Importance of Oocyte to Cumulus Cell Bi-directional Signalling on Oocyte and Subsequent Embryo and Foetal Development and Viability

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ABSTRACT

Oocyte in vitro maturation (IVM) possesses significant scientific and clinical benefits such as the elimination of dangerous side-effects like ovarian hyperstimulation syndrome. Unfortunately, due to a lack of understanding of the intricate processes involved, IVM success rates are low and cannot rival that of current IVF protocols involving hormonal stimulation. Recent scientific advancement has unveiled the existence of the oocyte-cumulus cell (CC) bi-directional regulatory loop and its importance to the development and survival of both cell types during folliculogenesis. This thesis therefore aimed to investigate the significance of these communication axes during IVM on oocyte and CC functions such as cumulus expansion, metabolism and oxidative stress levels and oocyte developmental competence into foetal development.

To target oocyte to CC signalling, growth differentiation factor 9 (GDF9), the primary identified oocyte paracrine factor in the mouse, and its SMAD2/3 signalling pathway were investigated. FSH/EGF were examined as modulators of CC to oocyte signalling, as these can only exert their effects on oocyte maturation through the CCs.

Maturation of mouse cumulus-oocyte complexes (COCs) in the absence of FSH/EGF and/or in the presence of SMAD2/3 inhibition resulted in the ablation of cumulus expansion whereas the addition of exogenous GDF9 to intact COCs significantly increased cumulus expansion. To assess the importance of cumulus expansion independent of oocyte and cumulus communications to embryo development, azaserine, an inhibitor of hyaluronan synthesis was used. Azaserine did not successfully attenuated cumulus

expansion in this system however subsequent blastocyst formation was severely diminished. Cumulus expansion was therefore not indicative of oocyte developmental competence.

Meiotic maturation was only affected by FSH/EGF but both signalling pathways affected sperm penetration. While blastocyst formation was unaffected, disrupted oocyte or cumulus signalling significantly decreased blastocyst inner cell mass (ICM) numbers. Conversely, addition of exogenous GDF9 with FSH/EGF during IVM increased ICM numbers. Significantly, exogenous supplementation of GDF9 during IVM increased foetal survival while inhibition of SMAD2/3 signalling had the opposite effect. Implantation rates and foetal weights were unaffected in both treatments.

The effect of GDF-9 and SMAD 2/3 signalling on metabolism of the COC was examined. The existence of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases (PFKFB) isoforms were discovered in COCs and along with other metabolic gene transcripts, were significantly altered in the absence of FSH/EGF and with exogenous GDF9 and FSH/EGF although SMAD2/3 inhibition had no effect. Glycolytic activity however, was decreased in the absence of FSH/EGF and with SMAD2/3 inhibition but increased with exogenous GDF9. TCA cycle activity was only affected by FSH/EGF. The absence of FSH/EGF, SMAD2/3 inhibition and azaserine during IVM all resulted in increased oxidative stress levels in the oocyte.

The work in this thesis also demonstrated that oocyte and CC signalling are co-dependent on each other as apart from cumulus expansion and slower developmental rates,

perturbations of both signalling pathways simultaneously did not have additive effects on oocyte developmental competence or on CC metabolic functions.

This thesis has therefore provided significance to the field of oocyte IVM through the evidence that oocyte-CC bi-directional communication during IVM is essential to oocyte viability and foetal outcomes.

DECLARATION

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 to Christine Xueling Yeo 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 text.

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* Data presented in Chapter 5 and 6 have published as listed respectively

a. **Yeo CX**, Gilchrist RB, Lane M. Disruption of Bi-directional Oocyte-Cumulus Paracrine Signalling during In-Vitro Maturation Reduces Subsequent Mouse Oocyte Developmental Competence. Biology of Reproduction 2009 May; 80(5):1072-80.
b. **Yeo CX**, Gilchrist RB, Thompson JG, Lane M. Exogenous Growth Differentiation Factor 9 in Oocyte Maturation Media Enhances Subsequent Embryo Development and Fetal Viability in Mice. Human Reproduction 2008 Jan;23(1):67-73.

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GLOSSARY/ ABBREVIATIONS

293H	293 human embryonic kidney cell line
ALK	Activin receptor like kinase
BMP	Bone morphogenetic protein
BMPRII	Bone morphogenetic protein receptor type II
cAMP	Cyclic adenosine monophosphate
CC	Cumulus cell
CEEF	Cumulus expansion enabling factor
COC	Cumulus oocyte complex
DO	Denuded oocyte
EGF	Epidermal growth factor
EMP	Embden-Myerhof pathway
FCS	Foetal calf serum
FGO	Fully grown oocyte
FSH	Follicle stimulating hormone
GC	Granulosa cell
GDF9	Growth differentiation factor 9
GFPT1	Glutamine-fructose-6-phosphate transaminase 1
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
HA	Hyaluronic acid
Has2	Hyaluronan synthase 2
HBP	Hexosamine biosynthesis pathway
ICM	Inner cell mass
IVF	In vitro fertilisation
IVM	In vitro maturation
Ldh	Lactate dehydrogenase
LH	Luteinising hormone
MAPK	Mitogen activated protein kinase
mGC	Mural granulosa cell

MI	Meiosis metaphase I
MII	Meiosis metaphase II
OHSS	Ovarian hyperstimulation syndrome
OOX	Oocytectomised cumulus complex
OSF	Oocyte secreted factor
PDE	Phosphodiesterase
PFKFB	6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase
PFKP	Phosphofructokinase platelet
PGE2	Prostaglandin E_2
PI3K	Phosphatidylinositol 3-kinase
PKA	cAMP dependent protein kinase A
PKC	cAMP dependent protein kinase C
PTGS2	Prostaglandin endoperoxide synthase 2
TCA	Tricarboxylic acid cycle
TE	Trophectoderm
TGFβ	Transforming growth factor beta
TGFBR1	Transforming growth factor beta 1 receptor

PUBLICATIONS AND CONFERENCE

PROCEEDINGS

Referred Journal Articles

Yeo CX, Gilchrist RB, Lane M. Disruption of Bi-directional Oocyte-Cumulus Paracrine Signalling During In-Vitro Maturation Reduces Subsequent Mouse Oocyte Developmental Competence. Biology of Reproduction 2009 May; 80(5):1072-80.

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Yeo CX, Gilchrist RB, Thompson JG, Lane M (2006) 6-Phosphofructo-2-kinase/fructose-2, 6-bisphosphatase (PFKFB) is present in mouse cumulus oocyte complexes and regulated by growth differentiation factor 9 (GDF9). Biology of Reproduction Special Issue 2006 Page 104-104

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C.X. Yeo, R.B. Gilchrist, J.G Thompson, M. Lane (2006) Exogenous Growth Differentiation Factor 9 During In Vitro Maturation of Oocytes Improves Subsequent Embryonic Development and Foetal Outcome. The Society For Reproductive Biology 2006 Annual Scientific Conference Abstract 303

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CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

The acquisition of oocyte developmental competence, defined as the ability of an oocyte to achieve successful fertilisation and develop into a viable embryo [2], depends on a variety of factors. A key regulator is the relationship between the oocyte and its surrounding somatic cells. The dependency of the oocyte on its surrounding follicular somatic cells has long been established however research over the past two decades has revealed that the oocyte also plays a principal regulatory role on the acquisition of its own developmental competence. This led to the relatively recent concept of an oocyte to cumulus bi-directional regulatory loop. The majority of the regulatory properties of this bi-directional loop have however been focussed on events prior to oocyte maturation with little known about the impact of this bi-directional communication during the duration of oocyte maturation (the time from germinal vesicle to mature metaphase II oocyte). Oocytes must complete both nuclear and cytoplasm maturation in order to sustain subsequent embryo development. While success of nuclear maturation can easily be determined by the extrusion of the first polar body, that of cytoplasmic maturation is still loosely defined. This contributes greatly to the decreased developmental competence of oocytes matured in vitro compared to those matured in vivo. Hence, although there are tremendous proposed benefits of in vitro maturation (IVM) both clinically and scientifically, the current low success rate renders the replacement of current in vitro fertilisation (IVF) procedures with IVM unfeasible. This review shall explore the roles of oocyte to cumulus cell bi-directional signalling in the oocyte's acquisition of developmental competence with emphasis placed on the events during oocyte maturation.

1.2 ASSISTED REPRODUCTIVE TECHNOLOGY (ART)

1.2.1 The Demand for ART

Since the birth of the first IVF baby in 1978, both development and demand for assisted reproductive technology (ART) have increased rapidly with treatment cycle numbers rising by 47% from 2002 to 2006 in Australia and New Zealand [3]. With changes in lifestyle, economics and financial choices, the report that 3% of all Australian live births result from ART is unsurprising considering around 15% of all Australian couples are infertile with female factor infertility accounting for 83.2% of these [3]. Alongside increasing demands, ART success rates have also increased over the years, however global success rates are still low, with only 19.6-27.2% of all initiated cycles resulting in live deliveries [3-5]. To counteract low success rates, more than one embryo are often transferred to the mother resulting in multiple gestation pregnancies with a high incidence of preterm birth rates, low birth weight and peri-natal death [3, 4].

1.2.2 Gonadotropins and Hormonal Stimulation Regimes

Humans are a mono-ovulatory species, thus standard IVF procedures routinely involve the use of hormonal stimulation regimes to achieve multiple pre-ovulatory oocytes to increase the number of good quality embryos can be obtained for transfer. In order to override normal hormonal controls, gonadotropin releasing hormone (GnRH) agonists or antagonists are used to suppress the secretion and release of endogenous gonadotropins from the pituitary. Follicle stimulating hormone (FSH) is administered to stimulate multiple follicle growth thereby increasing the number of large ovarian follicles from which fully grown,

developmentally competent oocytes can be obtained. Once multiple large follicles are simultaneously growing, oocyte maturation is then induced by an injection of human chorionic gonadotropin (hCG) approximately 34-36hrs prior to intra-follicular oocyte retrieval [6]. Aside from the high cost of the exogenous gonadotropins, patients frequently have to endure a level of discomfort, inconvenience and disruptions to daily life while undergoing these hormonal stimulation regimes. Frequent blood assays and trans-vaginal ultrasound scans are necessary for determining the patient's response to the ovarian stimulation and the progression of their ovarian follicle growth. These then dictate the type, dose and timing of gonadotropin to be administered. While most of the common side effects reported such as headaches, fatigue, nausea, ovarian tenderness and fluid retention are mild and bearable, the most serious complication of IVF, ovarian hyper stimulation syndrome (OHSS) can be fatal. Up to 33% of all IVF treatments have been reported to be associated with mild forms of OHSS while severe OHSS has been reported in 3-8% of IVF cycles [7].

1.2.3 In Vitro Maturation Of Oocytes

The ability to collect and mature immature germinal vesicle (GV) stage oocytes in vitro would reduce or eliminate the need for hormonal stimulation. Thus poses as an attractive alternative to conventional IVF protocols. This procedure is known as in vitro maturation of oocytes (IVM).

IVM first successfully produced live births in 1991 from donated immature oocytes retrieved from whole ovaries [8]. Shortly after, a method utilising trans-vaginal ultrasound guided follicular aspiration was designed, enabling maturation of a patient's own oocytes

[9] providing a safer option for patients susceptible to OHSS such as those with polycystic ovary syndrome (PCOS). Female fertility decreases sharply after the early to mid 30s [10] yet current trends have shown increasing average maternal ages with a 2% increase in the proportion of women over 40 seeking infertility treatment setting the mean age of all women undergoing ART at 35.6 years [3]. Successful IVM creates the possibility for the preservation of female fertility through the storage of immature oocytes to be matured and fertilised at a later stage in a woman's life. Furthermore, for cancer patients, cryopreservation of immature oocytes without the need for hormonal stimulation will not only negate the delay in chemotherapy treatment due to the long completion time of ovarian stimulation regimes but also eliminate potential complications in patients with certain cancers such as oestrogen-receptor positive breast cancer [11].

Unfortunately while there have been approximately 300 live births resulting from IVM [12], IVM success rate are still substantially lower than those following conventional IVF utilising in vivo matured oocytes [11, 13]. Not only are fewer morphologically good quality embryos available for transfer [14] but fertilisation, implantation and pregnancy rates of embryos derived from IVM are retarded compared to in vivo matured oocytes [13, 14]. With a pregnancy rate in non-PCOS patients of less than 25% per embryo transferred [15-17], IVM has yet to become routine clinical practice.

1.3 OOCYTE DEVELOPMENT

1.3.1 Folliculogenesis

The compromised developmental competence of IVM is largely attributed to the lack of knowledge of and hence ability to provide for the oocyte's intrinsic requirements during maturation. Unlike male spermatogenesis, mitotic proliferation of female primordial germ cells occur only as they migrate towards the gonadal ridges of the mesonephros of the developing embryo, hence females are born with a finite number of oocytes [18].

Meiosis is initiated at 11-12 weeks of gestation in the human foetal ovary up until meiosis prophase I at which oocytes meiotically arrest concurrently in association with around three to five pre-granulosa somatic cells forming a primordial follicle surrounded by a basement membrane [19]. This is possibly the first functional interaction between the oocyte and the ovarian somatic cells since oocytes not surrounded by pre-granulosa cells continue meiosis eventuating in cell death [20]. This process has also been shown to be governed by the germ cell specific ligand, folliculogenesis specific basic helix-loop-helix (FIGLA) as mice null for FIGLA cannot form primordial follicles [21]. Within the first week of birth and until the end of fertility a cohort of primordial follicles leave the resting pool and begin their journey into follicle growth. The signals initiating this transition are currently ill defined although the involvement of the somatic cell derived anti-mullerian hormone (AMH) has been implicated [22]. Pregranulosa cells transition from a squamous to cuboidal morphology and are now known as granulosa cells. They proliferate and associate with the oocyte to form a type 3a follicle of no more than 20 granulosa cells. By the 3b follicle stage the oocyte is surrounded by a complete single layer of more than twenty granulosa cells.

Oocytes also develop a glycoprotein coat known as the zona pellucida and are now connected to granulosa cells by cytoplasmic projections that transverse the zona pellucida to form specialised connections with the oolemma known as gap junctions [23]. Type 3a and 3b are classed as primary follicles [24].

Oocyte and granulosa cell interactions are critical to the transition of primodial to primary follicles. The phophatidylinositol 3 kinase (PI3K) pathway, which is activated upon the binding of granulosa cell specific Kit ligand to the oocyte expressed c-Kit receptor [25] and its down stream components such as forkhead box O3 (Foxo3) are imperative to normal primary follicle formation [26]. In the absence of Foxo3, global activation of primodial follicles occurs resulting in oocyte depletion and preamature ovarian failure in *Foxo3* deficient mice [27]. Ablation of oocyte specific phosphatase and tensin homolog (Pten), a negative regulator of the PI3K pathway [28], also results in premature ovarian failure due to abnormal primodial follicle activation [29]. Other factors such as Sohlh1 have also been identified to be involved in primodial follicle activation as *Sohlh1* null female mice are completely void of oocytes by 7 weeks of age from defective primodial to primary follicle transitions [30].

Follicles then progress into the secondary preantral stage (type 4 to 5b follicle), which is characterized morphologically by two or more layers of granulosa cells and the formation of an external layer of ovarian somatic cells known as the thecal layer which is separated from the granulosa cells by a basal lamina. It is also at this stage that the granulosa cells initiate expression of high-affinity receptors for FSH [31, 32]. Type 5b preantral follicles either undergo atresia or progress to the early antral stage which begins with the

accumulation of scattered fluid filled spaces between the granulosa cells. Once the oocyte has completed preantral follicle development, it is fully grown and capable of meiotic resumption [33].

Early antral follicular development marks the responsiveness and dependency of the follicle on extra-ovarian regulation governed by the hypothalamus-pituitary-gonadal axis [33]. FSH is secreted by the pituitary as a result of a low GnRH pulse from the hypothalamus. FSH promotes ovarian follicle growth, granulosa cell proliferation, LH receptor expression and expression of cytochrome P450 aromatase, which catalyses the conversion of androgens to oestrogen [34]. Once in the antral stages, follicles undergo rapid growth to eventuate into a preovulatory follicle containing two distinct populations of granulosa cells, mural granulosa cells (mGCs) which line the follicular wall and cumulus cells (CCs) which surround the now fully grown oocyte forming the cumulus oocyte complex (COC).

As follicle size increases, so does the level of oestrogen which intensifies the GnRH pulse frequency and sensitivity of the anterior pituitary to GnRH. This ultimately results in the ovulatory LH surge, which marks the start of a series of events resulting in the release of a cumulus oocyte complex consisting of a fully matured oocyte capable of fertilisation and development into an embryo.

In the mouse, only primordial follicles are present at birth. Follicle development commences shortly after birth and at postnatal day 10-12, the mouse ovary consists of various sized secondary follicles consisting of meiotically incompetent mid-growth stage oocytes with one or more complete layers of granulosa cells. Antral follicle formation

commences around day 14 and by age 18-24 days, fully grown meiotically competent oocytes are found residing in antral follicles at various stages of antrum formation [24, 35]. A schematic representation of folliculogenesis and follicle classifications is shown in Figure 1.1.

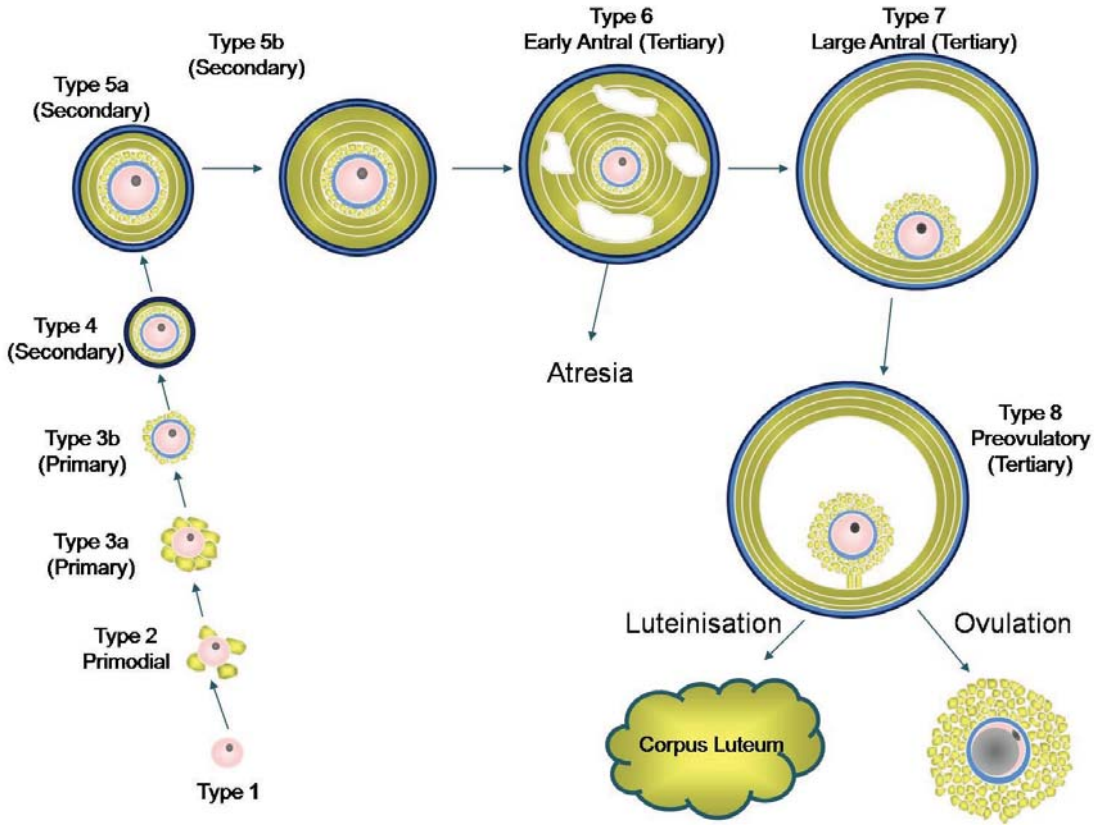


Figure 1.1. Schematic representation of folliculogenesis. At the Type 1 stage, oocytes are not yet associated with follicular somatic cells. Primordial follicles (Type 2) mark the start of oocyte and granulosa cell association. Once the oocyte is surrounded by a complete layer of cells it is known as a primary Type 3a follicle. The follicle gets classed as a Type 3b when there are more than 20 cells in a single layer around the oocyte. The oocyte also develops the zona pellucida at this stage. The formation of a second layer of granulosa cells and the formation of the thecal layer marks the progression of the follicle into the secondary stage (Type 4). Additional layers of granulosa cells appear as the follicle transitions into the Type 5a stage. At the Type 5b stage, oocytes are fully grown and the follicle either undergoes atresia or progresses to the tertiary antral stage. Once in the antral stages the oocyte becomes meiotically competent and fluid filled cavities start to develop between the granulosa cells at the Type 6 stage until a single cavity is formed at the Type 7 stage. At the type 8 preovulatory stage the follicle is fully grown and consists of two distinct populations of granulosa cells, the mural granulosa cells which line the follicular wall and the cumulus cells which are most closely associated with the oocyte. The two populations are connected by a distinct cumulus stalk. Upon the ovulatory signal, the oocyte and its cumulus cells are released from the follicle and the remainder of the follicle undergoes luteinisation to result in a corpus luteum. (Adapted and modified from [24, 33])

1.3.2 Oocyte Maturation

In order to achieve successful fertilisation and support subsequent embryo formation, oocytes must undergo both nuclear and cytoplasmic maturation processes. Oocytes are maintained in dictyate arrest at meiosis prophase I throughout folliculogenesis until the initiation of ovulation. However oocytes only acquire the ability to resume meiosis when they are fully grown at the antral stage. Prior to receiving ovulatory signals, CCs are connected to each other and to the oocyte by gap junctions which are composed of connexin proteins [36]. Connexin 43 makes up the majority of the gap junctions between follicular somatic cells [37-39] while connexin 37 comprises the oocyte-somatic cell gap junctions [37, 40]. These gap junctions extend from cellular projections of the corona radiata (layer of CCs closest to the oocyte) and transverse the oocyte's zona pellucida into the oolemma to allow the transport of small molecules like cyclic adenosine 3',5'-monophosphate (cAMP), metabolites and amino acids from the CCs to the oocyte [41, 42].

The regulation of cAMP levels plays a key role in oocyte maturation. Oocytes are maintained in meiotic arrest via elevated intracellular cAMP levels [43]. This is achieved by the constitutive activity of oocyte G-protein coupled receptor 3 (GPR3) which activates adenylate cyclase leading to the production of cAMP [44]. This works in conjunction with decreased oocyte phosphodiesterase activity (PDE), enzymes responsible for the degradation of cAMP. cAMP and other meiosis inhibiting factors such as hypoxanthine, a purine found in follicular fluid [45], are also transported by the CCs across gap junctions into the oocyte [46]. In contrast to decreased oocyte cAMP in response to the ovulatory LH signal, cAMP levels are risen in granulosa and CCs [34], processes fundamental for the activation of several key ovulatory genes [47]. This delicate balance of cAMP regulation is

likely achieved through the regulation of PDE activity as the oocyte and follicular somatic cells possess varied PDE isoforms. PDE4D has been found to be up-regulated in granulosa cells in response to LH and inhibition of the oocyte specific PDE3 results in meiotic arrest regardless of the addition of the meiotic inducers FSH or EGF [48, 49].

The regulation of gap junctional communication also plays a fundamental role in oocyte meitotic resumption. Mural granulosa cells are thought to transfer small molecules to the oocyte such as cGMP, which inhibit oocyte cAMP degradation, and hence maintain the oocyte in meiotic arrest [50]. Upon receiving the LH signal, MAP kinase-dependent phosphorylation of connexin 43 occurs, leading to a rapid but transient closure of gap junctions between cumulus and granulosa cells but not between the oocyte and CCs. This was shown to occur within 0.5 to 2hrs after LH exposure in intact mouse follicle cultures, immediately preceeding germinal veshicle breakdown (GVBD) [51].

The importance of gap junction communication to oocyte meiotic resumption has also been confirmed by in vivo studies. Mice lacking connexin 37 are anovulatory with oocytes that cannot resume meiosis [40]. Similarly, mice null for connexin 43, which is indispensible for gap junction communication between granulosa and CCs, have preantral stage arrested follicles with oocytes that cannot undergo meiotic maturation in vitro [52]. Furthermore, connexin 43 null oocytes when grafted to wild type granulosa cells are able to successfully complete meiotic maturation indicating normal developmental competence of these oocytes [53]. Hence gap junctional communications both within the layers of cumulus and granulosa cells and between the CCs and the oocyte have pivotal roles to the acquisition of meiotic competence and meiotic resumption of the oocyte.

As germinal vesicle fully grown oocytes (FGOs) can spontaneously resume meiosis once removed from the suppressive effects of the follicular environment [54], the reduction of gap junction communication is sufficient to induce oocyte meiotic resumption. However other mechanisms of meiotic resumption are also known to function in parallel since GVBD still occurs in the presence of LH despite inhibition of gap junction closure [51]. It is also important to note that the development of these spontaneously matured oocytes are significantly compromised compared to ligand induced maturation by FSH and/or EGF [55]. Spontaneous maturation can be prevented by altering cAMP levels in vitro through the use of phosphodiesterase inhibitors such as hypoxanthine and milirone [45] or adenylate cyclase activators such as forskolin [56] and FSH and EGF are both able to override these meiotic inhibitors [57]. As the use of FSH has been shown to increase the time of functional gap junction coupling [58], it is possible this persistence in gap junction communication allows sufficient time for the oocyte to acquire compounds necessary for cytoplasmic maturation from the CCs compared to spontaneous oocyte maturation models.

1.4 THE ROLE OF CUMULUS CELLS ON THE ACQUISITION OF OOCYTE DEVELOPMENTAL COMPETENCE

1.4.1 Cumulus Expansion

In response to the ovulatory signals, CCs produce a viscoelastic matrix that results in a characteristic spherical volumetric increase of the COC. This process is known as cumulus expansion and is closely associated with the breakdown of gap junction communication and the resumption of meiosis.

1.4.1.1 Signalling mechanisms involved in cumulus expansion

The formation of the COC matrix involves the induction of multiple intracellular signalling pathways within the cumulus and mGCs. In vivo, upon binding to its G-protein-coupled receptor in mGCs, LH activates adenylate cyclase which results in an increase in cAMP and activation of the cAMP-dependent protein kinase A (PKA) [34] which then leads to mitogen activated protein kinase 3/1 (MAPK3/1) also known as the extracellular regulated kinase 1/2 (Erk1/2) activation [59, 60]. While it had been known that cumulus expansion and meiosis required an intracellular rise in cAMP in the CCs and a decrease in the oocyte [48, 61], CCs lack LH receptors and are unresponsive to LH [62, 63]. Hence the mechanism by which LH mediates these effects in vivo remained a mystery until compelling evidence in a landmark study by Park et al., [64]in 2004 revealed the EGF-like growth factors amphiregulin (*Areg*), epiregulin (*Ereg*) and betacellulin (*Btc*) as secondary messengers of the LH signal [64]. These growth factors were shown to be rapidly induced

within 1-3 hours of hCG administration and undetectable prior to 6-12 hours post hCG administration in the mouse. Furthermore, Areg and Ereg mRNA were found localised exclusively in mGCs of mouse preovulatory follicles in vivo and able to induce cumulus expansion and meiotic maturation in whole mouse follicle cultures. These LH mediated effects were found to be further negated by inhibition of the EGF receptor [64]. This study therefore revealed the physiological role of EGF in cumulus expansion and ovulation. LH also activates the MAPK14 (also known as the p38MAPK) pathway independently of PKA [60, 65] and while the exact molecular mechanisms by which these EGF-like factors are produced by granulosa cells in vivo remain unknown, involvement of the MAPK14 pathway and prostaglandin 2 (PGE2) have been suggested [66]. Aside from MAPKs, LH also induces other effectors of cumulus expansion. Prostaglandin E_2 (PGE2) and progesterone are induced in the mGCs by LH and are vital for ovulation since mice null for the rate limiting enzyme of prostaglandin 2 synthesis, prostaglandin-endoperoxide synthase 2 (PTGS2) or the progesterone receptor (PR) are anovulatory with cumulus expansion defects [67-69].

While the mGCs play critical roles in cumulus expansion and meiotic maturation in vivo, cumulus expansion can also be induced in isolated pre-ovulatory COC complexes in vitro by EGF, EGF-like growth factors, FSH, prostaglandins or cAMP analogues such as forskolin in the presence of serum [57, 64, 70, 71]. Similarly to LH, FSH binds to a G-protein coupled receptor to increase cellular cAMP levels resulting in PKA-activated intracellular signalling. This is however dependent on gap junction communication as inhibition of gap junction communication prevents the FSH induced cAMP increase [72]. As illustrated in Figure 1.2, FSH also activates MAPK14 upstream of MAPK3/1 to result in

the expression of critical cumulus expansion genes hyaluronan synthase 2 (*Has2*), *Ptgs2*, tumour necrosis factor alpha induced protein 6 (*Tnfaip6*) and pentraxin 3 (*Ptx3*) and inhibition of either MAPK results in inhibition of cumulus expansion and gene expression.

MAPK3/1 activation by FSH but not MAPK14 is also dependent on oocyte secreted factors [73]. The expression of *Areg*, *Btc* and *Ereg* have been found to be up regulated by FSH in isolated mouse COCs through a *Ptgs2* and EGF receptor dependent pathway [66]. Inhibition of EGF receptor kinase activity also prevents meiotic resumption [74], *Ptgs2* expression and phosphorylation of MAPK14 by both FSH and forskolin [66]. Thus similarly to LH in vivo, the effects of FSH and forskolin in vitro on cumulus expansion appear to be mediated by EGF-like peptides in vitro.

Unlike FSH, EGF signals through a transmembrane tyrosine kinase glycoprotein receptor [75] to activate MAPK 3/1 and MAPK14 independently of one another resulting in the up-regulation of different cumulus expansion related genes (Figure 1.2). Also, inhibition of only MAPK 3/1 and not MAPK14 was found to prevent EGF-mediated cumulus expansion [73]. EGF-like growth factors also appear to affect cumulus expansion through the MAPK3/1 pathway as inhibition of MAPK3/1 negated AREG induction of *Ptgs2* [66].

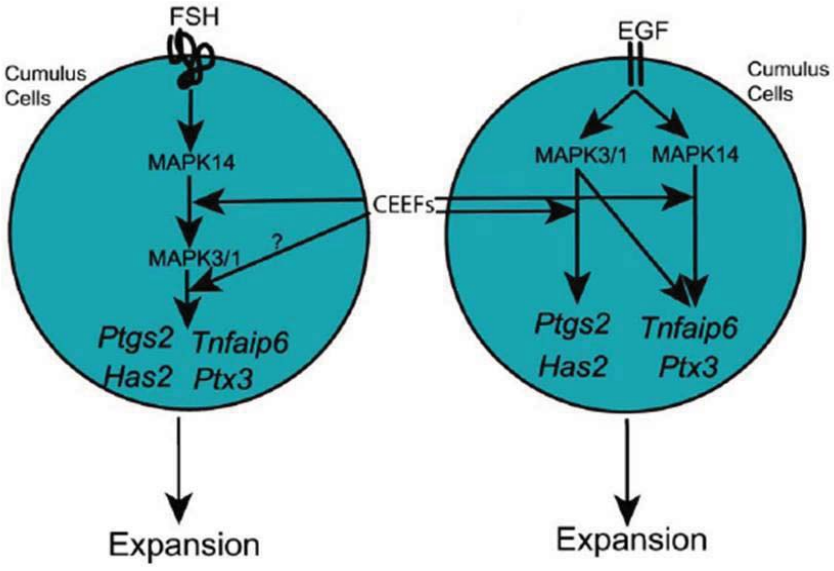


Figure 1.2. FSH and EGF signalling pathways involved in cumulus expansion gene expression. (From [73])

Inductors of cumulus expansion in vivo and in vitro therefore appear be dependent on several different intracellular signalling pathways. These pathways may run parallel to or downstream of one another and also dependent or independently of oocyte secreted factors [66, 73, 76]. The induction of cumulus expansion therefore is a well co-ordinated process dependent on several mechanisms.

1.4.1.2 Prostaglandins

One of the crucial events necessary for cumulus expansion is the presence of prostaglandins, namely PGE2, which is synthesized via transcription and translation of *Ptgs2* by both cumulus and granulosa cells. Prostaglandins are bio-molecules derived from fatty acids stored in cellular membranes and are key mediators of inflammation and ovulation [77]. The rate limiting enzyme of PGE2 production is PTGS2 which is significantly up-regulated in a rapid and transient manner 2-4 hours post hCG [67, 78] similar to that of the EGF-like growth factors [64, 66]. PTGS2 is also up-regulated in CCs

by EGF-like growth factors [64], EGF [79] and FSH by cAMP/PKA activation [78]. Activation of cAMP pharmacologically also results in an increase in PTGS2 expression which is sensitive to MAPK14 and MAPK 3/1 inhibition [70].

PTGS2 is crucial for the stabilisation of the cumulus matrix as mice null for PTGS2 have abnormal cumulus expansion and fail to ovulate. The expression of genes encoding for a key matrix component TNFAIP6 is also significantly decreased in *Ptgs2*$^{-/-}$ mice and mice null for the PGE2 receptor prostaglandin E receptor 2 (PTGER2) [80]. These mice also have abnormal implantation and decidualisation responses. However, infertility of *Ptgs2*$^{-/-}$ mice can be rescued by administration of PGE2 with gonadotropins [68]. Administrations of inhibitors of PTGS2 such as indomethacin block gonadotropin induced follicle rupture and impair or delay ovulation in both animal models and humans [81-84]. Despite the long established importance of PTGS2 to ovulation, little is known about the mechanisms by which PGE2 exerts its effects. It has been suggested that PTGS2 has a feedback autocrine regulation via the MAPK14 pathway on EGF-like growth factors as *Ptgs2*$^{-/-}$ mice have decreased *Areg* expression and inhibition of EGF receptor signalling can be rescued by PGE2 administration on *Areg* and *Has2* expression [66]. PGE2 may therefore act synergistically with LH or FSH to activate MAPK14 and MAPK 3/1 and sustain EGF-like growth factor levels necessary for inducing cumulus expansion genes and other ovulatory events.

1.4.1.3 Cumulus matrix composition

The cumulus matrix consists of a meshwork of proteins that are derived from a various number of sources including local production in the CCs and more distal production in

mGCs and serum. These include TNFAIP6, PTX3, the heavy chains of inter-α-trypsin inhibitor (IαI) and a large proteoglycan thought to be versican [85] (Illustrated in Figure 1.3). Each of these components play a pivotal role in the cumulus matrix construction as the absence of any one of these results in an inhibition of or deformed cumulus expansion resulting in sub or in-fertility [86-89]. The organisational structure of the cumulus matrix and the interactions between its components however, are complex and is still an intense area of research.

The scaffold of the cumulus matrix is made out of hyaluronan, a glycosaminoglycan composed of repeated disaccharides of glucuronic acid and N-acetyl-glucosamine. Hyaluronan is rapidly synthesized by CCs after the LH surge in vivo and is readily detectable around 4 hours after FSH stimulation in vitro [90]. Hyaluronan synthesis is predominantly regulated by transcription of *Has2* which codes for the enzyme responsible for polymerizing and elongating hyaluronan into the intercellular space [91]. *Has2* mRNA is absent in CCs prior to the LH surge but is rapidly induced within 1 hour of the ovulatory signal then down regulated prior to the end of hyaluronan synthesis [92]. While the exact roles of hyaluronan in the COC matrix are still poorly defined, hyaluronan has been shown to directly influence intracellular signalling upon binding to its receptor CD44 in tumour cells leading to anchorage-independent growth and cell survival [93, 94]. CD44 is also synthesized by CCs simultaneously with hyaluronan prior to ovulation by CCs [95-97] and is suggested to anchor hyaluronan to the cell surface [97]. However, unlike inhibition of hyaluronan synthesis which results in obliteration of cumulus matrix formation [88], CD44 deficient mice are fertile [98] which suggests that hyaluronan has other receptors on CCs or molecular binding mechanisms enabling unobstructed formation of the cumulus matrix.

Most of the known components of the COC matrix exhibit hyaluronan binding properties and are crucial for stabilisation of the matrix. IαI molecules are synthesized in the liver and found abundantly in blood circulation. They consist of a core protein known as bikunin which is connected via ester bonds to proteins known as heavy chains (HC) through a chondroitin sulphate chain [99]. Upon permeabilisation of the blood-follicle barrier after the LH surge, serum IαI enters the preovulatory follicle and its HCs are transferred to hyaluronan through a transesterification process and reformation of ester bonds [100]. This process is crucially catalysed by TNFAIP6 which functions as an intermediate via binding to the IαI chondroitin sulphate chain and displacing the HC protein [101-103]. TNFAIP6 and bikunin null mice have similar phenotypes consisting of unaffected hyaluronan synthesis but an unorganised array of dispersed CCs [87, 89]. TNFAIP6 also binds PTX3, an anti-inflammatory protein that does not possess a hyaluronan binding site but localises intensely to the COC matrix and mice lacking PTX3 are also severely subfertile [104]. TNFAIP6 and PTX3 are synthesized by CCs in the same rapid and temporal pattern as hyaluronan [92, 104]. Hence IαI, TNFAIP6 and PTX3 may serve to cross link the hyaluronan COC matrix thus stabilising it.

While proteins crucial for the morphological representation of cumulus expansion are synthesised by CCs or found in serum, mGCs have been shown to exclusively produce matrix proteins such as versican [105]. Versican is a large aggregating proteoglycan which has dual link-protein moieties in its C-terminal region that bind hyaluronan [106]. This C-terminal property also allows versican to interact directly with the link module of CD44 [107] and other cell surface proteins such as integrins [108]. As mice with a genetic

deletion of versican are embryonic lethal [109], it has yet to be concluded if versican is obligatory for COC expansion or fertility in vivo.

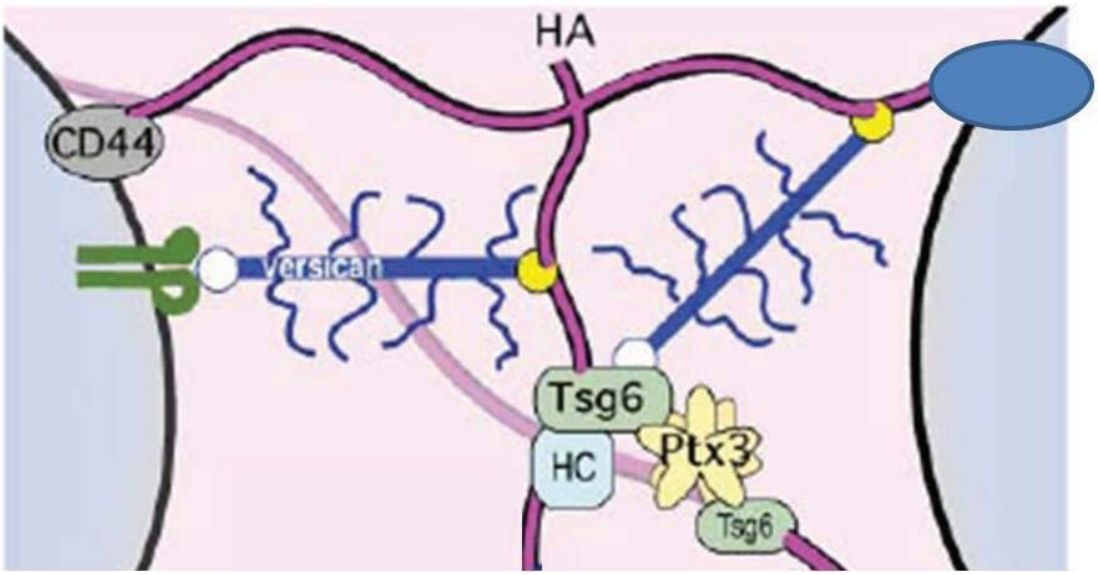


Figure 1.3. **Schematic representation of the molecular structure of the cumulus matrix**. Hyaluronan (HA) forms the backbone of the cumulus matrix and is bound to the surface of CCs by receptors such as CD44. Versican, TNFAIP6 and the heavy chains (HC) of IαI all bind to HA and along with Ptx3 serve to stabilise the matrix. Versican may also interact directly with the cumulus cell surface proteins to further stabilise the matrix. (Adapted and modified from [47]).

1.4.1.4 Functions of the cumulus matrix

The role of cumulus expansion to female fertility in vivo is obligatory given the sterility or severe sub-fertility of mice with deformed cumulus matrices [68, 88, 110]. The principal cause of infertility in these disrupted cumulus matrix mice models is the dramatically reduced rate of ovulation and in vivo fertilisation (reviewed in Russell and Robker, 2007 [47]). This is most likely because the polyionic characteristic of hyaluronan results in a highly hydrated matrix with viscoelastic properties that allow for the deformation of the COC enabling passage through the ruptured follicular wall during ovulation.

While the exact mechanistic functions of the cumulus matrix is still an intense topic of research, the observation that many cumulus matrix deficient mice models, develop entrapped oocytes in luteinised follicles [110] suggests that the formation of the cumulus matrix plays a role in the extrusion of the COC complex. To date, the only protease discovered in the cumulus matrix to be essential for follicle rupture is ADAMTS-1 (a disintegrin and metalloproteinase with thrombospondin-like repeats-1) which has been shown to selectively cleave versican [111]. ADAMTS-1 expression is significantly decreased in mice models with defective follicle rupture, such as progesterone knockout (PRKO) mice [112] and ADAMTS-1 null mice have reduced pregnancy rates resulting from the identical phenotype of entrapped oocytes in luteinised follicles [113]. Interestingly, ADAMTS-1 and versican which are both produced by mGCs and selectively localised to in vivo derived expanded COC complexes [111], were virtually undetectable in in vitro expanded cumulus matrices [114]. This suggests that the cumulus matrix is significantly altered in vitro although the components of the matrix which are vital to fertility in vivo may not be essential to embryo formation in vitro. Supportively, only expanded cumulus enclosed oocytes are efficiently transported through the oviductal lumen while denuded oocytes and unexpanded COCs are not [115, 116]. Cumulus expansion is therefore crucial to ovulation as it facilitates the extrusion of the oocyte though the ruptured follicular apex and the attachment of the COC complex to the ciliated infundibular epithelium of the oviduct.

The COC matrix also plays significant roles post ovulation in sperm capacitation and acrosome reaction [117, 118]. While the identities of the exact components mediating these

effects are unknown, it was recently found that incubation with hyaluronan, the main component of the COC matrix, resulted in sperm capacitation and acrosome reaction in a dose-dependent manner. Furthermore these capacitated sperm were able to penetrate and fertilise unexpanded COC complexes [119]. The plasma membrane of capacitated sperm is also rich in the hyaluronidase proteins such as sperm adhesion molecule 1 (SPAM1) in the acrosomal region [120] allowing sperm to burrow through the dense cumulus matrix. While the physiological acrosome reaction is directly co-ordinated by receptor binding at the oocyte zona pellucida [121, 122], interaction of PH20 with hyaluronan resulted in increased sperm basal calcium levels and responsiveness of acrosomal induction after zona pellucida binding [123]. Hence the cumulus may function as a selective barrier for sperm activity and quality. The cumulus matrix has also been suggested to have chemo attractive properties with a study showing the preferential migration of sperm towards media containing COCs [124]. Expanded human COC complexes were also found to contain a sperm attracting gradient with the highest level of attraction at the CCs closest to the oocyte [125]. Hence while the cumulus matrix may serve as a selective barrier, it may also function to increase the number of sperm around the oocyte therefore increasing the rate of fertilisation in vivo. This however, may not be relevant in IVF due to the non-physiological concentrations of sperm used since mice lacking PTX-3 were found to have severely impaired fertilisation in vivo but fertilisation was normal in vitro. PTX-3 is also a sperm binding protein which may serve to attract and secure sperm to the COC in the oviduct [104].

1.4.1.5 Cumulus expansion as a measure of oocyte quality

Cumulus expansion in the mouse is dependent on a cumulus enabling factor(s) only secreted by fully grown meiotically competent oocytes [126] hence it is unsurprising that the degree of cumulus expansion was thought to be a measure of oocyte developmental competence. Interestingly, studies in several other species such as the bovine, which do not require the presence of the oocyte for cumulus expansion, have also correlated the degree of cumulus expansion and the level of expression of matrix component transcripts to oocyte developmental outcomes. Increased inhibition of mouse cumulus matrix formation using 6-diazo-5-oxo-L-norleucine (DON), an inhibitor of hyaluronan synthesis, was negatively correlated to fertilisation success [88]. Culture conditions giving the highest percentage of bovine meiotic maturation also gave the highest degree of cumulus expansion [127]. Moreover, recent studies in the human found that nuclear maturation and embryo development were increased in oocytes surrounded by CCs with higher expressions levels of *Ptgs2* and *Has2* in patients undergoing IVF treatment [128, 129].

