The Role of the Cumulus Oocyte Complex During Ovulation

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"Just because something doesn't do what you planned it to do doesn't mean it's useless."

Thomas Edison

“Somewhere, something incredible is waiting to be known.”

Carl Sagan

Abstract

Ovulation is fundamentally crucial to the reproductive success of all mammals. Despite this fact there remain major knowledge gaps in our understanding of how the Luteinizing Hormone (LH) surge, which initiates ovulation, controls this process. There have been numerous theories regarding this phenomenon, yet the underlying mechanisms involved remain relatively unknown. In this thesis I sought to elucidate mechanisms involved in ovulation, with a particular focus on the role played by the expanded cumulus oocyte complex (COC). Specifically, I investigate whether the cumulus cells and their associated matrix following expansion could contribute actively to its own extrusion from the ovarian follicle during ovulation.

I developed a novel hypothesis whereby the cumulus cells transition to an adhesive, motile and invasive cell phenotype in response to an ovulatory stimulus, hCG an analog of LH. I investigate whether the cumulus cells from expanded COCs are capable of cell adhesion to various extracellular matrices found in the follicle wall, and whether this is dependent upon hormonal stimulation by comparison to cumulus cells from unexpanded COCs, not receiving such stimulation.

Further, I investigate whether the cumulus oocyte complex is capable of transitioning to a migratory cell phenotype. I tested this with established methods used in the study of cancer cell metastasis. I determine whether this phenotype is firstly dependent on an ovulatory stimulus, and whether it is cumulus cell specific. I attempt to elucidate the molecular mechanisms involved by investigating expression of the well-characterised CD44 cell migration pathway in COCs, during an ovulation time-course. I then use specific antagonists to this pathway, to inhibit cell migration.

The final step in our hypothesis involves the investigation of the invasive capacity of the expanded COC. I analyse whether the expanded COCs are capable of degrading an extracellular matrix barrier during migration assays, and I compare this ability to characterised invasive and non-invasive breast cancer cell lines. I also investigate possible mechanisms involved in the invasive phenotype by inhibiting the matrix metalloprotease system, proposed to play an important role in the degradation of the follicle wall during follicle rupture, and by examining the *Adamts1* null mouse, as Adamts1 is a protease shown to be crucial during ovulation.

This thesis demonstrates novel and exciting properties of the cumulus oocyte complex during ovulation; offering new insight into our understanding of this complex process. It shows that the oocyte and its surrounding cumulus cells are not merely a passive entity, as previously thought, but rather may play an active role during this vital reproductive process.

Declaration

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Table of Contents

List of Figures

List of Tables

Abbreviations

αMEM	Minimum Essential Medium alpha
Adamts	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif
Ambp	alpha 1 microglobulin/bikunin
ANOVA	analysis of variance
Ar	Androgen receptor
ART	artificial reproductive technology
bp	base pairs
BSA	bovine serum albumin
Bmp15	bone morphogenetic protein 15
CD44	CD44 antigen
cAMP	cyclic adenosine monophosphate
cDNA	Complementary DNA
Cebpb	CAAT/enhancer binding protein (C/EBP), beta
COC	cumulus oocyte complex
Csf2	colony stimulating factor 2 (granulocyte-macrophage)
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide
DNA	Deoxyribonucleic acid
eCG	equine chorionic gonadotropin
ECM	extracellular matrix
Egf	epidermal growth factor

Egf-L	Egf-like ligand
Egfr	epidermal growth factor receptor
EMT	epithelial to mesenchymal transition
ERK1/2	Extracellular-signal-regulated kinase 1 and 2
FCS	Fetal calf serum
F1	first filial
FSH	follicle stimulating hormone
GC	granulosa cell
Gdf9	growth differentiation factor 9
GDP	guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
h	hour
HA	hyaluronan
Has2	hyaluronan synthase 2
HC	heavy chain
hCG	human Chorionic Gonadotropin
Hmmr	hyaluronan mediated motility receptor (RHAMM)
HSC-3	human head and neck squamous carcinoma cell line
IαI	inter-α trypsin inhibitor
Ifna	interferon alpha
IL	interleukin
i.p.	intraperitoneal

IU	international units
IVF	invitro fertilisation
IVM	in vitro maturation
KO	knock out
LB	luria broth
LH	Luteinizing hormone
Lhcgr	luteinising hormone/choriogonadotropin receptor
LPS	lipopolysaccharide
Lyve1	lymphatic vessel endothelial hyaluronan receptor 1
MAPK	Mitogen-activated protein kinase
MI	metaphase I
MII	metaphase II
min	minute
mIU	milli international units
MMP	matrix metalloproteinase
mRNA	Messenger RNA
Nrip1	Nuclear receptor interacting protein 1
^{o}C	degrees Celsius
OSF	oocyte seceted factor
OSE	ovarian surface epithelium
PB	polar body
PBS	Phosphate Buffered Saline
Ptg	prostaglandin
Ptger2	prostaglandin E receptor 2 (subtype EP2)

PCR	polymerase chain reaction
Pde4d	phosphodiesterase 4D, cAMP specific
Pgr	progesterone receptor
Plg	plasminogen
Plat (tPA)	plasminogen activator, tissue
Plau (uPA)	plasminogen activator, urokinase
PGRKO	Progesterone receptor knockout
Ptgr2	prostaglandin E receptor 2, subtype EP2
Ptgs2	prostaglandin-endoperoxide synthase 2
Ptx3	pentraxin related gene
PVDF	polyvinylidene difluoride
Rac1	RAS-related C3 botulinum substrate 1
Rcf	Relative centrifugal force
RhoA	ras homolog gene family, member A
RNA	ribonucleic acid
Rock	Rho-associated coiled-coil containing protein kinase
Rpl19	ribosomal protein L19
Rpm	revolutions per minute
RT	reverse transcription
RT-PCR	reverse transcription polymerase chain reaction
SEM	standard error of the mean
SDS	Sodium Dodecyl sulphate
SDS-PAGE	Sodium Dodecyl sulphate - polyacrylamide gel electrophoresis
TBE	tris borate EDTA

Tgfb	transforming growth factor, beta
Tiam1	T-cell lymphoma invasion and metastasis 1
TIMP	tissue inhibitor of metalloproteinase
TLR	toll like receptor
Tnfaip6	Tumor necrosis factor alpha-induced protein 6
Tnfa	tumour necrosis factor alpha

Chapter 1

Introduction

1.1 BACKGROUND

Female fertility and reproductive success is completely dependent on the ability of an ovarian follicle to ovulate a mature cumulus oocyte complex that can be fertilised and ultimately establish a pregnancy. However, ovulatory defects and anovulation are common and unfortunately are major causes of female infertility. Conversely, a large number of women use oral steroids as contraception to prevent ovulation. Despite ovulation being a vital biological process and one which we have attempted to control for many years, the normal cellular mechanisms which fundamentally control this process remain relatively unknown.

The changes that occur within the ovarian follicle prior to ovulation, however, have been well characterised. Folliculogenesis, the process whereby a primordial follicle grows and progresses to a mature preovulatory follicle, is a gradual, staged maturation of the follicle and the oocyte it contains (Figure 1.1). A primordial follicle consists of an oocyte surrounded by a single layer of somatic cells. Primary follicles are recruited from the 'resting' primordial pool, which is characterised by the morphological change of granulosa cells from flattened to cuboidal and their proliferation (McGee and Hsueh, 2000). This is followed by the development of follicles to the pre-antral stage where granulosa cell layers surrounding the oocyte continue to increase in number, until finally in a mature or preovulatory follicle they become a stratified epithelial cell layer known as the mural granulosa cell layer. This stage is also accompanied by the development of another somatic cell layer, the theca (Edson, et al., 2009), which is separated from granulosa cells by a basement membrane. Subsequently a fluid filled antrum develops forming a physical separation between the granulosa cells lining the follicle wall and the somatic cells directly adjacent to the oocyte, which are known as the cumulus cells

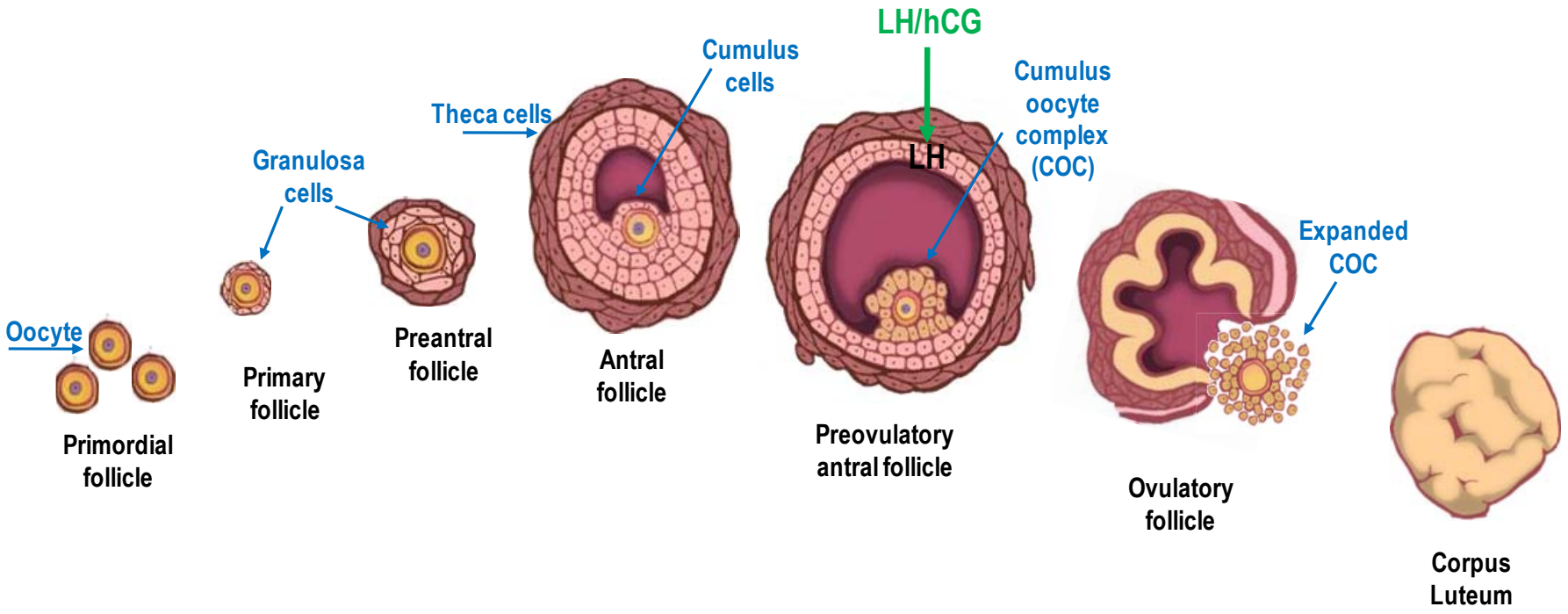

Figure 1.1 Schematic representation of folliculogenesis

In primordial follicles, oocytes are surrounded by a flattened layer of somatic cells and as folliculogenesis progresses, cells differentiate to form specific cell types. Primary follicles consist of a single granulosa layer around the oocyte, and following substantial proliferation of the granulosa cells, multiple layers surround the oocyte and the theca layer develops. A fluid filled cavity, the antrum, is formed and granulosa cells differentiate into cumulus cells, which immediately surround the oocyte and mural granulosa cells, which line the follicle. Granulosa cells are separated from theca cells by a basement membrane. These preovulatory antral follicles express the LH receptor on granulosa cells, and can respond to the LH-surge, which initiates expansion of the cumulus oocyte complex (COC) capable of ovulation and eventually fertilisation. Finally, the cycle of folliculogenesis is complete with the terminal differentiation or luteinisation of follicular cells to form a corpus luteum, important for pregnancy maintenance.

(Figure 1.1). Cumulus cells exist as a compact layer of approximately 1000-3000 cells (Salustri, et al., 1992) and are attached to the oocyte and each other via gap junctions, which are important for cell-cell communication by allowing transfer of small metabolites and other regulatory molecules (Buccione, et al., 1990, Lawrence, et al., 1978). Collectively, the oocyte and its surrounding cumulus cells are known as the cumulus oocyte complex (COC). Cumulus and granulosa cells respond differently to hormonal cues leading to distinct morphological changes and functions within these cell types, with each contributing to the physiology of the follicle in specific ways. Only preovulatory, antral follicles containing a COC are capable of responding to the ovulatory stimulus, which is a surge of luteinising hormone (LH) secreted from the pituitary. Granulosa cells expressing high levels of luteinising hormone receptor (Lhcgr) respond to the LH-surge, secreting epidermal growth factor-like (EGF-L) ligands to initiate the ovulatory cascade in the COC. Hallmarks of which are oocyte nuclear maturation and the biological phenomenon known as cumulus expansion, processes required for ovulation and subsequent fertilisation of the oocyte. The final stages of folliculogenesis involve the terminal differentiation of granulosa and theca cells, and invasion of blood vessels to form the corpus luteum, responsible for progesterone secretion for pregnancy maintenance.

Ovulation is a highly organised and heavily regulated physiological process that importantly coordinates nuclear maturation of the oocyte with its release from the follicle and in the case where fertilisation occurs, receptivity of the uterus for impending implantation. Ovulation requires both mechanical and molecular changes within the ovary. The mechanism of ovulation has remained a topic of intense debate over many years with numerous researchers developing diverse hypotheses. However, it is widely acknowledged that following the LH-surge several steps are critical, including the cascade of regulated gene expression, expansion of the COC, and rupture of the follicle at the apex. These processes will be discussed in detail below. Whilst the decades of research in this area have provided

important insight into the mechanisms controlling ovulation, there still exist many contradictory results and gaps in our knowledge. The majority of our understanding of the molecular mechanisms of ovulation comes from research in rodents, particularly mice. In mice ovulation is stimulated by eCG to stimulate follicle growth followed by hCG 44-48 hours later to mimic the LH surge and initiate the physiological events associated with ovulation, including the vital cumulus expansion. Although, mice are a polyovular species, and ovulate greater than one COC per cycle compared to humans, who typically ovulate one COC per cycle, both species respond similarly to ovulatory hormonal stimulation. Physiologically, both display similar processes during ovulation, including cumulus expansion, rupture at the apical wall of the antral follicle, and subsequent extrusion of the COC for fertilisation. The major difference in the ovary of a mouse is the presence of an ovarian bursa, a sac which encloses the ovary and the infundibulum, this structure is absent in the human.

This review will initially focus on the well-established processes that occur in the ovary prior to, during and after ovulation, and then will focus on the various hypotheses and unresolved questions surrounding this multifaceted event.

1.2 LUTEINISING HORMONE SIGNALLING TO INITIATE OVULATION

Luteinising hormone (LH) is a glycoprotein hormone that acts via its G-protein coupled transmembrane receptor (Lhgcr) which is present on granulosa cells of preovulatory follicles (Figure 1.2). A surge of LH from the pituitary is required to initiate ovulation. This occurs in response to rising estradiol levels secreted from large ovarian follicles, resulting in an increased LH pulse frequency and amplitude from the pituitary and subsequently a large surge in circulating LH levels (Goodman and Karsch, 1980, Karsch, 1987, Moenter, et al., 1990). LH signalling initiates a cascade of coordinated events which

ultimately leads to oocyte nuclear maturation, cumulus expansion, ovulation and granulosa cell luteinisation. The luteinising hormone receptor (*Lhgcr*) is highly expressed on granulosa cells of pre-ovulatory follicles with relatively low to undetectable levels present on cumulus cells (Peng, et al., 1991). The LH-surge initiates the induction of a cohort of EGF-like ligands (EGF-L) in granulosa cells which act as secondary signals upon the COC to initiate COC expansion and the resumption of meiosis and nuclear maturation of the oocyte (Park, et al., 2004).

Prior to the surge of LH the oocyte is surrounded by a compact layer of epithelial-like cumulus cells, which are connected to each other and the oocyte via gap junctions, which ensure continuous communication between the oocyte and cumulus cells. These gap junctions are critical in maintaining meiotic arrest via the transfer of cumulus cell synthesised cAMP to the oocyte. This transfer is required to maintain high levels of cAMP within the oocyte and prevent formation of the meiosis promoting complex and thus the resumption of meiosis (Bornslaeger, et al., 1986, Schultz, et al., 1983, Webb, et al., 2002). The LH-surge initiates increased synthesis of cAMP in cumulus cells. However, the concomitant breakdown of gap-junctions between the oocyte and cumulus cells results in declining cAMP levels within the oocyte and consequently meiosis is resumed (Dekel, et al., 1981). In addition to the breakdown of gap junctions, decreasing levels of cAMP within the oocyte is also attributable to the dissociation of cumulus cells from the oocyte during COC expansion (Hess, et al., 1999).

As well as initiating the resumption of meiosis, LH-induced EGF-L ligands also upregulate cumulus cell specific gene products, many of which are essential for COC expansion and ovulation (Hsieh, et al., 2007, Panigone, et al., 2008, Park, et al., 2004).

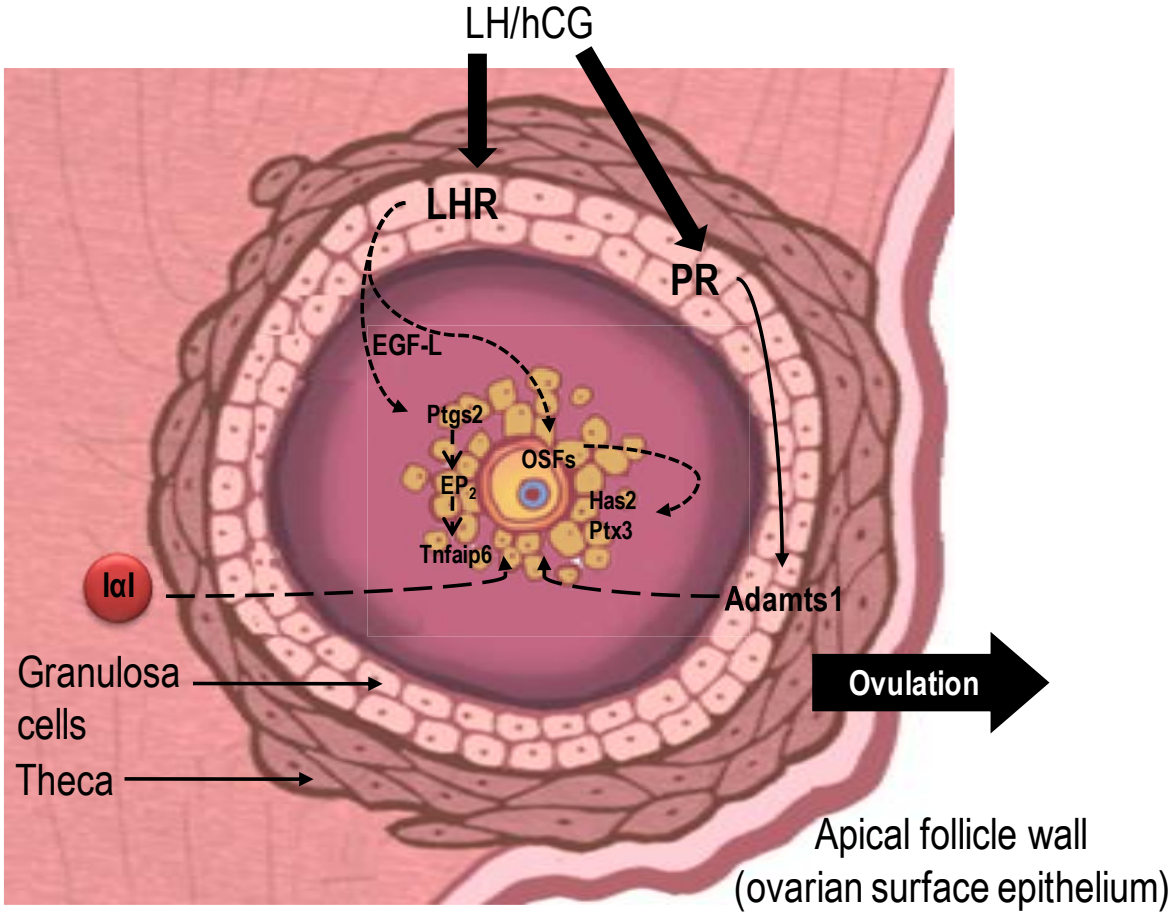

Figure 1.2 LH responsive gene expression in the ovulating follicle

The preovulatory follicle is able to respond to the luteinising hormone (LH) surge due to granulosa cells expressing the LH receptor (Lhgcr). This initiates a downstream signalling cascade involving induction of epidermal growth factor-like ligands (EGF-L), epiregulin and amphiregulin, which act on cumulus cells to induce synthesis of prostaglandin (via *Ptgs2* induction), whose signalling via the receptor, EP2 is essential for induction of the cumulus matrix gene tumour necrosis factor-induced protein 6 (*Tnfaip6*). The EGF-L ligands also act to induce cumulus matrix proteins important for cumulus oocyte complex (COC) expansion including pentraxin 3 (*Ptx3*). Induction of the cumulus matrix genes (hyaluronan synthase 2) *Has2*, *Ptx3* and *Tnfaip6* are dependent on both LH signalling and factors secreted from the oocyte (OSFs). Cumulus expansion requires the incorporation of products from serum (such as inter-alpha-trypsin inhibitor (Iαl)) and granulosa specific products (such as a disintegrin and metalloproteinase with thrombospondin motifs 1 (*Adamts1*)) into the expanding cumulus matrix. The LH stimulated process also initiates the resumption of meiosis and nuclear maturation of the oocyte allowing a mature expanded COC to be ovulated at the apical follicle wall.

1.2.1 Cumulus oocyte complex expansion

When the ovulatory stimulus of the LH-surge is received, the COC undergoes massive changes in its structure. This includes the rapid production of a viscous extracellular matrix (ECM) between cumulus cells, causing the entire complex to expand in size and dissociate from the follicle wall. A major component of the matrix of an expanded COC is hyaluronan (HA), which is synthesised in cumulus cells by hyaluronan synthase 2 (Has2), and is detectable shortly after exogenous human chorionic gonadotropin (hCG) administration, an analogue of LH (Tirone, et al., 1997). Hyaluronan is thought to be anchored to the cumulus cells by the HA receptor CD44 (Ohta, et al., 1999), which has been localised in the ovaries of many species including human, porcine and bovine (Kimura, et al., 2002, Ohta, et al., 1999, Schoenfelder and Einspanier, 2003, Yokoo, et al., 2002a, Yokoo, et al., 2007, Yokoo, et al., 2002b). The cumulus matrix is dependent on a number of matrix stabilising proteins and these have also been shown to play an important role during ovulation. One of these critical stabilising proteins is tumour necrosis factor-induced protein 6 (Tnfaip6), whose induction is dependent on both LH and factors secreted from the oocyte (OSFs) (Fulop, et al., 2003, Varani, et al., 2002, Yoshioka, et al., 2000). It has been reported that this HA-binding protein is important in matrix stabilisation via its interaction with Inter-alpha-trypsin inhibitor (IαI) (Carrette, et al., 2001, Chen, et al., 1996, Fülöp, et al., 1997, Kohda, et al., 1996, Wisniewski, et al., 1994). IαI is an integral component of the matrix but is not produced within the follicle; rather it enters via the circulation after the rise in vascular permeability following the LH-surge (Chen, et al., 1992, Hess, et al., 1999, Powers, et al., 1995). The heavy chains (HC) of IαI are covalently transferred onto HA, which is catalysed by Tnfaip6 (Chen, et al., 1996, Fulop, et al., 2003). The essential nature of such circulation-derived factors is demonstrated by the fact that expansion of mouse COCs in vitro only occurs in the presence of serum, which provides vital extrafollicular factors, including IαI (Chen, et al., 1992, Salustri, et al., 1989).

1.2.2 Role of cumulus cells and the extracellular matrix in ovulation

Cumulus expansion has been described in most mammalian species. Interestingly, what is now emerging is that a correctly organised matrix assembled around the oocyte is absolutely required for ovulation and fertilisation. This has been well demonstrated through the use of mice with null mutations in genes that express, or regulate the expression of, cumulus matrix components, which include *Tnfaip6*, *Ptx3*, *Bikunin*, *Adamts1*, *Pgr*, *Ptgs2*, *Ptger2*, *Nrip1*, *Pde4d* and *Cebpb* (Table 1.1). These null mutations result in a decrease, or complete failure in ovulation, which is associated with the absence of cumulus expansion or when expansion does occur, a compromised integrity of the cumulus matrix. Ablation of the matrix genes *Adamts1*, *Tnfaip6*, *Ptx3*, and bikunin (*Ambp*) lead to either the absence of expansion or abnormal and disorganised cumulus matrix structure, resulting in dissociation of the cumulus cells during expansion (Fulop, et al., 2003, Mittaz, et al., 2004, Salustri, et al., 2004, Sato, et al., 2001, Varani, et al., 2002, Zhuo, et al., 2001). Other nulls, such as *Ptgs2*, also have a reduced ovulation rate but in conjunction with a fertilisation failure due to a defect in cumulus matrix breakdown following ovulation (Davis, et al., 1999, Hizaki, et al., 1999). In contrast, null mutations in *Pgr*, *Nrip1* and *Erk1/2*, (MAPK3/1) are completely anovulatory, and often follicles contain entrapped oocytes following the LH-surge, suggesting a defect in follicular rupture (Fan, et al., 2009, Jin, et al., 1999, Lydon, et al., 1995, Park, et al., 2003, Robker, et al., 2000). Cumulatively, the results of these studies suggest that the cumulus-oocyte complex, and specifically a correctly assembled extracellular matrix during expansion, is vital for successful ovulation.

1.3 MECHANISMS OF OVULATION

The extrusion of an expanded COC occurs through a rupture pore at the apical wall of the preovulatory follicle in response to the LH-surge. The cause of this follicular rupture has been debated for a number of years, with various theories being investigated. The timing of follicle rupture and ovulation in response to hormonal stimulus varies across species, but what is consistent is the expanded cumulus oocyte complex always ovulates at the apex of the follicle in order to pass into the oviduct where fertilisation can take place. Despite the large number of anovulatory models it is still unclear how the process of follicular rupture occurs. Highlighted here are a variety of mechanisms proposed to be responsible for the breakdown of the follicle wall and eventual release of the COC. These include a role for inflammatory events, immune cells, proteolytic remodelling of the follicle wall, increased muscle contraction and intra-follicular pressure.

1.3.1 Inflammatory reactions at ovulation

The hypothesis likening ovulation to an inflammatory response was famously proposed by Prof. L. Espey (Espey, 1980) and since then, many researchers have followed his lead and further investigated this concept (Brännström and Enskog, 2002, Bukulmez and Arici, 2000, Richards, et al., 2002). The idea that ovulation is similar to inflammation is supported by the important role of prostaglandins and proteolytic enzyme activity in the ovary, with both of these also being associated with classic inflammatory responses. Like inflammation, ovulation is accompanied by major physical changes that include oedema and an increase in local blood volume, eventually leading to tissue degradation (follicular rupture) and tissue repair (luteinisation and remodelling).

Similarities between inflammation and ovulation are clearly evident following the ovulatory, LH stimulus. The LH-surge causes an increase in ovarian vascularisation and vascular permeability leading to accumulation of fluid within the follicle, a similar process to the increase in blood flow and hyperaemia to a site of inflammation in a tissue. This increase in blood flow and fluid into the follicle is rapid (Powers, et al., 1995), and remains until the time of follicle rupture. The influx of fluid into the follicle is necessary for the incorporation of serum products into the cumulus matrix, which is crucial for cumulus expansion (Zhuo, et al., 2001).

Additionally, prior to and during ovulation there is an accumulation of leukocytes in the ovary, similar to that occurring during inflammation where there is an influx of leukocytes into an injured tissue. Leukocytes are recruited into the ovary following the LH-surge via the ovarian vasculature and are localised predominantly to periovulatory follicles (Bjersing and Cajander, 1974, Brännström and Enskog, 2002). Also, the number of leukocytes present within the ovary peaks, and is significantly increased at ovulation (Oakley, et al., 2010). Leukocytes have been implicated in the ovulatory process since in vitro perfusion of leukocytes and LH into the ovary led to an increase in the number of ovulations compared to LH perfusion alone (Hellberg, et al., 1991). Neutrophils and macrophages have also been observed in the ovary, with a striking increase in their infiltration during ovulation (Brannstrom, et al., 1993, Van der Hoek, et al., 2000). Furthermore, neutrophils converge at the apex of ovulating follicles (Brannstrom, et al., 1993) and depletion of ovarian neutrophils or macrophages results in a reduced ovulation rate, further emphasising the importance of leukocytes during ovulation (Brännström, et al., 1995).

	Gene name	Gene symbol	Ovulation Rate (% null versus controls)	Comments	Reference
Extracellular matrix proteins	Tumour necrosis factor and inducible protein 6	*Tnfaip6 (Tsg6)*	48%	COC expansion abnormal	(Fulop, et al., 2003)
	Pentraxin 3	*Ptx3 (TSG-14)*	45% No difference	Denuded or poor matrix formation Denuded or poor matrix formation	(Varani, et al., 2002) (Salustri, et al., 2004)
	A disintegrin and metalloprotease with thrombospondin motifs 1	*Adamts1*	33% 40%	COC expansion observed, less extensive Abnormal matrix formation, reduced fertilisation	(Mittaz, et al., 2004) (Brown, et al., 2010)
	alpha 1 microglobulin (Bikunin)	*Ambp*	43% (natural mating) 14% (natural mating)	COC expansion abnormal Entrapped oocytes, COC expansion abnormal	(Zhuo, et al., 2001) (Sato, et al., 2001)
Cell signalling and regulators of transcription	Progesterone receptor	*PgR*	0% 0%	COC expansion normal COC expansion normal	(Lydon, et al., 1995) (Robker, et al., 2000)
	Nuclear receptor interacting protein 1	*Nrip1 (Rip140)*	0% (natural mating)	COC expansion abnormal	(White, et al., 2000)
	Androgen receptor	*AR*	62%	Poor cumulus expansion, dissociated cumulus	(Hu, et al., 2004)
	Prostaglandin-endoperoxide synthase 2, cyclooxegenase 2	*Ptgs2 (Cox2)*	<5% (natural mating) <20%	No fertilisation, disorganised cumulus expansion No fertilisation	(Davis, et al., 1999) (Lim, et al., 1997)
	Prostaglandin E receptor 2, subtype EP2	*Ptger2*	68%	COC expansion abnormal, reduced fertilisation	(Hizaki, et al., 1999)
	Phosphodiesterase 4D	*PDE4D*	<30% <15%	Entrapped oocytes Entrapped oocytes	(Jin, et al., 1999) (Park, et al., 2003)
	CCAAT/enhancer binding protein	*C/EBPβ*	12%	No CL formation	(Sterneck, et al., 1997)
	Extracellular signal-regulated kinase 1 and 2	*Erk1/2, (MAPK3/1)*	0%	COC expansion abnormal, entrapped oocytes	(Fan, et al., 2009)
	Liver receptor homolog 1	*LHR1 (Nr5a2)*	0% (natural mating) <10% (superovulation)	COC expansion absent	(Duggavathi, et al., 2008)
	Sulfotransferase family 1E, member 1	*Sult1E1*	50%	COC expansion absent	(Gershon, et al., 2007)

Table 1.1 Null mutations resulting in severe ovulatory defects and associated with defects in matrix formation or cell signalling events

The role leukocytes play during ovulation is not known but may include the secretion of cytokines, chemokines and various growth factors. Cytokines known to play a role during ovulation include interleukins (IL), interferon α (Ifna), tumour necrosis factor α (Tnfa) and granulocyte-macrophage colony stimulating factor (CSF2) (Brannstrom and Norman, 1993, Brannstrom, et al., 1994, Brannstrom, et al., 1993, Hurwitz, et al., 1991, Wang, et al., 1992). In particular, IL-1 is upregulated at ovulation (Brannstrom, et al., 1994, Hurwitz, et al., 1991), and has been found to be important for stimulation of progesterone synthesis and the induction of proteases, and may be important as a chemotactic factor to encourage migration of leukocytes into the follicle (reviewed in (Brannstrom and Norman, 1993)). Tnfa also appears key during ovulation. Bioactive Tnfa is secreted by the ovulating rat ovary (Brannstrom, et al., 1993) and is also found in human follicular fluid (Wang, et al., 1992). This cytokine is involved in the induction of both prostaglandin and progesterone synthesis (Brannstrom and Norman, 1993) both of which are critical for successful ovulation. Collectively, these studies offer some insight into the importance of cytokine secretion by leukocytes in the ovulatory process. Leukocytes, particularly macrophages, are additionally important for the secretion of growth factors, including epidermal growth factor (EGF) and transforming growth factor β (TGFβ) both of which are important for ovarian function (Ashkenazi, et al., 2005, Park, et al., 2004, Tsafriri, et al., 2005) .

