Regulation of Cumulus Expansion: Role of Oocyte-Secreted Factors and Transforming Growth Factor β Superfamily Signalling

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A thesis submitted to the University of Adelaide in total fulfilment of the requirements for the degree of Master of Medical Science

March 2006

Contents

Abstract

Oocyte paracrine signalling to cumulus cells (CCs) is essential for expansion of the mouse cumulus-oocyte complex (COC), which is required for ovulation and hence fertility. Previously in the field, researchers have focused on members of the transforming growth factor β (TGFβ) superfamily in order to identity the oocyte-secreted factor(s) (OSF) that account for the mouse cumulus expansion-enabling factor (CEEF). Currently, growth differentiation factor 9 (GDF9) is the prime candidate molecule for the elusive mouse CEEF.

This thesis exploits a range of experimental approaches and tools to characterise the mouse CEEF and the signalling pathways involved in regulating cumulus expansion. A major component of this thesis is a thorough investigation into the role of GDF9 in the regulation of cumulus expansion. Both GDF9 and oocytes stimulated expansion of oocytectomised (OOX) complexes. GDF9-induced expansion was completely abolished when treated with a specific monoclonal GDF9 neutralising antibody, mAb-GDF9-53. On the contrary, oocyte-induced cumulus expansion was unaffected. Moreover, examination of TGFβ and activin A and B revealed that none of these TGFβ superfamily members account for the CEEF.

To analyse the signalling pathways used by the CEEF, the requirement for the classic TGFβ signalling cascade in CCs was examined. A synthetic portion of the extracellular domain of the GDF9/bone morphogenetic protein type-II receptor (BMPR-II) partially attenuated oocyte-induced cumulus expansion. Furthermore, the small molecule kinase inhibitor, SB-431542, a specific antagonist of activin receptor-like kinase (ALK) 4/5/7, was tested for its ability to neutralise cumulus expansion. Treatment with SB-431542 completely abolished oocyte-induced expansion and significantly attenuated oocyte-induced OOX hyaluronan synthase-2, cyclooxygenase-2, tumour necrosis factor-α-stimulated gene-6 and pentraxin-3 mRNA expression.

The work presented in this thesis has provided an insight into the identity of the OSFs that account for the mouse CEEF. Collectively, these findings provide evidence that GDF9 alone does not account for the CEEF and suggests that the CEEF is comprised of multiple TGFβ superfamily members. Moreover, these results demonstrate that the CEEF signals via the SMAD 2/3 signalling cascade and utilises the BMPR-II amongst other receptors, to enable cumulus expansion.

Declaration

This thesis contains no material that has been accepted for the award of any other degree or diploma in any university or other tertiary institution except where cited in Chapter 2, which includes results, previously examined for the award of BSc. Hons and is included in the current thesis for completeness only. To the best of my knowledge and belief this thesis contains no material previously published or written by another person except where due reference has been made in the text.

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March 2006

Rebecca Dragovic

Acknowledgements

I must first offer my sincere thanks to the principal supervisor Dr. Robert Gilchrist, for allowing me the opportunity to undertake a Masters, in a field of research that I have grown to enjoy immensely. The completion of this Masters has been a challenging journey, but at the same time an enjoyable one. Many thanks for your support and enthusiasm for the project and for dealing with my so-called "quick" chats that would end up lasting for hours. You have been a fantastic mentor.

I would also like to thank my co-supervisor Prof. David Armstrong for additional support and guidance throughout this Masters project. You have brought extra expertise and knowledge to this work, and I am most grateful.

I must also give an enormous thankyou to Lesley Ritter for just about EVERYTHING! You are such a great asset to our lab and you have always been happy to dedicate so much of your time to help me with various experimental techniques. I will miss our morning chats at the microscopes and working with you in the lab. To me, you have been more than just a work colleague you are a great friend.

Thanks to all members of the Gilchrist/Armstrong group. A special mention should go to Samantha Schulz for assisting with experiments (you have truly become the expert cumulus expansion assessor) and for always providing a friendly smile around the lab. I would also like to thank Theresa Hickey, who has always been willing to help me, be it with experimental problems, or in the preparation of presentations and manuscripts. Thanks also to Fred Amato for purifying our supply of GDF9 and for helping me try my hand at Western blots. I am also thankful to Drs. Jeremy Thompson and Darryl Russell, who have offered great advice during the course of this project. A big thankyou to all the animal house staff, especially Adrian Hines (I feel that it will be the year of the crow)!

I must also thank my family for all the love and support they have provided throughout the course of my Masters. Mum and Dad, I am so fortunate to have such wonderful supportive parents and I appreciate everything that you have done to help me along the way. Thanks also to my brother Matt and sister-in-law Bec for all your love and support over the years. Thanks Bec for not just being a great sister-in-law, but for being such a great friend too and for

accompanying me on all of those shopping trips. I can't wait for you to visit me in Sydney soon so we can make up for all our lost shopping opportunities over the past few months.

Last, but by no means least, I must give a HUGE thankyou to my very supportive partner Steve. I am forever grateful for your continuous love and encouragement throughout my Masters. Thankyou for everything you have done for me, especially in these last few months. I love you very much and could not have completed this thesis without your support.

Abbreviations

293H	293 human embryonic kidney cell line
ActR-II	activin receptor type-II
ALK	activin receptor-like kinase
AR	amphiregulin
BMP	bone morphogenetic protein
BMPR-II	bone morphogenetic protein receptor type-II
BSA	bovine serum albumin
BTC	betacellulin
cAMP	cyclic adenosine monophosphate
CC	cumulus cell
CEEF	cumulus expansion-enabling factor
COC	cumulus-oocyte complex
COX2	cyclooxygenase-2
DO	denuded oocyte
DMSO	dimethyl-sulfoxide
eCG	equine chorionic gonadotrophin
EP_2	prostaglandin receptor subtype 2
EPI	epiregulin
ECD	extracellular domain
ECM	extracelluar matrix
EGF	epidermal growth factor
FCS	fetal calf serum
FSH	follicle stimulating hormone
GC	granulosa cell
GDF9	growth differentiation factor 9
GV	germinal vesicle
GVBD	germinal vesicle breakdown
HA	hyaluronan
HAS2	hyaluronan synthase-2
H-TCM-199	HEPES-buffered tissue culture medium-199
IVM	*in vitro* maturation
L19	ribosomal protein L19

LH	luteinising hormone
mAb-GDF-53	GDF9 monoclonal neutralising antibody
MAPK	mitogen activated protein kinase
MGC	mural granulosa cell
OOX	oocytectomised complex
OSF	oocyte-secreted factor
PG	prostaglandin
PTX3	pentraxin-3
rhFSH	recombinant human FSH
SB-431542	ALK4/5/7 kinase inhibitor
SMAD	mothers against decapentaplegic
TGFβ	transforming growth factor β
TGFβR-II	transforming growth factor receptor type-II
TSG6	tumour necrosis factor-α-stimulated gene-6
WAY	waymouth MB 752/1 medium

Scientific Publication Record

1. **Dragovic RA**, Ritter LJ, Amato F, Schulz SJ, Armstrong DT and Gilchrist RB. (2005) Role of oocyte-secreted growth differentiation factor 9 in the regulation of mouse cumulus expansion. *Endocrinology* 146(6): 2798-2806. (Cited 2 times, Impact Factor 5.06).

2. Gilchrist RB, Ritter LJ, Myllymaa S, Kaivo-Oja N, **Dragovic RA**, Hickey TE, Ritvos O and Mottershead DG. Molecular basis of oocyte-paracrine signalling that promotes granulosa cell proliferation. *J.Cell Sci.* (Submitted 20/03/06).

3. **Dragovic RA**, Ritter LJ, Schulz SJ, Thompson JG, Armstrong DT and Gilchrist RB. Oocyte-secreted factor activation of SMAD 2/3 signalling enables mouse cumulus expansion. Manuscript to be submitted to *Dev. Biol.* March 2006.

Other Journal Contributions

Dragovic RA, Ritter LJ, Schulz SJ, Armstrong DT and Gilchrist RB. (2005) Mouse oocyte paracrine signalling to cumulus cells by TGFβ superfamily molecules is indispensable for cumulus expansion. *Reprod. Fertil. Dev. 17 (Suppl):* 231 (Oral presentation).

Dragovic RA, Ritter LJ, Amato F, Scott SJ, Cranfield M, Groome N, Armstrong DT and Gilchrist RB. (2004) Regulation of mouse cumulus expansion by oocyte-secreted growth differentiation factor 9. *Reprod. Fertil. Dev. 16 (Suppl):* 251 (Oral presentation).

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Dragovic RA, Ritter LJ, Schulz SJ, Armstrong DT and Gilchrist RB. (2005) Mouse oocyte paracrine signalling to cumulus cells by TGFβ superfamily molecules is indispensable for cumulus expansion. *Proceedings of the North Western Adelaide Health Service Research Day.* Abstract 11. (Oral presentation).

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Provisional Patents

Hussein T, Gilchrist RB, Thompson JG, **Dragovic RA**. "Modulation of granulosa cell apoptosis" Australian Provisional Patent Application No. 2005903782. Filed 18/7/2005.

Scholarships, Prizes and Awards

2004-06	Awarded Faculty of Health Science Postgraduate Research Scholarship.
2004-06	Awarded The Queen Elizabeth Hospital Research Foundation Postgraduate Research Scholarship.
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2004	Research Centre for Reproductive Health Travel Award.
2004	Winner of the "Higher Degrees Junior Laboratory Prize" Competition, The Queen Elizabeth Hospital Research Day.
2004	Finalist in the New Investigator Award, Society for Reproductive Biology.
2004	Society for Reproductive Biology Travel Award.

Chapter 1

Literature Review

1.1 Introduction

Growth and development of the ovarian follicle occurs in a remarkably structured manner and is dependent upon bi-directional communication between the germ cell and somatic cells. Endocrine, paracrine and autocrine signalling are all essential for coordinating the growth and development of the ovarian follicle. Folliculogenesis begins with a large population of non-growing primordial follicles, from which oocytes are recruited until the end of reproductive life. Primordial follicles are continually recruited to develop into primary, secondary (pre-antral), early tertiary (peri-antral), late tertiary (antral) and eventually preovulatory follicles (Fig. 1). The primordial to secondary stage of follicle growth and development is independent of the gonadotrophins follicle-stimulating hormone (FSH) and luteinising hormone (LH), but thereafter there is an absolute requirement for gonadotrophins. A preovulatory follicle consists of a fluid filled antrum and two distinct sub populations of granulosa cells; the mural granulosa cells (MGCs), the cells lining the follicle wall; and the cumulus cells (CCs), the cells which intimately surround the oocyte, joined together by an intricate gap-junction network, forming the cumulus-oocyte complex (COC). MGCs and CCs each have their own functional characteristics and in response to the preovulatory surge of gonadotrophins undergo distinctive changes. In response to the LH surge, the CCs synthesise hyaluronan (HA), enabling them to disperse away from the oocyte and form a mucoid extracellular matrix (ECM). This process is referred to as cumulus expansion or cumulus mucification. Cumulus expansion causes the COC to disengage from the follicle wall, accompanied by further enlargement of the follicle forming a distinct bulge at the surface of the ovary. At ovulation, proteolytic enzymes secreted by the follicle cause the follicle wall to rupture, resulting in the release of the expanded COC (Fig. 1). MGCs respond to LH via LH receptors in a very different manner compared to that of the CCs. MGCs luteinise to become luteal cells. In conjunction with the theca cells they form mature corpus luteum and produce large amounts of progesterone, which is a requirement for embryo implantation and a successful pregnancy.

The unique structure of the COC complex permits communication between the oocyte and surrounding CCs to control oocyte growth and development. The oocyte and the CCs communicate via two distinct mechanisms; 1) communication occurs via gap-junction transmembrane channels and 2) by paracrine signalling (Fig. 2). The CCs penetrate the zona pellucida via trans-zonal cytoplasmic projections and adjoin the oocyte

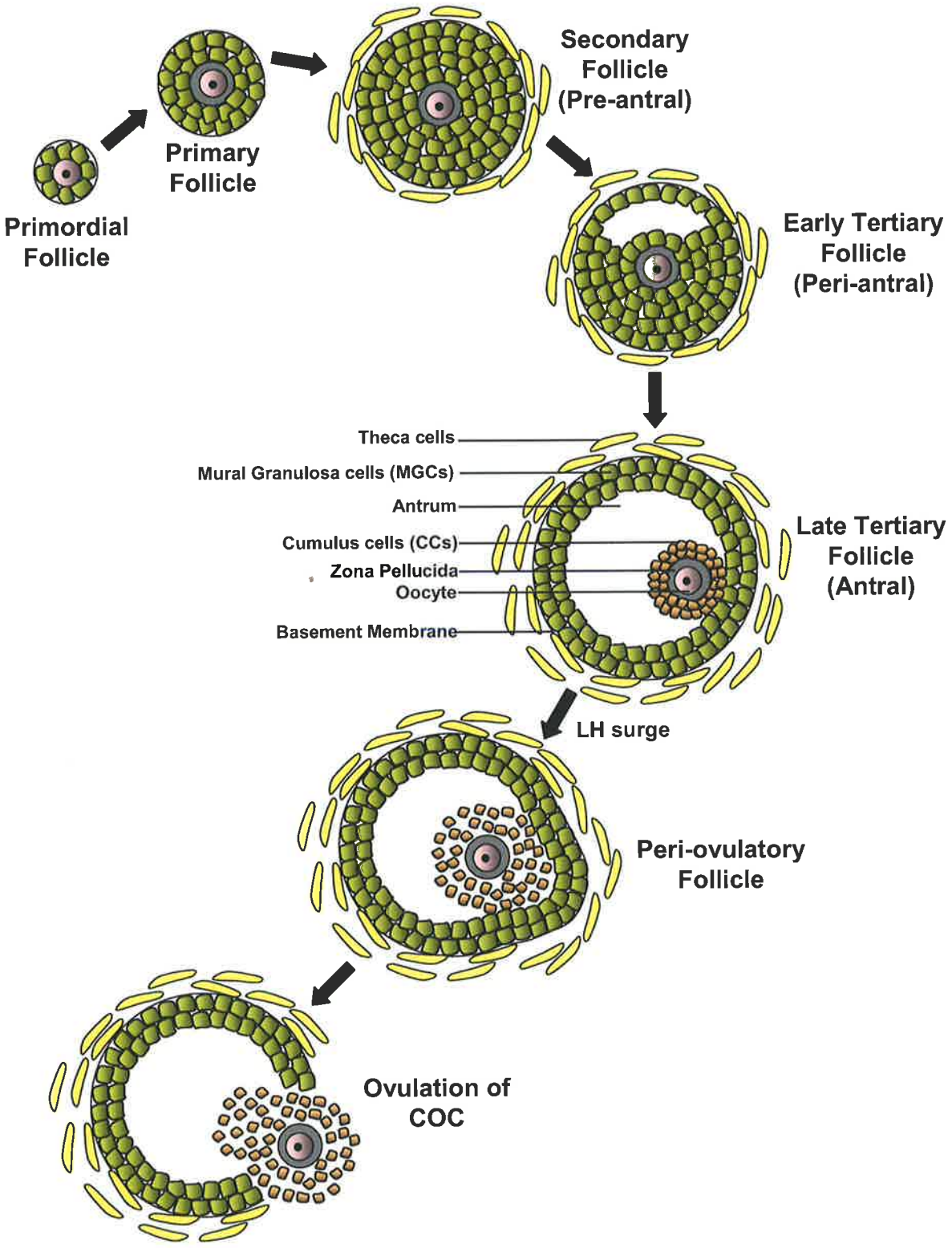

Figure 1. Schematic representation of folliculogenesis and ovulation. Follicles begin as primordial follicles and successively progress through to the antral stage of development. Antral follicles are comprised of 1) an oocyte intimately surrounded by specialised cells called CCs, forming the COC, 2) MGCs line the follicle wall, 3) theca cells surrounding the basement membrane and 4) a central fluid-filled cavity called an antrum. In response to the LH surge the COC undergoes expansion, a process critical for follicle rupture, ovulation and fertilisation.

membrane (1). Gap-junction transmembrane channels at the end of these projections provide a communication axis for low molecular mass molecules (< 1000Mr) including amino acids, nucleotides, metabolites and cyclic adenosine monophosphate (cAMP). The second form of communication occurs via paracrine signalling, this is a bi-directional communication axis between the oocyte and CCs (Fig. 2). Originally, the oocyte was considered to be a passive participant in the regulation of its growth and development. However, over the past 15 years it has become obvious that the oocyte actively influences the normal development of its own follicle. For example, in the absence of the oocyte the CCs fail to undergo expansion (2).

The identification of oocyte-secreted paracrine factors that regulate various GC and CC processes has become the focal point of numerous studies. Majority of the research has focused on members of the transforming growth factor β (TGFβ) superfamily, which consists of over 40 structurally related, yet functionally different proteins. Family members including; TGFβs, growth differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15), BMP6 and activins, have all been shown to be able to mimic the oocyte and control various GC and CC activities *in vitro* [reviewed in Ref. (3)]. GDF9, BMP15 and BMP6 are all expressed by the oocyte and for that reason an emphasis has been placed on these few molecules.

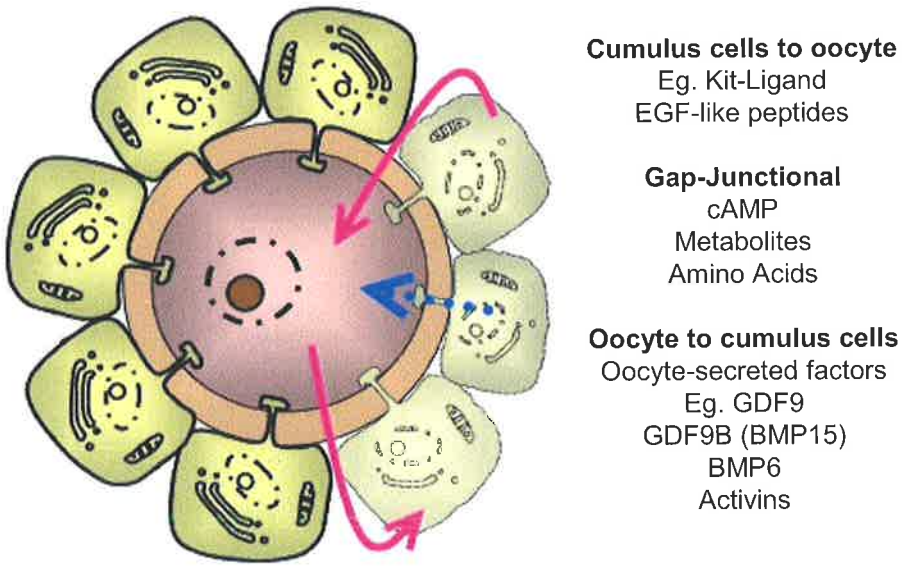

Figure 2. Oocyte-cumulus cell communication. A bi-directional communication axis exists between the oocyte and surrounding CCs to regulate oocyte growth and development. Communication occurs via gap-junction transmembrane channels, allowing the transport of small regulatory molecules (straight arrow) and via paracrine signalling (curved arrows). Adapted and modified from (4).

