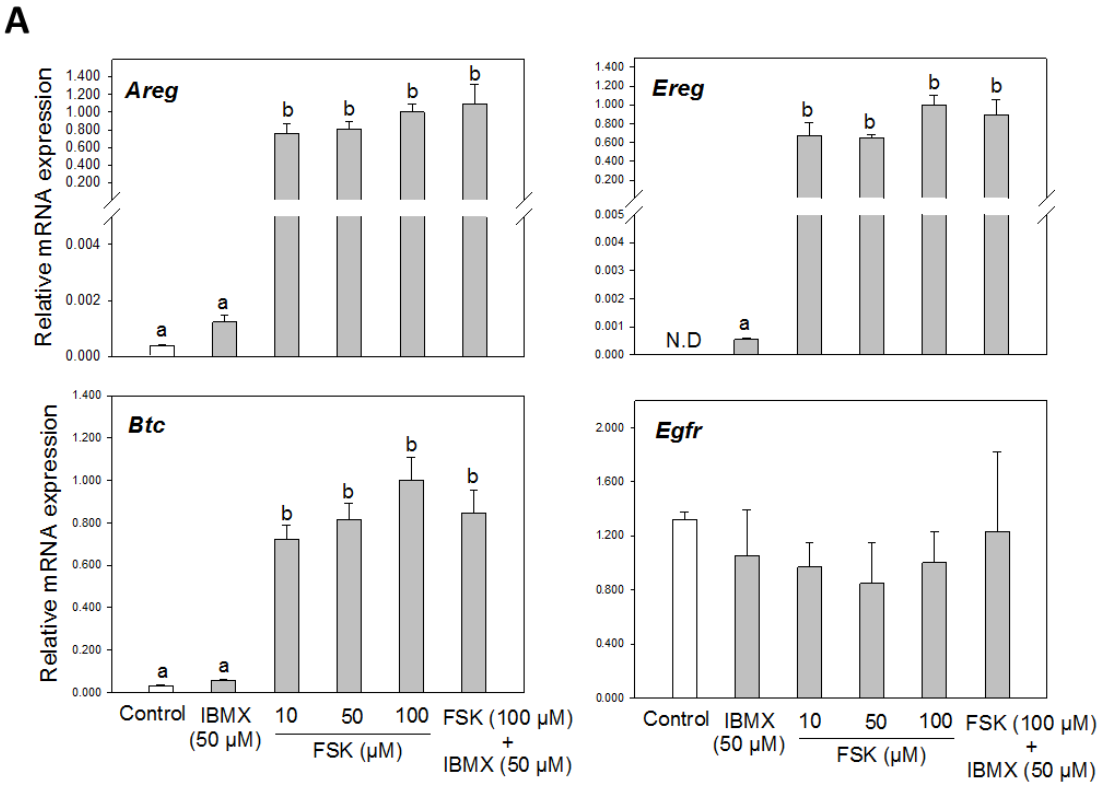

+ 2 h pre-IVM
+ 4 h pre-IVM
ICM cells (#)
% ICM
Total cells (#)
TE cells (#)
% Cleavage
% Blasts/cleaved (D6)
IVV
standard IVM
FSH
EGF
AREG
EREG
FSH
EGF
AREG
EREG

Figure 4.3: Effect of 2 h pre-IVM on EGF-like peptide and EGFR mRNA expression, and EGFR and ERK1/2 phosphorylation. COCs underwent 2 h pre-IVM culture in the presence of control (no treatment), or forskolin and/or IBMX at indicated doses. (A) Cumulus cell were separated from oocytes and their mRNA expression after 2 h pre-IVM was quantified using RT-qPCR and normalised to the geometric mean of the *Ppia* and *Mrpl19* housekeeper genes (n=3). (B) The relative levels of whole COC phosphorylated EGFR (pEGFR) and phosphorylated ERK1/2 (pERK1/2) were measured after 2 h pre-IVM via Western blotting (n=4); a representative blot is shown and the quantified densitometric values below it. Bars not sharing a common letter are significantly different ($P<0.05$); NS, not significant; N.D., below limit of detection.


A
Areg
Ereg
Btc
Egfr
Relative mRNA expression
N.D
Control
IBMX (50 μM)
10
50
100
FSK (μM)
FSK (100 μM) + IBMX (50 μM)

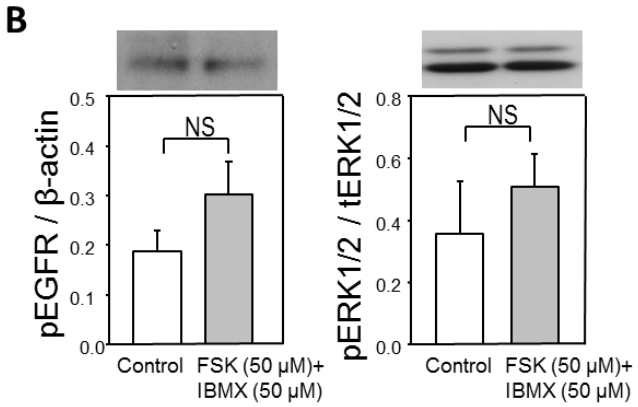

B
pEGFR / β-actin
pERK1/2 / tERK1/2
NS
Control
FSK (50 μM)+ IBMX (50 μM)

Figure 4.4: Effect of cAMP modulators during pre-IVM on EGF-like peptide and EGFR mRNA expression during IVM. COCs either underwent standard IVM with FSH (-pre-IVM), or were maintained for 2 h in the presence of forskolin and IBMX (+pre-IVM) followed by IVM with FSH, for 3 h, 6 h, or 12 h . COC mRNA expression was quantified using RT-qPCR and normalised to the geometric mean of the *Ppia* and *Mrpl19* housekeeper genes. (*) denotes a significant difference ($P<0.05$). Data is from 3 replicate experiments.