Macrophages are known to express toll like receptors (TLR) and a number of TLRs have been found to be induced at ovulation in the COC (Shimada, et al., 2006). COCs cultured with lipopolysaccharides (LPS), a TLR ligand, were found to increase expression of a number of immune-related genes, including *IL6*, *Ptgs2* and *Tnfα* (Shimada, et al., 2006). It has been suggested that TLRs play an important role during ovulation (Liu, et al., 2008). However, to date there is no information known about possible reproductive phenotypes of TLR null models, although aberrant TLR function may be related to infertility due to its associated pathways being important for cytokine production.

Macrophages and neutrophils are able to produce and secrete proteolytic enzymes, including cysteine proteases, the cathepsins, serine proteases including plasminogen activators (Plau and Plat), and matrix metalloproteinases (MMP) (Wu, et al., 2004). The role of proteases in the ovulation process will be discussed in more detail below, however, it is clear that proteolytic degradation is crucial in both an inflammatory response and during ovulation. Leukocytes may hone their actions to particular areas of the ovary, for example the apex during follicular rupture, and influence the ECM environment during ovulation by modifying its composition through the secretion of both cytokines and degradative enzymes (Vaday, et al., 2001).

Prostaglandins (PG) which are characteristically involved in pyrogenic (fever) responses are also known to have a key role in ovulation (Davis, et al., 1999, Espey, 1980, Murdoch, et al., 1986). Prostaglandins are produced ubiquitously throughout the body, and via their range of receptors, are involved in a variety of physiological processes, including control of inflammatory responses, smooth muscle cell contraction and renal function (Dubois, et al., 1998, Hao and Breyer, 2008, Horton, 1969). Prostaglandin E2 subtype (PGE2), which plays a role in vasodilation during inflammation, is produced in large amounts in the ovary in response to the ovulatory stimulus. This occurs via LH-induced expression of the prostaglandin synthase 2 gene (*Ptgs2*; synonym COX-2) (Brown and Poyser, 1984). *Ptgs2* is referred to as the inducible *Ptgs* isoform, and is associated with acute inflammatory responses, thus ovulation is likened to an acute rather than a chronic inflammatory response. Ptgs2 and in turn PGE2 are induced rapidly yet transiently in the ovary following the LH-surge or an ovulatory dose of hCG (Sirois and Richards, 1992). Inhibition of Ptgs2 in the ovary by administration of either indomethacin or NS398 (Ptgs2 inhibitors), leads to a significantly decreased ovulation rate (Gaytan, et al., 2006, Mikuni, et al., 1998). Similarly, the *Ptgs2* null mouse model, where PGE2 is not induced following an ovulatory stimulus, also results in a severely reduced ovulation rate (Davis, et al., 1999, Lim, et al., 1997).

Additionally, PGE2 replacement in *Ptgs2* null mice restores ovulation to rates comparable with wild-type controls (Davis, et al., 1999). Together, these studies support a role for prostaglandins in ovulation.

PGE2 acts through its cell surface receptors, in particular prostaglandin E2 receptor (Ptger2), in an autocrine manner. *Ptger2* expression increases in cumulus cells following hCG administration in mice, in a similar pattern to *Ptgs2* (Hizaki, et al., 1999). *Ptger2* null mice are sub-fertile and have a reduced litter size compared to controls (Hizaki, et al., 1999, Kennedy, et al., 1999). In these mice, ovulation rate is not as markedly reduced, as observed in the *Ptgs2* null mice, however, cumulus expansion is absent and fertilisation rate is severely affected (Hizaki, et al., 1999). *Ptger2* null mice also show both reduced mRNA and protein levels of the cumulus matrix component *Tnfaip6*, revealing a role for PGE2 signalling via *Ptger2* in cumulus matrix formation during COC expansion (Ochsner, et al., 2003). These data suggest that PGE2 is important for cumulus cell function and matrix production during COC expansion and that these processes are important for ovulation and subsequent fertilisation.

Inflammation often results in tissue damage and degradation, with serine proteases implicated in this process. For instance plasmin is an inflammatory serine protease that is involved in clotting and wound healing. In the ovary, plasmin has been investigated for its role in ovulation. Plasmin is produced by cleavage of its precursor plasminogen, which is a serum protein secreted by the liver. Plasminogen activators and inhibitors are hormonally regulated in the ovary at the time of ovulation (Beers, et al., 1975, Canipari and Strickland, 1985, Chun, et al., 1992, Liu, et al., 1991, Peng, et al., 1993, Reich, et al., 1985, Reich, et al., 1986). Whilst plasminogen deficient mice show a reduced ovulation rate, insinuating a role for this system in ovulation, the reduction is moderate and suggests compensatory proteolytic mechanisms are also present (Liu, et al., 2006, Ny, et al., 1999).

Whilst this inflammation model does offer insight into the mechanisms responsible for ovulation, a number of these typical 'inflammatory markers', as detailed above, have been shown to not be absolutely necessary or functionally redundant and possibly compensated for. Thus although inflammatory pathways obviously impact the process of ovulation, other processes are clearly at play.

1.3.2 Proteolytic degradation of the follicle wall

The favoured paradigm for ovulation is that a rupture pore is created at the apex of a follicle in response to proteolytic degradation of the extracellular matrix around the follicle. Schochet proposed in 1916, that ovulation was caused by something other than an increase in intra-follicular pressure, and suggested that the follicular fluid was capable of enzymatic degradation (Schochet, 1916). For follicle rupture to occur, the ECMs, which compose the follicle wall, must be breached, and this is most likely due to proteolytic degradation. The exact protease(s) required and their source within the ovary remain relatively unknown. Many other studies have proposed alternative mechanisms for the release of an expanded COC. These will be emphasised below.

Extracellular matrix of the ovarian follicle

The extracellular matrix (ECM) of the ovary provides crucial structural support for the follicles. Importantly in the ovary, the various ECMs present provide barriers and define compartments within the ovary. In addition to this structural role, the molecular constituents of ECM provide morphogenic signals and thereby are able to promote varying activities, such as adhesion, migration and invasion. Further, via specific interactions with cell receptors, as well as secreted proteins such as growth factors, the ECM can promote cell activity and differentiation.

The ovarian follicle is composed of various ECMs and these have been identified in a range of species and shown to have similar distribution patterns. Collagen IV has been localised in human follicles across folliculogenesis, mostly associated with theca and granulosa cells (Yamada, et al., 1999). Fibronectin, laminin and collagens I and IV have been localised in bovine ovaries to varying compartments including the basement membrane, theca and granulosa cell layers (Figueiredo, et al., 1995, van Wezel, et al., 1998, Zhao and Luck, 1995). In the rat, collagen IV has been localised to the theca and the basement membrane, and also the surface epithelium of preovulatory follicles (Fröjdman, et al., 1998). Despite the mouse being used extensively as a model to study folliculogenesis, the ECM composition of the mouse ovary is not as well characterised. However, collagen has been reported as a major component of the tunica albuginea, at the follicle apex (Berkholtz, et al., 2006, Bjersing and Cajander, 1974).

Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a specific class of proteolytic enzymes involved in tissue remodelling in both physiological and pathological events. There currently are 25 different MMPs described, belonging to four different classes (Birkedal-Hansen, et al., 1993, Curry and Osteen, 2003). Many similarities exist between the subtypes, with significant overlap in functionality. All MMPs are synthesised as pre-proenzymes and secreted in an inactive form. This latent zymogen requires extracellular activation either by other proteases, the plasminogen activator system or non-proteolytic agents such as denaturants, reactive oxygen species or a low pH environment. MMP activity is also controlled via inhibition by tissue inhibitors of metalloproteinases (TIMPs), which are abundant in reproductive tissues and hormonally regulated (Hagglund, et al., 1999, Li and Curry, 2009, Lind, et al., 2006).

Matrix metalloproteinases (MMPs) have been implicated in causing the breakdown of the follicular wall leading to rupture of the apex and extrusion of the COC. Several different MMPs are induced at various times during folliculogenesis and many appear to be LH regulated. MMPs shown to be upregulated in response to the LH-surge include MMP1 and 2 in rat and primate (Chaffin and Stouffer, 1999, Reich, et al., 1991), MMP7 in primate (Chaffin and Stouffer, 1999), MMP9 (Robker, et al., 2000) and MMP19 (Hagglund, et al., 1999) in mice, MMP13 in rat (Balbin, et al., 1996) and MMP14 in rat and bovine (Bakke, et al., 2002).

The MMPs also exhibit distinct localisation within different compartments in the ovary, and their association with the follicular apex would suggest a role during ovulation. Indeed, studies have confirmed that MMPs accumulate at the time of ovulation at the apical wall, in particular MMP1 and MMP2 (Curry, et al., 1992, Murdoch and McCormick, 1992, Tadakuma, et al., 1993). Studies using inhibitors of MMP activity indicate that these enzymes play an essential role in ovulation. Administration of collagenase-type (MMP1, MMP8, MMP13) MMP inhibitor SC44463 resulted in a 50% reduction in ovulation rate (Butler, et al., 1991). This inhibition was time-dependent with SC44463 able to block ovulation when administered up to 7h post LH induction. Administration of specific collagenase inhibitors into the ovarian bursa or ovarian perfusion also resulted in a dramatic reduction in ovulation (Brannstrom, et al., 1988, Ichikawa, et al., 1983), suggesting an important role for collagenases in the breakdown of collagen in the follicular wall during ovulation. These collagenase inhibitors however, show very poor specificity for the collagenase-type MMPs. Alternatively, administration of a broad spectrum metalloprotease inhibitor, such as Galardin (GM6001) led to a modest 20% reduction in ovulation rate in rodent studies (Liu, et al., 2006). Thus although multiple studies indicate that MMP activity is involved in ovulation, it is unclear which particular MMP(s) are the essential mediators.

MMP deficient mouse models have not revealed a specific MMP responsible for follicular rupture, as all studies report little or no loss in fertility. Specifically, MMP2, MMP11, MMP13 and MMP19 nulls have been reported as fertile (Itoh, et al., 1997, Masson, et al., 1998, Pendas, et al., 2004, Stickens, et al., 2004). Fertility defects have not been reported in MMP3 null mice (Mudgett, et al., 1998). MMP9 and MMP12 nulls show reduced breeding efficiency, but this has been attributed to a placental deficiency rather than an ovulation defect (Vu, et al., 1998). Thus although MMP activity, particularly collagenase, is likely to be involved in ovulation, the high degree of redundancy within the family suggests that there may not be one single gene regulating this aspect of follicle rupture and ovulation.

A disintegrin and metalloproteinase with thrombospondin motifs 1

A disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif (Adamts1) is a protease induced in granulosa cells following the LH-surge and is regulated by progesterone receptor (PgR). Adamts1 is synthesised in granulosa cells in an inactive pro-form, and is secreted, with the mature and active form localising to the extracellular matrix and cell surfaces of the COC (Russell, et al., 2003b). It has been demonstrated to play an important role during ovulation due to null mice having a severe ovulation defect, with approximately 70% reduction in ovulation rate following superovulation (Brown, et al., 2010, Mittaz, et al., 2004) and approximately 75% reduction in ovulated COCs following natural mating (Brown, et al., 2010). It appears that the subfertility of the *Adamts1* null females is due, at least in part, to follicles being unable to ovulate. This leads to speculation that this protease may have an important role in follicular rupture, as many follicles from *Adamts1* null mice contained entrapped oocytes, 16h after hCG administration (Mittaz, et al., 2004).

The mechanism by which Adamts1 causes follicular rupture is not known. Perhaps, Adamts1 is working to cleave and activate MMPs responsible for degradation of the follicle wall; however no MMP deficient

model has resulted in ovulatory defects, as described above. Thus Adamts1 could be acting to breakdown the follicle wall by interacting with it directly. Substrates of Adamts1 include collagen (Rehn, et al., 2007, Shindo, et al., 2000) present in the ovarian follicular wall (Berkholtz, et al., 2006) Another substrate of Adamts1 is versican another protein found in the matrix of an expanded COC (Sandy, et al., 2001). Studies have revealed a defect in follicular remodelling in Adamts1 null mice (Brown, et al., 2006). Versican is a proteoglycan produced by granulosa cells, which cross-links HA and may play a role in cumulus matrix organisation (Miura, et al., 1999, Ujita, et al., 1994, Wu, et al., 2002, Xiang, et al., 2006), and has been identified as a direct target of Adamts1-mediated cleavage in the ovary (Russell, et al., 2003b). Thus, the role of Adamts1 could be to cleave versican, which may be important for oocyte release and the subsequent breakdown of the matrix prior to fertilisation. The function of Adamts1 appears to be largely non-redundant. Despite the presence and LH induction of Adamts4 and Adamts5 in follicles (Richards, et al., 2005), null mice for these Adamts genes are fertile (Stanton, et al., 2005). Thus, to date, Adamts1 is the only protease which, individually, has a major role in ovulation.

Plasminogen activator (PA) system

The plasminogen activator (PA) system has been discussed briefly for its involvement in the inflammation hypothesis of ovulation. Proteolytic action of the plasminogen activator system is responsible for physiological and pathological events and it has been implicated in the ovulation process from a number of lines of evidence (Beers, et al., 1975, Leonardsson, et al., 1995, Liu, et al., 2006, Ny, et al., 1999, Reich, et al., 1985). These include the observation that plasminogen production increases prior to ovulation, is localised to preovulatory follicles and can be induced by FSH and LH in vitro (Beers and Strickland, 1978, Beers, et al., 1975, Strickland and Beers, 1976, Wang and Leung, 1983). Plasminogen must first be activated to form the proteolytic enzyme plasmin by its activators, tissue-type PA (Plat) and urokinase-type PA (Plau) (Saksela and Rifkin, 1988). The production of Plat and Plau is

modulated in various ways including by growth factors and hormones (Collen and Lijnen, 1991, Saksela and Rifkin, 1988, Vassalli, et al., 1984). In the mouse ovary Plat and Plau have been shown to be induced following hCG administration (Leonardsson, et al., 1995). However, despite the suggestion that plasminogen and the PA system could be important for proteolytic degradation of the follicular wall, knockout mouse studies have revealed they are not essential. Mice deficient in plasminogen, the inactive proform of the enzyme plasmin, show a mild, 13% reduction in ovulation rate compared to wild-type (Ny, et al., 1999) suggesting that plasminogen is not essential during follicle rupture and ovulation. Mice deficient in only a single PA gene show no defect in ovulation and have normal fertility compared to wild type mice (Leonardsson, et al., 1995). When mice lack both *Plat* and *Plau*, ovulation is only reduced by 25% compared to wild type controls (Leonardsson, et al., 1995).

The PA system also works in cooperation with MMPs, to produce broad spectrum proteolytic action thought to be important during matrix remodelling in the ovary and specifically ovulation (Ny, et al., 1993). To determine the relative contributions of these protease systems to ovulation, plasminogen deficient mice have been treated with the broad spectrum protease inhibitor GM6001. Treatment of knockout mice with the inhibitor, so that both the PA and MMP systems are compromised, caused a further 14% reduction in ovulation (Liu, et al., 2006), resulting in a 30% reduction compared to wild-type no treatment control. Therefore, although both MMPs and plasminogen appear to play some role in follicle rupture, these proteolytic enzymes acting individually or together do not appear to be wholly responsible for ovulation.

1.3.3 Smooth muscle cell contraction and intra-follicular pressure

Early theories developed on ovulation and in particular the rupture of the follicle apex, have considered the involvement of intrafollicular pressure, either caused by smooth muscle cell contraction or an

increase in antral fluid in the preovulatory follicle due to the increase in vascular permeability (Hess, et al., 1999, Powers, et al., 1995). Numerous studies have looked at facets of these hypotheses with conflicting results. Literature over many decades debated whether there were smooth muscle cells within the ovary, and where they are located. More recently, it has been reported that smooth muscle cells are present in the theca layer surrounding follicles prior to ovulation (Ko, et al., 2006, Ren, et al., 2009). Interestingly, the apex of the follicle conspicuously lacked staining for smooth muscle cells (Ko, et al., 2006). Further, ovarian tissue from follicles stimulated with hCG have been shown to exhibit contractile activity following treatment with endothelin (Ko, et al., 2006). However, endothelin receptor antagonists show varying effects on ovulation (Bridges, et al., 2010, Ko, et al., 2006, Palanisamy, et al., 2006). Despite the long held theory that smooth muscle cell contraction may be responsible for follicular rupture, there is no definitive evidence yet for it having such a functional role.

In conjunction with smooth muscle cell contraction, increased intra-follicular pressure has been implicated in causing rupture of an ovarian follicle during ovulation. The literature surrounding this claim is again controversial. Early studies observed only a moderate increase in follicular pressure, and concluded that the rise was not sufficient to cause the follicle to rupture (Espey and Lipner, 1963). However, increased intrafollicular pressure has been reported following either administration of an ovulatory stimulus or a stimulus to induce cell contraction (Coutinho, et al., 1974, Virutamasen, et al., 1976). Whilst there was a rise in intrafollicular pressure detected in these studies, it is difficult to ascertain whether this was in fact due to an increase in systemic pressure, as it has been shown that intrafollicular pressure is dependent on and directly proportional to arterial hydrostatic pressure (Espey and Lipner, 1963). More recently, studies have confirmed the short-term increase in intrafollicular pressure during ovulation, yet the increase was reported to be moderate (Matousek, et al., 2001). However, the increase in intrafollicular pressure occurs concurrently with an increase in follicle wall

contraction (Matousek, et al., 2001), which could be important in influencing the structure of the follicle prior to ovulation, specifically at the apex where COC extrusion occurs.

It is now generally accepted that intrafollicular pressure does moderately increase at the time of ovulation, consistent with the accumulation of fluid in an antral preovulatory follicle. Nevertheless, there is no evidence that this increase in pressure is sufficient to cause follicular rupture.

It is clear that prior to ovulation the ovarian surface epithelium is lost and the follicle wall thins and ruptures at the apex (Talbot, et al., 1987). The cause of this is unknown but it is likely to involve complex interactions between many if not all of the mechanisms described above, with each contributing in some part to the process. Although there is an increase in intrafollicular pressure and a contraction in the smooth muscle cells present in the preovulatory follicle, this process itself is not likely to cause the ovarian follicle to breakdown at the apex. Instead, this penultimate event is more likely to involve proteases at the apical wall of the follicle. However, the identity, localisation and regulation of such proteolytic activity is not understood. In addition, aspects of the inflammatory process, for instance prostaglandin production, are clearly essential to the process, but their mechanistic actions are unknown.

Whilst the aforementioned mechanisms are all important components required for successful ovulation, they still do not explain how the follicle ruptures. Thus far, all theories have focused on the follicular wall being crucial, whilst an active role for the COC is overlooked. However there is increasing evidence described in detail above (Section 1.2.2), that the expanded COC, particularly its matrix components, is also somehow involved. I propose a novel idea whereby the expanded COC interacts with the follicle

wall and through highly localised and tightly regulated mechanisms, plays an active role in facilitating its own ovulation.

1.4 AN ACTIVE ROLE FOR THE CUMULUS OOCYTE COMPLEX

Although some observations are contradictory, the basic physiological mechanisms involved in ovulation are accepted. However, many knowledge gaps still exist. Most importantly, there has been no investigation of the role of the cumulus oocyte complex in controlling ovulation despite cumulus expansion being identified as an essential process to enable ovulation to occur. I hypothesise that the cumulus cells of the expanded cumulus oocyte complex transition to an adhesive cell type with invasive migratory properties; that the complex adheres to the apical wall prior to ovulation and invades the follicular wall through proteolytic action, thus creating and migrating through a rupture pore. Our hypothesis is demonstrated schematically in Figure 1.3.

In this model, the follicle responds to the LH-surge, inducing cumulus specific genes responsible for cumulus expansion (Figure 1.3A). A number of these important ovulatory genes are also known mediators of cellular motility and invasive capacity. Production of HA and its assembly into a pericellular matrix is a well known step in the progression of epithelial to mesenchymal transition (EMT). Versican is also a strong promoter of this process (Kern, et al., 2006) and the interaction of HA with cells via the HA receptor CD44, has been widely demonstrated to activate cell motility mechanisms in other systems (Bourguignon, et al., 2006, Bourguignon, et al., 2007, DeGrendele, et al., 1997).

During cumulus expansion, CD44 may be upregulated and bind HA to cumulus cells. CD44 may then activate intracellular targets to enable cumulus cells to transition to a motile

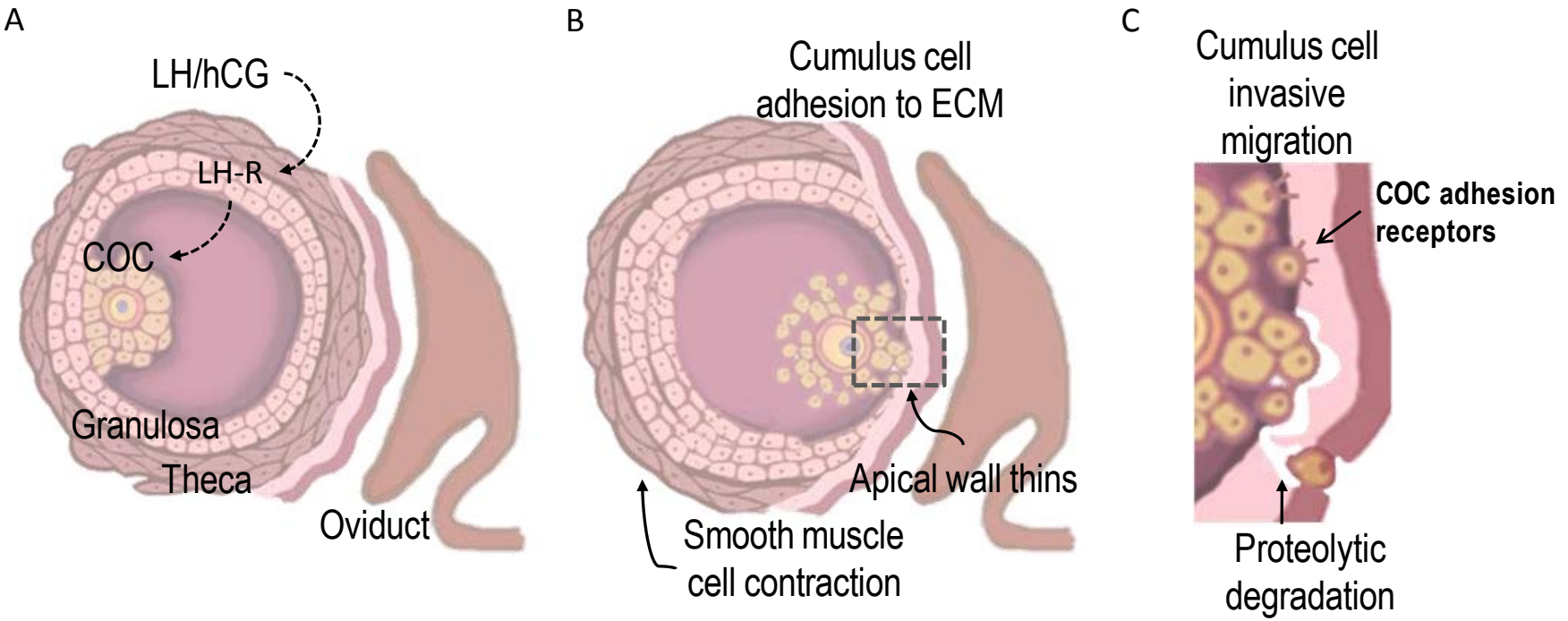

Figure 1.3 Novel hypothesis of an active role for the cumulus oocyte complex in ovulation

Ovulation begins with the LH-surge initiating a cascade of gene expression leading to cumulus oocyte complex expansion (A-B). The apex of the follicle thins due to contraction of smooth muscle in the basal region, causing thinning of the granulosa cell layers and revealing ECM epitopes in the follicular wall, which allows cumulus cell adhesion (B). Cumulus cells in the expanded COC then undergo epithelial to mesenchymal transition (EMT), responsible for cell migration. Just prior to ovulation the adhesion of cumulus cells to the apical wall triggers the proteolytic degradation of the ECM by cumulus cell derived proteases (C). Cumulatively this process allows the entire COC to invade and rupture the follicle. Box in (B) depicts area enlarged in (C).

phenotype. These targets may include the Rho family of GTPases, responsible for cytoskeletal rearrangement leading to cell migration (Hall, 1998). This model also involves the COC adhering to the apical wall of the follicle. Such adherence is a requirement for migration in other cell types. In the COC this might be mediated by the specific induction of adhesion receptors on cumulus cells. Alternatively or perhaps simultaneously, the thinning of the apical wall just prior to rupture, which includes marked thinning of the granulosa cell layers, may reveal follicular wall epitopes, ordinarily covered by granulosa cells. I propose that these components, namely extracellular matrices such as collagen, may be attractive to the COC allowing it to adhere at the apex of the follicle (Figure 1.3B). Following adhesion to the apical wall, the cumulus cells are capable of invasive migration, actively invading the follicular wall, through the actions of proteases in the COC such as Adamts1 creating a rupture pore to enable the release of the expanded COC (Figure 1.3C).

A fundamental validation of our hypothesis is the association of many of the critical COC genes with invasive cancer. In the ovulating ovary Adamts1 is induced in granulosa cells and incorporated into the expanded COC (Russell, et al., 2003b). Adamts1 has also been established as playing a role during cancer metastasis, including pancreatic, mammary and lung tumours (Liu, et al., 2005, Masui, et al., 2001). Versican is also induced during ovulation (Russell, et al., 2003a) and cleavage of versican has been shown to be mediated by Adamts1 (Russell, et al., 2003b, Sandy, et al., 2001). Versican is also a major component of tumour stromal matrix in prostate cancer and has been demonstrated to indicate the metastatic potential of tumours and prognosis as it also promotes EMT (Ricciardelli, et al., 1998). Growing evidence implicates Adamts1 proteoglycan processing in the invasive phenotype of cancer cells. The Adamts1 mediated cleavage of versican has been shown to enhance glioma invasion, whilst antibodies neutralising cleaved versican

can reverse this effect (Arslan, et al., 2007). Cleavage of versican by Adamts1 is additionally important physiologically during heart development by mediating cell migration to form heart valve and chamber structures (Kern, et al., 2007). Versican cleavage products are present in the COC during ovulation (Russell, et al., 2003b) whilst in *Adamts1* null mice, these cleavage products are reduced or absent in the expanded COC and basal follicular wall (Brown, et al., 2010). Combined with the severe ovulatory defect of *Adamts1* null mice, these results provide evidence that cleavage of versican by Adamts1 is crucial during ovulation.

Hyaluronan (HA) is another matrix protein consistently associated with both pathological and physiological mechanisms of cell migration and invasion (Fraser, et al., 1997, Knudson and Knudson, 1993, Turley, 1992). Overexpression of hyaluronan synthase (Has) in transfected cancer cell lines has been demonstrated to lead to accumulation of HA matrix (Itano, et al., 2002, Kosaki, et al., 1999), promotion of cell motility (Itano, et al., 2002), increased adhesion to ECM and increased tumourigenesis in mice in vivo (Kosaki, et al., 1999). The highly consistent association of HA and versican-containing matrices in cell migration and invasion suggest that the upregulation in Has2 and an accumulation of HA in the cumulus matrix could have a similar role during ovulation; promoting cumulus cell adhesion to follicle ECM and also cell migration. Furthermore, as mentioned previously, the HA receptor CD44 is implicated in cell migration and invasion following binding to its extracellular ligand. A comparable mechanism is proposed to act during epicardial cell differentiation during heart development. This mechanism involves the upregulation of Has2 by TGFB leading to HA production and binding to CD44, consequently inducing

intracellular signals, including activation of Rho family GTPases, responsible for changes in cell morphology and increased cellular migration and invasion (Craig, et al., 2010).

Pericellular matrices are HA and proteoglycan rich cellular coats that have been widely shown to facilitate motility of cancer and endothelial cells (Ricciardelli, et al., 2007). The COC matrix is an example of such matrix composition. Formation of a pericellular matrix has been suggested to be important for the regulation of cell adhesion, contributing to both adhesive and anti-adhesive cell behaviour (Evanko, et al., 2007). Such pericellular matrix has also been described in cancer cell models whereby the production of a hyaluronan-rich extracellular matrix increases cell migration (Evanko, et al., 2007, Ricciardelli, et al., 2007). Pericellular matrix production was increased in the prostate cancer cell line, PC3, when treated with recombinant versican (Ricciardelli, et al., 2007). Similarly, COC expansion involves versican incorporation into the cumulus matrix. Interestingly, this study also correlated expression of CD44 in PC3 cells to pericellular matrix production (Ricciardelli, et al., 2007). These results suggest an important role for CD44 in the metastatic capacity of prostate cancer. In further support of a role for CD44 and pericellular matrix formation for cell migration, research has revealed that COS-7 cells transfected with two different isoforms of CD44 form pericellular matrices, which are hyaluronan dependent. Pericellular matrix was increased around cells when exogenous hyaluronan was added to culture (Knudson, et al., 1993). Displacement of the pericellular matrix was observed upon the addition of either hyaluronidase or hyaluronan hexasaccharides, which compete for binding to hyaluronan receptors, such as CD44 (Knudson, et al., 1993). CD44 has been identified in the COC of many species (Furnus, et al., 2003, Kimura, et al., 2007, Kimura, et al., 2002, Ohta, et al., 2001, Ohta, et al., 1999, Schoenfelder and Einspanier, 2003, Yokoo, et

al., 2002a, Yokoo, et al., 2007, Yokoo, et al., 2002b), however little is known about its regulation in the mouse, and its importance during ovulation is yet to be elucidated.

A further mechanism by which CD44 interacts with hyaluronan to influence cell behaviour is during epithelial-to-mesenchymal transition (EMT), whereby a cell loses the characteristics of an epithelial cell type gaining mesenchymal features. EMT is evident in both physiological and pathological states. For example during heart development EMT contributes to cardiovasculature formation promoting epicardial cell invasion (Craig, et al., 2010). CD44 is considered characteristic of a mesenchymal cell, along with the expression of MMP9 (Takahashi, et al., 2010), which as has already been mentioned, is upregulated during ovulation in mice (Robker, et al., 2000).