However, while the degree of cumulus expansion may be reflective of or even directly impact oocyte development in vivo, recent reports have emerged demonstrating the redundancy of cumulus expansion in vitro to oocyte developmental competence. This may be because the most outstanding cause of infertility in mice with defective cumulus expansion is the inability to ovulate or decreased fertilisation rates (reviewed in Russell and Robker, 2007 [47]) rather than a defect in oocyte quality. This would explain why *Ptx3*-/- oocytes have normal embryonic development when fertilised through IVF [104]. Furthermore, while denuded and corona radiate (the inner most layer of CCs) enclosed mouse oocytes are unable to form blastocysts, co-culture of corona radiata enclosed oocytes

with a monolayer of CCs results in complete restoration of developmental potential similar to that of expanded COCs [130].

Hence while CCs may play a significant role in the acquisition of oocyte developmental competence, cumulus expansion itself may not necessarily have a direct influence on oocyte developmental competence. Rather it could be a measure of the level of endocrine and paracrine communication between the follicular somatic cells and the oocyte. Furthermore, culture conditions that promote optimal cumulus expansion such as the inclusion of serum, FSH and EGF also confer improved IVM and subsequent embryo outcomes [55, 131]. Hence the degree of cumulus expansion may be indicative of follicular maturity and oocyte quality.

1.4.2 The Hexosamine Biosynthesis Pathway (HBP)

1.4.2.1 Role of the HBP in cumulus expansion

Cumulus expansion may also be reflective of the nutrient consumption and metabolism of the CCs. Hyaluronan is synthesized from glucose by the hexosamine biosynthesis pathway (HBP) whose rate limiting enzyme is glutamine-fructose-6-phosphate transaminase 1 (GFPT1) [132]. GFPT1 catalyses the first irreversible step in the HBP converting fructose-6-phosphate to glucosamine-6-phosphate and this reaction is dependent on glutamine as a co-factor. Under normal conditions, the HBP accounts for 1-3% of cellular glucose metabolism [133]. GFPT1 has been shown to be inhibited by glutamine analogues such as o-diazoacetyl-L-serine (Azaserine) and DON (reviewed in Milewski et al., 2002 [134]) and the addition of DON results in complete inhibition of cumulus expansion in several species which have been often further correlated to a decrease in oocyte developmental competence [88, 119, 135]. This decrease in embryo development however was, as with any defect in cumulus expansion as previously mentioned, attributed to a decrease in fertilisation rates. There is currently little known about the effect of HBP inhibition and cumulus expansion on embryo development independent of fertilisation rates. However, the finding that addition of hyaluronic acid or heparin to IVF media reverses DON's inhibitory effects on fertilisation and blastocyst formation despite the lack of a cumulus matrix formation supports the idea that the absence of hyaluronan during maturation does not directly impact subsequent embryo formation if successful fertilisation can be achieved [119].

1.4.2.2 Resultant effects of o-linked glycosylation

Alterations in the HBP during IVM of cumulus enclosed oocytes however can directly impact oocyte developmental competence independent of cumulus expansion. The addition of glucosamine, an intermediate substrate of the HBP, resulted in a significant decrease in both bovine and porcine blastocyst formation despite no changes in cumulus expansion or cleavage rates [136]. This is most likely because the HBP results in protein o-linked glycosylation which potentially alters the activity and stability of key regulatory proteins and transcription factors [137]. The formation of other glycoproteins and peptidoglycans aside from hyaluronan may also play significant roles in oocyte development. Indeed increased HBP activity through glucosamine addition has also been correlated with depletion of ATP [138] and inhibition of the pentose phosphate pathway (PPP) leading to the depletion of reduced glutathione and increased oxidative stress [139]. Overstimulation of the HBP through the addition of glucosamine hence potentially disrupts the fine balance in metabolic pathway activity and intracellular signalling in COCs.

Interestingly, the HBP is also responsible for the activation of several key proteins involved in mediating cellular stress responses (reviewed in Chatham et al., 2008 [140]). Inhibition of o-linked glycosylation has been shown to increase sensitivity to stress and reduce cell survival [141, 142] while elevated o-linked glycosylation attenuates the lost of mitochondria membrane potential induce by oxidative stress [143]. Thus HBP activity appears to exist in a fine equilibrium controlled accordingly to environmental conditions with perturbations in either direction adversely affecting cell viability and survival.

The HBP has also been implicated as the mandatory pathway involved in the late pre-implantation embryo's ability to sense and utilise glucose. The nutrient requirements of the pre-implantation embryo changes as it progresses from an oocyte to a blastocyst. Prior to 8-cell formation, oocytes and embryos preferentially utilise pyruvate as their energy source [144], once at the 8-cell stage the embryo must acquire the ability to utilise glucose as its main energy source in order to fuel the high energy Na^+/K^+ ATPases that facilitate compaction [145]. The expression of SLCA3, a high-affinity high-capacity glucose transporter is correlated to this embryonic acquisition of glucose utilisation and ablation of SLCA3 inhibits blastocyst formation [146]. Inhibition of the HBP via azaserine significantly decreased SLCA3 expression and blastocyst formation which was shown to be rescued by the addition of the HBP intermediate substrate glucosamine [147]. The HBP thus appears to have a crucial role in metabolic programming of the early embryo; however whether this effect extends to the oocyte during the window of maturation remains to be investigated.

1.4.3 Follicle Stimulating Hormone And Epidermal Growth Factor

FSH and EGF have been found to have significant roles in vivo to female fertility and have been routinely used for IVM of several species since the late 1980s. Conceivably this is due to the abilities of both FSH and EGF to induce meiotic resumption in the presence of meiotic inhibiting compounds such as hypoxanthine [57] and improve subsequent fertilisation rates and embryonic development in comparison to spontaneously matured oocytes [148-151].

These effects however are strictly mediated by the CCs and their association with the oocyte. Unlike cumulus and granulosa cells that possess abundant levels of FSH receptors, oocytes lack FSH receptors [152-154]. While EGF receptors are present in both the CCs and in the oocyte [155-157], FSH, EGF and EGF-like growth factors have no effect on the developmental competence of oocytes removed from their CCs [57, 74, 158]. Thus the translation of FSH and EGF signals is a key component of CC to oocyte communication during IVM. The importance of FSH and EGF presence both in vivo and in vitro is discussed below.

1.4.3.1 The importance of FSH and EGF to female fertility

FSH is a glycoprotein synthesized by the pituitary gonadotrophs in response to the pulsatile stimulation of the hypothalamic hormone GnRH. It consists of an alpha (α)-subunit which is also common to the pituitary produced LH and thyroid stimulating hormone (TSH) and a unique beta (β)-subunit and requires heterodimerization to function. Mice with targeted

genetic disruptions to the α- and β- subunits and the FSH receptor have been produced and are viable but with severe female infertility [159-161]. Both male and female mice null for the FSH α-subunit (*FSH-α*$^{-/-}$) were infertile, growth restricted, hypothyroid and hypogonadal. While follicle development prior to sexual maturity was indistinguishable to that of the wild type, *FSH-α*$^{-/-}$ females were anovulatory with smaller ovaries that did not contain any antral follicles or corpus luteum [159]. Since these effects cannot be attributed to FSH alone due the absence also of LH and TSH, the specific effects of FSH must be elucidated from FSH β-subunit (*FSH-β*$^{-/-}$) and FSH receptor knockout (FORKO) mice. Notably, *FSH-β*$^{-/-}$ and FORKO female mice exhibit similar follicular defects with significantly smaller ovaries and a block to folliculogenesis prior to antral formation leading to an anovulatory phenotype. While the fertility defects of FORKO were attributed to the absence of oestrogen this is not the case with *FSH-β*$^{-/-}$ hence FSH must also affect folliculogenesis by other mechanisms independent of oestrogen [160, 161]. The ovulatory defects of *FSH-β*$^{-/-}$ mice could also be rescued by administration of FSH through standard superovulation protocols to give viable embryos [160]. Similar findings in the human have also been observed. Mutations in the *FSHβ* gene and in the FSH receptor both resulted in female infertility with a lack of antral follicle formation and ovulation defects [162-164]. While an FSHβ deficient patient achieved a successful pregnancy after ovarian stimulation [162], patients lacking functional FSH receptors had disrupted ovarian cycles and hypergonadotropic ovarian failure [163, 165]. Thus FSH is mandatory to female fertility through its involvement in the transition of ovarian follicles from the preantal to antral stage. This is unsurprising given that ovarian follicles are only responsive and dependent on extraovarian regulation governed by the hypothalamus-pituitary-gonadal axis after early antral follicular development [33]. The ability of FSH administration to rescue *FSH-β*$^{-/-}$

deficiency and produce viable embryos further supports the importance of FSH to antral follicle formation. Indeed, FSH administration has been shown to promote granulosa cell proliferation in vivo [166], up-regulate LH receptors and promote the synthesis of steroid hormones such as cytochrome P450, family 11, subfamily a, polypeptide 1 (CYP11A1) in granulosa cell cultures [167, 168].

EGF, a polypeptide first isolated from mouse submaxillary glands [169], is a member of a large family of closely related proteins that include transforming growth factor alpha (TGFα) and EGF-like growth factors AREG, EREG, BTC and neuregulins [170]. EGF is functional in a wide range of tissues. In the embryo, receptors are highly expressed from the 4-cell stage [171] through to all three germ layers of the developing embryo [172]. Due to its wide expression, mice null for the EGF receptor are embryo or neonatal lethal with impaired epithelial development in several organs [173-175]. Hence the specific effects of EGF absence on fertility cannot be elucidated in vivo.

However, EGF has been shown to increase granulosa cell proliferation [176] and is suggested to play a positive role in preantral to antral follicle growth in several species such as the mouse [177], cow [178], pig [179] and human [180]. EGF has also been shown to be involved in steroidogenesis [181, 182]. Importantly, a landmark finding by Park et al., [64] in 2004 found the EGF-like growth factors AREG, EREG and BTC to be the key mediators of the LH ovulatory signal in vivo [64]. This was further supported by later evidence where in vivo administration of the EGF receptor tyrosine kinase inhibitor AG1478 into rat ovarian bursas abolished the effects of hCG stimulated ovulation resulting in meiotic maturation and ovulation inhibition [183].

1.4.3.2 Cumulus cells as mediators of FSH and EGF functions during IVM

While the effects of FSH and EGF on oocyte maturation have been known for some time, the mechanisms mediating these effects are still an intense area of research. Cumulus cell MAPK activation is mandatory for FSH and EGF induced meiotic resumption [184-187] and MAPK has been shown to be activated by PKA, protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3K) [185, 187, 188]. FSH activates PKA through a cAMP dependent mechanism upon binding to its G-protein coupled receptor [189], protein kinase C (PKC) independently of cAMP [186] and phosphatidylinositol 3-kinase (PI3K) [190, 191]. Both PKA and PKC but not PI3K have been shown to be in involved in MAPK activation and the resumption of meiosis in the mouse [186, 187, 192, 193]. PKA exists as PKAI and PKAII in both the oocyte and the CCs [61], and the activation of PKAI in CCs maintains gap junction communication and the oocyte in dictyate arrest while PKAII activation results in the induction of GVBD [61]. This differential regulation of PKA subtypes therefore potentially allows a window of opportunity for the communication of other factors or activation of additional intracellular pathways implicated in the acquisition of oocyte developmental competence [194]. However, PKA inhibition does not affect the positive effects of FSH on blastocyst development [195]. Also induction of meiotic resumption by EGF however does not appear to be cAMP dependent [72, 196] nor does it cause an initial delay prior to meiotic resumption [57].

Although the mechanisms by which EGF and EGF-like growth factors induce meiotic maturation are still being uncovered, the activity of EGF appears to be mediated through PKC since inhibition of PKC negates EGF induced maturation in the pig [196]. PKC inhibition also negates FSH induction on cumulus cell MAPK activation and oocyte

maturation in vitro [186]. EGF activation of MAPK also requires the PI3K pathway and unlike the mouse, PI3K signalling was fundamental to porcine oocyte meiotic maturation [197]. Importantly, FSH has been shown to phosphorylate the EGF receptor [198] and induce expression of EGF-like growth factors AREG and EREG in COCs in vitro in identical patterns to that induced by LH in whole follicles [64, 66]. Inhibition of the EGF receptor kinase activity using AG1478, also prevents FSH induced meiotic maturation in both the mouse [74] and the pig [198]. However, AG1478 also inhibits pig spontaneous maturation [198] indicating that basal levels of EGF signalling are fundamental to oocyte meiotic resumption.

Hence it is possible that FSH mimics the in vivo functions of LH during IVM through the up regulation of EGF-like growth factors resulting in increased cumulus cell EGF receptor signalling. This along with direct EGF signalling causes an increase in PKC activity, resulting in activated MAPK signalling which eventuates in the resumption of oocyte maturation and improved oocyte developmental competence.

1.4.3.3 FSH and EGF effects on metabolism

FSH and EGF have been found independently to affect CC and/or oocyte metabolism and their metabolic requirements in vitro. FSH induction of meiotic maturation but not spontaneous maturation requires glucose [199, 200]. Mouse cumulus enclosed oocytes were shown to be able to resume meiosis with pyruvate equally in the presence or absence of FSH. However when pyruvate was replaced with glucose, cumulus enclosed oocytes required FSH to commence GVBD. Inhibition of glucose transport or replacement with a non-metabolisable glucose analogue also resulted in decreased oocyte meiotic resumption.

Although meiotic resumption occurs with pyruvate, oocyte viability was found to be higher with glucose as the energy source [199]. This was the first indication that stimulation of CC glycolysis by FSH is necessary for the supply of glucose products needed for oocyte meiotic resumption as well as viability.

The pattern of energy substrate utilisation in individual COCs has also been shown to be affected by FSH and is developmentally linked. In a meiotically uninhibited system, IVM with FSH compared to without FSH resulted in increased glucose uptake and lactate production while pyruvate was produced in a dose dependent manner by oocytes that had completed meiosis [193]. These FSH induced changes in metabolic profile were correlated to the stage of meiotic completion as FSH had no effect on the carbohydrate turnover of oocytes that had not commenced (GV stage) or completed meiosis (metaphase I stage). In the absence of FSH however, metaphase I and II oocytes metabolic profiles were indistinguishable to each other and to oocytes that failed to complete meiotic maturation with FSH. Inhibition of the PI3K pathway attenuated this FSH induced increase of glucose consumption and lactate production but had no effect on meiotic maturation [193] which suggests that the relationship between oocyte development and COC metabolic profile may involve other FSH stimulated pathways.

Detailed analyses utilising radioactive labelled substrates have further revealed the effects of FSH and EGF on the fates of glucose or pyruvate consumed and metabolic pathway activities. In support of the findings above, FSH significantly increased glycolytic and pentose phosphate pathway (PPP) activity but had no effect on the tricarboxylic acid (TCA) cycle over the course of maturation in COCs [201]. It is interesting to note that while FSH

caused the most substantial increase in COC glycolytic activity, inhibition of glycolysis barely affected meiotic resumption [202]. Instead the PPP was the metabolic pathway found to be mandatory for both FSH induced and spontaneous oocyte meiotic maturation in COCs [203]. Furthermore, stimulation of phosphofructokinase, a key regulatory enzyme of glycolysis was suggested not to be dependent on meiotic maturation in the rat [204]. Since glucose utilisation through the TCA cycle was negligible and the amount of glucose consumed by CC glycolysis was approximately 100x that of total glucose oxidation [201], it is possible that the FSH induced glycolytic increase in CCs may be linked to cytoplasmic maturation and hence developmental competence of the oocyte. FSH has also been shown to increase hexokinase activity in CCs [202]

While the effects of EGF alone or in combination with FSH on CC metabolic activity have not yet been analysed, EGF has also been shown to increase phosphofructokinase activity in rat COCs [204] and human preantral ovarian follicles [180]. Bovine oocytes isolated post maturation with EGF were also found to have increased pyruvate metabolism which was correlated to increased cleavage rates post fertilisation [205] compared to controls. Thus it is likely that EGF, like FSH, will have a positive role on CC metabolism. However this remains to be elucidated.

Although FSH and EGF alteration of CC metabolism has been correlated to oocyte nuclear maturation, there is little known about the resultant effects of FSH/EGF induced metabolic alterations during IVM on subsequent embryo development. It is possible that the alterations in metabolic substrate utilisation and pathway activity by FSH and EGF may

alter the microenvironment of the oocyte to not only improve meiotic completion and oocyte viability but also the acquisition of developmental competence.

1.4.3.4 FSH and EGF effects on subsequent embryo development

The independent and combined effects of FSH and EGF during IVM on subsequent embryo and foetal outcomes have been extensively studied, in various models and species. Several studies have investigated the addition of FSH and EGF during IVM on mouse oocyte developmental competence with varied reports on the extent of their effects. FSH has been shown to increase the percentage of mouse metaphase II oocytes in both induced maturation systems [49] and when inhibitors of spontaneous maturation were not used [193]. However in another study, FSH or EGF did not have an effect on first polar body extrusion over the controls but significantly increased the percentage of 2-cell embryos after IVF. These 2-cell embryos were then transferred to pseudo-pregnant mice and while EGF did not have a significant effect on foetal survival, FSH significantly increased the resultant number of foetuses. Implantation rates were not affected with FSH or EGF. As CCs were removed prior to IVF, these findings were attributed solely to the IVM oocyte's ability to support male pronucleus formation during fertilisation and subsequent embryo development independent of the effect of CCs [55]. Conversely, in another study, FSH and/or EGF significantly increased meiotic completion in FSH primed mice but only EGF or FSH with EGF increased 2-cell embryo numbers and blastocyst formation. However FSH and/or EGF did increase blastocyst cell number. Interestingly, aside from an increase in blastocyst cell number, FSH and/or EGF have no significant effect on IVM of COCs retrieved from unprimed mice [206]. The differences observed in these studies are likely due to the tenfold different concentrations of FSH and EGF used since the base IVM media

were similar. Merriman et al., [55] utilised 10μg/ml FSH and 1ng/ml EGF while 100 ng/ml FSH and 10 ng/ml EGF was used by De La Fuente et al., [206]. It is important to note also that high concentrations of FSH during IVM have been suggested to induce chromosomal abnormalities. Excessive concentrations of FSH of over 20 ng/ml results in increased aneuploidy over that of controls and 2 ng/ml FSH [207]. Hence the positive effects of FSH and EGF on oocyte developmental competence appear to be dose dependent where excessive doses can be detrimental.

In the bovine, addition of either FSH or EGF has been shown to increase fertilisation rates and 8-cell or blastocyst formation [195, 208-210]. Furthermore, EGF in combination with low doses of FSH significantly increases bovine blastocyst formation over conditions of FSH or EGF alone [149]. Similar findings were also noted in the pig where despite no significant differences in fertilisation, addition of EGF during IVM significantly enhances development to the blastocyst stage [211]. Combinations of FSH and EGF have also resulted in increased pig oocyte maturation and/or sperm penetration over that of EGF alone but not FSH [151, 212].

The effects of EGF have also been analysed in the human oocyte in both the presence and absence of CCs. When germinal vesicle stage oocytes retrieved from FSH and hCG stimulated patients were matured in vitro, EGF resulted in an increase in metaphase II oocytes only in denuded oocytes and not cumulus enclosed oocytes. However cumulus enclosed oocytes did have had a higher incidence of meiotic completion than denuded oocytes. Successful fertilisation, which was determined by the formation of a second polar body, was also higher in cumulus enclosed oocytes than denuded oocytes. However, IVM

with EGF increased fertilisation in cumulus enclosed oocytes but not denuded oocytes. EGF has also been shown to stimulate GVBD and first polar body formation in a dose dependent manner in human oocytes retrieved from patients that had not been stimulated with gonadotropins [213]

As shown from these studies, the evidence of the positive roles of FSH and EGF on cumulus expansion, meiotic maturation, fertilisation and resultant embryo viability and quality is unequivocal. Unfortunately little is known about the mechanistic physiological downstream effects of FSH and EGF that led to these improved developmental competences. Unravelling of these mechanisms will not only enable a deeper understanding of the in vitro functions of FSH and EGF but aid in determining the optimal concentrations of these factors during IVM.

1.4.4 The Importance Of Cumulus Cell Metabolism To The Oocyte

Oocytes and CCs exhibit significantly different metabolic profiles and acquisition of oocyte nuclear and cytoplasmic maturation are intrinsically linked to metabolism of both germ and somatic cells. Oocytes are highly deficient for several metabolic processes which in contrast are efficiently performed by the CCs. The reliance of the oocyte on its surrounding CCs and the metabolic processes involved in the acquisition of developmental competence are discussed in this section.

1.4.4.1 Provision of nutrients by cumulus cells to the oocyte

The fundamentality of cumulus cell metabolism to the oocyte was first discovered in 1967. Denuded GV-stage mouse oocytes, like pre-implantation mouse embryo development prior to the 8-cell stage [144, 214], cannot complete meiotic maturation with glucose as the only energy source unlike intact COCs [215]. Instead, denuded mouse oocytes were shown to preferentially utilise pyruvate although in COCs meiotic induction with glucose gave the largest percentages of GVBD [199]. The presence of glucose during IVM of intact COC's has also been shown to be important to the acquisition of oocyte developmental competence in several other species besides the mouse such as the cow and rhesus monkey [193, 216, 217].

Since glucose is important to oocyte development, CCs must provide the oocyte with products of glycolysis. Indeed subsequent experiments found that mouse CCs did produce pyruvate in vitro which was subsequently supplied to the oocyte [218, 219]. This supply of pyruvate by CCs as the oocyte's energy source has also been shown to be vital in vivo to

oocyte maturation as mice with defective pyruvate dehydrogenase enzymes are ovulated with incomplete nuclear maturation and gross abnormalities of the meiotic spindle and chromatin. These oocytes fail to develop after fertilisation and defects are even more pronounced with gap junction inhibition [220]. Hence gap junction communications appear to have a physiological role in the supply of pyruvate from CCs to the oocyte.

Bovine oocytes matured in vivo or isolated at the end of IVM with increased glucose metabolism have also been correlated to improved developmental competence [217, 221, 222]. Glucose consumption and lactate production of mouse COCs with oocytes that fertilised were found to be higher than those that did not. Denuded oocytes which failed to form blastocysts were also shown to have significantly lower glucose and lactate turnover which was rescued by co-culture with CCs but not to the levels of COCs. This suggests the role of gap junctions in the CC and the oocyte's metabolism during IVM. Glucose and lactate metabolic profiles also varied over the course of maturation for oocytes with different developmental competence and the effects of CC inclusion were found to be most pronounced at the end of maturation [223].

The metabolic pathways influencing meiotic maturation have been investigated. While glycolytic and PPP activity were found to increase when COCs were stimulated to mature in vivo and in vitro, there were no differences in TCA cycle activity compared to GV stage COCs. Despite their inability to mature with glucose, denuded oocytes were found to be capable of glycolysis, PPP and TCA cycle activity but contributed to less than 10% of COC metabolism. Importantly, the glucose consumed by oocytes and CCs have different fates. While almost all of the glucose consume by CCs is channelled into glycolysis for the

production of pyruvate/lactate, most of the glucose consumed by the oocyte is diverted to the PPP pathway [201]. This supports earlier findings where the PPP was found to be the key metabolic pathway in the oocyte involved in meiotic resumption [202, 203]. Supportively, oocytes have also been found to have higher levels of glucose-6-phosphate dehydrogenase than CCs in the bovine [224] which indicates higher PPP activity in bovine oocytes than CCs.

Aside from glucose metabolism, oocytes are also deficient in amino acid transport systems and are unable to transport amino acids such as L-alanine, L-histidine and L-lysine. However, antral stage CCs are equipped with the cellular machinery necessary to ingest these amino acids and transfer them to the oocyte via gap junctions prior to maturation [225-227]. While there is little known regarding the effects of CC amino acid transfer on oocyte maturation or development, the inclusion of amino acids in culture media has been shown to play substantial roles in meiotic completion of in vitro matured hamster oocytes [228] and mouse embryo culture and subsequent foetal outcomes [229]. Exclusion of amino acids dramatically alters in vitro cultured mouse blastocyst metabolism leading to non-physiological increases in glycolysis and decreased pyruvate oxidation. Inclusion of amino acids subsequently restored the balance between glycolysis and pyruvate oxidation to that of in vivo derived mouse blastocysts [230]. Many amino acids can be converted to intermediates of the TCA cycle [231] and utilisation of alanine inhibits pyruvate kinase which would result in low levels of glycolysis [232]. Hence the transfer of amino acids from CCs might play a key role in regulating the oocyte's intracellular metabolic balance, a necessity for meiotic and cytoplasmic maturation.

Oocytes also lack HDL- and LDL-cholesterol receptors and are unable to take up carrier-borne cholesterol [233, 234]. In addition, oocytes have compromised cholesterol synthesis systems and instead rely on CCs for the provision of synthesised cholesterol from acetate [235]. Embryos do not acquire the ability to synthesise cholesterol until the blastocyst stage [236], yet pharmacological depletion of cholesterol in mouse zygotes prevented development past the 2-4 cell stage [237]. Cholesterol stores are thus needed to support early embryo development and must be acquired by the oocytes from CCs prior to maturation.

While the role of CC metabolism to maturation of the human oocyte has not yet been investigated, human oocytes only express one glucose transporter isoform, solute carrier family 2 (facilitated glucose transporter), member 1 (SLC2A1) whereas human CCs express four [238] and have been shown exhibit a high level of glycolysis in vitro [239]. Human oocytes have also been shown to have low hexokinase activity and hence would be unlikely to utilise glucose efficiently [240].

CC metabolism at the end of maturation therefore potentially serves as a marker of oocyte developmental competence. However, specific CC metabolic pathway activity or gene expressions have not been correlated to subsequent embryo development. Furthermore, analyses of overall substrate utilisation have been limited to the blastocyst stage. Nonetheless, a relationship between CC metabolism and the oocyte meiotic competence has been shown by the above studies.

1.4.5 The Protective Role Of Cumulus Cells Against Oxidative Stress

Reactive oxygen species (ROS) are normal products of aerobic metabolism generated from the reduction of oxygen molecules in the intermediate steps of various metabolic processes such as oxidative phosphorylation [241]. All cells including oocytes are equipped with various antioxidant defence mechanisms including non-enzymatic antioxidants such as glutathione and enzymes such as glutathione peroxidise and superoxide dismutases [242, 243] which enable cells to manage the their ROS levels.

Excessive ROS levels can cause a variety of cellular damage such as DNA strand breaks, lipid peroxidation, enzyme inactivation, ATP depletion and mitochondrial perturbations. Oxidative stress has also been implicated as a major cause of embryonic developmental arrest and cell death (reviewed in Guerin et al., 2001 [242]). Oxidative stress during meiotic maturation has also been shown to cause chromosomal errors which may affect oocyte developmental competence [244]. However, ROS also have physiologically roles as signalling molecules. EGF has been shown to cause a sudden increase in ROS to promote cell growth [245]. ROS also function as secondary messengers regulating transcription factors such as nuclear factor alpha and hypoxia inducible factor 1 which play substantial roles in oocyte/embryo survival and development [246]. Hence a certain level of ROS may be needed for the acquisition of oocyte developmental competence. In support of this, certain cell-permeant antioxidants were found to inhibit spontaneous maturation of rat oocytes [247] while appropriate levels of ROS production during bovine IVM were associated with increased embryo development [248].

Unfortunately, aside from intracellular ROS, oocytes are subjected to extracellular ROS in their immediate environment both in vivo and in vitro. Follicular fluid contains a variety of ROS and antioxidants [249, 250] while in vitro manipulations of the oocyte inevitably exposes it to non-physiological factors such as atmospheric oxygen and visible light which can induce ROS [251, 252]. Indeed, increased hydrogen peroxide levels were recorded in mouse embryos in vitro compared to those derived in vivo [253].

As oocytes are closely associated with and ovulated with CCs, it is feasible that CCs would exert a protective role for the oocyte against oxidative stress [243, 254, 255]. The dependency of the oocyte on the CCs for metabolic substrates allows a relative quiescent metabolism of the oocyte which would reduce its levels of oxidative stress. Denuded porcine oocytes cultured in an oxidative stress induced system had irreparable DNA damage, decreased GSH levels and increased apoptosis compared to freshly isolated oocytes. Culture of intact COCs in the same system however, had no significant oocyte damage but conversely had increased GSH levels compared to freshly isolated oocytes [254]. GSH expression was also found to be higher in intact bovine COCs and lower in denuded oocytes. Moreover, culture with cysteamine, a precursor of GSH, had no effect on the GSH levels of denuded bovine oocytes but was significantly increased in denuded oocytes co-cultured with intact COCs however, GSH levels were still lower than that of oocytes matured as COCs [256]. Hence CCs may possibly have a paracrine effect on oocyte GSH synthesis but may also be involved in a direct supply of the antioxidant GSH to the oocyte via gap junctions.

Interestingly there have been conflicting reports about the protective role of CCs on oocyte oxidative stress levels. ROS levels were found to be equivalent in oocytes denuded prior to maturation or matured as intact COCs in standard bovine IVM. However, antioxidant enzymes were found to be higher in the CCs than in the oocyte [243]. Similar findings were also reported in the mouse where although GSH levels were increased over that of the GV stage oocytes, oocytes that were matured as intact COCs, denuded oocytes with or co-culture with CCs had similar GSH levels but were significantly lower than in vivo matured oocytes [257]. The reasons for these diverse findings are perplexing given that the same base media and oxygen levels were used in the conflicting reports.

Nonetheless, sub-optimal IVM conditions which alter CC or oocyte metabolism during the course of IVM may directly impact oxidative stress levels and have a resultant effect on subsequent embryo development.

1.5 OOCYTE CONTROL OF THEIR MICROENVIRONMENTS: REGULATION OF FOLLICULAR CELLS

While the dependency of the oocyte on its surrounding follicular cells had long been established, it was only acknowledged decades later that the oocyte is not a passive recipient but a principle regulator of its follicular environment. The roles of the oocyte and studies leading to the discovery of these roles shall be discussed in the following section. The mechanistic approaches utilised by the oocyte shall also be detailed with focus placed on one of the only discovered specific oocyte secreted factor growth differentiated factor 9 (GDF9).

1.5.1 Oocytes Are The Rate Limiting Factor Of Folliculogenesis

The oocyte's role in folliculogenesis begins with the formation of primordial follicles. Despite normal embryonic gonadogenesis and initiation of meiosis I, oocytes lacking the germ cell specific FIGLA fail to associate with somatic pre-granulosa cells to form primordial follicles. These *Figla*$^{-/-}$ oocytes fail to grow and diminish at birth so that FIGLA null female mice are completely void of oocytes by postnatal day 7 and hence infertile [21].

Other oocyte specific factors have also been shown to be involved in the various stages of follicle development. Inhibition of kit ligand-c kit receptor signalling in oocytes results in ovarian follicles arresting just before two layers of granulosa cells can be formed [258].

Mice deficient for the oocyte specific TGFβ superfamily member, growth differentiation factor 9 (GDF9), are infertile with a block in ovarian follicular development at the primary type 3b stage with no thecal cell layer [259].

Perhaps the most unequivocal evidence displaying the oocyte's regulatory property in folliculogenesis were the findings of an elegant experiment involving the re-aggregation of preantral follicular oocytes with the somatic cells of primordial follicles [35]. Isolated oocytes from preantral follicles of 12 day old mice were aggregated with primordial somatic follicular cells from new born age 0 neonatal mice, termed '12/0 ovaries', then surgically implanted beneath the renal capsules of ovariectomised host adult mice and compared with identically treated day 0 oocytes. Large antral follicles were noticed in the 12/0 ovaries but not the 0/0 ovaries in the time frame identical to normal follicle development. When isolated and matured in vitro, COCs from antral follicles of these 12/0 ovaries produced morphologically normal cumulus expansion and comparable rates of 2-cell and blastocyst embryo formation to that of COCs isolated from pre-pubertal 22 day old mice [35]. As oocytes are meiotically incompetent until the antral stage [260] and only CCs and not granulosa cells able to undergo expansion [126], the compartmentalization, differentiation and development of the oocyte and the various layers of follicle somatic cells of these 12/0 grafted ovaries must have been normal. These findings therefore show that the oocyte orchestrates folliculogenesis as the rate of follicular development appears to be evolved around the developmental program unique to the oocyte.

1.5.2 Oocytes Control Granulosa/Cumulus Cell Phenotype, Proliferation And Survival

Granulosa cells differentiate at the antral stage into two subpopulations separated by a fluid filled cavity, the mGCs that line the follicle wall and the CCs most closely associated with the oocyte. Upon ovulation the CCs are extruded out of the follicle with the oocyte while the mGCs remain in the follicle to undergo terminal differentiation into lutein cells of the corpus luteum [152]. mGCs and CCs have distinct gene expression patterns and functions which appear to be interchangeable unless maintained by the oocyte in a gradient pattern [261].

The finding that oocytes control the rate of follicle development was predated by research that demonstrated the oocyte's abilities in stimulating granulosa cell proliferation and differentiation. Vanderhyden et al. [262] discovered that removal of the mouse oocyte from preantral granulosa cells and COCs resulted in a decrease in DNA synthesis. Media conditioned with meiotically competent fully grown oocytes (FGOs) obtained from mouse antral follicles, were subsequently shown to restore levels of DNA synthesis to that of intact complexes and stimulate mural granulosa cell proliferation [262]. Similar results have also been reported in the cow [263], rat [264] and pig [265]. It was later found that the mitogenic property of the oocyte is highly dependent on its stage of development. A detailed study by Gilchrist et al., [266] illustrated the oocyte's degree of mitogenic capability throughout its various stages of development. Oocytes that are meiotically incompetent were found unable to elicit proliferation of preantral or mGCs. The ability of the oocyte to increase granulosa cell DNA synthesis is hence dependent on its ability to resume meiosis with GV stage arrested FGOs isolated from antral follicles being the most

mitogenic. The mitogenic properties of FGOs subsequently diminish as maturation progressed where MII and fertilized oocytes were found to have no mitogenic capacities [266]

The first evidence of the oocyte's role in controlling follicular somatic cell phenotype was shown in the premature luteinisation of rabbit follicles upon removal of the oocyte [267]. Oocytes have subsequently been shown to be responsible for the maintenance of the cumulus cell phenotype [63, 268]. Although cumulus and granulosa cells originate from the same precursors, they possess several key differences. Only CCs and not preantral or mGCs are able to undergo expansion [73, 126]. Although both cumulus and mGCs possess FSH receptors [152] , in vivo only mGCs respond to the FSH induction of LH receptors (LHR) due to the inhibition of luteinizing hormone/choriogonadotropin receptor (*Lhcgr*) expression by the meiotically competent oocyte [63]. Removal of the oocyte results in the expression of mural granulosa cell specific genes such as *Lhcgr*, *Cd34* and *Cyp11a1* in CCs in response to FSH stimulation. Conversely, co-culture with FGOs resulted in the suppression of these genes in mGCs even in the presence of FSH and increased the cumulus cell specific anti mullerian hormone gene *(Amh*). Interestingly, FSH also caused the suppression of the cumulus specific genes androgen receptor (*Ar*) which is involved in the formation of the corpora lutea [269], and solute carrier family 38, member 3 (*Slc38a3*) which encodes for an amino acid transporter, [226] in intact COCs [268]. This suggests that the characteristics of ovarian follicular somatic cells are governed by the opposing concentration gradients of oocyte paracrine factors and external endocrine factors to affect gene expression.

This oocyte paracrine concentration gradient also has an effect on somatic cell apoptosis. Preovulatory bovine COCs were found to have an apoptotic gradient with cells closest to the oocyte having the lowest incidence of apoptosis. Removal of the oocyte resulted in increased apoptosis which was reversed by co-culture with FGOs. The anti-apoptotic properties of the oocyte, which were even potent enough to counteract external apoptotic insults, were achieved through increased anti-apoptotic B-cell CLL/lymphoma 2 (BCL2) proteins and decreased pro-apoptotic BCL2-associated X protein (BAX) proteins in CCs [270].

Thus the oocyte is able to regulate the growth, development and survival of its surrounding somatic cells. This ability however appears entirely dependent on the oocyte's own stage of development and maturation. The oocyte's control of the phenotypic characteristics of somatic cells therefore indicates the oocyte's ability to alter its surrounding environment to meet its own requirements as it progresses through various stages of development.

1.5.3 The Role Of The Oocyte In Cumulus Expansion

Evidence of the oocyte's regulatory properties during the preovulatory stage can be found in its secretion of the cumulus expansion enabling factor (CEEF). Mouse oocytectomised (OOX) complexes, (COCs which have had their oocytes microsurgically removed leaving only the zona pellucida attached to the CCs and hence retention of the COC structure) have been shown by various studies to be unable to respond to gonadotropin or EGF induced cumulus expansion. This was however rescued by co-culture of OOXs with denuded oocytes, oocyte conditioned media or specific factors in a dose dependent manner [73, 271-273]. The CEEFs have also been shown to be mandatory for the expression of several genes involved in cumulus matrix formation such as *Has2*, *Tnfaip6* and *Ptx3* [73, 274].

Expression of the CEEF is dependent on the developmental stage of the oocyte as while fully grown meiotically competent GV arrested, maturing or MII mouse oocytes have detectable amounts of CEEFs, meiotically incompetent growing oocytes do not. The CEEF is also gradually lost upon embryo formation and undetectable from the 2-cell stage onwards [126]. The ability of follicular somatic cells to respond to the CEEF is also developmentally dependent. Preantral granulosa cells are unable to expand despite the up-regulation of *Has2* [73] or when they are grown in culture to the equivalent COC size [126], mGCs also cannot respond to CEEFs [126]. This suggests that association with the oocyte from the preantral to antral formation is indispensable for the acquisition of matrix formation in response to the CEEF. This is further supported by the finding that FSH could only stimulate the MAPK3/1 pathway in cumulus and not preantral granulosa cells even when FGOs were present [73]. Oocytes possibly transport factors through the gap junctions into the CCs which alter their functional characteristics to produce the cumulus cell phenotype.

As detailed previously, MAPK activation in CCs is vital for cumulus expansion and inhibition of MAPK3/1 prevents transcription of *Has2* and *Ptgs2*. The activation of MAPK by FSH however depends on oocyte secreted paracrine factors (OSFs) since OOXs have low FSH induced MAPK activity which is increased upon co-culture with FGOs [76]. Hence it is possible that in CCs, CEEFs mediate their effects by alternating the pathways necessary for the production of cumulus expansion transcripts.

It is important to note however that the mouse appears to be the only species dependent on CEEFs for cumulus expansion. OOXs in all species examined to date, the rat, pig [275, 276] and cow [277] were able to produce morphologically normal cumulus matrices when ligand stimulated. CEEFs are however still produced by these species as oocyte conditioned media induced mouse cumulus expansion [277, 278]. Furthermore like the mouse the expression of these CEEFs appears to be developmentally regulated [279]. Unfortunately to this date, it is still unclear which animal model the human resembles and whether human CCs require CEEFs to expand.

1.5.4 Oocytes Control Cumulus Cell Metabolism

In all the stages of follicle growth up to the initiation of maturation, oocytes are connected to their surrounding granulosa/cumulus cells via gap junctions. These gap junctions facilitate the direct passage of small molecular weight molecules such as metabolites [280]. While the dependency of oocytes on their surrounding somatic cells has been established in the 1960s [215], the regulation of cumulus cell metabolism by the oocyte was only established at the end of the last millennium.

1.5.4.1 Oocyte regulate cumulus cell glucose metabolism

Oocytes were first suggested to be involved in cumulus cell metabolism in the early 1990s when removal of the bovine oocyte negated an LH induced increased in cumulus cell metabolic activity [281].

Subsequently, suppression subtraction hybridisation screening found that several key glycolytic transcripts, including subunits of the rate liming enzyme of glycolysis

phosphofructokinase, phosphofructokinase platelet *(Pfkp*), and that responsible for lactate production, lactate dehydrogenase A (*LdhA*), were up-regulated in CCs of GV arrested oocytes compared to preantral granulosa cells and mGCs. These transcripts were dependent on the presence of the oocyte since OOXs had decreased mRNA levels which were rescued to the same levels as intact COCs when co-cultured with FGOs. Addition of FGOs to mGCs also significantly increased expression of these glycolytic transcripts expression over that of the controls. Gene expression levels of metabolic enzymes are often not true indicators of metabolic activity since their activities are often post-translationally regulated. Metabolic pathway activity was therefore subsequently investigated through the use of radio labelled substrates and glycolytic and TCA cycle activities were found to be significantly higher in intact COCs than OOXs. Similar to gene expression, co-culture of OOXs with FGOs increased these metabolic activities. Furthermore, the ability to control CC metabolism was developmentally acquired. Oocytes isolated from preantral granulosa cells could not stimulate glycolytic or TCA cycle metabolic activity in CCs. FGOs could stimulate an increase in preantral granulosa cell glycolysis but not TCA cycle activity [282]. Hence this suggested a development/differentiation acquired ability of follicle somatic cells to respond to OSFs.

This demonstrates that the co-operative metabolic roles of oocytes and their surrounding somatic cells are developmentally dependent and most likely regulated according to the changing needs of the oocyte as it progresses through the various developmental stages. Oocytes exhibit rapid growth from the preantral to early antral stage [148] hence it is possible that their metabolic requirements would be greatest at this time. Oocytes also undergo dramatic nuclear and cytoplasmic changes during maturation [283], however the

effects of oocyte control on cumulus cell metabolism during maturation in intact COCs and its effect on oocyte developmental competence have not yet be investigated.

There has however been conflicting reports of oocyte control on bovine cumulus cell metabolism. A study measuring glucose, lactate and pyruvate carbohydrate utilisation found no significant difference between COCs and OOXs during oocyte maturation with FSH [284]. This is in direct contrast to the LH-induced increase in glycolytic pathway activity of COCs but not oocyte-void CCs [281]. Measurements of resultant carbohydrate levels in spent media as opposed to the use of radioactive labelled substrates cannot account for the substrate's fate and therefore pathway activity. Hence it is possible that the lack of differences in carbohydrate utilisation may be due to glucose consumption by other metabolic pathways such as the HBP. Further investigations into the effect of oocyte paracrine factors on bovine and other species are hence warranted.

1.5.4.2 Oocyte regulate cumulus cell amino acid transport and cholesterol biosynthesis

Aside from the above glycolytic enzymes mentioned, expression of *Slc38a3* which encodes for a sodium-coupled amino acid transporter with a preference for L-glutamate, L-histidine and L-alanine [285] was also found to be up-regulated in mouse CCs over that of mGCs and preantral granulosa cells. *Slc38a3* expression was not found in the oocyte but appears to be oocyte regulated in vivo as it is expressed in CCs but not preantral GCs or mGCs of antral follicles. Removal of the oocyte resulted in a decrease in *Slc38a3* mRNA in CCs and preantral granulosa cells which was fully restored by co-culture with FGOs but not preantral isolated oocytes. OOXs also consumed less L-alanine, L-histidine and L-leucine than COCs which was increased by co-culture with FGOs but not preantral oocytes. Co-

culture of isolated preantral granulosa cells with FGOs also increased L-alanine uptake but not L-leucine suggesting that granulosa cells respond to OSFs differently depending on their differentiation/development levels. Interestingly unlike glucose metabolism, co-culture with FGOs could not restore amino acid levels in CCs to that of intact COCs suggesting that while paracrine factors play a role, gap junctions are also involved in amino acid metabolic cooperation.

Denuded mouse oocytes have also been found to contain significantly less L-alanine, L-histidine but not L-leucine compared to oocytes from intact COCs [226]. These findings support earlier studies which showed that denuded mouse FGOs are capable of ingesting certain amino acids such as leucine, valine and phenylalanine to the same extent as intact COCs but not several other amino acids including L-alanine, glutamic acid and L-lysine [227, 286]. Differences also exist between amino acids in their pattern of expression as L-alanine is decreased in denuded oocytes from preantral and antral follicles but L-glycine is only decreased in denuded oocytes from preantral follicles. Inhibition of gap junction communication also obscured the differences in L-glycine of preantral granulosa cell-enclosed versus denuded oocytes [227]. Similar to the control of amino acids, almost all transcripts involved in cholesterol biosynthesis are significantly reduced in mouse OOXs compared to intact COCs. Removal of the oocyte also results in decreased cholesterol biosynthesis and co-culture with FGOs rescues cholesterol biosynthesis but not to equivalent levels as COCs [235].

It is obvious from these studies that the mouse oocyte regulates specific amino acid consumption and cholesterol biosynthesis of its coupled granulosa/cumulus cells, although

little is known about the specific mechanisms employed. Unlike glucose metabolism however, these are partially dependent on gap junction communications which may be expected since oocytes also have deficient transporting systems for the amino acids they up-regulate in CCs [225] and do not express receptors for either HDL- or LDL-cholesterol [233, 234].

The amino acid cooperation is also developmentally regulated since the amino acid profiles of preantral granulosa enclosed oocytes differ to that of COCs. It has been found that at least for one amino acid, cycloleucine, uptake in sheep oocytes cultured as intact COCs was higher after maturation [287]. Activity of the PPP is fundamental to oocyte maturation which implicates the importance of amino acids during maturation [203]. Despite the oocytes inability to synthesise cholesterol, cholesterol stores appear essential for pre-implantation embryonic development [237]. Hence the possibility that oocyte-regulated CC provision of amino acids and cholesterol are fundamental processes in the acquisition of developmental competence. Knowledge of the extent of this amino acid as well as cholesterol cooperation during oocyte maturation would provide valuable insight into the oocyte's metabolic kinetics and assist in the development of oocyte maturation media.