This review will firstly examine the relationship between the oocyte and surrounding CCs and for the most part focus on the regulation of cumulus expansion by oocyte-secreted paracrine factors. Secondly, CC genes and the specific components of the cumulus oocyte ECM that appears to be crucial for ovulation and fertilisation will be discussed. Finally, this review will examine various members of the TGFβ superfamily with reference to their role in folliculogenesis and regulating such processes as cumulus expansion.

1.2 Cumulus Expansion

Prior to ovulation *in vivo*, the preovulatory follicle undergoes specific transformations in response to the LH surge that are essential for successful release of the oocyte and subsequent fertilisation. One of the most significant transformations that occur is cumulus expansion, a complex process that occurs in unison with oocyte maturation. The CCs surrounding the oocyte produce HA, which expands the spaces in between the CCs and embeds them into a spherical mucoid ECM. This process can be mimicked *in vitro* by culturing the COC with FSH, cAMP and the cAMP analogues, epidermal growth factor (EGF) and EGF-like peptides (5-8). Expansion causes an obvious enlargement of the COC, resulting in a 20-40 fold increase in the initial volume of the cumulus mass (Fig. 3).

The oocyte itself has been shown to play an essential role in the regulation of cumulus expansion in rodents (2, 5, 9), but plays a lesser role in ruminants (9-12). In the absence of the oocyte, mouse and rat CCs cannot undergo expansion, demonstrating they are dependent on an oocyte-paracrine factor(s), referred to as the cumulus expansion-enabling factor (CEEF). The CEEF is not required for cumulus expansion in ruminants, although it is secreted (11, 12).

Cumulus expansion is associated with the breakdown of the gap-junctional network between the oocyte and CCs. Maintenance of this gap-junctional communication up until cumulus expansion is important for the acquisition of developmental competence of the oocyte. Disruption of this network prior to cumulus expansion can affect the competence of the oocyte to undergo normal fertilisation, as oocyte growth and maturation will be severely affected. For example, oocyte development arrests prior to achieving meiotic competence in mice deficient for the ovary-specific gap-junction protein, connexin 37 (13). Cumulus expansion is also accompanied by oocyte maturation (14). As the CCs expand the oocyte resumes and completes meiosis I, characterised by germinal vesicle breakdown (GVBD) and

the extrusion of the first polar body. The oocyte then arrests at the metaphase stage of meiosis II where it awaits fertilisation by a spermatozoon.

At the time of ovulation, the expanded COC exits the follicle through the rupture site. The oviductal fimbria pick up the COC and guides it into the ampulla of the oviduct, ready to be fertilised. Assembly of the expanded CC mass surrounding the oocyte is necessary for female fertility (15). Cumulus expansion is required for the exit of the complex from the follicle and for pick-up by the fimbria. Failure of either of these processes results in fewer ovulations and oocyte transfer into the oviduct is compromised (16, 17). Mucification of the COC also appears to help guide sperm to the complex and favour fertilisation by providing the appropriate microenvironment. Sperm undergo an acrosomal reaction from which hyaluronidase is released from the sperm head, dispersing the CCs, allowing sperm to penetrate the oocyte (18).

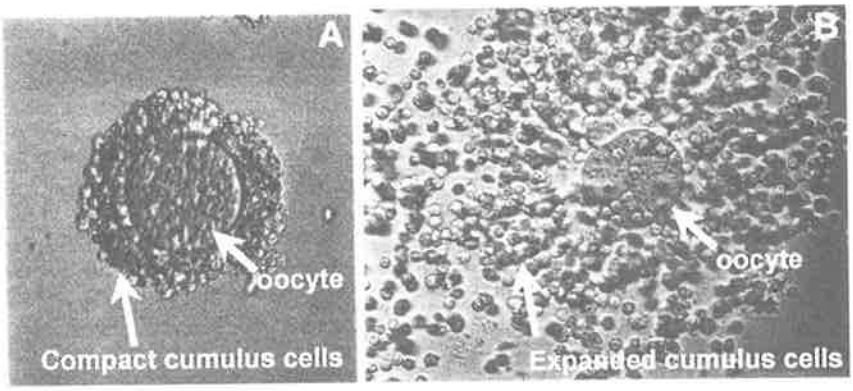

Figure 3. Cumulus expansion. (A) Mouse COC with compact layers of CCs. **(B)** Mouse COC has undergone prominent mucification and expansion of all CC layers. Figure adapted and modified from (19).

1.3 Cumulus Cell Extracellular Matrix Components and Ovulation

Multiple studies have shown that the formation of the ECM surrounding the oocyte is essential for female fertility (15-17, 20). The ECM protects the oocyte from proteolytic and mechanical stresses during follicle rupture and extrusion. The interaction of many proteins, including HA and several HA binding proteins are required for cumulus expansion and stabilisation of the ECM.

1.3.1 Hyaluronan

HA, a non-sulphated glycosaminoglycan of high molecular weight forms the principal structural backbone of the COC ECM (21). Hydration of HA expands the spaces between the CCs resulting in a gelatinous matrix surrounding the oocyte. HA synthesis in the CCs can be detected 2-3 hours after gonadotrophin stimuli in rodents. It reaches a maximum level at 4-10 hours after which time it begins to decline, reaching negligible levels by 18 hours (21). Fulop *et al.*, (22) showed HA synthesis is primarily controlled by the enzyme hyaluronan synthase-2 (HAS2) because of the strong correlation between the time frame of HAS2 mRNA expression and HA synthesis. Multiple HA-binding proteins, including; inter-alpha-trypsin inhibitor (IαI) (17), tumour necrosis factor-α-stimulated gene-6 (TSG6) (15, 23), pentraxin-3 (PTX3) (24), link protein (25) and versican (26) are all essential proteins involved in the organisation and maintenance of HA into a stable ECM.

1.3.2 Inter-alpha-trypsin inhibitor

IαI is described as a serum-derived protein that is primarily made by the liver and enters into the follicle during ovulation. IαI is composed of the light chain bikunin (also referred to as urinary trypsin inhibitor) and one or two of the three isoforms of heavy chains (HC1, HC2 and HC3), commonly referred to as serum-derived HA binding proteins (SHAPs). Bikunin covalently attaches to the SHAPs via a chondroitin sulphate chain. In order to stabilise the expanding ECM surrounding the oocyte, the heavy chains of IαI become covalently bound to HA via a transesterification reaction, releasing bikunin as a result (27). This provides a suitable explanation as to why *in vitro* culture of COCs requires serum or IαI to undergo expansion and organise HA into a stable ECM.

1.3.3 Tumour necrosis factor-α-stimulated gene-6

During the process of cumulus expansion, the HA binding protein TSG6 is accumulated into the ECM. TSG6 binds HA via its link module domain (28), forms stable complexes with the heavy chains of IαI during cumulus mucification (15, 29) and has also been shown to interact with chondroitin-4-sulphate side chains of proteoglycans, such as aggrecan and versican (26, 30, 31). Moreover, TSG6 is the protein accountable for a transesterification reaction, whereby the heavy chains of IαI are covalently transferred to HA (15, 29). In situ hybridisation studies of mouse ovaries revealed that TSG6 mRNA expression is rapidly induced in the CCs and the MGCs closest to the antrum, 4 h after an ovulatory dose of human chorionic gonadotrophin

(hCG). TSG6 mRNA levels remained high in the CCs 12 h after hCG, however mRNA levels in the MGCs were barely detectable (32). Varani *et al.*, (24) also examined TSG6 expression in the mouse ovary using in situ hybridisation, detecting TSG6 mRNA in both the CCs and MGCs as quickly as 1 h post hCG. TSG6 is only expressed after the preovulatory LH surge, suggesting that it plays no part in the early stages of folliculogenesis (33). In a subsequent study, Ochsner *et al.*, (34), demonstrated that TSG6 mRNA as well as protein are induced in COCs expanded *in vitro* in response to various cAMP stimuli. Furthermore, this study used a specific rat monoclonal antihuman TSG6 antibody, targeted to specific amino acids within the link module domain to examine the function of TSG6 in cumulus expansion. Using recombinant human TSG6 and recombinant human IαI, Western blot analysis not only showed that the antibody blocked HA from binding to recombinant TSG6, but it also showed that the antibody blocked the interaction of TSG6 with the heavy chains of the IαI and prevented proper cumulus expansion. The morphology of the cumulus expansion observed in these experiments was abnormal. In particular, as the CCs began to spread away from the oocyte, they began to detach from the matrix and disperse into the culture dish (34). These results provide evidence that formation and stability of the ECM surrounding the oocyte requires the interaction of TSG6 with IαI.

1.3.4 Pentraxin-3

PTX3 is a member of the Pentraxin superfamily of conserved proteins. To date, PTX3 is the only long pentraxin identified and is produced as a 10-20 subunit multimer protein. In addition to examining the localisation of TSG6 mRNA in the mouse ovary, Varani *et al.*,(24) examined PTX3 mRNA expression using in situ hybridisation. One hour after hCG, PTX3 mRNA was detectable in the MGCs that line the antral cavity. By 5 h post hCG, a few MGCs that line the antral cavity still expressed PTX3 and there was a prominent PTX3 mRNA signal apparent in the CCs. After 12 h post hCG, PTX3 mRNA expression was only localised to the expanding CCs. The incorporation of PTX3 in the expanding ECM is required to maintain HA molecules in the intercellular spaces, a process that is likely to be mediated by TSG6 (35). Interestingly, PTX3 mRNA was also detected in human CCs, along with the accumulation of the PTX3 protein in the ECM. Salustri *et al.*, (35) also made the observation that spermatozoa can bind both soluble and immobilised PTX3, suggesting a possible role for PTX3 in facilitating the interaction of sperm with the ECM during fertilisation.

1.3.5 Link Protein

The presence of link protein in the ECM of preovulatory follicles was discovered in 1999 (36, 37). This discovery directed the investigation into whether link protein played a stability role in the interaction between HA and IαI in the preovulatory follicle. The study carried out by Sun *et al.*, (25) used an *in vitro* culture system to test the function of link protein in the process of mouse cumulus expansion. Firstly, link protein was tested for its ability to induce cumulus expansion by substituting for IαI or serum. COCs cultured with IαI alone underwent cumulus expansion, however COCs cultured with purified link protein alone failed to expand. COCs cultured with both IαI and link protein improved the overall level of cumulus expansion in comparison to culturing COCs with IαI alone. Furthermore, COCs treated with a polyclonal neutralising link protein antibody, reduced cumulus expansion back to the level observed when culturing COCs with IαI alone, thus ablating the enhancing activity of link protein. Collectively, these results suggest that the role of link protein in the expanding ECM is to stabilise the IαI-HA complex required for expansion of the COC (25).

1.3.6 Versican

The proteoglycan versican is a member of the hyalectan family. Versican is comprised of an N-terminal link-module HA binding domain and a C-terminal lectin-like domain that may well bind cell surface or ECM molecules (26). In 2003, Russell *et al.*, (26) examined the expression profile of versican in the rodent ovary. Using RT-PCR and in-situ hybridisation, three isoforms of versican (V0, V1 and V3) were identified in mouse and rat ovaries throughout folliculogenesis. All isoforms were significantly up regulated in the MGCs, 4-12 h after an ovulatory dose of hCG. Using a specific antibody raised against human recombinant versican, localisation of versican protein was examined using immunohistochemistry. Results showed that versican protein was first detected in GCs of primary follicles and became increasingly intense in the ECM of expanding COCs, suggesting a role for versican in the early stages of folliculogenesis and in the formation of the expanding ECM surrounding the oocyte. Another essential component of the CC ECM is the disintegrin-like and metalloproteinase with thrombospondin type I motifs-1 (ADAMTS-1), responsible for cleaving the proteoglycans aggrecan and versican (38, 39). Russell *et al.*, (39) illustrated that ADAMTS-1 accumulates on the COC ECM and functions to cleave versican (isoform V1) during cumulus expansion.

1.3.7 Cyclooxygenase-2

Ovulation is one of many inflammatory processes that occur within the body, mediated by prostaglandins (PGs) (40, 41). PGs are synthesised from arachidonic acid by the rate-limiting enzyme, cyclooxygenase (COX), for which there are two isoforms; COX1 and COX2. COX2 is the critical enzyme involved in the synthesis of PGs in the ovary, such as PG E_2 (PGE_2). COX2 is up regulated in MGCs in response to LH (42, 43). Joyce *et al.*, (44) examined the kinetics of COX2 mRNA in mouse preovulatory and ovulatory follicles using an RNase protection assay and in situ hybridisation. COX2 mRNA levels were up regulated in both CCs and MGCs at 4 h post hCG. At 8 h post hCG COX2 mRNA expression had declined in both the CCs and MGCs. A second peak was observed at 12 h post hCG in the CCs and MGCs, although the expression levels were much higher in the CCs compared to that of the MGCs. Furthermore, results from this study showed that the increased CC COX2 mRNA levels observed at 12 h post hCG are probably due to a factor(s) produced by the oocyte as well as the follicle wall.

1.3.8 Prostaglandin E_2 receptor EP_2

PGE_2 is a dominant prostanoid in the ovary and is synthesised by COX2 (42). Prostanoid specific cell surface receptors, of which there are four subtypes; EP_1 EP_2, EP_3 and EP_4, mediate the actions of PGE_2 (45). Of the four receptor subtypes, EP_2 has been the focus of much research as EP_2 knockout mice exhibit reduced fertility (45, 46). Hizaki *et al.*, (45) used in situ hybridisation to examine the expression of EP_2 mRNA in preovulatory and ovulatory follicles. EP_2 mRNA was not induced in any other cell type except the CCs. EP_2 mRNA expression was up regulated at 4, 8 and 12 h after hCG, hence displaying a similar expression profile to that of COX2 (44, 45).

1.3.9 Extracellular matrix knockout mice phenotypes

Much of what is known about the importance of these ECM molecules to female fertility, particularly in reference to formation and stability of the matrix, has been determined by gene mutation studies. Inactivation of the bikunin gene, consequently leads to a loss of circulating Iαl. Examination of bikunin null female mice exhibit reduced fertility due to disorganisation of the cumulus ECM (20). Ovulation rates were reduced by more than half and oocytes were completely denuded by day 0.5 post coitus. Subsequently, oocytes were rarely fertilised, resulting in infertility.

TSG6 null female mice exhibit a similar phenotype to that of the bikunin null female mice. Fulop *et al.*, (15) illustrated that during the preovulatory stage of folliculogenesis, TSG6 plays a crucial role in the mucification and expansion of the COC. Although TSG6 deficient mice appear to be able to synthesise HA, they are unable to organise it into a stable ECM, therefore preventing proper cumulus expansion. The TSG6 null female mice were able to ovulate, although majority of the oocytes were denuded of their CCs, resulting in significantly reduced ovulation rates in comparison to the wild type or heterozygous mice. The oocytes were then tested for their ability to be fertilised *in vivo* by examining the capacity of the oocyte to reach the two-cell stage. No two-cell embryos were detected from the TSG6 null mice, demonstrating that their infertility is due to the failure of their oocytes to be fertilised, a consequence that stems from impaired cumulus expansion. COCs from TSG6 null mice cultured *in vitro* resulted in CCs detaching from the matrix and dispersing into the culture dish and so the oocytes were either completely or near completely denuded, the same phenotype as that observed *in vivo*. Normal CC expansion was fully restored when recombinant TSG6 was added into the culture, further supporting the important role of TSG6 within the formation and stability of the ECM.

COX2 also plays an important role in the formation and stability of the ECM. COX2 deficient female mice are unable to undergo proper cumulus expansion, as they cannot retain the structural organisation of the ECM (47). Subsequently, COX2 deficient mice display severely reduced ovulation and fertilisation rates (47-49). Ovulation rates can be restored in these mice by treatment with PGE_2 or interleukin-1β, indicating that PGs and other signalling pathways are required. In addition to the COX2 knockout, infertility is also compromised in the EP_2 knockout female mouse. These mice display defects in cumulus expansion, reduced ovulation rates and severe failure of fertilisation (45). Ochsner *et al.*, (32) identified the first ECM target of PG action by investigating the gene expression profiles of HAS2, TSG6 and IαI in COX2 deficient mice as well as the expression profiles of HAS2 and TSG6 in EP_2 deficient mice. RT-PCR and in situ hybridisation studies revealed TSG6 mRNA, but not HAS2 mRNA was down regulated in COX2 and EP_2 deficient mice. Furthermore, Western blot analysis illustrated that TSG6 protein was reduced in COCs of COX2 deficient mice, but was still covalently linked to IαI. Collectively, these results demonstrate that the HA binding protein TSG6 is a direct target of PG action. The lack of CC TSG6 in COX2 and EP_2 null mice may well be attributable to the defects in cumulus expansion and the reduction in ovulation and fertilisation rates observed.

Infertility observed in PTX3 knockout female mice is also associated with the incapacity to organise the CCs into a stable ECM (24, 35). PTX3 deficient mice display normal ovulation rates, but significantly reduced fertilisation rates (24, 35). The role of PTX3 in the formation of the cumulus ECM was studied in detail by culturing COCs from PTX3 deficient mice *in vitro* (35). COCs from PTX3 deficient mice were able to synthesise a normal amount of HA, but failed to organise it into a stable matrix, resembling the same phenotype as the TSG6 deficient mice. This phenotype was restored when COCs from PTX3 deficient mice were cultured in the presence of recombinant PTX3. Moreover, oocytes from PTX3 deficient mice were incapable of being fertilised *in vivo*, the same phenotype that was observed in the oocytes from TSG6 deficient mice (24, 35).

The knockout mice studies described here provide an insight into the importance of formation of an appropriate CC ECM during the course of cumulus expansion, as erroneous matrix formation affects ovulation, fertilisation and hence female fertility.

1.4 Regulation of Cumulus Expansion

One of the most exciting and informative discoveries in reproductive biology from the past 15 years is that the oocyte plays a pivotal role in regulating many cellular processes, one of those being cumulus expansion. The oocyte in conjunction with gonadotrophin stimuli and various ECM molecules work together to regulate cumulus expansion. Failure of any of these components will result in impaired cumulus expansion and as a result have an effect on female fertility.