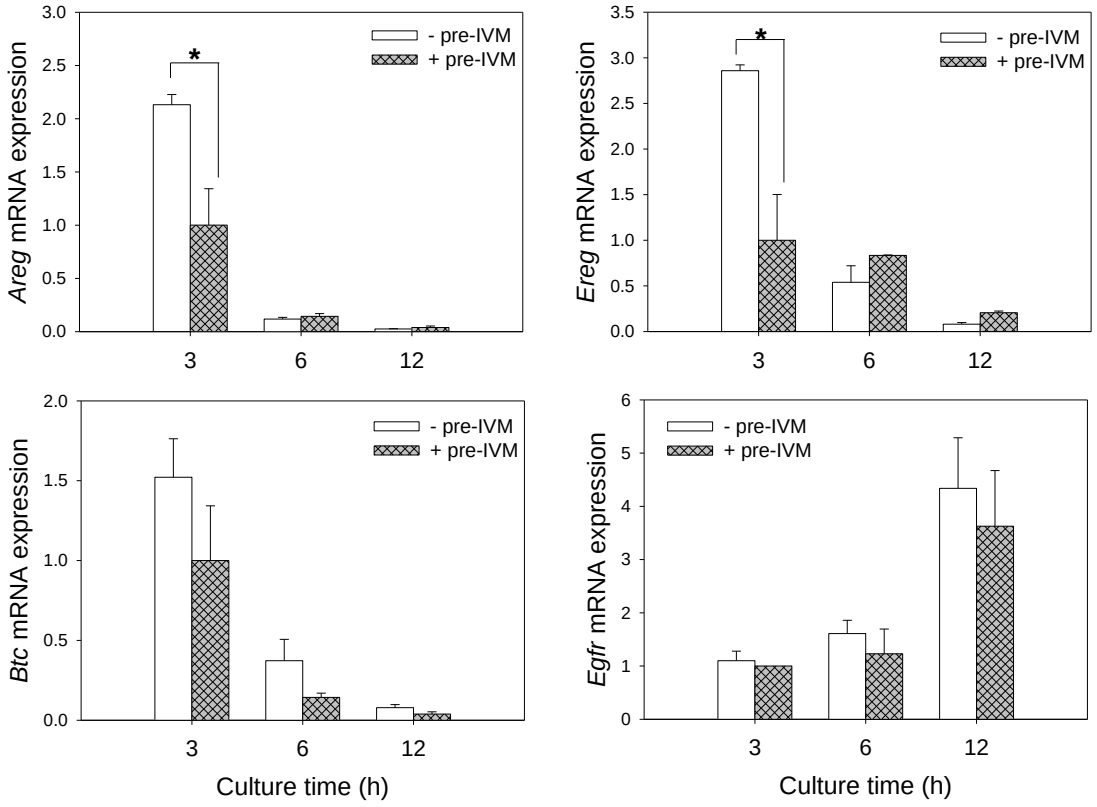

Areg mRNA expression
Ereg mRNA expression
Btc mRNA expression
Egfr mRNA expression
- pre-IVM
+ pre-IVM
*
3
6
12
Culture time (h)

Figure 4.5: Effect of 2 h and 4 h pre-IVM followed by IVM in the presence of EGF-like peptides, FSH, or EGF on EGF-Like peptide expression. COCs were either matured *in vivo* (IVV) for 6 h, under standard IVM conditions (no pre-IVM + FSH IVM) for 6 h, or were subjected to 2 h or 4 h pre-IVM culture in the presence of forskolin and IBMX, followed by IVM in the presence of FSH, EGF, AREG or EREG for 6 h. COC mRNA expression was quantified using RT-qPCR and normalised to the geometric mean of the *Ppia* and *Mrpl19* housekeeper genes. Bars not sharing a common letter are significantly different ($P<0.05$). Data is from 6 replicate experiments.