1.6 OOCYTE PARACRINE FACTOR (GDF9)

It is evident from the effects of oocyte conditioned media in the studies previously mentioned that the oocyte exerts its regulatory properties by secreted paracine factors (OSFs). While the specific identities and signalling mechanisms of these OSFs are still an intense area of research, members of the transforming growth factor beta (TGFβ) family such as growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15 or GDF9B), and the fibroblast growth factor (FGF) family have been identified [288-290] from their specific expression in the oocyte, abilities to mimic the oocyte on granulosa/cumulus cell functions and their importance to female fertility. Two signalling pathways, the SMAD2/3 and SMAD1/5/8 have also been the focus of research on OSFs.

1.6.1 Molecular Characteristics Of GDF9

Like all TGFβ super-family members, GDF9 is expressed as a pre-protein consisting of a signal peptide, a large pro-region and the biologically active c-terminal mature region [291]. Specific functions of the GDF9 pro-region are still being elucidated but have been thought to involve the regulation of the biological activity of the mature region by facilitating proper protein folding [292, 293].Unlike other TGFβ family members however, GDF9 along with BMP15 lack the conserved fourth cysteine residue in their mature region. Hence it is not known if dimerisation is necessary for their actions in vivo [294, 295]. However, both GDF9 and BMP15 have been shown to form non-covalently linked homodimers as well as heterodimers in vitro [296, 297]. Given that GDF9 and BMP15 are co-expressed in the ovary and the reports of synergy between them [298, 299], the formation of both homodimers and heterodimers are also likely in vivo.

1.6.2 GDF9 Signalling: The SMAD2/3 Pathway

TGFβ super-family members classically exert their effects through a receptor complex consisting of two membrane-bound serine-threonine kinases, type I and type II. There are currently seven known type I receptors (formerly known as activin A receptor type II-like kinase (ALK) 1-7) and five type II receptors (TGFBR2, ACVR2A, ACVR2B, BMPR2 and AMHR2). Each TGFβ member signals through a specific type I and type II receptor combination [300-302]. Ligand binding results in the kinase activation of the type II receptor which then phosphorylates the type I receptor leading to activation of the type I receptor's kinase. The activated type I receptor then phosphorylates and activates either the SMAD2/3 or SMAD1/5/8 signalling molecules which then associate with the common SMAD4 and translocates to the nucleus to regulate the transcription of target genes [300, 303].

SMAD2/3 is known to be activated by phosphorylation of the activin A receptor type IB (ACVR1B formerly ALK4) the transforming growth factor beta 1 receptor (TGFBR1 formerly ALK5), or activin A receptor type IC (ACVR1C formerly ALK 7) type I receptors by GDF9, TGFβ1, TGFβ2, TGFβ3, activin A, activin B, nodal and GDF8. Of these GDF9, TGFβ1 TGFβ2, nodal and the subunits of activin A and B have been found in the mouse oocyte [272, 304]. SMAD1/5/8 on the other hand is activated by the BMPs upon phosphorylation of the type I receptors: activin A receptor type 1 (ACVR1 formerly ALK 2), bone morphogenetic protein receptor type 1A (BMPR1A formerly ALK3) or bone morphogenetic protein receptor type 1B (BMPR1B formerly ALK6) and the type II BMPR2 receptor [291] and BMP6 and 15 have been found to be expressed by the oocyte [263, 305]. Hence the SMAD2/3 signalling pathway by TGFβ superfamily members is

commonly referred to as the TGFβ/activin signalling pathway while that of the SMAD1/5/8 is also known as the BMP pathway. (Illustrated in Figure 1.4) It is interesting to note however that unlike the other SMAD2/3 activating members, GDF9 signals through the same type II receptor as the BMPs, BMPR2, and the TGFBR1 type I receptor [306-308]. Members of the TGFβ super-family have also been shown to activate MAPK signalling although the mechanisms governing this are unknown [309]. Specifically, recombinant GDF9 was shown to activate MAPK3/1 signalling independently of FSH in mouse OOXs [76].

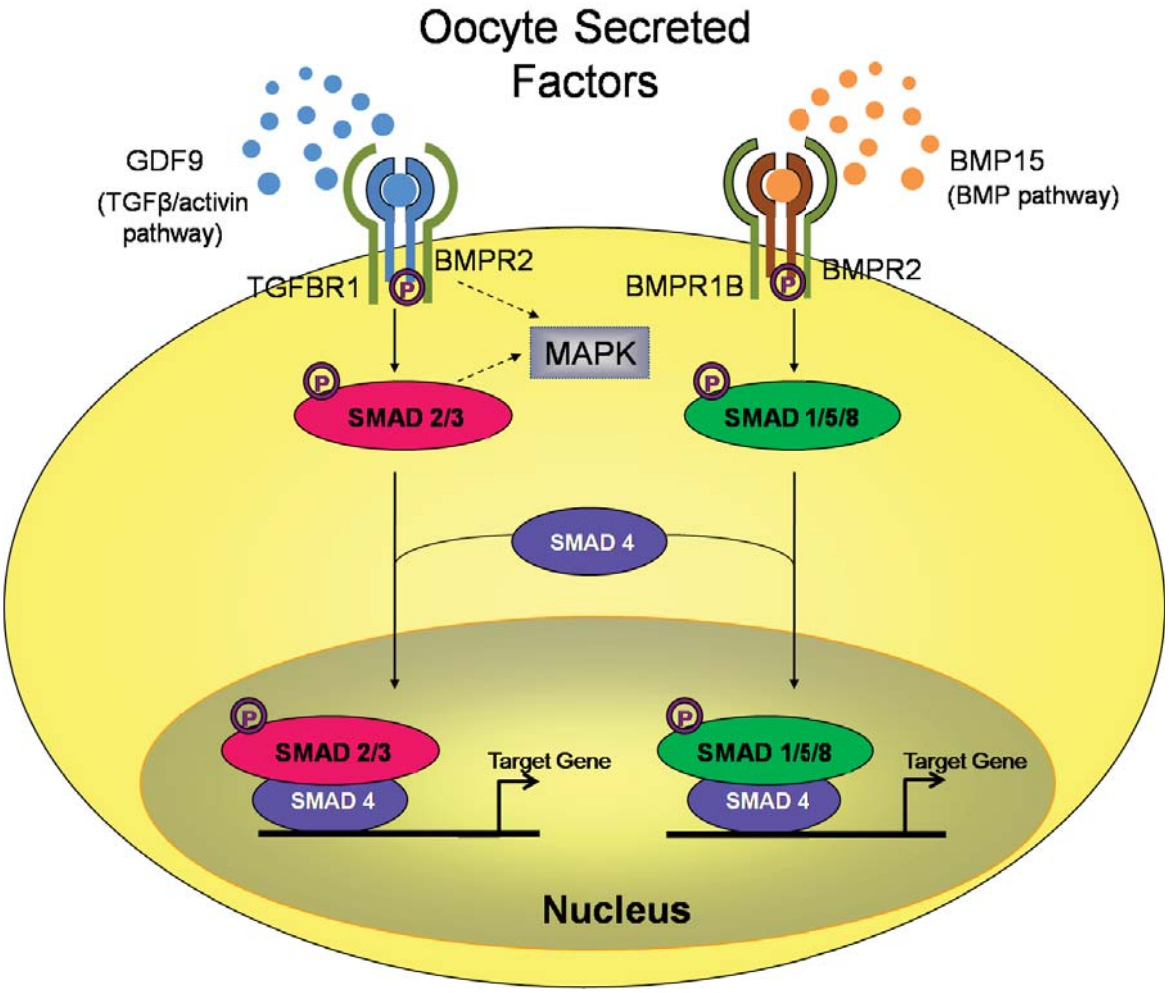


Figure 1.4. **Simplified diagram of the signalling pathways of oocyte secreted factors in a cumulus cell.** Like other ligands that activate the TGFβ/activin pathway, upon GDF9 binding, the BMPR2 is activated resulting in the phosphorylation of the type I TGFBR1 receptor. This in turn phosphorylates SMAD2/3. Similarly, BMP15 which is part of the BMP signalling pathway activates SMAD1/5/8. The activated SMAD2/3 or SMAD1/5/8 then associates with SMAD4 and translocates to the nucleus to induce the transcription of their specific target genes. GDF9 possibly also activates other pathways such as MAPK.

1.6.3 GDF9 Expression Patterns

There are several reasons that have led to the identification of GDF9 as one of the key molecules involved in oocyte paracrine signalling. Mouse GDF9 was the first discovered mammalian oocyte specific growth factor [310] and since then GDF9 mRNA and protein have also been found to be mainly restricted to the oocyte in several other species [311-313]. Although there are reports of GDF9 expression in the cumulus and granulosa cells of the pig and rhesus monkey [314, 315], these were all found to be significantly lower than in the oocyte. GDF9 has also been detected in the pituitary and testis [316, 317] however these extra-ovarian functions of GDF9 have not yet been elucidated. The expression patterns of GDF9 however appear to differ between species. Except for primordial follicles of both neonatal and adult mice, GDF9 mRNA and protein were detected at all stages of oocyte growth and even in fertilised zygotes in the mouse [310, 318]. Expression of GDF9 also initiated at the primary follicle stage of the rat [311]. In contrast, GDF9 transcripts were found in primordial follicles of the sheep [312], cow [319] and possum [313].

Expression of GDF9 is initiated at the primary follicle stage of the human [320]. Reports in its specificity however have been rather varied. GDF9 mRNA was first indicated to be exclusively expressed by the oocyte in the human ovary [320] but was later found also in human CCs although at significantly lower levels than the oocyte [321]. GDF9 protein has also been detected in human follicular fluid and interestingly was also found to be expressed in granulosa-lutein cells of ovulated follicles. Furthermore, this endogenous human granulosa-lutein cell expressed GDF9 was functional with an autocrine effect on the regulation of beta B subunit and inhibin B production. [322]. Still the relative contributions

of this somatic cell expressed GDF9 in relevance to that of the oocyte on normal ovarian function remains to be elucidated.

1.6.4 The Importance Of GDF9 To Female Fertility In Vivo

Despite the differences in expression patterns, GDF9 appears to play a critical role in female fertility to all species studied to date.

1.6.4.1 Mice knockout models

The fundamental role of GDF9 to female fertility has been made apparent from the complete infertility in GDF9 knockout female mice. Male and female heterozygote GDF9 mutant mice however are fertile. While gonadogenesis and primordial follicle formation are unaffected, ovarian follicles in GDF9 null mice fail to develop past the primary Type 3b stage with the maximum of a single layer of granulosa cells surrounding the growing oocytes. Although these Type 3b follicles appear morphologically normal, the granulosa cells lack the ability to proliferate or undergo apoptosis. Instead they remain inactive until the eventual demise of the oocyte where they differentiate into abnormal steroidogenic cells expressing both luteal and non-luteal markers. There is also no evidence of a thecal layer in any of the ovarian follicles despite the presence of thecal precursors in the ovarian interstitium. Corpora lutea is also not observed. At 10-12 weeks of age, GDF9 null oocytes are larger and show significant reduction in meiotic maturation after in vitro culture compared to controls. Ovaries from adult GDF9 null mice also contain multiple ovarian cysts and elevated levels of FSH and LH compared to control mice. GDF9 null female mice are thus hypogonadal and hypergonadotropic [259, 323]. GDF9 null ovaries also have aberrant expression of several genes compared to control mice of the same age. Inhibin beta-B, follistatin and PTGS2 transcripts were either absent or significantly decreased while

the granulosa cell specific kit ligand, which has been shown to stimulate oocyte growth in vitro [324], was drastically elevated [323].

Hence GDF9 fundamentally regulates ovarian gene expression and oocyte-granulosa cell interactions which are fundamental to secondary follicle and thecal formation despite being present in primary follicles. GDF9 thus appears not involved in the initiation of follicle growth or primordial and primary follicle formation despite its presence in primary follicles at least in the mouse [310]. This is further supported by a later study in the rat where in vivo administration of recombinant GDF9 resulted in increased primordial and primary follicle progression and up-regulation of the thecal cell marker cytochrome P450, family 17 subfamily A polypeptide 1 (CYP17A1) [325]

Knockouts for the GDF9 signalling molecules SMAD2 and SMAD3 have also been generated. SMAD2 deletion results in embryonic death [326]. SMAD3 null mice are viable but are sub-fertile with abnormal ovarian function and impaired folliculogenesis [327]. SMAD3 deficiency was also found to slow follicle growth, promote degeneration of the oocyte and atresia and decrease the expression of the anti-apoptotic protein BCL2. Similar to GDF9 deletion, SMAD3 null mice have low oestradiol and high FSH levels and no corpora lutea, and are unresponsive to ovulatory induction protocols [328].

It is interesting to note at this stage that mice null for another oocyte specific factor BMP15 have normal folliculogenesis. They are however subfertile with defects in ovulation and fertilisation. However, mice homozygous for BMP15 and heterozygous for GDF9 mutations exhibit fertility impairment due to defects in cumulus expansion and fertilisation

resulting in smaller and less frequent litters than BMP15 null mice [329]. This suggests that GDF9 may also play a significant role at the preovulatory and periovulatory stages of follicle development.

1.6.4.2 GDF9 mutations in mono-ovulatory species

Similar to the mouse model, sheep homozygous for naturally occurring GDF9 and BMP15 mutations or that have been immunised against these OSFs were found to be infertile with a block at the primary stage of follicle development. However unlike the mouse, heterozygous GDF9 and BMP15 mutant sheep have an increased rate of ovulation which eventuates in a larger number of offspring per pregnancy [330, 331].

Evidence of GDF9's significance to human fertility has also emerged where rare mutations in GDF9 along with BMP15 were found to contribute to premature ovarian failure [332-334]. GDF9 mutations have also been suggested to influence dizygotic twinning [335]. Furthermore, patients with polycystic ovarian syndrome were found to have aberrant expression of GDF9 [336].

1.6.5 GDF9 Is Able To Mimic Oocyte Functions In Vitro

While the exact functions of GDF9 on follicle stages past the primary stages cannot be determined in vivo, the development of recombinant GDF9, specific neutralising antibodies and signalling inhibitors have facilitated investigations into GDF9 functions at the later stages of oocyte development via in vitro culture systems. As such GDF9 was shown repeatedly in several species and independent studies to be able to replicate various oocyte functions as shall be discussed below.

1.6.5.1 GDF9 induces granulosa cell proliferation

The absence of a more than one layer granulosa follicles [259] and the finding that almost all of the granulosa cells are arrested at the G_0 stage of the cell cycle progression in GDF9 null mice suggests that GDF9 regulates granulosa cell proliferation [323]. Indeed, GDF9 conditioned media was found to stimulate follicle formation in cultured perinatal hamster ovaries [337]. Recombinant GDF9 also increases the growth of isolated preantral rat follicles [338] and follicle survival and progression to the secondary stage in cultured sections of human ovarian tissue [339]. These studies however cannot elucidate if GDF9 mediated these follicle growth effects independently or in concert with endogenous GDF9 and other oocyte factors given the presence of the oocyte. Hence the specific effects of GDF9 have to be assessed in isolated granulosa cell cultures.

Through the use of radiolabelled 3[H] thymidine as a measure of DNA synthesis and an isolated mural granulosa cell culture system, it was found that GDF9 and/or TGFβ1, TGFβ2 and activin-A all stimulated mouse and bovine mural granulosa cell proliferation [263, 340]. Neutralisation of only endogenous GDF9 attenuated oocyte mitogen activity, however at the same antibody dose that virtually obliterated granulosa cell proliferation with exogenous GDF9 that with denuded oocytes was only decreased by 15% [340]. This indicated that GDF9 was not the only oocyte mitogenic factor. A subsequent experiment found that BMPR2 the type II receptor for GDF9, BMP15 and BMP6 was the only type II receptor mandatory for oocyte induced mural granulosa as well as cumulus cell proliferation. However, oocyte induced proliferation did not appear to activate SMAD1/5/8. Conversely inhibition of SMAD2/3 with a specific inhibitor SB-431542, reduced mural granulosa/cumulus cell proliferation in a dose dependent manner [341]. Therefore while

GDF9 is not the sole mitogen factor, oocytes control the proliferation of their surrounding somatic cells through paracrine factors that bind to BMPR2 and activate the SMAD2/3 signalling pathway. It was also shown in the pig that GDF9 interacted with androgen to induced follicle growth [265].

GDF9 has also very recently been shown to have similar mitogenic effects in the human. Unfortunately these findings have limitations as the effects of recombinant human GDF9 were tested on luteinising granulosa cells from postovulatory follicles of patients that had been hormonally stimulated for IVF treatment. Nonetheless, exogenous GDF9 was shown to induce proliferation of these luteinising human granulosa cells in culture via the SMAD2/3 pathway and the MAPK (ERK 42/44) pathway which mediated their effects independently via different downstream mechanisms [342].

These studies therefore confirm that the GDF9 signalling pathway SMAD2/3 is mandatory for the oocyte's mitogenic properties and that GDF9 plays a role in proliferation of granulosa and CCs past the primary follicle stage.

1.6.5.2 GDF9 induces differentiation and maintains the cumulus cell phenotype and prevents steroidogenesis

While it can be inferred from the mouse knockout model that GDF9 is required for the transition of the follicle from primary to secondary stage, in vitro cultures of OOXs and mGC culture have revealed the role of GDF9 signalling at the antral stage in the differentiation and maintenance of these two subpopulation's phenotype.

The expression of LH receptors (LHR) signifies the mGC identity with the granulosa cells furthest away from the oocyte expressing the highest levels of *Lhcgr* [343, 344]. FGOs actively suppress the expression of LHR transcript *Lhcgr* [63] along with the FSH induced plasminogen activator urokinase (PLAU), a protease suggested to be involved in the degradation of the cumulus matrix, early corpus luteum formation and angiogenesis [345-347]. CCs on the other hand are categorised by their ability to express transcripts necessary for cumulus expansion such as *Has2* and the ability to synthesise the cumulus matrix [88].

Addition of recombinant mouse GDF9 to mGCs increased *Has2* and decreased *Plau* expression simultaneously between 3-9 hours and lost its effect by 24 hours [318] which is parallel to the time frame of cumulus expansion which commences approximately 3 hours after gonadotropin simulation to ovulation after 12-16 hours after induction [90]. GDF9 also decreases the FSH induced *Lhcgr* expression and increases the cumulus matrix related transcript *Ptgs2* in mGCs. In the absence or low doses of FSH (0-1 ng/ml), GDF9 increases progesterone production in mGCs but had no additional effects at high levels of FSH (10 ng/ml). It was also found that GDF9 and FSH stimulated progesterone production through different enzymes; GDF9 increases the expression of the steroidogenic acute regulator protein (StAR) while FSH increased synthesis of CYP11A1 mRNA synthesis [318]. It is important however to note that GDF9 has been reported to inhibit FSH induced progesterone production in both mouse OOXs [288] and porcine mGCs [265]. These discrepancies could however be due to the varying doses of GDF9 and FSH used since the oocyte and FSH function in opposing gradients [268].

Further evidence of GDF9's role in CC and mGC characteristics is shown by the inhibition of the GDF9 signalling SMAD2/3 pathway which attenuated the endogenous effects of FGOs on *Lhcgr* suppression, along with the suppression of *Cd34* and *Cyp11a1* in mGCs. Conversely these transcripts were increased when SMAD2/3 was inhibited in intact COCs. Furthermore the inhibition of SMAD2/3 also resulted in the loss of cumulus transcripts *Slc38a3*, *Amh* and *Ar* in intact COCs and stimulated *Amh* expression in mGCs [268].

Hence native oocytes utilise the GDF9 signalling pathway SMAD2/3, to determine and maintain the CC and mGC lineages. Exogenous recombinant GDF9 therefore appears to mimic the oocyte's role in CC and mGC differentiation and steroidogenesis.

1.6.5.3 GDF9 induces cumulus expansion

In the mouse, CEEFs secreted by the oocyte are mandatory for cumulus expansion [271] and many studies have been devoted to unravelling the identity of these CEEFs [71, 274, 288, 348]. GDF9 which was the first, along with TGFβ1, Activin A and Activin B induced cumulus expansion in mouse OOXs [274, 288, 318, 341]. GDF9 has also stimulated cumulus expansion related transcripts such as *Has2*, *Ptgs2 Tnfaip6* and *Ptx3* in OOXs [272]. Inhibition of the GDF9/TGFβ/activin common signalling pathway SMAD2/3 completely inhibited cumulus expansion and gene expression facilitated by co-culture with denuded oocytes [272].

There are however several conflicting reports on whether GDF9 is the sole CEEF. Use of the same GDF9 neutralising antibody that significantly decreased the mitogenic effect of native oocytes [340] with or without the TGFβ type II receptor inhibition, did not affect oocyte induced cumulus expansion in the presence of FSH. This suggests that neither

GDF9 nor TGFβ1 can account for the CEEF although the GDF9 type II receptor antagonist did partially decrease oocyte induced cumulus expansion which suggests some involvement of GDF9 in oocyte induced cumulus expansion [274].

In contrast to this, oocytes from GDF9 null mice cannot induce cumulus expansion [288]. While this may be attributed to their lack of maturity and compromised development, FGOs treated with an RNA interference knockdown approach for GDF9 and hence negative for GDF9 expression, could not induce cumulus expansion in the presence of FSH although knockdowns for another OSF BMP15 could [349]. This report indicates that GDF9 is mandatory for cumulus expansion and must be the CEEF.

Another fundamental difference in recombinant GDF9 experiments on mouse OOXs is the conflicting requirement for ligand stimulation. Several studies have demonstrated that GDF9 can induce cumulus expansion in the absence of FSH [76, 350] while others resemble the physiological situation and require the presence of gonadotropin stimulation [274]. Furthermore the recombinant GDF9 that stimulated cumulus expansion in OOXs without FSH could not stimulate GVBD in intact COCs unless FSH was included [76].

The recombinant GDF9 used in all studies to date are obtained from the conditioned media of GDF9 transfected cells of varying origin (reviewed in Pangas and Matzuk 2005, [351]). Different GDF9 preparations therefore potentially alter their protein conformation and functional properties resulting in varying levels of activity. Since little is known about the post translation processing of GDF9 and the actual molecular structure of receptor binding GDF9, comparisons of these biologically active recombinant GDF9 proteins to that in vivo

cannot be made. Since oocytes and FSH function in opposing gradients [352], it is also possible that the differences seen in cumulus expansion induction may be due to the different concentrations of bioactive GDF9 present in the different preparations.

1.6.5.4 GDF9 and cumulus cell metabolism

Given the importance of GDF9 to female fertility and its ability to replicate all the oocyte mediated functions so far mentioned, it is confounding that GDF9 was suggested not to be involved in oocyte regulation of cumulus cell glucose metabolism prior to maturation. Instead, recombinant BMP15 in combination with FGF8 were shown to significantly increase *Pfkp* and *LdhA* transcripts and glycolytic activity in OOXs to similar levels as that of FGOs. BMP15 or FGF8 alone or in combination with GDF9 could not achieve the same effect. Inhibition of the FGF receptor also decreased FGO induced increases in glycolytic activity which implied the importance of FGF signalling in CCs to oocyte metabolic regulation [290]. GDF9 however has been implicated in the regulation of cholesterol synthesis in CCs. As $BMP^{-/-}/GDF9^{+/-}$ mice have significantly reduced cholesterol synthesis compared to $BMP15^{-/-}$ mice [235].

However, as mentioned previously several different preparations with varied functions of recombinant GDF9 exist (reviewed in Pangas and Matzuk 2005, [351]). Furthermore the GDF9 used in the above study was recombinant human GDF9 on mouse OOXs and the use of non-homologous recombinant GDF9 has been suggested to generate in vitro artefacts [298]. More conclusive studies utilising various GDF9 preparations and /or SMAD2/3 inhibitors are needed before the GDF9 can be ruled out as an OSF involved in CC glucose metabolism.

Also, the possibility that GDF9, or similar SMAD2/3 signalling factors, synergistically adding to the FGF and BMP regulation of CC glycolysis must be considered. Comparisons of differentially regulated transcripts between *BMP*$^{-/-}$ mice and *BMP*$^{-/-}$/*GDF9*$^{+/-}$ mice reveal around 400 differentially expressed genes and most of these mutation affected genes were found to be involved in metabolism [235]. OSF regulation of CC metabolism during mouse oocyte maturation has also not yet been investigated and hence the role of GDF9 in regulating CC glucose metabolism as the oocyte acquires its last stage developmental competency is currently unknown.

1.7 OOCYTE COMMUNICATION AFFECTS OOCYTE DEVELOPMENTAL COMPETENCE

As established above and recently revised extensively by Gilchrist et al. [353], FGOs have distinct regulatory capabilities to that of growing oocytes. These regulatory properties such as the stimulation of proliferation, glycolysis, and amino acid uptake allow oocytes to control their immediate environment enabling oocyte survival. The extent of the ability of the oocyte to exert this control and the response of its surrounding somatic cells may indicate the oocyte's developmental status and its ability to support embryonic life.

Indeed a recent pioneering study has demonstrated the importance of OSFs to bovine oocyte developmental competence. Co-culture with denuded oocytes or recombinant GDF9 or BMP15 increased the blastocyst formation rates of IVM cumulus enclosed oocytes over that of respective controls. Inhibition of endogenous GDF9 or BMP15 signalling in intact

COCs subsequently resulted in decreased blastocyst formation [354]. This suggests that the deficiency of IVM compared to in vivo matured oocytes may be due to compromised levels or activities of OSFs in vitro since exogenous OSFs have a synergistic effect with that of endogenous OSFs. OSFs GDF9 and BMP15 therefore have a regulatory role in developmental programming of the oocyte at least to the blastocyst stage. Unfortunately whether or not these effects extend past the blastocyst stage into foetal development is unknown.

Further evidence supporting the importance of OSFs was established through IVM of COCs from *BMP15*$^{-/-}$*/GDF9*$^{+/-}$ double mutant mice which have reduced fertilisation rates compared to *BMP15*$^{-/-}$ mice [329]. While *BMP15*$^{-/-}$, *GDF9*$^{+/-}$ and *BMP15*$^{-/-}$*/GDF9*$^{+/-}$ double mutant mice all have slower developing blastocysts, double mutants exhibit significantly increased perturbations after IVM. Double mutant COCs were found to have decreased cumulus expansion even when co-cultured with wild type oocytes compared to the homozygous *BMP15* $^{-/-}$ mutants. Further analyses found that both the double mutant oocytes and CCs were deficient in promoting cumulus expansion or responding to CEEFs of wild type mice. Double mutants also have decreased 2-cell development indicating an inability to support normal fertilisation. Since nuclear maturation was unaffected in all the mutants, these findings demonstrate the importance of GDF9 and BMP15 to oocyte cytoplasmic maturation. Furthermore, as the fertility of double mutants is more severely affected than the BMP15 null mice, the role of GDF9 must be prevalent throughout folliculogenesis and during oocyte maturation. The effect of recombinant GDF9 or BMP15 during mouse IVM have not yet been investigated. Such studies would elucidate if the beneficial effects of GDF9 and BMP15 seen in bovine IVM were not just due to autocrine

effects on the oocyte since BMPR-II has been found in the cow and sheep but not detected in the mouse [302]. Since autocrine signalling of these factors are thus not possible in the mouse, the effects of recombinant OSFs would be entirely due to the oocyte-cumulus cell interactions.

While the importance of GDF9 and BMP15 to human fertility are still being unravelled. Two small studies have correlated the levels of GDF9 regulated CC gene expression *Ptgs2* and Gremlin (*Grem1)* with increased meiotic resumption and subsequent embryo development [129, 355]. These reports together with the findings in animal models provide a strong indication of the clinical benefits of OSFs on clinical IVM.

1.8 CONCLUSION

As the demand for ART increases, oocyte IVM which would eliminate rigorous IVF hormonal stimulation regimes and enable salvage of oocytes that would otherwise be lost in physiological processes, is becoming more essential. However there is still little known about the intricate demands of the oocyte as it acquires the ability to support embryonic and foetal development and hence IVM success rates are currently unacceptably low.

The bi-directional communication between the oocytes its surrounding somatic cells has been established to be fundamental to the survival, growth, and development of both cell types throughout follicle growth. OSFs have also been shown to play a determinant role during IVM on subsequent embryo development.

However, while maturation involves highly coordinated processes between the oocyte and the CCs, little is known about the mechanistic roles of this bi-directional communication to the acquisition of oocyte developmental competence during IVM. The importance of oocyte-cumulus bi-directional communication during maturation to long term programming has also not yet been assessed. Elucidating the importance of oocyte-cumulus bi-directional communication during IVM to subsequent pregnancy and foetal development will enable valuable insight into the regulatory processes involved in the acquisition of oocyte developmental competence.

Knowledge of the importance and functional effects of this regulatory control mechanism will eventuate in improved IVM systems and realise the potential of IVM as standard ART protocol.

1.9 AIMS

The overall aim of this thesis is therefore, to investigate the significance of oocyte to cumulus cell bi-directional communication during IVM on oocyte and cumulus cell functions in relation to oocyte developmental competence and subsequent foetal development.

The aims of individual experimental chapters are as listed below:

Chapter 3

To establish the developmental competence of IVM/IVF oocytes in relation to those matured in vivo and fertilised in vitro and those matured and fertilised in vivo in the experimental system of this thesis.

Chapter 4

To investigate the effects of inhibiting oocyte to cumulus cell bi-direcional communication and the addition of exogenous GDF9 on cumulus expansion, along with the assessment of cumulus expansion attenuation with azaserine on oocyte developmental competence.

Chapter 5

To investigate the importance of oocyte and cumulus cell bi-directional communication through the SMAD2/3 and FSH/EGF signalling pathways during IVM on oocyte maturation, fertilisation and subsequent embryo and foetal development.

Chapter 6

To investigate the effects of exogenous GDF9 during mouse IVM on subsequent embryonic development and foetal outcomes both in the presence and absence of FSH and EGF.

Chapter 7

To investigate the possible mechanisms resulting in affected oocyte developmental competence in earlier chapters with regards to metabolism and oxidative stress.

CHAPTER 2

EXPERIMENTAL METHODS

2.1 ANIMALS AND HORMONE ADMINISTRATION

All animals were purchased from Laboratory Animal Services (Adelaide, South Australia) and treated in accordance with the *Australian Code of Practice For The Care and Use of Animals for Scientific Purposes*. Mice were housed in a 14-hour light and 10-hour dark cycle and food and water were supplied *ad libitum*.

An intra-peritoneal injection of 5 IU pregnant mare serum gonadotropin (PMSG) (Folligon; Intervet, Bendigo, Victoria, Australia) was administered to each mouse to stimulate follicle growth. In cases where ovulation was induced, 5 IU of human chorionic gonadotropin (hCG) was administered 46-48 hours after PMSG injections, intra-peritoneally (Pregnyl; Organon, Oss, The Netherlands). See Appendix 2 for information for PMSG and hCG preparation.

2.2 COLLECTION AND CULTURE OF CUMULUS OOCYTE COMPLEXES/ZYGOTES

2.2.1. In Vitro Maturation

2.2.1.2 Isolation of ovaries

COCs intended for in vitro maturation (IVM) were obtained from 21-25 day old CBA/C57BL6 female mice 46-47 hours after administration of an intra-peritoneal injection of 5 IU pregnant mare serum gonadotropin (PMSG) (Folligon; Intervet, Bendigo, Victoria, Australia). Mice were sacrificed by cervical dislocation, and then placed on their backs and

their abdomens disinfected with 70% ethanol. All instruments used were sterilised by autoclaving and soaked in 70% ethanol immediately prior to use. Using a pair of forceps, the disinfected area was lifted so that a small incision could be made under the skin in the peritoneum at the ventral midline, this was then extended on either side towards the forearms and the skin pulled back towards the head to reveal the visceral organs which were moved aside to reveal the reproductive organs. Holding the utero-tubal junction with a pair of watchmaker forceps, the surrounding tissues were blunt dissected away from the uterus using the point of a pair of fine scissors. Whole ovaries were located and removed clean and free of adipose tissue by ripping the ovarian bursa using two fine watchmaker forceps.

2.2.1.2 IVM media

Serveral commercially available media are conventionally employed as the base media for IVM culture systems. The most commonly used are Eagle's Minimum Essential Media (MEM), MEM alpha (αMEM), Dulbecco's modification of Eagle's medium (DMEM), Nutrient Mixture F-12 (Ham F-12), TCM199 and Waymouth MB752/1. These media were however, initially designed to meet the needs of non-ovarian somatic cells and vary greatly in compound concentrations and composition. They have also been found to produce specie-dependent varied degrees of success at meeting the complex and dynamic requirements of the oocyte during maturation. Bovine oocytes matured in TCM199 and MEM have higher blastocyst development rates than those matured in Waymouth MB 752/1 or Ham's F-12 [356] the reverse was however, found for porcine oocytes which developed best in Waymouth MB 752/1 [357].

Relevant to the experimental animal model of this thesis, an elegant study comparing the developmental competence of mouse oocytes matured spontaneously with foetal calf serum in DMEM, MEM with non-essential amino acids, Waymouth MB 752/1, BGJb medium Fitton-Jackson modification (BGJb), Ham F-12 and TCM199 [358] found that only Waymouth MB 752/1 and MEM alpha significantly increased blastocyst formation over that of their routinely [359] used IVM culture system. Waymouth MB 752/1 was the only media that increased both 2-cell and blastocyst formation and produced significantly more live births after embryo transfer than that of the control [358].

Waymouth MB 752/1 was originally designed for culture of mouse L929 cells [360]. In comparison to the other commonly used IVM media, it contains high levels of cysteine, cystine and glutathione which have antioxidative properties and may play protective roles during oocyte maturation [357, 361]. Waymouth medium also has substaintially more glucose in an excess of 20 mmol/L which has been shown to be beneficial to bovine ooycte development, when matured at 5% oxygen [362]. Waymouth MB 752/1 with 5% foetal calf serum was therefore chosen as the IVM base medium with culture conditions maintained at 37°C in 6% CO_2, 5% O_2, 89% N_2 for the entirety of this thesis.

2.2.1.3 COC collection and maturation

After isolation, ovaries were placed into Waymouth MB 752/1 medium supplemented with 5% non-heat inactivated foetal calf serum (Invitrogen; Carlsbad, CA, USA) buffered with HEPES and sodium bicarbonate maintained at approximately 37ºC (IVM Handling medium; Appendix 1). All procedures following the removal of mice ovaries were done on warming stages so that oocytes were maintained at approximately 37 ºC. Ovaries were subsequently placed into a 35 mm Petri-dish containing approximately 3 ml of clean IVM

handling media and germinal vesicle (GV)-stage COCs were aspirated by puncturing large antral follicles under a dissecting microscope (Nikon SMZ1500 or Nikon SMZ800, Nikon Corporation, Japan). COCs were washed once in a second 35 mm Petri-dish containing clean IVM handling medium then cultured in groups of approximately 30-50 for 17.5-18 hours at 37°C in 6% CO_2, 5% O_2, 89% N_2 in 3 ml of IVM culture media (Appendix 1) containing various treatments. Culture media was equilibrated at 6% CO_2, 5% O_2, 89% N_2 approximately 4 hours prior to use. Only COCs with a uniform covering of compacted cumulus cells were utilised in experiments.

2.2.2. Collection Of Ovulated COCs

14-15 hours post hCG administration, 21-25 day old CBA/C57BL6 female mice were sacrificed and dissected as above. Oviducts were carefully removed by first making a cut between the ovary and the oviduct using a pair of fine scissors and forceps. A second cut was then made just below the utero-tubal junction and the removed oviduct was immediately placed into warm IVM handling media. Oviducts were then transferred into 35 mm Petri-dishes containing approximately 3 ml of IVM handling media. Masses of ovulated COCs were located in the swollen ampulla under a dissecting microscope (Nikon SMZ1500 or Nikon SMZ800, Nikon Corporation, Japan) and released into the IVM handling media by ripping the adjacent area of the oviduct using two pairs of fine forceps. All procedures following the removal of mice oviducts were done on warming stages ensuring that oocytes were maintained at approximately 37 ºC.

2.2.3 Collection Of 1-Cell Zygotes

Immediately after hCG administration, 21-25 day old CBA/C57BL6 female mice were mated overnight with 6-8 week old CBA/C57BL6 male mice. Male mice were housed

individually unless mating and no more than 2 females were placed into one cage with a male. A minimum of 2 days was allowed before the same male mouse was mated again. Female mice were then assessed for the presence of a vaginal plug the following morning which indicated successful mating. Approximately 22-24 hours after females were initially placed together with the males; these mated female mice were sacrificed by cervical dislocation and dissected as in Chapter 2.2.1. Removed oviducts were then placed into warm MOPS-G1 media (Appendix 1). Oviducts were subsequently transferred into 35 mm Petri-dishes containing approximately 3 ml of warm MOPS-G1 media. Masses of 1-cell zygotes were located under a dissecting microscope and released into the MOPS-G1 media by ripping the oviduct area adjacent to the zygotes using two pairs of fine forceps. Zygotes were then placed into 1 ml of warm hyaluronidase (Sigma Aldrich, St Louis, MO, USA) diluted to a concentration of 0.5 mg/ml with MOPS-G1 media and gently pipetted to remove any remaining cumulus cells for approximately 30-60 seconds. Zygotes were then washed once in 3 ml of fresh warm MOPS-G1 then twice in 20 µl pre-equilibrated drops of G-1 v3 (Vitrolife, Kungsbacka, Sweden) set up as described in Chapter 2.4. Zygotes were then cultured in groups of approximately 10 in 20 µl G-1 v3 drops, pre-equilibrated and over layered with mineral oil (Vitrolife, Kungsbacka, Sweden or Merck, Darmstadt, Germany) at 37°C in 6% CO_2, 5% O_2, 89% N_2. All procedures following the removal of mice oviducts were done on warming stages ensuring that oocytes were maintained at approximately 37 ºC.

2.3 TREATMENT OF COCs

2.3.1 FSH and EGF

15 µl of a 10 IU/ml stock solution of follicle stimulating hormone (FSH) (Puregon; Organon, Oss, The Netherlands; see Appendix 2) was added to 3 ml of IVM culture media to give a final working concentration of 50 mIU/ml. Epidermal growth factor (Sigma-Aldrich, St Louis, MO, USA) was made up to a 1 mg/ml stock solution (see Appendix 2) and used at a 1 in 100 dilution (30 µl in 3 ml) to achieve a final working concentration of 10 ng/ml.

2.3.2 SMAD2/3 Inhibitor SB-431542

SB-431542 was generously donated by GlaxoSmithKline (Stevenage, UK) and dissolved in dimethyl sulphoxide (DMSO) (see Appendix 2). The carrier control for SB-431542 was therefore the equivalent 0.04 % v/v DMSO (Hybri-Max, sterile filtered, endotoxin, hybridoma tested, Sigma-Aldrich, St Louis, MO, USA). SB-431542 has no toxicity to cells and acts as a competitive ATP binding site kinase inhibitor with high specificity towards ACVR1B (ALK4), TGFBR1 (ALK5) and ACVR1C (ALK7), the kinases that activate SMAD2/3 when used at <10 µM [363]. SB-431542 was used at 4 µM in all experiments as this concentration was shown to effectively prevent cumulus expansion in oocytectomised cumulus complexes [272].

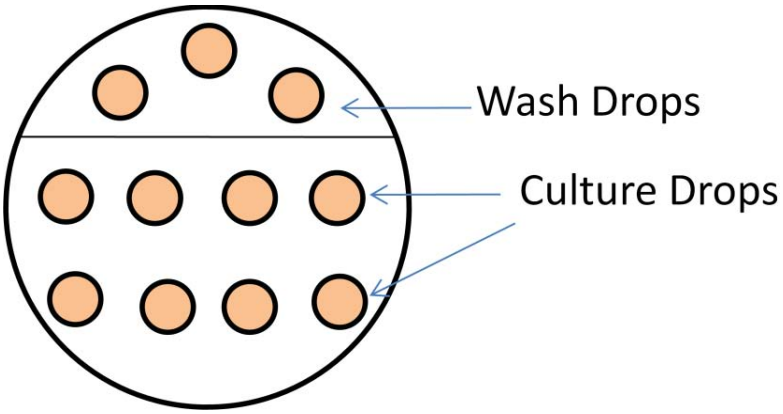
2.3.3 Exogenous Oocyte Secreted Factor GDF9

Recombinant mouse GDF9 was produced and partially purified as previously described [265, 274, 340, 364] and in Appendix 2. Briefly, human embryonic kidney 293H-cell lines were stably transfected with recombinant mouse GDF9 and the protein was partially

purified from the conditioned medium using hydrophobic interaction chromatography (HIC). Conditioned medium from non-transfected 293H cells was also subjected to the same HIC purification process and used as the negative control (designated; 293H). Recombinant mouse GDF9 was used at a concentration of 200 ng/ml chosen based on a does response assessment of its effects on cumulus expansion of mouse oocytectomized complexes [274]. The equivalent 7% v/v parent cell line 293H conditioned medium was used as the control for all experiments involving the use of recombinant GDF9.

2.4 EMBRYO CULTURE DISH SET UP

Embryo culture dishes were set up and equilibrated either the night before or for no less than 4 hours prior to use. Drops of 20 µl of culture medium were laid out on a 35 mm Petri-dish as illustrated below then overlayed with 3.5 ml of batch tested and proven embryo non-toxic mineral oil (Vitrolife, Kungsbacka, Sweden or Merck, Darmstadt, Germany).



2.5 IN VITRO FERTILISATION

Male CBA/C57BL6 mice with proven fertility were used as sperm donors for IVF. To ensure that sperm quality would not be compromised, male mice were mated no less than 2 days and no more than 5 days prior to IVF. On the day of insemination, male donors were sacrificed by cervical location approximately 13-14 hours after hCG administration to the experimental female mice or 16-17 hours after IVM. The male mouse was then placed ventral side upwards and its abdomen disinfected with 70% ethanol. A cut was made at the ventral midline through the skin and peritoneum and extended to expose the body cavity. The fat pads were moved aside to reveal the testes, epididymis and vas deferens. Using the point of a scissors and holding the vas deferens with a pair of watchmaker forceps, a cut was made in vas deferens after the connective tissue surrounding it had been blunt dissected. The epididymis was then carefully cut away from the testes and removed into warm (~37ºC) MOPS-G1 for transportation. Once on the warming stage, the epididymis and vas deferens were then transferred into a pre-equilibrated dish containing a 1 ml drop of fertilisation medium (Appendix 1) over layered with mineral oil. Spermatozoa were released from the cauda epididymis into the medium using a pair of fine forceps and a 'plucking' motion and left to capacitate for 1 hour. 10 µl of capacitated sperm was then added to pre-equilibrated 90µl drops of fertilisation medium over layered with mineral oil.

After 17.5-18 hours of IVM or 14-15 hours after hCG administration, COCs were washed twice in fertilisation medium then co-incubated with the capacitated sperm in groups of approximately 20 per each 100 µl fertilisation drop for 4 hours at 37°C in 6% CO_2, 5% O_2, 89% N_2. At the end of this co-incubation period, presumptive zygotes were washed once in MOPS-G1 then twice in G-1 v3 (Vitrolife, Kungsbacka, Sweden) using a fine glass pipette

to minimise transfer of sperm to the culture drops. Presumptive zygotes were then cultured in groups of approximately 10 (no more than 14 and no less than 5) in 20 µl G-1 v3 drops, over layered with mineral oil (Vitrolife, Kungsbacka, Sweden or Merck, Darmstadt, Germany) at 37°C in 6% CO_2, 5% O_2, 89% N_2.

2.6 EMBRYO CULTURE AND SCORING

Assessment of embryo development was performed on a warming stage set to maintain the temperature in the petri dish at 37^{o}C under 100 x magnifications on a dissecting microscope (Nikon SMZ1500, Nikon Corporation, Japan). Cleavage rates were determined by the number of 2-cell embryos which was assessed 20 hours after IVF or 24-26 hours after 1-cell zygote culture. Two-cell embryos were then transferred to fresh G-1 v3 drops for another 25-27 hours after which embryos were assessed for their rate of development as determined by the percentage of 8-cell and compacted embryos. For some experiments, the percentages of 2-cell arrested embryos were also simultaneously observed. All embryos were then washed twice in G-2 v3 (Vitrolife, Kungsbacka, Sweden) medium then cultured in 20 µl G-2 v3 drops over layered with mineral oil for 47-49 hours at 37°C in 6% CO_2, 5% O_2, 89% N_2. Embryonic morphology was assessed at the end of the culture period (96-100 hours of embryo culture; 5 days after IVM), to determine blastocyst development. Embryos were classified as blastocysts when they contained a blastocoel cavity greater than 1/3 of the total volume of the embryo. Hatching blastocysts were identified by the protrusion of the trophectoderm through the zona pellucida.

2.7 DIFFERENTIAL STAINING

In order to assess resultant embryo quality at the blastocyst stage, blastocyst inner cell mass (ICM) and trophectoderm (TE) cell numbers were determined using a differential nuclei staining protocol as described by Gardner et al., [365]. The resultant foetus is formed soley from the ICM [366] and hence a minimal number of ICM cells are required for foetal formation. Mouse blastocyst with increased ICM had increased foetal viability after transfer despite no differences in blastocyst formation rates [229]. Bovine embryos with poor morphology were found to form blastocysts with significantly less ICM [367] and abnormal allocations of cells to the TE were suggested to result in defective placenta development leading to pregnancy abnormalities such as hydroallantois [368, 369]. As such, blastocyst cell allocation analysis has been shown in several animal models such as the cow [367], pig [370], rabbit [371] and mouse [229] to be a viable measure of blastocyst quality and predicative of foetal development and viabilty.