1.4.1 Role of gonadotrophins and the EGF cascade

Ultimately, the oocyte regulates the CC's response to gonadotrophin stimuli. *In vivo*, the mid-cycle LH surge triggers the CCs to undergo prominent mucification to form a large spherical mass of expanded cumulus encapsulating the oocyte. Cumulus expansion can be mimicked *in vitro* by culturing COCs in the presence of either FSH (5), cAMP analogues (6), epidermal growth factor (EGF) (7) or as established more recently, by EGF-like peptides (8).

Importantly, COCs cannot undergo cumulus expansion *in vitro* when exposed to LH (50). CCs and oocytes have low to undetectable levels of LH receptors in the preovulatory follicle and are unresponsive to direct LH stimulation (8). This suggests that upon LH stimulation a

factor from the MGCs or the serum positively induces cumulus expansion. A recent study investigated the possibility of EGF-related proteins as the paracrine mediators transmitting the LH signal throughout the ovarian follicle (8). Park *et al.*, (8) demonstrated that *in vivo* hCG injection or *in vitro* culture of whole intact follicles with LH, initiated the rapid mRNA expression of three members of the EGF-like growth factor family; amphiregulin (AR), epiregulin (EPI) and betacellulin (BTC). AR-, EPI- or BTC-stimulated follicle culture mimicked the actions of LH, causing the CCs to undergo expansion. RT-PCR also showed the induction of three important CC genes; HAS2, TSG6 and COX2. Moreover, i*n vitro* culture of COCs with AR, EPI or BTC stimulated cumulus expansion as well as oocyte maturation via the EGF receptor. This landmark study demonstrates that the EGF- related growth factors are the paracrine mediators transmitting the LH signal in the follicle from the MGCs to the COC (8).

1.4.2 Mitogen-activated protein kinase pathway

The mitogen-activated protein kinases (MAPK) are a large family of serine/threonine protein kinases. MAPK pathways transduce a wide variety of external signals, leading to an extensive range of cellular responses, including growth, differentiation, inflammation and apoptosis. MAPK signalling cascades are composed of three intermediate stages (Fig 4). MAPK is phosphorylated and activated by MAPK kinases (MAPKKs), which in turn are phosphorylated and activated by MAPKK kinases (MAPKKKs). In oocytes, the MAPKKK MOS activates the MAPKK, MEK1/2, whereas in GCs the MAPKKK, RAS/RAF activates MEK1/2 to mediate MAPK signalling [reviewed in Ref. (51)]. Numerous studies have demonstrated that FSH activates the MAPK pathway, particularly ERK1/2 and p38 MAPK (52-55). In addition to FSH, EGF and cAMP can also activate ERK1/2 in mouse CCs (53).

MAPK studies carried out firstly in 2000 by Leonardsen *et al.*, (56) and soon after by Su *et al.*, (53, 57) illustrated that specific MEK inhibitors blocked gonadotrophin-induced oocyte GVBD and cumulus mucification. Because the same MEK inhibitors blocked MAPK activation in both the CCs and the oocyte, this made it impossible to determine in which cell type MAPK activation is required for gonadotrophin-induced COC maturation. Su *et al.*, (53) addressed this issue by culturing COCs from MOS null mice with specific MEK inhibitors both *in vivo* and *in vitro*. The results illustrated that MAPK was not activated in gonadotrophin-induced MOS null oocytes cultured *in vivo* or *in vitro*, thereby demonstrating that activation of MAPK in oocytes is not required for gonadotrophin-induced resumption of

meiosis. Furthermore, both GVBD and cumulus expansion were eliminated in wild type and MOS null oocytes, when treated with a specific MEK inhibitor (U0126). The results from this study demonstrate that gonadotrophin-induced cumulus expansion and GVBD require MAPK activation specifically in the CCs (Fig. 4).

Additional results from Su *et al.*, (58) illustrated that activation of MAPK alone in the CCs is insufficient to stimulate oocyte meiotic resumption and cumulus expansion. A paracrine OSF(s) is absolutely required to induce both GVBD and cumulus expansion (58). Moreover, the results show that MAPK activation is required for the expression of CC HAS2 and COX2. Ochsner *et al.*, (34) demonstrated that CC TSG6 mRNA expression also required the activation of MAPK, specifically p38 MAPK.

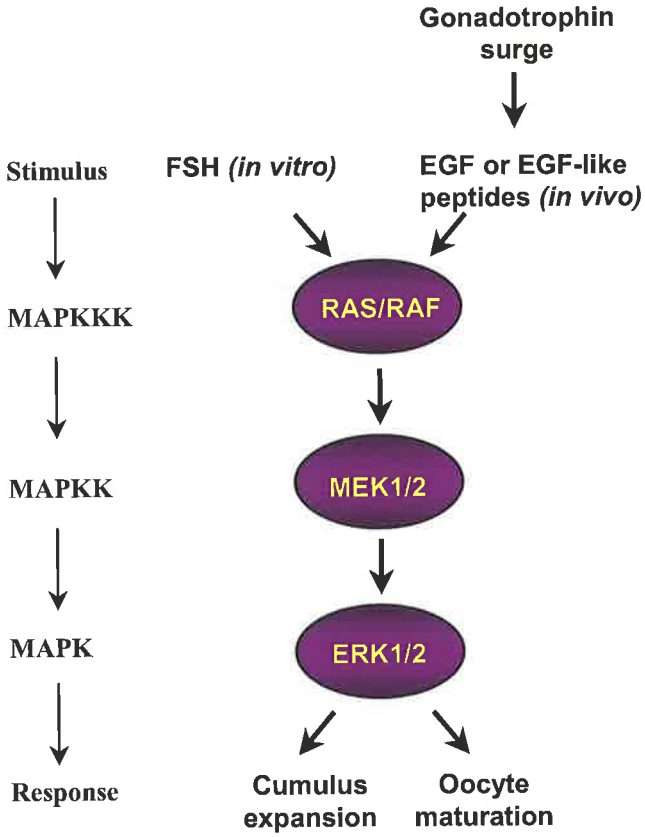

Figure 4. Schematic representation of the MAPK signalling pathway. The gonadotrophin surge stimulates EGF or EGF-like peptides (*in vivo*) to activate MAPKKK (RAS/RAF), which results in phosphorylation and activation of MAPKK (MEK1/2). MAPKK then activates CC MAPK (ERK1/2) to regulate cumulus expansion and oocyte maturation in the mouse. FSH can mimic this process *in vitro*.

1.4.3 The oocyte-secreted cumulus expansion-enabling factor

The pioneering studies of Buccione *et al.*, (2), Salustri *et al.*, (5) and Vanderhyden *et al.*, (19) have illustrated that cumulus expansion in the mouse is absolutely dependent on the oocyte. Microsurgical removal of the oocyte from the COC by oocytectomy generates a hollow ball of CCs, described as an oocytectomised (OOX) complex. Treatment of OOX complexes with FSH alone, synthesise low levels of HA and fail to undergo cumulus expansion (2, 5). Co-culture of OOX complexes with denuded oocytes (DOs) or oocyte-conditioned medium in the presence of FSH reverses the inhibitory effect of oocytectomy on cumulus expansion and restores HA synthesis. Results from these studies provide evidence that mouse oocytes produce a soluble CEEF that is essential for FSH to exert its actions on the CCs leading to expansion (2, 5, 19) (Fig. 5).

Experiments carried out *in vitro* on pig oocytes suggests that the pig oocyte also secretes a CEEF, however in comparison to the mouse, expansion of the pig COC appears to be independent of the CEEF (9-11). Vanderhyden *et al.*, (9) demonstrated *in vitro* that pig OOX complexes in the presence of FSH, cAMP or EGF underwent cumulus expansion, indicating that the presence of the oocyte is not required. The study also showed that pig oocyte-conditioned medium enabled the expansion of mouse and rat OOX complexes in response to FSH. Although pig COCs secrete a CEEF, cumulus expansion seems to be independent of this OSF(s), suggesting that the regulation of cumulus expansion in pig COCs is different to that in rodents. Bovine COCs behave identically to that of the pig, such that cumulus expansion does not depend on the oocyte-secreted CEEF (12). Moreover, mouse OOX complexes in the presence of FSH and bovine oocyte-conditioned medium underwent cumulus expansion, indicative that bovine COCs produce the CEEF even though they do not actually require it for expansion (12).

The ability of the oocyte to secrete the CEEF is dependent upon the stage of development of the oocyte (19). Mouse oocytes are first able to secrete the CEEF at the time they become competent to resume the first meiotic division and undergo GVBD. This is at approximately the end of the oocyte growth phase and formation of the follicular antrum (19). The mouse CEEF is continually secreted throughout the period of growth and maturation (19). The pattern of secretion of the CEEF in the pig differs to that of the mouse. Secretion of the CEEF by pig oocytes occurs only in germinal vesicle (GV) stage and transition to metaphase I (MI),

after this period the ability to secrete the CEEF is lost (59). The role or the mechanism of the oocyte-secreted CEEF in the human is unclear at this point in time.

The identity of the mouse oocyte-secreted CEEF is currently unknown and remains a controversial subject. Much of the research into identifying the OSF(s) that account for the CEEF have focused on the members of the TGFβ superfamily, due to their ability to mimic many oocyte-regulated CC and GC processes *in vitro*.

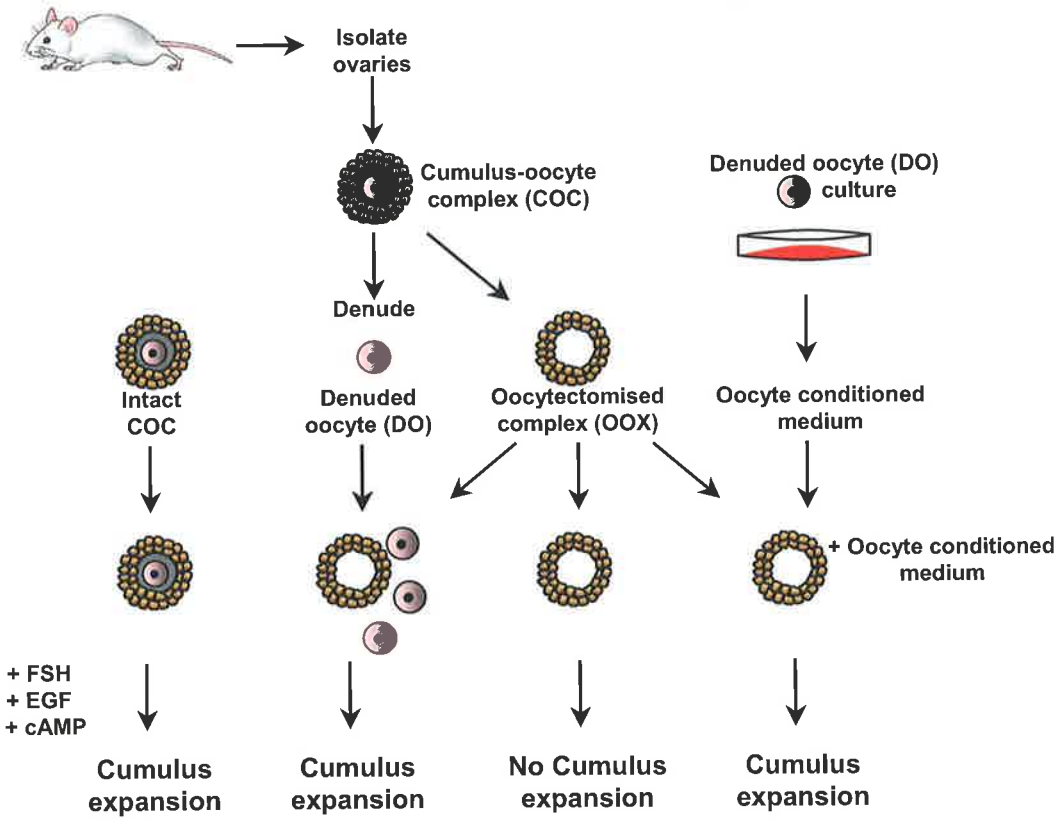

Figure 5. Standard procedures for examining the effects of oocytes on cumulus cell function. Oocytectomy mechanically removes the oocyte from the COC, generating a hollow ball of CCs surrounding an intact zona pellucida. Comparisons are made between the intact COCs, the OOX complexes either co-cultured with DOs or cultured in media conditioned for 24 hours with DOs. All of these complexes in the presence of FSH, EGF or cAMP undergo cumulus expansion. OOX complexes cultured alone in the presence of FSH, EGF or cAMP fail to undergo cumulus expansion. Figure adapted and modified from (60).

1.5 Transforming Growth Factor β Superfamily

The TGFβ superfamily is comprised of more than 40 growth factors, each similar in structure yet different in function. Members of the superfamily include TGFβ; which is present in three isoforms (TGFβ 1,2,3), anti-Mullerian hormone (AMH), two inhibins (inhibin A and B), three

activins (activin A, B and AB), 20 different bone morphogenetic proteins (BMP1 to 20) and nine growth differentiation factors (GDF1 to 9). Many members of the TGFβ superfamily are expressed in different cell types within the ovarian follicle, including activins, inhibins, AMH, TGFβ, BMPs 2, 3, 6, 7, 15 and GDF9. GDF9, BMP15 and BMP6 are the only members identified so far to be expressed in the oocyte.

1.5.1 TGFβ superfamily signalling pathways

Many studies have shown that members of the TGFβ superfamily, with the exception of inhibin, bind as homo-or heterodimers to membrane-bound type-I and type-II receptors. The type-I and type-II receptors are comprised of a cysteine-rich extracellular domain, a single membrane spanning domain and a serine-threonine kinase domain. To date, five type-II receptors (ActR-II, ActR-IIB, BMPR-II, TGFβR-II and AMHR-II) and seven type-I receptors (activin receptor-like kinase; ALK 1-7) have been identified. Furthermore, a third receptor type (TGFβR-III/betaglycan) has been identified and shown to enhance TGFβ signalling (61). Ligands bind to their respective type-I and type-II receptors on the cell surface, forming an oligomeric complex that is required for signal transduction. Upon ligand binding, the type-I receptor is transphosphorylated by the kinase domain of the type-II receptor. The activated type-I receptor then phosphorylates one or more intracellular substrate signalling molecules called receptor regulated SMADs (R-SMADs). R-SMADs include; SMAD 1, 2, 3, 5 and 8. SMADs 6 and 7 are inhibitory SMADs (I-SMADs) and can impede the phosphorylation of R-SMADs. Activated R-SMADs associate with a co-SMAD (SMAD 4) and then translocates to the nucleus where it interacts with transcription factors involved in the regulation of specific genes (62). Typically, BMPs activate the SMAD 1/5/8 signalling cascade, whereas, SMAD 2/3 is activated by TGFβ and activin (Fig. 6).

As a general rule, TGFβ/activin superfamily members bind their type-II receptors first, which in turn recruit and phosphorylate the type-I receptor. On the contrary, BMPs have a higher binding affinity for the type-I receptors. Recent evidence also suggests that BMP receptor mediated downstream signalling is dependent on whether the type-I and type-II receptors at the cell surface are preformed or not. BMPs that bind the type-I receptor and then recruit the type-II receptor into an oligomeric complex, leads to activation of the p38 MAPK pathway. Alternatively, BMPs that bind already preformed type-I and type-II receptors at the cell surface activate the SMAD 1/5/8 signalling cascade (63).

To date, SMADs are the only receptor substrates identified that mediate signals of the TGFβ superfamily members. However, recent studies have illustrated that members of the TGFβ superfamily can signal via the MAPK signalling pathways, therefore suggesting that there is crosstalk between these two pathways. Crosstalk between the SMAD and MAPK pathways can work in both directions depending on the cell type and experimental conditions (64).

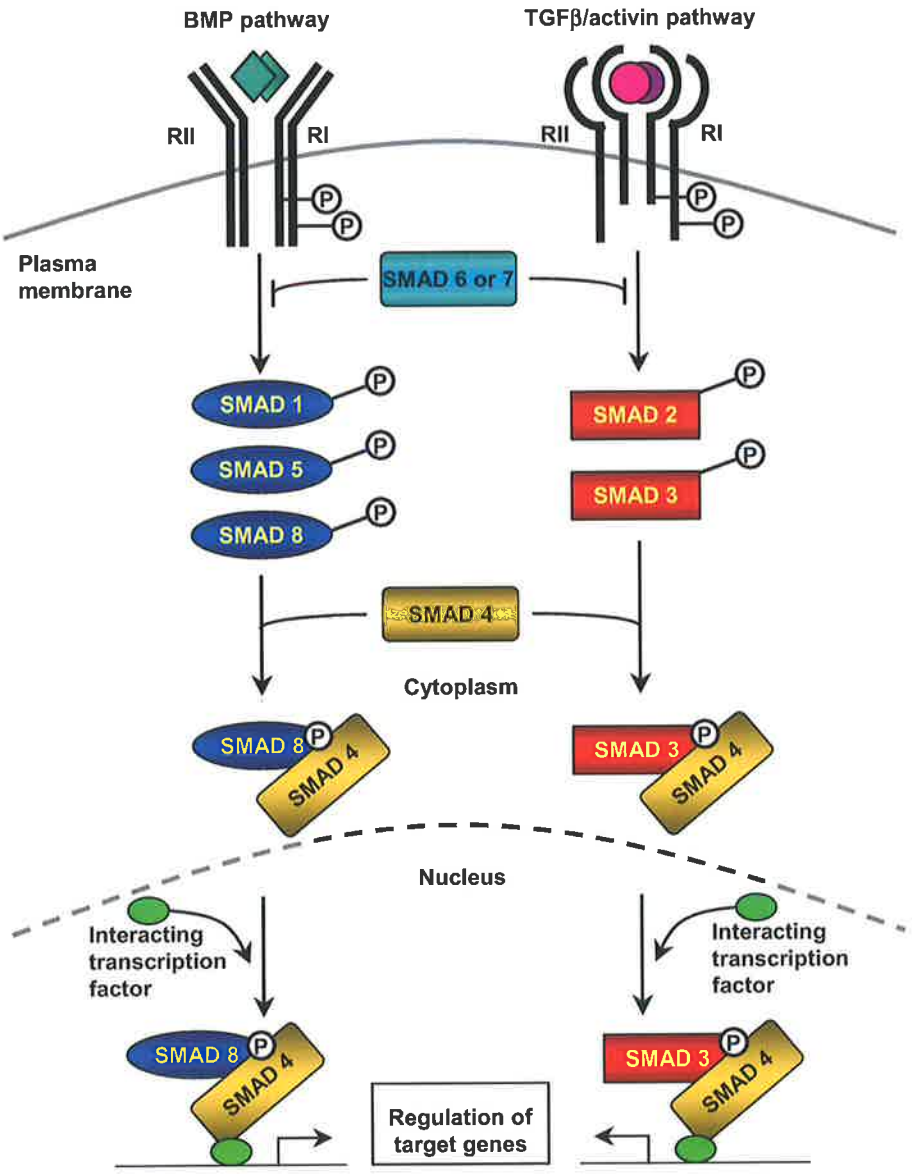

Figure 6. Schematic representation of the basic TGFβ superfamily members SMAD signalling pathway. Upon ligand binding, the type-I receptor (RI) and type-II receptor (RII) form a tetrameric signalling complex. RI, phosphorylated by RII in turn phosphorylates receptor regulated SMADS (R-SMADs). SMAD 1/5/8 is phosphorylated by BMPs, whereas SMAD 2/3 is phosphorylated by activin/TGFβ. These activated R-SMADs then associate with the co-SMAD (SMAD 4), at which stage the SMAD complex translocates into the nucleus where it interacts with various transcription factors to regulate the target genes of the TGFβ superfamily members. Adapted and modified from (65, 66).