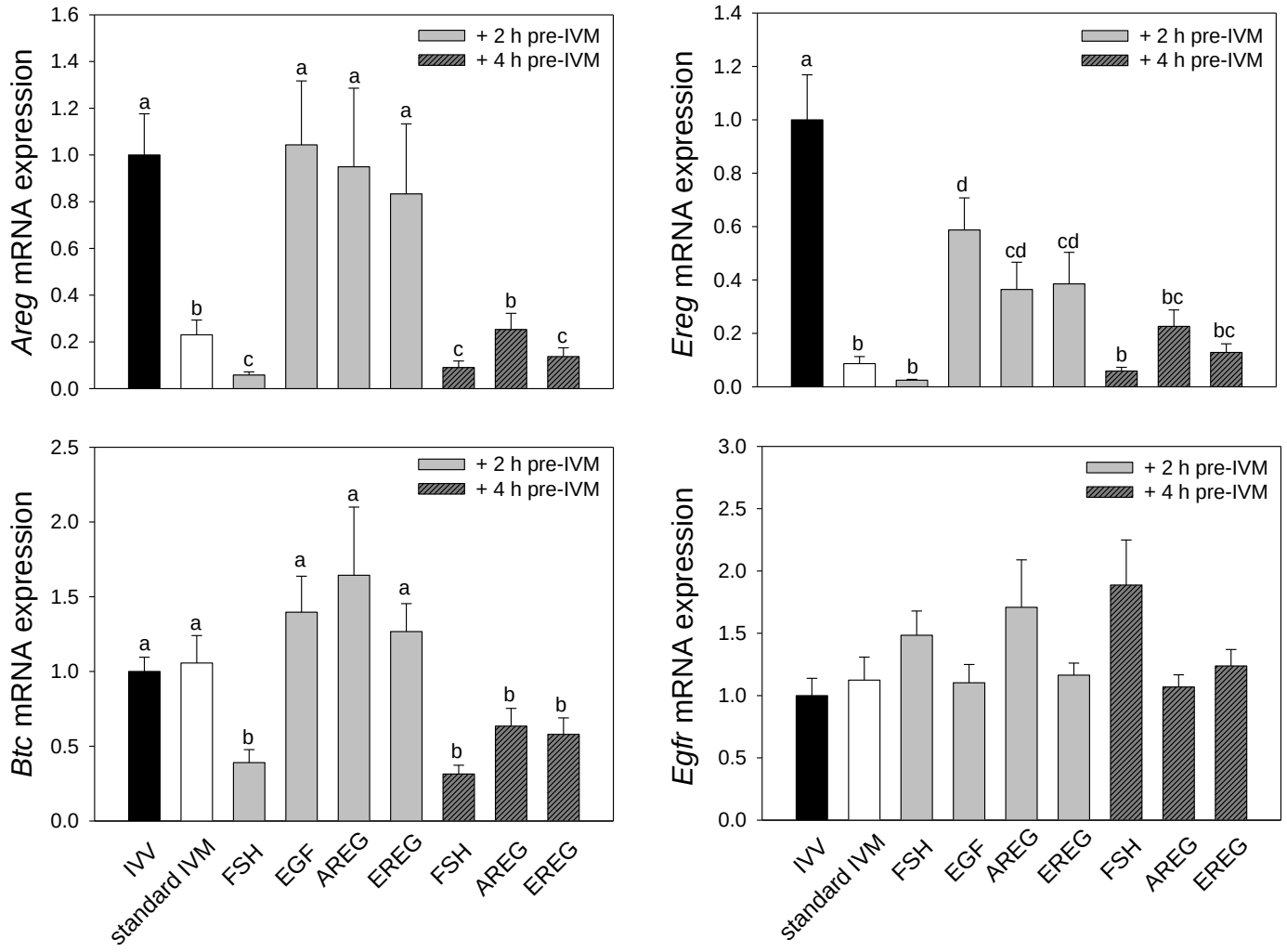
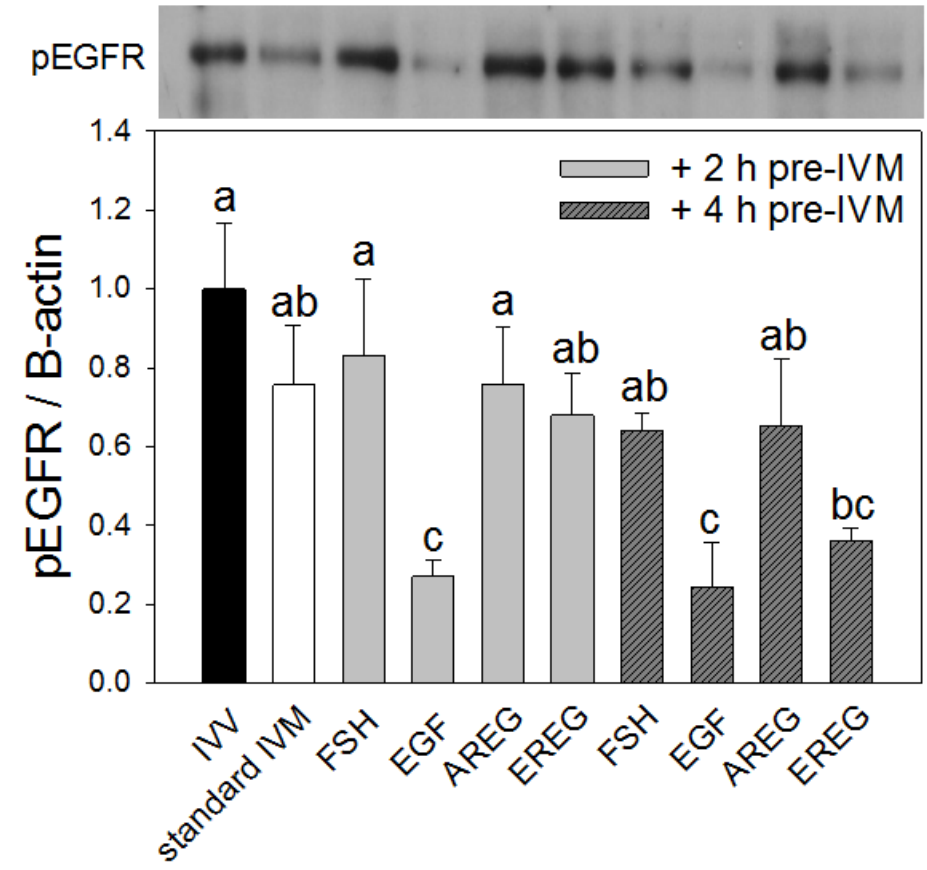
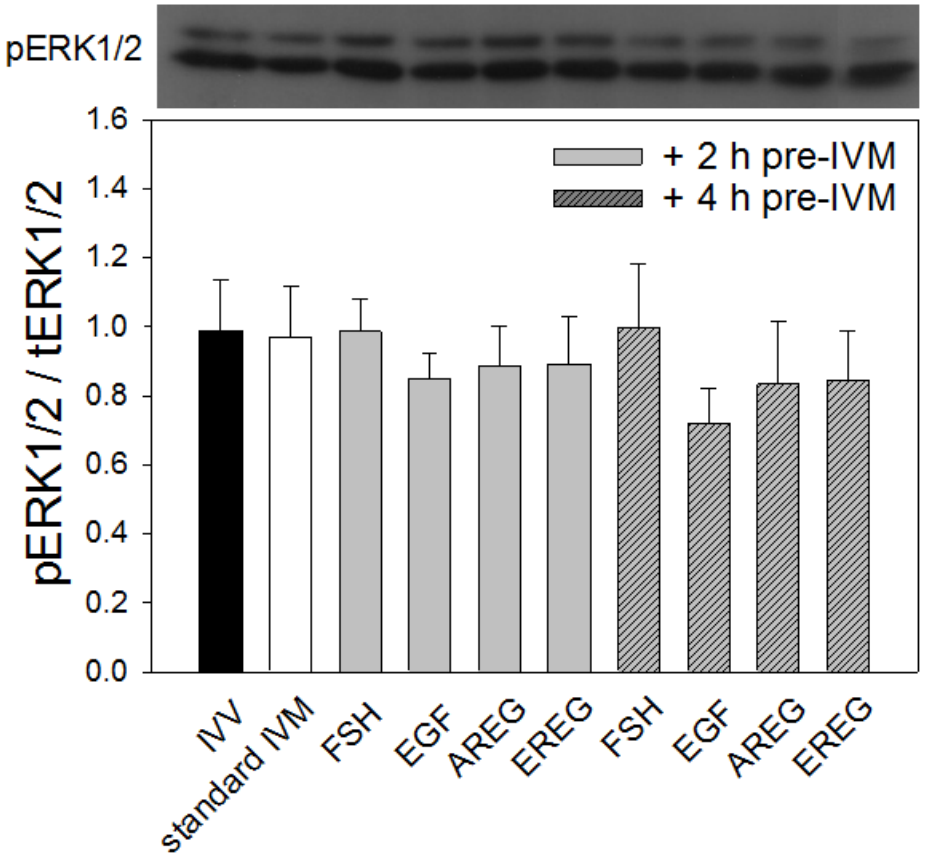