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich (St Louis Mo, USA) and laid out in the form of 50 µl drops on a 35 mm Petri-dish over layered with 3.5 ml mineral oil and left to equilibrate at the appropriate temperatures as subsequently listed for approximately 4 hours prior to commencing the experiment. At the end of culture, blastocysts of the same treatment were transferred in groups of 5 to 10 into 0.5% pronase at 37°C for approximately 1-2 mins to dissolve their zona pellucida. Once the zona pellucida had completely dissolved, as determined through visualisation at 70 to 100 x magnification under a dissecting microscope (Nikon SMZ1500 or Nikon SMZ800, Nikon Corporation, Japan), blastocysts were then incubated for 10 mins in 10 mM 2,4,6-trinitrobenzenesulfonic acid (TNBS) at 4°C. After which they were transferred into 0.1

mg/ml anti-dinitrophenyl-BSA (α-DNP BSA) for 10 mins at 37°C with two washes in HEPES buffered modified G1 medium (H-SG1; Appendix 1) at 37°C in between each step. Blastocysts were then placed in guinea pig serum with propidium iodide for 5 mins at 37°C and then transferred to a 4 well Nunc plate (1 well per treatment) containing 400 µl bisbenzimide in ethanol at 4°C overnight. Blastocysts were then washed in 100% alcohol the following day then mounting in a glycerol drop on a siliconized slide. Individual blastomeres were then counted under a fluorescent microscope at 200x magnification, where ICM and TE cells appeared blue and pink respectively under UV light.

2.8 EMBRYO TRANSFERS

2.8.1 Vasectomy of Male Mice

6-8 week old male CBA/C57BL6 mice were given an intra peritoneal injection of 2% Avertin (0.015 ml/g body weight; see Appendix 3) then tested for successful anaesthetisation by the lack of response when gentle pressure was applied to one foot. Each anaesthetised mouse was then placed ventral side upwards and its abdomen disinfected with 70% ethanol. A small incision was then made in the scrotum followed by the separation of the underlying connective tissue to reveal the body cavity. The testes were gently eased out one at a time from the abdomen towards the incision then removed from their surrounding bursa out of the body cavity using two pairs of fine forceps. The vas deferens was located and a 10 mm length was removed by an incision at either end of its attachment. The testis was then eased back into the bursa and the body cavity. This procedure was then repeated on the other testis. The mesentery and skin wound were both sutured and the mouse was placed on a 37 ºC warming plate to facilitate recovery.

After a minimum of 3 weeks, each male was mated with two super-ovulated 3-4 week old CBA/C57BL6 females and successful mating was confirmed by the presence of a vaginal plug the next morning. Plugged females were then monitored for 3 weeks to check for pregnancy. A male was determined to be sterile once he had plugged 3 females that did not become pregnant.

2.8.2 Embryo Transfer Procedure

6-10 week old Swiss female mice were used as recipients for embryo transfers and naturally mated with vasectomised male mice. Successful mating was determined by the presence of a vaginal plug the next morning and this was termed day 1 of pseudo-pregnancy.

On day 3.5 of pseudo-pregnancy, plugged females were anaesthetised with an intra-peritoneal injection of 0.7-0.8 ml Avertin (0.015 ml/g body weight; see Appendix 3) prior to embryo transfer. Once successful anesthetisation had been confirmed through the lack of response after applying gentle pressure to one foot, the mouse was placed under a dissecting microscope on its abdomen and its back was disinfected with 70% ethanol. A 5 mm longitudinal incision was made in the midline below the last rib and the skin was moved so that the incision was above the ovary and adjoining fat pad. The underlying connective tissues were gently dissected away using a pair of scissors and fine forceps, then an incision of less than 5mm was made into the peritoneum directly above the fat pad. The fat pad along with the ovary, oviduct and uterus were then pulled from the body cavity using a pair of fine forceps and secured to the back of the female with an ovarian retractor. Six day 4.5 hatching and expanded blastocysts from the same treatment were then picked

up in a flame polished glass pipette in a medium volume of less than 1µl. A sterile 26-gauge needle was then used to make a small hole in the wall of the lower third of one uterine horn, which was held taut by a pair of fine forceps, into the lumen. The pipette containing the embryos was then inserted into the hole and the pipette gently slid back and forth to ensure that it was in the lumen. The embryos were then gently expelled and the glass pipette was then checked to ensure all the embryos had been deposited in the uterus. The reproductive tract was eased back into the body cavity using blunt forceps avoiding contact with the rest of the uterus.

This procedure was then repeated with blastocysts from a different treatment for the contralateral uterine horn. Once embryo transfer had been completed, the skin wound was sealed with Michel clips (Becton-Dickson, USA) and the mouse was gently placed on a 37ºC heating stage to facilitate recovery before being returned to her cage.

2.8.3 Post-Mortems and Isolation of Foetuses

On day 15 or 18 of pregnancy, females were sacrificed via cervical dislocation then placed on their backs. Their abdomens were sterilised with 70% ethanol and an incision was made along the ventral midline followed by 2 subsequent incisions up towards each forearm revealing the peritoneum. The uterine horns containing the foetuses were eased and dissected out one at a time. The uterine wall was then cut to reveal the foetuses and implantation sites. The number of foetuses and implantations including resorption sites, identified by rudimentary embryonic tissue (dark protrusions) in the uterine wall, were recorded. Individual foetuses and their corresponding placentas were then isolated using a pair of forceps and fine scissors. All analyses were performed blinded to the treatment of origin. Each foetus and placenta was then weighed and the foetal crown to rump length

determined by a pair of callipers. Foetal weight, size and gross morphology, as determined by limb, structural and overall appearance were considered normal if they fell within the parameters of published information [372] of in vivo derived mouse foetuses of the same age.

2.9 RNA EXTRACTION

2.9.1 Whole Tissues and COC Standards

RNA extracted from the heart and half a brain of a single 27 day old female CBA/C57BL6 mouse was used as the positive control in gene expression analysis for selected genes. After the removal of the ovaries as described in Chapter 2.2.1, the incision at the ventral midline was extended into the rib cages. These were moved apart to facilitate the removal of the heart by dissecting away the surrounding connective tissue. A series of careful incisions were then performed around and through the skull, facilitating the removal of the mouse brain. Both tissues were placed into warm IVM handling medium immediately and washed once more in fresh IVM handling medium before being frozen in liquid nitrogen then stored at -80ºC. Homogenisation of these tissue samples was performed on ice using a sterile glass homogeniser. 1 ml of Tri® Reagent (Sigma Aldrich, St Louis, MO, USA) was used per tissue according to the manufacturer's instructions to homogenise and extract total RNA. The homogeniser was stringently washed twice with the following steps prior to the homogenisation of each sample: Two 1M sodium hydroxide washes followed by two 1% w/v sodium dodecyl sulphate SDS washes and two 1% v/v Triton X wash. All solutions were made up in autoclaved Milli Q water. Extracted total RNA was resuspended in 40 µl of RNase free water.

For COC standards, a total of approximately 600 ovulated COCs were obtained from 15 CBA/C57BL6 21-25 day old mice and pooled. RNA was extracted using 1 ml of Tri® Reagent (Sigma Aldrich, St Louis, MO, USA) according to the manufacturer's instructions. 20 µg of a 20 mg/ml stock solution of Glycogen (Roche Diagnostics, Germany) was also added to facilitate RNA precipitation. Extracted Total RNA was resuspended in 30 µl of RNase free water. All extracts were DNase treated using Ambion DNA-free kit (Applied Biosystems/Ambion, Austin, TX, USA) according to the manufacturer's instructions and stored at -80°C.

2.9.2 Oocyte/COC/Embryo Samples

Total RNA was extracted using RNeasy® Micro Kit (Qiagen Pty Ltd, Victoria, Australia) according to the manufacturer's protocols. Briefly, groups of approximately 10 or 20 oocytes/cumulus cells/COCs/embryos were collected in 0.6 ml eppendorf tubes and 4 ng/µl of the supplied carrier RNA was used to aid in precipitation. Samples were Dnase treated as part of the protocol and total RNA was resuspended in 14 µl aliquots of RNase free water and stored at -80°C.

2.10 QUANTIFICATION OF RNA

Quantification of RNA was done after DNase treatment (Qiagen Pty Ltd, Victoria, Australia), by UV absorbance at 260 nm using a Nanodrop ND-1000V3.1.0 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Readings were only accepted if the 260 nm absorbance reading was greater than 0.15 and the ratio of absorbance at 260nm to 280nm was between 1.9-2.3.

2.11 Reverse Transcription

First strand complementary DNA (cDNA) was synthesized from the appropriate amount (2000ng, 5000ng, 20000ng for COC, Heart and Brain standards respectively) or the maximum volume (11µl for samples) of total RNA for samples, using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and 250 ng of random primers (Invitrogen, Victoria, Australia) according to the manufacturer's protocols. Briefly, 1µl of a 10 mM dNTP mix and 1µl of a 250 ng/µl random primer mix were added to appropriate volumes of the RNA samples and made up to a total volume of 13 µl with Rnase free water. This was then heated to 65ºC for 5 mins and placed on ice for a minimum of 1 min. 4 µl of the 5x first-strand buffer, 1 µl of 0.1M DTT and 1 µl of recombinant Rnase inhibitor (40 units/µl) (Invitrogen; Carlsbad, CA, USA) and 1µl of the Superscript III reverse transcription enzyme (200 units/µl) were then added to the sample and mixed well by a quick vortex. This was then incubated at 25ºC for 5 mins then at 50ºC for 60 mins. First strand cDNA samples were then stored at -20ºC.

2.12 REAL TIME POLYMERASE CHAIN REACTION (PCR)

2.12.1 Primer Design and Validation

Specific primers for target genes were designed against published coding mRNA sequences in the NCBI Pubmed database (www.ncbi.nlm.nih.gov), using Primer Express software (PE Applied Biosystems, Foster City, CA, USA). Intron and exon sequences were found using Ensembl (www.ensembl.org) and primers were chosen to span an intro/exon boundary

where possible to ensure replication of mRNA and not genomic DNA. Primers were obtained from Geneworks Pty Ltd (Adelaide, Australia) and made up to a stock solution of 1 µg/ml with Rnase free water and stored at -20ºC. An aliquot of stock solution was then diluted to 10 pmol/µl of which 0.5µl was used per PCR reaction to the final amount of 5 pmol each of reverse and forward primer per PCR reaction.

Prior to analysing gene expression in treatment samples, primer efficiency was validated through the formation of a standard curve using serial dilutions of cDNA from tissues or cells of which the target gene was known to be abundantly expressed. For analysis of gene transcripts involved in cumulus expansion or/and abundantly produced by cumulus cells, 2 mg of COC standard total RNA were reversed transcribed to give a concentration of 100 ng/µl cDNA which was then diluted with Rnase free water to a stock solution of 50 ng/µl. Serial dilutions of 1 in 10 were then performed to give cDNA aliquots of 5, 0.5, 0.05 and 0.005 ng/µl. For genes with unknown expression in COCs, 6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase 2 and 3 (*Pfkfb2 and Pfkfb3*) mouse heart and brain cDNA were used as the standards respectively as these tissues have been shown to abundantly express the respective genes [373]. 5 mg heart and 20 mg brain total RNA were reversed transcribed to give the final cDNA concentrations of 250 ng/µl and 1 mg/µl respectively. These were diluted into stock concentrations of 50 ng/µl and serially diluted to give cDNA aliquots at 25, 5, 0.5, 0.05 and 0.025 ng/µl. cDNA concentrations included the assumption of 100% reverse transcription efficiency.

Real time RT-PCR was performed as described below in Chapter 2.12.2 and the mean Ct of duplicate/triplicate samples were calculated and plotted against the log input of cDNA to

create a standard curve. Correlation coefficients (R^2) confirmed the linear relationship between the threshold cycle (Ct) and the logarithm of the cDNA amount while the slope of the standard curve indicated the primer efficiency. A slope value of -3.32 implied a primer efficiency of 1 (100%). Primer efficiency was calculated by the formula Primer Efficiency = $10^{[-1/slope]}$-1 [374, 375] manually or through the Corbett Rotor Gene-6000 software (Corbett Life Science, Qiagen Pty Ltd., Victoria, Australia). Primers of target genes were only accepted when their correlation coefficients were approximately 0.99 and efficiencies were within 10% of that of the endogenous housekeeper as shown in Appendix 5.

2.12.2 Real Time PCR

Real time Reverse Transcription (RT)-PCR was performed in duplicates or triplicates where sample amount permitted on a Corbett Rotor Gene-6000 (Corbett Life Science, Qiagen Pty Ltd., Victoria, Australia) or a GeneAmp 5700 Sequence Detector (Applied Biosystems, Australia) with a thermal cycling program of 50°C for 2 mins followed by 95°C for 10 mins, then 40 amplification cycles of 15s at 95°C (denaturation) and 1 min at 60°C (annealing/extension). PCR reactions were ran at a total volume of 20 µl made out of 5pmol (0.5µl) of each forward and reverse primer with the exception of *Rpl19* which had 10 pmol/reaction, 10 µl SYBR Green Mix (Applied Biosystems, Foster City, CA, USA), 2 µl cDNA per reaction and the appropriate amount of Rnase free water. Ribosomal protein L19 (*Rpl19)* and 18s ribosomal RNA (*Rn18s*) were chosen as the endogenous references as their expressions were not known to be affected by any experimental treatments and have been used extensively as housekeeping genes in the literature [114, 376-378]. A non-template negative control (PCR mix without any cDNA) for every primer set analysed was included on each PCR run and found to give no amplification of gene transcripts. At the

end of every PCR run, a dissociation curve analysis was performed to ensure that only one peak was observed indicating the absence of non-specific amplification or the presence of primer dimmers. The baselines were set from approximately 2 to 12 cycles to reduce background readings and the thresholds were set to ensure that the resultant reported Ct values were in the exponential linear phase of the amplification cycles. Analysis of relative gene expression was done by calculating the average threshold cycle (Ct) for each duplicate/triplicate PCR sample then obtaining the ΔCt value by subtracting the average Ct value of the endogenous *Rpl19* or *Rn18s* control from the average Ct of the target gene. The ΔCt of the respective corresponding experimental reference control sample (calibrator control) was then subtracted from the ΔCt of each treatment samples to give the ΔΔCt value. Gene expression for each treatment was then expressed relative to the calibrator control through the $2^{-\Delta\Delta Ct}$ method [379].

CHAPTER 3

EFFECTS OF IN VITRO MATURATION FERTILISATION AND CULTURE ON OOCYTE DEVELOPMENTAL COMPETENCE

3.1 INTRODUCTION

The first successful human birth from IVF occurred in 1978. Since then, millions of births have resulted from IVF worldwide, yet global success rates are still low, with only 19.6-27.2% of all initiated cycles resulting in live deliveries [3-5]. Aside from infertility factors, this low success rate may be also attributed to the handling and culture of pre-implantation embryos in vitro. In vivo, oocytes reside in enclosed protected environments, moving upon ovulation from the follicle to the fallopian tube where it is fertilized then into the protected uterine environment for implantation. Removal of oocytes/embryos from their natural environment unavoidably exposes them to factors that they would not encounter in vivo compromising their physiology, gene expression and development [380]. Moreover, a recent study has found changes in genetic imprinting in in vitro cultured embryos [381]. Consequently, there have been numerous reports of decreases in developmental rates and blastocyst formation and quality of embryos cultured in vitro compared to in vivo derived embryos [382, 383].

However, since the establishment of technologies for the culture of mammalian pre-implantation embryos in the late 1950s [384], knowledge of the requirements of the pre-implantation embryo as it transcends through its various developmental status has advanced significantly. Improvements in embryo culture media and laboratory conditions resulting from this have seen the production of mouse blastocysts cultured from zygotes in vitro of similar quality as those obtained in vivo [385] and as high as 80% pregnancy rates following transfer of no more than 2 in vitro developed blastocyst on a donor oocyte model in humans [386].

Unfortunately another concerning factor of IVF is the use of rigorous hormonal stimulation regimes to achieve multiple pre-ovulatory oocytes to increase success rates. Aside from the high cost and lifestyle disruptions involved, these hormonal stimulation regimes can cause several side effects the worse being ovarian hyper stimulation syndrome (OHSS), which can be fatal. Up to 33% of all IVF treatments have been reported to be associated with mild forms of OHSS while severe OHSS has been reported in 0.5-5% of IVF cycles [7].

The ability to mature an oocyte in vitro is therefore an attractive infertility treatment option as it would reduce the costs and side effects of gonadotropin stimulation currently involved in routine IVF treatment cycles. Unfortunately, the developmental competence of in vitro matured (IVM) oocytes is still far lower than that of in vivo matured oocytes. Embryos derived from IVM have been found to have decreased cleavage rates, increased embryo growth retardation and poor implantation and pregnancy success rates compared to in vivo matured oocytes [11, 13, 14]. This is most likely due to the lack of understanding of the intricate requirements of an oocyte as it undergoes the final stages of both nuclear and cytoplasmic maturation and thus the inability to replicate this in vitro. It is also probable that the increased handling and culture time resulting from IVM may result in exposure to negative environmental and physical factors contributing to decreased developmental competence.

This chapter thus sought to establish the developmental competence of IVM/IVF oocytes in relation to those matured in vivo and fertilised in vitro and those matured and fertilised in vivo in our experimental system.

3.2 EXPERIMENTAL DESIGN/ MATERIALS AND METHODS

3.2.1 Developmental Competence of In Vivo Obtained Zygotes

After ovulation induction as in Chapter 2.1, 4-6 21-25 day old CBA/C57BL6 female mice were mated with males of similar strain and 1-cell zygotes were obtained following the procedures in Chapter 2.2.3. These were then washed twice in G-1 v3 (Vitrolife, Kungsbacka, Sweden) then cultured in groups of approximately 10 in 20 μl G-1v3 drops, over-layered with mineral oil at 37°C in 6% CO_2, 5% O_2, 89% N_2. Culture protocols and embryo development assessment protocols can be referred to in Chapters 2.4 and 2.6. A total of 4 experimental replicates were performed giving a total of 392 presumptive zygotes in 40 groups of approximately 10 embryos.

3.2.2 Developmental Competence of In Vivo Ovulated and In Vitro Matured COCs

Ovulated COCs were obtained as in Chapter 2.2.2. A total of 5 mice were used per experiment. In vitro maturation of COCs was performed with 50 mIU/ml FSH and 10 ng/ml EGF as described in Chapter 2.2.1 with 6 mice sacrificed per experiment. In order to avoid variance in sperm quality, IVF for both ovulated and IVM COCs were done simultaneously following protocols in Chapter 2.5. Embryos were cultured and assessed as

in Chapters 2.4 and 2.6. A total of 252- 254 COCs were obtained from 4 experimental replicates.

3.2.3 Statistics

Two cell, on-time and blastocyst embryo development along with 2-cell embryo arrest were analysed using a Kruskal Wallis non-parametric test as there were more than 3 treatment groups and the data set was not normally distributed. A univariate general linear analysis of variance utilising least significant differences was used to analyse the development of hatching blastocysts as the data set was normally distributed and had equal error variance between treatments. Differences between experiment days within treatments were also analysed and accounted for as a covariate when a univariate general linear analysis of variance was used or tested for significant variation using a Kruskal Wallis non-parametric test. A P value of <0.05 indicated statistical significance. All statistical analyses were performed using SPSS version 13.0 for windows.

3.3 RESULTS

3.3.1 Effects of IVM, IVF and Culture on Embryo Developmental Competence

3.3.1.1 Two cell development

Upon successful penetration of the sperm into the oocyte's plasma membrane, a series of events occur including the release of cortical granules, decondensation of the sperm head and fusion of the paternal and maternal pronuclei. This eventually leads to the first cleavage stage resulting in the formation of a 2-cell embryo. Fertilisation success was therefore measured by the presence of a 2-cell embryo. Embryos were assessed for the formation of a 2-cell embryo the following day (~20 hrs) after IVF or collection of in vivo fertilised zygotes. Analysis of the percentage of 2-cell embryos revealed no difference between the three experimental groups. There was thus no significant difference in the ability of embryos derived from IVM to complete the first cleavage stage in comparison to in vivo derived zygotes or to embryos derived from in vivo ovulated COCs (N=25 – 40 replicates of groups of 10 oocytes from 4 independent experiments; no differences were found between experimental replicates within each treatment $P>0.05$) (Figure 3A).

3.3.1.2 Rate of development

Faster growing embryos have been associated with increased pregnancy rates [387] hence the rate of development was used as a measure of embryo development. Mouse embryos are either at or past the 8-cell stage of preimplantation embryo development 48 hours post fertilisation [388] thus on-time embryonic development was determined by the percentage

of 8-cell and compacted embryos on the third day of embryo culture. To eliminate the influence of fertilisation success, the percentage of on time developed embryos were expressed per 2-cell embryo and not per isolated oocyte/zygote. As shown in Figure 3B, although in vivo obtained zygotes appeared to have a faster rate of development, no significant differences were found in the rate of embryo development between any of the experimental groups.

3.3.1.3 Two cell embryo development arrest

Sub-optimal culture conditions have been shown to result in the formation of 2-cell developmentally arrested mouse embryos [389]. Hence the percentage of 2-cell arrested embryos was used as a measure of developmental ability. Assessment of 2-cell developmental arrest showed that similar to the rate of development, despite the appearance of a linear increase in the percentages of 2-cell arrested embryos from in vivo fertilised zygotes to in vivo ovulated/ IVF oocytes to IVM /IVF oocytes, no statistical significance was observed between the experimental groups (Figure 3C).

3.3.1.4 Blastocyst Formation

The formation of a fluid filled cavity known as the blastocoel marks the final stage of preimplantation embryo development known as the blastocyst stage. As the fluid cavity expands and the number of blastocyst cells increase, cells of the blastocyst eventually break through the zona pellucida to form a hatching blastocyst. Both blastocyst formation and hatching blastocyst formation were therefore used as the final measures of embryo developmental competence in this study. To eliminate any bias from fertilisation success rates, the percentage of blastocysts and hatching blastocysts were expressed per 2-cell embryo and not per oocyte/zygote. Blastocyst formation appeared to be lower in IVM

derived embryos compared to those developed from in vivo obtained zygotes and in vivo ovulated and IVF oocytes. There was however, similar to the other embryo development parameters measured, no significant differences in the ability of the fertilised oocytes to develop into blastocyst or hatching blastocyts in any of groups (Figure 3D).

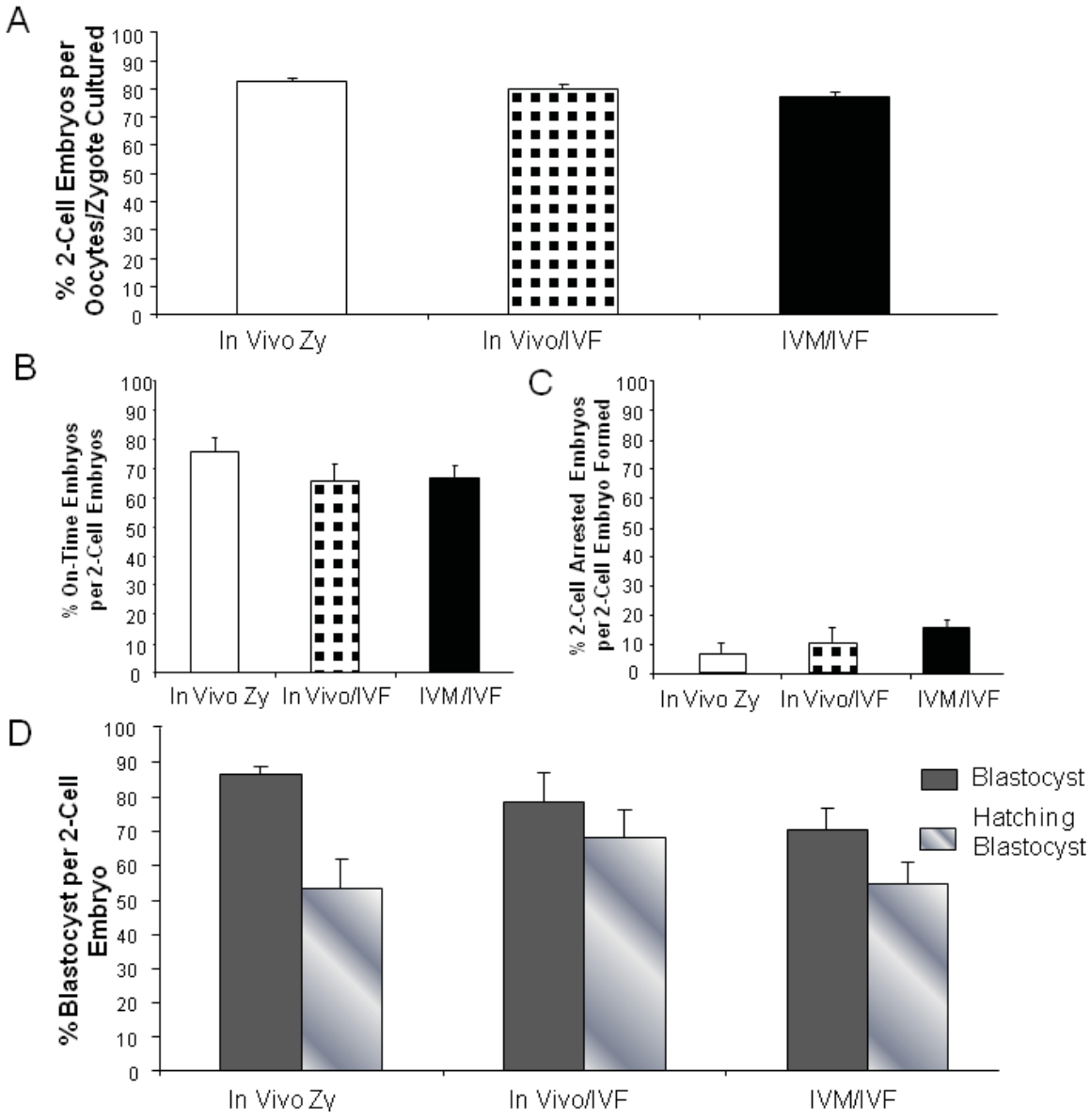


Figure 3. Effects of IVM, IVF and culture on developmental competence of embryos. 1-cell zygotes (In Vivo Zy) were obtained 24 hours after mating while in vivo ovulated COCs (In Vivo/IVF) and in vitro matured COCs (IVM/IVF) were fertilized in vitro then cultured. A) 2-cell embryo formation was determined by the percentage of 2-cell embryo per presumptive zygote or COC cultured. Rate of development was observed on day 3 of development and assessed by the percentage of 8-cell/compacted embryo (B) and 2-cell arrested embryos percentage (C) per 2-cell embryo developed. D) Blastocyst and hatching blastocyst were determined on day 5 of development. Data represents the mean ± SEM of approximately 400 presumptive in vivo obtained zygotes and 252-254 COCs cultured in groups of 10 from a total of 4 experimental replicates.

3.4 DISCUSSION

The success of in vitro fertilisation (IVF) procedures and embryo viability are dependent on the quality of the oocyte from which the embryo resulted [390]. This chapter therefore sought to investigate the differences in developmental competence between embryos obtained through various sources and thus establish the effects of in vitro maturation, in vitro fertilisation and in vitro culture on mouse embryo developmental competence in the experimental model of this thesis.

Three key stages, 2-cell embryo formation, rate of development and blastocyst formation, were chosen as key check points of oocyte developmental competence. Formation of a 2-cell mouse embryo marks the transition from maternal gene to zygotic gene dependence [391] and is the stage when zygotic gene activation occurs [392]. Oocytes arrest at meiosis metaphase II upon meiotic maturation, progressing only when fertilised to produce a pronucleate embryo which then has the ability to develop into a 2-cell therefore 2-cell formation was correlated to fertilisation rates. Mouse 1-cell zygotes also often form developmental blocks at the 2-cell stage when cultured in suboptimal culture conditions. This has been suggested to result from in vitro stresses since this 2-cell developmental arrest has not been reported to occur in vivo [389] hence the percentage of 2-cell arrested embryos were also observed. Rate of development was chosen as an assessment parameter as faster growing embryos have been shown to have increased pregnancy rates [387]. Unexpectedly, there were no significant differences in any of the parameters observed although there was a trend towards a decrease in the rate of embryo development and blastocyst formation and an increase in the percentage of developmentally arrested 2-cell

embryos in both in vivo and in vitro matured oocytes compared to in vivo fertilised zygotes.

It is important to note that due to the underlying aim of this thesis, which is to improve current IVM success rates, the IVM and embryo culture conditions in our experimental model had been optimised to the best possible level according to existing literature. This potentially could account for the lack of observable significant differences between the developmental competences of oocytes obtained from the various sources.

C57BL6/CBA hybrid mice were chosen as they have been shown to produce blastocysts with a low apoptotic index [393] and primed with PMSG which has been shown to have a beneficial effect on oocyte developmental competence [394]. IVM in Waymouth media produces maximal rates of blastocyst formation in the mouse [358] and IVM with serum has been shown to increase blastocyst formation [395] and blastocyst cell numbers [396]. COCs and embryos were cultured at 5% and not ambient oxygen concentration which has been shown to be detrimental to embryo viability (reviewed in [397]). Large culture media volumes and single embryo culture have been shown to negatively impact mouse blastocyst formation and cell numbers as embryos have been suggested to secrete autocrine and paracrine factors beneficial to their development [398-401]. Therefore, embryos were cultured in groups at the embryo/volume range of 0.70-0.25 embryo/µl media. As the requirements of the embryo changes as it progresses through the various developmental stages (reviewed in [402]), embryos were cultured in a sequential embryo culture system [403] utilising commercially available embryo culture media (G-1 and G-2 v3 Vitrolife, Kungsbacka, Sweden). Furthermore, all culture and handling media were pre-equilibrated

and handled on warming stages and culture media drops were over layered with mineral oil. This was done to minimise oocyte and embryo stress caused by sudden changes pH and temperature.

In conclusion, there were no statistically significant differences observed in developmental competence between in vivo obtained 1-cell zygotes, in vivo matured/IVF and IVM/IVF derived embryos, in 2-cell development, the percentage of 2-cell developmental arrest, rate of development and blastocyst formation in the employed experimental model. However, this was most likely because of the robust IVM and embryo culture system employed. It is also plausible that while there was no difference in the ability of these embryos to develop into blastocysts, there may have been a difference in blastocyst quality as denoted by blastocyst cell number and allocation [229]. Blastocyst quality was therefore investigated in all future experiments involving embryo development assessment in this thesis.

CHAPTER 4

THE ROLE OF CUMULUS EXPANSION ON OOCYTE DEVELOPMENTAL COMPETENCE

4.1 INTRODUCTION

In mammalian Graafian follicles, mural granulosa cells line the follicular wall while a set of highly specialized somatic cells known as cumulus cells surround the oocyte, forming the cumulus oocyte complex (COC). These layers of granulosa and cumulus cells mediate external endocrine signals to the oocyte enabling oocyte growth and maturation.

Prior to the ovulatory luteinizing hormone (LH) surge, cumulus cells are connected to the oocyte by trans-zonal cytoplasmic processes that facilitate intercellular gap junction communication [404]. Through these gap junctions, cumulus cells are able to transport amino acids and key metabolites that are essential for oocyte growth and development [353] and signalling molecules such as cyclic adenosine monophosphate (cAMP) maintaining the oocyte arrested at meiotic prophase 1. While the oocyte had been previously thought to be a passive recipient, findings in the last decade [405, 406] have now established the existence of a bi-directional communication loop between the oocyte and its surrounding cumulus cells. Oocytes have been shown to be the key regulators of follicular development [35] and shown to influence cumulus cell glycolytic activity [282] as well as amino acid transport [226]. Oocytes also secrete mitogens eliciting cumulus and granulosa cell proliferation [265, 341, 407], lower the apoptotic index of cumulus cells [364] and prevent cumulus cell luteinisation [408], thereby maintaining the distinct cumulus cell phenotype [407].

One of the functions dependent on this oocyte to cumulus bi-directional communication is cumulus expansion, a process where cumulus cells form a hyaluronan rich viscoelastic matrix in response to the appropriate ovulatory signals, leading to an eventual loss in gap

junction communication and resumption of meiosis [271, 409, 410]. Cumulus expansion facilitates ovulation and is critical for female fertility in vivo as mice with defective cumulus cell matrix formation are anovulatory and have compromised fertility [47].

In vitro, cumulus expansion can be stimulated through the use of follicle stimulating hormone (FSH) and epidermal growth factor (EGF) ligands [57, 73]. However, these alone are insufficient to induce cumulus expansion without an oocyte-secreted cumulus enabling factor since mouse COCs with surgically removed oocytes are unable to expand unless co-cultured with denuded oocytes or with recombinant oocyte secreted factors such as growth differentiation factor 9 (GDF9) [271, 274, 318]. Similarly, studies in our laboratory have shown that the oocyte-secreted cumulus expansion enabling factor is unable to induce cumulus expansion on its own without ligand (e.g. FSH) stimulation [274]. Cumulus expansion thus can be regarded as a successive measure of the ability of oocytes and cumulus cells to communicate during maturation.

IVM routinely involves the transfer of COCs from their enclosed follicular environment into culture media, which potentially compromises the complexity of somatic-oocyte cellular interactions that occur in vivo. This chapter thus sought to investigate the effect of 1) inhibiting endogenous oocyte to cumulus cell bi-directional communication then 2) adding exogenous recombinant GDF9, on cumulus expansion in order to understand its contribution to oocyte developmental ability investigated in later chapters.

To inhibit oocyte to cumulus communication, the SMAD2/3 signalling pathway was targeted since activation of SMAD2/3 signalling is crucial for cumulus expansion [411].

This was achieved through the use of SB-431542, a small molecule that acts as a competitive ATP binding site kinase inhibitor highly specific for the receptor kinases that activate SMAD2/3 signalling when used at <10μM [363]. These are the type I receptors, transforming growth factor beta 1 receptor (TGFBR1), also known as the activin receptor-like kinase 5 (ALK5) which GDF9 signals through, ACVR1B (ALK4) and ACVR1C (ALK7) [300]. Since cumulus cells translate external ligand stimulatory signals such as FSH and EGF to the oocyte, cumulus to oocyte communication was inhibited through the absence of these stimulatory ligands.

It is however important to note that while cumulus expansion could potentially reflect the degree of bi-directional communication between the oocytes and their surrounding cumulus cells, it is unclear if the physical act of expansion itself is necessary for oocyte developmental competence given recent findings [58, 119, 130, 412] that suggest that while there is a fundamental need for cumulus cells during oocyte maturation, cumulus expansion itself is not mandatory for oocyte development in vitro.

In order to establish if the oocyte developmental competence results observed in later chapters were dependent on the physical act of cumulus expansion, cumulus expansion was also inhibited through inhibition of hyaluronic acid (hyaluronan) synthesis. Hyaluronic acid is the main component of the COC extracellular matrix and is synthesised through the hexosamine biosynthesis pathway (HBP) (Figure 4.1) from glucose or glucosamine [90, 413]. Glutamine-fructose-6-phosphate transaminase 1 (GFPT1), formerly known as Glutamine: Fructose-6-Phosphate Amidotransferase (GFAT), converts fructose-6-phosphate to glucosamine-6-phosphate using glutamine as the amide donor and is both the

first and rate-limiting enzyme of the HBP [414]. O-diazoacetyl-L-serine (azaserine) a glutamine analogue which acts as a competitive inhibitor has been shown to inhibit GFPT1 activity and thus the production of HBP end products such as hyaluronic acid [133, 147, 415].

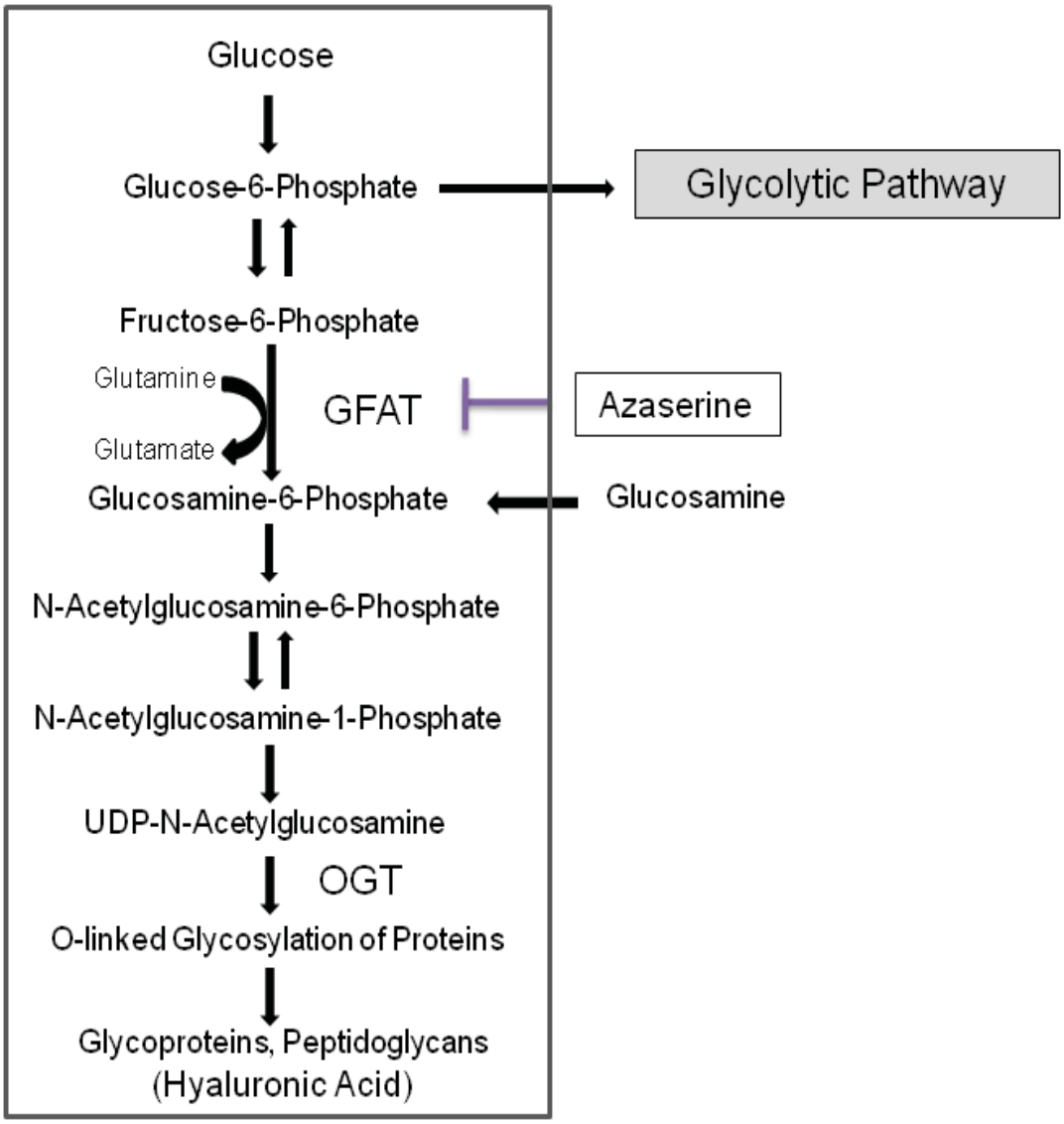


Figure 4.1. Hexosamine Biosynthesis Pathway

Previous work from our laboratory found that cumulus expansion and expression of some of the cumulus matrix genes was substantially decreased when COCs were matured with azaserine (Figure 4.2) [1]. This chapter thus also sought to investigate the effect of attenuation of cumulus expansion with azaserine on oocyte developmental competence.

Figure 4.2. Previously observed cumulus expansion and Has2 gene expression. Adapted from the Honours Thesis of Kathryn Gebhardt [1]

4.2 MATERIALS AND METHODS

4.2.1 Collection and Culture of COCs

COCs were collected and cultured as in Chapter 2.1 and 2.2.1 of General Experimental Methods.

4.2.2 Treatments

Treatment 1: Inhibition of Oocyte to Cumulus Bi-directional Signalling

Cumulus to oocyte signalling was enabled by culture of COCs were cultured in the presence of FSH and EGF and compared to maturation in the absence of 50 mIU/ml FSH and 10 ng/ml EGF. Oocyte to cumulus signalling was disrupted through the inhibition of SMAD2/3 signalling using 4μM SB-431542 and compared to the equivalent 0.04% DMSO carrier control. See Chapter 2.3.1 and 2.3.2 for details of FSH/EGF and SB-431542 respectively.

Experiment 2: Addition of Exogenous GDF9

COCs were cultured with 200 ng/ml of recombinant GDF9 or the equivalent 7% v/v of its 293H parent cell line control conditioned media both in the absence and presence of 50 mIU/ml FSH and 10 ng/ml EGF. See Chapter 2.3.3 for exogenous GDF9 details.

Experiment 3: Inhibition of Cumulus Expansion using Azaserine

O-diazoacetyl-L-Serine (azaserine) was purchased from Sigma-Aldrich Chemical Co, (St Louis, MO, USA) and dissolved in MilliQ water to a stock concentration of 10 mM and stored at -20°C according to the manufacturer's instructions. Azaserine was used at a final

concentration of 10 µM and 100 µM in 3 ml maturation media in the presence of 50 mIU/ml FSH and 10 ng/ml EGF.

4.2.3 Cumulus Expansion Assessment

After 17.5-18 hours of culture, culture dishes were placed under an Olympus Ck2 ULWCD 0.30 inverted microscope (Olympus Corporation, Japan) at 100x magnification for analysis of cumulus expansion. COCs were not removed from their culture environment to prevent mechanical perturbations to the cumulus matrix. Cumulus expansion was assessed blinded to treatments according to the Vanderhyden 0-4 scale and the cumulus expansion index (CEI) was calculated as described [126, 416]. Using this scale, score 0 indicates no detectable response characterized by the detachment of cumulus cells from the oocyte to assume a flattened monolayer of fibroblastic appearance. A score of +1 indicates the minimum observable response where COCs remain spherical, cumulus cells have a glistering appearance, and most cells remain compacted around the oocyte. For score +2 complexes, only the outer most layers of cumulus cells have expanded, score +3 complexes have all layers except the corona radiata (cells most proximal to the oocyte) prominently expanded and a score of +4 indicates the maximum degree of expansion including the corona radiata [126].

4.2.4 Analysis of Cumulus Gene Transcripts

Total RNA was extracted from groups of 20 COCs and reversed transcribed as in Chapters 2.9.2 and 2.11. First strand cDNA samples were then diluted 1 in 10 with Rnase free water and 2 µl of this was analysed per PCR reaction. Each PCR reaction thus contained cDNA from approximately 0.2 of a COC. Gene expression was then analysed through real time PCR using the $2^{-\Delta\Delta Ct}$ method [379] as described in Chapter 2.12. The genes analysed were

Hyaluronan Synthase 2 (Has2) and *prostaglandin-endoperoxide synthase 2(Ptgs2)* while *Ribosomal protein L19 (Rpl19)* was used as the housekeeping gene. Primer pairs and sequences are as listed below in Table 4.1. Relative gene expression is expressed as the mean $2^{-\Delta\Delta Ct}$ value of all experimental replicates.

Table 4.1. Murine Real Time PCR Primer Sequences

Gene	Oligonucleotide Sequence (5'-3')	Amplicon Length (Base Pairs)	Genbank Accession Number
Rpl19	F: TTC CCG AGT ACA GCA CCT TTG AC R: CAC GGC TTT GGC TTC ATT TTA AC	103	NM_009078
Has2	F: GCA TTG GTT ACC CAT GAA TTC R: GGC TGT GTC CAG TGC ATA AGC	105	NM_008216
Ptgs2	F: AGA TCC ACA GCC TAC CAA AAC AG R: CTC AGT TGA ACG CCT TTT GAT TAG	103	NM_011198

4.2.5 Determination of Oocyte Developmental Competence after Maturation with Azaserine

At the end of the oocyte maturation period, COCs underwent in vitro fertilization, embryo culture and differential staining of blastocyst to determine oocyte developmental competence. Refer to Chapters 2.4-2.7 for experimental procedures.

4.2.6 Statistics

Cumulus expansion, embryonic development and real time PCR results were analysed using a univariate general linear analysis of variance with experiment day as a covariate to analyse the degree of and account for variance between experiments. Least significant differences (LSD) was used when treatments had equal error variance and a Dunnett T3 post hoc was used when treatments had unequal error variance. Treatments with a P value

of <0.05 were taken to be significantly different. All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) version 13.0 for windows. Data are expressed as mean ± SEM.