1.5.2 Oocyte-secreted factors

Paracrine oocyte-secreted factors (OSFs) play a pivotal role in the regulation of ovarian function. OSFs are involved in the regulation of many GC and CC processes important for normal folliculogenesis and fertility (Fig. 7). Much of what we know about these OSFs has originated from, (i) sheep and mouse loss of function, in which the genes for GDF9 and BMP15 have either been made inactive or have natural mutations, and (ii) utilising *in vitro* GC and CC bioassays, whereby MGCs or OOX complexes are co-cultured with fully-grown DOs [reviewed in Ref. (3)].

By using these techniques, researchers have begun to unravel the profound effects that OSFs have on GC and CCs. For example, oocytes regulate the expression of the tissue remodelling protein, urokinase plasminogen activator (uPA) prior to and after the preovulatory gonadotrophin surge (67). Ovulation also requires cumulus expansion (2, 5, 19, 68), HA production (5, 69), as well as HAS2 (58), COX2 (58), and PTX3 (35) expression, all of which are regulated by oocyte-secreted paracrine factor(s). Oocytes also secrete mitogenic factors that potently stimulate GC and CC growth (70-75). The actions of FSH, insulin like growth factor-I (IGF-I) and androgens interact with the oocyte secreted mitogenic factor(s) to enhance their growth-promoting actions (71, 72, 76, 77). Whilst stimulating proliferation, oocytes also function to prevent cell death. Recently, oocytes were illustrated to secrete a morphogenic gradient to prevent CC apoptosis (78). Furthermore, another important function of OSFs is the role they play in the regulation of GC and CC steroidogenesis, by maintaining oestrogen production and inhibiting progesterone production (60, 72, 79). Oocytes also regulate the suppression of FSH-induced LH receptor (80), the GC inhibin-activin-follistatin system (81) and the expression of kit-ligand (KL) (82).

These studies clearly illustrate that oocyte paracrine signalling to follicular GCs and CCs is crucial for regulating appropriate somatic cell growth and differentiation and hence for normal folliculogenesis and fertility. Members of the TGFβ superfamily are the most likely candidate oocyte-secreted molecules that regulate such processes, due to their ability to mimic these *in vitro* [reviewed in Refs. (3, 60)].

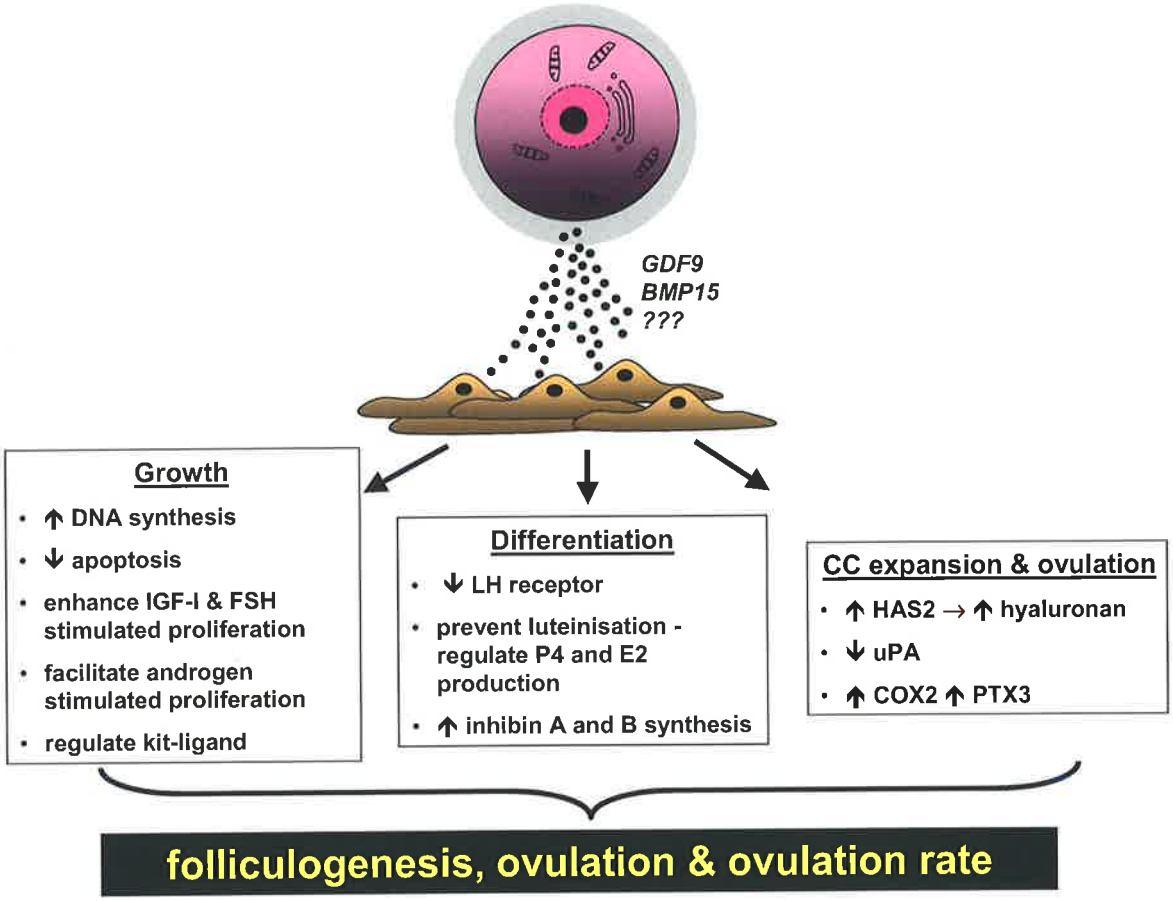

Figure 7. Oocyte-regulated granulosa and cumulus cell processes necessary for folliculogenesis and fertility. Oocyte secreted paracrine factors regulate a wide variety of ovarian functions including growth, differentiation, cumulus expansion and ovulation. The exact identities of these oocyte-secreted factors are elusive, however GDF9 and its most closely related homologue BMP15 are important OSFs that can mimic many of these processes *in vitro*. Adapted and modified from (3).

1.5.3 Transforming growth factor β

Three isoforms of TGFβ; TGFβ1, TGFβ2 and TGFβ3 are expressed by mammalian ovarian cells. TGFβ signals by binding the TGFβR-II, which in turn recruits and phosphorylates ALK5, followed by the phosphorylation of the SMAD 2/3 complex (83). Numerous studies have shown that both TGFβ1 and TGFβ2 are able to completely substitute for the oocyte and mimic many oocyte-regulated GC and CC functions *in vitro*. Vanderhyden *et al.*, (60) investigated the actions of TGFβ1 on cumulus steroid hormone production and compared this to the actions of oocytes. Both oocytes and TGFβ1 were shown to promote oestradiol production. The addition of a TGFβ neutralising antibody could only neutralise the TGFβ1 effects on oestradiol production and not the oocyte effects, indicating that TGFβ1 alone is not the OSF mediating this process. Moreover, Gilchrist *et al.*, (74) demonstrated that TGFβ2 can mimic the oocyte and promote bovine MGCs proliferation. Using a TGFβ pan specific

neutralising antibody, the TGFβ2 effects were eliminated, yet the antibody was unable to neutralise the oocyte effects on GC proliferation.

In addition to steroidogenesis and proliferation, TGFβ can also enable cumulus expansion. Studies by Salustri *et al.*, (69) and Vanderhyden *et al.*, (60) demonstrated that TGFβ1 mimics the CEEF secreted by mouse oocytes *in vitro*. Culturing OOX complexes with FSH or TGFβ1 alone had no effect on cumulus expansion, however culturing OOX complexes together with FSH and TGFβ1 enabled expansion of the CCs. Neutralising antibodies to TGFβ were effective in neutralising TGFβ1-induced, but not oocyte-induced cumulus expansion. These results indicate that TGFβ1 does not account for the CEEF in the mouse (Table 1).

Table 1. TGFβ1 mimics the effects of the mouse oocyte-secreted CEEF

	Degree of Expansion	
Treatment	**COC (n)**	**OOX (n)**
FSH	**+3 to +4 (60)**	**0 (70)**
TGFβ1	**–**	**0 (30)**
FSH + TGFβ1	**+3 to +4 (20)**	**+2 to +3 (110)**
FSH + TGFβ1-Ab	**+3 (40)**	**0 (20)**
FSH + TGFβ1 + TGFβ1-Ab	**+3 (40)**	**0 (70)**
OCM + FSH	**+3 to +4 (60)**	**+3 to +4 (60)**
OCM + FSH + TGFβ1-Ab	**+3 (50)**	**+3 (70)**

TGFβ1 can mimic the oocyte and enable FSH-induced cumulus expansion. Treatment with the TGFβ1 neutralising antibody (TGFβ1-Ab) ablated TGFβ1-induced but not oocyte-induced cumulus expansion. The degree of cumulus expansion was determined on a scale of 0 (no expansion) to +4 (maximum expansion). COC: cumulus-oocyte complex; OOX: oocytectomised complex; TGFβ1-Ab: neutralising antibodies to TGFβ1; OCM: oocyte-conditioned medium, media collected from plates of denuded oocytes cultured for 24 h; –: treatment not performed. Adapted and modified from (60).

Taken together, these multiple lines of evidence demonstrate that TGFβ can mimic the actions of the oocytes but does not account for the OSF(s) responsible for regulating proliferation, steroidogenesis, nor is it accountable for the mouse CEEF. Further studies continue to investigate other TGFβ superfamily members as candidates for such processes.

1.5.4 Growth differentiation factor 9

The TGFβ superfamily member GDF9 is a homodimeric protein that was first discovered in 1993 by McPherron and Lee (84). GDF9 binds the BMPR-II, followed by the association with the TGFβ type-I receptor ALK5, leading to its phosphorylation and further phosphorylation of the SMAD 2/3 complex (85, 86).

GDF9 mRNA and protein have been identified in the oocytes of numerous species [reviewed in Ref. (87)]. It was originally thought that GDF9 expression was found exclusively in the oocyte, however studies have shown that GDF9 mRNA is present in human CCs (88), as well as mRNA and protein can be found in the GCs in the rhesus monkey (89). GDF9 is also found in other tissues besides the ovary. GDF9 mRNA is expressed in the testis and in the hypothalamic-pituitary axis (90). Moreover, the expression pattern of GDF9 in the oocyte appears to be species dependent. In mice, rats and humans, GDF9 expression first commences in oocytes of small primary follicles (91-93), whereas GDF9 expression in bovine, ovine, possum and hamster oocytes is detectable from the primordial stage of folliculogenesis (94-96).

Characterisation of both the male and female GDF9 knockout mouse in 1996 by Dong *et al.*, (97) revealed that male GDF9 deficient mice had normal testis size and displayed normal fertility rates. On the contrary, female GDF9 deficient mice were infertile due to severe defects in folliculogenesis. The ovaries from the female homozygous mice were smaller than those from the wild type mice. More importantly, the homozygous ovaries exhibited primordial and primary one-layer follicles, but a complete block in folliculogenesis was apparent beyond the primary one-layer follicle stage. Similarly, ewes with natural mutations in the GDF9 gene or immunised against GDF9, were also found to be infertile because of a block in folliculogenesis beyond the primary stage of development (98, 99). Interestingly, ewes heterozygous exhibited increased ovulation and fertility rates (98), in contrast to GDF9 heterozygous mice, which have normal fertility (100).

GDF9, like TGFβ, can mimic many of the oocyte-secreted processes described thus far, specifically steroidogenesis, growth and differentiation, as well as cumulus expansion [reviewed in Refs. (3, 60, 101)]. Gilchrist *et al.*, (102) utilised an OSF bioassay in conjunction with a specific GDF9 neutralising monoclonal antibody to determine to what

extent GDF9 accounts for the mitogenic effects of oocytes on GCs. The results showed that although GDF9 promotes proliferation of MGCs, GDF9 only accounts for approximately half of the mitogenic activity of oocytes. Gilchrist *et al.*, (75) recently conducted a follow-up study, demonstrating that the oocyte-secreted mitogenic factors utilise the BMPR-II and signal via the SMAD 2/3 pathway to regulate GC DNA synthesis. Elucidation of the OSF(s) responsible for the remaining mitogenic activity requires further investigation.

Cumulus expansion in the mouse requires an oocyte-secreted paracrine factor(s) and at present GDF9 is the most likely candidate molecule. *In vitro* studies have demonstrated that GDF9 can substitute for the oocyte in inducing cumulus expansion and promotes expression of several downstream target genes important for ECM formation and stability. Recombinant GDF9 regulates HAS2 (24, 103), COX2 (24, 44, 104), PGE_2 (105), TSG6 (24) and PTX3 (24) mRNA expression in GC and CCs.

Table 2. Effect of GDF9 $^{+/-}$ and $^{-/-}$ denuded oocytes on cumulus expansion of OOX complexes

Control/treatment group	No. of oocytes used for conditioning	No. of complexes assessed	Degree of expansion (0 to +4)
COC	0	15	3.3 ± 0.3
OOX	0	15	0
OOX + GDF9 $^{+/-}$	25	20	3.3 ± 0.2
OOX + GDF9 $^{-/-}$	25	20	0

OOX complexes were cultured in the presence of GDF9 heterozygous $^{+/-}$ and homozygous $^{-/-}$ oocytes. Control and treatment groups were cultured in maturation media for 16 h in the presence of FSH. Degree of cumulus expansion was determined on a scale from 0 (no expansion) to +4 (maximum response). Values represent the mean ± SEM of three experiments. Adapted and modified from (60).

Researchers have used various techniques to examine the role of GDF9 in the regulation of mouse cumulus expansion. Elvin *et al.*, (103) observed cumulus expansion when OOX complexes were treated with recombinant mouse GDF9, therefore concluding that because GDF9 could mimic the actions of the oocyte, GDF9 must account for the mouse CEEF. Moreover, a study conducted by Vanderhyden *et al.*, (60) used GDF9 knockout mice to investigate the role of GDF9 in cumulus expansion. Oocytes obtained from the GDF9 $^{-/-}$ mice were unable to induce cumulus expansion of OOX complexes cultured *in vitro* (Table 2).

Most recently, an RNA interference approach was used to show that cumulus expansion was significantly reduced when oocytes were injected with GDF9 double-stranded RNA (106).

Based on the results above, these studies suggest that GDF9 is the oocyte-secreted CEEF in the mouse. However, the results obtained from the GDF9 knockout mice study are inconclusive due to the likelihood that GDF9 is not the only factor missing from the oocytes obtained from the GDF9 deficient mice. As acknowledged by the authors (60), it is highly likely these oocytes are deficient in a multitude of developmentally regulated transcripts as a consequence of their aberrant follicle growth. Furthermore, the oocyte-secreted CEEF requires FSH to induce expansion and regulate the all-important ECM genes, whereas recombinant GDF9 can regulate these processes in the absence of FSH (53, 103). This result demonstrates that GDF9 is behaving differently to that of the CEEF secreted by the mouse oocyte. One line of evidence to suggest that GDF9 is not the mouse oocyte-secreted CEEF is based on the results showing preantral follicles do not produce the CEEF (19), even though GDF9 is expressed at this stage of folliculogenesis (107). This result suggests that either GDF9 is not the mouse CEEF or GDF9 may be present in an inactive form. Taking all these studies into consideration, whether GDF9 accounts for the mouse CEEF still remains an open-ended question. An alternate approach to address this question would be to use a specific GDF9 neutralising antibody, which would significantly facilitate additional studies in this area.

Until recently, little was known about the biochemical signalling pathways involved in the initiation of cumulus expansion, especially those stimulated by GDF9. Su *et al.*, (53) investigated whether recombinant GDF9 mimicked the oocyte in its requirement for MAPK activation in the CCs to induce cumulus expansion. OOX complexes treated with recombinant GDF9 underwent cumulus expansion, however this expansion was completely abolished by a specific MEK inhibitor (U0126) (Table 3). This result indicates that the MAPK signalling cascade is required for cumulus expansion and occurs downstream of GDF9 (53).

Table 3. Effect of MEK inhibitor (U0126) on GDF9 induced cumulus expansion of OOX complexes

Treatment	n	0 (%)	1 (%)	2 (%)	3(%)	4(%)	CEI
OOX	67	67 (100)					0
OOX + GDF9	73				15 (21)	58 (79)	3.79
OOX + GDF9 + U0126	70	50 (71)	20 (29)				0.29
OOX + GDF9 + U0124	70				11 (16)	59 (84)	3.84

OOX complexes were cultured in maturation medium for 15 hours. OOX complexes were cultured alone, treated with GDF9 with and without the MEK inhibitor U0126 or U0124 (inactive analogue of U0126) CEI: Cumulus expansion index, this represents the mean expansion value for the complexes, with 0 equal to no expansion, and 4 equal to maximum expansion. Values are the results from three independent experiments. Adapted and modified from (53).

Because MAPK activity is required for GDF9 to promote cumulus expansion, this advocates that the MAPK pathway interacts with the GDF9-signal transduction cascade (53). Moreover, the MAPK pathway is known to crosstalk with the TGFβ superfamily SMAD signalling pathway (108). Gilchrist *et al.*, (75) recently demonstrated that OSFs activate predominantly SMAD 2/3 to regulate GC DNA synthesis. Therefore, the question remains whether the OSF(s) that regulate cumulus expansion activate SMAD 2/3 and if so, precisely where in the pathway these two signalling cascades interact.

1.5.5 Bone morphogenetic protein 15

BMP15, also referred to as GDF9B, shares the highest homology with GDF9 amongst the TGFβ superfamily members. BMP15 was first discovered in the mouse oocyte five years after GDF9 (93, 109). BMP15 is a homodimeric protein that binds the same type-II receptor as GDF9 (BMPR-II), but a different type-I receptor (ALK6). Phosphorylated ALK6 in turn activates the SMAD 1/5/8 complex, which then associates with SMAD4 and the complex translocates into the nucleus to regulate target genes (110). Hence, in contrast to GDF9, BMP15 elicits a classical BMP intracellular response (Fig 6.). Like GDF9, BMP15 mRNA and protein are found in the oocyte of numerous species [reviewed in Ref. (87)], as well BMP15 mRNA has been detected in the pituitary (111). BMP15 expression noticeably increases in the primary stage of follicle development in mice, rats, humans and sheep and continues throughout folliculogenesis (91, 93, 112, 113).