Areg mRNA expression
Ereg mRNA expression
Btc mRNA expression
Egfr mRNA expression
+ 2 h pre-IVM
+ 4 h pre-IVM
IVV
standard IVM
FSH
EGF
AREG
EREG
FSH
AREG
EREG

Figure 4.6: Effect of 2 h and 4 h pre-IVM followed by IVM in the presence of EGF-like peptides, FSH, or EGF on EGFR and ERK1/2 activation. COCs were either matured *in vivo* (IVV) for 6 h, under standard IVM conditions (no pre-IVM + FSH IVM) for 6 h, or were subjected to 2 h or 4 h pre-IVM 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.


pEGFR
pEGFR / B-actin
+ 2 h pre-IVM
+ 4 h pre-IVM
a
ab
a
c
a
ab
ab
c
ab
bc
IVV
standard IVM
FSH
EGF
AREG
EREG
FSH
EGF
AREG
EREG


pERK1/2
pERK1/2 / tERK1/2
+ 2 h pre-IVM
+ 4 h pre-IVM
IVV
standard IVM
FSH
EGF
AREG
EREG
FSH
EGF
AREG
EREG

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CHAPTER FIVE

FINAL DISCUSSION

5.1 FINAL DISCUSSION & FUTURE DIRECTION

Knowledge of how to mature oocytes *in vitro* from unstimulated ovaries has existed for more than four decades. Whilst oocyte *in vitro* maturation (IVM) is highly utilised and decidedly successful in domestic animal, particularly cattle, assisted reproduction, IVM has not been widely embraced in human assisted reproduction despite its obvious financial and medical advantages over *in vitro* fertilisation (IVF). The lower efficiency of IVM, including pregnancy and live birth rates, appears to be the only evident barrier to its adoption into routine clinical practice. There have been significant advances in the field of mammalian oocyte biology over the last decade, which have clarified long-standing controversies regarding oocyte maturation. Clinical and laboratory IVM practices, however, have not adapted to this newfound knowledge, and it is imperative that clinicians and scientists capitalise on this knowledge to modernise and improve IVM methodologies in order to transform this technology into a mainstream fertility treatment.