4.3 RESULTS

4.3.1 Effects of FSH/EGF and SMAD2/3 Signalling During IVM on Cumulus Expansion

As expected and shown in Figure 4.3, cumulus expansion did not occur in the absence of FSH/EGF (CEI = 0.3 ± 0.2 (N=20 COCs) vs. 2.6 ± 0.2 (N=25 COCs) for +FSH/EGF). Addition of SB-431542 in the presence of FSH/EGF also significantly reduced cumulus expansion (CEI = 0.6 ± 0.2 (N=26 COCs)) to levels similar to that when FSH/EGF were absent. Interestingly, SB-431542 and absence of FSH/EGF did have an interactive combined negative impact on the morphology of the COC complexes observed at the end of the maturation period. Almost all complexes had total detachment of the cumulus cells from the oocytes to assume a flattened monolayer of fibroblastic appearance adhered to the bottom of the culture dish rendering most oocytes completely denuded. As such an observation had not been previously described within the Vanderhyden scoring system [126], this treatment was excluded from cumulus expansion analysis. There was no significant difference in cumulus expansion between FSH/EGF (2.6 ± 0.2; N=25 COCs) and the carrier control (DMSO + FSH/EGF; 3.1 ± 0.3 (N=27 COCs), $P>0.05$).

4.3.2 Effect of Exogenous GDF9 During IVM on Cumulus Expansion

As illustrated in Figure 4.4, exogenous GDF9 during in vitro maturation significantly increased ($P<0.05$) cumulus expansion in the presence of FSH and EGF. The CEI was 3.1 $\pm$ 0.2 (N=36 COCs) with exogenous GDF9 compared to a CEI of 2.4 $\pm$ 0.1 (N=39 COCs) for

the 293H control. Although minimal cumulus matrix was observed in the absence of FSH/EGF, exogenous GDF9 resulted in a higher number of COCs staying intact with a CEI of 0.6 $\pm$ 0.1 (N=28 COCs), compared to the 293H control which had more partially denuded oocytes resulting in most of the cumulus cells attaching to the substratum (CEI= 0.2 $\pm$ 0.1 (N=20 COCs)) ($P<0.05$). No significant differences were observed between the IVM and 293H controls.

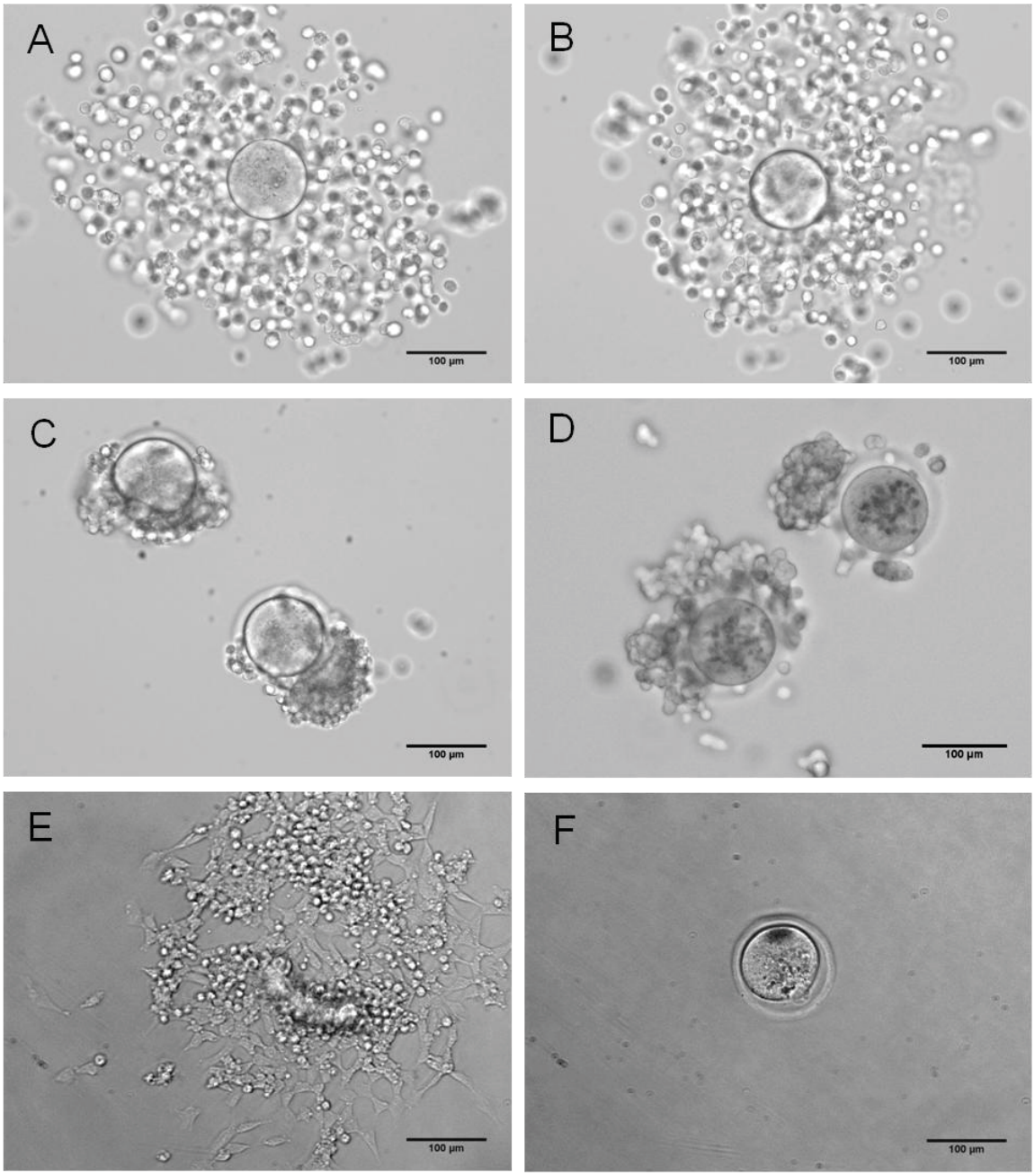


Figure 4.3. Effects of FSH/EGF and/or SMAD2/3 signalling during IVM on cumulus expansion. COCs were matured for 18 hours in the various treatments, IVM with 50 mIU/ml FSH and 10 ng/ml EGF (A), DMSO carrier control: 4% v/v DMSO + FSH/EGF (B), IVM without FSH/EGF (C), SB-431542 + FSH/EGF (D) and SB-431542 without FSH/EGF (E and F). Images were captured on a Nikon Eclipse TE-2000E inverted microscope (Nikon Corporation, Japan) 18 hours after IVM at 20x magnification.

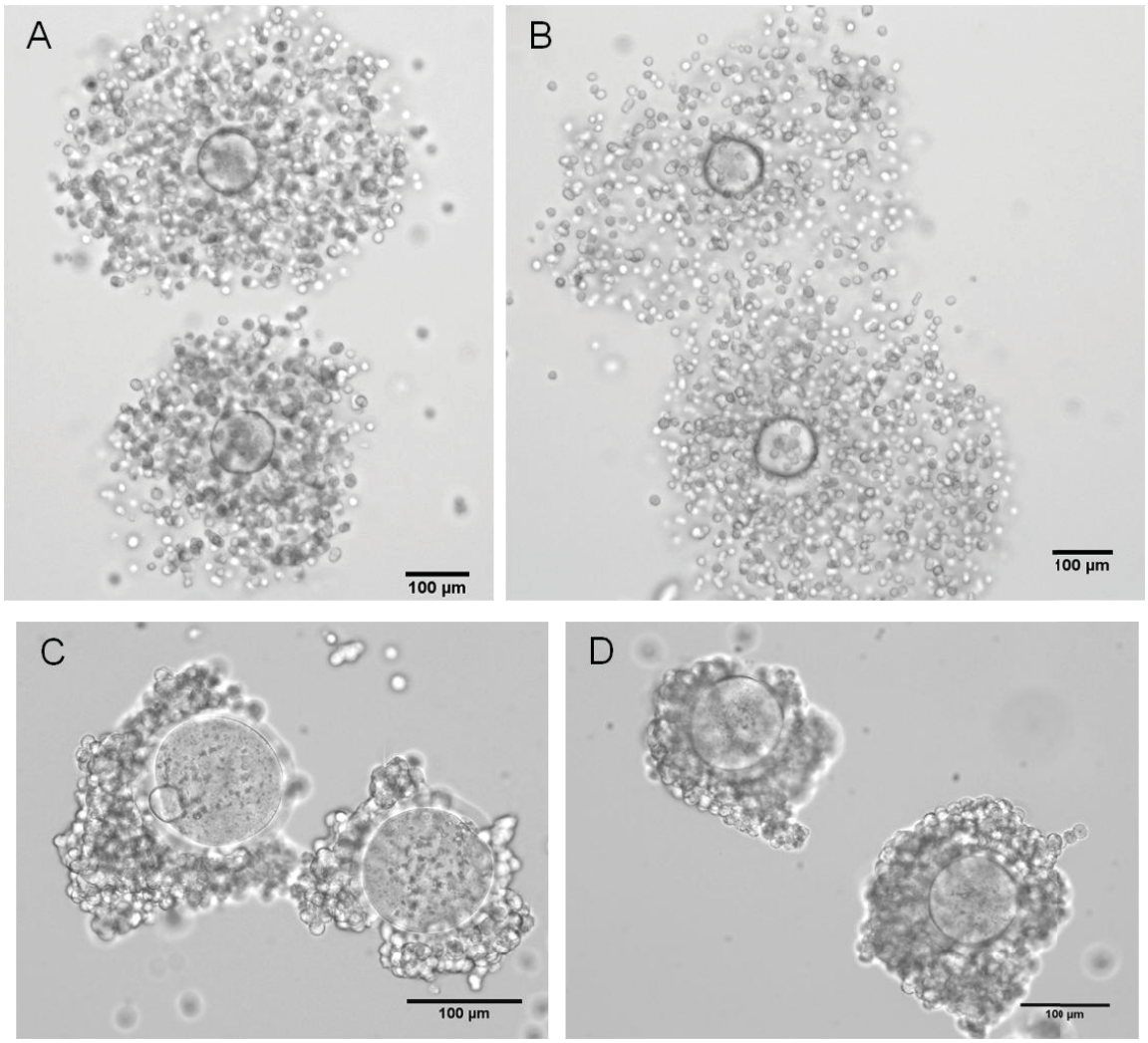


Figure 4.4. Effect of exogenous GDF9 on cumulus expansion. COCs were cultured with either the equivalent 7% v/v 293H parent cell line control conditioned media or exogenous GDF9 (200 ng/ml) in the presence or absence of 50 mIU/ml FSH and 10 ng/ml EGF. Images were captured on a Nikon Eclipse TE-2000E inverted microscope (Nikon Corporation, Japan) 18 hours after IVM at 10x magnification for 293H+FSH/EGF (A) and GDF9+FSH/EGF (B) and at 20x magnification for 293H-FSH/EGF (C) and GDF9-FSH/EGF (D).

4.3.3 Effect of Azaserine on Cumulus Matrix Gene Transcripts

Addition of azaserine both at 10 µM and 100 µM during IVM did not have an observable effect on cumulus expansion in our IVM system. This contradicts previous observed results where addition of azaserine at these concentrations during IVM significantly decreased cumulus expansion (Figure 4.2). COCs were thus collected for the analysis of gene transcripts *Has2* and *Ptgs2* which protein products are essential for cumulus expansion (N=3 independent experimental replicates). Using IVM with FSH/EGF as the calibrator control, we found no significant differences in either *Has2* or *Ptgs2* gene expression in COCs matured in the presence of 10 or 100 µM azaserine compared to those matured without azaserine (Figure 4.5).

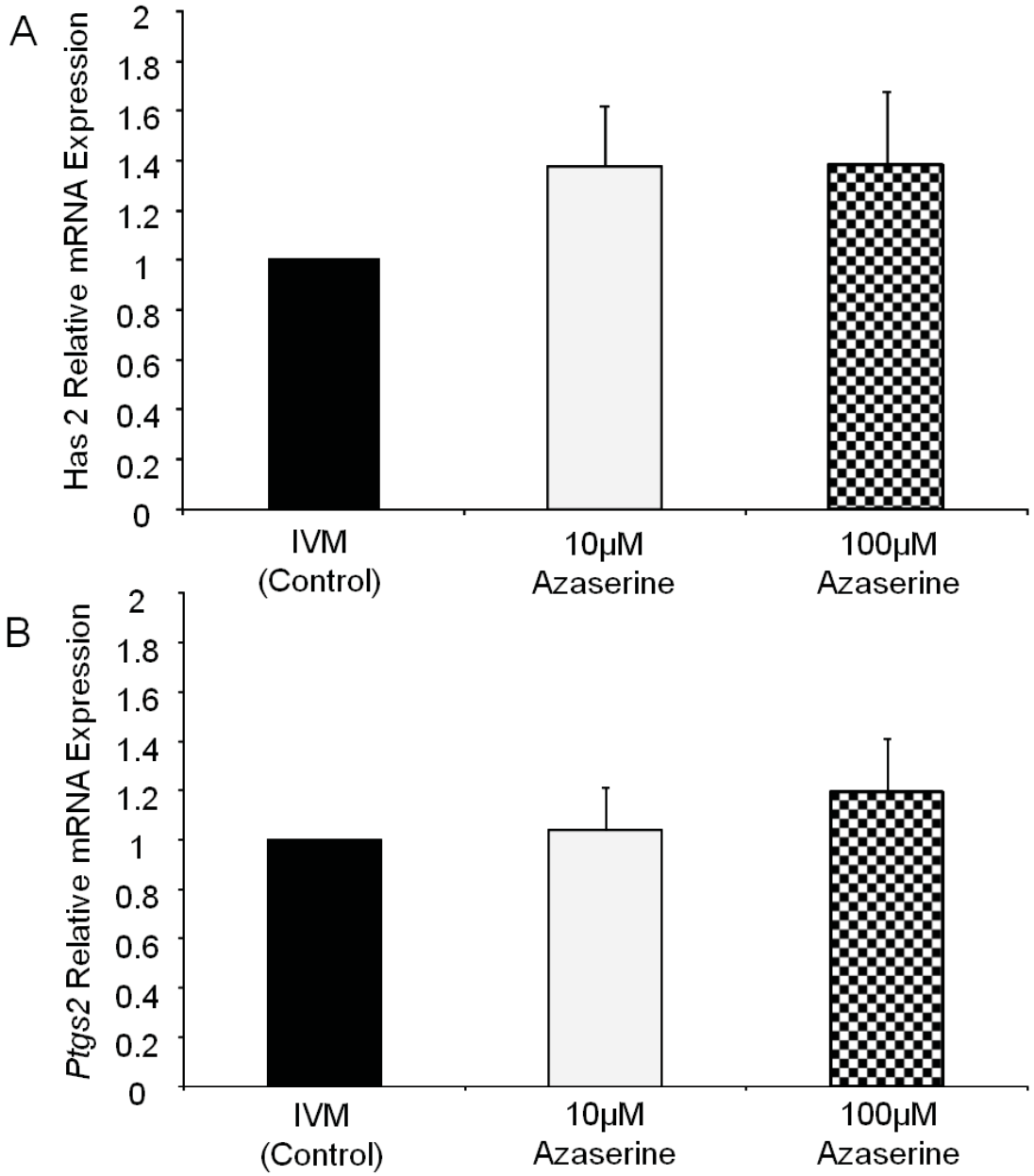


Figure 4.5. Expression of cumulus cell transcripts Has2 and Ptgs2 after IVM with azaserine. mRNA expression of *Has2* and *Ptgs2* extracted from groups of 20 COCs matured with either 50 mIU/ml FSH and 10 ng/ml EGF (IVM Control), FSH/EGF + 10 μM azaserine (10 μM Azaserine) or FSH/EGF + 100 μM azaserine (100 μM Azaserine) reverse transcribed and analysed by real time PCR. The mRNA expression of (A) *Has2* and (B) *Ptgs2* are normalised to the *Rpl19* endogenous control and presented as mean ± SEM (n = 3 independent experiments) *Has2* and *Ptgs2* are expressed relative to the calibrator (IVM Control) which was set at 1.

4.3.4 Effect Of Azaserine During IVM On Subsequent Embryo Development

Oocytes matured in the presence of either 10 or 100 µM of azaserine were then fertilised to investigate the effect of azaserine on subsequent embryo development. While 10 µM azaserine had no observable effect on the ability of embryos to form 2-cells or blastocysts, it significantly reduced the percentage of on-time developed embryos at the 8-cell or compacted embryo stages after 49-51 hours of culture. Maturation with 100 µM of azaserine however, not only significantly decreased 2-cell embryo formation despite the lack of an observable effect on cumulus expansion, but drastically reduced on-time embryo development and blastocyst formation compared to when no or only 10 µM of azaserine was used (N=10 – 14 replicates from 3 independent experiments; no statistical difference was found between independent experimental within each treatment P>0.05) (Table 4.2).

Table 4.2. Effect of azaserine during IVM on subsequent embryo development.

Treatment	2-cell Embryos After 20 h of Culture Per IVM Oocyte (%)	8-cell/Compacted Embryos After 49-51 h of Culture Per 2-cell Embryos (%)	Blastocyst After 96-100 h of Culture Per 2-cell Embryo (%)	Hatching Blastocyst After 96-100 h of Culture Per 2-cell Embryo (%)
IVM (Control)	64.0 ± 8.0 ^{a}	67.0 ± 4.4 ^{d}	77.1 ± 5.1 ^{g}	57.2 ± 6.1 ^{i}
10µM Azaserine	49.8 ± 4.4 ^{a,b}	49.3 ± 6.3 ^{e}	72.1 ± 7.7 ^{g}	40.8 ± 10.8 ^{i}
100µM Azaserine	48.9 ± 4.3 ^{b}	1.8 ± 1.8 ^{f}	18.9 ± 7.8 ^{h}	7.2 ± 3.6 ^{j}

Data represents average percentage ± SEM of at least 10 replicates representing a minimum of 100 oocytes (approximately 10 oocytes per replicate) from 3 separate experiments. All COC's were matured in the presence of FSH and EGF. Values with different superscripts $^{a-j}$ represent statistically significant differences (P<0.05).

4.3.5 Effect Of Azaserine During IVM On Blastocyst Quality

Blastocysts were then differentially stained to determine blastocyst cell numbers and types as an indicator of blastocyst quality as shown in Table 4.3. No significant differences were observed in total, ICM or TE cell numbers in embryos derived from oocytes matured with 10 µM azaerine compared to the control. Very small numbers of blastocysts were produced from oocytes that were matured with 100 µM azaserine and therefore statistical comparisons could not be performed for this group however the blastocysts that were produced had lower numbers of total, and TE cells (N=2-16 blastocyst from 2 independent experiments; no statistical difference was found between independent experimental within each treatment $P>0.05$).

Table 4.3. Effect of azaserine during IVM on blastocyst quality

Treatment	Total Cell Numbers	ICM Cell Numbers	TE Cell Numbers
IVM (Control)	45.8 ± 3.4	13.3 ± 1.0	32.4 ± 2.8
10µM Azserine	52.8 ± 6.9	13.5 ± 2.1	39.3 ± 5.2
100µM Azaserine	31.0 ± 8.0	12.0 ± 6.0	19.0 ± 2.0

Data represents mean cell number ± SEM of 2-16 blastocysts derived from 2 separate experiments. All COCs were matured in the presence of 50 mIU/ml FSH and 10 ng/ml EGF.

4.4 DISCUSSION

In vivo, the formation of a viscoelastic cumulus matrix in response to ovulatory signals is vital for female fertility as mice defective for any of the components necessary for the formation of this cumulus matrix develop entrapped COCs or ovulate partially denuded oocytes and thus are either sterile or severely sub-fertile [87-89, 104, 417]. Many studies have positively correlated the degree of cumulus expansion [418, 419] or the expression of genes associated with cumulus expansion [128] with oocyte developmental competence after IVF. Yet recent research have suggested that it is not cumulus expansion itself but the relationship between cumulus cells and the oocyte during oocyte maturation that affects oocyte developmental competence, since blocking gap junction communication decreased subsequent blastocyst formation despite normal cumulus expansion and fertilisation [58] and maturing oocytes enclosed with only the corona radiata on a monolayer of cumulus cells rescued subsequent embryo formation to levels equal to that of intact COCs [130]. Hence it is plausible that while the presence of cumulus cells is important to acquisition of oocyte developmental competence, the act of expansion itself is not [58, 130].

In vitro, FSH and EGF are commonly used to trigger cumulus expansion. FSH and EGF induce cumulus expansion by up-regulating transcripts necessary for matrix formation, such as hyaluronan synthase 2 (*Has2*) and prostaglandin synthase 2 (*Ptgs2*) [73], however importantly, these FSH and EGF mediated functions are critically dependent on the presence of oocyte-secreted factor signalling in cumulus cells, as cumulus expansion cannot occur in the absence of an oocyte [126, 271] or recombinant oocyte secreted factors such as growth differentiation factor 9 (GDF9) [271, 274, 318]. Similarly, the oocyte-secreted cumulus expansion enabling factor is unable to induce cumulus expansion on its own

without ligand (e.g. FSH) stimulation [274]. Cumulus expansion thus can be regarded as a successive measure of the ability of oocytes and cumulus cells to communicate during maturation.

The aim of this chapter was therefore to investigate the effect of oocyte and cumulus bi-directional signalling on cumulus expansion and the effect of inhibiting cumulus expansion independent of this signalling on oocyte developmental competence.

The effects of GDF9 are mediated through activation of the SMAD2/3 pathway upon binding to its serine/threonine kinase type-1 receptor, TGFBR1 [308]. The effects of inhibiting SMAD2/3, with and without the effects of cumulus mediated FSH and EGF signalling were thus investigated utilising the small molecule inhibitor SB-431542. SB-431542 is a competitive ATP binding site kinase inhibitor and was first identified as a potent inhibitor of TGFBR1 (ALK5) [420]. A subsequent study characterising the specificity of SB-431542 showed that ACVR1B (ALK4) and ACVR1C (ALK7), the other kinases that activate SMAD2/3 signalling [300], were also specifically inihibited. Although other TGFβ type I receptors ACVRL1 (ALK1) and ACVR1 (ALK2), and ERK, JNK or MAPK signalling pathways were unaffected by SB-431542, when used at 10 μM, BMPR1A, BMPR1B and the p38α MAPK signalling pathway were weakly influenced [363]. SB-431542 is thus a potent specific inhibitor of SMAD2/3 signalling and has been extensively used both in oocyte studies [272, 341, 354] and in other cell types [317, 421, 422]. As SB-431542 had previously been shown to have the highest specific inhibition of cumulus cell expansion gene expression at 4 μM [272] and was also used at 4 μM to study

oocyte paracrine signalling in bovine COCs [354], 4 μM was the chosen concentration used in this thesis.

In accordance with previous findings [411], inhibition of SMAD2/3 with SB-431542 in the presence of FSH and EGF significantly decreased cumulus expansion to levels similar to that when FSH and EGF were not used. Inhibition of SMAD2/3 in the absence of FSH/EGF had an additive effect, resulting in almost all cumulus cells assuming fibroblastic appearances, plating down at the end of the maturation period leaving fully denuded oocytes. Stimulation of the MAPK pathway through increased cAMP levels in cumulus cells leads to cumulus expansion and both GDF9 and FSH/EGF have been shown to activate MAPK through independent pathways [66, 76, 187], increasing cumulus expansion gene transcripts such as *Ptgs2* and *Has2* [47]. However, FSH and/or EGF together with an oocyte-secreted cumulus expansion enabling factor, such as GDF9, must be present for mouse cumulus expansion [271, 274] and it has been suggested that oocyte paracrine factors 'license' MAPK activation by cAMP in cumulus cells [76]. Taken together, this could explain the combined negative effect on cumulus expansion.

To investigate the possibility that the complexities of somatic-oocyte cellular interactions that occur in vivo are impeded in vitro during the routine transfer of COCs from their enclosed follicular environment into culture, exogenous GDF9 was then added during IVM. Indeed, exogenous GDF9 did significantly increase cumulus expansion in the presence of FSH and EGF and, in accordance with published literature [274], while it did not stimulate cumulus expansion in the absence of FSH and EGF, it did result in more cumulus enclosed oocytes after IVM when compared to the control.

In order to establish if the oocyte developmental competence results observed in later chapters may be dependent on the physical act of cumulus expansion, cumulus expansion was then obstructed via a method independent of cumulus to oocyte bi-directional communication. Azaserine, a glutamine analog, was used to inhibit production of the key component of the cumulus matrix, hyaluronic acid (hyaluronan) through competitive inhibition of GFPT1, the key regulatory enzyme of the hexosamine biosynthesis pathway (HBP) of which hyaluronan is an end product. Interestingly, contrary to previous findings [1], addition of 10 or 100 μM of azaserine had no observable effect on cumulus expansion or expression of cumulus gene transcripts *Has2* and *Ptgs2*. The lack of observable attenuation in cumulus expansion could be attributed to firstly, the substantially higher concentration of 27.8 mM glucose in our maturation media (Waymouth MB 752/1) and secondly the more than doubled amount of 2.4 mM glutamine compared to that previously used (Defined Maturation Media: 0.5 mM glucose and 1.0 mM glutamine) [1]. Given the vast supply of glucose and higher substrate competition from glutamine in Waymouth medium, hyaluronan synthesis may not have been sufficiently perturbed to warrant a noticeable effect on the expanded cumulus matrix.

Surprisingly, despite the seemingly unperturbed cumulus expansion, subsequent embryo development was severely compromised as shown by the significant decrease in 2-cell formation and drastic reductions in developmental rate and the ability to form blastocyst when 100 μM of azaserine was used during IVM. While 10 μM of azaserine had no significant effect on 2-cell or blastocyst formation, a significant reduction in rate of development was also noted with this lower dose of azaserine. This is in agreement with a

previous report of decreased blastocyst formation in bovine COCs matured with another GFPT1 inhibitor, 6-diazo-5-oxo-L-norleucine (DON) [119].

Although the HBP only accounts for 1-3% of cellular glucose metabolism [133], it's primary end product, uridine 5'-diphospho-N-acetylglucosamine (UDP-GlcNAc), is the substrate for O-linked glycosylation of various proteins including transcription factors, signal transduction proteins and nuclear hormone receptors, altering their activity through O-linked glycosylation of serines and threonines that could otherwise be phosphorylated [423]. Indeed, changes in HBP flux has been shown to alter the activity of critical regulatory kinase pathways such as protein kinase A and C [415, 424]. The HBP has also been implicated to be the nutrient sensing pathway of the early embryo [147]. Zygotes cultured without glucose cannot form blastocysts unless exposed to glucose or glucosamine prior to the blastocyst stage [147, 425], however addition of azaserine negated the effect of glucose but not glucosamine on blastocyst formation and the activation of SLC2A3, a high-affinity glucose transporter essential for blastocyst formation [147]. Hence the HBP has a crucial role in metabolic programming of the early embryo.

It is also important to note that the HBP has been implicated to be the first line of defence against cellular stress and trauma in mammalian somatic cells [140] since stress stimuli increased protein O-linked glycosylation levels [141] and inhibition of GFPT1 resulted in decreased cell survival after heat stress [142]. However, over stimulation of the HBP through addition of glucosamine has the potential to inhibit the pentose phosphate pathway and increase oxidative stress [139]. Bovine oocytes also showed decreased blastocyst development despite no differences observed in cumulus expansion when matured in the

presence of glucosamine [136]. The HBP pathway must thus function within a finely balanced equilibrium since alterations either way perturbs cellular health.

Thus while cumulus expansion was not affected by the addition of azaserine during IVM, alterations in o-linked glycosylation, and hence signalling regulatory pathways, and perturbations in metabolic programming potentially accounts for the decrease in oocyte developmental competence.

In conclusion, the findings of this chapter have shown that while cumulus expansion is a successive reflection of oocyte to cumulus bi-directional signalling, in agreement with published reports [119, 130, 136], the physical act of expansion itself is independent of oocyte developmental competence in vitro.

CHAPTER 5

DISRUPTION OF BI-DIRECTIONAL OOCYTE-CUMULUS PARACRINE SIGNALLING DURING IN VITRO MATURATION REDUCES SUBSEQUENT EMBRYO DEVELOPMENT AND FOETAL VIABILITY

Data presented in this chapter has been published: **Yeo CX**, Gilchrist RB, Lane M. Disruption of Bi-directional Oocyte-Cumulus Paracrine Signalling During In-Vitro Maturation Reduces Subsequent Mouse Oocyte Developmental Competence. Biology of Reproduction 2009 May; 80(5):1072-80.

Yeo, C.X., Gilchrist, R.B. and Lane, M. (2009) Disruption of Bidirectional Oocyte-Cumulus Paracrine Signaling During In Vitro Maturation Reduces Subsequent Mouse Oocyte Developmental Competence
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CHAPTER 6

ADDITION OF EXOGENOUS GROWTH DIFFERENTIATION FACTOR 9 DURING OOCYTE MATURATION ENHANCES SUBSEQUENT EMBRYO DEVELOPMENT AND FOETAL VIABILITY

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6.1 INTRODUCTION

In vitro maturation of oocytes (IVM) poses an attractive alternative to the use of exogenous hormonal stimulation for assisted reproductive techniques (ART) which has a number of adverse side effects, the most serious being ovarian hyperstimulation syndrome. Unfortunately, oocytes matured in vitro result in embryos with compromised development compared to oocytes matured in vivo [438, 439] and result in lower pregnancy rates per embryo transfer [12, 440, 441]. Hence, there is a great need to improve our understanding of what regulates an oocyte's developmental potential, as well as to improve oocyte IVM efficiency.

As shown in the previous chapters, oocyte to cumulus bi-directional signalling directly impacts subsequent blastocyst quality and foetal survival. IVM routinely involves the transfer of COCs from their enclosed follicular environment into culture media, which potentially compromises the complexity of somatic-oocyte cellular interactions that occur *in vivo* due to dissipation of native oocyte-secreted factors into the surrounding media. Thus the reduced developmental competence of IVM oocytes may possibly be attributed to inappropriate levels or composition of oocyte-secreted factors. Indeed, the addition of native or recombinant oocyte-secreted factors during bovine IVM improved post-IVF pre-implantation embryo development, although subsequent developmental potential post-transfer was not examined [354]

The specific oocyte-secreted factors that are involved in this bi-directional regulatory loop remain currently unclear, however, a prime candidate is growth differentiation factor 9

(GDF9), a member of the transforming growth factor beta (TGFβ) superfamily. GDF9 is an oocyte-specific paracrine factor expressed throughout most stages of folliculogenesis [310, 318] and persists after fertilisation through pre-implantation embryo development [442]. Previous studies have demonstrated the ability of GDF9 to mimic certain oocyte functions in the absence of the oocyte, including regulating cumulus cell progesterone synthesis [443], suppressing luteinizing hormone (LH) receptor expression [318] promoting granulosa cell growth [341] and regulating cumulus expansion [274, 318]. In accordance with these studies, results from Chapter 4 demonstrated a significant increase in cumulus expansion with exogenous GDF9 addition in intact COCs during oocyte IVM. These known regulatory roles demonstrate that GDF9 is a key regulator of normal cumulus cell function. Furthermore, the previous chapter has shown that disruption of the SMAD2/3 pathway, of which GDF9 signals through, resulted in decreased embryo quality and foetal development. Therefore, there is strong implication that GDF9 is a key element in the oocyte-cumulus regulatory loop.

The aim of this chapter was thus, to investigate the effects of exogenous GDF9 during mouse IVM on subsequent embryonic development and foetal outcomes both in the presence and absence of the common IVM additives FSH and EGF.

6.2 MATERIALS AND METHODS

6.2.1 Collection and Culture of COCs

COCs were collected and cultured as in Chapters 2.1 and 2.2.1 of General Experimental Methods.

6.2.2 Treatments

COCs were cultured for 18 hours with 200 ng/ml of recombinant GDF9 or the equivalent 7% v/v of its 293H parent cell line control conditioned media both in the absence and presence of 50 mIU/ml FSH and 10 ng/ml EGF. See Chapter 2.3.1 for exogenous GDF9 details.

6.2.3 Determination of Oocyte Developmental Competence after Maturation

At the end of the oocyte maturation period (18 hours), COCs underwent in vitro fertilization, embryo culture and differential staining of blastocysts to determine oocyte developmental competence and blastocyst quality as reflected by blastocyst cell number and allocation to the inner cell mass (ICM) and trophectoderm (TE). Refer to Chapters 2.4 to 2.7 for experimental procedures.

6.2.4 Determination of Subsequent Foetal Outcomes

Swiss female recipient mice were mated with vasectomized CBA/C57BL6 males (See Chapter 2.8.1 for details on male mice vasectomy) and anaesthetized on Day 3.5 of pseudopregnancy prior to embryo transfer. Refer to Chapter 2.8.2 for embryo transfer

experimental procedures. Six blastocysts from one treatment were randomly assigned to each uterine horn. A total of 48 embryos were transferred per treatment to a total of 8 recipient mothers. On day 15 of pregnancy, the percentage of implantations, foetal development, foetal and placental weight and foetal crown to rump length were assessed as described in Chapter 2.8.3.

6.2.5 Statistics

Embryonic development was analysed using a three by two-way and/or univariate general linear analysis of variance utilising least significant differences when treatments had equal error variance and a Dunnett T3 post hoc when treatments had unequal error variance. Experiment day was used as a covariate to analyse the degree of and account for variance between individual experiments. Foetal outcomes were assessed using Chi-square or paired student t-tests. Treatments with a P value of <0.05 were taken to be significantly different. All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) version 13.0 for windows. Data is expressed as mean ± SEM and represents 13 or more replicates from a minimum of 6 separate experiments except where stated.

6.3 RESULTS

6.3.1 Effect of Exogenous GDF9 During IVM on Embryonic Development

Fertilisation rates, determined by the percentage of cleaved embryos at 20 hours of culture after IVF, were not different with the addition of GDF9 during maturation compared to the controls in the presence of FSH and EGF (Table 6.1). In the absence of FSH and EGF, a significant difference ($P<0.05$) was noticed between GDF9 and its 293H control. Three by two way ANOVA analyses showed an interaction between FSH/EGF and the other treatments on fertilisation rates.

Compared to the 293H controls, addition of GDF9 to the maturation medium in the presence of FSH and EGF significantly ($P<0.05$) increased the rate of embryo development to the 8-cell or morula stage but had no effect on rate of embryo development after 49-51 hours of culture in the absence of FSH and EGF (Table 6.1).

There was no effect of exogenous GDF9 in the oocyte maturation medium on blastocyst development in the presence or absence of FSH and EGF (Table 6.1). However, the percentage of hatching blastocysts per cleaved embryo was significantly increased ($P<0.05$) with exogenous GDF9 both in the presence and in the absence (Table 6.1) of FSH and EGF.

There were no significant differences observed in any of the parameters assessed between the IVM and 293H controls either in the presence or absence of FSH and EGF. However, embryo development in all aspects was significantly ($P<0.05$) compromised in the absence of FSH/EGF compared to when FSH and EGF were present. Three by two way ANOVA analyses also showed no interaction between FSH/EGF and the various treatments on developmental outcomes aside from fertilisation rates. (N=15-19 groups of ~10 oocytes for treatments not inclusive of embryo transfers and N=40 and 41 groups of ~10 oocytes for 293H+FSH/EGF and GDF9+FSH/EGF respectively. As no statistical significant difference was observed between individual experiments, the data set was pooled and presented in Table 6.1)

Table 6.1. Effects of exogenous GDF9 during oocyte IVM on embryonic development.

	FSH/EGF$^{\Psi}$	2-cell Embryos After 20 h of Culture Per IVM Oocyte (%)	8-cell/Morula Embryos After 49-51 h of Culture Per 2-cell Embryos (%)	Blastocyst After 96-100 h of Culture Per 2-cell Embryo (%)	Hatching Blastocyst After 96-100 h of Culture Per 2-cell Embryo (%)
IVM	+	85.1 ± 4.6	16.4 ± 5.4 ^{a,b}	73.0 ± 7.3	37.2 ± 5.7 ^{a,b}
293H	+	84.6 ± 2.3	16.3 ± 2.5 ^{a}	70.4 ± 4.0	37.6 ± 3.8 ^{a}
GDF9	+	87.6 ± 2.3	24.0 ± 3.0 ^{b}	77.8 ± 2.5	50.4 ± 4.0 ^{b}
IVM	-	69.5 ± 5.3 ^{c,d}	3.5 ± 2.8	50.3 ± 8.3	29.2 ± 6.8 ^{c,d}
293H	-	79.1 ± 5.6 ^{c}	2.6 ± 1.8	49.3 ± 5.1	19.1 ± 3.9 ^{c}
GDF9	-	59.3 ± 4.8 ^{d}	4.9 ± 1.9	62.2 ± 6.6	36.7 ± 6.7 ^{d}

IVM : COCs cultured in maturation media with or without 50 mIU/ml FSH and 10 ng/ml EGF; 293H: COCs cultured in media compositions identical to that of IVM controls but with 7% v/v control parent cell line 293H conditioned medium; GDF9: COCs cultured in media compositions identical to that of IVM controls plus 200 ng/ml recombinant GDF9. Data represents the mean ± SEM of at least 13 experimental replicates and includes embryos cultured for subsequent embryo transfers as there was no statistical difference (P>0.05) between datasets. N = 152-186 oocytes for treatments not inclusive of embryo transfers and n = 395 and 410 oocytes for 293H+FSH/EGF and GDF9+FSH/EGF respectively.

$^{\Psi}$ +, present; -, absent. $^{a-d}$ Values with different superscripts within the same FSH/EGF treatment groups represent statistical significance (P < 0.05).

6.3.2 Effect of Exogenous GDF9 on Blastocyst Quality

In the presence of FSH and EGF, GDF9 addition during IVM significantly (P<0.05) increased blastocyst total cell numbers over the 293H control (N=44 and 28 blastocysts respectively) (Table 6.2). ICM cell numbers were significantly (P<0.05) increased over both controls while exogenous GDF9 had no effect on TE cell numbers (Table 6.2).

In the absence of FSH and EGF, no differences in total cell numbers or the numbers of ICM or TE cells were observed with exogenous GDF9 (N=22 blastocysts) compared to either

control. However both the 293H and GDF9 treatments had significantly (P<0.05) fewer TE cells than the IVM only control (Table 6.2).

There were no significant differences observed in blastocyst total, ICM or TE cell numbers between the IVM and 293H controls in the presence of FSH and EGF (N=32 and 28 blastocysts respectively). However, the 293H control had significantly (P<0.05) fewer total and TE cell numbers compared to the IVM control in the absence of FSH/EGF (N=28 and 21 blastocysts respectively). Blastocyst total, ICM and TE cell numbers were all significantly decreased in the absence of FSH/EGF compared to when FSH and EGF were present. Three by two way ANOVA analyses showed no interaction between FSH/EGF and the various treatments for total and TE cell numbers but a significant (P<0.05) interaction between FSH/EGF and the treatments on ICM cell numbers.

Table 6.2. Effects of exogenous GDF9 during oocyte IVM on blastocyst cell numbers.

	FSH/EGF$^{\Psi}$	Total Cell Numbers	ICM Cell Numbers	TE Cell Numbers
IVM	+	40.2 ± 3.0 ^{a,b}	11.9 ± 1.1 ^{a}	28.3 ± 2.5
293H	+	37.3 ± 2.8 ^{a}	7.1 ± 0.8 ^{a}	26.4 ± 2.2
GDF9	+	46.8 ± 1.8 ^{b}	15.0 ± 0.7 ^{b}	31.8 ± 1.6
IVM	-	30.3 ± 1.7 ^{c}	5.4 ± 0.6	24.9 ± 1.9 ^{c}
293H	-	24.9 ± 1.2 ^{d}	7.1 ± 0.5	17.8 ± 1.0 ^{d}
GDF9	-	26.1 ± 1.9 ^{c,d}	7.4 ± 0.8	18.7 ± 1.4 ^{d}

IVM: COCs cultured in maturation media with or without 50 mIU/ml FSH and 10 ng/ml EGF; 293H: COCs cultured in media compositions identical to that of IVM controls but with 7% v/v control parent cell line 293H conditioned medium; GDF9: COCs cultured in media compositions identical to that of IVM controls plus 200 ng/ml recombinant GDF9. Data represents the mean ± SEM of at least 13 experimental replicates. N > 20 blastocysts per treatment. $^{\Psi}$ +, present; -, absent. $^{a-d}$ Values with different superscripts within the same FSH/EGF treatment represent statistical significance (P < 0.05).

6.3.3 Effect of Exogenous GDF9 on Subsequent Pregnancy Outcomes

Since embryo development was impaired in the absence of FSH/EGF during IVM and most effects of exogenous GDF9 were noticed in the presence of FSH and EGF, a second set of experiments was conducted with oocytes matured only with GDF9 or the 293H conditioned media in the presence of FSH and EGF. Resultant embryos (N=48 embryos per treatment) were then transferred to pseudo-pregnant recipients.

There was no difference in the percentage of transferred embryos that established implantations (Figure 6.1 A). However there was a significant ($P<0.05$) increase in the number of foetuses that developed from these implantations when GDF9 was added during IVM (Figure 6.1B).

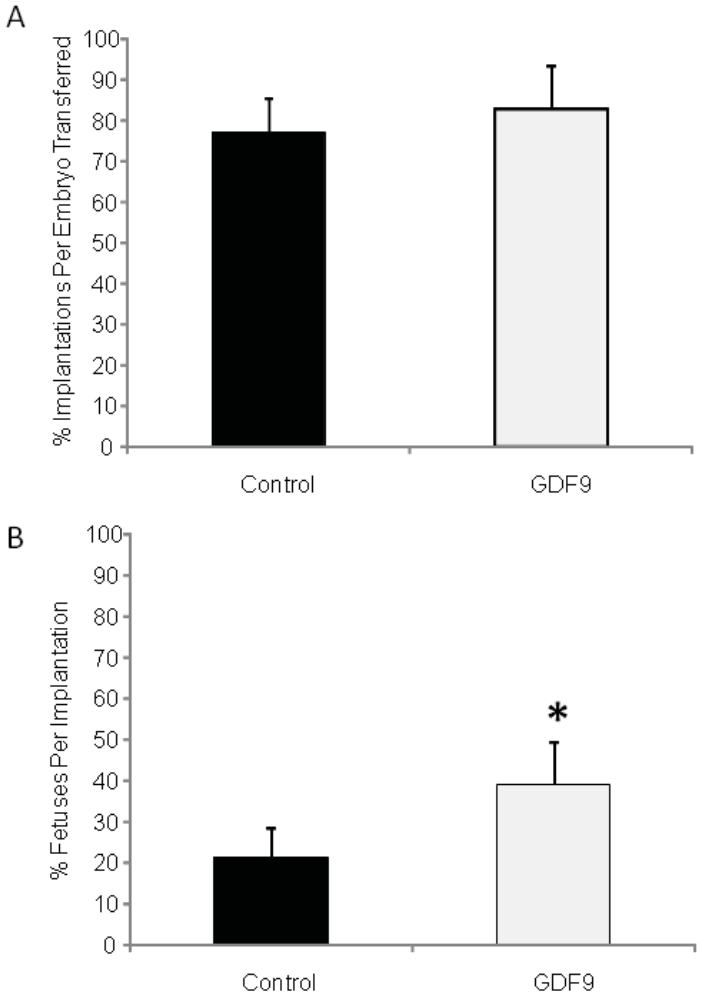


Figure 6.1. Effects of GDF9 supplementation during IVM on embryo viability. Implantation (A) and foetal development (B) rates after embryonic transfer of blastocysts after 96-100 hours post fertilisation derived from oocytes matured in vitro with exogenous GDF9 or 293H control media containing 50 mIU/ml FSH and 10 ng/ml EGF. Data represents mean ± SEM. N = 48 embryos transferred per treatment. * Significantly different from control, $P<0.05$.

Analysis of the foetuses determined that there were no differences in weight (Figure 6.2A) or crown to rump length (Figure 6.2B) in the foetuses derived from oocytes matured with exogenous GDF9 and the controls. There was also no difference in placental weight when oocytes were matured with or without GDF9 (Figure 6.2C) nor were there any other morphological differences. Foetuses from both treatments were within the normal weight,

size and gross morphology, as determined by limb, structural and overall appearance, when compared to published information [372] of in vivo derived mouse foetuses of the same age.

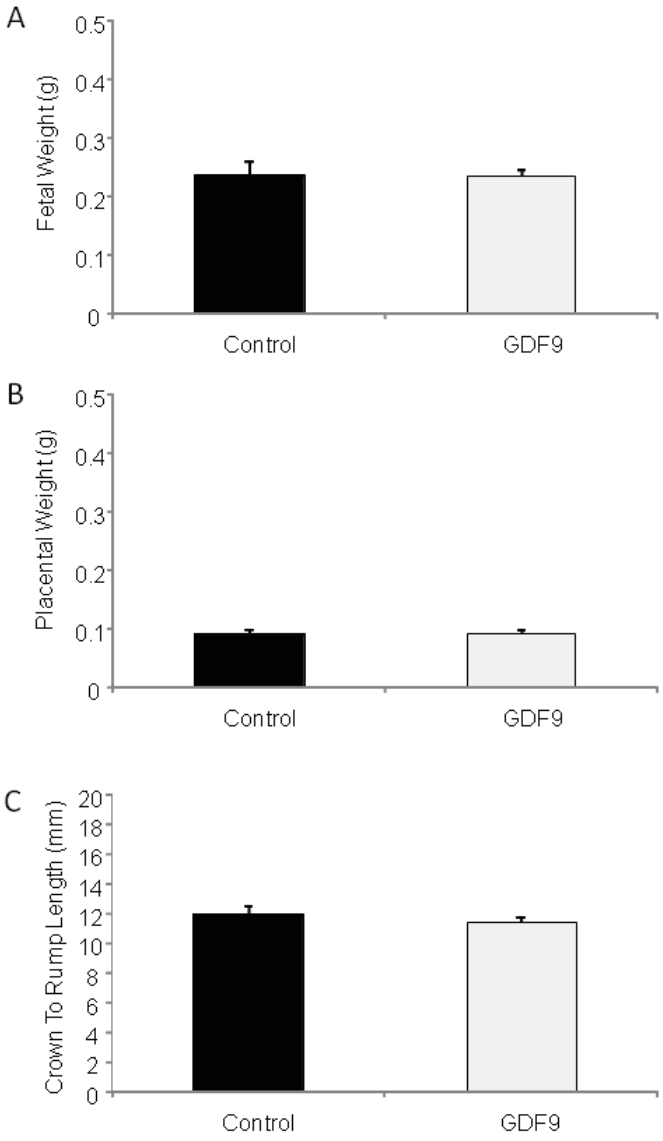


Figure 6.2. Effect of GDF9 supplementation during IVM on foetal outcomes. Foetal weight (**A**), placental weight (**B**) and foetal crown to rump length (**C**) of foetuses and placentas developed from embryos transferred after IVM of oocytes with exogenous GDF9 or 293H control media in the presence of 50mIU/ml and 10ng/ml EGF. Data represents mean ± SEM. N ≥ 8 Foetuses/Placentas per treatment.