Taken together I propose that the combined gene expression and morphological changes in ovulatory COCs generate a compelling argument for cumulus cells transitioning to an adhesive, migratory and invasive cell type during ovulation. I speculate that resultant production of ECM degradation enzymes, ECM remodelling, and promotion of cell migration act in unison to enable COC release from the follicle during the ovulation process.

1.5 SUMMARY

Numerous studies have highlighted the importance of the COC in the process of ovulation by showing that defects during cumulus expansion and matrix production all lead to severe infertility phenotypes including anovulation. However, to date research investigating ovulation has mainly focused on the follicle itself including increasing intrafollicular

pressure, smooth muscle cell contraction, and proteolytic enzymes localised to the follicle wall. Yet, conclusive evidence is lacking to show that one or a combined series of these events are exclusively accountable for the release of a mature COC.

My general hypothesis is that the cumulus oocyte complex, through specific action of the cumulus cells, is able to actively contribute to ovulation by transitioning to an adhesive, invasive and migratory cell type.

To test this general hypothesis it is necessary to establish whether the COC expresses molecular components of a cell migration pathway following an ovulatory stimulus. Specifically the CD44 cell migration pathway has been implicated in playing a role in cancer cell metastasis and is known to be upregulated in cumulus cells following hormonal stimulation, in other spcies but has yet to be specifically studied in mice. However, no genes and proteins involved downstream of a CD44-HA interaction required for cell invasion, nor the adhesive, migratory or invasive capacity of the COC have been the subject of any previous investigation.

I hypothesise that COCs responding to an ovulatory stimulus upregulate components of the CD44 cell migration pathway.

An essential initial step in activating invasive migratory phenotypes in cells is their contact and adhesion to extracellular matrix. Just prior to ovulation the granulosa cell layer at the apex of the follicle thins revealing the follicle wall ECM. This may permit adherence of the expanded COC to the follicular wall - a key first step for cellular invasion.

I hypothesise that preovulatory expanded COCs will have the capacity to adhere to specific extracellular matrices found at the apex of the ovarian follicle wall.

The final key event in the model is that following cumulus cell adhesion to the follicle wall, the cumulus cells have the capacity to transition from epithelial cells to invasive migratory cells. Following cumulus cell adhesion to the follicle wall ECM, the COC, may be capable of invading through the follicle wall leading to its ovulation.

I hypothesise that the cumulus cells have an active role in ovulation, specifically, that they transition to an invasive motile cell type following an ovulatory stimulus.

This novel hypothesis of ovulation incorporates evidence accumulated from previous studies including the importance of proteases, follicle wall contraction and cumulus-specific gene expression, to explain the role of the cumulus oocyte complex in ovulation.

1.6 SPECIFIC HYPOTHESES AND AIMS

Hypothesis 1: Preovulatory expanded COCs are able to adhere to specific extracellular matrices of the ovarian follicular wall.

To address this hypothesis the following aims were addressed:

- Determine whether cumulus cells of expanded preovulatory COCs from ovarian follicles which had received an ovulatory stimulus, were more adherent than cumulus cells of unexpanded COCs.
- Determine whether cumulus cells of preovulatory expanded COCs showed an affinity for adhering to specific extracellular matrices known to be present in the ovarian follicle wall.

Hypothesis 2: Preovulatory expanded COCs express the CD44 migratory molecular complex, which is induced at the time of ovulation.

To address this hypothesis the following aims were addressed:

- Determine whether CD44 is temporally regulated in COCs following an ovulatory stimulus.
- Determine which variant isoforms of CD44 are expressed in COCs.
- Determine whether downstream targets of CD44 are temporally and spatially regulated in the ovary following an ovulatory stimulus.

Hypothesis 3: The COC transitions to a motile cell type following an ovulatory stimulus and this phenotype is mediated by CD44 signalling.

To address this hypothesis the following aims were addressed:

- Determine whether COCs are capable of cell migration following an ovulatory stimulus.
- Determine whether this migratory phenotype is cumulus cell specific by establishing whether granulosa cells are capable of a similar transition.

- Determine whether the migratory phenotype of cumulus cells is CD44-mediated by blocking CD44 and downstream targets.

Hypothesis 4: The COC is capable of invading an extracellular matrix barrier.

To address this hypothesis the following aims were addressed:

- Determine whether preovulatory expanded COCs are capable of invading an extracellular matrix.
- Determine whether COC invasion is comparable to characterised invasive and non-invasive cell lines.
- Determine whether COC invasion is protease dependent by utilising an MMP inhibitor and *Adamts1* null mice.

Chapter 2

Matrix adhesive properties of the cumulus oocyte complex

2.1 INTRODUCTION

It is known that following the LH surge a number of changes occur in the ovary. Most profoundly, a cascade of cumulus cell-specific regulated gene expression leads to the production of an extracellular matrix, which surrounds the cumulus cells. This process is known as cumulus expansion and is required for ovulation and reproductive success (Chen, et al., 1993, Fulop, et al., 2003, Hess, et al., 1999, Salustri, et al., 2004). Following cumulus expansion, the cumulus oocyte complex (COC) is released from the follicle and into the oviduct where it can be fertilised. However, it is still unknown how follicle rupture occurs and the role played by expanded COC matrix. The extracellular matrix of a follicle maintains its cellular organisation and is also important for its structural integrity. However, throughout folliculogenesis the growth and development of the follicle requires continual remodelling of the extracellular matrix of the follicle wall. This is particularly evident during ovulation whereby the follicle wall breaks down enabling release of the ovulating COC.

The localisation of ECM during mouse folliculogenesis is not well characterised, however it has been reported that collagen is a major component of the follicular structure, mostly within the tunica albuginea the most dense part of the follicle apex (Berkholtz, et al., 2006, Bjersing and Cajander, 1974). Collagen IV and laminin have both been identified in the basement membrane of the follicle (Rodgers, et al., 1998), whilst, collagen II has been identified in the granulosa and theca layers of the ovary (Saha, et al., 2007). Fibronectin has been localised to the follicular fluid of antral follicles (Berkholtz, et al., 2006). Figure 2.1, a schematic representation of the wall of an follicle, shows the follicle wall prior to ovulation and illustrates where particular extracellular matrices reside.

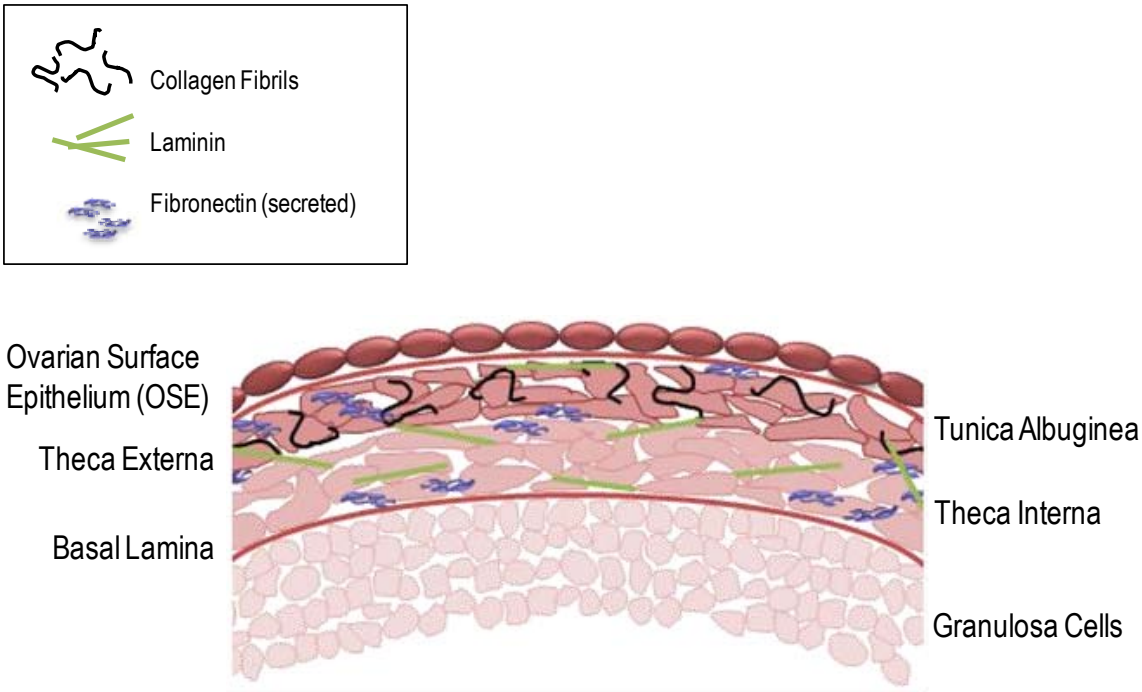

Figure 2.1 Schematic representation of a cross section of the ovarian follicle wall

The ovarian follicle wall is made of a number of different cellular layers that perform distinct roles during folliculogenesis. The outermost layer is the ovarian surface epithelium (OSE), which is shed prior to ovulation. The tunica albuginea is the next layer adjacent to the OSE, this layer consists of thick connective tissue and collagen I fibrils. The next cell layers are the theca externa and theca interna. The theca externa contain abundant collagen fibres and provide a supportive role, whilst the theca interna cells express LH receptors and respond to it by producing steroids, particularly androgens. The basal lamina is a specific extracellular matrix between the granulosa and theca cell layers and is composed mostly of laminin and collagen IV. The innermost cell layers of the ovarian follicle are the mural granulosa cells, which are important for steroid production, primarily estrogen. These cells are in direct contact with the follicular fluid of the follicle antrum. The follicular wall is composed of different extracellular matrices dependent on the stage of follicular development. This schematic represents an antral follicle, which is abundant in collagen fibrils in the theca externa layers, laminin is present in both theca layers and the basal laminaa, and fibronectin is secreted in the theca layers and the follicular fluid.

What has been established by a number of studies, is that the follicle wall at the apex of the ovary thins (Bjersing and Cajander, 1974, Espey, 1967, Martin and Talbot, 1987) and is devoid of surface epithelial cells just prior to ovulation (Murdoch and McCormick, 1992, Talbot, et al., 1987). Images demonstrating this thinning just prior to ovulation are shown in Figure 2.2. The panels show the follicle wall following stimulation with either eCG alone or a subsequent ovulatory dose of hCG. In the eCG treated ovary the follicle has a thick granulosa and theca cell layer at the apical side, consisting of multiple compact cell layers. Whilst the apex of the follicle just prior to ovulation (post hCG), thins dramatically, and consists of a single theca cell layer and a dispersed granulosa cell layer. This phenomenon only appears to occur at the apical side of the follicle wall. Further, it has been demonstrated that collagen degradation is greatest at the apex of the follicle at time of ovulation (Murdoch and McCormick, 1992), including in the human (Postawski, et al., 1996).

The mechanism leading to follicle rupture and ovulation has been surrounded by much debate. I hypothesise that the COC plays an active role in facilitating its own release in a directional manner at the apex of the follicle. As described in the working model (Figure 1.3) I hypothesise that prior to ovulation, the thinning of both cellular layers and extracellular matrix at the apical wall of the follicle may expose distinct ECM moieties that were previously protected by granulosa layers. Furthermore, I anticipate that the ECM presents adhesive receptors to the expanded COC, and this could lead to adherence of the COC and provide directional cues for ovulation to proceed reliably toward the follicle apex and hence oviduct. This kind of directed cell adhesion is known to occur in biological systems such

Figure 2.2 Representative example of mouse ovarian follicles following hormonal stimulation

These photomicrographs show ovarian follicles following ovulatory hormone treatment with (A) eCG and (B) eCG + hCG. (A) Unexpanded COCs with a compact layer of cumulus cells closely associated with the mural granulosa cells (mGC) at the basal wall of the follicle. (B) Example of expanded COC associated with the apical wall of the follicle, just prior to ovulation. The apical wall of follicles becomes thinned dramatically when compared to follicles prior to the ovulatory stimulus. The mGC layer at the apex becomes only a couple of cell layers, but increases in thickness at the basal wall. Images modified from (Russell, et al., 2003b) and unpublished data of D Russell.

as axon guidance, where axons recognise substrates such as extracellular matrix proteins, and adhere to specific targets or adhesion molecules in order to establish neuron connections (Thomas, et al., 1990, Zhang, et al., 2008). These adhesion molecules include integrins, which can be found on neighbouring cells. In a disease model system such as tumourigenesis, cell adhesion is vital for the progression of cancer cells to distal sites. For cancer cells to invade surrounding tissue, first they must adhere to the extracellular matrix of that tissue. They may upregulate a number of integrins on surrounding cells by secreting growth factors, thus adhere to these sites. The preovulatory expanded COC could act in a similar way, by adhering to extracellular matrix following the reception of a particular guidance cue, or perhaps by the upregulation of integrins on the cell surface by secretions of surrounding cells. Additionally, the ECM laminin has been shown in previous studies to be an important substrate for promoting the cellular adhesion and subsequent migration and outgrowth of neurons (Baron-Van Evercooren, et al., 1982, Hall, et al., 1990, Hammarback, et al., 1988, Thomas, et al., 1990, Zhang, et al., 2008) and laminin is a component of the follicle wall (Berkholtz, et al., 2006). Adhesion by cumulus cells to the ECM of the follicle wall is a necessary initial step for the proposed COC migratory invasive mechanism.

I hypothesise that the expanded COC will adhere to ECM substrates found predominantly in the follicle wall and that adhesion to extracellular matrices is specific to cumulus cells after ovulatory stimulus and not preovulatory cumulus nor granulosa cells. This phenotype would allow the preovulatory expanded COC to recognise substrates revealed at the apex of the follicle wall following thinning and be directed to where ovulation is to take place, at the apex of the follicle. To elucidate whether the expanded COC is capable of adhering to ECM found in the ovarian follicle wall, adhesion assays of cumulus cells against a number of ECMs were employed The results demonstrate that cumulus cells from expanded COCs compared to those from unexpanded COCs have varying adhesive properties.

2.2 MATERIALS AND METHODS

2.2.1 Animals and hormonal stimulation protocol and Tissue Collection

All animals were treated in accordance with the NHMRC Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NHMRC, 2004). Ethics approval was obtained from The University of Adelaide Animal Ethics Committee. The F1 C57Bl6 X CBA mouse line were housed at the University of Adelaide Animal Facility and maintained on a 12h/12h day/night cycle with rodent chow and water provided *ad libitum.* Mice were superovulated by i.p injection with 5 IU Equine chorionic gonadotropin (eCG) (Professional Compounding Centre of Australia, Sydney, NSW, Australia) at 1400 h on day 21-23 of age to stimulate follicle growth. This was followed 44 – 48 hours later with an injection of 5 IU Human Chorionic Gonadotropin (hCG) (Organon Australia, Sydney, NSW, Australia) to initiate ovulation. Cumulus oocyte complexes were punctured from follicles of ovaries at 44 – 48 h post eCG and10 h post hCG, in hepes buffered α-MEM with 5% FCS using 30 gauge needles (BD Biosciences) (Figure 2.3).

2.2.2 Adhesion Assays

Commercially available ECM Cell Adhesion Array Kits (Millipore, North Ryde, NSW, Australia) were used as per manufacturer's instructions. The plate consisted of wells pre-coated with purified human collagen I, II and IV, fibronectin, laminin, tenascin and vitronectin, with a bovine serum albumin (BSA) coated well as a negative control. Briefly, plate strips containing extracellular matrices were rehydrated with 200 µL of PBS for 10 min at room temperature. Cumulus oocyte complexes were isolated as described from eCG + 10 h hCG treated mice (n=10 mice/experiment). Cumulus cells were dissociated

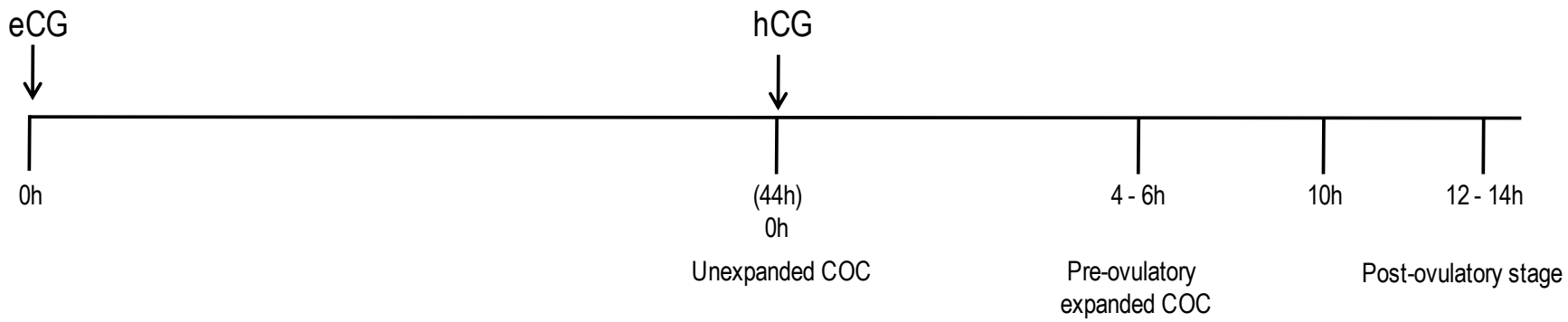

Figure 2.3 Time-course of hormonal stimulation protocol

Time-course of hormonal stimulation protocol. Groups of prepubertal mice (C57Bl6, F1 C57Bl6 X CBA or *Adamts1*) were stimulated with eCG to initiate follicle growth followed 44h later by an ovulatory dose of hCG. Mice were sacrificed and ovaries removed. Cumulus oocyte complexes (COC), mural granulosa cells (mGC) and residual ovaries were collected for various analyses.

from complexes either by vortexing or hyaluronidase treatment. COCs from eCG treated animals were vortexed for approximately 4 min to dissociate the cumulus cells (method presviously described (Dragovic, et al., 2007)). Whilst, COCs from hCG treated animals were dissociated using 50 µL of hyaluronidase (10µM) and constant pipetting. Viable cell numbers were determined using the trypan blue exclusion assay, where stained cells visible in the field of a haemocytometer were deemed unviable and not included in the counts of unstained cells. Cell counts were performed and a single cell suspension was prepared in DMEM. In 100µL, $10x10^3$ cells were placed in each well in duplicate. The plates were incubated for 1 h at 37°C/5% CO_2 in air. After incubation media was gently aspirated and the wells were washed 2-3 times with 200µL of Assay Buffer (Cell Adhesion Array Kit, Millipore, North Ryde, NSW, Australia). Cells were fixed with absolute methanol for 2 min. After fixation cells were stained using the DiffQuik (Fronine Laboratory Supplies, Lomb Scientific, SA, Australia) differential staining protocol. Briefly, solution A (eosin) was added for 30 seconds followed by solution B (methylene blue) for 30 seconds, wells were washed in H_2O. Wells were then visualised on a microscope and cells manually counted.

2.2.3 Statistics

A 2-Way ANOVA was performed on log transformed data to normalise distribution with a Student Newman Keuls Multiple Comparison post hoc test. Statistics were performed using GraphPad Prism software (GraphPad Software Inc, La Jolla, CA, USA). Significance was accepted at $p<0.05$.

2.3 RESULTS

2.3.1 Adhesion of cumulus cells to ECM substrates following hormonal stimulation.

Adhesion assays were performed with dissociated cumulus cells from expanded and unexpanded COCs across a variety of extracellular matrices (Figure 2.4) There was a significant difference between adherence of cumulus cells isolated from unexpanded versus expanded COCs ($p<0.001$) but no significant difference in adherence to the type of matrix ($p=0.6$). Cumulus cells derived from expanded COCs were significantly more adherent to all matrices, with the exception of tenascin and the BSA negative control, compared to those derived from unexpanded COCs ($p<0.05$). Whilst not statistically significant, cumulus cells from expanded COCs appear to have a greater affinity for the collagen matrices over the other matrices examined. The negative control wells, coated with BSA, contained very few adherent cells as expected and confirms the selective affinity of cumulus cells for ECM proteins.

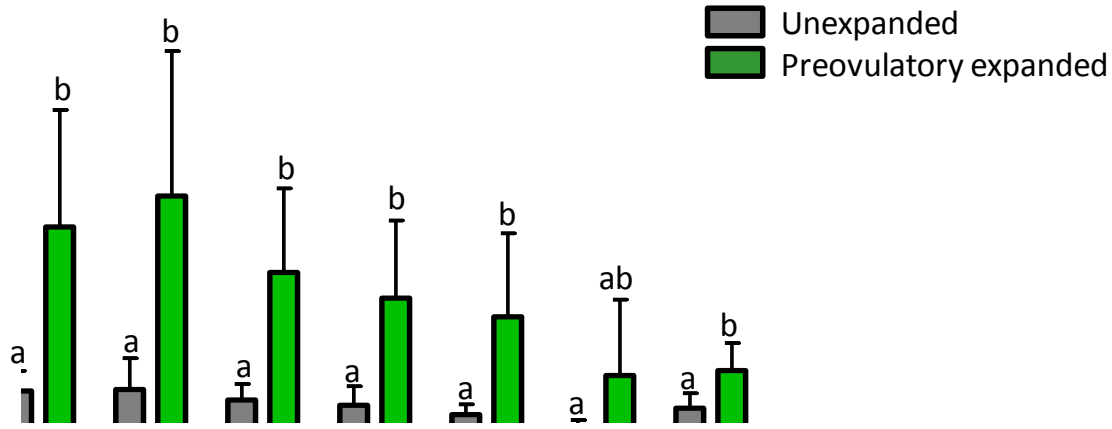

Figure 2.4 Adhesion assay to investigate cumulus cell affinity for extracellular matrices

Dissociated cumulus cells from unexpanded COCs (*grey*, eCG treated mice) and expanded COCs (*green*, eCG + 10 h hCG treated mice) were assessed for their binding affinities for a range of extracellular matrices. The number of cells which adhered to the various extracellular matrices is presented on the y-axis. Data is presented as mean adherent cell number (mean ± SEM). A 2-Way ANOVA with a Student Newman Keuls Multiple Comparison post hoc test was performed on data that was log transformed to normalise distribution. Different letters represent statistical significant difference ($p<0.05$). n=3 replicates (3 independent experiments performed in duplicate).

2.1 DISCUSSION

In many biological systems, cell adhesion is the foundation for cell migration. Cells must attach at their leading edge, and detach at the trailing edge in order to project themselves forward. I have demonstrated that following expansion the cumulus cells are adhesive, which may be reflective of a change in cell morphology or indicative of cellular motility. The precise cellular mechanisms involved in the adhesive properties of the cumulus cells requires further investigation, for instance assessments of the breakdown of focal adhesion complexes. As part of our working model (Figure 1.3), the cumulus cells of expanded COCs are hypothesised to adhere to specific matrices in the follicle wall as an initial step towards ovulation through the apex. I investigated this by examining whether cumulus cells dissociated from expanded COCs were more adhesive to ECM than cumulus cells dissociated from unexpanded COCs. Further I examined whether cumulus cells from expanded COCs, would adhere preferentially to matrices found in the apical follicle wall specifically, collagen, laminin and fibronectin. Expanded COCs are always found at the follicle apex as ovulation approaches (illustrated in Figure 1.1), at least in the mouse where this is practical to investigate.

The mechanism for this may be explained by my observations that cumulus cells from preovulatory expanded COCs were significantly more adherent to all ECM substrates tested compared to cumulus cells dissociated from unexpanded COCs. Comparisons between individual ECM substrates were made, and whilst not statistically significant due to variability in the numbers of adherent cells, cumulus cells from preovulatory expanded COCs tended to show a greater affinity for collagen than the other ECMs. The result is an important biological observation, as collagen has been reported as the most abundant extracellular matrix in the follicle wall (Berkholtz, et al., 2006, Bjersing and Cajander, 1974). Furthermore, together with the collagens, laminin and fibronectin appeared particularly receptive to the expanded COCs. These two extracellular matrices are also present in the follicle wall of the ovary, and

could play an important role for the adhesion of the expanded COC In support of this concept, in other systems adhesion has shown to be promoted via an interaction between cells and particular moieties of laminin (Baron-Van Evercooren, et al., 1982, Hall, et al., 1990, Hammarback, et al., 1988, Thomas, et al., 1990, Zhang, et al., 2008).

How the cumulus cell may adhere to extracellular matrices of the follicle wall would be an important future focus. Specific cell adhesion molecules, including integrins, enable direct interaction between cells and the extracellular matrix. A major component of the follicle wall is collagen and this is a ligand for a number of different integrins, in particular the Beta1 integrins which interact with various alpha chains. Integrin expression has been investigated in the ovary, (Burns, et al., 2002, Fujiwara, et al., 1998, Fujiwara, et al., 1995, Honda, et al., 1995, Honda, et al., 1997, Nakamura, et al., 1997, Tamba, et al., 2008, Yamada, et al., 1999) however little is known about expression on the COC during ovulation, as most studies have involved the characterisation of granulosa cell integrin expression during follicle growth and luteinisation. It has been reported that α2 (Yamada, et al., 1999), α5 (Honda, et al., 1997), α6 (Burns, et al., 2002, Nakamura, et al., 1997), α_v , β1, β3 and β5 (Burns, et al., 2002) integrin subunits are expressed on granulosa cells and theca cells during follicle growth, ovulation and subsequent corpus luteum formation. The integrin subunit β1 has been reported to be expressed on cumulus cells and thought to interact with ECM, specifically fibronectin and collagen IV, prior to fertilisation (Tamba, et al., 2008). Further investigations into integrin expression specifically during ovulation are required, as much of the focus to date has been on their involvement during luteinisation and fertilisation.

Prior to ovulation, the e basement membrane of the follicle breaks down and the granulosa and theca cell layers thin (Bjersing and Cajander, 1974, Martin and Talbot, 1987) possibly revealing ECM proteins,

which previously would have been concealed by granulosa cell layers (Martin and Talbot, 1987). }. It is possible that smooth muscle cell contraction causes the follicle wall thinning, and in the process reveals the ECM to the expanded COC. In support of this when the development of smooth muscle cells are inhibited in the ovary, the follicle develops normally but ovulation fails (Ren, et al., 2009). Smooth muscle cells are induced to contract predominantly by the Endothelin receptor system. Endothelin 2 mRNA specifically has been shown to increase just prior to ovulation, additionally follicular tension increases following treatment with endothelin 2 (Ko, et al., 2006). Furthermore, using an endothelin receptor antagonist, tezosentan, to block smooth muscle cell contraction results in a loss of follicular tension and decreased ovulation rate due to an absence of follicular rupture (Ko, et al., 2006). However, the inhibition of individual endothelin receptors alone is not sufficient to block ovulation, suggesting redundancies amongst the receptors of endothelin (Bridges, et al., 2010). These findings suggest an important role of smooth muscle cell contraction during ovulation. Smooth muscle cell contraction and apical thinning are likely to expose adhesive epitopes in this region to compliment the elevated adhesive capacity of the COC.

Enhanced adhesion of cumulus cells to ovarian ECM moieties satisfies the first requirement of our working model and provides a potential mechanism for how the COC becomes localised to the apical region of the follicle at the time of ovulation. I hypothesise further that the expanded COC attaches at the apical wall of the follicle and then initiates invasive qualities to form a rupture pore and invade through the ovarian surface, thus contributing to the ovulation process. In further chapters, I will explore this concept further, including investigating whether the COC has migratory and invasive properties during ovulation. However, the adhesive capacity of the COC is the first step in the migratory invasive cell phenotype I propose in the following chapters. In summary, our results have shown that the dissociated cumulus cells from expanded COCs acquire the ability to adhere to an extracellular matrix

surface. These expanded cumulus cells showed a greater affinity for the collagen substrates, which are major components of the follicle wall.

Chapter 3

Expression of Migratory Genes in the Cumulus Oocyte Complex during Ovulation

3.1 INTRODUCTION

Our overarching hypothesis is that expansion of the cumulus oocyte complex (COC) mediates ovulation by transitioning to an adhesive migratory and invasive cell type. Having shown that ECM adhesion properties are indeed increased in an expanded COC just prior to ovulation, I next sought to determine whether the COC expresses genes involved in migratory pathways and whether these are hormonally regulated during ovulation.

The observed transition of cumulus cells to a more adhesive phenotype following expansion (Chapter 3) is an important initial step if these cells are to migrate. Whether components known to be important for cellular motility are present and induced in cumulus cells during the ovulation process will be investigated herein.

3.1.1 Cell migration mechanism

Cell migration occurs in response to extracellular chemokine or growth factor signals and involves the formation of attachment sites at the leading edge of the cell and detachment from rear adhesion sites. Typically, a cell capable of migration shows alterations to its shape, through remodelling of the actin cytoskeleton, which is structurally linked to the extracellular surroundings via interaction with transmembrane adhesion receptors (Lauffenburger and Horwitz, 1996, Vicente-Manzanares, et al., 2005). Cell migration is a highly orchestrated process. One well-characterised pathway is CD44-mediated cell migration, involving the transmembrane glycoprotein CD44.

CD44 is a transmembrane hyaluronan (HA) receptor (Underhill, et al., 1987), and HA is a major component of the extracellular matrix of an expanded COC. CD44 activates cell migration via interaction with its downstream intracellular targets; these include T-cell lymphoma invasion and metastasis 1

(Tiam1), Rho family GTPases Ras-related C3 botulinum substrate 1 (Rac1) and Ras homolog gene family, member A (RhoA) (Bar-Sagi and Hall, 2000, Bourguignon, 2001, Bourguignon, et al., 1995, Bourguignon, et al., 2000), (Figure 3.1). The Rho GTPases are mediators of actin cytoskeleton regulation during cell migration They are dependent on intracellular signalling molecules known as Guanine nucleotide exchange factors (GEFs), such as Tiam1, which control the activity of Rho GTPases by exchanging GDP on the inactive molecule for GTP leading to activation of the protein (Heasman and Ridley, 2008).

3.1.2 CD44 isoform specific functions

The CD44 gene consists of 20 exons (Figure 3.2A), which can be alternatively spliced to give rise to twelve variant mRNA and protein isoforms (Figure 3.2B). Several of these variant forms of CD44 have been shown to be associated with tumour progression and metastasis (Bourguignon, 2001, Screaton, et al., 1992) (Figure 3.2). Alternative splicing of exons in CD44 occurs in the extracellular region of the protein (Figure 3.1), whilst the standard form of CD44 (CD44s) contains no alternative exons, and is identified by the presence of exons 1 - 5 and exons 15 – 19, only (Figure 3.2B). The amino terminal region has conserved homology between species, and contains the HA binding “link protein” domain, with some homology with other HA binding proteins such as cartilage link protein and the COC matrix protein Tnfaip6 (Kahmann, et al., 2000). The implications of various CD44 isoforms in tumourigenesis has been extensively studied in breast cancer, where overexpression of specific isoforms was shown to activate downstream targets resulting in increased cell motility and metastasis (Bourguignon, et al., 2000 reviewed in Bourguignon, et al., 2008).