Although the complete array of signals that promote oocyte maturation in the follicle are still incompletely understood, great strides in elucidating the chain of events involved have been made in recent years. These include the discovery of the role of C-type natriuretic peptide (CNP) and cyclic GMP (cGMP) in regulating meiotic maturation (Norris et al. 2009; Vaccari et al. 2009; Zhang et al. 2010). Another breakthrough discovery was the critical role played by epidermal growth factor (EGF)-like peptides in mediating the LH signal within the follicle to induce oocyte maturation and cumulus expansion. The molecular mechanisms by which EGF-like peptides signalling elicit these effects remain unclear, however new evidence suggests that this key signalling pathway is also involved in cGMP regulation (Conti et al. 2012; Tsuji et al. 2012).

For many years, EGF was proposed to be a mediator of the ovulatory LH signal as it induced oocyte maturation and cumulus expansion *in vitro* in several mammalian species (Das et al. 1992; Dekel and Sherizly 1985; Goud et al. 1998; Prochazka et al. 2000; Rieger et al. 1998), however evidence to confirm this remained inconclusive as EGF was scarcely detectable in pre-ovulatory follicles subjected to LH stimulation (Reeka et al. 1998; Westergaard and Andersen 1989). A seminal study by Marco Conti's group in the mouse finally shed light on this dilemma by demonstrating that other members of the EGF family, and not EGF, in fact acted as LH mediators via the EGF receptor (EGFR) (Park et al. 2004). Three members of the EGF family of proteins, the EGF-like peptides amphiregulin,

epiregulin, and betacellulin are now known to be up-regulated in response to the LH surge, and to be important for oocyte maturation and ovulation *in vivo* in the mouse; the involvement of EGF-like peptides in oocyte maturation has since been shown to be important in several mammalian species, including human (Ashkenazi et al. 2005; Ben-Ami et al. 2006; Freimann et al. 2004; Hsieh et al. 2007; Motola et al. 2008; Sekiguchi et al. 2004; Shimada et al. 2006; Zamah et al. 2010). The main source of follicular EGF-like peptides following the LH stimulus is the mural granulosa cells, which are the major epithelial component of the follicle and the source of a significant proportion of the EGF-like peptides produced and secreted in the follicle. Hence, one of the earliest signals the cumulus-oocyte complex (COC) receives to resume maturation and prepare for embryonic development is from EGF-like peptides produced by mural granulosa cells.

I hypothesised that EGF-like peptide signalling in IVM COCs is perturbed, as these complexes are removed from their follicular environment and matured in 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. The first study of this thesis (chapter 2) investigated the effect of standard IVM on EGF like peptide signalling. This study was the first to show that EGF-like peptide expression and signalling is perturbed as a consequence of IVM (Table 5.1). This study demonstrated that the common IVM stimulants, FSH and EGF, are inadequate propagators of the EGF-like peptide signalling cascade in IVM cumulus cells when compared to their *in vivo* counterparts. Although FSH, the IVM additive of choice, is a known stimulator of EGF-like peptide expression in IVM cumulus cells (Downs and Chen 2008), this study shows that FSH does not promote sufficient cumulus cell gene and protein expression of the EGF-like peptides when compared with levels seen *in vivo* (Fig. 5.1). In addition, both FSH and EGF do not maintain an adequate level of activation of the EGFR throughout IVM in comparison to EGFR activity seen *in vivo*. A deficiency in EGF-like peptide signalling during IVM likely adversely impacts oocyte developmental competence.

FSH is universally used as the IVM additive of choice since it is a stimulator of IVM oocyte maturation and improves oocyte developmental competence in comparison with spontaneous IVM (Freimann et al. 2004; Lindner et al. 1974; Park et al. 2004; Zamah et al. 2010). EGF is also occasionally used in animal IVM systems, usually in combination with FSH, as it is also a potent inducer of oocyte maturation and improves oocyte maturation relative to spontaneous IVM (Dekel and Sherizly 1985; Goud et al. 1998; Prochazka et al.