6.4 DISCUSSION

GDF9 is a member of the transforming growth factor beta family and is one of just two known oocyte-specific paracrine growth factors. Females that are genetically deficient in GDF9 are infertile with ovarian follicles that do not form past the primary stage [323] [330]. GDF9 has also been shown to mimic oocyte functions in oocytectomised cumulus complexes (COCs with removed oocytes), such as suppression of luteinizing hormone receptor expression [318], stimulation of cumulus cell proliferation [341] and regulation of cumulus matrix gene expression such as cyclooxygenase 2 (*COX2*) and hyaluronan synthase 2 (*HAS2*) leading to cumulus expansion [274, 318, 444]. Addition of exogenous GDF9 to intact COCs also resulted in increased cumulus expansion as demonstrated in Chapter 4.

GDF9 thus has a fundamental role to female fertility, particularly in the function of cumulus cells. Given that Chapter 5 established that disruption of GDF9's signalling pathway significantly compromised subsequent embryo and foetal viability, the hypothesis of this chapter was therefore that addition of GDF9 to the developing oocyte during IVM would improve subsequent embryonic quality and viability.

Indeed, in the presence of FSH and EGF, exogenous GDF9 increased the proportion of on time developed embryos and hatching blastocysts, despite having no effect on the percentage of embryos reaching the blastocyst stage. Total blastocyst cell numbers have been shown to reflect embryo viability as demonstrated by increased capacity to develop into a viable foetus after embryo transfer [229], and hence blastocyst cell numbers were quantified as a reflection of blastocyst quality. Exogenous GDF9 during IVM resulted in

increased blastocyst total cell numbers, with the most significant observations found in increased inner cell mass cell numbers, the portion of the blastocyst that derives the foetus. Concordantly, foetal number was almost doubled after embryonic transfer but no differences were found in implantation rates. This supports Chapter 5's findings, where disruption of SMAD2/3 signalling during mouse IVM resulted in decreased ICM cell numbers and subsequent foetal survival rates with unaffected implantation rates

Hence, while GDF9 addition during IVM did not affect the ability of embryos to develop into blastocysts, it did significantly enhance blastocyst quality as established by increased foetal survival. Thus GDF9 addition during the short window of oocyte maturation appears to have a profound effect on developmental programming of the oocyte, a legacy that persists through late pre-implantation development and into foetal development.

The concept of addition of oocyte-secreted factors during IVM is new and has only very recently been proposed. A recent study demonstrated increased blastocyst formation after exposure to native oocyte-secreted factors, or treatment with recombinant GDF9 or BMP 15, another oocyte-specific factor, during bovine IVM [354]. The results of this chapter confirm these findings in another species, and extend them by demonstrating for the first time improved foetal development and survival post-transfer. Furthermore, the observation that the foetuses and their placentas derived from GDF9 treatment during IVM have normal gross morphology, suggested no obvious adverse effect on development.

It is important to note at this point that the recombinant GDF9 is only partially purified by chromatography [265] and that contaminating factors originating from the human

embryonic kidney 293H parent cell line, present in both the GDF9 preparation and the 293H control, have adverse effects on embryo development [354]. Hence positive effects of the exogenous GDF9 on some parameters of embryo development over that of conventional IVM conditions may be masked by the 293H contaminants in the preparation. In spite of this, exogenous GDF9 during IVM with FSH and EGF still resulted in a significant increase of inner cell mass cell numbers over the IVM experimental control. Hence the positive effects of exogenous GDF9 on oocyte developmental competence might be even more pronounced should a purified active product become available.

It would be interesting to study the effects of other oocyte specific factors on embryo viability as it is likely that the oocyte utilises a variety or combination of factors to regulate its environment for optimal developmental competence. Recombinant ovine BMP15 has been shown to improve bovine IVM oocyte developmental competence [354]. It is relevant to note, however, the varied degree of importance of BMP15 in different species [298, 445]. Unlike GDF9 null mice [259] and GDF9 and BMP15 deficient sheep [330, 331] which are all sterile, BMP15 null mice undergo folliculogenesis, but exhibit mild defects in cumulus expansion and pre-implantation embryo development [299] leading to sub-fertility [329]. Recent studies have shown that BMP15 expression is up-regulated in peri-ovulatory mouse follicles with oocytes producing active BMP15 only after an ovulatory signal [445, 446]. Thus it appears that GDF9 is most crucial for follicular function and oocyte-somatic cell communication throughout folliculogenesis across species, whereas BMP15 has a more subtle role in the mouse later in folliculogenesis. Unfortunately to date, recombinant murine BMP15 cannot be produced [447] hence the effects of homologous exogenous BMP15 on mouse IVM cannot be elucidated.

In the current study the combination of FSH and EGF during IVM dramatically improved oocyte maturation and developmental competence, as evidenced by increased cumulus expansion, on-time embryo development, development to blastocyst, blastocyst hatching rates and increased blastocyst cell numbers. This is in accordance with findings from the previous chapters and not surprising as FSH and EGF have long been known to be beneficial to oocyte meiotic and cytoplasmic maturation and hence developmental competence [55, 57, 213], and as such, these hormones are common IVM additives. Through different signalling receptors, FSH and EGF both stimulate the MAP kinase pathways in cumulus cells, activating a cascade of cellular consequences. For example, FSH positively influences aspects of cumulus metabolism such as glycolysis [193, 201], while EGF has also been shown to up-regulate glycolysis in human ovarian follicles [180] and other cell types [448, 449]. FSH and EGF also induce cumulus expansion by up-regulating transcripts necessary for matrix formation, such as *HAS2*, *COX2* and tumour necrosis factor induced protein 6 *(TNFAIP6)* [73], however importantly, these FSH and EGF mediated functions are critically dependent on the presence of oocyte-secreted factor signalling in cumulus cells, as cumulus expansion cannot occur in the absence of an oocyte [126, 271]. Upon binding to its serine/threonine kinase type-1 receptor, TGFBR1, the effects of GDF9 are mediated through activation of the SMAD2/3 pathway [308]. Recent findings demonstrate that oocyte-secreted factor activation of SMAD2/3 signalling in cumulus cells is essential to enable FSH or EGF to induce cumulus expansion [444], suggesting critical interactions between the SMAD2/3 and MAPK signalling pathways. It is unclear from the results of the present study if such an interaction also regulates oocyte developmental competence. The most notable effects of GDF9 on oocyte competence were

observed in the presence of EGF and FSH, yet GDF9 also improved oocyte development to some extent in their absence as seen by an increase in percentage of hatched blastocysts, a response which differs from the regulation of cumulus expansion.

Although live births have resulted from oocytes matured in vitro in clinical applications, [450-453] current IVM success rates are significantly reduced compared to peri-ovulatory oocytes collected after superovulation stimulation with gonadotrophins [13, 452]. Current IVM procedures also result in reduced embryo quality with embryos displaying frequent cleavage retardation and blockage in development [283, 454]. Given the current limitations of IVM, it is thus essential that systems be devised to improve the viability of individual cumulus-oocyte complexes in order to advance IVM proficiency in the human. Therefore, the observation that the addition of an oocyte-secreted factor GDF9 to the medium increased embryo development and foetal viability is an important novel finding that may provide insight into the formulation of maturation media for the human cumulus-oocyte complex.

Unfortunately, the role of GDF9 and other identified oocyte-secreted factors in human oocyte biology is still unclear. The only implication of their importance is suggested by the observation that some of the cumulus cell genes up-regulated by GDF9 are reflective of oocyte developmental competence in a small study involving only 8 patients [128]. Hence, human studies are necessary to identify oocyte-specific paracrine factors actively involved in the human oocyte to cumulus bi-directional regulatory loop. This potentially has great implications for our fundamental understanding of human oocyte biology as well as for the development of IVM media and the improvement of clinical IVM success rates.

CHAPTER 7

IMPORTANCE OF OOCYTE-CUMULUS BI-DIRECTIONAL SIGNALLING TO METABOLIC ACTIVITY, GENE EXPRESSION AND OXIDATIVE STRESS

7.1 INTRODUCTION

The metabolism of both oocytes and embryos are intrinsically linked to their development, health and viability. Oocytes with higher developmental potential such as those matured in vivo, have been found to have greater glucose metabolism compared to those with compromised development such as immature and IVM oocytes [222, 455]. Aberrations resulting in decreased foetal development have also resulted in alterations in embryo metabolic activity and profile [456] while non-invasive assessments of glucose consumption and glycolytic activity have been successfully used as predictors of embryo viability [457, 458].

Although oocytes are incapable of metabolising glucose efficiently for ATP production, instead utilising pyruvate as their main energy source [215], glucose plays a key role in oocyte maturation. Oocyte maturation and subsequent embryo development are improved when glucose is present in the maturation medium [193, 216, 217]. However this only occurs in the presence of follicular cells as denuded oocytes cannot resume meiosis with glucose as the only energy source [215]. Furthermore, a rare study assessing COC metabolism during IVM, revealed a correlation between increased glycolytic activity and fertilization success rates [223].

While it has long been established that the oocyte relies on its surrounding follicular cells for the nutritional support necessary for its growth and development [218, 219, 280, 459], the knowledge of the oocyte's regulatory role in follicular cell metabolism for this provision has only recently been discovered [226, 282]. Co-culture of fully grown

meiotically competent oocytes with oocytectomised cumulus cells (OOX) prior to oocyte maturation increased critical glycolytic gene transcripts such as phosphofructokinase (*Pfkp*) and lactate dehydrogenase (*LdhA*) and glycolytic activity in cumulus cells compared to OOXs alone [282]. Interestingly, only oocytes derived from antral and not preantral follicles were capable of influencing cumulus cell metabolism [282] suggesting that the oocyte's control on cumulus cell metabolism could be an indicator of the oocyte's growth and developmental competence. However, the influence of specific oocyte secreted factors such as GDF9 on cumulus cell metabolism of intact COCs during IVM has not yet been investigated. Given the effects on embryo and foetal survival in the previous chapters, it is highly probable that oocyte-cumulus cell paracrine regulation of metabolism involves the SMAD2/3 pathway to affect the acquisition of oocyte developmental competence and subsequent embryogenesis.

It is important to note that while the level of gene expression often indicates protein activity, the most potent allosteric regulator of the rate limiting enzyme dictating glycolytic flux, phosphofructokinase (PFK), is fructose-2-6-bisphosphate (F2,6BP) [460-462]. The intracellular levels of F2,6BP are in turn regulated by a single homodimeric bi-functional enzyme family known as 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases (PFKFB) which is responsible for both the formation and degradation of F2,6BP from/to fructose-6-phosphate via its kinase and bisphosphatase domains respectively [463-465]. This is illustrated in Figure 7.1.

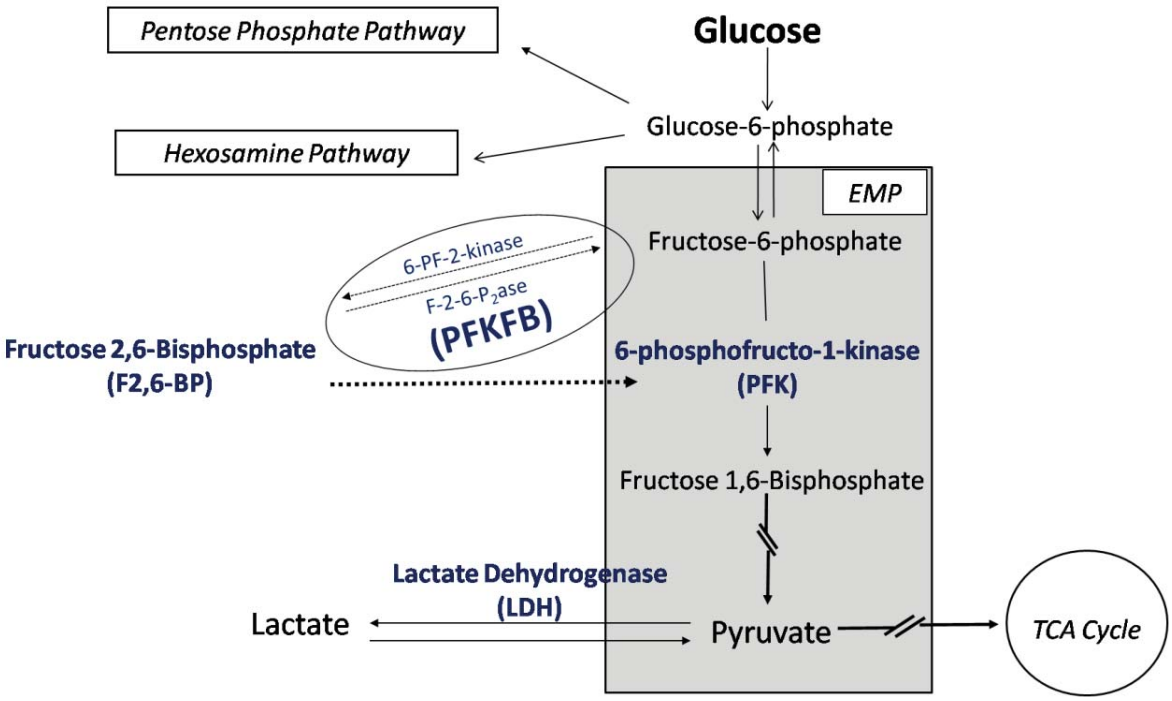


Figure 7.1. Overview of Glucose Metabolism. The Embden-Meyerhof pathway (EMP) is represented in the shaded box. Dashed lines represent enzymatic regulation and broken arrows represent 2 or more substrate conversions. Once converted to glucose-6-phosphate, glucose will be metabolised by one of 3 specific pathways, EMP, pentose phosphate pathway or the hexosamine pathway. The first non-reversible and hence rate limiting step for the commitment of glucose into the glycolytic pathway (conversion of glucose to lactate) is the conversion of fructose-6-phosphate to fructose 1, 6-bisphosphate. This is catalysed by 6-phosphofructo-1-kinase (PFK) which is allosterically regulated by fructose 2, 6-bisphosphatase (F2, 6-BP) which in turn is synthesized and degraded from/to fructose-6-phosphate by 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases (PFKFB) by its 6-phosphofructo, 2-kinase (6-PF-2-kinase) and fructose-2,6-bisphosphatase (F-2,6-P_2ase) domains respectively. Once glucose has entered glycolysis and is converted to pyruvate, lactate dehydrogenase (LDH) is responsible for the conversion of pyruvate to lactate.

To date, 4 isoforms of the PFKFB family have been discovered, PFKFB1, first isolated in the liver [466] and also in skeletal muscle [467, 468], PFKFB2 (heart) [469, 470], PFKFB3 (brain) [471] and PFKFB4 (testis) [472]. Each of these isoforms PFKFB1-4 is coded on 4 independent genes and while their expression is dependent on the tissue type and development [464, 465, 473], more than one isoform has been found in the same tissue [373]. This may be in part due to the different regulatory properties of each isoform due to the great variance in their N- (kinase) and/or C-terminal (bisphosphatase) regions which

differ in length, compositions and number of protein kinase phosphorylation sites, despite the high degree of similarity in the sequence of their core catalytic domains [463, 464]. Although the disruption of PFKFB3 has been found to be embryonic lethal [474], the expression of the PFKFB isoforms in COCs and their possible roles in oocyte-cumulus metabolic regulation has not yet been investigated.

One of the consequences of perturbed metabolism is the accumulation of reactive oxygen species (ROS) resulting in alterations in cellular redox state and increased oxidative stress [475, 476]. ROS, which directly affects a cell's cytosolic redox state, is produced when oxygen molecules acquire additional electrons and is generated endogenously as a by-product of oxidative phosphorylation (OXPHOS), NADPH oxidase and xanthine oxidase [241, 477]. Under normal physiological conditions cells, including embryos and oocytes, are equipped with several defence mechanisms such as; non-enzymatic antioxidants, superoxide dismutases and enzymes such as glutathione peroxidase and catalase, which enable them to manage a certain level of ROS [242]. However, perturbations in cellular metabolism may potentially result in the production rate of ROS through OXPHOS to exceed that of its removal. Excessive ROS has been shown to result in DNA damage, mitochondrial alterations and apoptosis [478, 479] and increased ROS levels in human follicular fluid has been correlated to decreased embryo formation [249].

Apart from intracellular ROS, oocytes are also exposed to extracellular ROS in their immediate micro environment both in vivo and in vitro. Follicular fluid has been shown to contain both ROS and antioxidants [249, 250] while in vitro manipulations of the oocyte inevitably exposes it to environmental factors such as atmospheric oxygen and visible light

which can induce ROS [251, 252]. It is unsurprising therefore that cumulus cells have been found to exert a protective role on the oocyte against oxidative stress [243, 254, 255]. Culture of denuded porcine oocytes resulted in irreparable DNA damage, decreased GSH levels compared to freshly isolated oocytes and increased apoptosis [254]. However, intact COCs cultured in the same system had no significant oocyte damage but had increased GSH levels compared to freshly isolated oocytes. GSH expression was also found to be higher in intact bovine COCs and lower in denuded oocytes. Moreover, culture with cysteamine, a precursor of GSH, had no effect on the GSH levels of denuded oocytes but GSH was significantly increased when denuded oocytes were co-cultured with COCs. However, this was still significantly lower than that of oocytes obtained from intact COCs [480]. This suggests an oocyte to cumulus cell paracrine regulatory role on oxidative stress defence mechanisms as well as the possible involvement of gap junctions in facilitating this role. Indeed the expression of another antioxidant, peroxiredoxin 6 (PRDX6), was found to be dependent on oocyte to cumulus bi-directional communication where the presence of oocytes up-regulated PRDX6 in cumulus cell clusters but gap junction communication was necessary for up-regulation of PRDX6 in oocytes. Furthermore, recombinant GDF9 was shown to increase PRDX6 expression in cumulus cells [481].

Previous chapters of this thesis have established the importance of oocyte-cumulus bi-directional communication to cumulus expansion, oocyte developmental competence and subsequent foetal outcomes. This chapter will therefore investigate the possible mechanisms resulting in the affected oocyte developmental competence with regards to metabolism and oxidative stress.

7.2 MATERIALS AND METHODS

7.2.1 Collection and Culture of COCs

COCs were collected and cultured as in Chapters 2.1 and 2.2.1 of General Experimental Methods.

7.2.2 Treatments

Treatment 1: Inhibition of Oocyte to Cumulus Bi-directional Signalling

Cumulus to oocyte signalling was enabled by culture of COCs in the presence of FSH and EGF and compared to maturation in the absence of 50 mIU/ml FSH and 10 ng/ml EGF. Oocyte to cumulus signalling was disrupted through the inhibition of SMAD2/3 signalling using 4 µM SB-431542 and compared to the equivalent 0.04% DMSO carrier control. See Chapters 2.3.1 and 2.3.2 for details of FSH/EGF and SB-431542 respectively.

Treatment 2: Addition of Exogenous GDF9

COCs were cultured with 200 ng/ml of recombinant GDF9 or the equivalent 7% v/v of its 293H parent cell line control conditioned media both in the absence and presence of 50 mIU/ml FSH (1 mIU= 0.1 ng FSH) and 10 ng/ml EGF. See Chapter 2.3.3 for exogenous GDF9 details.

Treatment 3: Disruption of Cumulus Metabolism Independent of Oocyte-Cumulus Bi-directional Communication

Cumulus metabolism through the hexosamine biosynthesis pathway (HBP) was disrupted independently of oocyte-cumulus bi-directional communication through the use of O-

diazoacetyl-L-Serine (azaserine). As described in Chapter 4, azaserine is an inhibitor of GFPT1, the rate limiting enzyme of the HBP. Azaserine was purchased from Sigma-Aldrich Chemical Co, (St Louis, MO, USA) and dissolved in MilliQ water to a stock concentration of 10 mM and stored at -20°C according to the manufacturer's instructions. Azaserine was used at a final concentration of 100 µM in 3ml maturation media in the presence of 50 mIU/ml FSH and 10 ng/ml EGF.

7.2.3 Analysis of Gene Transcripts

7.2.3.1 RNA extraction, quantification and reverse transcription

To obtain large amounts of RNA for primer validation and preliminary assays, COC, heart and brain RNA standards were created. Total RNA was extracted from a pool of approximately 600 ovulated COCs from 15 21-25 day old CBA/C57BL6 mice and the heart and half a brain of a single 27 day old female CBA/C57BL6 mouse and quantified as described in Chapters 2.9.1 and 2.10. For sample analysis, total RNA was extracted from groups of 15-20 COCs (see Chapter 2.9.2) and the maximum volume of 11 µl was reverse transcribed for all reactions. For more details see Chapter 2.11. 2 mg of COC standard total RNA was reverse transcribed to give a concentration of 100 ng/µl cDNA which was then diluted with Rnase free water to a stock solution of 50 ng/µl. Serial dilutions of 1 in 10 were then performed to give cDNA aliquots of 5, 0.5, 0.05 and 0.005 ng/µl. A total of 5 mg heart and 20 mg brain total RNA were reverse transcribed to give final cDNA concentrations of 250 ng/µl and 1 mg/µl respectively. These were diluted into stock concentrations of 50 ng/µl and serially diluted with Rnase free water to give cDNA aliquots at 25, 5, 0.5, 0.05 and 0.025 ng/µl.

7.2.3.2 Primer sequences and determination of PFKFB1-4 expression in COCs

As there is no evidence in the literature of the existence of PFKFB1-4 in COCs, a preliminary experiment was conducted to investigate the presence of PFKFB1-4 in COCs. The maximum amplification cycles employed was 40 hence Ct values above 40 or that of the no template control verified the presence of the gene transcripts. Primers were designed as described in Chapter 2.12. Primer sequences of all genes analysed in this chapter are listed below in Table 7.1. Due to the limitation of sample sizes, 0.1 to 0.2 of a COC is loaded per PCR reaction; hence the expression of target genes must be readily detectable at 1 ng (~0.06 of a COC) for comparable expression analysis between treatments. Concentrations of 100, 10, 1, and 0.1 ng of reverse transcribed COC RNA were analysed in a total volume of 2 µl per PCR reaction and performed in duplicates. Refer to Chapter 2.12 for more details on real time PCR.

Table 7.1. Real Time PCR Primer Sequences

Gene	Oligonucleotide Sequence (5'-3')	Amplicon Length (Base Pairs)	Genbank Accession Number
Rpl19	F: TTC CCG AGT ACA GCA CCT TTG AC R: CAC GGC TTT GGC TTC ATT TTA AC	103	NM_009078
Rn18s	F: AGA AAC GGC TAC CAC ATC CAA R: CCT GTA TTG TTA TTT TTC GTC ACT ACC T	91	AF176811
Pfkp	F: ATT TTG GCC AGC CGT ATG G R: CTG TAC ATC CTG GGT CAT TTG C	147	NM_019703
LdhA	F: GGA CAG TGC CTA CGA GGT GAT C R: GCA CCC GCC TAA GGT TCT TC	107	NM_010699
Pfkfb1	F: ACC TTA GAG GCC GCA TTG G R: TGT GGC TAG TCC ACA CCT TCA G	123	NM_008824
Pfkfb2	F: TGG ATA AAG GCG CAG ATG AGT R: GCC ACA GGA GTA AGT TTG AAG ATG	73	NM_008825
Pfkfb3	F: GGG ACC TAA CCC GCT CAT G R: AAG CCA CAC GCT CCT CAA AG	101	NM_172976
Pfkfb4	F: ACC CGA AGG GTG AGT CCT ATG R: CAG CCT GGT GGC AAA TGA C	102	NM_173019

7.2.3.3 Primer sequences and validation

As detailed in Chapter 2.12, in accordance with the use of the $2^{-\Delta\Delta Ct}$ method for the analysis of gene expression, the efficiency of the target gene primers must be of similar efficiency to that of the relative housekeeper gene [374, 379, 482]. The efficiencies of target gene primers to that of the housekeeper genes *Rn18s* and *Rpl19* were tested using a set of serially diluted cDNA of known concentrations. COC cDNA standards were used for *Pfkp* and *LdhA* at 100, 10, 1, 0.1 and 0.01 ng of reverse transcribed RNA in a total volume of 2 µl per PCR reaction. *Pfkfb2* has been shown to be strongly expressed in the heart while *Pfkfb3* has been shown to be expressed in the brain [373]. Heart and brain standards were used at 50, 10, 1 0.1 and 0.05 ng of reverse transcribed RNA in a total volume of 2 µl per PCR reaction. As shown in Appendix 5, all target gene primers ran at the same efficiency as both

housekeepers, hence the $2^{-\Delta\Delta Ct}$ method was used for all subsequent gene expression analysis.

7.2.3.4 Analysis of metabolic gene transcripts Pfkp, LdhA and Pfkfb2 and Pfkfb3 expressions in COCs

After first strand cDNA synthesis, samples were diluted 1 in 10 with Rnase free water and 2 µl of this was analysed per PCR reaction. PCR reactions were performed in duplicate or triplicates, where sample size permitted, with each PCR reaction containing cDNA from approximately 0.2 of a COC. Real time PCR results were then analysed as the relative gene expression level relative to that of a standard exogenous control after normalisation to the internal endogenous control using the $2^{-\Delta\Delta Ct}$ method [379] as described in Chapter 2.12. *Rpl19* and *Rn18s* were used as the endogenous control and IVM in the presence of 50 mIU/ml FSH and 10 ng/ml EGF was the exogenous control. Relative gene expression is therefore presented as the mean $2^{-\Delta\Delta Ct}$ value of all experimental replicates with normalisation to the mean delta Ct value of all IVM + FSH/EGF (exogenous control) experimental replicates.

7.2.4 Analysis of Metabolic Pathway Activity: The Hanging-Drop Assay

7.2.4.1 Overview of the hanging-drop assay

The metabolism of glucose through specific pathways results in the release of its carbon and hydrogen atoms in the form of carbon dioxide (CO_2) and water (H_2O). As illustrated in Figure 7.2, entry into the Embden-Meyerhof pathway (EMP) results in glucose C-1 and C-6, C-2 and C-5, C-3 and C-4 becoming pyruvate C-3, C-2 and C-1 respectively and the

release of the hydrogen attached to glucose C-5 as H_2O. Pyruvate which is then converted to acetyl-CoA, enters the mitochondria and releases its C-2 and C-3 atoms as CO_2 after 2 or more cycles through the tricarboxylic acid (TCA) cycle.

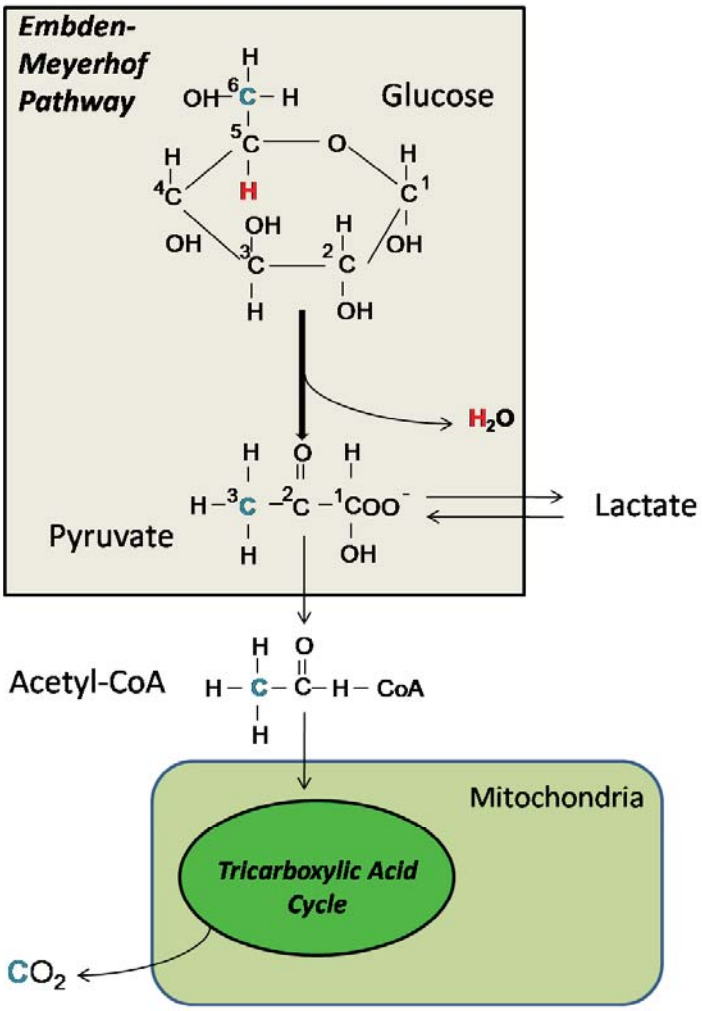


Figure 7.2. The fate of glucose carbon and hydrogen atoms during metabolism.
When metabolised through the Embden-Meyerhof pathway, the hydrogen atom attached to glucose C-5 is released as H_2O. Glucose C-6 is converted to pyruvate C-3 which enters the mitochondria as acetyl-CoA and is released as CO_2 after 2 or more subsequent cycles through the TCA cycle. Measurements of H_2O and CO_2 amounts therefore reflect metabolic activity of the respective pathways.

The hanging-drop assay [231, 483] utilises these conversions of the carbon and hydrogen atoms of energy substrates when they are metabolised as an indicator of metabolic activity. Using this technique, a single embryo/COC is picked up in a small media volume (2 µl) containing the radio labelled energy substrate and placed on the severed lid of a 1.5ml microtube filled with a pre-equilibrated reservoir trap consisting of either a sodium

hydroxide (NaOH) or bicarbonate ($NaHCO_3$) solution. The air space between the drop and the reservoir is then equilibrated with the appropriate gas mixture if necessary before the cap is fitted. The hanging-drop apparatus is illustrated in Figure 7.3. Due to the large volume difference between the incubation drop and the reservoir, the labelled ^{14}C or ^{3}H released as $^{14}CO_2$ and $^{3}H_2O$ as a result of embryo/COC metabolism, is captured by the reservoir trap. At the end of the incubation period, 1 ml of the reservoir trap is removed into a scintillation vial and sodium hydroxide (NaOH) is added and left at 4ºC for a minimum 24 hours to facilitate the conversion of CO_2 to carbonate. Scintillation fluid is then added just prior to analysis through a scintillation counter. The activity of metabolic pathways can therefore be determined by the amount of radioactive carbon or hydrogen molecules.

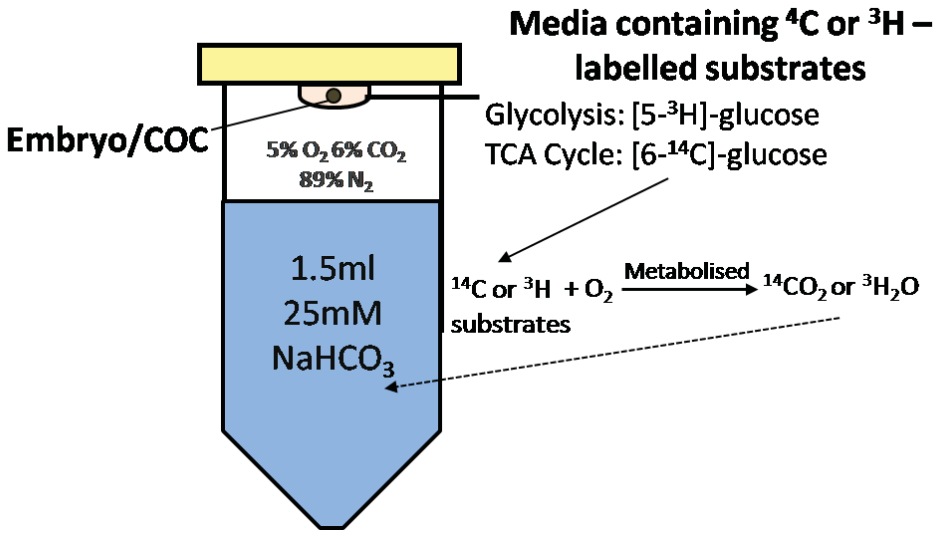


Figure 7.3. Schematic Representation of the Hanging-Drop Assay

7.2.4.2 Reagents

All reagents were purchased from Sigma-Aldrich (St Louis, MO, USA) unless otherwise specified. 4 g of NaOH and 2.1 g of $NaHCO_3$ were dissolved in a volumetric flask with 1

litre of MilliQ water under aseptic conditions to give a final concentration of 0.1 M and 25 mM respectively. These were then stored in sterile glass bottles at room temperature.

D-[5-^{3}H] glucose (pack size of 250 µCi at a concentration of 1.0 mCi/ml, specific activity of 16.0 Ci/mmol, 98.4% radiochemical purity) and D-[6-^{14}C] glucose (pack size of 50 µCi, concentration of 200 µCi/ml, specific activity of 55.0 Ci/mmol, 99.6% radiochemical purity), were purchased from Amersham (Amersham, GE Healthcare UK Limited, Buckinghamshire, United Kingdom). The nominal working concentrations of each labelled substrate was 0.25 µCi/µl [231]. However, the amount of labelled substrate must be sufficient to produce detectable amounts of $^{3}H_20$ and $^{14}CO_2$ but not result in a significantly greater amount of total energy substrate than that which is normally used for culture. As the glucose concentration of the IVM media (Waymouth MB 752/1) is 27.8 mM, an unachievable amount of radioactive substrates would be needed to compete with the unlabelled glucose in the media to produce detectable levels of radioactivity. Since this was a comparative assay investigating subsequent effects on COC metabolism after IVM, it was not necessary to maintain the IVM culture conditions for the duration of the hanging-drop experiment. Hence, G1.2 media (Appendix 1) with a glucose concentration of 0.5 mM glucose was used as the hanging-drop incubation media.

For D-[5-^{3}H] glucose, 0.25 µCi/µl is equivalent to 0.0156 mM of glucose. This would result in an increase of less than 5% of the total glucose concentration hence no modification of the G1.2 media was necessary. Since D-[5-^{3}H] glucose was supplied at 1 mCi/ml, a 1 in 4 dilution was needed to achieve a working concentration of 0.25 µCi/µl. As per the manufacturer's recommendations, D-[5-^{3}H] glucose must be dried and reconstituted

prior to use. Therefore, a day before use, 80 µl D-[5-^{3}H] glucose solution was added to a 5 ml tube and dried by passing a gentle steady stream of 98% nitrogen gas through a glass pipette over the surface of the solution for approximately 30 mins. This was then stored at 4ºC. On the day of the experiment, 240 µl of warm pre-equilibrated G1.2 was added to the dried D-[5-^{3}H] glucose and left at 37ºC for 30 mins to dissolve.

For D-[6-^{14}C] glucose, 0.25 µCi/µl is equivalent to 4.55 mM of glucose. As this would significantly alter the normal glucose concentration of G1.2, a lower nominal radioactive concentration and a glucose free medium was employed. Modified glucose free G1.2 was constituted as described in Appendix 1 with Stock A containing all components except glucose. To obtain a total concentration of 0.5 mM, 43.2 µl of D-[6-^{14}C] glucose was added to 276.8 µl of warm pre-equilibrated glucose free G1.2 and mixed well prior to use. The resulting radioactive concentration was 0.027 µCi/µl.

7.2.4.3 Recovery efficiencies

Previous experiments in the lab have determined the recovery efficiencies of the ^{14}C- and ^{3}H- labels by using solutions containing known amounts of Na$H^{14}CO_2$ and $^{3}H_2O$. 50 hanging-drop systems containing 1.5 ml of 25 mM $NaHCO_3$ reservoirs were set up with 2 µl of these solutions suspended on the microtube lids as would be for the embryos/COCs. 1 ml of the $NaHCO_3$ reservoir was collected from 5 tubes every 30 mins from 0 to 300 mins and added to 200µl NaOH in a scintillation vial. These were then stored at 4ºC for a minimum overnight, after which 5ml of scintillation fluid was added and radioactivity levels determined using a liquid scintillation counter. The levels of radio labelled $^{14}CO_2$ and $^{3}H_2O$ recovered were close to maximal at 3 hours (180 mins) of incubation. Recovery

efficiencies were estimated by the percentage of total radioactivity that was recovered and found to be 92% and 94% respectively at 3 hours of incubation.

7.2.4.4 Validation of the hanging-drop assay on embryo development

In order to verify that the metabolic measurements were not confounded by adverse effects of the hanging-drop conditions on embryo health and viability, a preliminary experiment was conducted to validate the procedure. There are two variations of the hanging-drop, the first consisting of a bi-carbonate free system with NaOH as the reservoir trap [483], and the second with a bicarbonate buffered system employing $NaHCO_3$ as the reservoir trap [231]. The use of NaOH would eliminate the need for the 6% CO_2 gas phase, however, this would mean that the culture system would be completely void of CO_2 and bicarbonate which have been suggested to favour embryo development by mechanisms unrelated to pH [484]. Both hanging-drop culture conditions were assessed for their impact on embryo development in comparison to standard culture conditions as detailed in Chapters 2.2.3 and 2.6.

To assess embryo viability, 1-cell zygotes were collected from 3 mice as described in Chapter 2.2.3. Once the surrounding cumulus cells were removed, zygotes were washed once in 3 ml of fresh warm MOPS-G1 then randomly divided into three groups. Two groups were washed twice in G1.2 drops (Appendix 1) pre-equilibrated at 37°C in 6% CO_2, 5% O_2, 89% N_2 under mineral oil. For the experimental control, zygotes from one of these groups were then placed in groups of approximately 10, into fresh pre-equilibrated G1.2 drops (Appendix 1) and cultured under standard conditions as described in Chapter 2.2.3. The remaining zygotes in the G1.2 media were then picked up individually in 2 µl volumes and placed on the severed lid of a 1.5 ml microtube. The lid was then inverted and gently placed on top of the microtube containing 1.5 ml of $NaHCO_3$. The air space between the lid

and the bicarbonate buffered reservoir was replaced by gently streaming a 6% CO_2, 5% O_2, 89% N_2 gas mixture through a glass pipette for approximately 5 seconds. The glass pipette was then swiftly removed and the lid sealed shut ensuring minimal disturbance to the drop. Zygotes allocated to the last group were washed once more in fresh warm MOPS-G1 then picked up individually in 2 µl volumes and placed on the severed lid of a 1.5 ml microtube containing 1.5 ml of NaOH. The lids were inverted and carefully sealed shut. All hanging-drop tubes were placed at 37ºC for 3 hours. All zygotes were then removed and cultured in fresh pre-equilibrated G-1 v3 drops (Vitrolife, Kungsbacka, Sweden) as described in Chapter 2.2.3. Embryo development was assessed as described in Chapter 2.6. As shown Table 7.2, while no significant differences were noted, there was a trend towards a reduction in all stages of development of zygotes from the bicarbonate free hanging-drop system compared to both the control and the bicarbonate buffered hanging-drop system. There was no apparent difference in development of zygotes from the bicarbonate buffered hanging-drop system compared to zygotes cultured under standard conditions. Hence the bicarbonate buffered hanging-drop system [231] was employed.

Table 7.2: Effect of the hanging-drop culture system on embryo development.

	2-cell Embryos After 24h of Culture Per Zygote (%)	8-cell/Morula Embryos After 49-51h of Culture Per 2-cell Embryos (%)	Blastocyst After 96-100h of Culture Per 2-cell Embryo (%)	Hatching Blastocyst After 96-100h of Culture Per 2-cell Embryo (%)
Control	80.0	93.8	93.8	87.5
$NaHCO_3$	84.6	90.9	100.0	90.0
NaOH	68.4	61.5	76.9	61.5

Data represents approximately 20 1-cell zygotes cultured in groups of ~10.

7.2.4.5 Metabolic activity assessment of COCs

Microtubes containing 1.5 ml of $NaHCO_3$, 100 µl G1.2 wash drops with or without glucose overlayed with mineral oil (Vitrolife, Kungsbacka, Sweden or Merck, Darmstadt, Germany) and 35 mm Petri dishes containing 3 ml of mineral oil were equilibrated at 6% CO_2, 5% O_2, 89% N_2 overnight. After 18 hours of maturation in their respective treatments, COCs were washed once in 3 ml of MOPS-G1 then three times in their respective G1.2 wash drops to ensure complete removal of the IVM media. Medium containing the radioactive labelled substrate D-[5-^{3}H] glucose or D-[6-^{14}C] glucose was then pipetted under the pre-equilibrated mineral oil. Groups of approximately 15 COCs were then transferred in 2 µl volumes into the radioactive medium. Individual COCs were then picked up in 2 µl volumes and placed onto the severed lid of a microtube. The air space between the lid and the $NaHCO_3$ reservoir trap was then replaced with a gas mixture of 6% CO_2, 5% O_2, 89% N_2 as previously described and the lids sealed shut with minimal disturbance to the drop. For each experiment, a minimum of 5 sham control tubes (2 µl drop of radioactive medium with no COC) were set up to determine the non-specific exchange of the radioactive label from the drop to the reservoir. A minimum of 5 total count control tubes

(2 µl of the radioactive medium added directly into the $NaHCO_3$ reservoir trap) were also set up to measure total radioactivity. Microtubes were then incubated for 3 hours at 37ºC. At the end of the culture period, 1 ml of $NaHCO_3$ from each microtube was transferred to a 6 ml scintillation vial containing 200 µls of 0.1 M NaOH and stored at 4ºC for a minimum of 24 hours. The exact incubation period for each microtube until the transfer of $NaHCO_3$ to the vial was recorded.

5 ml of scintillation fluid (Optiphase Hisafe 3, PerkinElmer USA) was added to each vial on the day of counting. The vials were then vortexed and placed into a liquid scintillation beta counter. Each vial was counted for 20 mins and the raw counts were corrected for quenching and converted to disintegrations per minute (dpm).

As shown in the equation below, the amount of substrate metabolised per COC was determined by first subtracting the mean dpm of the sham controls from the dpm of each vial to correct for non-specific counts. This was then multiplied by the amount of labelled substrate present, the recovery efficiency and the concentration of total substrate in the medium. The result was then divided by the mean dpm of the total count controls multiplied by the concentration of the labelled substrate. This value was then divided by the time of incubation in hours to obtain the amount of substrate metabolised in pmol/COC/hour.

$$\text{Amount of Substrate Metabolised} = \frac{\text{(Dpm COC – Dpm sham) x Labelled Substrate Amount (pmols) x Recovery Efficiency (\% coverted to a number by / 100) x Total Substrate Concentration (pM)}}{\text{Dpm Total Counts x Labelled Substrate Concentration (pM)}}$$

7.2.5 Oxidative Stress Measurement

At the end of the 18 hour maturation period, 3 µl of the 0.5 mM RedoxSensor Red CC-1 (Molecular Probes, Oregon, USA) (Appendix 4) stock solution was added directly into the maturation dish containing the COCs to give a final working concentration of 0.5 µM. The dish was then swirled to mix the probe and incubated for 15 mins at 37°C in 6% CO_2, 5% O_2, 89% N_2, after which COCs were removed and placed into warm IVM handling media (Appendix 1). COCs were then washed once in MOPS-G1 (Appendix 1) and oocytes were then denuded by repeated gentle pipetting in MOPS-G1 (Appendix 1). Oocytes were then pipetted in groups of 5-10 in a row onto a large glass coverslip containing a 5 µl drop of MOPS-G1 (Appendix 1). The coverslip was then placed under a Leica DMIRB microscope attached to a Leitz MPV COMBI (Wetzlar, Germany) at 100x magnification and the fluorescence intensity of a fixed area of each oocyte was measured. Treatments were staggered in 10 min intervals to minimise the post maturation time differences for each treatment's analysis and to maintain uniformity of the 15 min staining period. The fluorescence reading of each oocyte was then analysed relative to the mean fluorescence reading of all the oocytes of the individual daily experimental control and results were expressed as the level of oxidative stress relative to the controls.