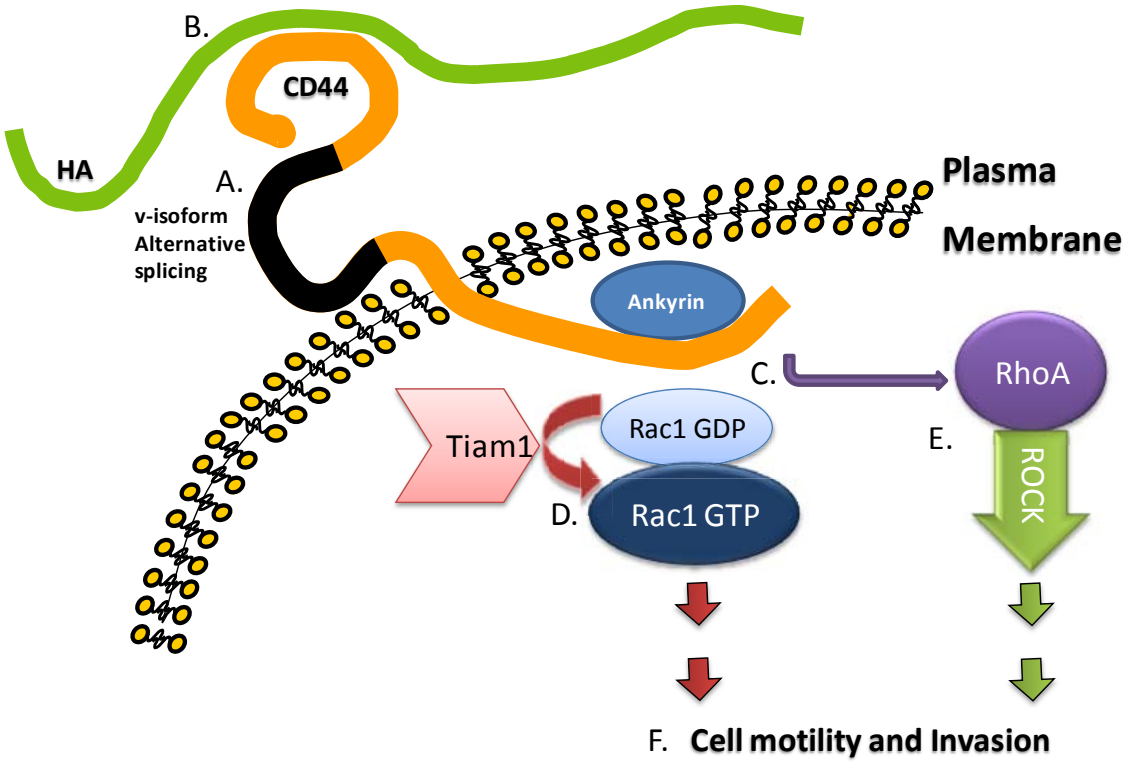

Figure 3.1 Known mechanisms of CD44-mediated motility in mammalian cells

CD44 is a glycoprotein which undergoes alternate splicing (A) events. CD44 is a known hyaluronan (HA) receptor (B) which upon binding leads to activation of its downstream targets (C). Downstream targets include the guanine-exchange factor (GEF) Tiam1 which leads to the activation of the small GTPase, Rac1, by the exchange of GDP for GTP (D). An alternate pathway is the activation of another small GTPase, RhoA which acts via its kinase Rock (E). Both of these activated cellular complexes trigger cytoskeleton changes leading to cell motility and invasion (F).

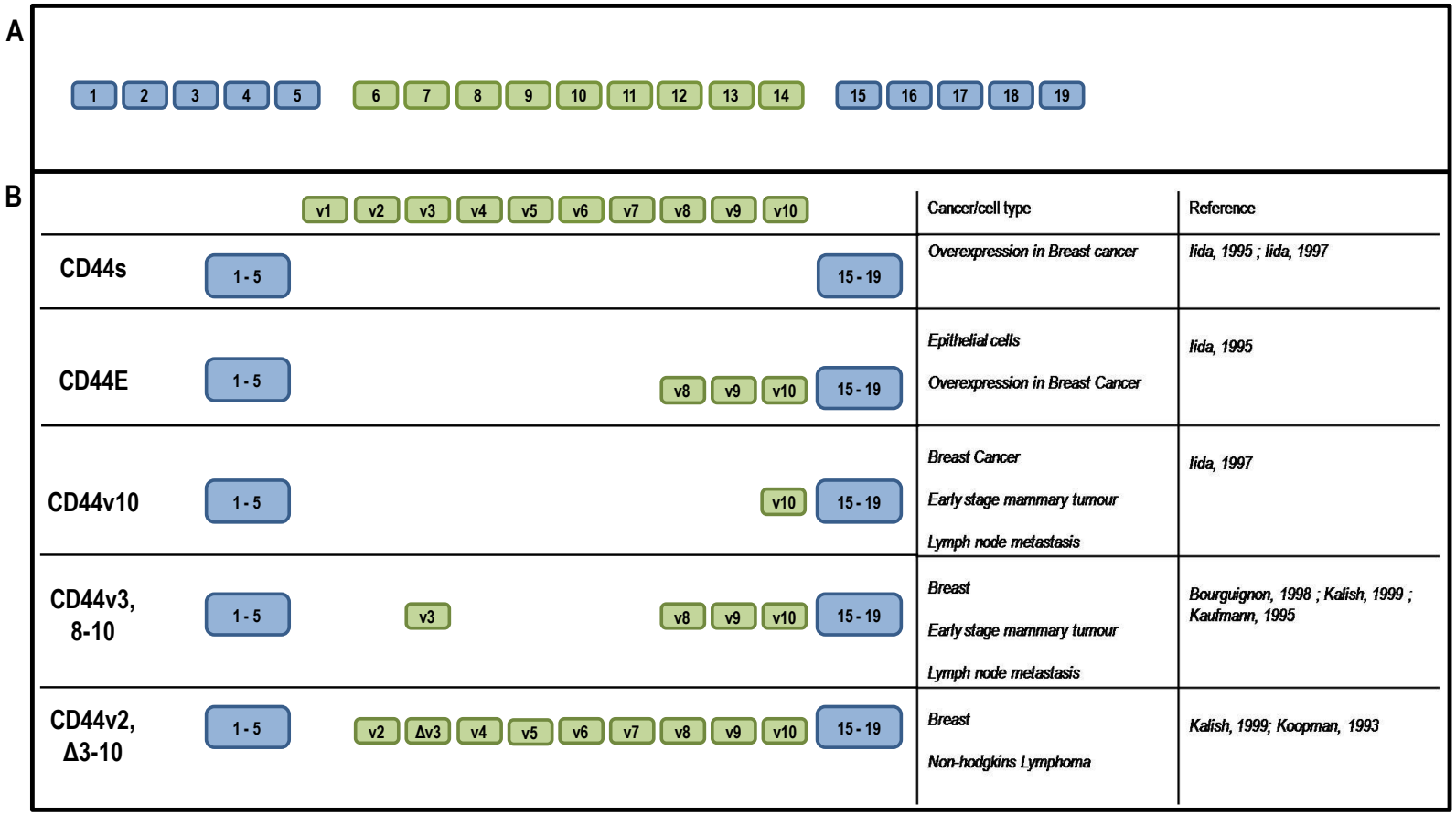

Figure 3.2 Schematic representation of the alternatively spliced isoforms of CD44 and their association with metastatic cancers

(A) CD44 can be alternatively spliced into 12 isoforms or variant forms by splicing in exons (green) between exons 5 and 15. Exons 1-5 and 15-17 make up the standard isoform of CD44 (blue). Specific isoforms are known to be associated with various metastatic tumours. (B) The schematic describes the exons which are found in each of the described isoforms. The table highlights their association with metastatic cancer types (Bourguignon, et al., 1998, Iida and Bourguignon, 1995, Iida and Bourguignon, 1997, Kalish, et al., 1999, Kaufmann, et al., 1995). CD44s: standard isoform; CD44E: epithelial CD44

3.1.3 CD44 in the ovary

In addition to the extensive investigation of CD44 in cancer metastasis, CD44 has also been identified in ovarian cells of pig (Kimura, et al., 2002, Yokoo, et al., 2002a, Yokoo, et al., 2007, Yokoo, et al., 2002b), cow (Furnus, et al., 2003, Schoenfelder and Einspanier, 2003) and human (Ohta, et al., 2001, Ohta, et al., 1999). Its expression in the mouse ovary has not been reported. In the porcine ovary CD44 has been localised to cumulus cells of expanded COCs, and appears to be absent from unexpanded COCs (Furnus, et al., 2003, Kimura, et al., 2002, Schoenfelder and Einspanier, 2003, Yokoo, et al., 2002a). Whilst present in human granulosa cells, the expression of *CD44* appears to be higher in cumulus cells (Ohta, et al., 1999). Although *CD44* is known to be expressed in granulosa cells and the COC, its expression in the mouse ovary has not been examined, furthermore an investigation of the variant isoforms of CD44 nor its downstream targets, Rac1, Tiam1 and RhoA has not been examined in the mammalian ovary.

3.1.4 Rationale

Previous studies showing that *CD44* expression in the ovary is hormonally regulated and greater in cumulus cells compared to other ovarian cells suggest that CD44 may play an important role in the cumulus complex and ovulation. I hypothesise that induction of CD44 in the COC activates one or more of its downstream targets, controlling migratory processes in the cumulus cells. By adopting a mesenchymal phenotype, in response to the ovulatory stimulus, the COC may have an active role in facilitating ovulation via invasion and migration through the ovarian surface.

I have investigated whether the components of this CD44-mediated cell migration pathway are present, and whether they are hormonally induced in ovarian cells of the mouse during ovulation. I also characterised which isoforms of CD44 are present in COCs. Using a hormonally stimulated mouse

model, the expression of migratory genes in COCs and granulosa cells were examined at various times during the ovulatory cascade to determine the expression profile of *CD44* and its interacting partners *Tiam1*, *RhoA*, and *Rac1*.

3.2 MATERIALS AND METHODS

3.2.1 Animals, hormonal stimulation protocol and tissue collection

All animals were treated in accordance with the NHMRC Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NHMRC, 2004). Ethics approval was obtained from The University of Adelaide Animal Ethics Committee. All C57Bl6 and F1 C57Bl6 X CBA mouse lines were housed at the University of Adelaide Animal Facility and maintained on a 12h/12h day/night cycle with rodent chow and water provided *ad libitum*. Mice were superovulated by i.p injection with 5 IU Equine chorionic gonadotropin (eCG) (Professional Compounding Centre of Australia, Sydney, NSW, Australia) at 1400 h on day 21-23 of age to stimulate follicle growth. This was followed 44 – 48 h later with an injection of 5 IU Human Chorionic gonadotropin (hCG) (Organon Australia, Sydney, NSW, Australia) to initiate ovulation. Ovaries and oviducts μre collected at 0 h and 44 h post-eCG administration and 4 h, 6 h, 10 h, 12 h and 16 h post-hCG administration (Figure 2.3). Cumulus oocyte complexes were punctured from follicles of ovaries, or dissected from oviducts 16 h post hCG in hepes buffered α-MEM with 5% FCS using 30 gauge needles (BD Biosciences). Granulosa cells and residual ovaries were also collected at all time-points

3.2.2 RNA isolation and reverse transcription (RT)

Total RNA was isolated using Trizol (Invitrogen Australia Pty Ltd, Mulgrave, Victoria, Australia) as per manufacturer's instructions, with the addition of an overnight precipitation in isopropanol and the inclusion of 7.5ug blue Glycogen (Ambion Inc., Austin, TX, USA) during precipitation, to aid with

visualisation of the pellet. Total RNA was then treated with Dnase (Ambion Inc., Austin, TX, USA) as per manufacturer's instructions. First-strand complementary DNA (cDNA) was synthesised from total RNA using random hexamer primers (Geneworks, Hindmarsh SA, Australia) and Superscript III Reverse Transcriptase (Invitrogen Australia Pty. Ltd, Mulgrave, Victoria, Australia). Briefly, 10µL of RNA, 2µL random hexamer primers, 2µL dNTPs (10mM) and 18.9µL H_2O, were all added to a microcentrifuge tube. The mixture was heated to 65° for 5 min and incubated on ice for 1 min. Contents were briefly centrifuged followed by the addition of 1µL SuperScript III reverse transcriptase (RT), 12µL 5x first-strand buffer, 3µL 0.1M DTT, 0.5µL RNase Inhibitor and 10.6µL H_2O. Samples were then incubated for 5 min at 25°C, followed by 50°C for 60 min, and finally 70°C for 15 min. cDNA was kept at -20°C.

3.2.3 Polymerase Chain Reaction (PCR)

For reverse transcription polymerase chain reaction (RT-PCR), the following method was used, 5µL of template, containing 100ng of cDNA, was added together with 5µL of GoTaq Flexi® buffer (Promega Corporation, Annandale, NSW, Australia), 2µL dNTPs (10mM), 1µL each of forward and reverse primer (50µM), 0.2µL GoTaq® polymerase (Promega Corporation, Annadale, NSW, Australia) and water to make a total reaction volume of 50µL. PCR cycling was performed on a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems, Mulgrave, Vic, Australia) with the following program; 94°C for 2 min, then 30 cycles at 94°C for 1 min, 60°C for 1 min, 72°C for 5 min, and finally 72°C for 5 min.

3.2.4 Agarose Gel Electrophoresis

To visualise PCR products samples were resolved by agarose gel electrophoresis. Briefly, 1% (w/v) agarose (Promega Corporation, Annandale, NSW, Australia) containing 0.5x TBE buffer (44.5mM Tris, 44.5mM Boric acid, 1mM EDTA pH 8.0), and 2µg/mL ethidium bromide. To each PCR sample, 15µL of 5X loading buffer (25% glycerol; 50mM EDTA; 0.25% bromophenol blue) was added and from this 15µL was loaded to wells of the gel. 5µL of 100bp ladder (Promega Corporation, Annandale, NSW, Australia)

was also run in each gel. Gel electrophoresis was performed in 0.5x TBE buffer at 80V. Gels were visualised using a UV transilluminator and imaged using gel documentation system (Kodak DC120).

3.2.5 Quantitative real-time RT-PCR (qRT-PCR)

Specific gene primers for qRT–PCR were designed against published mRNA sequences (NCBI Pubmed database) using Primer Express software (PE Applied Biosystems, Foster City, CA, USA) and synthesised by Sigma Genosys (Sigma-Aldrich Pty. Ltd, Castle Hill, NSW, Australia). Primer pairs and sequences for murine *CD44*, *Tiam1*, *Rac1, RhoA, Has2, Tnfaip6, Ptgs2* and *RpL19* are shown in Table 3.1. Real-time qRT-PCR was performed in triplicate for each sample on a Corbett Rotorgene 6000 (Qiagen, Doncaster, Victoria, Australia). In each 20 µL reaction there was 2µL of cDNA (equivalent to 5ng/µL of RNA), 0.2µL forward and reverse primers, 10µL SYBR Green master mix (Qiagen, Doncaster, Victoria, Australia) and 7.6µL H_2O. All primers were used at a concentration of 25µM except for CD44 primers, which were used at 50µM. This was determined by primer optimisation and primer efficiency results (data not shown). PCR cycling conditions were 50ºC for 2 min, 90ºC for 10 min, followed by 40 cycles of 90ºC for 15 seconds and 60ºC for 1 min. Controls included reaction mixture as described with the omission of cDNA template (No template control). This result showed no amplification of product or presence of primer dimers. Gene expression was normalised to the RpL19 internal control then expressed relative to calibrator gene expression using the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001). Calibrator sample was a 6h hCG stimulated whole ovary sample.

3.2.6 General methods for subcloning PCR product sequences

3.2.6.1 Polymerase Chain Reaction (PCR)

For reverse transcription polymerase chain reaction (RT-PCR), the following method was followed. 5µL of sample cDNA was added at 100ng together with 5µL of 10x PCR buffer, 2µL dNTPs (10mM), 1µL

each of forward and reverse primer at 50μM, 0.2μL GoTaq polymerase (Promega Corporation, Annadale, NSW, Australia) and enough water to make a total reaction volume of 50μL. PCR was completed on a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems, Mulgrave, Vic, Australia); 94°C for 2 min, cycling for 30 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 5 min, a final extension of 72°C for 5 min step to cease reaction.

3.2.6.2 Gel extraction of PCR product purification

Following conventional PCR of genes of interest, all PCR products were cloned to confirm gene sequence. Conventional PCR products were purified by gel extraction using the Qiagen MinElute Gel Extraction Kit (Qiagen Australia, Doncaster, Victoria, Australia). Briefly, the PCR product band was excised from the agarose gel with a clean, sharp scalpel. The gel slice was weighed and 3x the amount of QG buffer was added to a tube which was then incubated at 50°C for ten min and vortexed frequently, until gel was dissolved. One gel volume of isopropanol was added and the tube was inverted. To a collection tube, 800μL of sample was added, centrifuged for 1 min at 15,000g, the flow through discarded and the process repeated until the entire sample had been processed. QG

Gene	Forward Primer	Reverse Primer	Amplicon size (bp)	Accession number
Tiam1	TTCACTCCATCCTGGTTCTGTCTAC	CCAGTTGATGTGTCTTGCAAATG	111	NM_009384.2
Rac1	CATCAGTTACACGACCAATGCA	AACATTGGCGGAATAGTTGTCA	72	NM_009007.2
RhoA	AGCACACGAGACGGG	TCTAAACTATCAGGG	96	NM_016802.3
CD44s (exon 4 – exon 5)	CAGTCACAGACCTACCCAATTCC	GTGTGTTCTATACTCGCCCTTCTTG	100	NM_009851.2
CD44s (exon 16 – exon 17)	ACTGTGACTCATGGATCCGAATTAG	TCTGGAATCTGAGGTCTCCTCATAG	100	NM_009851.2
CD44v3 (exon 4 – exon 7)	TGTACATCAGTCACAGTCACAGACCTACCC	GACTCTGATGGTTGAAATAACCATGAAAACC	482	NM_009851.2
CD44v10/CD44E (exon 8 – exon 16)	TGGGAGCCAAATGAGGAAAATGAAGATG	GATCCATGAGTCACAGTGCGG	1030	NM_009851.2
CD44v3 (exon 5 – exon 7)	CTATTGTCAACCGTGATGGTACTCGC	GACTCTGATGGTTGAAATAACCATGAAAACC	430	NM_009851.2
CD44 * (exon 5 – exon 16)	CTATTGTCAACCGTGATGGTACTCGC	GATCCATGAGTCACAGTGCGG	1524 or 279	NM_009851.2
RpL19	TTCCCAGTACAGCACCTTTGAC	CACGGCTTTGGCTTCATTTTAAC	99	NM_026490.2
Has2	CAGACCTTCTCACATGCACAATGAG	TCAGTTACAGTTCGCCCATGTAAAC	80	NM_008216.2
Ptgs2	CCCTTCCTCCCGTAGCAGAT	TGAACTCTCTCCGTAGAAGAACCTTT	110	NM_011198.3
Tnfaip6	ATACAAGCTCACCTACGCCGAA	ATCCATCCAGCAGCACAGACAT	122	NM_009398.2

Table 3.1 Murine PCR primer sequences (*primer will detect CD44s and any alternate isoforms of CD44 present in cumulus oocyte complexes

(500µL) buffer was added to the spin column, centrifuged for 1 min and the flow through discarded, followed by an additional 1 min spin at 15,000 rcf. PE (750µL) buffer in ethanol was used to wash the spin column with a 1 min centrifuge at 15,000 rcf, after which the flow through was discarded and the spin column was centrifuged for an additional min. Subsequently, the sample was eluted by the addition of 10µL of EB (elution buffer) to the centre of the spin column, which was allowed to stand for 1 min and then centrifuged at 15,000 rcf for 1 min.

3.2.6.3 Subcloning of PCR product sequences

Gel purified PCR products were subcloned utilising the TOPO TA cloning kit (Invitrogen Australia Pty. Ltd, Mulgrave, Victoria, Australia). Briefly, 4µL of purified PCR product, 1µL of salt solution and 1µL of pCR4-TOPO vector were mixed gently and incubated for five min at room temperature. The reaction was then placed on ice in preparation for transformation into competent TOP10 cells (Invitrogen Australia Pty. Ltd, Mulgrave, Victoria, Australia).

3.2.6.4 Transformation of E. Coli

Chemically competent TOP10 cells (Invitrogen Australia Pty. Ltd, Mulgrave, Victoria, Australia) were thawed on ice. When cells were thawed, 2µL of TOPO cloning reaction was placed in one vial of TOP10 cells and incubated on ice for 30 min. Afterwards, cells were heat-shocked in a 42°C water bath for 30 seconds. Then 250µL of room temperature SOC media (2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) (Invitrogen Australia Pty. Ltd, Mulgrave, Victoria, Australia) was added to the transformed cells, which were shaken horizontally for 1 h at 37°C at 220 rcf. Following this, 150µL of the transformed cells were spread on pre-warmed selective LB-agar plates (10g/L tryptone, 5g/L yeast extract, 5g/L NaCl, 15g/L agar) containing ampicillin (100µg/mL, Sigma Aldrich Pty. Ltd., Castle Hill, NSW, Australia) and incubated at 37°C overnight.

Following overnight incubation positive colonies were identified by performing gene specific conventional PCR on single colonies.

3.2.6.5 Plasmid midi-preparations

Positive colonies were screened by preparing small scale mini plasmid preparations using the Wizard® Plus Minipreps DNA Purification System (Promega Corporation, Annandale, NSW, Australia). Firstly, overnight cultures were prepared by the addition of single colonies to 2mL LB (10g/L tryptone, 5g/L yeast extract, 5g/L NaCl) containing ampicillin (100µg/mL, Sigma Aldrich Pty. Ltd., Castle Hill, NSW, Australia) and overnight horizontal incubation on a shaking incubator at 37°C. Subsequently, 1mL of each overnight culture was centrifuged at 15,000 rcf for 5 min to achieve a pellet. Each pellet was resuspended with 250µL of cell suspension solution, and then 250µL of cell lysis solution was added to each sample and inverted. Alkaline protease solution (10µL) was added, samples inverted and then incubated for 5 min at room temperature. Next, neutralisation solution (350µL) was added and mixed by inversion and samples were centrifuged at 15,000 rcf for 10 min. A spin column was inserted in the collection tube and the cleared lysate was decanted into the spin column and centrifuged at 15,000 rcf for 1 min, after which the flow through was discarded. Wash solution (750µL) containing ethanol was added to the spin column and centrifuged at 15,000 rcf for 1 min and the flow through discarded. This process was repeated with 250µL wash solution and centrifuged for 2 min. The spin column was transferred to a sterile 1.5mL tube, 100µL sterile water was added and the tube centrifuged at 15,000 rcf for 1 min. Plasmid DNA was stored at -20°C.

3.2.6.6 Restriction Digest

To ensure the gene insert had been successfully transformed, restriction digest of the plasmid DNA was performed. DNA (5µL) was added to a tube containing 1µL 10x buffer, 1µL EcoR1 enzyme and 3µL

dH_2O. The reaction was incubated for 1 h at 37°C. The digest was run on a 1% (w/v) agarose gel, and compared to the conventional PCR product to confirm a size shift.

3.2.6.7 Sequencing

Sequencing of plasmids was performed using Big Dye Terminator 3.1 kit (Applied Biosystems, Mulgrave, Victoria, Australia). The sequencing reactions contained 190ng/µL of plasmid DNA, 0.4µL Big Dye, 2µL buffer and 2.4pmol of designated primer. Conventional PCR was performed with the following cycling conditions: 96°C 1 min (1 cycle), 96°C 10 seconds, 50°C 5 seconds, 60°C 4 min (25 cycles). Sequencing products were precipitated by the addition of 80µL of 75% isopropanol, vortexing and precipitation for 15 min at room temperature. Reactions were then spun in a microcentrifuge for 20 min at 15,000 rcf and supernatants were aspirated with a pipette and discarded. The pellets were washed with an additional 250µL of 75% isopropanol, vortexed briefly and re-centrifuged at 15,000 rcf for 5 min. Supernatants were aspirated and residual isopropanol was removed by evaporation by placing tubes, caps open, on a 90°C heat block for 1 min. Samples were analysed at the Molecular Pathology Sequencing Facility (Institute of Medical and Veterinary Science (IMVS), Adelaide, Australia) by an ABI 3730 Capillary sequencer (Applied Biosystems, Mulgrave, Victoria, Australia). Chromatograms were aligned and compared with published sequences for gene sequence homology.

3.2.7 Statistical Analysis

Real-time PCR expression data was normalised to *Rpl19*, expressed relative to the calibrator sample (whole ovary following treatment with eCG + 6 h hCG). Data are presented as fold change from the calibrator which was set as 1. Data is presented as mean ± SEM (n≥3 independent experiments) and analysed by one way-ANOVA or two way-ANOVA with post hoc test as described in figure legend. Statistical significant difference was accepted at $p<0.05$. Statistics were performed using Graph Pad Prism Software (GraphPad Software Inc, La Jolla, CA, USA).

3.3 RESULTS

3.3.1 Identification of CD44 isoforms in the hormonally stimulated mouse ovary

To determine whether CD44 and its variant isoforms were expressed in cumulus oocyte complexes, mice were stimulated with eCG + 6 h hCG after which ovaries were removed and expanded COCs analysed for their expression of CD44 isoforms by RT-PCR. Primer pairs were designed to cross exon boundaries to recognise specific variant isoforms. Location of primer pairs in relation to exon boundaries, the CD44 isoforms they detect and size of product they amplify are summarised in Table 3.1. Figure 3.4 is a gel electrophoresis showing the results of this experimental approach. Primers for *RpL19* amplified a positive control band of the expected length (lane 1). Primers for CD44ex5 amplified a band of expected length (lane 2). Two sets of primers designed to amplify the CD44v3 isoform (lanes 3 and 4) failed to amplify products of the predicted size (Table 3.1, Figure 3.4), suggesting that these were non-specific products. Additionally, a primer pair located within exon 5 and exon 16, were employed to detect all CD44 spliced variants. PCR products generated using these primers would differ in size depending on which exons were present and thus identify the number of CD44 isoforms present in COCs.) These primers amplified a distinct band of approximately 300bp in size (lane 5) corresponding to the expected size for CD44s. This product was sequenced and verified as being CD44 (data not shown). No product was detected with the use of primers for CD44v10 (lane 6). Using the strategies employed here, only the standard isoform of CD44 (CD44s) was detected in expanded COCs.

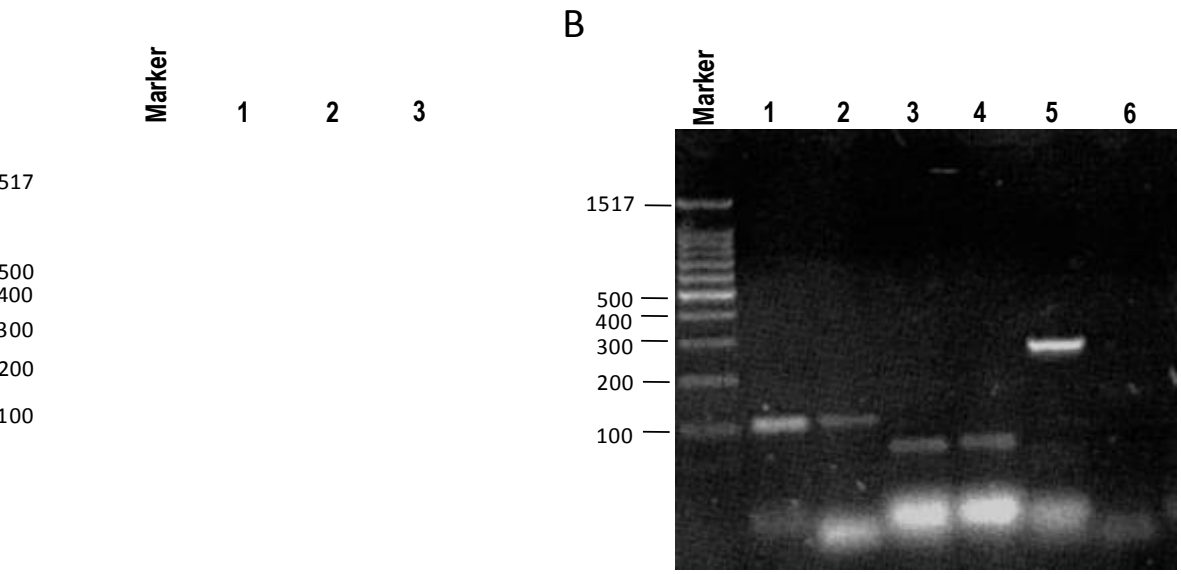

Figure 3.3 Detection of CD44 isoforms

Gel electrophoresis of PCR products identifying isoforms of CD44 in expanded COCs (eCG + hCG 6 h). Primer pairs were designed to identify alternate isoforms of CD44 (*methods section*). *RpL19* (lane 1; control primer pair), *CD44ex5* (lane 2), *CD44v3* (lane 3), CD44v3 (lane 4), all CD44 isoforms (lane 5; primer pair that span exon 5 - 16) and *CD44v10* (lane 6).

3.3.2 Temporal induction of *CD44s* in the cumulus oocyte complex during the periovulatory period

Following the identification of the standard form of *CD44* I investigated whether this gene was hormonally regulated during the ovulatory period by performing quantitative real time RT-PCR on COCs at four time-points during expansion and ovulation.Primer pairs spanning exons 4-5 and exons 16-17 were used to allow robust quantitative measurement of *CD44s* in COCs. There was no significant difference in the level of *CD44s* expression between COCs isolated from untreated versus eCG treated mice using either primer pair (Figure 3.5 A and B). Induction of *CD44s* was observed in COCs at 6 h post hCG (Figure 3.5 A), when COC expansion is occurring, however this was not significant when using primers against exons 16 – 17 (Figure 3.5 B). Levels of *CD44s* expression at 12 h post hCG were significantly higher compared to unexpanded eCG treated COCs but not significantly different to 6 h post hCG, using either primer pair (Figure 3.5). Thus, *CD44* mRNA is upregulated 6h post hCG administration, and this level of expression is maintained at the time of ovulation, 12h post hCG administration.