2000; Rieger et al. 1998). However, both these stimulants are not physiological inducers of oocyte maturation. Chapter 2 shows that the use of amphiregulin or epiregulin, instead of FSH or EGF, during IVM increases mouse oocyte developmental competence, as evidenced by a ≥18% increase in embryo yield by epiregulin, and a ≥36% increase in the percentage of blastocyst inner cell mass (ICM) cells, relative to FSH and EGF. This is encouraging as we have previously shown that, from IVM oocytes, small increases in blastocyst rate (as little as 6%) or inner cell mass cell number (7 cells) have a large impact on mouse fetal yield (≥ 44%) (Albuz et al. 2010; Sudiman et al. 2014). Prochazka et al. (2011) also independently report an increase in porcine embryo yield and size from IVM COCs matured with amphiregulin or epiregulin compared with FSH or LH; they did not compare the effect of EGF. These data suggest that bioactive EGF-like peptides, rather than FSH or EGF, are more suitable IVM additives, as such an approach may represent a more physiological form of IVM that recreates an environment more closely resembling that found *in vivo*. This is supported by the fact that amphiregulin and epiregulin activate signalling pathways slightly different from those activated by EGF (Wilson et al. 2009; Wilson et al. 2012). This thesis and the study by Prochazka et al. (2011), do not examine developmental parameters following the pre-implantation period. Furthermore, they do not provide comprehensive information regarding the optimal doses and combinations of EGF-like peptides to yield the greatest improvement in developmental outcomes. Hence, further investigation into the safety and efficacy of EGF-like peptides in an IVM system is required. I predict that, since they are the physiological inducers of oocyte maturation, EGF-like peptides will prove to be safe for use during IVM. A study by Akaki et al. (2009) provides encouragement as they successfully produced 11 healthy piglets from 3 mothers using a bi-phasic IVM system devoid of gonadotropins that used amphiregulin with EGF and dibutyryl cyclic AMP (cAMP) for the first 20 h of maturation, and no treatment for the remainder of maturation.

The work in this thesis is the first to investigate potential mechanisms by which EGF-like peptides influence oocyte developmental competence. Chapter 3 focussed on the differential effects of these stimulators on oocyte and cumulus cell metabolism, particularly glucose metabolism and oocyte mitochondrial activity and REDOX state. This chapter shows that EGF-like peptides and EGF induce greater metabolic activity of the hexosamine biosynthesis pathway than FSH, enabling more hyaluronic acid synthesis (and consequently greater cumulus expansion) and global protein β-O-linked glycosylation (Table 5.1). Future work could be conducted to characterise the different β-O-linked

glycosylation targets within COCs matured with these treatments in order to identify differentially targeted pathways during maturation; this may provide new insight into differential control of oocyte competence. This study also shows that EGF-like peptides induce significantly more oocyte mitochondrial activity than FSH and EGF. These metabolic alterations may be a mechanism by which EGF-like peptides increase oocyte developmental competence since COC metabolism is an important component of oocyte developmental competence (Krisher 2004).

The work in this thesis highlights the differential effects of EGF and the EGF-like peptides on COC function, despite being closely related and acting via a common receptor. EGF endows significantly lower oocyte developmental competence than epiregulin and amphiregulin (chapter 2). Additionally, EGF does not elicit the same level of COC EGFR phosphorylation as amphiregulin or epiregulin; however this may be due to differences in the temporal pattern of EGFR activation or differential tyrosine residue phosphorylation (chapter 4). Furthermore, EGF induces significantly lower oocyte mitochondrial activity than the EGF-like peptides (chapter 3). These data indicate that substitution of other members of the EGF family of growth factors for EGF-like peptides would not suffice in an IVM system, and highlight the importance of elucidating optimal EGF-like peptide combinations for different species. The work in this thesis suggests epiregulin to be the most suitable EGF-like peptide for use during mouse IVM as it yields the best developmental and metabolic outcomes.

Figure 5.1: Schematic summary of the differential effects of EGF-like peptides versus FSH on cumulus cell and oocyte function, as identified in this thesis. The number of arrows is qualitatively indicative of the extent of up-regulation in response to the EGF-like peptide or FSH stimuli. Horizontal arrows indicate no differential effect by stimuli.