7.2.6 Oxidative Stress Localisation

A dual stain of RedoxSensor Red CC-1 and MitoTracker Green FM stain (Molecular Probes, Oregon, USA) (Appendix 4) was employed to visualise the localization of oxidative stress. Immediately prior to use, 1 µl of a frozen 1mM MitoTracker Green FM aliquot was added to 1 ml of warm IVM handling media (Appendix 1). 100 µl of this was then added to another 400 µl of warm IVM handling media. 350 µl of this was then added to a tube containing 10 µl of the 0.5 mM RedoxSensor red CC-1 stock solution and 350 µl of warm IVM handling media to give the final working dual stain solution. This was then laid down as 100 µl drops in a 35 mm Petri dish and overlayed with 3 ml of mineral oil (Vitrolife, Kungsbacka, Sweden or Merck, Darmstadt, Germany). At the end of maturation, COCs were placed into these drops and left in the dark at 37°C in 6% CO_2, 5% O_2, 89% N_2 for 15 mins. COCs were then washed once in MOPS-G1 (Appendix 1) then denuded by gentle repeated pipetting and placed in groups of 5-10 oocytes into a 5 µl drop of MOPS-G1 on a glass coverslip with paraffin wax at the edges. A smaller coverslip was then placed on top of the paraffin wax and the oocytes were imaged at 400-600x magnification under a Nikon Eclipse TE2000-E confocal microscope.

7.2.7 Statistics

Gene expression, metabolic activity and oxidative stress measurements were analysed using a univariate general linear analysis of variance utilising least significant differences when treatments had equal error variance and a Dunnett T3 post hoc when treatments had unequal error variance. Experiment day was used as a covariate to analyse the degree of and account for variance between experiments. Treatments with a P value of <0.05 were

taken to be significantly different. All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) version 13.0 for windows. Data is expressed as mean ± SEM and represents 3 or more experimental replicates unless otherwise stated.

7.3 RESULTS

7.3.1 Detection of PFKFB Isoforms in COCs

All isoforms of PFKFB were detected in COCs however, in varying abundance as shown in Table 7.3. PFKFB1 and 4 were only weakly expressed in untreated COCs with Ct values above 30 at the loading concentration of 1 ng of COC and close to 40 at the next lowered concentration of COCs. No template real time PCR controls had Ct values of 40 for all genes analysed. As the genes must be readily detected at 1 ng of COC due to limited sample sizes, only PFKFB2 and 3 were analysed in subsequent treatment samples.

Table 7.3. PFKFB1-4 Expression in COCs

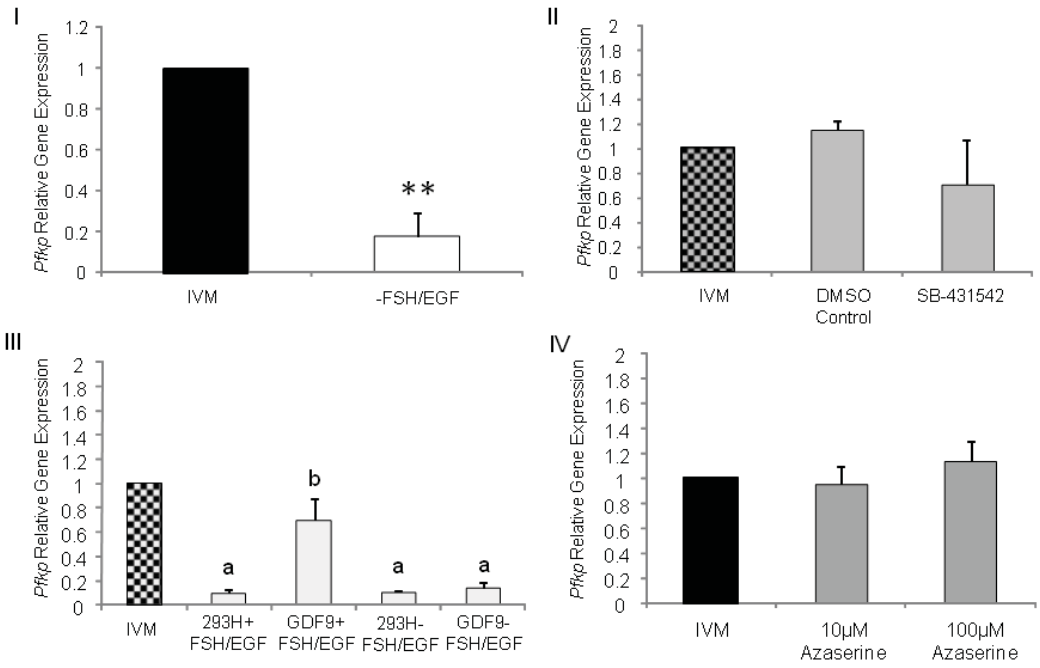
	PFKFB1	**PFKFB2**	**PFKFB3**	**PFKFB4**
100ng (~6 COCs)	26.45 ± 1.07	23.82 ± 0.20	20.65 ± 0.05	25.97 ± 0.08
10ng (~0.6 COCs)	30.55 ± 0.23	26.05 ± 0.07	24.69 ± 0.14	29.50 ± 0.11
1ng (~0.06 COCs)	33.19 ± 0.17	29.03 ± 0.06	27.37 ± 0.13	31.55 ± 0.51
0.1ng (~0.006 COCs)	40 ± 0.0	32.61 ± 0.35	30.25 ± 0.22	34.44 ± 0.235

Data represents mean Ct values ± SEM of duplicate PCR reactions. Values of above 40 indicate the presence of the gene transcript.

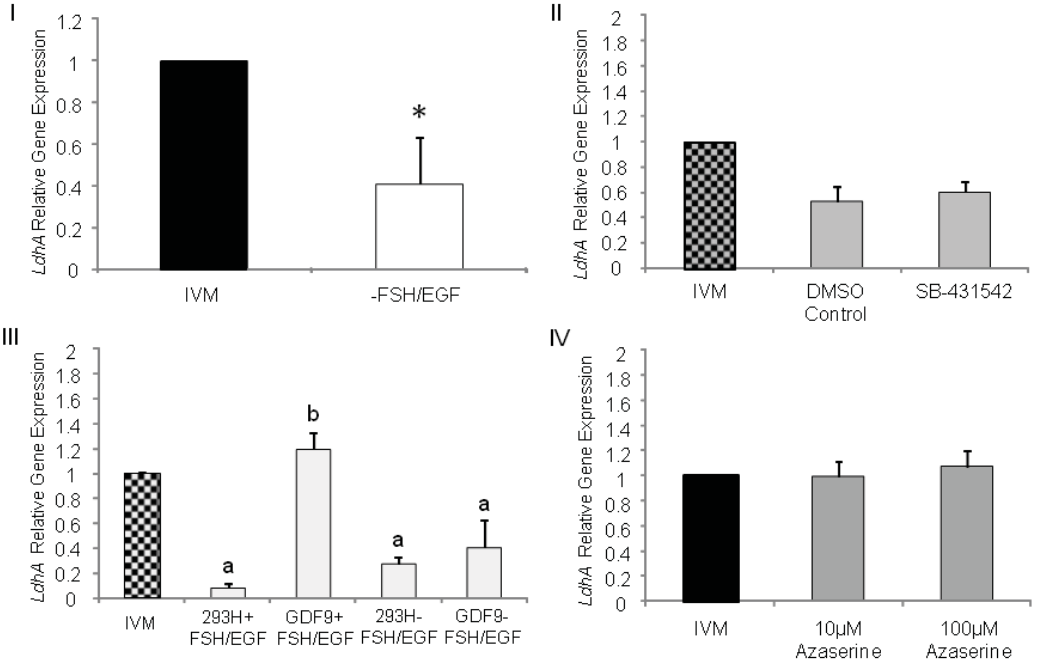
7.3.2 Effect of Oocyte-Cumulus Bi-directional Communication on Metabolic Gene Transcripts

As shown in Figure 7.4 A-D, IVM in the absence of FSH/EGF resulted in a significant decrease in the expression of all the metabolic gene transcripts analysed (Pfkp ($P \leq 0.001$), LdhA ($P < 0.05$), Pfkfb2 ($P \leq 0.001$), and Pfkfb3 ($P < 0.05$) (N=5 independent experiments)) compared to IVM in the presence of FSH/EGF. Inhibition of SMAD2/3 signalling in the presence of FSH and EGF had no effect on the levels of any of the gene transcripts analysed compared to the DMSO control (N=5 independent experiments). In contrast, the addition of exogenous GDF9 significantly ($P < 0.05$) increased Pfkp, LdhA and Pfkfb2 expression over that of the 293H control in the presence of FSH/EGF. However GDF9 addition had no effect on any of the genes analysed in the absence of FSH/EGF. Pfkfb3 expression was also unaltered with exogenous GDF9 addition (N=4 independent experiments). There was no effect on the expression of any of the metabolic genes analysed when COCs were matured with 10 µM or 100 µM of azaserine (N=3 independent experiments).

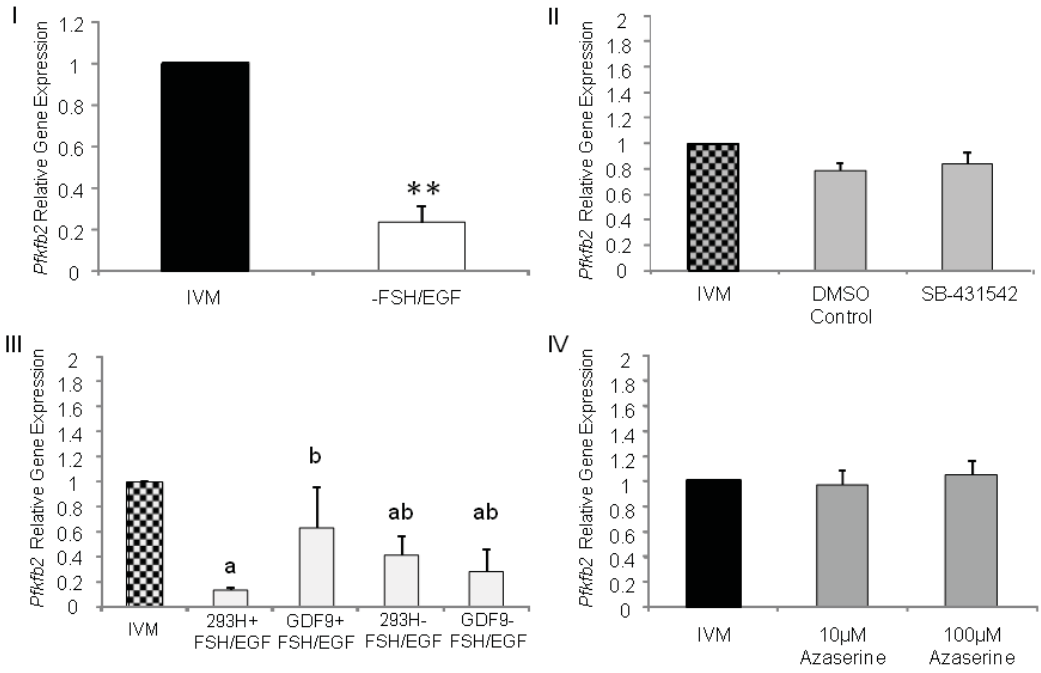
A


I
Pfkp Relative Gene Expression
1.2
1
0.8
0.6
0.4
0.2
0
**
IVM
-FSH/EGF
II
Pfkp Relative Gene Expression
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
IVM
DMSO Control
SB-431542
III
Pfkp Relative Gene Expression
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
b
a
a
IVM
293H+ FSH/EGF
GDF9+ FSH/EGF
293H- FSH/EGF
GDF9- FSH/EGF
IV
Pfkp Relative Gene Expression
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
IVM
10μM Azaserine
100μM Azaserine

B


I
LdhA Relative Gene Expression
1.2
1
0.8
0.6
0.4
0.2
0
*
IVM
-FSH/EGF
II
LdhA Relative Gene Expression
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
IVM
DMSO Control
SB-431542
III
LdhA Relative Gene Expression
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
b
a
a
IVM
293H+ FSH/EGF
GDF9+ FSH/EGF
293H- FSH/EGF
GDF9- FSH/EGF
IV
LdhA Relative Gene Expression
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
IVM
10μM Azaserine
100μM Azaserine

C


I
Pfkfb2 Relative Gene Expression
1.2
1
0.8
0.6
0.4
0.2
0
**
IVM
-FSH/EGF
II
Pfkfb2 Relative Gene Expression
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
IVM
DMSO Control
SB-431542
III
Pfkfb2 Relative Gene Expression
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
b
ab
ab
IVM
293H+ FSH/EGF
GDF9+ FSH/EGF
293H- FSH/EGF
GDF9- FSH/EGF
IV
Pfkfb2 Relative Gene Expression
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
IVM
10μM Azaserine
100μM Azaserine

D

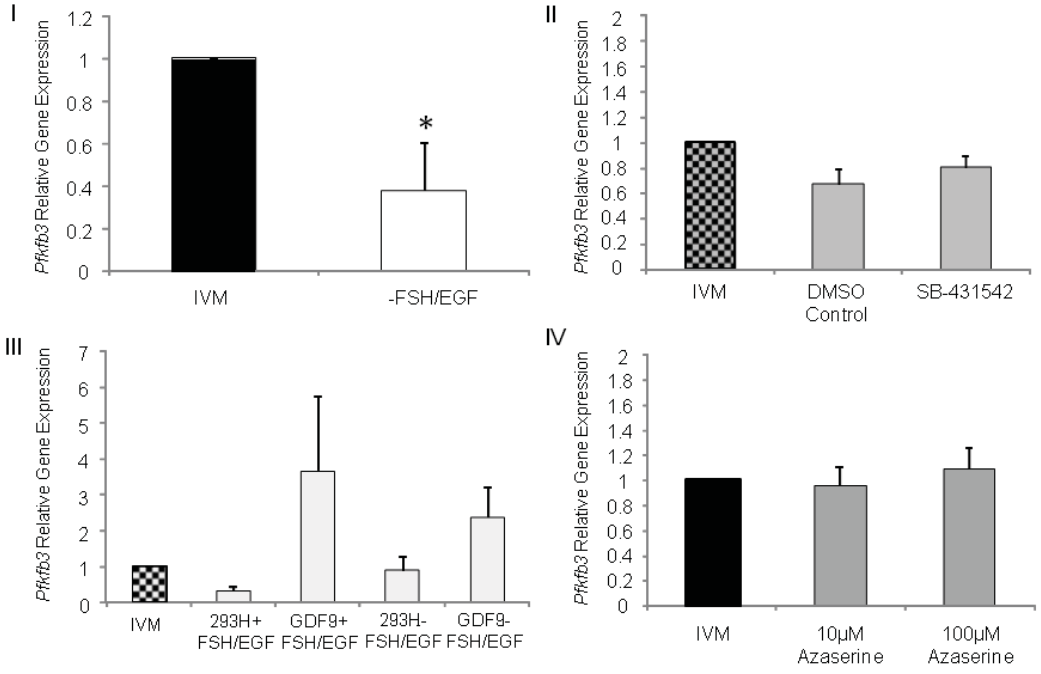

I
Pfkfb3 Relative Gene Expression
1.2
1
0.8
0.6
0.4
0.2
0
*
IVM
-FSH/EGF
II
Pfkfb3 Relative Gene Expression
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
IVM
DMSO Control
SB-431542
III
Pfkfb3 Relative Gene Expression
7
6
5
4
3
2
1
0
IVM
293H+ FSH/EGF
GDF9+ FSH/EGF
293H- FSH/EGF
GDF9- FSH/EGF
IV
Pfkfb3 Relative Gene Expression
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
IVM
10μM Azaserine
100μM Azaserine

Figure 7.4. Relative expression of metabolic genes in COCs after IVM in various treatments. Relative gene expression of *Pfkp* **(A)**, *LdhA* **(B)**, *Pfkfb2* **(C)** and *Pfkfb3* **(D)** of COCs matured for 18 hours **(I)** in the absence (-FSH/EGF) compared to the presence of FSH and EGF (IVM), **(II)** with the SMAD2/3 inhibitor SB-431542 (4 µM) or the DMSO control (0.04% v/v) in the presence of FSH/EGF, **(III)** with exogenous GDF9 addition or the 293H control in the presence (GDF9+FSH/EGF; 293H+FSH/EGF) or absence (GDF9-FSH/EGF; 293H-FSH/EGF) of FSH and EGF, **(IV)** with 10 µM or 100 µM of azaserine in the presence of FSH and EGF. FSH and EGF were used at the concentrations of 50 mIU/ml and 10 ng/ml respectively. Gene expression was analysed by real time PCR. IVM with FSH/EGF (IVM) was used as the exogenous relative control. Values of all experimental replicates were first normalised to their individual endogenous controls *Rpl19* or *Rn18s* then made relative to that of IVM and expressed as the mean ± SEM. Statistical comparisons were only made between treatments of the same experiment. Solid IVM bars indicate IVM as both the experimental and exogenous control while checker IVM bars indicate its use as the exogenous control only. $^{a.b}$ Columns with different superscripts represent statistically significant differences ($P<0.05$); *= $P<0.05$; **= $P\leq0.001$.

7.3.3 Effect of Oocyte-Cumulus Bi-Directional Communication on Metabolic Activity

7.3.3.1 Glycolytic Activity

Metabolic activity was analysed using radio labelled glucose substrates via the hanging drop method. Glycolytic activity of individual COCs through the Embden-Myerhof pathway (EMP) was analysed through the metabolism of D-[5-^{3}H] glucose to radioactive $^{3}H_2O$ and expressed as the mean amount of total glucose metabolised through the EMP in pico moles per COC per hour. As shown in Figure 7.5, the absence of FSH and EGF during IVM (N=13 COCs) significantly decreased ($P \leq 0.001$) glycolytic activity compared to COCs matured with FSH/EGF (N=12 COCs) (Figure 7.5A). Inhibition of oocyte signalling through the SMAD2/3 pathway also resulted in a substantial significant decrease ($P \leq 0.001$) in glycolytic activity compared to the DMSO control in the presence FSH/EGF. However, there was no additional effect on glycolytic activity when SMAD2/3 was inhibited in the absence of FSH/EGF (N=15 COCs per treatment) (Figure 7.5 B). Addition of exogenous GDF9 during IVM resulted in a significant increase in glycolytic activity both in the presence ($P \leq 0.001$) or absence of FSH/EGF ($P < 0.05$) over that of the 293H control. There was also a significant increase ($P < 0.05$) in glycolytic activity in COCs matured with both exogenous GDF9 and FSH/EGF compared to COCs matured with exogenous GDF9 in the absence of FSH/EGF (N=14 COCs per treatment) (Figure 7.5 C).

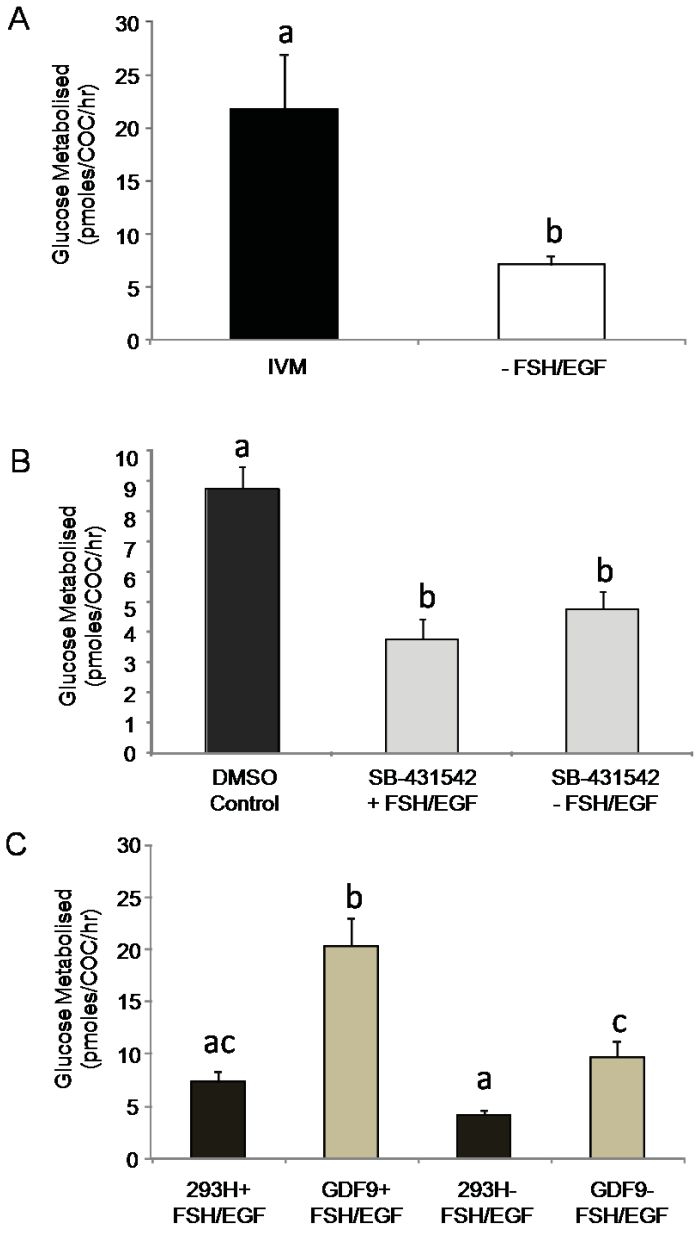


Figure 7.5. Effect of oocyte-cumulus bi-direction signalling on glycolytic activity. Glycolytic flux through the Embden-Myerhof pathway was assessed via the metabolism of D-[5-^{3}H] glucose into $^{3}H_2O$ of individual COCs for 3 hours after 18 hours of maturation in their respective treatments. Data represents the mean amount of glucose metabolised per COCs per hour. COCs were matured **(A)** in the absence (-FSH/EGF) and compared to the presence of FSH and EGF (IVM), **(B)** with the SMAD2/3 inhibitor SB-431542 (4 µM) in the presence (SB-431542+FSH/EGF) or absence (SB-431542-FSH/EGF) of FSH and EGF or with 0.04% v/v DMSO with FSH/EGF (DMSO control), **(C)** with exogenous GDF9 addition or the 293H control in the presence (GDF9+FSH/EGF; 293H+FSH/EGF) or absence (GDF9-FSH/EGF; 293H-FSH/EGF) of FSH and EGF. FSH and EGF were used at the concentrations of 50 mIU/ml and 10 ng/ml respectively. Values are expressed as the mean ± SEM of 12-15 individual COCs. [a.b] Columns with different superscripts represent statistically significant differences ($P<0.05$);

7.3.3.2 TCA Cycle Activity

Metabolic activity of the tricarboxylic acid (TCA) cycle was analysed through the conversion of D-[6-^{14}C] glucose to radioactive $^{14}CO_2$ and expressed as the mean amount of total glucose consumed through the TCA cycle in pico moles per COC per hour. As shown in Figure 7.6, the absence of FSH and EGF during IVM (N=12 COCs) significantly ($P<0.05$) decreased TCA cycle activity compared to COCs matured with FSH/EGF (N=18 COCs) (Figure 7.6 A). However, inhibition of oocyte signalling through the SMAD2/3 pathway had no effect on TCA cycle activity of COCs matured with or without FSH/EGF compared to the DMSO control (N=14 COCs per treatment) (Figure 7.6 B). Addition of exogenous GDF9 during IVM also had no effect on COC TCA cycle activity regardless of the presence or absence of FSH and EGF compared to the 293H control (N=14 COCs per treatment) (Figure 7.6 C).

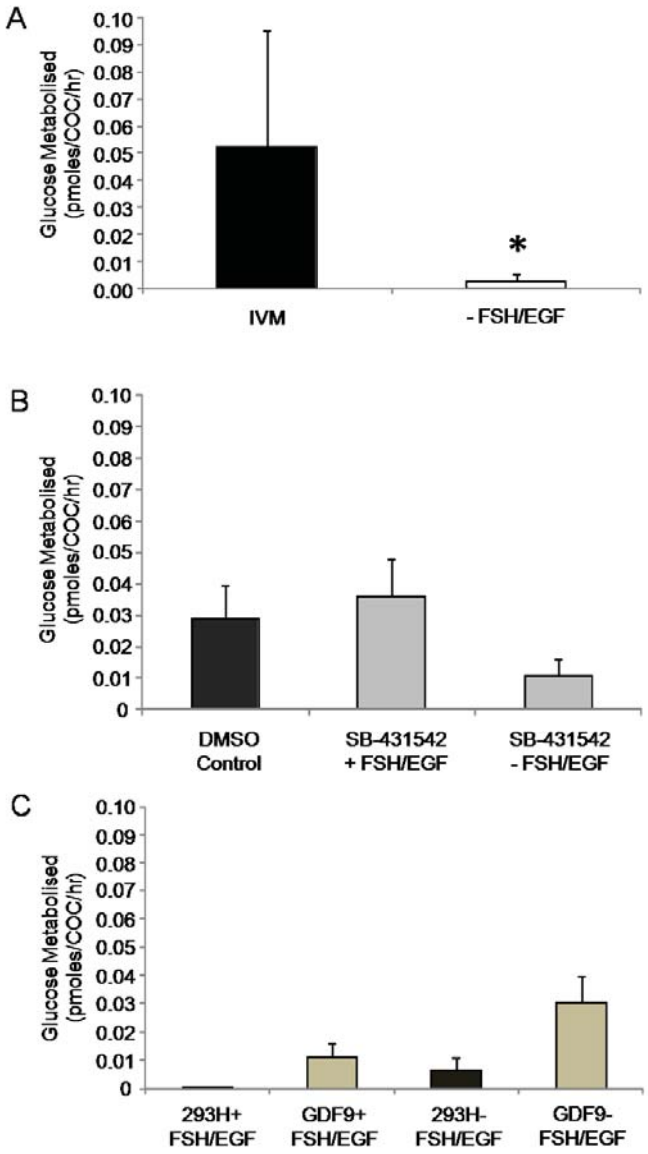


Figure 7.6. Effect of oocyte-cumulus bi-direction signalling on TCA cycle activity. The total amount of glucose committed to TCA cycle metabolism was analysed through the conversion of D-[6-^{14}C] glucose to $^{14}CO_2$ of individual COCs for 3 hours after 18 hourd of maturation in their respective treatments. Data represents the mean amount of glucose metabolised per COC per hour. COCs were matured **(A)** in the absence (-FSH/EGF) and compared to the presence of FSH and EGF (IVM), **(B)** with the SMAD2/3 inhibitor SB-431542 (4 µM) in the presence (SB-431542+FSH/EGF) or absence (SB-431542-FSH/EGF) of FSH and EGF with 0.04% v/v DMSO with FSH/EGF (DMSO control) as the experimental control. **(C)** COCs were matured with exogenous GDF9 addition or the 293H control in the presence (GDF9+FSH/EGF; 293H+FSH/EGF) or absence (GDF9-FSH/EGF; 293H-FSH/EGF) of FSH and EGF. FSH and EGF were used at the concentrations of 50 mIU/ml and 10 ng/ml respectively. Values are expressed as the mean ± SEM of 12-14 individual COCs. * Denotes statistical difference where $P<0.05$.

7.3.4 Effect of Oocyte-Cumulus Bi-Directional Communication on Oxidative Stress

7.3.4.1 FSH/EGF Cumulus to Oocyte Signalling

At the end of the 18 hour maturation period, COCs were denuded and the oocyte of individual COCs were assessed for oxidative stress levels using RedoxSensor Red CC-1 (Molecular Probes, Oregon, USA) (Appendix 4), a stain which fluorescence increases with the amount of oxidative stress. Localisation of oxidative stress was also visualised using dual staining of the mitochondria (MitoTracker Green FM stain (Molecular Probes, Oregon, USA) (Appendix 4)) which has a green fluorescence and RedoxSensor Red CC-1 which has a red fluorescence, dual stained areas should appear orange/yellow. As shown in Figure 7.7, maturation of COCs in the absence of FSH and EGF (N=42 oocytes) resulted in a 2-4 fold significant increase ($P \leq 0.001$) in oxidative stress levels in the oocyte compared to IVM with FSH/EGF (N=34 oocytes). The areas of oxidative stress appeared to be evenly distributed between the lysosomes in the cytosol and in the mitochondria as indicated by more orange/yellow stained areas than individual green or red areas.

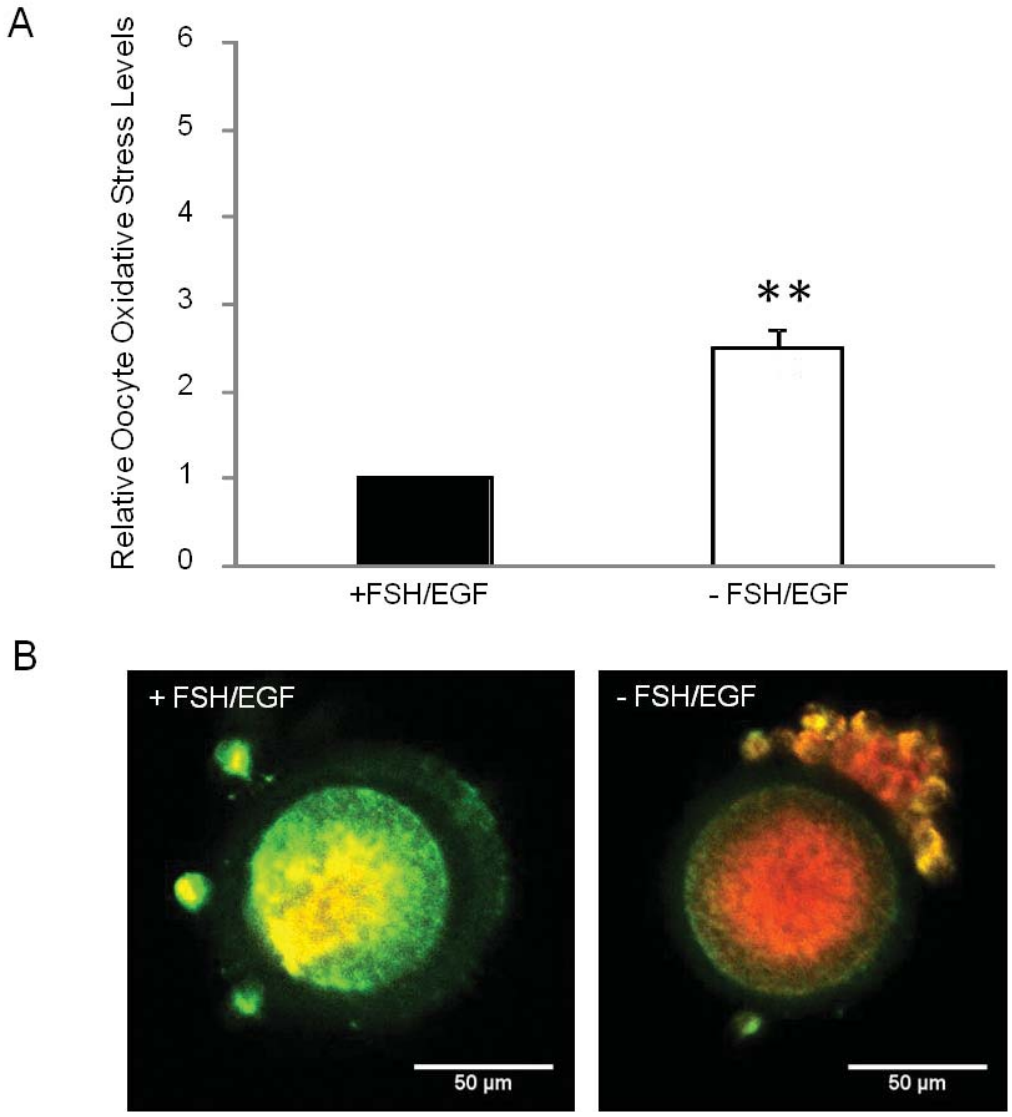


Figure 7.7. Effect of FSH and EGF during IVM on oxidative stress. (A) COCs were matured for 18 hours in the presence (+FSH/EGF) or absence (-FSH/EGF) of 50 mIU/ml FSH and 10ng/ml EGF. Cumulus cells were then removed by repeated pipetting and oocyte oxidative stress levels were quantified. Oxidative stress levels of oocytes matured without FSH/EGF made relative to those matured with FSH/EGF. Data is expressed as mean ± SEM. **Denotes statistical significance where P≤0.001. N= 34-42 oocytes from 3 indenpendent experiments. **(B)** Representative fluorescent photomicrographs of oxidative stress patterns. Mitochondria are stained green, red staining indicates oxidative stress regions and yellow/orange staining indicate dual stained regions. Images were captured on a Nikon Eclipse TE-2000E inverted microscope (Nikon Corporation, Japan) at 20x magnification under UV light.

7.3.4.2 Disruption of Oocyte to Cumulus Signalling

Oocyte to cumulus signalling was disrupted through the use of SB-431542, an inhibitor specific for SMAD2/3 signalling. As shown in Figure 7.8, maturation of COCs with the SMAD2/3 inhibitor in the presence of FSH and EGF resulted in a significant approximate 2 fold increase ($P \leq 0.001$) in oxidative stress levels in the oocyte (N=14 oocytes) compared to the control (N=12 oocytes). Oxidative stress appeared to be evenly distributed between the lysosomes in the cytosol and in the mitochondria as indicated by more orange/yellow stained areas than individual green or red areas.

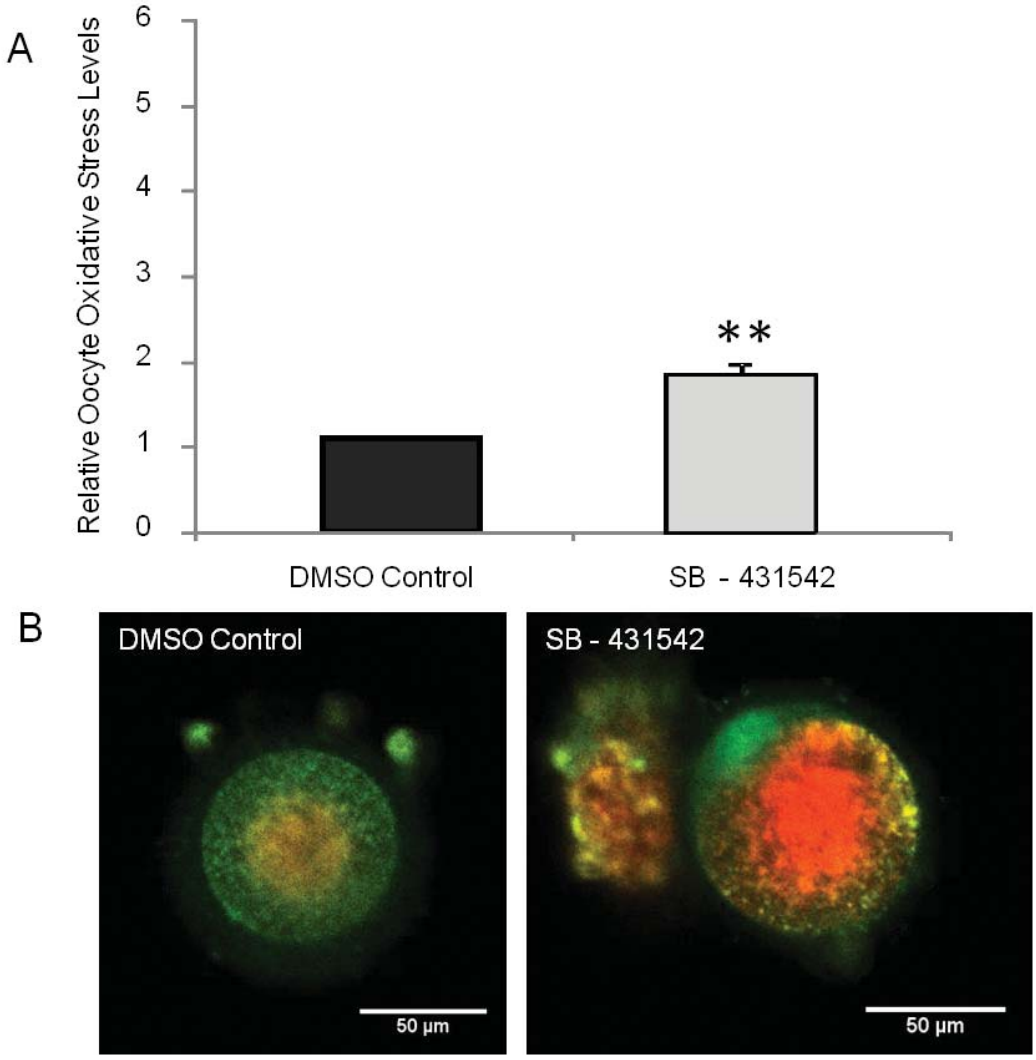


Figure 7.8. Effect of SMAD2/3 inhibition during IVM on oxidative stress. (A) After 18 hours of maturation with 4 µM SB-431542 or the equivalent 0.04%v/v DMSO control in the presence of 50 mIU/ml FSH and 10ng/ml EGF, cumulus cells were removed by repeated pipetting and oocyte oxidative stress levels were quantified. Oxidative stress levels of oocytes matured with 4 µM SB-431542 were then made relative to those matured with the DMSO control. Data is expressed as mean ± SEM. **Denotes statistical significance where P≤0.001. N= 12-14 oocytes. **(B)** Representative fluorescent photomicrographs of oxidative stress patterns. Mitochondria are stained green, red staining indicates oxidative stress regions and yellow/orange staining indicate dual stained regions. Images were captured on a Nikon Eclipse TE-2000E inverted microscope (Nikon Corporation, Japan) at 20x magnification under UV light.

7.3.4.3 Disruption of COC Metabolism Independent of Oocyte-Cumulus Bi-directional Signalling

Azaserine, an inhibitor for the rate limiting enzyme of the hexosamine biosynthesis pathway, GFPT1, was used as the model for disruption of COC metabolism independent of oocyte-cumulus bi-directional signalling. As shown in Figure 7.9, maturation of COCs with azaserine resulted in a significant ($P \leq 0.001$) increase in oxidative stress levels in the oocyte (N=30 oocytes) compared to the control (N=23 oocytes). Oxidative stress appeared to be localised in the lysosomes rather than in the mitochondria as indicated by more green stained areas than yellow/orange areas.

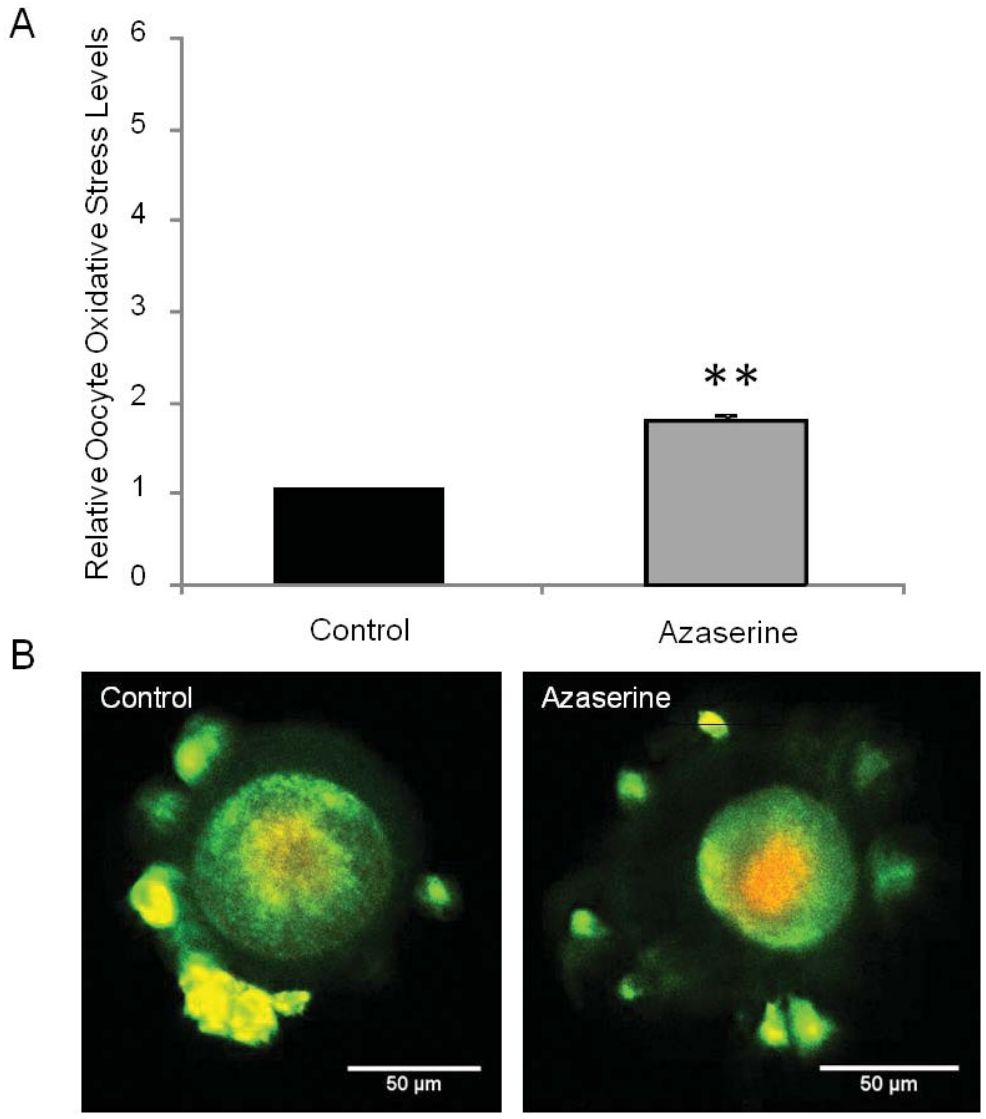


Figure 7.9. Effect of Azaserine during IVM on oxidative stress. (A) After 18 hours of maturation with 100 µM Azaserine plus 50 mIU/ml FSH and 10ng/ml EGF (Azaserine) cumulus cells were then removed by repeated pipetting and oocyte oxidative stress levels were quantified. Oxidative stress levels oocytes matured with azaserine were then made relative to those of the IVM control. Data is expressed as mean ± SEM. **Denotes statistical significance where $P \leq 0.001$. N= 30-34 oocytes from 2 independent experiments **(B)** Representative fluorescent photomicrographs of oxidative stress patterns. Mitochondria are stained green, red staining indicates oxidative stress regions and yellow/orange staining indicate dual stained regions. Images were captured on a Nikon Eclipse TE-2000E inverted microscope (Nikon Corporation, Japan) at 20x magnification under UV light.

7.4 DISCUSSION

Oocytes have been shown to directly regulate key glycolytic transcripts and metabolic activity of cumulus cells prior to maturation since they cannot utilise glucose efficiently [215] and are reliant on cumulus cells for products of glycolysis [218]. This regulatory role is dependent on the oocyte's developmental stage since only fully grown oocytes were able to exert this effect [282]. Hence the ability of an oocyte to regulate cumulus cell physiology may be reflective of its developmental competence.

Previous chapters in this thesis have established the importance of oocyte to cumulus bi-directional communication during the short period of oocyte IVM on subsequent embryo and foetal outcomes. The aim of this chapter was therefore to investigate the influence of oocyte-cumulus cell bi-directional communication during IVM on COC metabolic gene expression and activity and to elucidate the possible mechanisms by which oocyte developmental competence is affected.

The presence of FSH and EGF during IVM resulted in a significant increase in all the metabolic gene transcripts; *Pfkp, LdhA, Pfkfb2* and *Pfkfb3,* analysed. The inhibition of SMAD2/3 signalling in the presence of FSH and EGF did not have a significant effect on metabolic gene expression but the addition of exogenous GDF9 with FSH and EGF during IVM resulted in a significant increase in *Pfkp, LdhA* and *Pfkfb2* over that of the 293H control. It is important to note however that similar to the embryo development shown in Chapter 6, inhibitory factors may reside in the 293H control conditioned media as all the metabolic gene transcripts appear to be down regulated compared to that of the IVM

control with GDF9 increasing relative gene expression only to that of the IVM control. Unfortunately, direct comparisons to standard IVM cannot be made as only the 293H control was included in these experiments investigating the effect of exogenous GDF9 since all components in the 293H control conditioned media are expected to also be present in the exogenous GDF9 conditioned media. IVM with azaserine had no effect on metabolic gene expression.

Pfkp and *LdhA* are isoforms of two key regulatory glycolytic enzymes phosphofructokinase (PFK) and lactate dehydrogenase (LDH) respectively. PFK is the rate limiting enzyme of glycolysis as it catalyses the first non-reversible step of the entry of glucose into the Embden-Meyerhof pathway and LDH is the enzyme responsible for the conversion of pyruvate to lactate. Both *Pfkp* and *LdhA* were shown to be rapidly down regulated in oocytectomised COCs and dependent on oocyte secreted factors for up-regulation in cumulus cells prior to maturation [282]. This chapter's findings demonstrated that this oocyte paracrine control exists throughout maturation but is bi-directionally controlled, since the absence of FSH and EGF negated the effect of exogenous GDF9. Surprisingly while the inhibition of SMAD2/3 prevented cumulus expansion and decreased oocyte developmental competence, as shown in Chapters 4 and 5, it did not have an effect on metabolic gene expression. This is possibly due to the high level of redundancy of the huge repertoire of oocyte secreted factors such as the bone morphogenetic proteins (BMPs) and fibroblast growth factors (FGFs). Recombinant oocyte secreted factors BMP15 and FGF8 were only able to increase cumulus *Pfkp* and *LdhA* transcription in OOXs when used in combination but not alone. Recombinant GDF9 alone or in combination with BMP15 or FGF8 however did not affect cumulus metabolic gene expression in OOXs [290]. Although

the recombinant GDF9 used in that study [297] was from a different origin and has varying properties in that it induced cumulus expansion in the absence of FSH [187] to that used in this thesis, it is possible that SMAD2/3 signalling is not critical for the up-regulation of Pfkp and LdhA gene expression during IVM but acts synergistically in combination with other oocyte secreted factors other than BMP15 and FGF8 to increase these metabolic gene transcripts.