3.3.3 Spatial and temporal expression of genes involved in CD44 mediated migratory complex

Following the identification of *CD44s* I next sought to analyse downstream interacting molecules in the CD44 migratory pathway (depicted schematically in Figure 3.1) and whether these are hormonally regulated in ovarian cells during the periovulatory period. Thus, quantitative real time RT-PCR was used to determine the spatial and temporal regulation of *Rac1*, *Tiam1* and *RhoA* in mouse COCs and granulosa cells (GC) at four time-points across a time-course of ovulation. *Rac1* was constitutively expressed in both COCs and GCs (Figure 3.6A). *Tiam1* was also constitutively expressed in both COCs

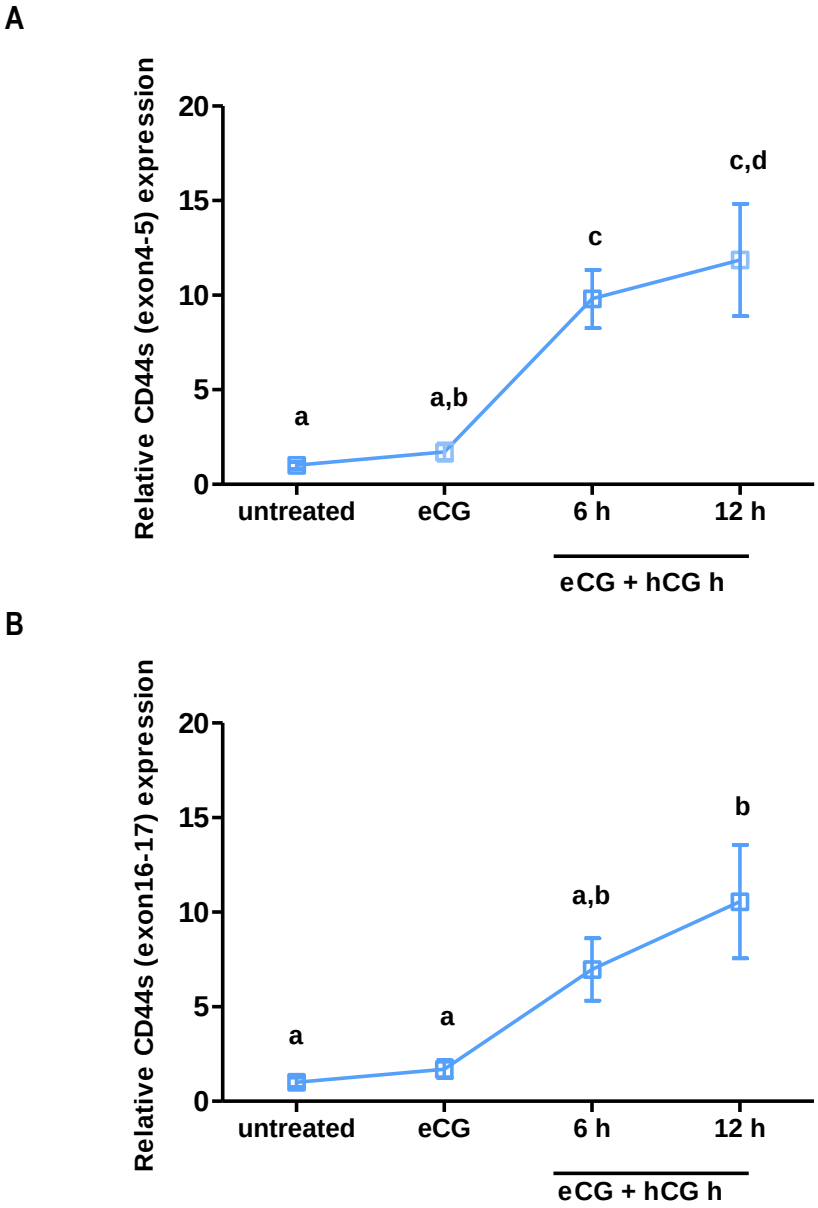

Figure 3.4 CD44 is significantly induced in cumulus oocyte complexes in response to ovulatory hormones.

CD44 expression was analysed in cumulus oocyte complexes (COC) isolated from mice that were either untreated, following 44 h equine chorionic gonadotropin (eCG) stimulation or following subsequent human chorionic gonadotropin (hCG) administration at 6 (pre-ovulatory expanded) or 12 h (post-ovulatory). Analysis of mRNA expression of the standard isoform of *CD44* (*CD44*) was performed utilising two primer pairs designed to cross intron-exon boundaries at exons 4-5 (A) and exons 16-17 (B). Data is presented as mean±SEM (n≥3 independent experiments) and analysed by one way-ANOVA with Tukey post hoc test with different superscripts signifying statistical difference ($p<0.05$).

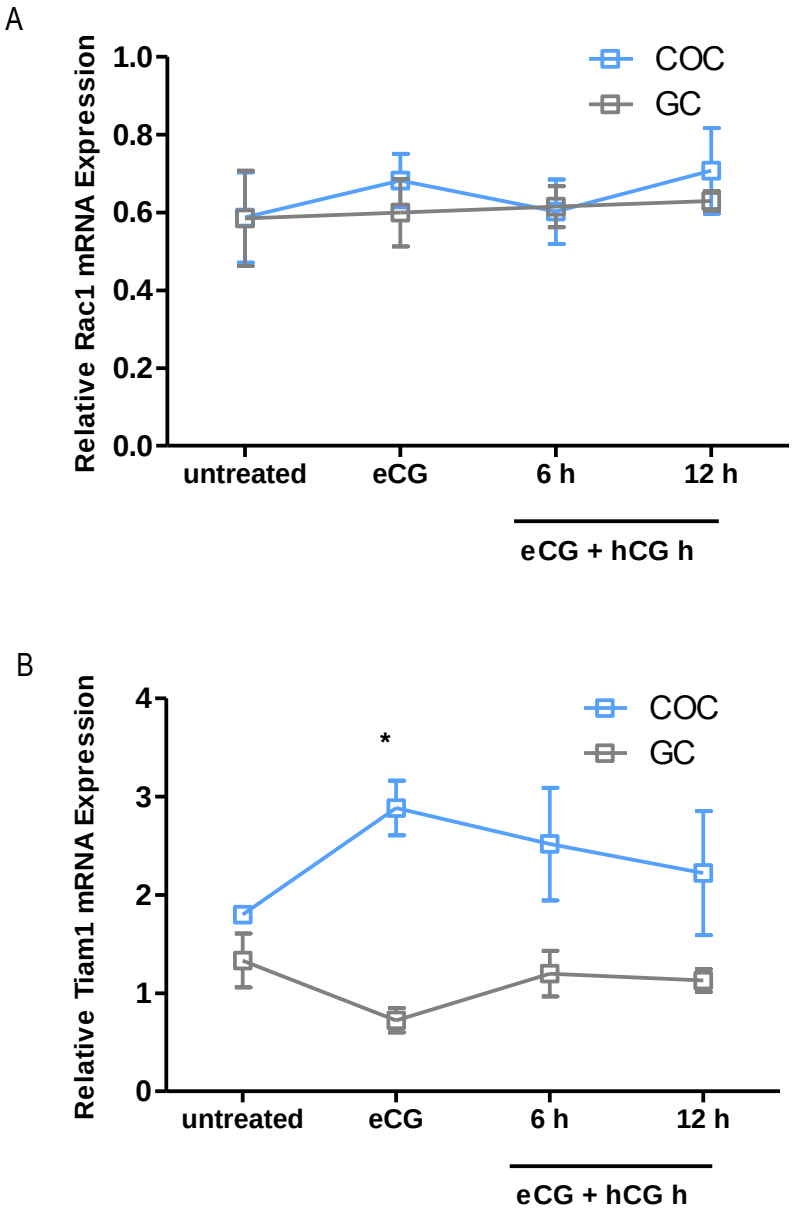

Figure 3.5 Rac1 and Tiam1, essential components of the CD44 mediated migratory complex, are constitutively expressed in both the COC and granulosa cells during the periovulatory period.

Analysis of *Rac1* (A) and *Tiam1* (B) expression in COCs (blue) and granulosa cells (grey) following no treatment or 44 h post administration of equine chorionic gonadotropin (eCG) or following subsequent administration of human chorionic gonadotropin at 6h (pre-ovulatory expanded) or 12 h (post-ovulatory). Expression data was normalised to *Rpl19* internal control and presented as mean±SEM (n≥3 independent experiments) Data is expressed relative to calibrator (whole ovary following treatment with eCG + 6h hCG) and presented as fold change from the calibrator which was set as 1. Data was analysed by two way-ANOVA with Bonferroni post hoc test (asterisk indicates statistical difference between cell types, $p<0.05$).

and GCs following hormonal stimulation (Figure 3.6B). At 44 h post eCG stimulation, there was significantly higher expression of *Tiam1* in COCs compared to GCs ($p<0.05$) but the cell types did not maintain significant differences in expression levels following hCG administration.

RhoA expression was also detected in both COCs and GCs (Figure 3.7). In COCs, *RhoA* expression was significantly induced 4 h post hCG compared to eCG, and this level of expression was maintained at 10 h and 14 h post hCG (Figure 3.7A). In GCs there was a similar pattern of expression (Figure 3.7B), where *RhoA* expression increased following hCG administration at 4 h, with a significant induction observed at 10 h post hCG compared to eCG treated COCs. However at 14 h post hCG, after ovulation, *RhoA* expression was down-regulated to levels seen in unexpanded eCG treated COCs (Figure 3.7B).

Thus, I have shown that the downstream mediator of CD44, Rac1 and Tiam1, are present and constitutively expressed across the ovulatory time-course, in both COCs and GCs. RhoA was found to be change across the cycle and was significantly induced following an ovulatory dose of hCG in both COCs and GCs. In COCs this increased level of *RhoA* expression was maintained throughout COC expansion and ovulation.

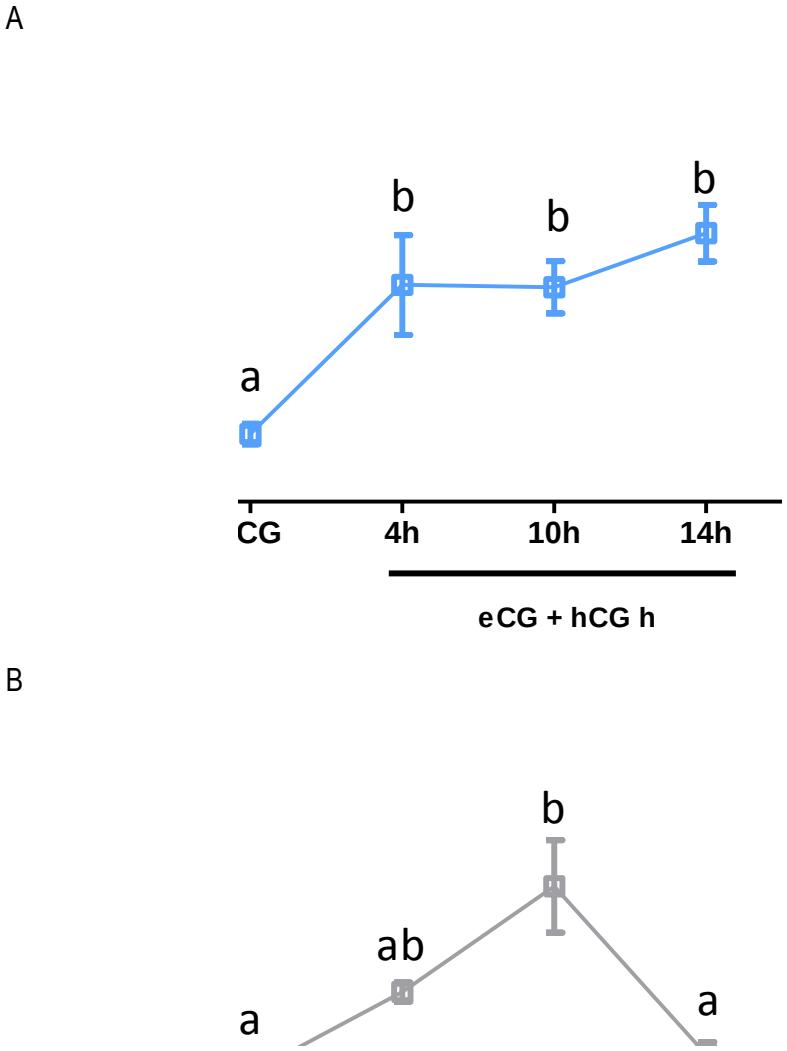

Figure 3.6 Expression of RhoA, a downstream target of CD44, is hormonally regulated in the cumulus oocyte complex and mural granulosa cells.

Analysis of *RhoA* mRNA expression in A) COCs (blue) and B) granulosa cells (grey) following 44 h post administration of equine chorionic gonadotropin (eCG) or following subsequent administration of human chorionic gonadotropin (hCG) at 4 h (pre-ovulatory), 10 h (pre-ovulatory) or 14 h (post-ovulatory). Expression data was normalised to Rpl19 internal control and presented as mean±SEM (n=3 independent experiments) Data is expressed relative to calibrator (whole ovary following treatment with eCG + 6h hCG) and presented as fold change from the calibrator which was set as 1. Data was analysed by One way-ANOVA with Tukey post hoc test (different letters indicates significant difference $p<0.05$).

3.4 DISCUSSION

CD44 mediated cell migration has been investigated in a number of models and is thought to play a role in cancer cell metastasis. For this reason, and because the CD44 ligand HA, is the major component of the ovulating COC matrix, I investigated the expression and hormonal regulation of the CD44 migratory machinery in the COC during the ovulatory period. I investigated the expression *CD44* and its downstream targets in COCs and granulosa cells, and found that the standard isoform of CD44 (*CD44s*) was significantly upregulated in COCs following stimulation with hCG. The downstream targets of CD44, *Tiam1* and *Rac1* were found to be constitutively expressed in both COCs and granulosa cells. In contrast, *Rho*A was hormonally regulated and significantly induced following ovulatory hCG treatment.

The expression of *CD44* standard isoform was upregulated within 6 h of hCG stimulation in COCs, peaking at 12 h post hCG administration. This is the first report of hormonal regulation of *CD44* in the mouse COC and supports previous studies which observed greater expression of *CD44* in mature compared to immature COCs, in pig (Yokoo, et al., 2002a, Yokoo, et al., 2007, Yokoo, et al., 2002b) and human (Ohta, et al., 2001, Ohta, et al., 1999). The different isoforms of CD44 can have important differences in cell function so it was important to determine which were present in the COC. In this study I detected the standard form of CD44 only and found no evidence of alternatively spliced isoforms. This was investigated via a conventional PCR approach using primers against different exons unique to each isoform. The standard isoform of CD44 has also been identified in the human ovary (Ohta, et al., 1999). Interestingly in the same study, the detection of the isoforms CD44v9 and CD44v3-10 were also examined however, there was no evidence that human cumulus or granulosa cells expressed these variant isoforms (Ohta, et al., 1999). I conclude that the standard isoform of CD44, CD44s, is the only isoform present in mouse cumulus cells and that this gene is LH-responsive.

It has been suggested that CD44 plays a role in COC expansion and/or oocyte maturation. A previous study sought to assess the role of CD44 in cumulus expansion in pig COCs, by adding a neutralising antibody during COC in vitro maturation; however, no differences in cumulus expansion were observed (Yokoo, et al., 2007). In vitro matured COCs show poor cumulus expansion and poor developmental competence (Dunning, et al., 2007, Eppig, et al., 2009, McKenzie, et al., 2004), however they express similar levels of *CD44* as in vivo matured COC, but the translated protein from these two conditions are of different molecular weight (Yokoo, et al., 2007). This is due to an increase in sialic acids on the terminal end of CD44 of in vitro matured COCs. Sialic acids are known the interfere with the binding of hyaluronan to CD44 (Katoh, et al., 1999, Katoh, et al., 1995), which could offer an explanation for the poor developmental competence observed following in vitro maturation of COCs (Yokoo, et al., 2007).

Stability of the COC matrix is essential for ovulation as mouse studies have revealed that null mutations in cumulus matrix genes such as *Ptx3* (Salustri, et al., 2004, Varani, et al., 2002), *Ptgs2* (Lim, et al., 1997), *Tnfaip6* (Fulop, et al., 2003) and *Adamts1* (Mittaz, et al., 2004) frequently leads to a failure in cumulus expansion and anovulation. Although ovarian function has not been specifically investigated in *CD44* null mice, they have not been reported to have an overt fertility defect (Schmits, et al., 1997). Thus unlike other cumulus and granulosa specific genes which when absent lead to poor matrix formation or complete lack of expansion, CD44 may not be acting as a matrix stabiliser. Most likely however there are other hyaluronan receptors present in the COC that could functionally compensate for CD44, for instance, lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) or hyaluronan mediated motility receptor (Hmmr, synonym; RHAMM). The role that CD44 plays during folliculogenesis and ovulation therefore, remains unknown.

Interestingly, soluble CD44 was found in the follicular fluid of patients undergoing infertility treatment, and higher CD44 levels were correlated with better embryo outcomes (Ohta, et al., 2001). Follicular fluid levels of CD44 have also been shown to correlate with IVF success (Ohta, et al., 2001) These studies suggest CD44 may play an important role in oocyte maturation and developmental competence. Additionally, CD44 may play an important role in the COC in promoting cell motility. The association between CD44 and hyaluronan initiates cell-cell and cell-matrix interactions but also promotes cancer cell metastasis (reviewed in (Heldin, et al., 2008)) and perhaps a similar process takes place within the expanded cumulus mass. The intracellular signalling mechanism activated by CD44 in the COC has not been studied.

Cell signalling targets of the CD44 mediated motility pathway were also investigated using real-time RT-PCR analysis. The results showed constitutive expression of the small GTPase *Rac1* across the ovulation period in COCs. The expression also did not differ between cell types, with both COCs and granulosa cells showing constitutive *Rac1* expression in response to an ovulatory stimulus. In contrast, *Tiam1* expression in COCs upon eCG administration was significantly different to the expression in granulosa cells at the same time-point however neither cell type showed significant induction in response to ovulatory hormone administration. *Tiam1* message may be increased in COCs over granolusa cells at this early time point to prepare cumulus cells to initiate activation of Rac1 rapidly after the LH surge. This result is the first known investigation of Tiam1 and Rac1 in mouse ovarian cells. In pig COCs Rac1 protein levels were reported to be stable prior to and during cumulus expansion, (Yi, et al., 2008). These results concur with our own study, which found constitutive expression of *Rac1* message prior to and during cumulus expansion, and also constitutive protein levels at 44 h post eCG treatment and at eCG + hCG 12 h treatment (Appendix 7.2). By assessing the level of GTP-bound Rac1 in COCs, I could determine whether this protein was active at the time of ovulation thus I also sought to

investigate the activation status of Rac1 in response to an ovulatory stimulus. When expanded COCs at 12 h post hCG were compared to eCG treated unexpanded COCs, Rac1 protein was detected in both COC groups (Appendix 7.2) and a very faint band, corresponding to active Rac1 was observed in expanded 12 h hCG COCs but not in unexpanded COCs. Thus while this assay was successful it was not reliably reproducible and was technically prohibitive due to the large amount of material required.

RhoA was found to be expressed in both COCs and granulosa cells and was significantly induced in both cells types following hCG. This mRNA expression correlates with reports of RhoA protein levels and activity in COCs at 14h post hCG in a previous study (Yodoi, et al., 2009). Recently *RhoA* mRNA has been detected in mouse ovaries and oocytes, and found to localise to the oocyte during spontaneous maturation (Elbaz, et al., 2010). Furthermore, when Rock, the downstream target of RhoA, was inhibited, extrusion of the first polar body was blocked (Elbaz, et al., 2010) pointing to the potential importance of this signalling cascade during oocyte nuclear maturation. It is likely that the regulation of RhoA shown in this study, by LH, is via an indirect mechanism, as there is no evidence in the literature of RhoA containing hormone response elements in its promoter region. Additionally, another study found that RhoA activity is regulated by prostaglandin signalling, which is an essential signalling cascade for COC expansion and ovulation in response to LH (Yodoi, et al., 2009). Overall, this evidence indicates a role for RhoA downstream of CD44 in the peri-ovulatory COC.

CD44 and RhoA have both been shown to have an association with oocyte developmental competence, but this pathway is also commonly involved in cell motility. Our findings show that mouse COCs express the machinery required for CD44 mediated cell migration and suggest that this pathway may be involved in the COC maturation process and during ovulation. The functional significance of this pathway in the

ovary during this time-period remains to be elucidated however and is the subject of further investigation.

Chapter 4

Migratory Capacity of the Cumulus Oocyte Complex during Ovulation

4.1 INTRODUCTION

Whether the cumulus cells are able to actively facilitate ovulation is the focus of this thesis, specifically whether the cumulus oocyte complex (COC) is able to transition to a motile cell phenotype following the ovulatory stimulus. I hypothesised that the HA receptor CD44 may contribute to this process, and in Chapter 3 I show that genes involved in the CD44 cell motility mechanism are present in the mouse ovary. However, it is unknown whether this pathway in cumulus cells indeed promotes a migratory phenotype.

Cell migration is a highly orchestrated process involving a series of events that can be the result of various regulated and overlapping pathways. Cell migration requires changes to cell shape, which is typically associated with actin cytoskeleton remodelling. The cytoskeleton of a cell is structurally linked to the extracellular surroundings, ie adjacent cells and matrix, and the migration of cells involves the detachment from rear adhesion sites and the formation of attachment sites at the leading edge of the cell (Ilina and Friedl, 2009, Vicente-Manzanares, et al., 2005). The leading edge of the cell initiates cell motility via cellular extensions known as lamellipodia or filopodia, protrusions that have integrin-containing contacts, which interact with extracellular matrix and form sites known as focal adhesion complexes (Ezratty, et al., 2005, Raftopoulou and Hall, 2004, Small, et al., 2002). Simultaneously, at the rear of the migrating cell, it is necessary for integrin dependent adhesion sites to be dispersed. This results in the reduction of focal contact size at the rear and the cell being propelled forward in the direction of migration. The process of cell migration and the changes observed occurs as the result of a cell responding to extracellular chemokine or growth factor signals and possibly hormonal regulation of pathways responsible for cell migration, adhesion and invasion (Ferretti, et al., 2007, Knofler, 2010).

4.1.1 CD44 and cell migration

CD44 mediated cell migration pathway primarily involves the small GTPases Rac1 and RhoA, which are members of the Rho family, and the guanine exchange factor Tiam1 which is responsible for the activation of Rac1 through exchange of GDP for GTP. To determine their relative contributions to cell migration, a number of studies have utilised inhibitors of particular components of the pathway.

CD44 neutralising studies have been completed in tumour cell models and found cell migration was reduced when cells were pre-treated with a neutralising antibody against CD44 (Bourguignon, et al., 2006, Bourguignon, et al., 2007)(Figure 4.1). The Rac1 inhibitor NSC23766 has been used to investigate the role of this small GTPase during cell migration (Figure 4.1). Extravillous trophoblasts, a highly motile and invasive cell type, pretreated with NSC23766, showed reduced migration in transwell migration assays (Nicola, et al., 2008). A number of studies have reported decreases in cell migration and invasion upon treatment with NSC23766 (Desai, et al., 2008, Jaffe and Schwartz, 2008, Ramesh, et al., 2007, Wang, et al., 2009) as well as changes in motile cell characteristics including loss of stress fibres (Ramesh, et al., 2007), loss of lamellipodial extensions (Jaffe and Schwartz, 2008), decreased active Rac1 (Desai, et al., 2008), and a decrease in adhesion molecule expression (Ramesh, et al., 2007). These cumulative observations demonstrate Rac1 with its associated activator Tiam1 are important components in cell motility.

RhoA activation of cell motility can be inhibited using the agent Y-27632, which inhibits RhoA activity, preventing phosphorylation and activation of its downstream target, Rho-associated coiled-coil containing protein kinase (Rock) (Ishizaki, et al., 2000)(Figure 4.1). The inhibition of Rock activation by Y-27632 blocks cytoskeletal remodelling and has been reported to decrease cancer cell (Chang, et al., 2010, Imamura, et al., 2000) and trophoblast cell migration (Nicola, et al., 2008).

The use of this inhibitor also leads to changes in known characteristics of cell morphology associated with cell adhesion and migration (Imamura, et al., 2000, Ishizaki, et al., 2000, Uehata, et al., 1997) and decreased levels of specific matrix metalloproteases (MMPs), including MMP9 and MMP2 (Chang, et al., 2010). These reports demonstrate an involvement of RhoA, and its associated cell-signalling pathway in cell migration and invasion, which requires proteolytic activity. However, other experiments using RhoA inhibition in various cell migration models have demonstrated the complexity of this pathway. Some reports show Y27632 promotes cell migration (Chang, et al., 2010, Darenfed, et al., 2007, Imamura, et al., 2000, Ishizaki, et al., 2000) and invasion while other studies report that migration is reduced (Fafet, et al., 2008, Lee and Kay, 2006, Salhia, et al., 2005, Tabu, et al., 2007). Cumulatively the studies demonstrate important effects of cell context whereby RhoA can mediate either actin remodelling and therefore promote motility or in other cases restrain the rate of motility by increased adhesion at the rear of the cell.

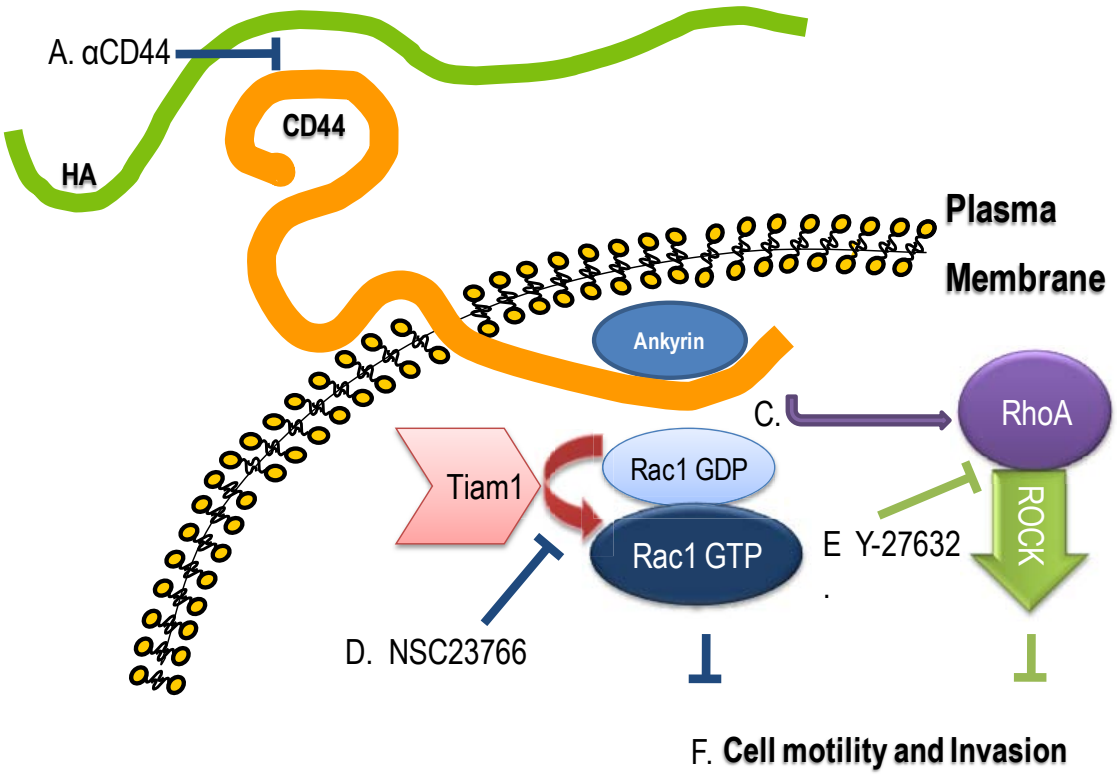

Figure 4.1 Schematic representation of sites of action of characterised inhibitors of CD44-mediated motility in mammalian cells.

CD44 is a known hyaluronan (HA) receptor which upon binding leads to activation of its downstream targets. In this chapter, a neutralising antibody against CD44 (A) was used to inhibit CD44 and its downstream effects. Downstream targets include the guanine-exchange factor (GEF) Tiam1 that leads to the activation of the small GTPase, Rac1, by the exchange of GDP for GTP. NSC23766 is a small molecule Rac1 inhibitor used to block activation of Rac1 by Tiam1 (D). An alternate pathway is the activation of another small GTPase, RhoA, which acts via its kinase ROCK. Y-27632 is an inhibitor against ROCK, the kinase downstream of RhoA (E). Each of these three inhibitors was hypothesised to decrease cell migration.

I have used the selective antagonists within this chapter to investigate the involvement of CD44 and its associated pathway components during cell migration in our model. Namely CD44, Rac1 and RhoA actions were neutralised in mouse COCs in an attempt to determine whether they are essential for a cumulus cell migratory phenotype.

Using migration assays, similar to those utilised in cancer cell migration studies, and a hormonally stimulated mouse model, I have investigated cumulus cell motility prior to and following COC expansion. I have investigated whether this pathway is cumulus cell specific. I have also explored the function of the CD44 pathway specifically via inhibiting components of this pathway.

I hypothesise that following ovulatory LH, COC expansion will lead to activation of cell migration, specifically in cumulus cells.

4.2 MATERIALS AND METHODS

4.2.1 Animals, hormonal stimulation protocol and tissue collection

All animals were treated in accordance with the NHMRC Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NHMRC, 2004). Ethics approval was obtained from The University of Adelaide Animal Ethics Committee. The F1 C57Bl6 X CBA mouse line were housed at the University of Adelaide Animal Facility and maintained on a 12h/12h day/night cycle with rodent chow and water provided *ad libitum.* Mice were superovulated by i.p injection with 5 IU Equine chorionic gonadotropin (eCG) (Professional Compounding Centre of Australia, Sydney, NSW, Australia) at 1400 h on day 21-23 of age to stimulate follicle growth. This was followed 44 – 48 h later with an injection of 5 IU Human Chorionic Gonadotropin (hCG) (Organon Australia, Sydney, NSW, Australia) to initiate ovulation. Cumulus oocyte complexes were punctured from follicles of ovaries in hepes buffered α-MEM

with 5% FCS using 30 gauge needles (BD Biosciences). Granulosa cells were also collected from follicles at the time of COC collection (Figure 1.1). Granulosa cells were spun at 1500 g and a single cell suspension was prepared in DMEM. Cell counts of viable cells were performed using the trypan blue exclusion assay and a haemocytometer (as previously described in Section 2.2.2). Granulosa cell numbers were matched to cumulus cell numbers assuming that each COC contains approximately 2500 cells.

4.2.2 Transwell migration assays

Migration assays were performed utilising 12µm polycarbonate transwell inserts (Millipore, North Ryde, NSW, Australia). Inserts were placed in 24-well Costar® cell culture plates (Corning Life Sciences, Mount Martha, Victoria, Australia). The bottom well contained αMEM supplemented with 5% Fetal calf serum (FCS) and 3ng/mL Epidermal Growth Factor (Egf; Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia) to serve as chemoattractant. Twenty intact COCs were placed in the top well in 100µL αMEM supplemented with 5ng/mL Follicle Stimulating Hormone (FSH; Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia), with or without appropriate inhibitors (Section 4.2.4). Migration assays are depicted schematically in Figure 4.2. Transwell migration assays were incubated at 37°C/5% CO_2 in air for 4 h after which time polycarbonate filters were removed and non-motile cells were scraped from the upperside of the filter using a cotton swab. Filters were fixed in absolute methanol for 2 min, followed by the DiffQuik (Fronine Laboratory Supplies, Lomb Scientific, SA, Australia) differential staining protocol. Briefly, solution A (eosin) was added for 30 seconds followed by solution B (methylene blue) for 30 seconds, filters were washed in H_2O. Filters were placed on microscope slides and processed using a NanoZoomer Digital Pathology technology (Hamamatsu Photonics K.K., Japan). Images of the entire filter were captured at 5x magnification and phase analysis was performed using AnalySIS LS Professional Software (Olympus Australia Pty. Ltd., Mt Waverly, Victoria, Australia) (Section 4.2.3; Figure 4.3).

4.2.3 Phase analysis quantification of migration and invasion assays

Using AnalySIS LS Professional Software (Olympus Australia Pty. Ltd., Mt Waverly, VIC, Australia) phase analysis was performed. Photomicrographs were taken at 5x magnification using the Nanozoomer NDP software (Hamamatsu Photonics, K.K., Japan Hamamatsu, Japan) and the entire filter area was captured (Figure 4.3A). Following this, photos were subjected to phase analysis whereby pixels were selected based on a pre-determined colour threshold. The program was able to determine a total filter area and a total area of purple pixels, thus determining a percentage of cell migration (Figure 4.3B-C). These results are expressed as a cell migration index. Data was normalised to a baseline group (eCG + hCG) as indicated.