Like TGFβ and GDF9, BMP15 can also mimic the oocyte and regulate several GC and CC processes *in vitro*. In 2000, Otsuka *et al.*, (113) identified GCs as the first target cells for

BMP15 by demonstrating that recombinant BMP15 stimulates proliferation of rat GCs *in vitro*. Furthermore, this study also illustrated that BMP15 regulates FSH-induced progesterone production. In addition to the growth promoting effects of BMP15, it appears that BMP15 is also involved in the regulation of CC apoptosis. Treating OOX complexes with recombinant BMP15 significantly reduced bovine CC apoptosis (78). It is also worth noting that bovine CC apoptosis was equally attenuated by co-culture with DOs or treatment with BMP6, but not GDF9. An additional important function of BMP15 is its ability to suppress FSH receptor expression. In conjunction with FSH, BMP15 inhibits steroidogenic acute regulatory protein (StAR), P450 aromotase, LH receptor and the inhbin/activin subunits mRNA expression in rat GCs (114). Furthermore, unlike GDF9, which inhibits KL expression, BMP15 stimulates the expression of KL in rat GCs (115). In 2001, Otsuka *et al.*, (116) made the major finding that the activin-binding protein follistatin also functions to antagonise the actions of BMP15. To date, follistatin is the only BMP15 antagonist described.

Elvin *et al.*, (103) was the first to examine the role of BMP15 in the regulation of mouse cumulus expansion. OOX complexes treated with recombinant BMP15 failed to undergo cumulus expansion. Furthermore, in the recent study by Gui and Joyce (106) who used a RNA interference approach to show that cumulus expansion was significantly reduced when oocytes were injected with GDF9 double-stranded RNA, also examined the effect of BMP15. Cumulus expansion was not impaired in oocytes injected with BMP15 double-stranded RNA. These findings suggest that BMP15 does not account for the mouse oocyte-secreted CEEF.

Not only are dramatic species differences observed for GDF9 in the regulation of fertility (97-99), but also they are apparent for BMP15. In 2001, Yan *et al.*, (100) aimed to define the function of BMP15 in mice by generating a BMP15 knockout mouse model. The BMP15 null males were normal and had no fertility defects. In contrast, the BMP15 null females were subfertile, displaying reduced ovulation and fertilisation rates. These findings were confirmed by Su *et al.*, (117), who also made the additional finding that the female mice exhibited a decrease in CC HAS2 mRNA expression. Contrasting the BMP15 homozygous mice, ewes homozygous for BMP15 or actively immunised against BMP15 are sterile, displaying streak ovaries and primary ovarian failure (98, 112, 118). Moreover, ewes heterozygous for BMP15 or with reduced BMP15 concentrations through passive immunisation have an increased ovulation rate and a higher incidence of multiple pregnancies (98, 112, 118). On the contrary, BMP15 heterozygous mice do not display this same phenotype, as heterozygosity has no

affect on mice fertility (100). Furthermore, the BMP15 type-I receptor, ALK6 appears to be necessary for fertility in mice because ALK6 null mice are infertile due to multiple defects in reproductive function, including severe abnormalities in cumulus expansion (119). Conversely, ALK6 heterozygous ewes are fertile and have increased ovulation rates compared to that of the wild type ewes (120). What is more astounding is that ALK6 homozygous ewes have an even higher ovulation rate than the heterozygotes (120). At this point in time there is no clear explanation as to why these phenotypes differ between mice and sheep. It has however been acknowledged, that the differences may be reflections of the mono- vs. poly-ovulatory nature of these two species or the requirement for these OSFs may be different in mice and sheep (99, 121).

In a study investigating the interactions between GDF9 and BMP15, it was revealed that these two growth factors are capable of forming a GDF9/BMP15 heterodimer (122). Moreover, in a recent study carried out by Hashimoto *et al.*, (123) it was illustrated that fully processed mouse BMP15 cannot be secreted as an intact homodimer. This result suggests that mouse oocytes may not secrete BMP15 as a homodimer, therefore implying that BMP15 in the mouse oocyte may be secreted as a GDF9/BMP15 heterodimer. In support of this finding, recombinant GDF9 and BMP15 co-operate synergistically to stimulate GC proliferation and to regulate GC inhibin and progesterone production *in vitro* (124, 125). Moreover, examination of COCs from $BMP15^{-/-}GDF9^{+/-}$ double mutant mice found that cumulus expansion was severely impaired in comparison to the BMP15 homozygous mice, demonstrating that these two growth factors act in a synergistic manner to enable cumulus expansion in the mouse (100, 117). To date, little is known about the receptor complex that the GDF9/BMP15 heterodimer might utilise, however it has been predicted that it may consist of two BMPR-II molecules in association with one ALK5 and one ALK6 molecule and in turn phosphorylate the SMAD 2/3 and SMAD 1/5/8 complex (126). Further studies are warranted to investigate GDF9 and BMP15 interactions including potential heterodimerisation.

1.5.6 Activins and Inhibins

Activins are TGFβ superfamily members closely related to, and signalling much like, TGFβ1 and GDF9. The dimerisation of β-subunits generates three isoforms of activin, activin A (βA-βA), activin AB (βA-βB) and activin B (βB-βB) (Fig. 8). To date, four types of β-subunit

genes (βA, βB, βC and βE) have been identified, however only activin A, activin B and activin AB are biologically active and important to reproductive function [reviewed in Refs. (127, 128)]. Multiple antagonists of activin have been identified, including follistatin, follistatin-related gene and cripto [reviewed in Ref. (128)]. Inhibins are heterodimers of an α subunit linked to either a βA or βB subunit to generate inhibin A (α-βA) or inhibin B (α-βB) (Fig. 8). Activins signal by binding their respective type-II receptors, ActR-II (for activin A) or ActR-IIB (for activin B) which inturn recruit and phosphorylate the type-I receptor, ALK4. Phosphorylated ALK4 activates SMAD 2/3, which then binds SMAD4 and the complex translocates into the nucleus to regulate target genes. Inhibin can antagonise activin signalling by binding via its β-subunit to the activin type-II receptor. This process is mediated by the inhibin co-receptor, betaglycan (TGFβ type-III receptor) (129).

Activins and inhibins were first identified as gonadal peptides found in mammalian follicular fluid, whereby activin stimulates and inhibin inhibits pituitary FSH production (130). Activin subunits βA and βB mRNA has been detected in Xenopus oocytes (131), however there are conflicting reports as to whether the activin subunits are expressed in mouse oocytes (88, 132). The inhibin α-subunit is prominently expressed in human CCs, however it is barely detectable in human oocytes and is not expressed at all in mouse oocytes (88). Human oocytes, CCs and luteal GCs all express the activin type-I and type-II receptors (88, 133). Both receptor types have also been detected in mouse oocytes and CCs (88), as well as in rat GCs (134).

Like other members of the TGFβ superfamily, activin can mimic the effects of the OSFs in regulating many GC and CC processes *in vitro*. For example, activin can induce proliferation of GCs in many species, including mice, rats and human (102, 135, 136). Although activin can mimic the oocyte and stimulate GC proliferation, Gilchrist *et al.*, (75) recently illustrated that activin does not account for the mouse oocyte-secreted mitogenic factor(s). Studies in numerous species also support a role for activin in the regulation of GC steroidogenesis as experiments carried out on rat and bovine GCs have shown that activin increases P450 aromotase activity and oestradiol activity, while suppressing progesterone production (135, 137). Vanderhyden *et al.*, (60) investigated activin as a candidate for the OSF that inhibits

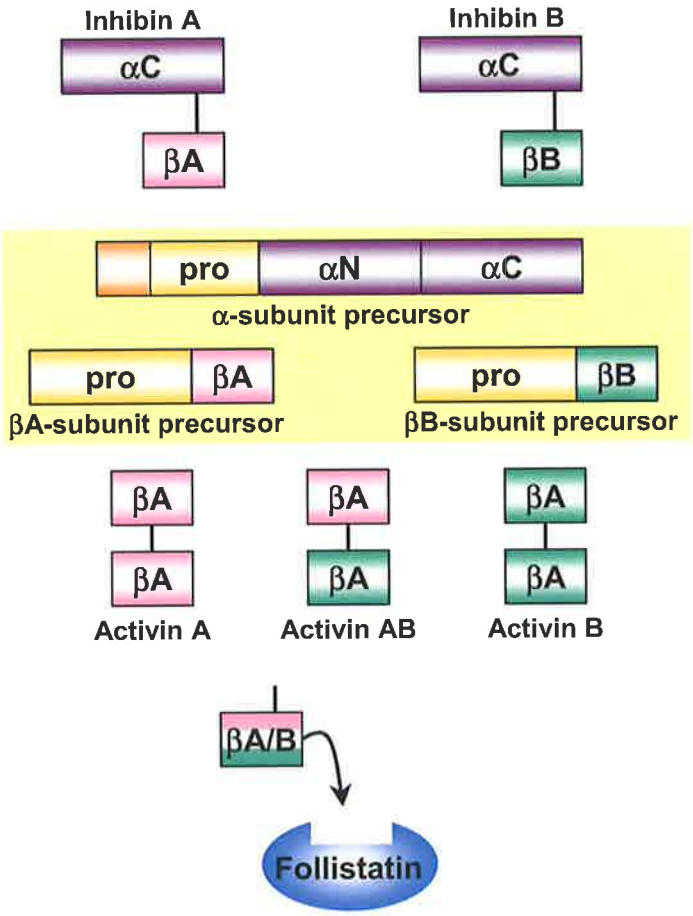

Figure 8. Schematic diagram of the basic molecular structures of the different isoforms of inhibin and activin. Inhibins A and B are heterodimers of a α subunit associated with either a βA (inhibin A) or βB (inhibin B) subunit. Activins are homo- or heterodimers of two β subunits; activin A (βA-βA), activin AB (βA-βB) and activin B (βB-βB). Each subunit is derived from a separate precursor molecule. Follistatin is a high affinity activin binding protein, which antagonises the biological activities of activins by forming inactive complexes. Adapted and modified from (138).

progesterone production, finding that activin A could not substitute for the oocyte in suppressing progesterone production. Furthermore, activins as well as inhibins have been implicated in the regulation of nuclear and cytoplasmic maturation of oocytes in numerous species [reviewed in Ref. (138)]. From these studies it is clear that activin can mimic the oocyte in the regulation of many reproductive functions, even though it does not account for the OSF(s) regulating proliferation or progesterone inhibition (60, 75). Nonetheless, activin should be considered as a possible candidate for the elusive oocyte-secreted CEEF in the mouse. To date, no experiments have been carried out to examine this possibility.

The generation of activin and inhibin knockout or knock-in mouse models have provided an insight into the role these growth factors play in folliculogenesis, however some models have

proved to be more beneficial than others [reviewed in Refs. (127, 139)]. Mice deficient in inhibin α exhibit an increase in serum FSH, hence supporting the role of inhibin in the regulation of FSH production (140). This increase in FSH results in the overproduction of activin and is associated with irrepressible GC proliferation and ovarian tumour growth. Over expression of inhibin A in wild type mice exhibit infertility due to a reduction in plasma FSH levels and a block at the early antral stage of folliculogenesis (141). Analysis of the ovaries from these mice revealed a number of atretic follicles and no corpora lutea, indicative that these mice fail to ovulate. Mice null for Act-RII are also infertile due to a decrease in plasma FSH levels and block in folliculogenesis at the early antral stage of development (142). Mice deficient in activin βB are viable and fertile (143), yet have defects in eyelid closure at the time of birth (144). In contrast, activin βA knockout mice have craniofacial anomalies and die within 24 hours after birth and for this reason is an unsuitable model to examine the role of activin βA in folliculogenesis (145). In 2000, Brown *et al.*, (146) created activin βB knock-in mice to illustrate that activin βB influences female fertility. The activin βA knock-in mice were able to rescue the phenotypic effects seen in the activin βA knockout mice, suggesting that activin βB can substitute for βA in early development. Mice homozygous (two knock-in alleles) for the knock-in allele contained follicles at varying stages of development, resulting in reduced fertility. Mice hemizygous (one knock-in allele and one null allele) for the knock-in allele had smaller ovaries compared to the control mice and contained fewer preovulatory follicles. These findings are indicative that activin βA appears to be important in ovarian growth and development, however activin βB cannot compensate for all aspects of βA during folliculogenesis.

1.6 Summary

Research investigating growth and development of the ovarian follicle has continued to progress immensely, and more and more studies have demonstrated that the oocyte plays a significant role. A multitude of studies has shown that paracrine OSFs are imperative for folliculogenesis and for female fertility. This review has provided an extensive overview of the significant role these OSFs play in folliculogenesis, particularly focusing on facilitating cumulus expansion. Knockout mouse models have identified CC genes and specific ECM components that are dependent upon the secretion of oocyte-secreted paracrine factors. The identities of the oocyte-secreted paracrine factors regulating these CC genes and ECM molecules have not been established with certainty at this point in time, however members of

the TGFβ superfamily are likely candidates. As additional matrix molecules continue to be identified it is gradually becoming evident as to how many ECM molecules are involved and the way in which they interact to form the stable matrix surrounding the oocyte. Clearly, further research is required to identity the OSFs responsible for regulating the ECM molecules and will undoubtedly facilitate in the discovery of additional ECM molecules.

Exploitation of GC and CC bioassays have demonstrated that numerous members of the TGFβ superfamily, including TGFβ, GDF9, BMP15 and activin can mimic the actions of the oocyte on GC and CCs *in vitro*. Null expression of GDF9 or BMP15 in mice and sheep, or immunising sheep against either of these growth factors, has clearly demonstrated that these OSFs are critical regulators of folliculogenesis and female fertility. Nonetheless, our understanding is far from complete and further studies are required to entirely elucidate the role these OSFs play in regulating processes such as cumulus expansion.

At present, our knowledge regarding the role of these OSFs in determining human fertility is very limited. However, it is highly likely they play a significant role in regulating different aspects of folliculogenesis, as impaired folliculogenesis can lead to conditions such as premature ovarian failure and polycystic ovarian syndrome (PCOS). A study in women diagnosed with PCOS demonstrated that GDF9 mRNA expression in oocytes was decreased compared to controls (147), therefore suggesting that impaired oocyte-secreted GDF9 may contribute to the aberrant folliculogenesis observed in these women. The primary cause of infertility in patients with PCOS is anovulation, a condition that can result from anomalies in cumulus expansion.

Further studies investigating oocyte-secreted paracrine factors will facilitate our understanding of which growth factors are responsible for regulating GC and CC processes. Ultimately, the identification of the OSF(s) enabling cumulus expansion, in addition to the signalling pathways involved, would contribute significantly to our understanding of female fertility.

1.7 Hypotheses and Aims for Masters Project

1.7.1 Hypotheses

1. GDF9 is a key OSF regulating cumulus expansion in the mouse.
2. TGFβ superfamily signalling through the SMAD 2/3 pathway is responsible for mediating the CEEF paracrine signal from the oocyte to the CCs.

1.7.2 Aims

Two mains aims are proposed for the project. Ultimately, the goal is to identify the OSF(s) responsible for enabling cumulus expansion in the mouse and to identify the signalling pathway(s) utilised by the CEEF.

The first main aim of this study is to determine whether GDF9 accounts for the mouse CEEF. A few different approaches will be used to achieve this aim. Firstly, the effect of recombinant GDF9 on mouse cumulus expansion will be examined. Secondly, given that previous reports have suggested that recombinant GDF9 does not require FSH to enable mouse cumulus expansion, the interaction between FSH and GDF9 will be investigated. Thirdly, the effect of a specific GDF9 monoclonal neutralising antibody as well as a TGFβ superfamily antagonist will be tested for its ability to neutralise recombinant GDF9- and oocyte-induced cumulus expansion. The work associated with achieving this aim forms the basis of Chapter 2.

The second main aim of this study is to determine whether signalling through the SMAD 2/3 pathway is responsible for mediating the CEEF paracrine signal from the oocyte to the CCs. Several different approaches will be used to accomplish this aim. Firstly, given that members of the TGFβ superfamily can mimic the oocyte and enable cumulus expansion, activin A and B will be examined to establish whether they account for the mouse CEEF. Secondly, the effect of a TGFβ superfamily antagonist on cumulus expansion and the expression of various ECM molecules will be investigated. Work associated with achieving this aim is presented in Chapter 3.

Chapter 2

Role of Oocyte-Secreted Growth Differentiation Factor 9 in the Regulation of Mouse Cumulus Expansion

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Endocrinology 2005 146(6): 2798-2806

† Contributions to this work by each author listed in Appendix 7

2.1 Abstract

Oocyte-secreted factors (OSFs) are required for expansion of the mouse cumulus-oocyte complex (COC), which is necessary for ovulation. Oocyte-secreted growth differentiation factor 9 (GDF9) signals through the bone morphogenetic protein receptor-II (BMPRII) and is currently the primary candidate molecule for the cumulus-expansion enabling factor (CEEF). This study was conducted to determine whether GDF9 is the mouse CEEF. COCs were collected from mice and the oocyte was microsurgically removed to generate an oocytectomised (OOX) complex. OOX complexes treated with FSH alone or recombinant mouse GDF9 alone failed to expand, whereas expansion was induced in the presence of FSH by GDF9, TGFβ1 or co-culture with oocytes. A specific GDF9 neutralising antibody, mAb-GDF9-53, neutralised the expansion of OOX complexes in response to GDF9, but not the expansion of OOX complexes co-cultured with oocytes. Using real-time RT-PCR, hyaluronan synthase-2 (HAS2) mRNA expression by OOXs was up-regulated 4- to 6- fold by oocytes and GDF9. Monoclonal neutralising antibody- GDF9-53 attenuated GDF9-induced OOX HAS2 expression but not oocyte-induced HAS2 expression. A TGFβ antagonist neutralised TGFβ-induced, but not oocyte-induced, expansion of OOX complexes, and when combined with monoclonal neutralising antibody-GDF9-53 also failed to neutralise oocyte-induced expansion. Furthermore, a soluble portion of the BMPRII extracellular domain, which is a known GDF9 antagonist, completely antagonised GDF9-induced expansion but only partially neutralised oocyte-induced expansion. This study provides further evidence that like TGFβ, GDF9 can enable FSH-induced cumulus expansion, but more importantly, demonstrates that neither GDF9 nor TGFβ alone, nor the two in unison, account for the critical OSFs regulating mouse cumulus expansion.