While PFK has been shown to be regulated at the level of transcription [485], the most potent regulator of PFK is fructose-2-6-bisphosphate (F2,6BP) [460, 461] which in turn is controlled by a single homodimeric bifunctional enzyme family known as 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases (PFKFB) [463-465]. Four known PFKFB isoforms have been discovered in different tissues, each encoded by a separate gene [464]. This chapter detected for the first time the presence of all PFKFB isoforms PFKFB1-4 in COCs and found that PFKFB2 and 3 were more abundantly expressed. The PFKFB isoforms differ in their net catalytic ability which is dependent on the ratio of their individual kinase and bisphosphatase domains [486], it is however been shown for PFKFB2 that this ratio can substantially change in response to external stimuli such as insulin and hypoxia [487, 488]. PFKFB3 has the highest affinity for F2,6BP out of all the isoforms, with a substantially increased kinase and decreased bisphosphatase activity [489, 490] and is also the most highly induced isozyme by hypoxia [373]. PFKFB3 knockouts are embryonic lethal [474] and PFKFB3$^{-/+}$ heterozygous mutant mice fibroblast cells have decreased intracellular F2-BP [491]. This indicates that the roles of each of the PFKFB isoforms are independent of one another and have little redundancy at least in the case of PFKFB3. Given these regulatory properties of PFKFB2 and 3 and their ability to increase kinase

activity in response to external stimuli such as hypoxia, it is plausible that they would play a key role in the metabolism of follicular somatic cells. Indeed, the expression of *Pfkfb2* and *Pfkfb3* were found to be similar to *Pfkp* and *LdhA*. Both genes were up-regulated in the presence of FSH and EGF and exogenous GDF9 with FSH and EGF also increased *Pfkfb2* significantly and had a trend towards an increase in *Pfkfb3* expression. An increase in these isoforms would tend to increase the levels of F2,6BP and would result in further increases in the activity of PFK and therefore glycolysis.

The activity of individual metabolic pathways were then directly analysed through the use of radio labelled substrates. D-[5-^{3}H] glucose and D-[6-^{14}C] glucose were used to determine the activity of the EMP and TCA cycle respectively. Reflective of glycolytic gene expression, the absence of FSH and EGF during IVM resulted in a substantial decrease in glucose metabolism through the EMP. While gene expression was not affected in COCs after maturation with SMAD2/3 inhibition in the presence of FSH/EGF, glycolytic activity was significantly reduced. The absence of FSH/EGF did not have an additive effect to that caused by SMAD2/3 inhibition to glycolytic activity. Addition of exogenous GDF9 significantly increased glycolysis over that of the 293H control both in the presence and absence of FSH/EGF. Hence while SMAD2/3 signalling did not significantly influence the expression of key glycolytic enzymes, glycolytic activity of COCs matured with alterations in this pathway was affected. This is unsurprising given that while the level of gene transcript may be reflective of its enzyme activity, metabolic enzymes are often regulated by post translational modifications such as phosphorylation.

An exceptionally small proportion (range from 0.03 - 5%) of glucose consumed through by the EMP was directed into the TCA cycle for all treatments indicating that glycolysis plays a more substantial role in COC metabolism during IVM than the TCA cycle which is in agreement with published findings [201]. Unexpectedly, the metabolism of glucose through the TCA cycle was decreased in COCs matured without FSH and EGF compared to when FSH/EGF were present. FSH has previously been found to have no effect on TCA cycle activity despite a substantial increase in the percentage of oocytes that resumed meiosis and EMP and pentose phosphate pathway (PPP) activity from 0-6 hours of maturation [201]. An investigation into carbohydrate usage patterns of individual mouse COCs during IVM with EGF found that while there were significant changes in glucose and lactate levels as maturation progressed, pyruvate levels in the media remained consistent throughout the 16 hours of maturation [223]. However, when used in an excess of 200 ng/ml, 40 times greater than that used in this study, FSH was found to increase the amount of pyruvate in the maturation media of individually cultured COCs from non-gonadotrophin stimulated mice. Furthermore, COC pyruvate metabolism was related to meiotic maturation with pyruvate consumption occurring in COCs with oocytes that failed to reach meiosis metaphase II while pyruvate was produced by those that completed nuclear maturation after 16 hours of culture [193]. Meiotic maturation was affected by the presence of FSH and EGF as shown in Chapter 5 and although it is the PPP that has been shown to be one of the important pathways mediating oocyte meiotic maturation [203], it is plausible that the combined effect of FSH and EGF and the difference in glucose concentration of the maturation media in this study to those of the above reports, mediated a significant effect on TCA cycle activity of COCs after maturation in the presence or absence of FSH and EGF.

SMAD2/3 inhibition or the addition of exogenous GDF9 during IVM both in the presence or absence of FSH and EGF did not have a subsequent effect on the TCA cycle activity of COCs. Hence, although oocyte secreted factors have been shown to up-regulate cumulus cell TCA cycle activity in oocytectomised COCs [282] , this may not be the case in intact COCs during or after IVM. The possibility that SMAD2/3 signalling does not directly influence cumulus cell TCA cycle activity must also be considered.

Azaserine, an inhibitor of GFPT1, the rate limiting enzyme of the HBP as described in Chapter 4, was used to disrupt cumulus metabolism independently of oocyte-cumulus bi-directional communication through the HBP. While there have been no studies that have directly measured the activity of individual metabolic pathways after perturbations in HBP activity, addition of glucosamine, a HBP substrate resulted in less glucose being incorporated into the bovine COC matrix [413]. However, addition of glucosamine during IVM subsequently decreased embryo developmental competence [136] which was unexpected since this should theoretically allow more glucose to be used by other metabolic pathways such as glycolysis and increased COC glycolytic activity has been positively correlated to oocyte developmental competence [221]. As only approximately 1-3% of cellular glucose metabolism is directed into the HBP [133] and cumulus expansion was not attenuated in this study's system, it is unlikely that changes in glucose metabolism through the EMP or TCA cycle would account for the decreased oocyte developmental competence reported in Chapter 4. Indeed, azaserine addition during IVM had no effect on cumulus glycolytic gene expression. It is plausible that like the lack of observable attenuation in cumulus expansion in Chapter 4, the absence of an azaserine effect on cumulus cell metabolic gene transcripts could be attributed to the substantially higher

concentration of glucose in the maturation medium (Waymouth MB 752/1: 27.8 mM glucose) hence the HBP was not sufficiently inhibited to result in an increase in glycolysis.

Increased and decreased HBP activities however, have been directly correlated to cellular oxidative stress [139, 140]. Hence the level of oxidative stress in the oocyte was measured after IVM with azaserine. Indeed, COCs matured with azaserine had significantly increased oocyte oxidative stress which was accumulated in the lysosomes and not the mitochondria. The HBP is responsible for activating, via o-linked glycosylation, an array of proteins involved in mediating cellular stress responses [140] hence it is likely that the increase in oxidative stress was not due to production of ROS by the TCA cycle but perhaps due to perturbations in ROS removal mechanisms.

Oocyte oxidative stress levels were also assessed in the experimental models where embryo development was reduced as a result of disruptions to oocyte-cumulus bi-directional communication as observed in Chapters 5 and 6. Indeed the absence of FSH and EGF during IVM resulted in a significant increase in oxidative stress which accumulated in both the oocyte mitochondria and the cytosolic lysosomes. This suggests that the oxidative stress was due to both perturbations in cellular metabolism resulting in the production rate of ROS through OXPHOS to exceed that of its removal and to disruptions in cellular ROS removal mechanisms. The increase in oxidative stress levels of the oocytes could also have been attributed to the decreased number of cumulus cells surrounding the oocytes at the end of maturation. Cumulus cells have been found to exert a protective role on the oocyte against oxidative stress [243, 254, 255] most likely through the transfer of antioxidants such as GSH through gap junctions. Since spontaneous maturation in the absence of the

FSH and EGF ligand stimuli results in an earlier breakdown of gap junction communication [492], oocytes may not have received the appropriate levels of antioxidants allowing them to effectively manage intracellular oxidative stress. The inhibition of SMAD2/3 signalling during IVM also increased oxidative stress in a similar distribution pattern to the absence of FSH and EGF. Hence it is likely that the increase in oxidative stress from inhibition of SMAD2/3 signalling during IVM was a causative factor for the reduced oocyte developmental competence observed in Chapter 5.

In summary, lack of cumulus signalling through the absence of FSH and EGF during IVM resulted in decreased expression of key glycolytic gene transcripts *Pfkp, LdhA and Pfkfb2 and Pfkfb3,* glycolytic and TCA cycle activity and increased oxidative stress. SMAD2/3 inhibition similarly decreased glycolytic activity and increased oxidative stress while the addition of exogenous GDF9 in the presence of FSH and EGF increased *Pfkp, LdhA and Pfkfb2* expression and glycolytic activity over that of the 293H control. Azaserine during IVM significantly increased oxidative stress levels despite having no effect on metabolic gene activity.

This is the first study that has detected the presence of Pfkfb1-4 in COCs and shown that oocyte-cumulus cell bi-directional communication is a significant regulatory factor of COC metabolism throughout maturation. The findings of this chapter have hence demonstrated the possible causes for the decreased oocyte developmental competence and subsequent foetal outcomes observed in Chapters 4 to 6.

Chapter 8

Final Discussion and Concluding Remarks

Successful in vitro maturation of oocytes poses an attractive alternative to current IVF procedures as the need for rigorous hormonal stimulation regimes would be eliminated, reducing cost, inconvenience and discomfort and health risks to patients. Unfortunately IVM success rates are still significantly lower than that of conventional IVF utilising in vivo matured oocytes [11, 13] and as such, it has yet to become routine clinical practice. This reduced success rates are largely attributed to a lack of understanding of the complex processes involved and the intrinsic requirements of the oocyte as it acquires the capacity for both nuclear and cytoplasmic maturation and hence the inability to mimic or support them in vitro. This thesis therefore aimed to further our knowledge of the interactions between the oocyte and its surrounding cumulus cells during IVM with focus on the oocyte secreted factor GDF9 and its signalling pathway, FSH and EGF and their contributions to oocyte developmental competence.

Research in the last few decades has revealed the existence of the oocyte to cumulus bi-directional regulatory loop fundamental to the function, growth and survival of both cell types [35, 64, 282, 341, 406]. While the precise composition of oocyte-secreted factors involved in this bi-directional regulatory loop remains unclear, one of the identified factors is GDF9, a member of the TGF-β super-family which signals through the SMAD2/3 signalling pathway [274, 318, 340, 443]. FSH and EGF have long been used to induce oocyte meiotic maturation but are only effective in cumulus enclosed oocytes mediating their effects on the oocyte via cumulus cells [57]. Hence for the entirety of this thesis the GDF9 signalling pathway, SMAD2/3, was targeted as the mode for oocyte to cumulus communication while FSH and EGF signalling were targeted as the mode for cumulus to oocyte communication.

One of the earliest discovered cumulus functions dependent on oocyte regulation was cumulus expansion [271]. Cumulus expansion requires both external stimuli such as FSH and EGF ligands [57, 73] and an oocyte-secreted cumulus expansion enabling factor in the mouse [274], hence can be regarded as a successive measure of the ability of oocytes and cumulus cells to communicate during maturation. Chapter 4 therefore investigated the effects of altering oocyte-cumulus bi-directional communication on cumulus expansion and the effect of inhibiting cumulus expansion independent of oocyte-cumulus bi-directional signalling on oocyte developmental competence.

As previously reported [411], the absence of FSH and EGF or the inhibition of SMAD2/3 signalling prevented cumulus expansion. FSH and EGF absence in conjunction with SMAD2/3 inhibition had an additive effect resulting in complete detachment of the cumulus cells to assume a flattened fibroblast appearing monolayer resulting in fully denuded oocytes. Cumulus expansion was then targeted independently of oocyte to cumulus bi-directional signalling through the use of azaserine, an inhibitor of the hexosamine biosynthesis pathway (HBP) and hence hyaluronan synthesis. Unexpectedly azaserine at the maximal concentration of 100 μM had no observable effect on cumulus expansion nor did it affect the expression of two key cumulus matrix transcripts hyaluronan synthase 2 (*Has2*) and prostaglandin synthase 2 (*Ptgs2*). This was most likely due to higher glucose and/or glutamine concentrations in the IVM media in comparison to previous reports where inhibition of the HBP with 6-diazo-5-oxo-L-norleucine (DON) or azaserine negated cumulus expansion [88, 119]. Despite unperturbed cumulus expansion, oocytes matured with 100 μM azaserine had significantly decreased embryo development rate and

two-cell embryo and blastocyst formation. Hence while cumulus expansion may be mandatory for successful ovulation and fertility in vivo (reviewed in [47]), the physical act of cumulus expansion itself is not indicative of oocyte developmental competence in vitro. Instead, cumulus expansion appears to indicate the ability of the oocyte to communicate with the cumulus cells and the ability of the cumulus cells to respond to that signal.

Since cumulus expansion is therefore a marker of the oocyte's regulatory ability, the importance of this this paracrine pathway along with that of the reverse pathway of cumulus cell to oocyte signalling on oocyte viability were subsequently explored. As investigated in Chapter 5, meiotic completion was only affected in the absence of FSH/EGF while perturbation of oocyte-cumulus bi-directional signalling in either direction resulted in decreased two-cell embryo formation and although blastocyst formation was unaffected, blastocysts had severely decreased inner cell mass cell numbers but unaffected trophectoderm cell numbers. Pregnancy outcomes supported the observations of blastocyst cell numbers, with a significant decrease in foetal survival but no difference in implantation rates of embryos derived from oocytes matured with the SMAD2/3 inhibitor in the presence of FSH/EGF. There were also no differences observed in foetal and placental weight or morphologies. Interestingly, while lack of both oocyte and cumulus signalling further perturbed cumulus expansion in Chapter 4, there were no major additive effects of these two treatments on subsequent embryo development. This indicates that the effects of oocyte-cumulus bi-directional communication during IVM on oocyte developmental competence are inter-dependent and the disruption of either signalling pathway adversely affects subsequent embryo development.

Having established that signalling through the SMAD2/3 pathway of COCs during IVM is important for subsequent foetal survival, the next chapter investigated the possibility that addition of exogenous GDF9 during IVM could increase oocyte developmental competence of intact COCs. Exogenous GDF9 addition during IVM in the presence of FSH and EGF improved the development rate and percentage of hatching blastocysts. Blastocyst quality was enhanced with significant increases in the total and inner cell mass cell numbers, although trophectoderm cell numbers were not affected. GDF9 addition in the absence of FSH and EGF had no effect on embryo development or blastocyst quality aside from an increase in the percentage of hatching blastocysts, highlighting again the inter dependency of oocyte and cumulus cell signalling on oocyte developmental competence. Foetal survival was doubled in embryos arising from oocytes matured with exogenous GDF9 while implantation rates and embryo and placental weight and morphologies were unaffected. These findings directly mirrored those observed in the previous chapter where oocyte signalling to CCs through SMAD2/3 was inhibited in the presence of FSH and EGF. Chapters 5 and 6 therefore demonstrate that oocyte to cumulus bi-directional signalling during the short duration of oocyte maturation has profound long-term impacts on developmental programming of the oocyte and the ability to develop successfully into a viable foetus. These findings have significant clinical relevance as it may be possible that the decreased viability of IVM oocytes may be attributed to deficient oocyte paracrine signalling. Hence the addition of exogenous GDF9 during IVM may potentially increase the success rate of IVM oocytes resulting in increased pregnancies and live deliveries.

After establishing that oocyte and cumulus bi-directional signalling have a profound effect on oocyte developmental competence, the next aim of this thesis was to investigate the

possible mechanisms responsible for these outcomes. The metabolism and oxidative stress levels of an oocyte have been shown to be intrinsically linked to its health and viability [222, 252, 455] and are greatly influenced by cumulus cells. [215, 223, 254, 282, 493]. Chapter 7 thus investigated the possibility that the alterations in oocyte developmental competence observed in the previous chapters may have resulted from changes in cumulus metabolism and oxidative stress levels and also determined the presence of a key glycolytic regulator 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases (PFKFB) in COCs.

PFKFB is responsible for both the formation and degradation of fructose-2-6-bisphosphatase (F2,6BP), which regulates the glycolytic rate limiting enzyme phosphofructokinase, from/to fructose-6-phosphate via its kinase and bisphosphatase domains respectively [463-465]. Although all 4 isoforms of PFKFB were present in ovulated mouse COCs, PFKFB2 and PFKFB3 were found to be most abundant. mRNA transcripts for all 4 key metabolic enzymes investigated *Pfkp*, *Ldha*, *Pfkfb2* and *Pfkfb3* were significantly decreased in cumulus cells from COC's matured in the absence of FSH and EGF. Inhibition of SMAD2/3 signalling in the presence of FSH and EGF had no effect on cumulus cell metabolic gene expression. Although unexpected, these findings do correlate with that of a recent study where recombinant GDF9 of a different origin had no effect on *Pfkp* and *Ldha* expression of OOXs [290] prior to maturation. Interestingly exogenous GDF9 addition in the presence but not the absence of FSH and EGF significantly increased the cumulus cell expression of *Pfkp*, *Ldha* and *Pfkfb2*over that of the 293H control. It is hence possible that while SMAD2/3 may not be a critical pathway affecting metabolic gene expression, GDF9 may act synergistically with other oocyte secreted factors to regulate expression of metabolic genes. Individual COC glycolytic and TCA cycle activities were

then measured directly using radioactive labelled glucose. In agreement with the decrease in gene expression, cumulus cell glycolytic activity was significantly reduced in the absence of FSH and EGF and when SMAD2/3 was inhibited, despite unaffected gene expression. Exogenous GDF9 addition also resulted in a significant increase in glycolytic activity both in the presence or absence of FSH/EGF over that of the 293H controls. Importantly, similar to the developmental competence results obtained in Chapter 5, the effect of the oocyte's regulation on cumulus cell glycolytic activity was also dependent on cumulus cell communication since inhibition of SMAD2/3 signalling in the absence of FSH/EGF did not further decrease glycolytic activity.

Interestingly, TCA cycle activity of the COC was only affected by the absence of FSH and EGF and not by oocyte to cumulus signalling. Oocytes require pyruvate to complete meiosis I [215] and COC pyruvate metabolism has been related to meiotic maturation success [193]. Thus although these findings contradict that of OOXs prior to maturation where co-culture with fully grown oocytes increased TCA cycle activity [282], it is in agreement with the results of Chapter 4 where meiotic maturation was only decreased by the absence of FSH and EGF and unaffected by SMAD2/3 inhibition. Furthermore while FSH alone has been shown to have no effect on TCA cycle activity of the COC during IVM [201] and EGF has been shown to have no effect on media pyruvate levels throughout maturation [223], it is plausible that the combined effect of FSH and EGF and the difference in maturation media in this study to those of previous reports, mediated a significant effect on TCA cycle activity of COCs after maturation with or without FSH and EGF.

Increased oxidative stress levels have the potential to negatively impact oocyte health through DNA damage, mitochondrial alterations and apoptosis [478, 479]. Furthermore, increased ROS levels in human follicular fluid have been correlated to decreased embryo formation [249]. Hence the levels of oocyte oxidative stress were measured in the experimental models that resulted in decreased embryo development. Both the absence of FSH and EGF or SMAD2/3 inhibition in the presence of FSH/EGF significantly doubled oocyte oxidative stress levels which may have resulted from the negative influence of perturbed COC glucose metabolism. However, cumulus cells have been shown to exert a protective role on the oocyte against oxidative stress in intact COCs [243, 254, 255]. Hence alternatively, the formation of the cumulus matrix and/or extended maintenance of gap junction communication between the oocyte and cumulus cells as a result of functional oocyte-cumulus bi-directional signalling may have a protective role independent of glucose metabolism on the regulation of oocyte oxidative stress levels. COCs matured with 100 µM azaserine also had increased oocyte oxidative stress levels. This is unsurprising since the HBP is responsible for activating, via o-linked glycosylation, an array of proteins involved in mediating cellular stress responses [140]. Hence it is likely that while cumulus expansion and glycolytic gene expression were unaffected, ROS removal mechanisms may have been disrupted eventuating to the decreased embryo development observed in oocytes matured with 100µM of azaserine.

In conclusion, the cross talk between the oocyte and cumulus cells during IVM exerts a profound regulatory effect on COC glycolytic activity and oxidative stress levels that persist long after the inhibitors have been removed into subsequent embryo and foetal development. This therefore potentially accounts for the profound effects that oocyte-

cumulus bi-directional communication has in the short span of IVM, on the long term developmental capacity of the oocyte.

Future Directions

Although this thesis has demonstrated the importance of GDF9 and SMAD2/3 signalling to oocyte developmental competence and cumulus cell functions, the exact composition and secretion patterns of the vast array of oocyte paracrine factors during IVM has yet to be determined. Other oocyte secreted factors such as bone morphogenetic protein (BMP15) which signals through the alternative SMAD1/5/8 pathway, have also been shown to mimic oocyte functions such as cumulus expansion in some species [445]. Oocyte secreted factors BMP15 and GDF9 have been reported to work in synergy with one another to affect oocyte developmental competence in mice [299] while a member of another family of oocyte secreted growth factors fibroblast growth factor 8 (FGF8) was shown to be able to affect cumulus metabolism in combination with BMP15 [290]. To date, no studies have been performed elucidating the effects of these other oocyte secreted factors on foetal outcomes. It would therefore be enlightening to investigate their roles individually and in combination with recombinant GDF9 on oocyte developmental competence.

The recombinant GDF9 used in this thesis was also only partially purified by chromatography [265] and contaminating factors originating from the human embryonic kidney 293H parent cell line, present in both the GDF9 preparation and the 293H control, have been shown to exert adverse effects on embryo development [354]. Hence, it is likely that positive effects of our exogenous GDF9 on some parameters of embryo development over that of conventional IVM conditions may be masked by the 293H contaminants in the preparation. Therefore the positive effects of exogenous GDF9 on oocyte developmental competence and metabolism might be even more pronounced should a purified active product become available.

While oocyte to cumulus signalling has been shown to exist in various animal models, individual identified oocyte paracrine factors appear to have species dependent regulatory properties and varied degrees of importance to female fertility. While GDF9 null mice and sheep are sterile with ovarian follicles that do not develop past the primary stage [259, 330], BMP15 homozygous mutant mice are only sub-fertile [329]. Furthermore, sheep with heterozygous mutations in GDF9 or BMP15 have increased ovulation rates [330]. While co-culture with recombinant GDF9 or BMP15 increased bovine oocyte developmental competence [354], the oocyte is not fundamental for cumulus expansion in the cow [277]. Although humans with rare mutations in GDF9 and BMP15 have premature ovarian failure [333, 334] and mutations in GDF9 have been associated with dizygotic twinning [48, 49], there is limited knowledge of the roles of oocyte to cumulus bi-directional signalling in the human. It is therefore fundamental to elucidate which animal model the human most closely represents so that the appropriate findings can be extrapolated and translated. Perhaps the most practical approach in resolving this would be the analysis of cumulus expansion in oocytectomised (OOX) surplus human COCs from non-stimulated patients. All species examined to date, the rat, pig [275, 276] and cow [277] are able to produce morphologically normal cumulus matrices without oocyte secreted factors but still produce the cumulus expansion enabling factors (CEEFs) necessary for mouse cumulus expansion [277, 278]. Therefore, examination of whether human OOXs require the presence of the oocyte to expand and analysis of human oocyte conditioned media on mouse cumulus expansion will elucidate if the human requires and produces similar oocyte secreted CEEFs.

Conclusion:

As illustrated in Figure 8.1, the studies in this thesis are the first to determine that the bi-directional communication between the oocyte and its surrounding cumulus cells during the short duration of IVM irreversibly impacts foetal survival. These findings therefore have significant potential to influence clinical practice by improving the outcomes of IVM techniques making then a more viable option for patients seeking fertility treatment. Several variations of recombinant bioactive human BMP15 and GDF9 and other oocyte secreted factors have recently been successfully manufactured [297, 494, 495]. Therefore, it may be possible to perform similar studies to those outlined in this study to begin elucidating the regulatory roles of these oocyte secreted factors on the acquisition of oocyte developmental competence in the human. Characterisation of the impact of oocyte-cumulus bi-directional signalling on human oocytes during IVM would enable significant advances to current IVM conditions and potentially result in the abolishment of rigorous hormonal stimulation regimes and the use of IVM as standard clinical practice.

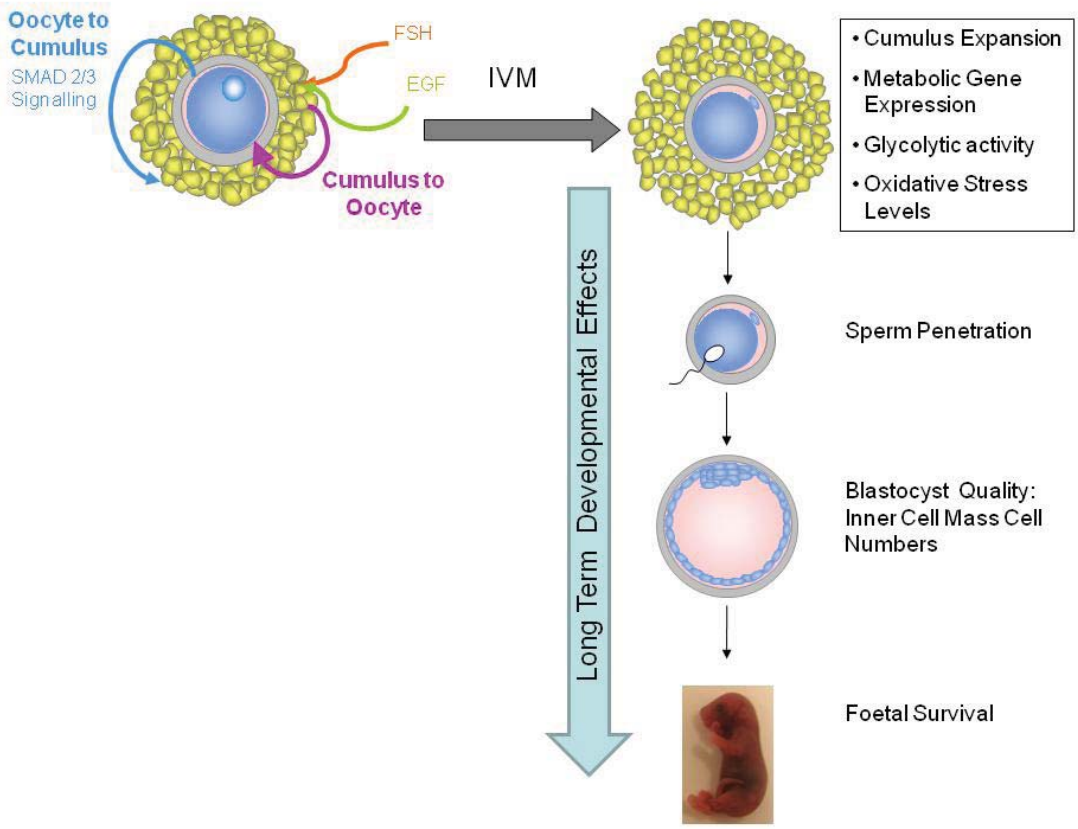


Figure 8.1. Summary of Thesis Findings. Oocyte-cumulus bi-directional signalling through the SMAD2/3 and FSH/EGF pathways affect both cumulus and oocyte functions such as cumulus expansion, metabolic gene expression, glycolytic activity and oxidative stress levels. These in turn likely affect subsequent fertilisation and blastocyst quality which have long term developmental consequences into foetal survival.

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APPENDICES

APPENDIX 1: MEDIA FORMULATION AND PREPARATION

All chemicals and reagents were purchased from Sigma-Aldrich (St Louis, MO, USA) unless otherwise stated.

IVM Handling Medium

1x Waymouth MB 752/1 packet (14 g) dissolved in 1 litre of Mill Q water
To 500 ml of Waymouth solution added
0.210 g $NaHCO_3$ (0.420 g/l, 23.5 mM)
2.992 g HEPES (5.984 g/l)

PH to 7.4 using sodium hydroxide pellets (Univar, WA, USA) dissolved in Waymouth solution

Added 5% non-heat treated foetal calf serum (FCS) (Invitrogen; Carlsbad, CA, USA). Ensured PH is now between 7.2-7.35.

Filtered through a 0.2 µM filter (Pall, MI USA) and stored at 2-4°C for up to 12 months

IVM Culture Medium

1x Waymouth MB 752/1 packet (14 g) dissolved in 1 litre of Mill Q water
To 500 ml of Waymouth solution added
1.050 g $NaHCO_3$ (2.1 g/l, 25 mM)
2.992 g HEPES (5.984 g/l)

Added 5% non-heat treated foetal calf serum (FCS) (Invitrogen; Carlsbad, CA, USA).
Filtered through a 0.2 µM filter (Pall, MI USA) and stored at 2-4°C for up to 12 months

IVF Fertilisation Medium

1x α-Minimal Essential Media (α-MEM) (Gibco^{TM} Invitrogen, Vic, Australia) packet (10.1 g) dissolved in 1 litre of Mill Q water

To 500 ml of α-MEM added
1.100 g $NaHCO_3$ (2.2 g/l)
0.025 g Streptomycin Sulphate (50 mg/l)
0.0375 g Penicillin G (75 mg/l)

A day prior to use 3 mg/ml bovine serum albumin (BSA) was added and left to dissolve then filtered through a 0.2 µM (Pall, MI USA) filter.

Oocyte/Embryo Media Stock Solutions

Composition in g/l

Stock A (x10 Concentration)

NaCl	5.552
KCl	0.41
NaH_2PO_4-H_2O	0.035
$MgSO_4$-$7H_2O$	0.246
Na Lactate (L)	1.176
Glucose	0.09

Stock B (x10 Concentration)

	MOPS	*G1 or G2*
$NaHCO_3$	0.42	2.101
Phenol Red	0.001	0.001

Stock C (x100 Concentration)

Sodium Pyruvate	0.035

Stock D (x100 Concentration)

$CaCl_2$-H_2O	0.265

Stock G (x100 Concentration)

Alanyl-Glutamine	10ml glutamax solution

Stock H (x10 Concentration)

MOPS	4.186
Phenol Red	0.001
NaOH	5 pellets

Stock N (Non-Essential Amino Acids) (x100 Concentration)

L-alanine	0.089
L-asparagine-H2O	0.15
L-aspartic acid	0.133
L-glutamic acid	0.147
Glycine	0.075
L-proline	0.115
L-serine	0.105

Stock T (x100 Concentration)

Taurine	0.0125

Stock EDTA (x10 Concentration)

EDTA	0.029
NaOH solution	0.4

Preparation of Stock Solutions

Stock A and B

Weighed out individual components into a 100 ml flask
Added 50 ml Milli Q water
Mixed well until all dissolved
Made up to 100 ml
Mixed well
Filtered through 0.2 μm filter
Stored at 2-4°C

Stocks C, D, T

Weighed components into a 10 ml tube
Added 10 ml Milli Q water
Mixed well until dissolved
Filtered through 0.2 μm filter
Stored at 2-4°C

Stock H

Weighed out MOPS and phenol red into a 100 ml flask
Added 50 ml Milli Q water and mixed well until dissolved
Made up to 100ml with water
Placed NaOH pellets into a 10ml tube
Added 10 ml MOPS solution to NaOH and mixed
PH MOPS solution to 7.4 using NaOH
Filtered through 0.2 μm filter
Stored at 2-4°C

Stock N

Weighed out components except L-serine into 10 ml of Milli Q water in a beaker on a stirrer Stirred until dissolved
Added the L-serine to 2 ml of 1M HCL and mixed until dissolved
Added serine/HCL mix to amino acid/water mixed and stirred
Filtered through 0.2 μm filter
Stored at 2-4°C
Ensured that pH of stock N is approximately 5.5-6.

Stock EDTA

Weighed out EDTA into a 10 ml tube
Weighed out NaOH into a separate 10 ml tube
Added 10 ml Milli Q water to NaOH and mixed well until dissolved
Added 200 μl NaOH solution to EDTA and mixed well until dissolved
Added 9.8 ml Milli Q water to the NaOH and EDTA mixture
Added 90 ml of Milli Q water to a beaker
Added the above 10 ml NaOH and EDTA solution to a beaker containing the 90 ml of Milli Q water to make up a total of 100 ml
Filtered through 0.2 μm filter
Stored at 2-4°C

Storage Life of Stock Solutions

All stocks were stored for 6 months except for stock B and EDTA which were utilised within 1 week and 1 month respectively from their preparation dates.

Embryo Culture Media G1.2:

The following were added under sterile conditions to a 10 ml tube, mixed well and stored at 2-4ºC for a maximum of 3 months.

1 ml of stock A
1 ml of stock B
0.1 ml of Stock C
0.1 ml of Stock D
0.1 ml of Stock G
0.1 ml of Stock T
1 ml of EDTA
0.1 ml of Stock N
6.5 mls of Milli Q water

Embryo Handling Media: MOPS-G1

Weighed out all components of stock A, B (MOPS), C and H into a 1 L volumetric flask and dissolved with around 300 ml of Milli Q water.
Weighed out stock D into a 15 ml tube and dissolved with 10 ml Milli Q water then added this to flask.
Added 10 ml glutamax solution (stock G) and 10 ml stock N
Added Milli Q to make up to 1 L of solution
Added 4 g of BSA and left to dissolve
Using NaOH pellets dissolved in a small amount of MOPS solution, the pH of the solution was then brought to approximately 7.4.
Filtered MOPS-G1 through 0.2μm filter (Pall, MI USA) and stored at 2-4°C for a maximum of 6 months.

Hepes Buffered Modified G1 Medium (H-SG1):

Prepared as MOPS-G1 above without phenol red (stock B) and BSA.

APPENDIX 2: REAGENTS

All chemicals and reagents were purchased from Sigma-Aldrich (St Louis, MO, USA) unless otherwise stated.

0.9% Saline Solution

0.9% sterile saline solution was prepared by dissolving 0.9 g of ultra pure (minimum 99.5% pure) sodium chloride in 100 ml of Milli Q water. The solution was then passed through a 0.2 µM filter (Pall, MI USA) into a sterile container for immediate use.

Pregnant Mare Serum Gonadotropin (PMSG)

PMSG (Folligon; Intervet, Bendigo, Victoria, Australia) arrived as a lyophilized powder and was dissolved in 0.9% saline solution under sterile conditions to a concentration of 50 IU/ml and stored for a maximum of 1 week at 2-4°C or 3 weeks at -20°C.

Human Chorionic Gonadoropin (hCG)

hCG (Pregnyl; Organon, Oss, The Netherlands) arrived as a lyophilized powder and was dissolved in 0.9% saline solution under sterile conditions to a concentration of 50 IU/ml and stored for a maximum of 1 week at 2-4°C or 3 weeks at -20°C.

Follicle Stimulating Hormone (FSH)

Recombinant human follicle stimulating hormone (FSH) (Puregon; Organon, Oss, The Netherlands) was purchased as a lyophilized powder. A 10 IU/ml stock solution was made by dissolving 75 IU in 7.5 ml sterile 0.9% saline solution containing 0.1% BSA (1mIU= 0.1ng FSH). Aliquots of 100 µl were stored at -20ºC and were used at the final working concentration of 50 mIU/ml.

Epidermal Growth Factor (EGF)

Tissue culture grade epidermal growth factor (EGF) derived from mouse sub maxillary glands, was purchased as a 1 mg lyophilized powder. EGF was reconstituted by the addition of 1 ml sterile 0.9% saline solution containing 0.5% BSA to achieve a stock solution of 1 mg/ml. EGF was stored as 10 µl aliquots at -20ºC and used at the final working concentration of 10 ng/ml.

293H Conditioned Media

293H conditioned media was produced in house. Culture media from untransfected 293 human embryonic kidney cells (Gibco Life Technologies, Paisley, UK) were concentrated approximately 20-fold by ultrafiltration using an YM100 membrane (10000 MWCO; Millipore, Bedford MA) then partially purified using hydrophobic interaction chromatography (HIC). Fractions were pooled, concentrated and dialyzed with phosphate buffered saline using a Centriprep concentrator (10000 MWCO; Millipore, Bedford MA) sterile filtered (Millipore, Bedford MA) and stored in aliquots at -80ºC.

Recombinant mouse GDF9

Fully processed and bioactive recombinant mouse GDF9 was produced and qualified in house. Culture media from recombinant human embryonic kidney-293H cells expressing mouse GDF9, kindly donated by O.Ritvos (Helsinki), were concentrated approximately 20-fold by ultrafiltration using an YM100 membrane (10000 MWCO; Millipore, Bedford MA) then partially purified using hydrophobic interaction chromatography (HIC). Fractions were pooled, concentrated and dialyzed with phosphate buffered saline using a Centriprep concentrator (10000 MWCO; Millipore, Bedford MA), sterile filtered (Millipore, Bedford MA) and stored in aliquots at -80ºC. The concentration of recombinant mouse GDF9 was determined by immunoanalysis against a rat GDF9 standard. GDF9 was used at a final working concentration of 200 ng/ml.

SB-431542

SB-431542 was generously donated by GlaxoSmithKline (Stevenage, UK). A 10 mM stock solution was prepared by dissolving 5.67 mg SB-431542 in 1.35 ml of DMSO and stored as 2 µl aliquots at -20ºC. Upon experimental setup, 198 µl of IVM medium was added to the stock aliquot to get a 100 µM solution. This was then used at a 1 in 25 dilution to get to a 4 µM final working solution

Mineral Oil

Batches of mineral oil (Vitrolife, Kungsbacka, Sweden or Merck, Darmstadt, Germany) which had been proven to be non-embryo toxic were stored in the dark at 2-4 ºC for a maximum of 6 months.

APPENDIX 3: AVERTIN FORMULATION

All chemicals and reagents were purchased from Sigma-Aldrich (St Louis, MO, USA) unless otherwise stated.

Stock Solution

1.0 g 2,2,2-tribromoethanol
1.0 ml Tert-amyl alcohol

The stock solution was prepared and left to dissolve in the dark at room temperature for approximately 48 hours before filtration under sterile conditions. The sterile stock solution was stored in an aluminium foil covered glass bottle at 2-4ºC for a maximum of 2 months.

Working Solution

0.24 µl stock solution
20 ml sterile saline

The working solution was prepared in an aluminium foil covered glass bottle then left to dissolve overnight at room temperature. After which it was kept at 2-4ºC up to 2 months but warmed to room temperature for a minimum of 0.5 hour prior to use.

Dosage

0.7-0.8 ml of avertin (working solution) was administered per mouse equating to approximately 0.015 ml avertin per gram body weight of each mouse.

APPENDIX 4: REDOXSENSOR RED AND MITOTRACKER GREEN STOCK SOLUTIONS

RedoxSensor Red CC-1 and Mitotracker Green FM stains were purchased from Molecular Probes (Oregon, USA). Dimethylsulphoxide (DMSO) used was high grade Hybri-Max DMSO purchased from Sigma-Aldrich (St Louis, MO, USA).

RedoxSensor Red

Description
The fluorescent intensity of RedoxSensor Red CC-1 stain is based on a cell's redox potential. It passively enters live cells as a non-fluorescent probe and oxidises in the cytosol to generate a red-fluorescent product that accumulates either in the mitochondria or the lysosomes depending on the metabolic status of the cell. RedoxSensor Red CC-1 stain (MW= 434.4) was supplied frozen as a 50 µg lyophilized powder in a light protected vial and was stored at -20ºC until use.

Stock Solution
As RedoxSensor Red CC-1 is light sensitive, all procedures were performed in a laminar flow hood with the lights off. A 0.5 mM was stock solution was prepared by adding 230 µl DMSO directly into the vial which was then gently swirled to dissolve the stain. The stock solution then stored as 7.5 µl aliquots in aluminium foil covered microtubes and frozen at -20ºC for no longer than 6 months.

MitoTracker Green

Description
MitoTracker Green FM stain is a green-fluorescent stain which permeates the mitochondria regardless of the mitochondrial membrane potential. MitoTracker Green is non-fluorescent in aqueous solution and only fluoresces when it is accumulates in the lipid environment of the mitochondria. MitoTracker Green FM was supplied frozen as a 50 µg lyophilized powder in a light protected vial and was stored at -20ºC until use.

Stock Solution
As MitoTracker Green FM is light sensitive, all procedures were performed in a laminar flow hood with the lights off. To prepare a 1 mM stock solution, a 50 µg vial of MitoTracker Green FM was first allowed to come to room temperature. 94 µl DMSO was then added directly into the vial and gently swirled to dissolve the stain. The stock solution then aliquoted into 10 µl aliquots in aluminium foil covered microtubes. Aliquots were kept frozen at -20ºC for use within 6 months.

APPENDIX 5: REAL TIME PCR PRIMER EFFICIENCY VALIDATION

Gene	Correlation Coefficient (R^2)	Primer Efficiency
Rn18s	0.995	1.02
Rpl19	0.999	0.98
Has2	0.994	1.06
Ptgs2	0.999	1.01
Pfkp	0.991	0.93
LdhA	0.998	0.97
Pfkfb2	0.986	0.92
Pfkfb3	0.995	1.08

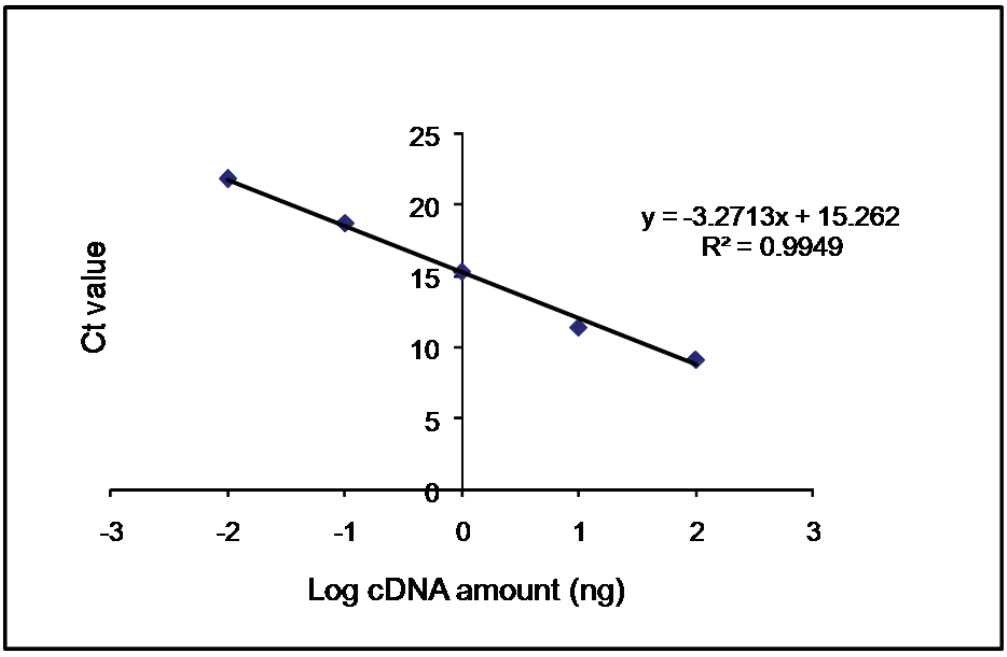

Figure A5.1. Example of a primer efficiency standard curve (18s) created using the logarithm of the amount of cDNA (ng) to its average Ct value.

APPENDIX 6: PUBLISHED VERSION OF CHAPTER 5

Disruption of Bi-directional Oocyte-Cumulus Paracrine Signalling During In-Vitro Maturation Reduces Subsequent Mouse Oocyte Developmental Competence.

Christine X. Yeo, Robert B. Gilchrist, Michelle Lane.

Biology of Reproduction 2009 May; 80(5):1072-80.

STATEMENT OF AUTHORSHIP CONTIBUTIONS

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Designed and performed all experiments, interpreted and analysed data and wrote manuscript.

Robert B. Gilchrist

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Michelle Lane

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Yeo, C.X., Gilchrist, R.B. and Lane, M. (2009) Disruption of Bidirectional Oocyte-Cumulus Paracrine Signaling During In Vitro Maturation Reduces Subsequent Mouse Oocyte Developmental Competence
Biology of Reproduction, v. 80 (5), pp. 1072–1080, May 2009

APPENDIX 7: PUBLISHED VERSION OF CHAPTER 6

Exogenous Growth Differentiation Factor 9 in Oocyte Maturation Media Enhances Subsequent Embryo Development and Fetal Viability In Mice.

Christine X. Yeo, Robert B. Gilchrist, Jeremy G. Thompson, Michelle Lane.

Human Reproduction 2008 Jan;23(1):67-73.

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Yeo, C.X., Gilchrist, R.B., Thompson, G.J. and Lane, M. (2008) Exogenous growth differentiation factor 9 in oocyte maturation media enhances subsequent embryo development and fetal viability in mice.
Human Reproduction, v. 23 (1), pp. 67-73, January 2008