4.2.4 Inhibitors of migration

Inhibitors included Rac1 inhibitor NSC23766 (Tocris Bioscience, Abacus ALS, East Brisbane, QLD, Australia), used at 5μM, 10μM and 25μM, CD44 neutralising antibody (Merck Pty. Ltd., Victoria, Australia), used at 5μg/mL and 10μg/mL and Rock inhibitor Y-27632 (Merck Pty. Ltd., Victoria, Australia), used at 10μM, 25μM and 50μM. NSC23766 and Y-27632 were both dissolved in sterile water. These were all added at the required concentration at the start of the migration assay in a final volume of 100μL of αMEM containing 20 intact COCs, prior to appropriate assay.

4.2.5 Quantitative real-time RT-PCR (qRT-PCR)

As outlined in Section 3.2.5. Primer pairs given in Table 3.1.

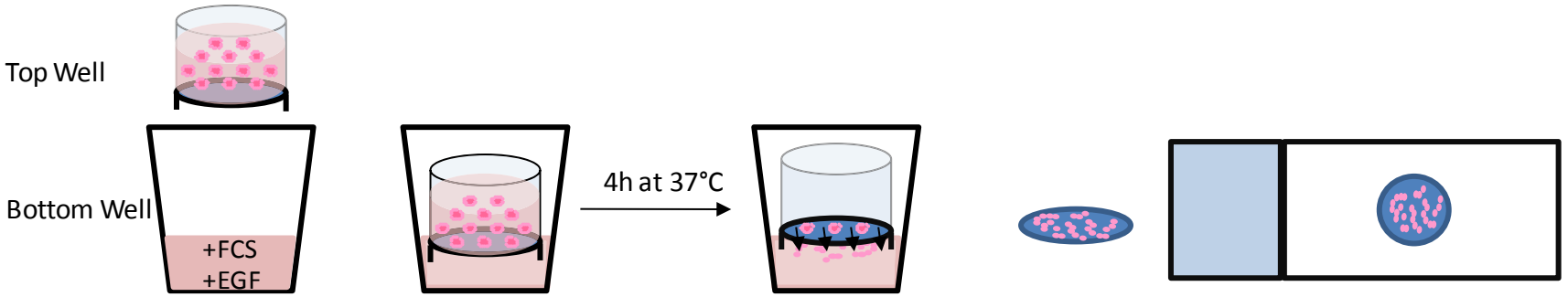

Figure 4.2 Cumulus cell migration/invasion assay protocol.

Cumulus cell migration assays were performed utilising commercially available membrane filters. The experimental protocol consisted of a top well containing media plus intact COCs, with or without inhibitors. The wells were placed in a 24 well plate containing media plus chemoattractant. Assay was incubated for 4 h at 37°C. Each treatment was repeated in duplicate. For invasion assays filters were coated with matrigel, and compared to a non-coated control.

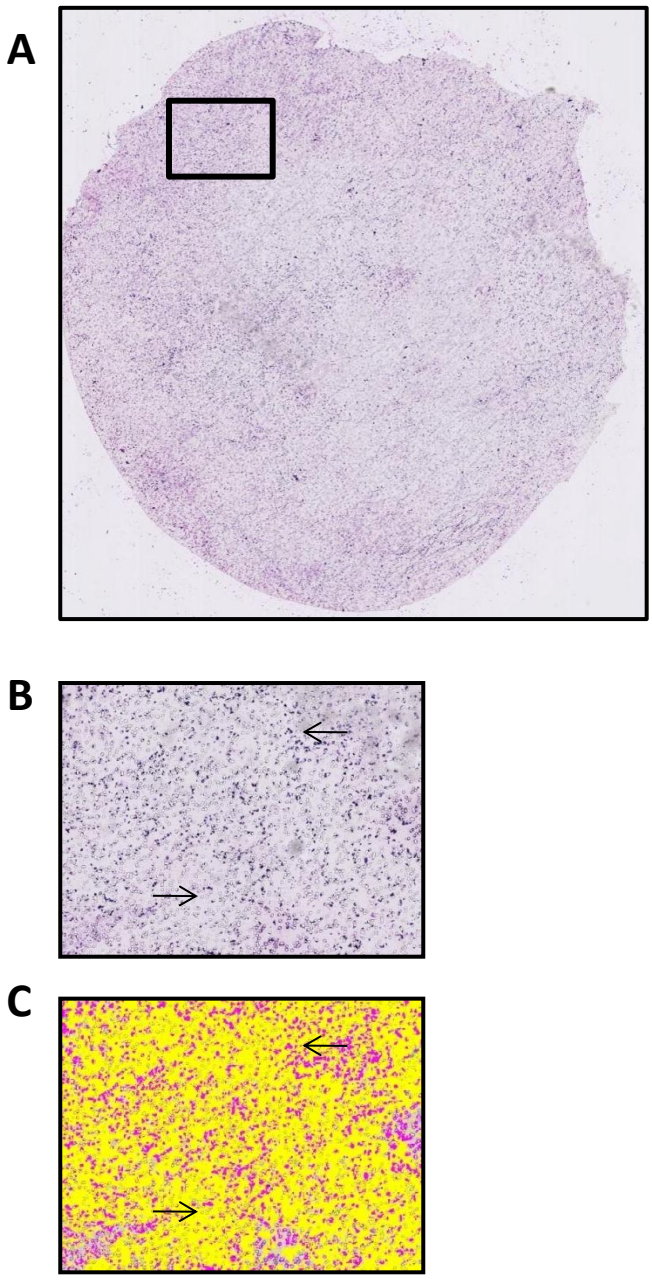

Figure 4.3 Method of phase analysis to determine percentage of migratory/invasive cells.

Phase Analysis was performed using AnalySIS LS Professional Software (Olympus Australia, Pty. Ltd., Mt Waverly, VIC, Australia).Images were captured across the entire filter area (A). Figure B depicts a typical image of that captured in A (rectangle), arrows point to migratory cells (B). Figure C depicts phase analysis image with arrows pointing to migratory cells labelled purple (C).

4.2.6 Statistics

Data was analysed by unpaired t-test and significance was accepted at $p<0.05$. One Way ANOVAs were performed. Significance was accepted at $p<0.05$. Statistics were performed using Graph Pad Prism software (GraphPad Software Inc, La Jolla, CA, USA).

4.1 RESULTS

4.1.1 Cumulus specific gene expression in cumulus cells following an extended culture period.

Cell migration and invasion assays typically use dissociated cells in culture. Initially it was necessary to determine whether dissociated cumulus cells could be used in the migration assays or whether intact COCs were required to maintain cumulus cell phenotypes. Thus, preliminary experiments were performed to investigate the effect of in vitro culture on cumulus cell gene expression to determine whether dissociated cumulus maintain cumulus cell characteristics for the extended period which would be required for the migration assays. Figure 4.4 describes the role of oocyte secreted factors (OSFs) in regulating the expression of cumulus specific genes. I investigated three well-characterised cumulus specific genes associated with the matrix of an expanded COC, *Has2*, *Ptgs2* and *Tnfaip6*, which are all known to be regulated by OSFs (Eppig, 1980, Gilchrist, et al., 2008, Joyce, et al., 2001, Salustri, et al., 1992, Varani, et al., 2002). The experimental protocol utilised is presented in Figure 4.4B. Intact COCs and dissociated cumulus cells underwent a 4 h culture period, followed by quantitative RT-PCR to

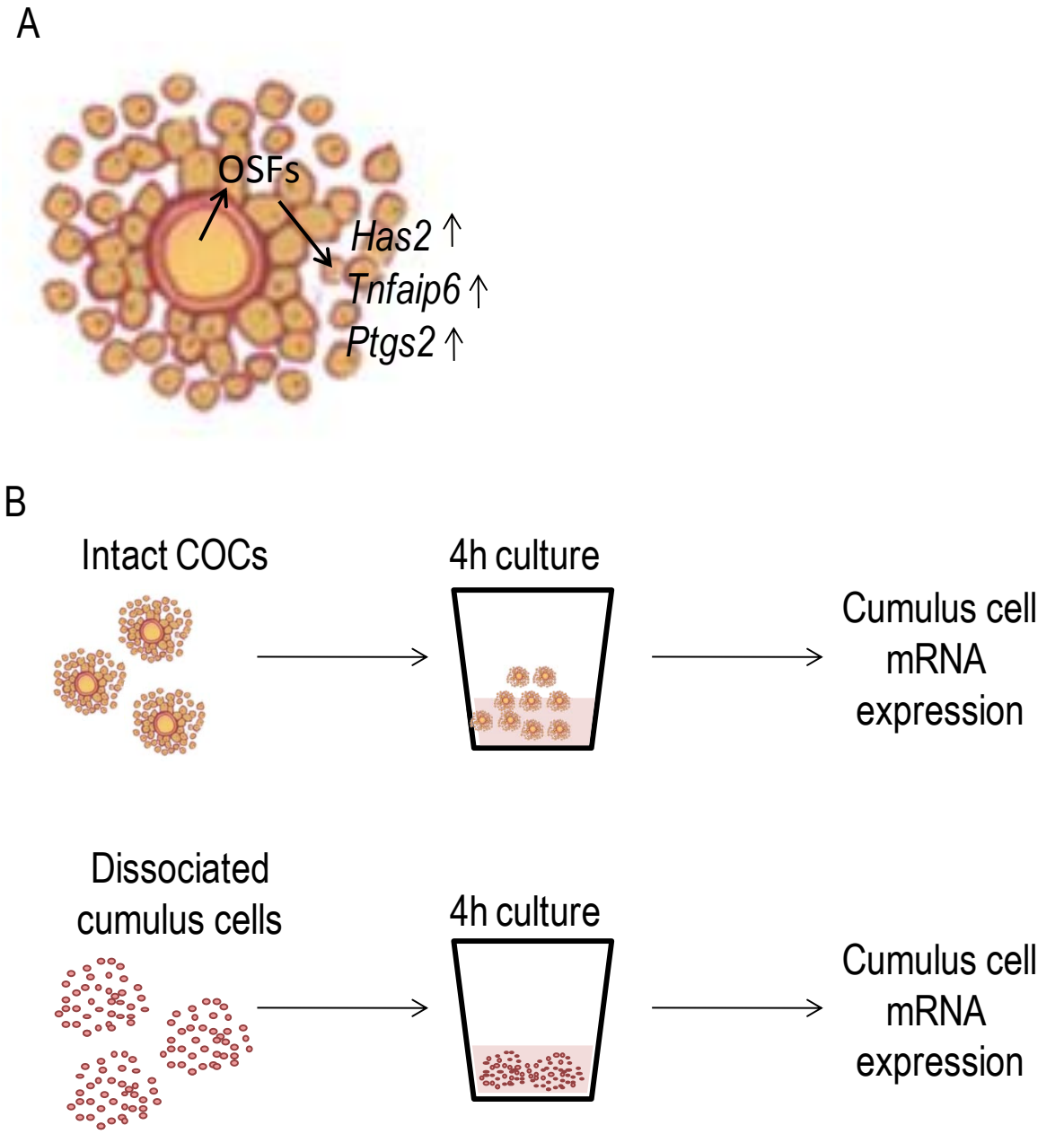

Figure 4.4 Schematic representation of experimental design to determine culture conditions for subsequent migration assays.

Prior to the development of a migration assay protocol, I determined culture conditions, which would ensure cumulus cells maintained a cumulus cell phenotype. A) Schematic representation of the regulation of cumulus cell genes (*Ptgs2*, *Has2* and *Tnfaip6*) in response to oocyte secreted factors (OSFs). B) Experimental design schematic. Either intact COCs or dissociated cumulus cells from mice administered with eCG + hCG 10h were placed in culture for 4 h, followed by real-time RT-PCR analysis to detect mRNA of cumulus specific genes shown in (A).

measure the mRNA levels of *Has2*, *Ptgs2* and *Tnfaip6*. The results show that following the culture period, dissociated cumulus cells had dramatically decreased levels of all three cumulus specific genes, compared to the intact COCs (Figure 4.5). The down-regulation of these genes was despite the presence of EGF and FSH, which are also required for COC expansion. These results suggest that dissociated cumulus cells do not maintain a cumulus cell phenotype and require factor(s) secreted from the oocyte, thus making it inappropriate to use dissociated cumulus cells for the extended culture period required in the migration assay. Therefore, intact COCs were utilised in cell migration (Chapter 4) and invasion (Chapter 5) assays.

4.1.2 Cumulus cell morphology following migration assay of expanded and unexpanded cumulus oocyte complexes

To determine whether cumulus cells have the ability to migrate across a porous membrane, I devised an experimental assay based on cancer cell migration assays. Cumulus oocyte complexes were isolated from follicles from mice, which had been treated with eCG 44h (unexpanded) or eCG + hCG 10h (expanded preovulatory). The capacity of COCs to migrate was assessed by transwell filter assays and subsequent image analysis performed. The immediate difference between filters following migration of expanded and unexpanded COCs was the morphology between the cumulus cells. Representative filters are shown in Figure 4.6. Filters from wells which contained unexpanded COCs (Figure 4.6A), had cumulus cells almost exclusively contained within the pores of the filter, and very few cells had transversed to the other side (migrated). In contrast, on filters from wells which contained expanded COCs (Figure 4.6B) cumulus cells were clearly visible, frequently some distance from the pores on the underside (migratory side) of the filters. Another obvious distinction was the morphological differences between the cumulus cells. Cumulus cells from unexpanded COCs were rounded in shape, whilst cumulus cells from expanded COCs had an elongated shape with cellular projections. These cellular projections are typical of the projections from migratory cells.

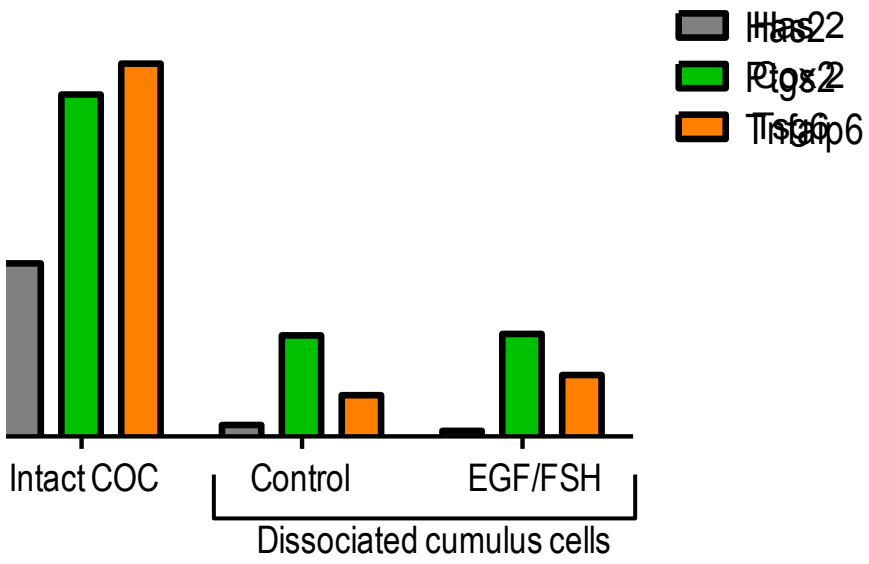

Figure 4.5 Dissociated cumulus cells do not retain cumulus specific gene expression of intact COCS after culture.

Real-time RT-PCR analysis of cumulus specific gene expression in intact cumulus oocyte complexes and dissociated cumulus cells cultured for 4h in α-MEM in the presence or absence of EGF (3ng/mL) and FSH (50mlU/mL). Cumulus specific genes examined were, *Has2 (grey), Ptgs2* (*green)* and *Tnfaip6 (orange)*. Expression data was normalised to *Rpl19*, expressed relative to the calibrator (whole ovary following treatment with eCG + 6h hCG) and presented as fold change from the calibrator which was set as 1 (n=1).

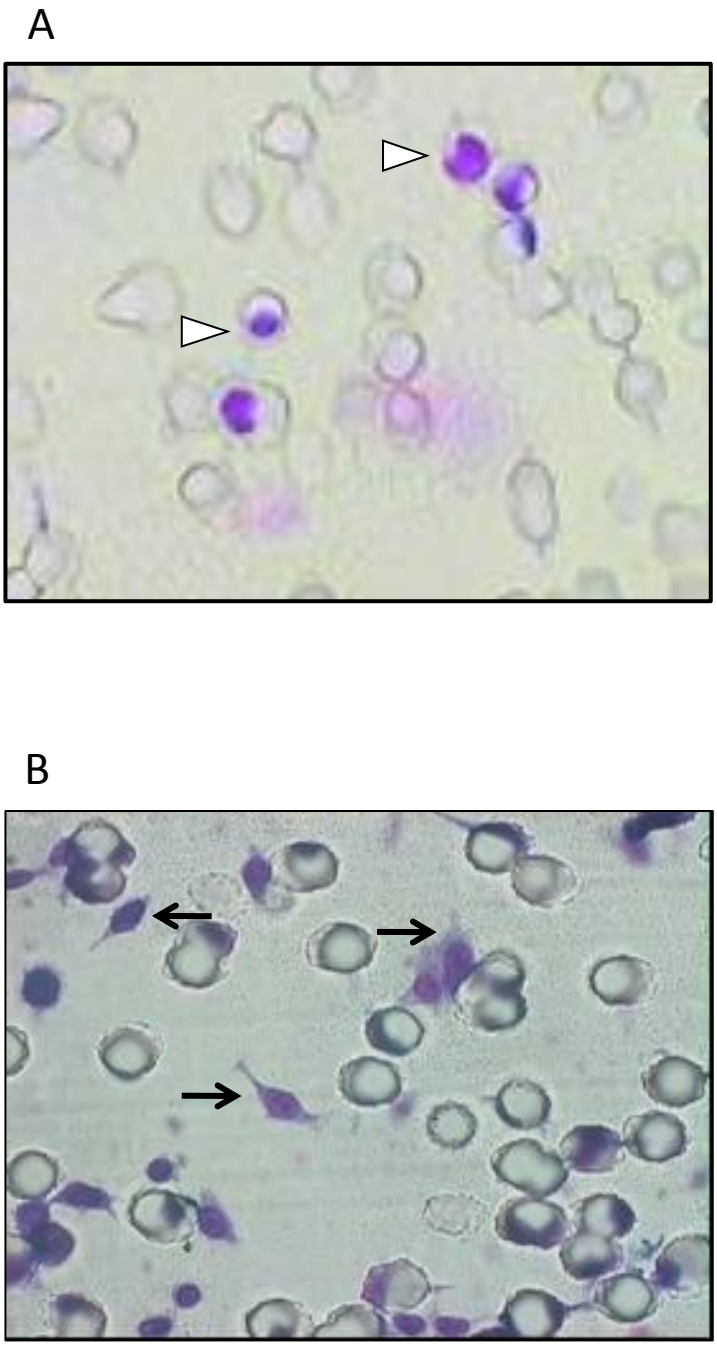

Figure 4.6 Morphology of cumulus cells from unexpanded and pre-ovulatory expanded cumulus oocyte complexes on the underside of migration assay filters.

Representative photomicrographs of typical filters from migration assays containing cumulus cells from unexpanded (A) and expanded (B) cumulus oocyte complexes. Pores are clearly visible on the filter surface. (A) Arrowheads point to cumulus cells that remain in the pores of the filter and no cumulus cells have moved away from the pores. (B) Arrows point to cumulus cells, which have moved away from pores, and have cellular projections. Images were taken at 5x magnification using NanoZoomer NDPview software (Hamamatsu Photonics, K.K.,Japan).

4.1.3 Migratory capacity of cumulus cells following ovulatory hormones

Migration of COCs was quantified by image analysis of the filters, which measured the area of purple stained cell nuclei (ie cell number) on the underside (migratory side) of each filter. Expanded COCs were compared with unexpanded COCs in transwell migration assays and relative cell migration indices are presented in Figure 4.7. Cumulus oocyte complexes from mice treated with hCG had a significantly greater (approximately 6.5 fold increase) migratory index compared to unexpanded COCs from mice treated with eCG only ($p=0.03$). The results demonstrate that COCs have a migratory phenotype that is hormonally regulated.

To determine whether the migratory phenotype observed in COCs was cumulus cell specific, the migratory capacity of COCs was compared to that of granulosa cells. Both cell types were retrieved from mouse ovarian follicles following administration with eCG + 10 h hCG. The results, shown in Figure 4.8 demonstrate that cumulus cells from pre-ovulatory expanded COCs have a significantly greater migratory index compared to that of granulosa cells collected at the same time, approximately 8-fold higher ($p=0.01$). Thus in follicles that have responded to ovulatory hCG, cell migration occurs specifically in COCs.

4.1.4 Treatment of cumulus oocyte complexes with inhibitors to block cell migration

Cumulatively, the above experiments demonstrate that cumulus cells from preovulatory follicles exhibit a migratory phenotype. I next sought to identify mechanisms regulating this phenotype. For these experiments I utilised well characterised inhibitors of the cell migratory machinery previously demonstrated to be present in cumulus cells (Chapter 3).

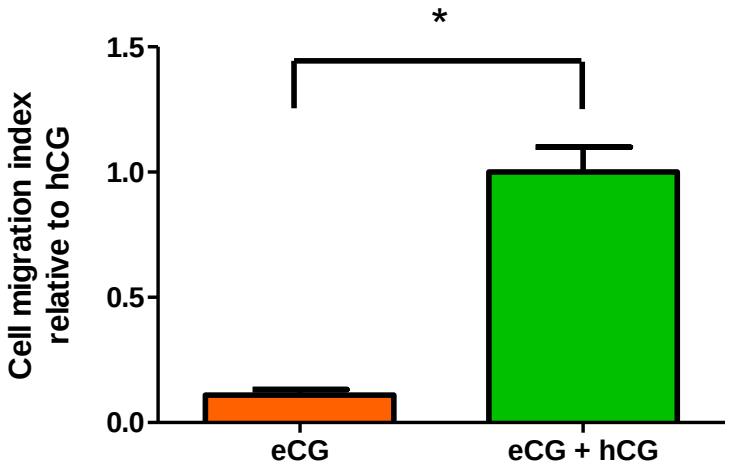

Figure 4.7 Expanded cumulus oocyte complexes show significantly greater migration than unexpanded cumulus oocyte complexes.

Cumulus oocyte complexes from mice which were hormonally stimulated with eCG alone (unexpanded, *orange*) or eCG + hCG for 10 h (pre-ovulatory expanded, *green*) were investigated in COC migration assays. Data is expressed relative to eCG + 10 h hCG COCs, which were set at 1, and presented as mean ± SEM. Data was analysed by unpaired t-test and asterisk denotes statistical significance (p=0.03; n=4 independent experiments).

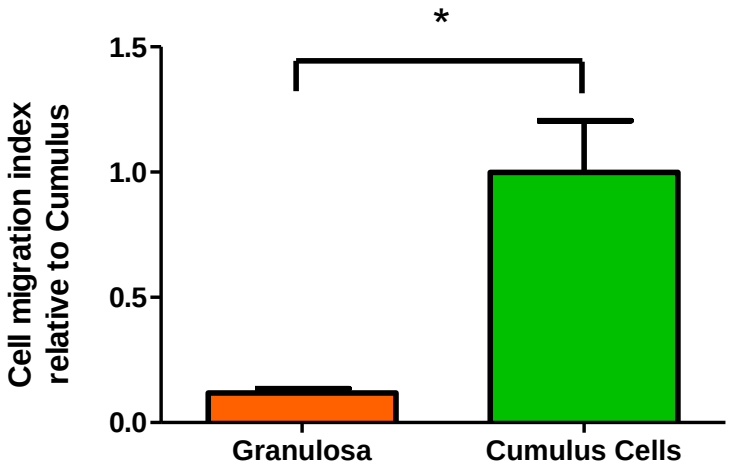

Figure 4.8 Migration of preovulatory expanded cumulus oocyte complexes is greater than migration of granulosa cells.

Mice were stimulated with eCG + 10 h hCG and COCs (green) were compared to granulosa cells (orange) isolated from the same ovarian follicles. Migration assays were performed with equal numbers of both cell types. Migration index is expressed relative to COCs and presented as mean ± SEM. Data analysed by unpaired t-test and asterisk denotes statistical significance (p=0.01; n=4 independent experiments).

The CD44 standard isoform is induced in COCs in response to ovulatory hCG (Chapter 3). To determine whether CD44 plays a role in the cumulus migratory phenotype, the CD44 neutralising antibody was added to COC migration assays at concentrations ranging from 5µg/ml to 10 µg/ml, based on similar published assays, that were able to block cell migration in other systems (Raheja, et al., 2008). Cumulus migration was assessed and the results are presented in Figure 4.9 with CD44 neutralising antibody treated groups represented relative to the untreated control. Cumulus oocyte complex migration under control conditions had the highest cell migration index, and although step-wise reductions in cell migration in response to increasing concentrations of CD44 neutralising antibody (5 or 10µg/mL) were observed, this was not statistically significant.

I identified the small GTPase Rac1 as a constitutively expressed gene during the ovulatory period (Chapter 3). I sought to elucidate the involvement of Rac1 in the migratory capacity of the COC by blocking its effects in vitro using NSC23766, a specific small molecule inhibitor of Rac1 activity. During the migration assays COCs were cultured in the presence of NSC23766 at three different concentrations, 5µM, 10µM and 25µM; based on previously used concentrations in the literature, that successfully inhibited cell migration (Grewal, et al., 2008, Ramesh, et al., 2007, Wang, et al., 2009). Results are shown in Figure 4.10 and data is presented as cell migration index relative to the control. Cell migration of COCs treated with NSC23766 at any concentration used here was not different to COCs under control conditions.

By utilising Y-27632, an inhibitor of Rock, I next sought to determine whether this inhibitor would inhibit cell migration mediated by RhoA, thus revealing it as a potential downstream target of CD44 necessary

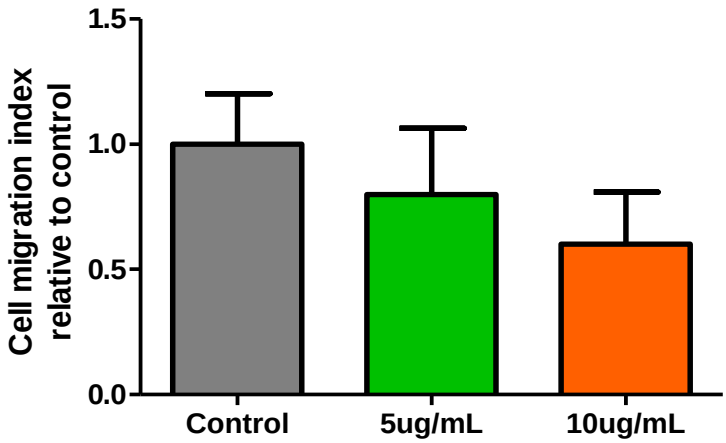

Figure 4.9 Migration of cumulus oocyte complexes was not inhibited with the addition of a neutralising antibody against CD44.

Pre-ovulatory expanded COCs were incubated in control conditions without antibody (grey) or in the presence of CD44 neutralising antibody 5µg/mL (green) or 10µg/mL (orange). Data is expressed relative to the control and presented as mean ± SEM (n=3 independent experiments) Data analysed by One way ANOVA.

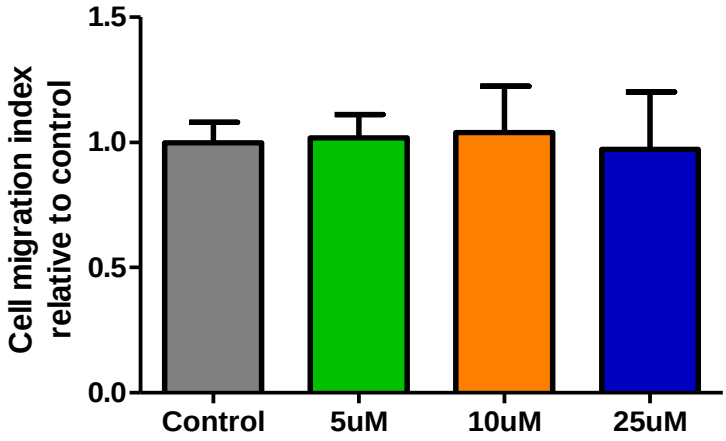

Figure 4.10 Migration of cumulus oocyte complexes was not inhibited by the addition of a NSC23766, a small molecule inhibitor of Rac1.

Pre-ovulatory expanded COCs were incubated in control conditions without inhibitor (grey) or in the presence of 5μM (green), 10μM (orange) or 25μM (blue) of NSC23766, during migration assays. Data is expressed relative to the control and presented as mean ± SEM (n=4 independent experiments). Data analysed by One-Way ANOVA.

for COC migration. During the migration assays COCs were cultured in thepresence of Y-27632 at three different concentrations, 10μM, 25μM and 50μM. These concentrations were used based on their ability to inhibit cell migration in the literature (Imamura, et al., 2000, Nicola, et al., 2008). Results are presented in Figure 4.11 and data is presented as cell migration index relative to untreated control. Modest increases in cell motility of COCs after Y27632 treatment were consistently observed, but not statistically significant.

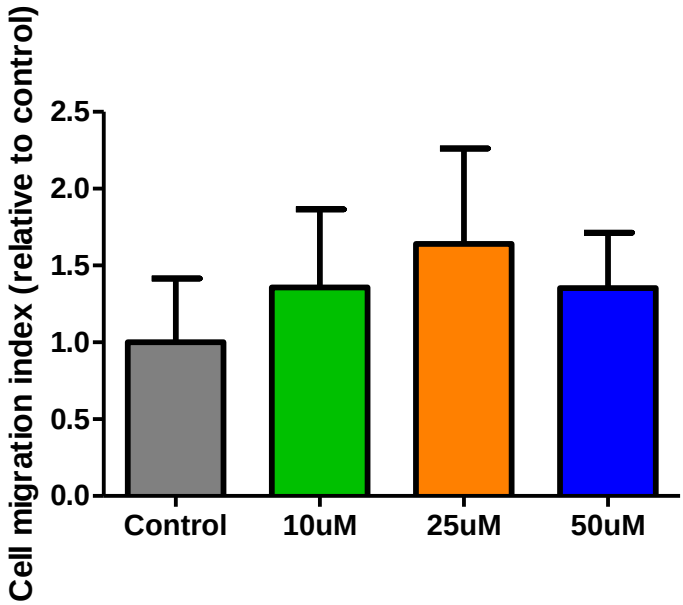

Figure 4.11 Migration of cumulus oocyte complexes was not inhibited by the addition of Y-27632, an inhibitor of Rock.

Pre-ovulatory expanded COCs were incubated in control conditions without inhibitor (grey) or in the presence of 10μM (green), 25μM (orange) or 50μM (blue) of Y-27632, during migration assays. Data is expressed relative to the control and presented as mean ± SEM (n=6 independent experiments). Data analysed by One-Way ANOVA.

4.1 DISCUSSION

The overarching hypothesis of this thesis is that the COC itself may play an active role in ovulation via transitioning to a migratory cell phenotype which may facilitate its release from the ovarian follicle. Excitingly the experiments and results described in this chapter, clearly demonstrate that the COC is able to transition to a motile cell phenotype, which is cumulus cell specific, and that ovulatory hormones induce this transition.