2.2 Introduction

In vivo, the mid-cycle LH surge initiates the ovulatory cascade resulting in expansion of the cumulus cell (CC) mass surrounding the oocyte. Cumulus expansion facilitates the release of the oocyte into the abdominal cavity, capture of the oocyte by the oviductal fimbria, sperm penetration and subsequent fertilisation. Correct cumulus expansion is a critical physiological process as impaired expansion leads to sterility (1). *In vitro*, cumulus expansion is not induced by LH, but can be mimicked by FSH, epidermal growth factor (EGF) or EGF-like peptides (2-4). These hormones induce CCs to secrete a number of extracellular matrix (ECM) molecules including hyaluronan (HA), the production of which is primarily controlled by the enzyme hyaluronan synthase-2 (HAS2), resulting in an expanded mucified ECM surrounding the oocyte (5).

Development of the ovarian follicle is controlled by bi-directional communication between the germ cell and somatic cells (6). Endocrine, paracrine, autocrine and gap-junctional signalling are responsible for the growth and development of the follicle (7). It is now well established that oocyte paracrine signalling to CCs is essential for mouse cumulus expansion. Mouse oocytes secrete a soluble factor(s) that enables CCs to produce matrix molecules in response to FSH (2, 8-10). Removal of the oocyte from the cumulus-oocyte complex (COC) by microsurgery (oocytectomy) eliminates cumulus expansion. However, by co-culturing oocytectomised (OOX) complexes with fully grown denuded oocytes (DOs), cumulus expansion is restored (2, 8). This demonstrates that mouse oocytes produce a soluble cumulus expansion-enabling factor (CEEF) that is absolutely required for cumulus expansion (2, 8, 9).

Since these pioneering studies on the role of the oocyte in cumulus expansion, it is now widely recognised that oocyte paracrine factors regulate a multitude of other processes involved in folliculogenesis [reviewed in Refs. (11, 12)], including; regulation of granulosa cell (GC) proliferation (13-15) and steroidogenesis (16); modulation of inhibin, activin and follistatin synthesis (17, 18); regulation of expression of kit-ligand (19), LH receptor (20) and urokinase plasminogen activator (21, 22). Clearly oocyte paracrine signalling to follicular GCs is essential for regulating normal folliculogenesis and fertility, yet to date the exact identities of these oocyte-secreted factors (OSFs) are unknown. This represents a clear deficiency in our current understanding of fundamental mechanisms regulating ovarian biology and fertility.

Members of the TGFβ superfamily, in particular growth differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15), and BMP6, are currently the prime candidate molecules for OSF, due to their ability to mimic the actions of oocytes on GCs *in vitro* (12, 23). Many studies have now demonstrated that both TGFβ1 and TGFβ2 are able to completely substitute for the oocyte and mimic many oocyte-regulated GC functions, including oestradiol production (23), mural GC proliferation (23-25) and CC expansion (23, 26). However, in all these studies, the addition of a specific TGFβ-neutralising antibody could only attenuate the specific effects of recombinant TGFβ1 and TGFβ2 and not the effects of oocytes, demonstrating that TGFβ1 and TGFβ2 alone are not the critical OSF mediating these granulosa events.

Until very recently, experimental neutralisation of OSF has not been possible for the more recently identified members of the TGFβ superfamily, GDF9 and BMP15. Both GDF9 and BMP15 are homodimeric proteins, expressed primarily in gametes of which oocyte expression is essential for female fertility in a species-specific manner (27-30). GDF9 uses the bone morphogenetic protein receptor type-II (BMPRII) and the TGFβ type I receptor such that GDF9 elicits a TGFβ-like intracellular response (31-34). Importantly, GDF9 and BMP15, like TGFβ1/β2, are able to mimic most oocyte-regulated GC activities described so far (35, 36). However, it remains to be shown whether GDF9 and BMP15 are acting like TGFβ1/β2 and mimicking OSF or whether these molecules are in fact the key OSF. Until recently these questions have remained unanswered, in part due to the lack of GDF9 and BMP15 experimental reagents. Recently our laboratory characterised a GDF9 monoclonal-neutralising antibody (mAb-GDF-53) specific within the TGFβ superfamily to GDF9 (25). mAb-GDF9-53 is a potent GDF9 antagonist, eliminating all recombinant GDF9 and approximately 50% of oocyte GC mitogenic activity (25).

Currently, GDF9 is considered to be the most likely candidate molecule for the CEEF in the mouse. Cumulus expansion is induced when OOX complexes are treated with recombinant GDF9 (22, 37). Hence, to some extent, GDF9 acts like TGFβ1, mimicking the expansion-stimulating properties of oocytes. However, it is puzzling that recombinant GDF9 promotes cumulus expansion in the absence of FSH, in contrast to oocyte-induced expansion, which requires FSH. Furthermore, oocytes obtained from GDF9 null mice were unable to induce cumulus expansion of OOX complexes *in vitro* (23). More recently, an RNA interference

approach was used to show that cumulus expansion was significantly reduced when oocytes were injected with GDF9 double-stranded RNA (38). The current inference from the literature is that GDF9 alone is the mouse CEEF (22, 35, 38), although based on these results and other evidence outlined above, this remains a controversial and an open question.

This study examines the hypothesis that GDF9 is the key OSF responsible for enabling cumulus expansion in the mouse. To determine whether oocyte-secreted GDF9 is the CEEF in the mouse we attempted to antagonise oocyte-induced cumulus expansion and HAS2 expression using a novel GDF9 neutralising antibody and a previously described GDF9 antagonist. Findings from this study provide evidence against the hypothesis that GDF9 alone is the mouse CEEF.

2.3 Materials and Methods

Unless specified, all chemicals and reagents were purchased from Sigma (St Louis, MO).

2.3.1 Isolation of COCs

Mice used in this study were maintained at the Queen Elizabeth Hospital animal house. The study was approved by local Animal Ethics Committees and was conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Twenty-one- to 28-d-old 129/SV mice were injected with 5 IU equine chorionic gonadotrophin (eCG; Folligon, Intervet, Castle Hill, Australia) and ovaries were collected 46 h later. Ovaries were cleaned free of adherent adipose and connective tissues and placed in HEPES-buffered tissue cultured medium-199 (H-TCM-199; ICN Biomedicals Inc., Costa Mesa CA) supplemented with 0.1% (wt/vol) BSA (H-TCM-199/BSA). COCs were isolated by puncturing antral follicles with 27-gauge needles and collected in H-TCM-199/BSA. Only COCs with a uniform covering of compacted CCs were used in this study.

2.3.2 Culture of COCs and OOX complexes

Microsurgical removal of the oocyte from the COCs was performed using a micromanipulation apparatus on an inverted microscope as previously described by Buccione *et al.*, (8). Approximately 200 OOX complexes were generated per hour. Treatment drops of 15 µl of Waymouth MB 752/1 medium (WAY) supplemented with penicillin G (100 U/ml),

streptomycin sulphate (100 mg/ml), 5% (vol/vol) fetal calf serum (FCS) (Trace Biosciences, Castle Hill, New South Wales, Australia); 50 mIU/ml recombinant human FSH (Puregon; Organon, Oss, Netherlands); and with or without treatment reagent were set up and overlaid with mineral oil in falcon petri dishes. COCs or OOX complexes were transferred in a 10 µl volume to the 15 µl drops to give a total volume of 25 µl. Ten COCs or OOX complexes were cultured per 25 µl drop. The complexes were cultured for 20 h at 37°C, 96% humidity in 5% CO_2 in air, before being assessed for degree of cumulus expansion.

2.3.3 Treatment of OOX complexes

Denuded oocytes

Oocytes were denuded of their surrounding CCs by rapidly agitating COCs using a vortex mixer for approximately 4 min in 2 ml H-TCM-199/BSA. Groups of 20 DOs and 10 OOX complexes were transferred together in a 10 µl volume to the 15 µl treatment drops. This results in a concentration of 0.8 DO/µl, which preliminary experiments revealed to generate maximal cumulus expansion.

2.3.4 TGFβ superfamily growth factors

Production of recombinant mouse GDF9 used in this study has previously been described (25, 39). In brief, recombinant mouse GDF9 was produced in-house using a transfected 293 human embryonic kidney cell line (293H), generously donated by Dr. Olli Ritvos (University of Helsinki, Helsinki, Finland). Control conditioned medium from untransfected 293H cells inhibited cumulus expansion and raw conditioned medium from GDF9-transfected cells did not promote cumulus expansion. Consequently, conditioned media were subjected to partial purification using hydrophobic interaction chromatography (40), which was effective at removing the inhibitory factors from the 293H parent cell line. These techniques generate a partially pure, mostly processed GDF9 of 17.5 kDa. Recombinant human TGFβ1 and recombinant human BMP6 were obtained (R&D Systems Minneapolis, MN) and were used at concentrations previously described (22, 23).

2.3.5 Growth factor antagonists

Attempts were made to antagonise recombinant and oocyte-secreted GDF9 bioactivity using a recently described GDF9 neutralising monoclonal antibody, mAb-GDF9-53, which was generously donated by Prof. Nigel Groome (Oxford Brookes University, Oxford, UK) (25). This mouse mAb was raised against a 32-amino acid peptide at the C-terminus of human GDF9. mAb-GDF9-53 has strong immuno-affinity for recombinant mouse GDF9 and has very weak cross reactivity with other members of the TGFβ superfamily, including BMP15. mAb-GDF9-53 specifically neutralises the mitogenic activity of recombinant mouse GDF9 and partially antagonises that of mouse OSF (25). A synthetic portion of the BMPRII extracellular domain (ECD) and the TGFβ receptor II extracellular domain (TGFβRII) ECD fused to the human IgG-Fc region, were both obtained from R&D Systems. These solubilised receptors act as antagonists by binding their respective ligands, thereby dramatically reducing ligand interaction with the native type II receptor. The BMPRII ECD presumably antagonises the many ligands utilising this receptor and it has been shown to neutralise the bioactivity of the recombinant forms of the key putative OSF, GDF9 (31) and BMP15 (32). Importantly, the BMPRII ECD completely neutralises the GC growth-promoting bioactivity of mouse oocytes (41).

2.3.6 Assessment of cumulus expansion

Cumulus expansion of COC and OOX complexes was recorded after a 20 h culture period. This was a blinded assessment to eliminate bias. The degree of cumulus expansion was assessed according to a subjective scoring system (0 to +4). In brief, Score 0, indicates no expansion and score +4, indicates complete expansion of all CC layers. A cumulus expansion index (CEI; 0.0 to 4.0) was calculated as previously described (9, 42).

2.3.7 Real-time RT-PCR

RNA isolation

An experiment was conducted to examine the effect of neutralisation of oocyte-secreted GDF9 on CC HAS2 mRNA levels after 6 h. OOX complexes were cultured in WAY supplemented with 5% (vol/vol) FCS + FSH (50 mIU/ml) and one of the following treatments: 1) 0 (control), 2) oocytes (0.8/μl), 3) oocytes + mAb-GDF9-53 (40 μg/ml), 4) GDF9 (250 ng/ml), or 5) GDF9 + mAb-GDF9-53. Ten OOX complexes were cultured per

treatment group, each treatment group was in quadruplicate and the experiment was replicated on five separate occasions. After the 6 h incubation, the DOs were removed and the OOX complexes were washed in H-TCM-199/BSA. The OOX complexes were transferred to Eppendorf tubes (40 OOX per tube) on ice and RNA was isolated using a micro RNA isolation kit (QIAGEN, Victoria, Australia). This included addition of 20 ng of carrier RNA to each sample before homogenisation, and all samples were DNase treated to eliminate any contaminating genomic DNA. RNA was quantified using a Ribogreen RNA quantification kit (Molecular Probes, Eugene, OR) according to the manufacturer's protocol.

2.3.8 Real-time RT-PCR analysis

Ninety nanograms of RNA was reverse transcribed using random primers (Roche Molecular Biochemicals, Mannheim, Germany) and a Superscript II RT kit (Life Technologies, Inc., Grand Island, NY) according to the manufacturer's instructions. A negative RT control substituting water for RNA was included. Primer pairs were designed for mouse ribosomal protein L19 (L19), and HAS2 using Primer Express software (PE Applied Biosystems, Foster City, CA), and synthesised by Geneworks (Adelaide, Australia). The sequence for each primer pair was as follows; L19 sense 5'-GAAAGTGCTTCCGATTCCA-3' and anti-sense primer 5'-TGATCGCTTGATGCAAATCC-3' based on mouse L19 sequence (accession no. NM_026490) and HAS2 sense primer 5'-CATTCCCAGAGGACCGCTTAT-3' and antisense primer 5'-AAGACCCTATGGTTGGAGGTGTT-3' based on mouse HAS2 sequence (accession no. U52524).

To consider L19 as an appropriate housekeeping gene, the critical threshold (C_T) value for all samples should not vary significantly across treatment groups. Using an ABI GeneAmp 5700 machine (PE Applied Biosystems), L19 mRNA levels were measured in triplicate and were then normalised to total RNA measurements for each sample. There were no significant differences in L19 mRNA levels between treatment groups ($P>0.05$). Primer amplification efficiencies were also examined to ensure that the housekeeping gene L19, and the target gene HAS2, primed with the same amplification efficiency. Each primer set was run with serially diluted cDNA and the slopes of each primer set were determined using the C_T values plotted against log dilutions of the cDNA. Slopes for each gene were determined and the L19 slope was statistically comparable to that of HAS2 ($P>0.05$).

Each experimental sample was run in triplicate on an ABI GeneAmp 5700 sequence detection system. Each sample consisted of: 3 µl of diluted cDNA sample (1:9), 10 µl of 2x SYBR green master mix (PE Applied Biosystems) and 10 pmol of each primer. Samples were treated at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of amplification at 95°C for 15 sec and 60°C for 1 min. No template controls, substituting water for cDNA, and a negative RT were used in each run. HAS2 gene expression was calculated for each sample relative to the housekeeping gene, L19, using the $2^{-\Delta\Delta C_T}$ method as described in Ref. (43). After RT-PCR amplification, a dissociation analysis was run on all products to ensure that a single product was produced during the PCR process. Products were then run on a 2% (wt/vol) agarose gel for confirmation of single, correctly sized products. Finally, the identity of the each PCR product was verified by sequencing. The L19 amplicon was 97% homologous to mouse L19 (accession no. NM_026490) and the HAS2 amplicon was 98% homologous to mouse HAS2 (accession no. U52524).

2.3.9 Data analyses

Each experiment was replicated three to five times (see figure legends). Treatment effects on cumulus expansion were examined using a Kruskal-Wallis one-way ANOVA on ranks and differences between means were detected using Dunn's method *post-hoc* comparisons or *t* tests. Real-time RT-PCR data underwent log transformation to satisfy ANOVA criteria and were then subjected to one-way ANOVA followed by Tukey comparisons. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using the software package SigmaStat for Windows version 2.03 (Jandel Corp., San Ramon, CA).

2.4 Results

2.4.1 Oocyte regulation of cumulus expansion is mimicked by certain members of the TGFβ superfamily

To examine the effect of OSF and TGFβ superfamily OSF candidate molecules on cumulus expansion, COC and OOX complexes were cultured alone, as positive and negative controls, or OOX complexes were treated with DO, GDF9, TGFβ1, BMP6 or 293H (GDF9 negative control conditioned media) in the presence of FCS and FSH. As expected, intact COC expanded to a degree of 2.7 ± 0.1, and the negative control OOX complexes cultured alone

failed to expand (Fig. 1A). However, expansion of OOX complexes was significantly ($P<0.05$) induced by co-culture with oocytes (2.6 ± 0.2), GDF9 (3.4 ± 0.2) or TGFβ1 (2.0 ± 0.2) (Fig. 1A). OOX complexes treated with 293H-conditioned medium or BMP6 failed to undergo expansion. GDF9 enabled OOX cumulus expansion in a dose-dependent manner, restoring expansion to COC levels at 250 ng/ml (Fig. 1B). The data presented in Fig. 1B was generated from work carried out towards the degree of BSc. Hons (44) and is included in the current thesis for completeness only.

2.4.2 GDF9 stimulated cumulus expansion requires FSH

This experiment was conducted to examine the requirement for FSH during GDF9-induced cumulus expansion. OOX complexes treated with FSH alone or GDF9 alone, failed to expand (score 0; Fig. 2). Expansion was significantly ($P<0.05$) induced with the combined presence of FSH and GDF9 (3.4 ± 0.2), mimicking the action of oocytes in co-culture, which require FSH to induce cumulus expansion (2.6 ± 0.2; Fig. 2).

2.4.3 Neutralisation of oocyte-secreted GDF9 does not prevent cumulus expansion

To determine whether GDF9 is the CEEF in the mouse, a specific GDF9 neutralising antibody mAb-GDF9-53 was tested against GDF9 and oocyte-induced cumulus expansion. Intact COC underwent cumulus expansion when cultured in medium supplemented with FCS and FSH (Fig. 3A). As expected, untreated OOX complexes failed to expand and expansion was restored when co-cultured with oocytes or treated with GDF9. The GDF9 neutralising antibody, mAb-GDF9-53, antagonised the expansion of OOX complexes stimulated by GDF9 in a dose dependent manner ($P<0.05$; Fig. 3A). In contrast, there was no significant effect of mAb-GDF9-53 on the expansion of OOX complexes stimulated by oocytes. The neutralising actions of mAb-GDF9-53 were not caused by general antagonist actions of the class of immunoglobulins, as mouse IgG (40 μg/ml) had no inhibitory effect on GDF9-induced or oocyte-induced cumulus expansion (2.9 ± 0.3, 2.4 ± 0.2, respectively). The data presented in Fig. 3A was generated from work carried out towards the degree of BSc. Hons (44) and is included in the current thesis for completeness only.