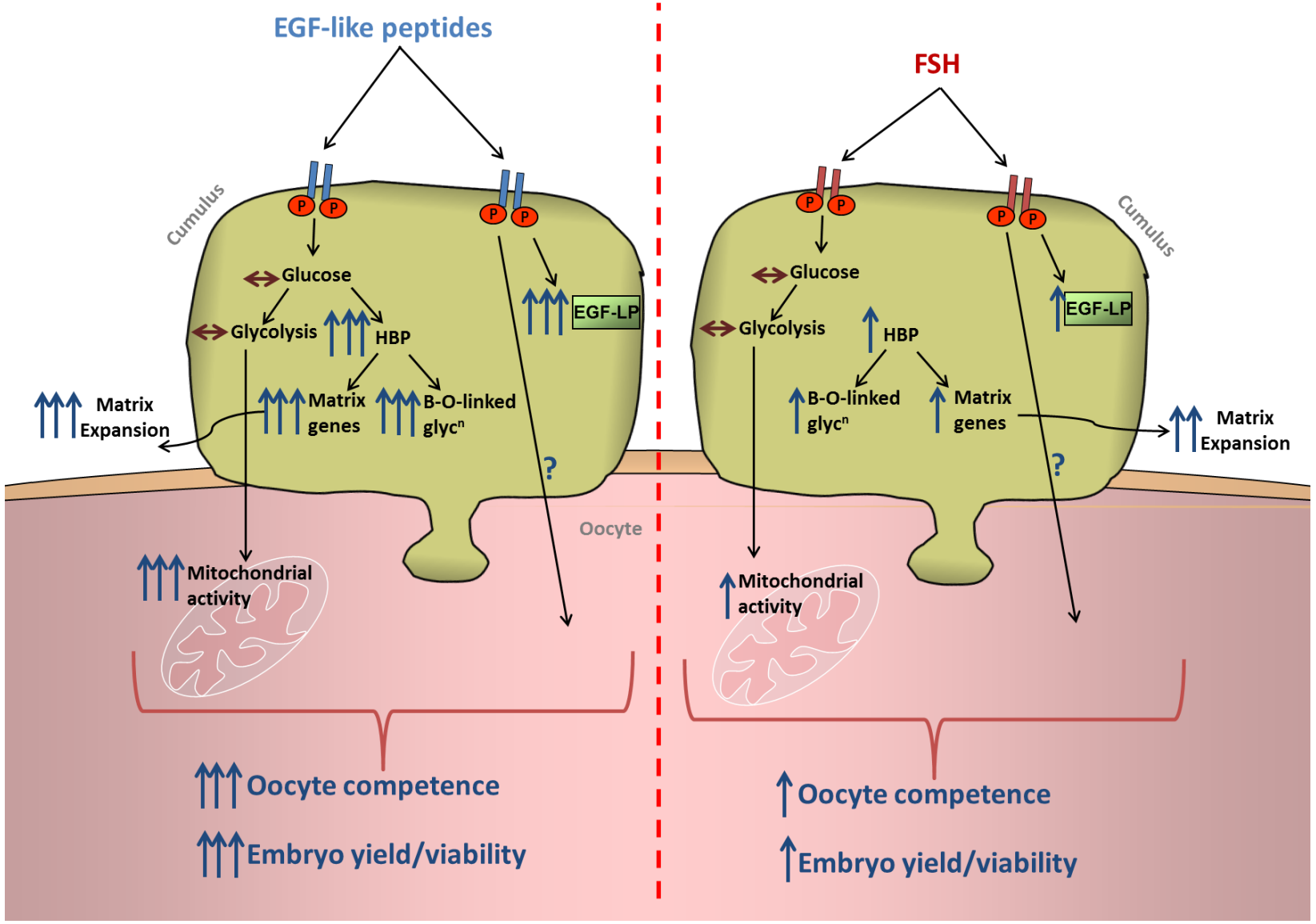
EGF-like peptides
FSH
Cumulus
Cumulus
P
Glucose
Glycolysis
HBP
EGF-LP
Matrix Expansion
Matrix genes
B-O-linked glyc^n
?
Oocyte
Mitochondrial activity
Oocyte competence
Embryo yield/viability

Table 5.1: Summary of the relative effects of IVM additives and *in vivo* maturation on COCs. The number of ticks indicates the relative level of stimulation and cross (X) denotes no stimulation. HBP, hexosamine biosynthesis pathway; ICM, inner cell mass.

		IVM						
	In vivo	**Spontaneous**	**FSH**	**EGF**	**AREG**	**EREG**	**BTC**	**Chapter**
EGF-Like peptide mRNA expression	✓✓✓	X	✓	✓✓✓	✓✓✓	✓✓✓	✓✓	2
EGFR mRNA expression	✓	✓✓✓	✓	✓	✓	✓	✓	2
EGFR phosphorylation	✓✓✓	X	✓	✓	✓	✓		2
ERK1/2 phosphorylation	✓✓✓	✓	✓✓✓	✓✓✓	✓✓✓	✓✓✓		2
Cumulus expansion		X	✓	✓✓✓	✓✓✓	✓✓	✓✓	3
Cumulus matrix mRNA expression		X	✓	✓✓✓	✓✓✓	✓✓✓	✓✓	3
Glycolysis		✓	✓✓✓	✓✓✓	✓✓✓	✓✓✓	✓✓✓	3
HBP activity		✓	✓	✓✓✓	✓✓✓	✓✓✓	✓✓✓	3
β-O-linked glycosylation		✓✓✓	✓	✓✓✓	✓✓✓	✓✓✓	✓✓	3
Oocyte mitochondrial activity		✓	✓	✓	✓✓✓	✓✓✓	✓✓✓	3
Blastocyst rates	✓✓✓		✓	✓	✓✓	✓✓✓		2, 4
Blastocyst quality (ICM)	✓✓✓		✓	✓	✓✓✓	✓✓✓		2, 4

The improvement to IVM oocyte developmental competence through cAMP modulation has strong precedence in the literature (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. 2004; Vanhoutte et al. 2009a; Vanhoutte et al. 2009b; Zeng et al. 2013). Prevention of the marked drop in COC cAMP that is characteristic of standard IVM prolongs gap-junctional communication between the oocyte and cumulus cells, thereby prolonging the vital bi-directional exchange of regulatory molecules; this prevents the aberrant spontaneous maturation of the oocyte *in vitro* (Albuz et al. 2010). There are two main approaches by which cAMP modulation of the IVM COC has been achieved. The first is the prevention of the drop in cAMP of COCs through the addition of specific or non-specific phoshphodiesterase (PDE) inhibitors that prevent cAMP degradation (Downs et al. 1986; Nogueira et al. 2003a; Nogueira et al. 2003b; Thomas et al. 2004; Vanhoutte et al. 2009a; Vanhoutte et al. 2009b). The second is IVM culture with agents such as forskolin or dibutyryl cAMP that lead to a large increase in COC cAMP, mimicking the spike in cAMP seen *in vivo* (Albuz et al. 2010; Funahashi et al. 1997; Guixue et al. 2001; Luciano et al. 1999; Rose et al. 2013; Shu et al. 2008; Zeng et al. 2013). Our group has previously developed a novel IVM system that incorporates a short pre-maturation phase that exposes COCs to cAMP modulators that cause a spike in, and maintains, cAMP in the oocyte and cumulus cells, that is then followed by IVM in the presence of FSH (Albuz et al. 2010). This pre-maturation phase attenuates spontaneous oocyte maturation and prolongs 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).