Expanded COCs were subjected to migration assays and compared to unexpanded COCs, what I found was that the cell migration was significantly increased in ovulatory, expanded COCs compared to unexpanded COCs. This result indicates that the cumulus cells acquire a motile cell phenotype following hCG administration. This phenotype is cumulus cell specific as granulosa cells from follicles receiving hCG, did not migrate across a porous membrane, in fact their cell migration index was similar to that of unexpanded COCs. The morphology of expanded and unexpanded cumulus cells following migration assays was also different. Cumulus cells from expanded COCs that successfully migrated through the filters and away from the pores displayed an elongated cell shape typical of a migrating cell, whilst in comparison, cumulus cells from unexpanded COCs maintained a rounded cell shape. I conclude that the cumulus cells transition to a motile cell within the expanded COC in response to hCG administration. Hyaluronan may play a part in the migratory phenotype of the expanded COC. Hyaluronan is a major component of the extracellular matrix of an expanded COC and without expansion ovulation does not occur. CD44 is a hyaluronan receptor, as discussed in the previous chapter, and is upregulated during COC expansion and ovulation. Further, addition of hyaluronan to cell migration assays has been reported to increase cell migration in human squamos carcinoma cells (HSC-3) (Bourguignon, et al., 2006). Hyaluronan is able to enhance cell migration by binding to CD44 and upregulating the intracellular migration pathway (Bourguignon, et al., 2006). The interaction between hyaluronan and

CD44 exists in other biological systems, including lymphocyte honing during an immunological response, whereby CD44-hyaluronan interactions target lymphocytes to specific sites of infection enabling cell adhesion (DeGrendele, et al., 1997). To further elucidate the role of hyaluronan it would be crucial to test hyaluronan directly. By adding exogenous hyaluronan to migration assays we could determine whether migration of cumulus cells is enhanced. Despite treatment with anti-CD44 having little effect on cell migration, hyaluronan may be acting via different receptors present on cells, for example RHAMM.

The expanded COC ovulates at the apical wall of the follicle, where it has been shown that the follicle wall thins and a rupture pore forms (Bjersing and Cajander, 1974, Martin and Talbot, 1987). Why the COC is ovulated at this apical side is unknown, but an explanation could lie in the directed cell migration of the cumulus cells. Such a mechanism would then involve the apical wall of the follicle being receptive to the expanded COC, leading to adhesion (as demonstrated and discussed in Chapter 2) followed by invasion through the follicle wall by the motile COC (Chapter 5).

In Chapter 3 the presence of CD44 and its associated intracellular targets, Tiam1, Rac1 and RhoA, were investigated during the time-course of ovulation. Results showed that *CD44s* was significantly upregulated in COCs following an ovulatory stimulus. *RhoA* was also found to be hormonally regulated in both COCs and granulosa cells, whilst *Rac1* and *Tiam1* were each constitutively expressed in both COCs and granulosa cells. In this chapter I show that COCs transition to a migratory phenotype following expansion and I further investigated whether the CD44 mediated cell migration pathway was responsible for COC migration.

The addition of inhibitors to components of the CD44-mediated cell migration pathway did not alter COC migration significantly. The neutralising antibody against CD44 tended to reduce cell migration however, providing some preliminary evidence that CD44 may play a role in the migratory phenotype of COCs. Previous studies using inhibitors of this cell migration mechanism, including inhibitors against Rac1 and Rock have yielded conflicting results. Whilst a number of studies have revealed that cell migration is decreased when an inhibitor is applied, other studies have found that inhibiting components of this pathway can in some instances lead to increases in cell migration. When the Rac1 inhibitor NSC23766 was applied to highly motile cancer cell lines, cell migration increased; while in contrast the same study demonstrated that Rac1 inhibition in less motile cancer cell lines decreased migration (Zuo, et al., 2006). In our study the Rac1 inhibitor had no effect on cumulus cell migration suggesting that this downstream target of CD44 is not responsible for cumulus cell migration. Reports of Rock inhibition are also contradicting, with some studies reporting an increase in cell migration (Fafet, et al., 2008, Lee and Kay, 2006, Salhia, et al., 2005, Tabu, et al., 2007) and others a decrease (Chang, et al., 2010, Darenfed, et al., 2007, Imamura, et al., 2000, Ishizaki, et al., 2000). Our results were suggestive of increased cumulus cell migration in the presence of the Rock inhibitor Y-27632, yet this apparent increase in cell migration was not statistically significant.

Given our results show no reduction in cumulus cell migration when three distinct and well-characterised inhibitors of cell migration are applied I conclude that the CD44 cell migration pathway is unlikely to be important for the migratory capacity of the ovulatory COC. Another strong possibility is that the antagonist treatment is being applied too late in the 'ovulatory process' to have an effect on the cumulus cell migratory phenotype. Our assay system is performed with COCs removed from follicles 10 h post hCG administration, used to induce ovulation. It may be that the cell migration machinery is already well established at this time, considering *CD44s* was upregulated at 6 h post hCG and *RhoA* was

upregulated at 4 h post hCG in COCs (Chapter 3). It may be necessary to perform cell migration assays with COCs removed from follicles shortly after receiving the ovulatory hCG stimulus. Related experiments from our laboratory have demonstrated that the COC matrix at 10 h post hCG is refractory to the penetration of solutes, similar in physical properties as the antagonists used in this study, thus in this system the antagonist may not be able to penetrate the COC to elicit their maximal effects. Of course, the problem of extended culture of COCs and the challenge of retaining a cumulus cell phenotype also exists. This may be overcome by using in vitro matured (IVM) COCs, whereby the ovulatory stimulus is administered to unexpanded COCs in culture, allowing us to use COCs at any stage during cumulus expansion in the migration assay. This would permit a more thorough investigation of when the migratory machinery is switched on in cumulus cells and more comprehensive exposure to antagonists. However, IVM COCs show poor expansion and development of the extracellular matrix, with a number of components known to be absent compared to in vivo matured COCs (Dunning, et al., 2007). Interestingly, preliminary experiments show that migration of IVM COCs is not as robust as in vivo matured COCs, supporting our general hypothesis that there is a role for extracellular matrix and additional follicular factors, in the cell migration phenotype. Alternatively, treatment of mice in vivo with inhibitors of the pathway prior to ovulation may demonstrate whether these components are important for ovulation. We could then determine whether any defect is associated with impaired cell migration by removing COCs from the ovaries of treated mice and evaluating migration.

Similar to the process of tumour metastasis, I have established that the periovulatory expanded COC transitions to an adhesive, motile cell phenotype, possibly by the activation of the CD44 mediated cell migration pathway. The next stage in the ovulatory cascade is extrusion of the COC through a rupture pore. Metastatic tumour cells degrade surrounding ECM and will invade surrounding tissue in order to

migrate to distal sites, and in a similar mechanism, I propose that the expanded COC may facilitate the formation of a rupture pore. This is explored in Chapter 5.

Chapter 5

The Invasive Capacity of the Cumulus Oocyte Complex

5.1 INTRODUCTION

Extrusion of an expanded cumulus oocyte complex (COC) from a preovulatory follicle requires that a rupture pore is formed at the apical wall of the follicle. How the COC ultimately penetrates the follicle wall to be ovulated remains unknown. However, I have established that cumulus cells following hCG administration, aquire adhesive properties that allow binding to various extracellular matrices (ECMs) that may become exposed by apical thinning. Additionally I have shown that the expanded COC transitions to a migratory cell phenotype in response to ovulatory hormones. The final step in our hypothesis, depicted schematically in Figure 5.1, is that the expanded COC develops invasive properties allowing it to actively penetrate the follicle wall.

The generally held hypothesis is that proteases derived from granulosa and/or theca cells act at the apical wall of the follicle to cause degradation of the follicle wall structure, through which the expanded COC may pass during ovulation.

There is a substantial amount of compelling evidence that proteases are involved in the ovulation process. Studies thus far have identified protease activity in the ovary at the time of ovulation, specifically the matrix metalloproteases (MMPs) have been the most extensively studied and found to be localised to the apical region of an ovulating follicle (Curry, et al., 2001). MMPs are organised into four distinct classes, the collagenases, the gelatinases, the stromelysins, and the membrane-associated metalloproteinases with each acting upon various ECM substrates including collagen, laminin and fibronectin, all important components of the ovarian follicle wall (Berkholtz, et al., 2006, Bjersing and Cajander, 1974, Rodgers, et al., 1998, Saha, et al., 2007). Follicle rupture is thought to be caused via MMP-mediated breakdown of the ECMs of the follicle wall, and a number of MMPs have been implicated including MMP1, MMP2, MMP19 and MMP14 (Chaffin and Stouffer, 1999, Hagglund, et al.,

1999, Ichikawa, et al., 1983, Jo and Curry, 2004). The broad-spectrum metalloprotease inhibitor GM6001, or galardin, has previously been used to attempt to inhibit ovulation in mice (Liu, et al., 2006). Assessment of MMP activity in GM6001 treated mice showed that active forms of MMP2 and MMP9 were efficiently and dose dependently reduced, however this resulted in only a moderate, however significant, 20% reduction in ovulation rate (Liu, et al., 2006). The plasminogen system has also been implicated in the proteolytic process involved in ovulation as plasminogen null mice exhibit reduced ovulation rates (Ny, et al., 1999). To elucidate the contribution of both the plasminogen and MMP systems in ovulation, GM6001 was administered to plasminogen deficient mice prior to ovulation but did not lead to a further reduction in ovulation (Liu, et al., 2006). As GM6001 is a broad spectrum MMP inhibitor, these results suggest that mechanisms in addition to the MMP system are involved in follicle wall breakdown, subsequent rupture and ovulation of the COC. However, to date no specific proteases have been identified as being individually or entirely responsible for ovulation; as knockout mice of any one protease, have no or very few reproductive defects. More specifically null mice have been generated for MMPs, including MMP2 (Itoh, et al., 1997), MMP3 (Alexander, et al., 2001), MMP7 (Wilson, et al., 1997), MMP9 (Vu, et al., 1998), MMP11 (Masson, et al., 1998), MMP12 (Shipley, et al., 1996) , MMP13 (Inada, et al., 2004, Stickens, et al., 2004), MMP14 (Holmbeck, et al., 1999), MMP19 (Pendas, et al., 2004), MMP20 (Bartlett, et al., 2004), however ovulation defects have not been reported in any of these models. This suggests that individual MMPs are redundant and can be compensated for and that the plasminogen and MMP systems are not absolutely essential for ovulation to occur. While it is intuitive that ECM degradation is important, what is lacking is definitive information about the cellular source of ovulatory proteolytic activity and how it is focused at the apex of the follicle

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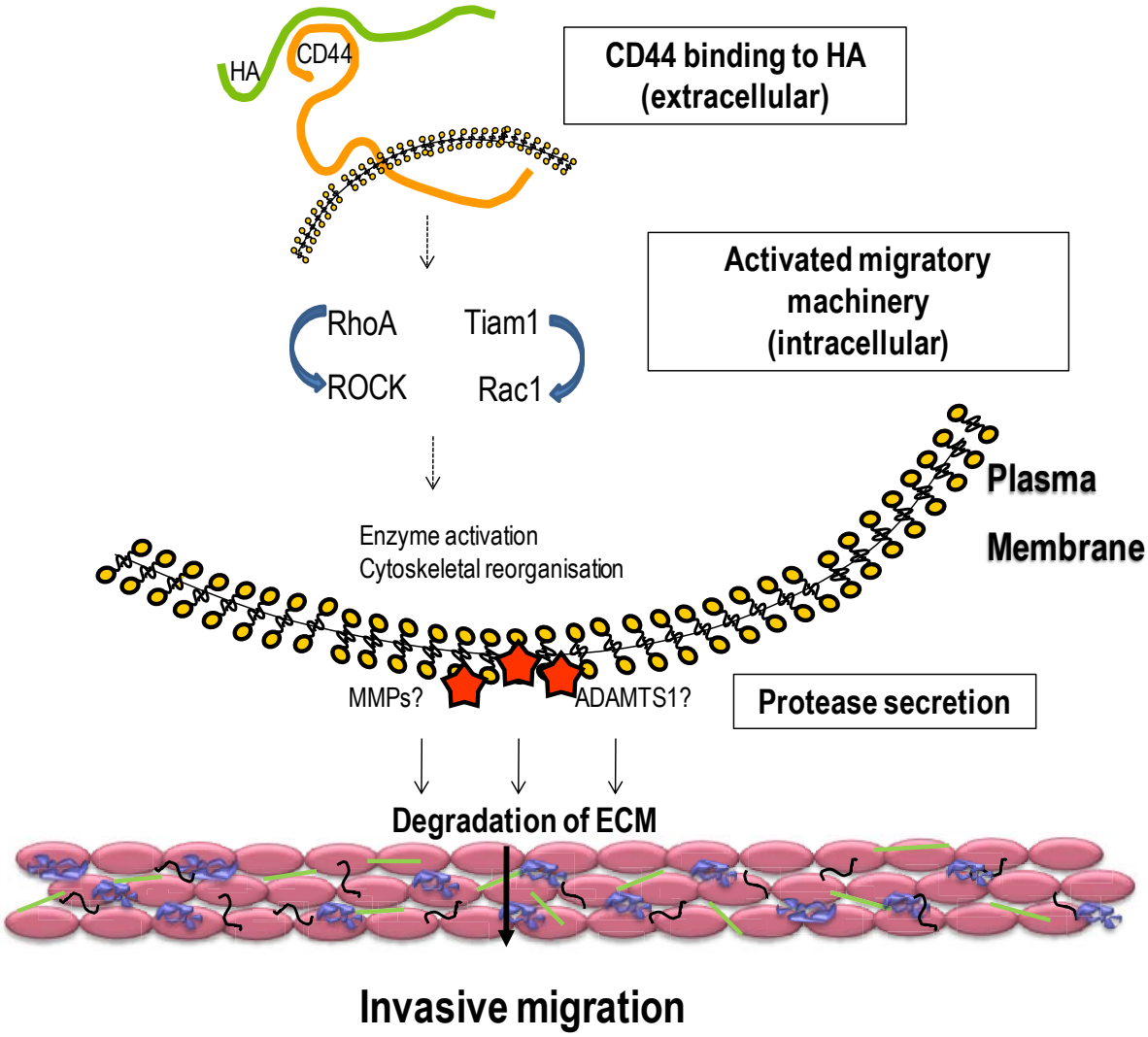

Figure 5.1 Schematic representation of proposed invasive action of the cumulus oocyte complex.

This schematic depicts the proposed action of an invasive migratory cumulus oocyte complex. Following the binding of HA to its receptor CD44, activation of intracellular migratory machinery begins, leading to consequent cellular secretion of proteases, which I hypothesise could be Adamts1 and/or MMPs. These proteases break down the ECM of the follicular wall, and subsequently the COC is ovulated through the rupture pore formed.

Adamts1 (A disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif 1) is a metalloprotease and the only protease shown to individually play a role in the ovulation process (Brown, et al., 2010, Mittaz, et al., 2004, Shindo, et al., 2000). A number of studies have reported fertility defects in *Adamts1* null mice, which have a significantly reduced ovulation rate (approximately 60% of wildtype littermates) (Brown, et al., 2010, Mittaz, et al., 2004, Shindo, et al., 2000). Furthermore, entrapped COCs are present in follicles at 16 h following an ovulatory dose of hCG, demonstrating a defect in follicular rupture in these mice (Mittaz, et al., 2004). A recent study, showed that the basal wall of periovulatory follicles in *Adamts1* null mice fail to remodel, with significantly more follicles displaying a disorganised structure in these mice (Brown, et al., 2010). Induction of *Adamts1* in the follicle occurs following the LH surge and is dependent on Progesterone receptor (Pgr) expression (Robker, et al., 2000). *Adamts1* expression, as detected by in situ hybridisation, is restricted primarily to the granulosa cell layer, with proteolytically active Adamts1 protein secreted from granulosa cells and incorporated into the expanded COC matrix (Russell, et al., 2003b). Just prior to ovulation, Adamts1 cleaves the cumulus matrix protein versican within the expanding COC matrix (Russell, et al., 2003b). In the *Admats1* null mouse, cumulus expansion has been reported to be abnormal and less extensive suggesting a defect in COC matrix structure and or function compared with wild-type mice (Mittaz, et al., 2004, Russell, et al., 2003b). Thus it is possible, and in line with our working hypothesis, that Adamts1-mediated matrix remodelling within the COC may also facilitate the process of follicle rupture.

In previous chapters, I demonstrated adhesive and migratory properties of the COC, processes that are necessary if a cell is to transition into an invasive cell type. Within this chapter, I sought to determine whether an expanded COC is able to invade an extracellular matrix. The ECM used in the invasion assays was Matrigel, which consists mainly of laminin and collagen IV. This system somewhat mimics an in vivo ovulation scenario where the follicle wall, which also contains collagen and laminin, provides a

barrier between the oviduct and the ovulating COC. As positive controls for invasive cellular migration, I compared COC invasion against known invasive and non-invasive cell lines, MDA-MB-231 and MCF7 breast cancer cell lines (Nagaraja, et al., 2005).

Two strategies were utilised determine whether protease activity was required for the COC to invade an ECM barrier (Matrigel). Firstly, the broad-spectrum inhibitor of invasion, GM6001 was used in invasion assays. Secondly, I investigated invasion by COCs from *Adamts1* null mice.

I hypothesised that the preovulatory expanded COC as well as acquiring cell motility, transitions to an invasive cell phenotype capable of invading the follicular ECM barrier. Further that the COC achieves this by exerting protease activity on the ECM and that COCs from *Adamts1* null mice will have an inability to degrade an ECM barrier and thus a reduced invasive capacity.

5.2 MATERIALS AND METHODS

5.2.1 Animals and hormonal stimulation protocol and Tissue Collection

All animals were treated in accordance with the National Health and Medical Research Council Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NHMRC, 2004). Ethics approval was obtained from The University of Adelaide Animal Ethics Committee. All F1 C57Bl6 X CBA and *Adamts1* null mutant mouse line were housed at the University of Adelaide Animal Facility and maintained on a 12h/12h day/night cycle with rodent chow and water provided *ad libitum.* The *Adamts1* null mutant mouse line used in this study is on a 129SvJ/C57Bl6 genetic background, and has been described previously (Mittaz, et al., 2004). Heterozygous female (*Adamts1*$^{-/+}$) and homozygous male (*Adamts1*$^{-/-}$) crosses were maintained as described above. At days 19-22 of postnatal age, mice

were weaned and tail tips were biopsied from anaesthetised mice for determination of genotype by PCR (Section 5.2.2). Mice were superovulated by i.p injection with 5 IU Equine chorionic gonadotropin (eCG) (Professional Compounding Centre of Australia, Sydney, NSW, Australia) at 1400 h on day 21-23 of age to stimulate follicle growth. This was followed 44 – 48 h later with an injection of 5 IU Human Chorionic gonadotropin (hCG) (Organon Australia, Sydney, NSW, Australia) to initiate ovulation. Cumulus oocyte complexes were punctured from follicles of ovaries, in hepes buffered α-MEM with 5% FCS using 30 gauge needles (BD Biosciences) (Figure 2.3).

5.2.2 Genotyping of *Adamts1* null mouse line

Genotyping of Adamts1 offspring was performed by PCR analysis of tail DNA. Genomic DNA was extracted by incubation at 55°C for 1 h (130 rpm shaking) in buffer (10mM Tris-HCL pH 7.8, 75mM NaCl, 25mM EDTA, 1% (W/V) SDS) containing 0.16µg/µL Proteinase K. To the digested tails, 250µL of phenol chloroform was added and mixed followed by centrifugation at 15,000 rcf for 10 min. Approximately 200µL of the upper, aqueous phase was transferred to a new tube and DNA was precipitated by adding 500µL of ice-cold absolute ethanol. Precipitated DNA was pelleted by centrifugation at 15,000 rcf for 20 min. The precipitated DNA was resuspended in 100µL of nuclease-free H_2O. The DNA was then used in two PCRs, the oligonucleotide pairs are shown in Table 6.1. The first PCR contained the primer pair Adamts1 ex2 (F) and Adamts1 ex3 (R) (WT reaction) which span exons 2 and 3. These primers amplified a product of 576bp from the wild type gene when exon 2 is present, identifying the progeny as carrying at least one wild type allele. The second reaction contained primer pairs Adamts1 KO (F) and Adamts1 KO (R) (KO reaction), which span across intron 1 and exon 3. A product of 278bp was amplified when exon 2 was deleted and an additional 1323bp product is amplified in the presence of exon 2. A reaction where the 278bp product was amplified in the presence of Adamts1 KO reaction, but no product was amplified in the presence of the Adamts1 WT reaction, indicated a homozygous *Adamts1*$^{-/-}$ progeny. Genotyping PCRs contained 5µL 5X GoTaq Flexi® buffer

(Promega Corporation, Annandale, NSW, Australia) 3mM $MgCl_2$, 0.5mM each dNTP, 5ng of each primer, 1.25 units of GoTaq® polymerase and H_2O to 25µL. Cycling conditions used were 94°C, 5 min (1 cycle); 94°C, 1 min; 60°C, 1 min; 72°C, 1 min (35 cycles), 72°C, 5 min (1 cycle). Amplified products were visualised by agarose gel electrophoresis.

5.2.3 Cell lines

MDA-MB231 metastatic human breast cancer cell line and MCF-7 breast cancer cell lines were used in invasion assays. These cell lines are considered as being invasive (MDA-MB231) and non invasive (MCF-7) cell types (Nagaraja, et al., 2005). MDA-MB231 cell lines were maintained in RPMI (Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia) supplemented with 10% fetal calf serum (FCS, Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia). MCF7 cell lines were maintained in RPMI (Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia) with 10% FCS. All cell lines were maintained at 37°C in a humidified incubator at 5%CO_2 in air. Cells were cultured until 80-90% confluence, trypsinised, centrifuged and resuspended in DMEM/5% FCS (Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia). Cell counts were performed using a haemocytometer before cells were added to invasion assays. Numbers of breast cancer cells were matched to cell number of 20 COCs, approximately 2000 cells/COC.

5.2.4 Transwell invasion assays

Invasion assays were performed utilising commercially available assay kits (Millipore, North Ryde, NSW, Australia). Invasion assays were performed as described for migration assays (Section 4.2.2; Figure 4.2), however transwell inserts were pre-coated with matrigel (Millipore, North Ryde, NSW, Australia) and cells transitioning across the ECM barrier were compared to migration on a non-coated insert (migration) to yield an index of invasive capacity normalised for migratory capacity. Phase analysis quantification was used to quantitative invasive and migratory cell number using AnalysisPro

software (Olympus Australia Pty. Ltd., Mt Waverly, VIC, Australia) (Section 4.2.3; Figure 4.3). Invasion indices were calculated by dividing the invasion result by the migration result, providing a percentage of migratory cells that were also invasive.

5.2.5 MMP inhibitor

GM6001 (Millipore, North Ryde, NSW, Australia) was dissolved in 10% DMSO and used at a final concentration of 25μM, a concentration previously shown to effectively inhibit cell migration in other systems (Bullard Dunn, et al., 2009, Rofstad, et al., 2006). In control wells 10% DMSO was included as a vehicle control.

5.2.6 Statistics

One Way ANOVA were performed and with a Newman-Keuls post hoc test Unpaired t-tests were applied as described in the figure legends. Statistics were performed using Graph Pad Prism software (GraphPad Software Inc, La Jolla, CA, USA). Significance was set at $p<0.05$.

5.1 RESULTS

5.1.1 Invasive capacity of cumulus cells compared to characterised breast cancer cell lines.

To investigate the invasiveness of cumulus cells, the number of cumulus cells penetrating a matrigel barrier was compared to that of well-characterised breast cancer cell lines. MDA-MB-231 cells are considered an invasive cell line and MCF-7 cells are considered a non-invasive cell line (Nagaraja, et al., 2005). The results of this experiment (Figure 5.2) showed that cumulus cells have a similar invasive index to MDA-MB231 cells.

Forward Primer	***Sequence (5' - 3')***	***Intron/Exon nucleotide number (NC_000082.5)***	***Reverse Primer***	***Sequence (5'-3')***	***Intron/Exon nucleotide number (NC_000082.5)***	***Predicted product size (bp)***
Adamts1 ex2 (F)	AGTTACCTCCAATGCAGCTCTCA	Exon 2 (2893-2915)	Adamts1 ex3 (R)	ATCCCCGAGAGTGTCACACGTG	Exon 3 (3448-3469)	576
Adamts1 KO (F)	TCCTCAAGCCCCACCCCTTGG	Intron 1 (2181-2201)	Adamts1 KO (R)	TCCTGCTGGGGTCACATACAG	Exon 3 (3484-3504)	1323 (WT) 278 (KO)

Table 5.1 Primers used in genotyping of Adamts1 null mutant mouse line

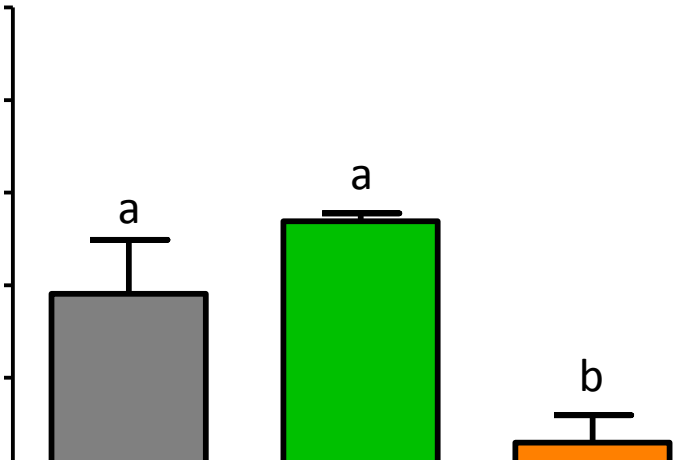

Figure 5.2 Pre-ovulatory expanded cumulus oocyte complexes are as invasive as a known invasive cancer cell line.

Expanded COCs from eCG 44h + hCG 10h treated mice were analysed for their ability to invade an extracellular matrix barrier, Matrigel. COCs (*cumulus cells*, grey) were compared to well-characterised cancer cell lines; MDA-MB231 cells (green), which are considered an invasive cell type and MCF-7 cells, which are considered a non-invasive cell type (orange). Data is expressed as mean invasion index ± SEM (n=3 experiments). One Way ANOVA performed with Newman-Keuls post hoc test. Different superscripts denote significant difference ($p<0.05$).

5.1.2 Inhibiting cell invasion by treatment with a protease inhibitor and utilising the protease deficient *Adamts1* null mouse.

To establish whether the invasive migration of cumulus cells is protease dependent I performed invasion assays in the presence of a broad-spectrum metalloprotease inhibitor, GM6001 that has been shown to block cell invasion in a number of cell types in Matrigel invasion assays at doses similar to that used here (Bullard Dunn, et al., 2009, Rofstad, et al., 2006). Results are presented in Figure 5.3. There was no significant difference in invasion of COCs between those treated with GM6001 and the no treatment control. This result indicates that cumulus cell invasion of a Matrigel ECM barrier is MMP independent.

COCs from mice lacking the *Adamts1* gene were subjected to matrigel invasion assays to determine whether this protease is important for the ability of cumulus cells to invade through this ECM. *Adamts1* null COCs were compared to those from heterozygous littermates. Data is presented in Figure 5.4. Invasion of COCs from knockout animals was slightly reduced. However, these experiments are inconclusive due to a lack of available KO mice that prohibited the analysis of larger numbers.

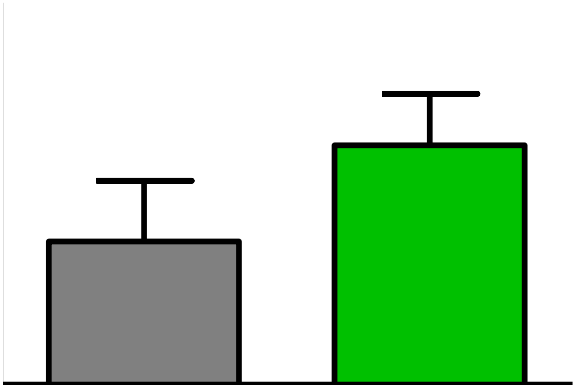

Figure 5.3 Treatment of cumulus oocyte complexes with the broad-spectrum protease inhibitor GM6001 did not block cell invasion.

Analysis of cell invasion of pre-ovulatory expanded COCs in control conditions without inhibitor (grey), or in the presence of the broad spectrum protease inhibitor GM6001 (green). Data is presented as mean invasion index ± SEM (n=3 experiments, duplicate wells per experiment). Unpaired t-test performed (p=0.4).

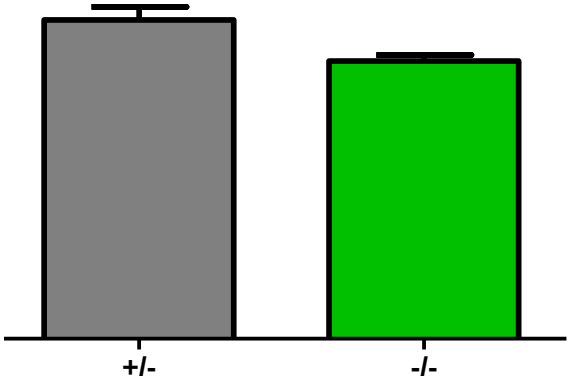

Figure 5.4 Invasion of Adamts1 null versus Adamts1 heterozygous cumulus oocyte complexes.

Analysis of invasive ability of cumulus oocyte complexes from *Adamts1* heterozygous (grey) and *adamts1* null (green) following hormonal stimulation of mice with eCG 44 h + 10 h hCG Data is represented as mean ± SD (n=2 independent experiments, with duplicate wells of each genotype)

5.1 DISCUSSION

Ovulation is a process involving dramatic extracellular matrix degradation and tissue remodelling. This process might be considered similar to tumour progression, whereby tumour cells may secrete, activate or associate with a number of proteases to break down extracellular matrix and invade surrounding tissue in order to metastasise. I hypothesised that a similar method may be utilised by cumulus cells of an expanded COC in order to facilitate the ovulation process, ie cumulus cells produce or accumulate proteases that breakdown and consequently rupture the follicle wall, through which a mature COC can be extruded. A number of studies have associated various protease systems, such as MMPs and plasminogen, with ovulatory tissue degradation, and Adamts1 is one protease that has been identified as playing a significant role in ovulation. However, exactly how proteolytic activity mechanistically triggers ovulation remains to be elucidated. I propose that cumulus cells themselves focus tissue remodelling to the follicular apex, to initiate breakdown of the follicle wall when they attach at the apical wall of the follicle. This model may be considered similar to embryo implantation, whereby trophoblast cells of the blastocyst attach to a basement membrane in the uterus (Campbell, et al., 1995), containing components also found in the ovary such as laminins and collagens (Aplin, 1997), invading the tissue to implant and establish a pregnancy.

In this chapter, I investigated the invasiveness of cumulus cells by subjecting them to invasion assays, which required them to penetrate through an extracellular matrix barrier, Matrigel. Cumulus cells were compared to an invasive breast cancer line, MB-MDA231 cells and shown to be equally as invasive; whilst the non-invasive performance of MCF-7 cells demonstrates the effective barrier formed by the matrigel. I hypothesised that the invasive capacity would most likely depend on proteases present in cumulus cells at ovulation. Previous studies have shown that MMPs and the plasminogen system are involved in the ovulation process, as deficiencies in these components in vivo resulted in moderate

reductions in ovulation rates (Liu, et al., 2006, Ny, et al., 1999). I investigated specifically the MMP contribution to cumulus cell invasion in our invasion system by administering GM6001, a broad-spectrum MMP inhibitor, during the invasion assay. However, there was no observed effect on invasion of cumulus cells with the use of this inhibitor. This could be due to a range of reasons. Perhaps, the inhibitor does not block all ovulatory proteases, and consequently they were able to compensate for each other. From these results, it is unlikely that MMPs alone mediate this invasive property across an ECM barrier, as the broad-spectrum MMP inhibitor was unable to impede invasion of the COCs in this system. It is possible that further studies are required using different concentrations of the inhibitor to exhibit an effect on cumulus cell invasion. Further to this it would be interesting to investigate the timing of inhibitor administration as perhaps the mechanisms involved have been initiated prior to 10h post hCG, which is when we remove COCs from the ovarian follicle.