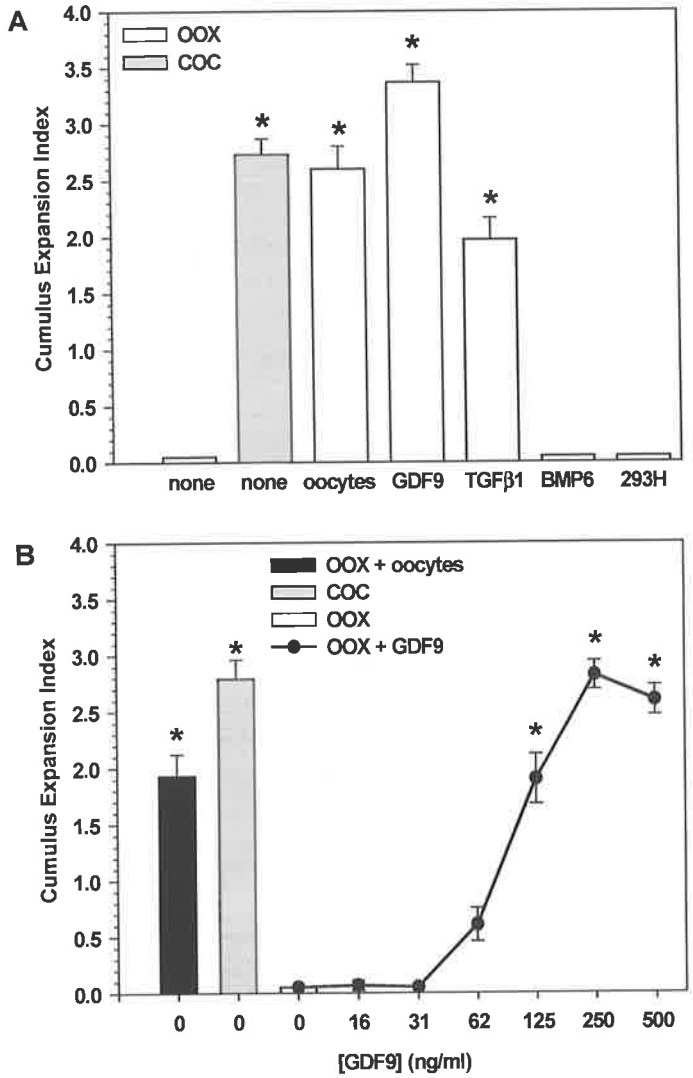

Figure 1. Oocyte-secreted factors and TGFβ superfamily members that enable CC expansion. (A) OOX complexes were cultured alone, with denuded oocytes (0.8/μl), GDF9 (250 ng/ml), TGFβ1 (10 ng/ml), BMP6 (50 ng/ml) or 293H (20% vol/vol; conditioned medium from the untransfected 293H parent cell line). OOX complexes and COCs were cultured in media supplemented with 50 mIU/ml FSH and 5% FCS. (B) OOX complexes were cultured in the presence of 50 mIU/ml FSH with an increasing dose of recombinant GDF9 (16-500 ng/ml). The degree of cumulus expansion was determined after 20 h of culture using a scale of 0 (no expansion) to +4 (maximal expansion). Results represent the mean ± SEM from three experiments, each with a total of 10 complexes (OOX or COC) per treatment group. An *asterisk* represents a significant difference to OOX alone ($P<0.05$). Data presented in Fig. 1B carried out towards the degree of BSc. Hons (44).

To verify that the morphological cumulus expansion results measured in the above experiment are associated with changes in HA production, treatment effects of GDF9 and mAb-GDF9-53 on the expression of HAS2 mRNA were examined using Real-time RT-PCR after 6 h of culture. CC HAS2 expression was up-regulated approximately 6-fold by recombinant GDF9 and approximately 5-fold by oocytes, compared to untreated OOX complexes (Fig. 3B). The GDF9 neutralising antibody, mAb-GDF9-53 significantly ($P<0.05$)

antagonised GDF9-induced OOX HAS2 expression, but not oocyte-induced HAS2 expression. This result confirms that treatment-induced morphological changes of the cumulus are related to a change in gene expression.

A second and well-known GDF9 antagonist was examined for its capacity to neutralise oocyte-induced CC expansion. It has previously been documented that BMPRII is the type-II receptor for GDF9 and that an (ECD) of BMPRII completely antagonises recombinant GDF9 bioactivity (31) as well as the mitogenic effects of OSF on GCs (41). OOX complexes co-cultured with oocytes were treated with an increasing dose of BMPRII ECD. The BMPRII ECD caused a dose dependent partial neutralisation of oocyte-induced cumulus expansion. GDF9-induced cumulus expansion was notably antagonised by BMPRII ECD in a dose-dependent manner. At a dose of 10 μg/ml of BMPRII ECD, which completely abolished GDF9- induced expansion, oocyte-induced expansion was still comparable to the COC positive control and was significantly higher than the GDF9 treatment at that dose ($P<0.05$; Fig. 4).

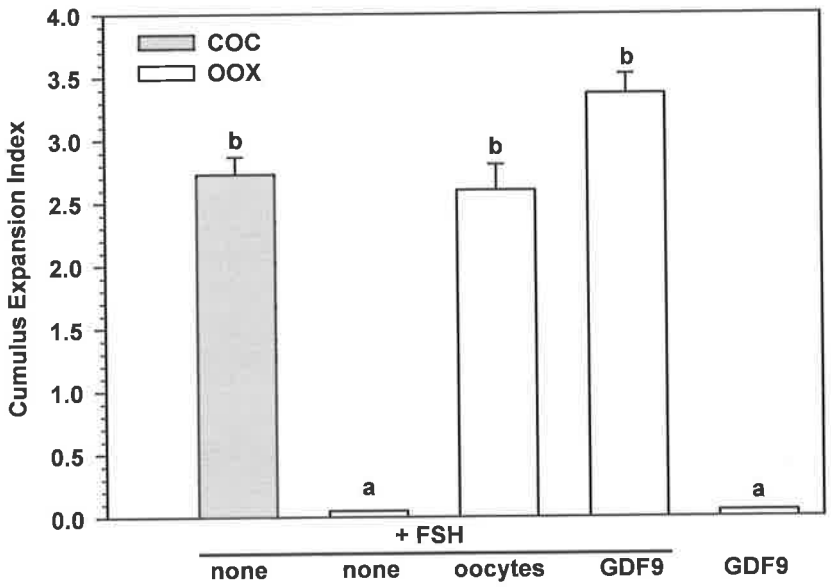

Figure 2. GDF9 enables FSH-stimulated CC expansion. OOX complexes were cultured with 50 mIU/ml FSH with or without denuded oocytes (0.8/μl), or with recombinant GDF9 (250 ng/ml) with or without FSH. FSH is required for GDF9-induced cumulus expansion. The degree of cumulus expansion was determined after 20 h of culture using a scale of 0 (no expansion) to +4 (maximal expansion). Results represent the mean ± SEM from 3 experiments each using a total of 10 complexes (OOX or COC) per treatment group. *Bars with different superscript letters* are significantly different ($P<0.05$).

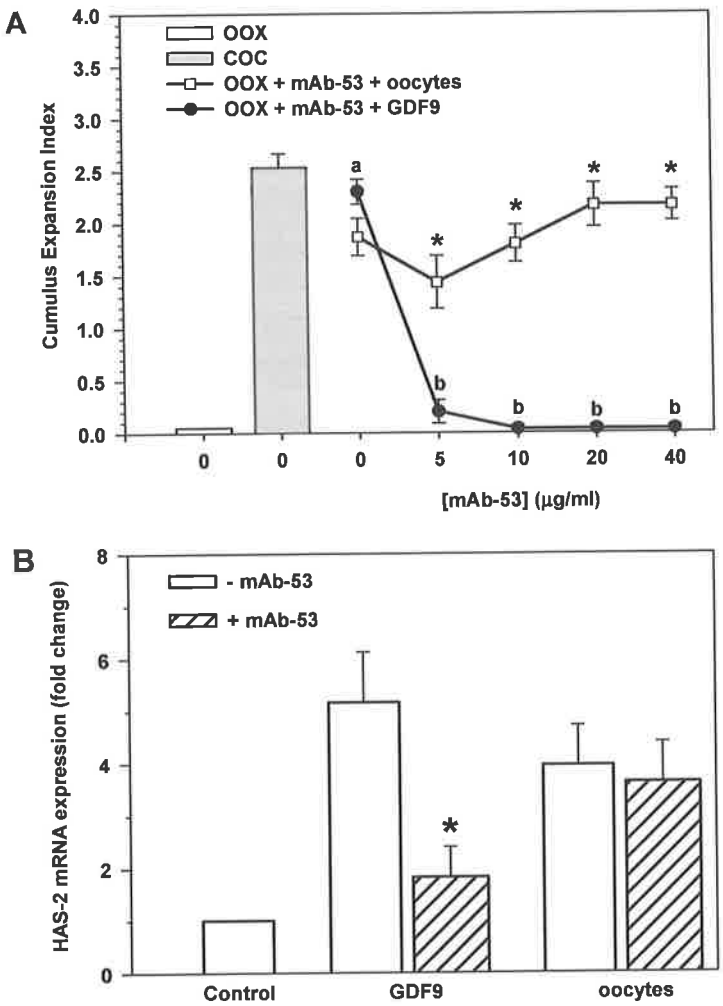

Figure 3. Effect of anti-GDF9 mAb-GDF9-53 on cumulus expansion and CC HAS2 mRNA expression. (A) OOX complexes were co-cultured with oocytes (0.8/µl) or treated with GDF9 (250 ng/ml) to induce cumulus expansion and with an increasing dose (0-40 µg/ml) of mAb-GDF9-53. All control and treatment groups were cultured for 20 h in media supplemented with 50 mIU/ml FSH and then cumulus expansion was measured according to the subjective scoring system; 0 (no expansion) to +4 (maximal expansion). The GDF9 neutralising antibody antagonised GDF9-induced, but not oocyte induced, cumulus expansion. Means within a line graph with *different superscript letters* are significantly different ($P<0.05$). *Asterisks* indicate the two means at that dose of antagonist are significantly different ($P<0.05$). (B) Fold differences in CC expression of HAS2 mRNA after 6 h culture in the presence of 50 mIU/ml FSH. Real-time RT-PCR analysis was performed using primer sets for HAS2 and L19 using RNA from CCs (OOX) cultured alone, with GDF9 (250 ng/ml) or with DOs (0.8/µl), either in the presence or absence of 40 µg/ml mAb-GDF9-53. Results are from five independent experiments and are expressed as a fold change from mRNA levels in OOX complexes cultured alone (control). All samples have been normalised to L19 mRNA content. An *asterisk* denotes a significant effect of mAb-GDF9-53 ($P<0.05$). Data presented in Fig. 3A carried out towards the degree of BSc. Hons (44).

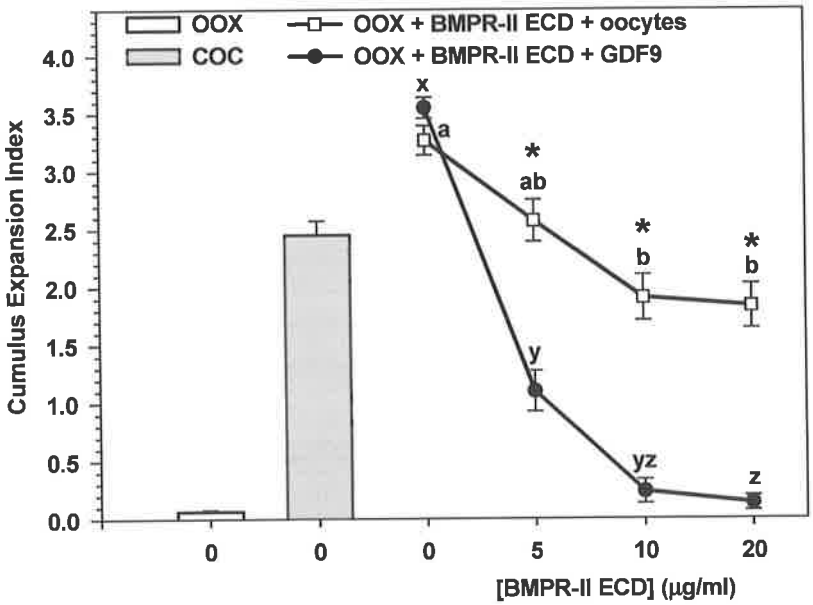

Figure 4. Effect of BMPRII Ectodomain on expansion of mouse OOX complexes. OOX complexes were cultured in the presence FSH either with oocytes (0.8/µl), recombinant GDF9 (250 ng/ml) and treated with an increasing dose of BMPRII ECD (5-20 µg/ml). The degree of cumulus expansion was measured using a scale of 0 (no expansion) to +4 (maximal expansion). Means within a line graph with *different superscript letters* are significantly different ($P<0.05$). *Asterisks* indicate the two means at that dose of antagonist are significantly different ($P<0.05$).

2.4.4 Neutralisation of oocyte-secreted TGFβ and/or GDF9 does not prevent cumulus expansion

These experiments were designed to assess whether GDF9 and TGFβ operate in a redundant manner to enable cumulus expansion. To do this, a known TGFβ antagonist, TGFβRII ECD, was tested for its capacity to neutralise TGFβ1-induced and oocyte-induced cumulus expansion, in the presence and absence of the GDF9 antagonist, mAb-GDF9-53. As expected intact COC cultured in the presence of FCS and FSH underwent cumulus expansion to a degree of 2.4 ± 0.2 (Fig. 5A). The addition of oocytes or TGFβ1 significantly ($P<0.05$) stimulated OOX complexes to expand to 2.3 ± 0.2 and 2.6 ± 0.2, respectively. The TGFβRII ECD was effective at neutralising the response of OOX complexes to TGFβ1 ($P<0.05$), but had no significant effect on the expansion of OOX complexes co-cultured with oocytes or on the expansion of COCs, indicating that TGFβRII ECD does not impede the actions of the oocyte-secreted CEEF (Fig. 5A). This result is consistent with the previous findings of Salustri *et al.* (26). A similar pattern of results were observed using the GDF9 antagonist mAb-GDF9-53, which effectively neutralised GDF9-induced OOX expansion, but had no significant effect on oocyte-induced expansion or expansion of intact COCs (Fig. 5B). OOX

complexes stimulated to expand by co-culture with oocytes but treated simultaneously with the GDF9 antagonist, mAb-GDF9-53, and the TGFβ antagonist, TGFβRII ECD, nonetheless underwent expansion to levels equivalent to the positive controls (P>0.05; Fig. 5C). The experiments performed that led to the results displayed in Fig. 5A and 5B were performed during the degree of BSc Hons (44) and these are included in the current thesis for completeness only.

2.5 Discussion

Expansion of the COC at ovulation is required for fertility (1). For cumulus expansion to occur in the mouse, there is an absolute requirement for a soluble OSF (2, 8, 26). The identity of the mouse CEEF has remained elusive for the past 15 years, and currently the primary candidate molecule is GDF9 [reviewed in Ref. (23)]. The current study was undertaken to investigate the role oocyte-secreted GDF9 plays in enabling cumulus expansion in the mouse. A well established cumulus bioassay was used to measure cumulus expansion (9). This entails a scoring system that is a total morphological measure of all ECM components that contribute to this process *in vitro*. The results of this study confirm that recombinant GDF9 and TGFβ1 are able to mimic the paracrine actions of oocytes and enable cumulus expansion; however neither antagonism of GDF9 alone (using two different approaches), TGFβ alone, or the two together did not neutralise oocyte-induced cumulus expansion. These findings provide strong evidence against the hypothesis that oocyte-secreted GDF9 is the key molecule regulating cumulus expansion in the mouse.

In vitro, cumulus expansion requires both the oocyte and stimulation, either by FSH or EGF (2, 3). Microsurgical removal of the oocyte from the COC ablates FSH-stimulated cumulus expansion, which is restored by co-culture of OOXs with oocytes but only in the presence of FSH (8). Our results show that OOX complexes treated with GDF9 in the absence of FSH do not undergo cumulus expansion but do in the presence of FSH, demonstrating that our GDF9 is behaving in the same manner as the oocyte in enabling cumulus expansion. Curiously, other groups (22, 45) recently reported that GDF9 in the absence of FSH stimulates cumulus expansion *in vitro*. Reasons for this discrepancy are unclear and warrant further study. One

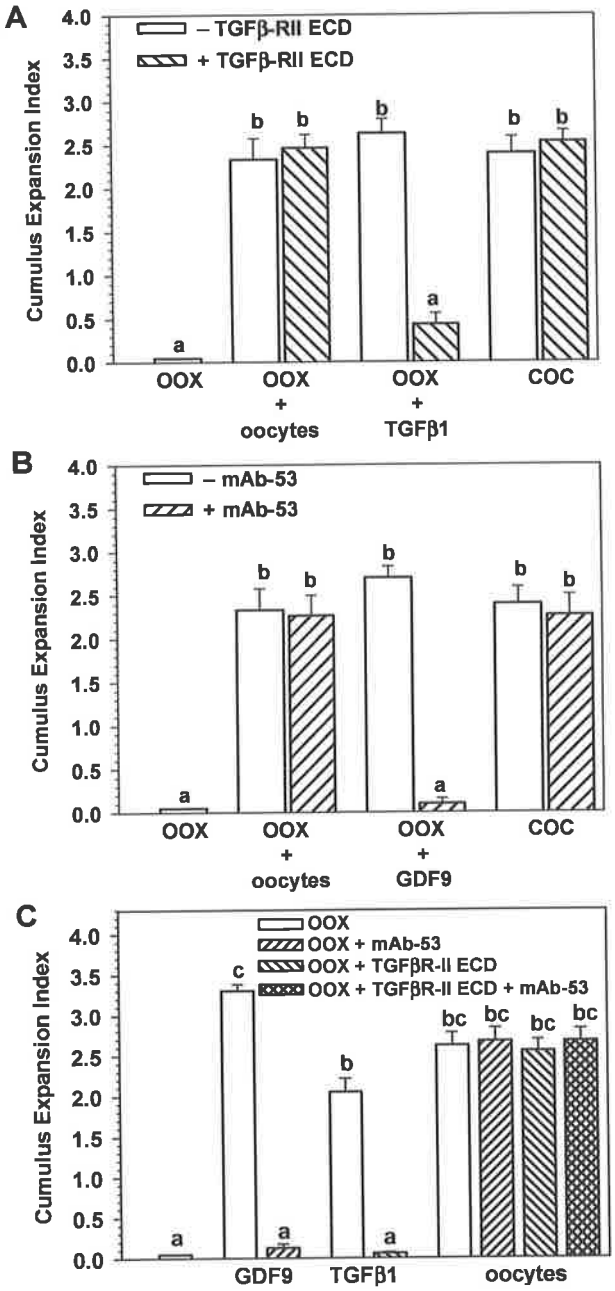

Figure 5. Effect of TGFβ ectodomain and/or mAb-GDF9-53 on cumulus expansion. All treatments consisted of 10 OOX/COC complexes cultured in media supplemented with 50 mIU/ml FSH. (A) OOX complexes were cultured with oocytes (0.8/μl) or 10 ng/ml TGFβ1 in the presence or absence of 200 ng/ml TGFβRII ECD. COCs were also cultured with or without TGFβRII ECD. (B) OOX complexes were cultured with denuded oocytes (0.8/μl) or 250 ng/ml GDF9 in the presence or absence of 40 μg/ml mAb-GDF9-53. COCs were also cultured either with or without mAb-GDF9-53. (C) OOX complexes were cultured alone, with GDF9 or with TGFβ1 in the presence or absence of mAb-GDF9-53 or TGFβRII ECD, respectively. OOX complexes were co-cultured with oocytes and treated with mAb-GDF9-53, TGFβRII ECD or the two antagonists together. After 20 h of culture the degree of cumulus expansion was assessed using the subjective scoring system; 0 (no expansion) to +4 (maximal expansion). Results show the mean ± SEM of three-four individual experiments and *bars within a graph with different superscript letters* are significantly different ($P<0.05$). Data presented in Fig. 5A and 5B carried out towards the degree of BSc. Hons (44).

possible explanation is that the chemical nature of the GDF9 used in these two studies somehow differs. GDF9 is not commercially available, and the production conditions in different laboratories will ultimately lead to different molecular forms of GDF9. The GDF9 from our laboratory is partially purified using hydrophobic interaction chromatography, a process that is required to demonstrate GDF9 bioactivity using the cumulus expansion bioassay. Moreover, the proregion of the GDF9 that we produce has been proteolytically cleaved to generate mostly mature processed GDF9 (25, 39). It has previously been hypothesised that cumulus expansion requires a FSH-stimulated CC product that alters the structure of native GDF9, *e.g.* processes the proregion, making it biologically active and thereby enabling expansion (45). In this case, recombinant GDF9, which is produced in this modified processed form, would therefore not require the FSH-dependent factor produced by CCs (45). Our results do not support this hypothesis as they illustrate that fully processed recombinant GDF9, just like OSF, requires FSH to stimulate cumulus expansion.