Work in chapter 4 of this thesis sought to adopt a novel approach that combines this prior knowledge of cAMP with the newfound knowledge generated in chapter 2 showing the benefits of the use of EGF-like peptides in place of FSH or EGF during IVM. Indeed, data in this thesis confirm that a cAMP up-regulation during a pre-maturation phase using pharmacological agents forskolin and IBMX improves subsequent IVM oocyte developmental competence (evidenced by increased blastocyst inner cell mass size) regardless of IVM conditions. Moreover, I have shown that a pre-maturation phase in conjunction with IVM in the presence of EGF-like peptides, particularly epiregulin in the mouse, endows even greater oocyte developmental competence as it leads to increased embryo yield and/or quality relative to FSH and EGF. This novel approach may represent a more physiological IVM system as it likely mimics key *in vivo* signalling events more

closely. Data in this thesis has shown that a minimum of two hours is required to observe a significant difference in oocyte developmental competence in the mouse, however the ideal duration of pre-maturation requires further optimization as this study has only investigated the effect of relatively short pre-maturation durations (up to four hours) and longer pre-maturation periods might prove to be beneficial.

Chapters 2 and 4 compared the effects of EGF-like peptides, EGF, and FSH on ERK1/2 activity as it is the classical and most studied EGFR signalling effector. Surprisingly, no difference in ERK/12 activity was observed in response to these stimuli. I suggested that these stimuli differentially regulate other known EGFR activated pathways. EGFR ligands have distinct binding specificities and affinities, and depending on ligand interaction, EGFR activation can initiate several signal transduction pathways, including the PI3K-AKT, PAK-JNKK-JNK, and Src pathways (Mendoza et al. 2011; Oda et al. 2005; Yarden and Sliwkowski 2001). Further analysis of alternative EGFR activated pathways may elucidate a mechanism by which EGF and the EGF-like peptides differentially regulate EGFR signalling. In support of this, a new study by Chen et al. (2013) has shed some light into the mechanism by which EGF-like peptides in the cumulus cells elicit oocyte maturation; they have shown, both *in vitro* and *in vivo*, that amphiregulin signalling regulates the translation of a subset of mRNAs within the mouse oocyte via the PI3K-AKT-mTOR signalling pathway, and perturbation of amphiregulin-mediated translation impacts fecundity. Hence, it appears imperative that future work into EGF-like peptide signalling within follicular cells investigates EGFR downstream effectors other than ERK1/2.

In conclusion, the work in this thesis indicates that EGF-like peptides are more appropriate IVM additives than EGF or FSH, and highlights that further investigation into their safety, efficacy, and combination in IVM systems is warranted before clinical application. The use of exogenous EGF-like peptides ameliorates their deficiency in IVM cumulus cell, improves oocyte metabolic parameters, and significantly increases oocyte developmental competence relative to EGF and/or FSH. Such an approach may represent a more physiological IVM system as it likely mimics key *in vivo* signalling events more closely than existing IVM clinical approaches.

The application of the outcomes of this thesis will have relevance to both the human clinical and livestock breeding ART sectors, as both would benefit greatly from an increase

in IVM success. The livestock industry has incorporated, and become highly dependent on, IVM technologies for breeding and reproductive cloning. In a clinical setting, increased efficiency of IVM will make it a viable tool for a wide range of patients, and will bring substantial cost and health benefits and simplify fertility treatment.

5.2 REFERENCES

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CHAPTER SIX

APPENDICIES

6.1 APPENDIX 1- Published format of Chapter 2

Richani D, Ritter LJ, Thompson JG, Gilchrist RB. 2013. Mode of oocyte maturation affects EGF-like peptide function and oocyte competence. Mol Hum Reprod 19(8):500-509.

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6.2 APPENDIX 2- Published format of Chapter 3

Richani D, Sutton-McDowall ML, Frank LA, Gilchrist RB, Thompson JG. 2014. Effect of Epidermal Growth Factor-Like Peptides on the Metabolism of In Vitro Matured Mouse Oocytes and Cumulus Cells. Biology of reproduction 90(3):49.

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6.3 APPENDIX 3- Published format of Chapter 4

Richani D, Wang X, Zeng H, Smitz JE, Thompson JG, Gilchrist RB. 2014. Pre-maturation with cAMP modulators in conjunction with EGF-like peptides during in vitro maturation enhances mouse oocyte developmental competence. Molecular Reproduction and Development 81(5):422-435.

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6.4 APPENDIX 4- Somatic Guidance for the Oocyte

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