To try to elucidate further the function of proteases in the process of cumulus cell invasion I investigated the possible role of the protease Adamts1. Mice null for this gene exhibit severe ovulatory defects (Brown, et al., 2010, Mittaz, et al., 2004) supporting a potential role of Adamts1 in ovulatory ECM remodelling. I utilised knockout mice to test the ability of Adamts1 deficient COCs to invade an extracellular matrix. Unfortunately, I was unable to conclude whether Adamts1 is necessary for the invasive phenotype of the COC due to lack of experimental animals and unexpected loss of this mouse colony. The results do however, show a slight trend for COCs from *Adamts1* null mice to be less invasive than the heterozygous COCs. It would be prudent, however, to repeat these experiments to clarify this result when the mouse line can be re-established.

Alternatively, Matrigel may not be a sufficient barrier to COCs and GM6001 treated or *Adamts1* null COCs might be deficient in their ability to invade alternative matrices, specifically collagen I, as it is a

major component of the follicle wall (Berkholtz, et al., 2006). The differences in ability of invasive cells to invade different ECM barriers is shown in a study comparing Matrigel and collagen I as potential barriers to ovarian cancer cell invasion. It was discovered that MMP-mediated proteolysis was not required for invasion of Matrigel as GM6001 was unable to inhibit cell invasion in a 3D system (Sodek, et al., 2008).

It is also possible that Adamts1 deficiency could be functionally compensated for by other Adamts family members, known to be present in both COCs and granulosa cells 12 h post hCG, at the time of ovulation (Richards, et al., 2005, Russell, et al., 2003b). Adamts4 is induced by hCG but shows a slightly different regulation pattern to Adamts1 (Richards, et al., 2005). Additionally, Adamts4 is localised to the COC and granulosa cells, although protein levels appear equally as abundant in the two cell types whereas Adamts1 appears to accumulate in the COC prior to ovulation (Richards, et al., 2005, Russell, et al., 2003b). However it is not likely that Adamts4, is compensating for the loss of Adamts1 during ovulation as *Adamts4* null mice do not display an ovarian or ovulatory defect (Brown, et al., 2010, Stanton, et al., 2005). It is indeed possible that in the absence of Adamts1, Adamts4 is compensating for its loss in a more crucial and yet to be elucidated role, however, this does not appear to be within the ovary. Adamts5 is another member of this family which is present in the ovary, however studies reveal that this protein is not hormonally regulated unlike Adamts1 and 4, demonstrating it is unlikely to be involved in ovulation (Richards, et al., 2005). The progesterone receptor (*Pgr*) null mouse has a severe ovulation defect where COC expansion appears normal however the mice exhibit a complete failure to ovulate (Lydon, et al., 1995, Robker, et al., 2000). In these mice, Adamts1 is reduced to approximately 10% of WT mice and this defect likely contributes to the absence of follicular rupture and entrapped oocytes in preovulatory antral follicles of these mice. Additionally, *Pgr* null mice show no changes in Adamts4 localisation or abundance in COCs and granulosa cells, demonstrating that Adamts4 is not regulated by PR unlike Adamts1 (Russell, et al., 2003b). This suggests that Adamts4 is not important

during ovulation. *Pgr* null mice also have reduced expression of another protease cathepsin L and it has been reported that progesterone regulates ovarian proteolytic enzymes involved in degradation of the follicular wall (Iwamasa, et al., 1992, Lydon, et al., 1995). Thus, Pgr may regulate a cohort of proteases with different specificities and sensitivity to different inhibitors, that together mediate ovulatory ECM degradation; and *Pgr* null COCs will be an important model system to test in our in vitro COC invasion system.

I have shown in this chapter that the preovulatory expanded COC is able to transition to an invasive cell type, and has the ability to transverse an ECM barrier, in this case matrigel. This supports our working model indicating that COCs have the mechanistic capacity to mediate degradation and rupture of the ovarian follicle apex. I attempted to further elucidate whether metalloproteases, or Adamts1 specifically mediate the proteolytic degradation of ECM using the broad-spectrum protease inhibitor GM6001 and *Adamts1* null mice. Unfortunately, I was unable to show that the invasive actions of cumulus cells is protease dependent, however further studies are required to clarify the role of Adamts1, including a detailed time-course study.

I have shown that preovulatory expanded COCs are able to transition to an invasive cell type, and this invasion is comparable to the MDA-MB-231 cancer cells, which have the capacity to invade blood vessels and established tissue structures in vivo (Yang, et al., 2001). This is the final step in our hypothesis, supporting an active role for the COC in facilitating ovulation.

Chapter 6 Conclusions and Future Directions

6.1 The active role of the cumulus oocyte complex during ovulation

Ovulation is a complex process of which the mechanisms involved are not well understood. For a number of decades now researchers have attempted to elucidate the intricate details involved in this process, and whilst a number of them have been illuminated there remain vital components that continue to be a topic for debate. Studies have provided evidence showing certain mechanisms are crucial, but none of these appear to be exclusively involved in the process. Specifically, the ultimate release of a mature oocyte from the follicle appears to involve a number of factors, including, muscle cell contraction, intrafollicular pressure and protease activity but none have been conclusively shown to act on their own to cause ovarian follicle rupture. What has emerged is the important role of the cumulus oocyte complex (COC), and this has been the focus of this thesis. I hypothesised the induction of CD44 and its associated cell migration pathway would play a role in the COC transitioning to an adhesive, invasive migratory cell type. I investigated the regulation of genes involved with CD44 mediated cell migration and invasion in cumulus cells and whether the cumulus cells exhibited a migratory cell phenotype.

A number of different mechanisms have been suggested as responsible for ovulation. These are highlighted schematically in Figure 6.1 which integrates the established cellular processes involved in ovulation whilst incorporating our novel hypothesis that the COC is also actively involved. Inflammation has been suggested to play a major role, and I highlight its importance in allowing COC expansion to occur through increased vascular permeability and the incorporation of serum molecules into the cumulus matrix during expansion. I know that COC expansion and the expression of cumulus specific genes mediating formation of the expanded matrix are vital for ovulation, due to defects observed in

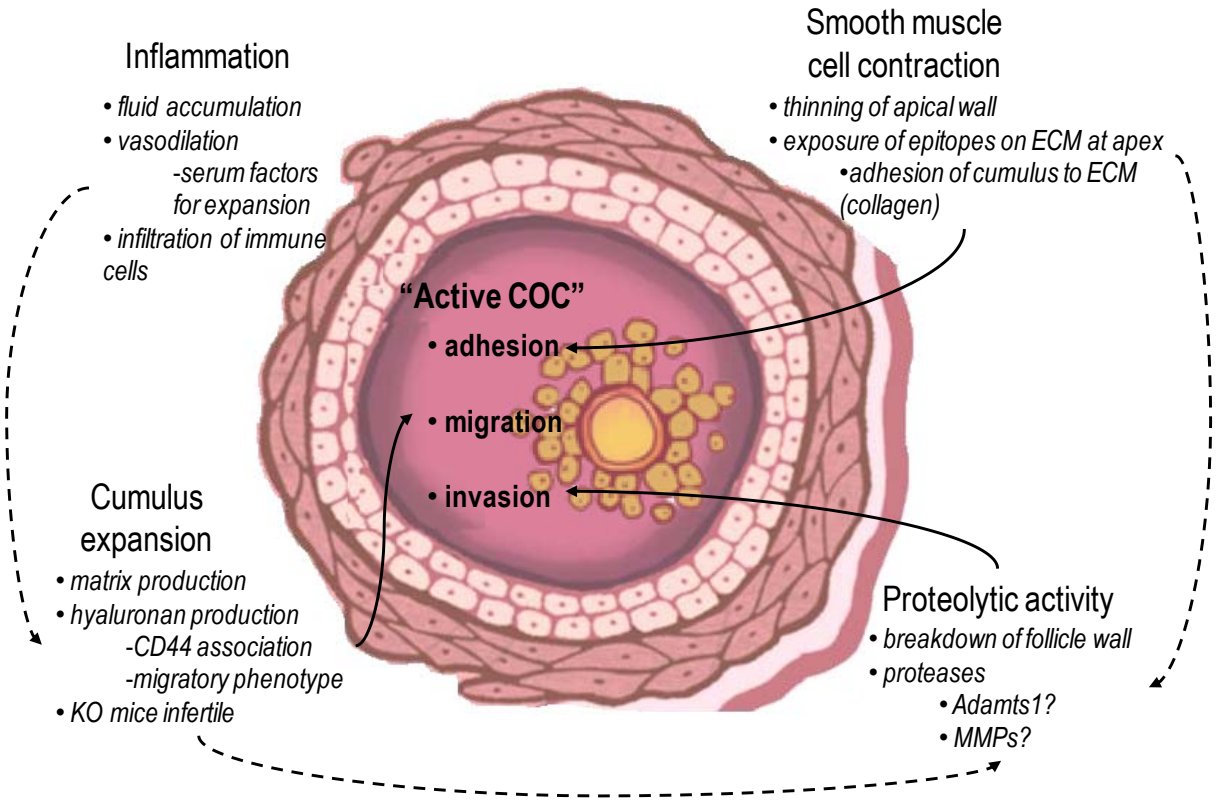

Figure 6.1 Schematic representation of ovulation hypotheses including the novel "active cumulus oocyte complex" hypothesis.

Various mechanisms of ovulation have been ideated, including ovulation as an inflammatory response, the involvement of smooth muscle cell contraction and intrafollicular pressure, and proteolytic degradation of the follicular wall. Few studies have focused on the vital role of the COC despite studies suggesting that cumulus expansion and matrix production are crucial for ovulation. All components mentioned appear essential but not conclusively causative of ovulation. Our novel "active COC" hypothesis of ovulation integrates these theories however with a focus on the COC, which involves the COC transitioning to an adhesive, invasive migratory cell type.

knockout mouse models. Cumulatively these studies suggest the COC plays a much more active role than first considered.

I have shown that *CD44* expression is upregulated prior to ovulation and during expansion in mouse COCs and speculate that it is the interaction between CD44 and hyaluronan, a major component of the expanded COC matrix, which activates an intracellular mechanism in cumulus cells mediating their transition to a motile cell phenotype through the downstream targets of CD44. Alternatively, other mechanisms of cell migration machinery may be at play in the COC. Another receptor of hyaluronan may be involved in the transition of cumulus cells to a motile and invasive phenotype; for example Hyaluronan mediated motility receptor (Hmmr), which has also been identified to functionally compensate for CD44 mediated motility in other systems (Nedvetzki, et al., 2004, Turley, et al., 2002) will be discussed in more detail later in the discussion.

The COC must ovulate from the apex of the follicle to reach the oviduct. I propose that mechanistically, cumulus cells acquire adhesive properties and smooth muscle cell contraction contributes to ovulation by weakening the apex leading to the wall breaking down and the COC being expelled. I speculate that smooth muscle cells contract to reveal epitopes to the COC in the follicle wall, such as a collagen rich extracellular matrix, and the COC then adheres to the follicle wall with the ability to exhibit proteolytic activity to degrade the apex. I have shown that the COC has the capacity to adhere to collagen and other extracellular matrices (ECMs) following stimulation with ovulatory hormones. Proteolytic degradation has also been clearly implicated in the breakdown of the follicular wall and ovulation. This putative proteolytic activity is generally thought to be derived from the granulosa and/or theca cells however, the cellular source has not actually been definitively shown. Our model proposes that proteases on the surface of the COC mediate the proteolytic degradation of the follicle wall and indeed

the protease Adamts1, even though produced by granulosa cells, is localised to cumulus cells immediately prior to ovulation. Adamts1 has been implicated in tumourigenesis by promoting metastatic behaviour of tumour cells by proteolyic degradation of ECM (Liu, et al., 2005).Ipropose that using a similar mechanism but in a completely different context, Adamts1 could be contributing to ovulation by degrading the ECM of the follicle wall.Ialso demonstrated that periovulatory expanded COCs were capable of invading an extracellular matrix, and that this invasion was comparable to invasive breast cancer cell lines. These findings further suggest that cumulus cell migration may be similar to cancer cell metastasis.

I showed the upregulation of the standard form of *CD44*, which is able to bind hyaluronan. A major component of the ECM of the expanded COC is hyaluronan, and it has been demonstrated that CD44 binding to hyaluronan correlates with tumour cell invasiveness (Bourguignon, et al., 2006, Bourguignon, et al., 2007) and reviewed in (Bourguignon, 2008). Results in this thesis have shown the migratory machinery to be present and regulated prior to and during ovulation, and that the COC transitions to a motile cell phenotype.

These results culminate in a convincing argument for an adhesive migratory phenotype for the cumulus cells during ovulation. Following the LH surge, the CD44 migration pathway is switched on in COCs, and although there is not yet evidence confirming its importance in directing ovulation, the upregulation of the pathway components corresponds with the migratory phenotype transition of the cumulus cells. Through binding to the ECM of the apical wall the COC may be able to facilitate degradation of the follicle wall by producing proteolytic enzymes, invasive behaviour eventually resulting in its release from the follicle.

6.2 Future Directions

My research, investigating whether the COC plays an active role during ovulation, discovered that the COC has the capacity to transition to a migratory cell type, which is able to invade an extracellular matrix in response to ovulatory hormones.

I have also shown that expanded COCs are adherent to various extracellular matrices, in particular collagen, found in the wall of the ovarian follicle. These results cumulatively show that the COC may play a much more dynamic role than first considered. Further investigation of the migration pathway involved may reveal the vital components for the migratory transition. Furthermore, a comprehensive time-course of COC migration would be useful in determining the time point during the ovulatory cascade that the cumulus cells transition to a migratory cell type and how long this is sustained.

Further exploration of the mechanics of the cumulus cell migration are warranted including investigation into whether the transition represents an epithelial to mesenchymal transition (EMT). If cumulus cells are considered an epithelial cell type, as is suggested, then their transition to a mesenchymal cell type may be due to EMT.Ihave shown that following hCG stimulation cumulus cells initiate cell migration and show increased association with extracellular matrices, specifically via adhesion to collagen, typical of a mesenchymal phenotype. An interesting study would be to determine polarity in cumulus cells following ovulatory stimulus, as a loss of polarity is characteristic of EMT. Further, a study investigating EMT markers in the ovary, including epithelial marker E-cadherin and mesenchymal markers, such as N-cadherin and vimentin, would offer insight into whether cumulus cells do have the capacity of EMT. The marker E-cadherin has been associated with non-metastatic MCF-7 breast cancer cells, whilst the markers N-cadherin and vimentin were found to be present in the aggressive MD-MB-231 breast cancer line, whilst E-cadherin expression was low (Uchino, et al., 2010).

A detailed investigation which examines the adhesive properties of the COC would be a worthwhile next step. Exploring integrin expression within the ovary during ovulation could offer valuable insight into this process. Furthermore, this knowledge would allow us to then block these components in the in vitro adhesion assay system to determine their importance in the adhesive phenotype of cumulus cells. Similarly, additional experiments, which focus on the localisation pattern of extracellular matrices in the ovary, would be important in determining the interaction between integrins and particular ECMs during ovulation. Given the results of the adhesion assays we might suggest the collagens to be vital in this process, however detail on the localisation of this and other ECMs is lacking in the literature.

A targeted approach to examine the effects of blocking ovulation in vivo would be interesting to determine whether CD44 specifically is involved in this process. Whilst CD44 null mice have been reported to be viable, their reproductive capacity has not been studied extensively (Protin, et al., 1999). Experiments which focus on blocking CD44 in vivo at the time when cumulus cell migration is initiated, as determined by comprehensive time-course studies, would be useful in determining the contribution of the CD44 pathway. Further, it may be useful to examine alternative pathways including another hyaluronan receptor Hmmr. Although CD44 and Hmmr coexist in some cell types, CD44 is able to out compete Hmmr for HA binding, however when CD44 is absent Hmmr has been reported to maintain cell motility (Nedvetzki, et al., 2004). Hmmr has been found expressed and localised in COCs, although mRNA expression was steady compared to CD44, which was upregulated during in vitro maturation of COCs, (Schoenfelder and Einspanier, 2003) perhaps any cell migrationIobserve when blocking CD44 in vitro is due to functional compensation by Hmmr.

Detailed analysis of in vivo localisation of molecular components of cell migration pathways in the ovary would be useful to correlate with cell migration time-course data, to determine exactly when this

pathway is switched on, how long it is sustained and in what ovarian cell types. Determining where CD44 is localised within the mouse ovary during ovulation would be important in elucidating the role of this molecule during ovulation. Especially given CD44 can undergo post-translation modification to produce variant isoforms with different modes of action (Lesley, et al., 1997). Coupling these investigations with studies of proteolytic degradation and a detailed time-course of cumulus cell adhesion, would build on our hypothesis of cumulus cell migration, invasion and adhesion, and assist in elucidating what is occurring in vivo during ovulation in the mouse. In addition, in vivo administration of compounds to simultaneously block multiple migratory pathways, may help further elucidate the role of cumulus cell migration and invasion in ovulation.

Cumulus cells may be responding to a signal from a chemoattractant and thus displaying directed cell migration, known as chemotaxis. The chemoattractant may be coming from the apical wall or even from outside the follicle, perhaps the oviduct, signalling the expanded COC to ovulate at the follicle apex. It is less likely but possible that the cumulus cells may be displaying random cell migration or chemokinesis, whereby a cell responds to a signal and makes a change in relation to its migration, not necessarily in any particular direction. This could be happening in the ovarian follicle whereby the expanded COC is responding to a signal coming from within the follicle - perhaps follicle fluid or prostaglandins- and switching on its migratory mechanism. This scenario of random cell migration is less likely than directed cell migration as ovulation is a precise process involving directionality. Certainly, further investigation is required to determine whether cumulus cell migration is the result of chemotaxis or chemokinesis

6.3 Significance

Ovulation remains fundamental to the reproductive success of all mammalian species, however the cellular processes regulating the event are not completely understood. What is emerging however is

that the COC appears to play an essential role and I hypothesised that, via transitioning to a motile and invasive cell type, it could actively facilitate its own release from a follicle. The widely accepted mechanisms of ovulation are highlighted in Figure 6.1. Our work however, incorporates the role of the COC into these processes and suggests that the invasive migratory phenotype of the COC also contributes to ovulation (Figure 6.2). This novel property of the COC could possibly control its release during ovulation; and offers a new perspective on this fundamental reproductive process. The further identification of crucial events involved in the ovulation process, and specifically the involvement of the cumulus cells and their associated matrix could be important for future implications for treatment of infertility but also a possible contraceptive target for medication to inhibit the process. Research into mechanisms of ovulation could offer valuable insight into the development of therapeutic targets. Anovulation is a major cause of infertility for women, with more than 50% of infertility in women attributed to an ovulatory defect (Healy, et al., 1994). On the contrary, vast numbers of women across the world block ovulation by using hormonal therapies such as the oral contraceptive pill. Thus, ovulation is one process which is of great concern to the female population, with women either wanting to prevent the process or many unfortunately suffering from infertility caused by anovulation.

Understanding the basic mechanisms involved in ovulation may enable us to develop new strategies for targeting drugs to block the key regulatory pathways, avoiding the long-term ingestion of steroid hormones for contraception. Conversely, new information about the basic mechanisms of ovulation may inform new methods for ovulation induction that would avoid the use of exogenous hormones, as well as the associated complications and large financial cost of the gonadotropin stimulation portion of ART treatment.

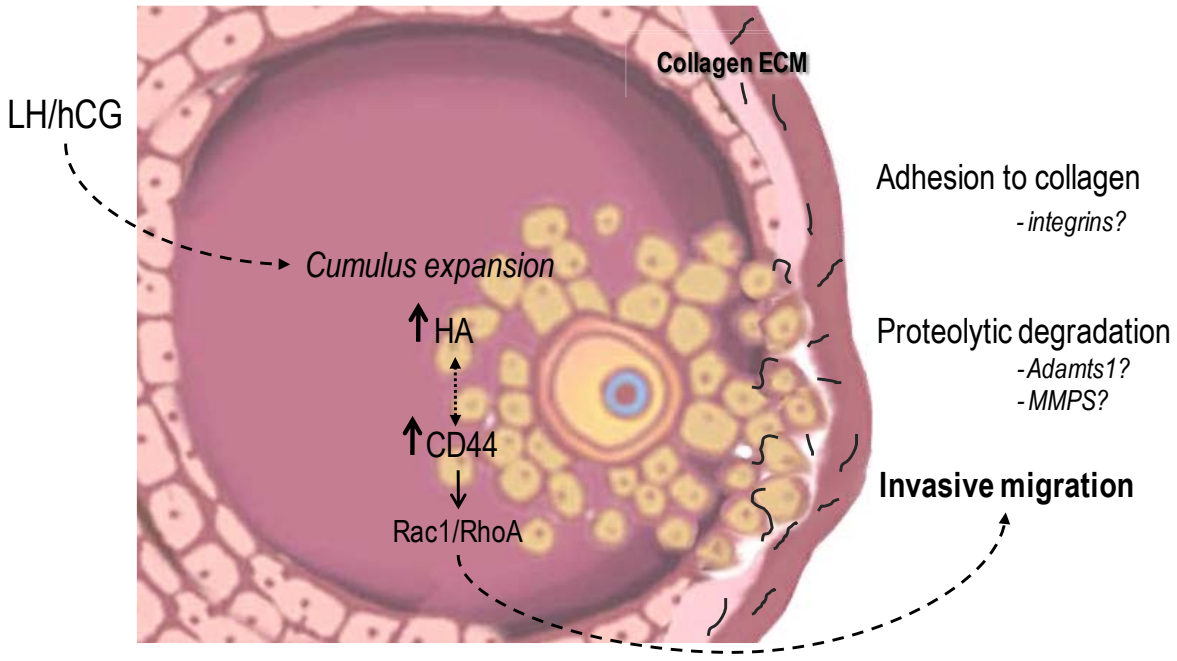

Figure 6.2 Schematic representation of the "active cumulus oocyte complex" hypothesis following an ovulatory stimulus

I have shown that following an ovulatory stimulus cumulus cells have the capacity to transition to an adhesive motile cell type, capable of invading an extracellular matrix (ECM) barrier. Following the LH surge, or hCG administration, cumulus expansion, including hyaluronan synthesis, is initiated.I have shown the HA receptor CD44 is upregulated following the LH surge which may lead to intracellular activation of its downstream targets, Rac1 and RhoA. Whether these GTPases are responsible for the observed cell migration is unknown.I have also observed increased cumulus cell adhesion to ECMs, predominantly collagen, following hormonal stimulation.I reported in Chapter 6 that cumulus cell invasion was comparable to an invasive cancer cell line, but whether proteases such as Adamts1 or MMPs are causing this degradation is unclear.

In a broader sense, it could be that by controlling its own release during ovulation the COC is the final checkpoint whereby only the most competent oocytes are ovulated. This concept is supported by some evidence. Firstly, secretion of factors from the oocyte control cumulus matrix expression (Eppig, 1980, Gilchrist, et al., 2008, Joyce, et al., 2001, Salustri, et al., 1992, Varani, et al., 2002). Further, several studies have linked the degree of cumulus expansion as well as the expression of cumulus matrix genes with oocyte quality, i.e. its ability to be fertilised and establish a pregnancy (Adriaenssens, et al., 2010, Ball, et al., 1983, Cillo, et al., 2007, McKenzie, et al., 2004, Somfai, et al., 2004, Zhang, et al., 2005). Lastly, based on the phenotypes of null mouse models the cumulus matrix is clearly essential for ovulation (Brown, et al., 2010, Fulop, et al., 2003, Mittaz, et al., 2004, Varani, et al., 2002, Zhuo, et al., 2001) . Cumulatively these observations suggest that only high quality oocytes with good developmental potential exhibit robust expansion, and that by inducing its cumulus matrix an oocyte thus determines its own ovulation.

The observations documented in this thesis support this concept by identifying mechanisms via which this could occur; by documenting that the COC has adhesive, migratory and invasive properties specifically at ovulation.

Chapter 7 Appendix

7.1.1 Rac1 activation pull-down assay

The amount of active Rac1 protein present in a sample was determined by a Rac1 activation pull-down assay (Chemicon, Temecula, CA, USA). Active GTP-bound Rac1 was detected by probing for Rac1 protein by western blot following a pull-down assay, as per manufactures instructions. Briefly, Pooled cumulus oocyte complexes were washed in tris-buffered saline (TBS) (10mM Tris [pH 7.5], 150mM NaCl) and pelleted with gentle centrifugation, supernatant was discarded and 1mL ice cold MLB (125mM HEPES, pH 7.5, 750mM NaCl, 5% Igepal CA-630, 50mM $MgCl_2$, 5mM EDTA, 10µg/mL leupeptin (Sigma), 10µg/mL aprotinin (Sigma), 10% glycerol) added to cell pellet to lyse cells by repeated pipetting. Lysates were transferred to microcentrifuge tubes on ice and 100µL glutathione agarose was added, and lyastes incubated for 10 min at 4°C with gentle agitation. Agarose beads were collected by pulsing for 5 seconds at 14,000g. For positive and negative controls, 0.25mL of cell extract was aliquoted to microcentrifuge tubes and 10µL of 0.5M EDTA added to each tube. For the positive control 5µL of GTPγS (to activate all Rac1 protein) was added to the cell lyaste, for negative control 5µL of GDP (to deactivate all Rac1 protein) was added to the cell lysate. Controls were incubated at 30°C for 15 min with agitation. For the Rac1 pull-down assay, 0.5mL of cell lysate was added to a microcentrifuge tube with 10µL of Rac/cdc42 Assay reagent (PAK-1 PBD, agarose). Lysate was incubated at 4°C for 60 min with gentle agitation. Once all lysates had been incubated, agarose beads were collected by pulsing for 5 seconds at 14,000g. The supernatant was discarded and beads were washed 3 times with 0.5mL MLB. Agarose beads were resuspended in 40µL of 2X Laemmli reducing sample buffer (126mM TRIS/HCl; 20% Glycerol; 4% SDS; Bromophenolblue; 2-mercaptoethanol; pH 6.8) and boiled for 5 min then collected by microcentrifuge pulse and used in the western blot analysis for Rac1.

7.1.2 Western blot detection of active Rac1

The supernatant and agarose pellet from the Rac1 pull-down were mixed and 20µL of each sample was separated by electrophoresis in 4-15% gradient reducing polyacrylamide gels prestained protein Mwt markers (Bio-rad Laboratories, Sydney, NSW). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed and protein was transferred to polyvinylidene diflouride (PVDF) membrane (Immobilon, Millipore Corp.), which was washed twice with water. Blotted PVDF membrane was blocked in phosphate-buffered saline (PBS; 80mM Na_2HPO_4, 20mM NaH_2PO_4, 100mM NaCl) with 3% non-fat dry milk (PBS-MLK) for 30 min at room temperature with constant agitation. Following, nitrocellulose was then incubated with 1µg/mL of anti-Rac1 clone 23A8 (Chemicon, Temecula, CA, USA) (diluted in PBS-MLK) overnight at 4°C with agitation. Nitrocellulose was washed twice with water and then incubated with goat anti-mouse HRP conjugated IgG (Chemicon, Temecula, CA, USA) (in PBS-MLK) for 1.5 h at room temperature with agitation. Nitrocellulose was washed twice with water and then in PBS-0.05% Tween (PBST) followed by rinsing in water. Protein visualisation was performed using enhanced chemiluminescence (ECL) western blot detection kit (GE healthcare) according to manfacturer's instructions.

7.2 IDENTIFICATION OF ACTIVE GTP-BOUND RAC1 DURING OVULATION

Following identification of Rac1 and Tiam1 mRNA I wanted to investigate whether Rac1 was in fact activated in COCs at the time of ovulation. To do this I utilised a Rac1 activity assay whereby active GTP-bound Rac1 was identified using a pull-down assay. Figure 7.1 shows a western blot for Rac1 protein. Samples of untreated COCs and 12 h hCG COCs were probed for total Rac1 protein (lanes 1 and 2) or just the active form in a pull-down assay (lanes 3 and 6). Included were positive and negative controls, which were samples where Rac1 was either activated or deactivated by GTP or GDP

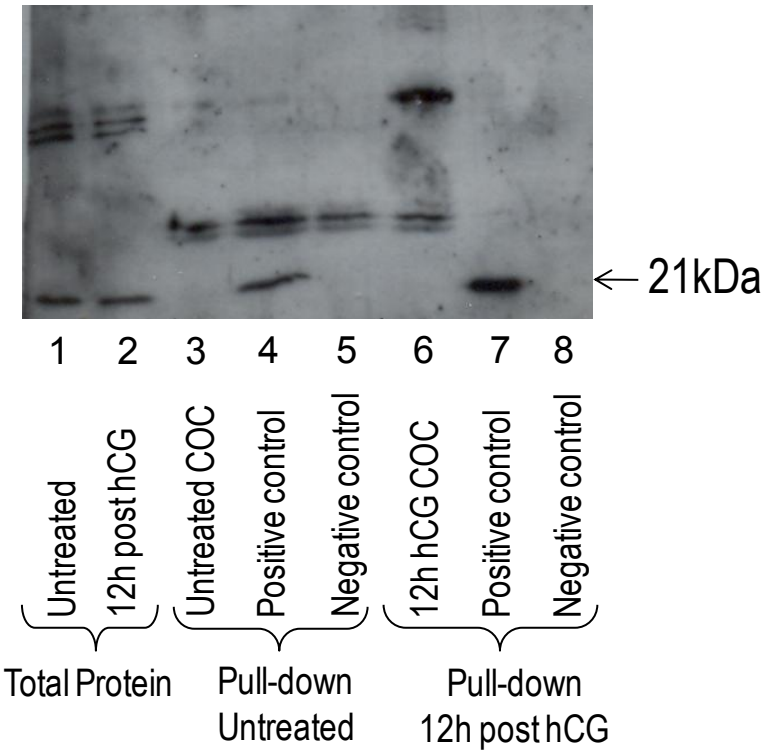

Figure 7.1 Rac1 activation pull-down assay

Pull-down assay to determine activation state of Rac1 protein in untreated and 12h post hCG cumulus oocyte complexes. A band corresponding to Rac1 ~21kDa, was detected in untreated COCs (lane 1, *black arrow*) and 12h post hCG COCs (lane 2, *black arrow*), which was a measure of protein present in the samples. Lanes 3 – 8 depict the pull-down assay to detect active Rac1 protein. A band corresponding to Rac1 protein ~21kDa was detected in positive controls for both untreated COCs (lane 4, *black arrow*) and 12h hCG COCs (lane 7, *black arrow*). No band corresponding to Rac1 was detected in negative control samples of either cell type (lanes 5 and 8).

exchange, respectively. A band corresponding to Rac1 ~21kDa was present in positive control lanes (lanes 4 and 7).

Although there are no bands present in either experimental sample in Figure 7.1, when the experiment was repeated a very faint positive band was identified in COCs at 12 h post hCG, when ovulation would usually occur (data not shown). However, this assay was not easily reproducible due to technical difficulties and the large quantities of protein required. Thus no conclusive information was obtained about regulation of Rac1 activity in the COC.

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