Although GDF9 specifically induces cumulus expansion, this does not mean that GDF9 is the key OSF that normally mediates this process. This is most clearly illustrated by the fact that just like GDF9, TGFβ1 and TGFβ2 can also behave in this manner and mimic a diverse range of oocyte-regulated GC/CC processes, including cumulus expansion, proliferation and steroidogenesis. However, TGFβ1/β2 are not the OSF regulating these processes (23, 24, 26). This has now been demonstrated in many studies using TGFβ neutralising antibodies, which are unable to inhibit oocyte-paracrine effects on GCs (23-26). Until recently this kind of experimental approach has not been possible with regard to GDF9 because GDF9 is a newly described molecule, and specific reagents and experimental tools are still limited.

This study is the first to use a GDF9 neutralising antibody in the cumulus expansion assay. The antagonist mAb-GDF9-53 is a highly specific monoclonal neutralising antibody recently characterised in detail in our laboratory (25). In the current study, mAb-GDF9-53 effectively inhibited recombinant GDF9-induced cumulus expansion but did not antagonise oocyte-induced cumulus expansion. COCs treated with the GDF9 neutralising antibody also underwent cumulus expansion. Failure of mAb-GDF9-53 to antagonise expansion of an intact complex is perhaps not surprising, as the IgG may not effectively penetrate the cumulus mass to reach the source of the OSF. However, there can be little doubt the antibody has access to OSF in the OOX + DO cultures. This is the first example of antibody-mediated neutralisation

of GDF9-induced cumulus expansion, although this was anticipated based on the characteristics of the antibody. mAb-GDF9-53 is a mouse monoclonal raised against a 32-amino acid peptide at the C-terminus of human GDF9 (25). Epitope mapping and alignment of the binding sequence indicate that the motif is highly conserved across species and so the neutralising activity is unlikely to be species specific. Alignment of the epitope with related members of the TGFβ superfamily illustrates low homology with BMP15 and no homology with the next closest member of the superfamily, and as such, mAb-GDF9-53 has low immuno-affinity for BMP15 and does not antagonise TGFβ1 or activin A bioactivity on GCs (25). Importantly, mAb-GDF9-53 does recognise the molecular form of GDF9 secreted by mouse oocytes and furthermore does partially antagonise oocyte-stimulated GC proliferation (25), even though in the current study this antibody failed to inhibit oocyte-induced cumulus expansion.

To confirm that the measured changes in cumulus morphology are associated with quantitative changes in CC gene expression, HAS2 was assessed, which is the major HA synthase enzyme involved in regulating cumulus expansion. Cumulus expansion involves the formation of a mucoid ECM surrounding the oocyte of which HA is a major structural component (22). The present study demonstrates that both oocytes and GDF9 can up-regulate HAS2 expression. Confirming the cumulus morphological observations, the GDF9 neutralising antibody antagonised GDF9-induced HAS2 expression, but did not neutralise oocyte-induced HAS2 expression. This suggests there is likely redundant regulation of HAS2 by other OSF as well as by GDF9, further supported by the observation that COCs from BMP15 null mice exhibit reduced HAS2 expression (46). Together, these provide additional evidence against the hypothesis that GDF9 is the sole oocyte factor enabling cumulus expansion.

To provide an additional line of evidence, an alternative GDF9 antagonist was tested for its capacity to antagonise oocyte-induced cumulus expansion. Previously it has been shown that GDF9 binds the BMPRII and that a solubilised portion of the ECD (BMPRII ECD) neutralises GDF9 bioactivity (31) and, importantly, also completely eliminates oocyte growth-promoting activity (41). In the current study, BMPRII ECD completely antagonised GDF9-induced cumulus expansion, but only partially neutralised oocyte-induced cumulus expansion. The latter suggests that signalling through BMPRII may be an important, but not

an exclusive, feature of the cumulus expansion process, and hence suggests that other receptors and their ligands are likely to be involved.

If neither GDF9 nor TGFβ alone is the CEEF and oocyte factors other than those utilising BMPRII are involved in cumulus expansion, we further considered whether GDF9 and TGFβ operate in a redundant manner to enable cumulus expansion. GDF9 and TGFβ use different type-II receptors, but they use a common type-I receptor, activin receptor-like kinase 5, and hence a common intracellular signalling pathway, both activating mothers against decapentaplegic-2 and –3 (Smad 2/3) (34, 39). Here we demonstrate that simultaneous antagonism of GDF9 and TGFβ using mAb-GDF9-53 and TGFβRII ECD, fail to neutralise oocyte-induced cumulus expansion. Together these results suggest that the mouse CEEF is composed of multiple OSFs, which may include GDF9 and TGFβ among others.

The conclusion that GDF9 is not the sole constituent of the CEEF may appear to contradict some very recent studies. Vanderhyden *et al.*, (23) showed that oocytes from GDF9-deficient mice are unable to promote expansion of OOX complexes. However as acknowledged by the authors, results obtained from the GDF9 knockout mice should be interpreted with caution due to the likelihood that GDF9 is not the only factor missing from these oocytes. It is highly likely these oocytes are deficient in a multitude of developmentally regulated transcripts as a consequence of their abnormal growth and development (47). Gui and Joyce (38) used GDF9 double-stranded RNA interference to successfully knock down oocyte-GDF9 expression and thereby eliminate cumulus expansion and as a result concluded that GDF9 alone is the mouse CEEF. A key experimental approach in the current study was to neutralise oocyte-GDF9 using mAb-GDF9-53, although it is conceivable that this antibody may be less effective against the form of GDF9 secreted by oocytes as it is against recombinant GDF9. Recombinant GDF9 is mostly produced in a mature processed state, whereas preliminary data suggests native GDF9 may be secreted (*in vitro* at least) with its proregion intact (25). In addition, glycosylation status may differ between native and recombinant GDF9. mAb-GDF9-53 may have a lower affinity for oocyte-secreted GDF9, nonetheless this antibody does partially antagonise the growth-promoting effects of oocyte-secreted GDF9 (25). Furthermore, the lack of complete neutralisation of oocyte-induced cumulus expansion by the GDF9 antagonist, BMPRII ECD, provides additional evidence that GDF9 alone is not the

CEEF. Despite 15 years of research in this area the elusive CEEF in the mouse still remains controversial.

All of the major putative OSFs, GDF9, BMP15 and BMP6, as well as other members of the TGFβ superfamily, use BMPRII as their primary type-II receptor, and as the BMPRII ECD partially antagonised oocyte-induced expansion, this suggests that one or a number of these growth factors contribute to the CEEF. Results from the current study suggest that neither GDF9 alone nor BMP6 alone regulate oocyte-induced cumulus expansion. Other possible molecules contributing to the mouse CEEF may include BMP15 and other BMPs. Like GDF9, oocyte-secreted BMP15 regulates a wide range of differentiation processes of GCs attributed to OSF (48, 49). BMP15 null mice display decreased ovulation and fertilisation rates (50), however it is unclear whether the BMP15 null mice are subfertile due to assembly failure of their cumulus ECM or for other reasons. RNA interference of oocyte BMP15 failed to prevent cumulus expansion (38), although BMP15 null mice exhibit lower CC expression of HAS2 (46). A recent study by Su *et al.*, (46) examined COCs from BMP15 $^{-/-}$ GDF9 $^{+/-}$ double mutant mice, suggesting that these molecules may act in a synergistic manner. Whereas cumulus expansion was not overtly impaired in COCs from BMP15 null mice, it was in the double mutant COCs, suggesting that these two OSF may be working synergistically to promote expansion, either as independent homodimers or alternatively as a GDF9/BMP15 heterodimer (51). Even though some type of GDF9-BMP15 interaction appears necessary for cumulus expansion because both these factors require BMPRII and cumulus expansion was not prevented by the BMPRII ECD in the current study, oocyte factors in addition to these molecules must be involved.

In conclusion, the present study, together with other studies, demonstrated that it is common for several members of the TGFβ superfamily to mimic the paracrine actions of oocytes on GCs or CCs *in vitro*. It is apparent that recombinant TGFβ1 and GDF9 can mimic the oocyte and promote cumulus expansion, yet in both cases, specific and more generalised antagonists to TGFβ and GDF9 fail to prevent the expansion-inducing action of oocytes. The findings from this study provide strong evidence against the hypothesis that GDF9 is the sole oocyte-secreted factor regulating cumulus expansion in the mouse. This study supports the argument that the mouse CEEF is composed of multiple TGF-β superfamily molecules, including at

least one of which is a BMPRII ligand(s) and one or more that are not. The results reported here provide a better understanding of the process of cumulus expansion.

2.6 Acknowledgements

RA Dragovic is supported by Faculty of Health Sciences, Adelaide University and The Queen Elizabeth Hospital Research Foundation. RB Gilchrist is the recipient of the FTT Fricker Medical Research Associateship Adelaide University. This project was supported by a National Health and Medical Research Council (NHMRC) of Australia Program Grant and the Research Centre for Reproductive Health. The authors would like to thank Dr. Olli Ritvos (University of Helsinki) for generously donating the GDF9-expressing cell line and Prof. Nigel Groome (Oxford Brookes University) for kindly providing the mAb-GDF9-53. We would also like to thank Jim Wang, Jeremy Thompson, Darryl Russell and Theresa Hickey for helpful discussions.

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Chapter 3

Oocyte-Secreted Factor Activation of SMAD 2/3 Signalling Enables Mouse Cumulus Expansion

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To be submitted to Developmental Biology, March 2006

3.1 Abstract

Expansion of the mouse cumulus-oocyte complex (COC) is dependent on one or more oocyte-secreted paracrine factors. TGFβ superfamily molecules are prime candidates for the cumulus expansion-enabling factor (CEEF), and we have recently determined that growth differentiation factor 9 (GDF9) alone is not the CEEF. The aim of this study was to examine oocyte paracrine factors and their signalling pathways that regulate mouse cumulus expansion. Using RT-PCR oocytes were found to express the two activin subunits, inhβA and inhβB. Furthermore, activin A and activin B both enabled FSH-induced cumulus expansion of oocytectomised (OOX) complexes. Follistatin, an activin antagonist, neutralised activin-induced expansion, but had no effect on oocyte-induced expansion. The type-I receptors for GDF9 and activin are activin receptor-like kinase 5 (ALK5) and ALK4, respectively, both of which activate the same SMAD 2/3 signalling pathway. We examined the requirement for this signalling system using an ALK4/5/7 kinase inhibitor, SB-431542. SB-431542 completely neutralised GDF9-, activin A-, activin B- and oocyte-induced cumulus expansion. In addition, using real-time RT-PCR, SB-431542 attenuated GDF9-, activin A- and oocyte-induced OOX expression of hyaluronan synthase-2, tumour necrosis factor-α-stimulated gene-6, cyclooxygenase-2 and pentraxin-3. This study demonstrates that the CEEF is composed of TGFβ superfamily molecules and that SMAD 2/3 signalling is required to enable cumulus expansion.

3.2 Introduction

Oocyte paracrine signalling to cumulus cells (CCs) is required for the mucification and expansion of the CCs surrounding the oocyte. Cumulus expansion is a highly coordinated process that occurs a few hours prior to ovulation and involves the production of a complex extracellular matrix (ECM), crucial for ovulation, fertilisation and hence fertility (1). Hyaluronan (HA), a non-sulphated glycosaminoglycan, forms the major structural backbone of the matrix and is synthesised by the enzyme hyaluronan synthase-2 (HAS2) (2, 3). Other important components of the ECM include the HA binding proteins tumour necrosis factor-α-stimulated gene-6 (TSG6) and pentraxin-3 (PTX3). Both TSG6 and PTX3 deficient mice synthesise normal amounts of HAS2, however they are infertile due to their incapability to organise HA into a stable matrix (1, 4). In addition, prostaglandin (PG) signalling is important for assembly of the CC matrix, as knockout mice for the PG rate limiting enzyme, cyclooxygenase-2 (COX2) and one of the PG EP_2 receptors are infertile due to cumulus expansion failure (5-7).

Expansion of the mouse cumulus oocyte complex (COC) is critically dependent upon two signalling events; 1) stimulation by gonadotrophins or epidermal growth factors (EGFs), and 2) a paracrine signal secreted by the oocyte, termed the cumulus-expansion enabling factor (CEEF), which acts on its neighbouring CCs enabling these cells to respond to the afore mentioned stimuli (8-11). While the gonadotrophin/EGF signal is now well described, the CEEF is poorly understood and remains a controversial topic. *In vivo,* gonadotrophin stimulation of cumulus expansion is clearly initiated by the mid-cycle luteinising hormone (LH) surge. *In vitro*, when mouse COCs are treated with LH they fail to undergo cumulus expansion as both CCs and oocytes have low to undetectable levels of LH receptors in the preovulatory follicle and therefore do not respond to direct LH stimulation (12). Mural granulosa cells however express LH receptors and it has recently been shown that the LH surge induces highly regulated expression of three EGF family members; amphiregulin, epiregulin and betacellulin (13). These EGF-like peptides then stimulate cumulus expansion in COCs via the EGF receptor demonstrating that these molecules are the paracrine mediators transmitting the LH signal to the COC in the ovarian follicle (13). Cumulus expansion can also be mimicked in *vitro* by follicle stimulating hormone (FSH), the cyclic adenosine monophosphate (cAMP) analog, 8-Br-cAMP and EGF (8, 14, 15), suggesting that the requirement for the gonadotrophin signal is mediated through the mitogen activated protein

kinase (MAPK) pathway. In support of this, Su *et al.*, (16) illustrated that MAPK activity in the CCs is required for cumulus expansion by successfully preventing FSH-, 8-Br-cAMP- or EGF-stimulated expansion of COCs from Mos (MAPKKK) null oocytes, using a specific MAPK inhibitor.

In addition to gonadotrophin/EGF activation of the MAPK cascade, expansion of the mouse COC requires an oocyte-secreted paracrine signal. Microsurgical removal of the oocyte from the COC (generating an oocytectomised (OOX) complex) eliminates FSH-induced CC expansion, however expansion can be restored by co-culturing these OOX complexes with denuded oocytes (DOs), demonstrating the secretion of and requirement for oocyte CEEFs (9). The identity of the CEEFs is controversial however it seems likely some combination of TGFβ superfamily molecules are responsible, as the CEEFs can be mimicked or substituted by TGFβ1 or growth differentiation factor 9 (GDF9) (17-19). These studies suggest that oocyte-paracrine signalling via the classic TGFβ superfamily signalling cascade is required for cumulus expansion. TGFβ superfamily ligands bind as homo- or heterodimers to their respective transmembrane bound serine/threonine kinase type-I receptor (activin receptor-like kinase; ALK) or type-II receptor, forming an oligomeric complex. The phosphorylated type-I receptor then phosphorylates receptor regulated SMADs (R-SMADs) which subsequently associate with the co-SMAD (SMAD4). This activated heterodimeric SMAD complex then translocates from the cytoplasm into the nucleus where it regulates transcription of target genes. TGFβ superfamily ligands signal through one of two distinct intracellular cascades; they either activate the SMAD 2/3 or the SMAD 1/5/8 signalling pathway, depending on which type-I and type-II receptors they utilise. The TGFβs, activins, GDF9, myostatin (GDF8) and nodal all activate SMAD 2/3 via their respective type-II receptors and the recruitment and phosphorylation of ALK4, 5 or 7 [reviewed in Refs. (20, 21)]. Conversely, the bone morphogenetic proteins (BMPs) activate SMAD 1/5/8 via the BMP type-II receptor (BMPR-II) and ALK 2, 3 or 6.

In the current study, we hypothesise that TGFβ superfamily signalling through the SMAD 2/3 pathway is responsible for mediating the CEEF paracrine signal from oocyte to CCs. This hypothesis is formulated on the basis that OSFs activate predominately SMAD 2/3 (22), and that stimulation of this pathway by TGFβ1 (17) or GDF9 (19) mimics the CEEF and enables CC expansion, but stimulation of the BMP pathway using BMP6 does not (19). In this study,

we show that activin A and B, which are expressed in the oocyte and which activate SMAD 2/3, also enable cumulus expansion, but in isolation do not account for the CEEF. Our results show that OSF-stimulated expression of HAS2, TSG6, PTX3 and COX2 in CCs is mediated by SMAD 2/3 and that SMAD 2/3 signalling is essential for mouse cumulus expansion.

3.3 Materials and Methods

Unless specified, all chemicals and reagents were purchased from Sigma (St Louis, MO).

3.3.1 Isolation and culture of COCs and OOX complexes

This study was approved by Local Animal Ethics Committees and was conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Ovaries were collected 46 h after injecting 21 to 26-d-old 129/SV mice with 5 IU equine chorionic gonadotrophin (eCG; Folligon, Intervet, Castle Hill, Australia). Ovaries were cleaned free of adherent adipose and connective tissues and placed in HEPES buffered tissue cultured medium-199 (H-TCM-199; ICN Biomedicals Inc., CA, USA) supplemented with 0.1% (wt/vol) BSA (H-TCM-199/BSA). COCs were isolated by puncturing antral follicles with 27-gauge needles and collected in H-TCM-199/BSA. Only COCs with a uniform covering of compacted CCs were used in this study.

Oocytectomy was performed using a micromanipulation apparatus on an inverted microscope as previously described by Buccione *et al.*, (9). Complexes were cultured in 50 µl drops of Waymouth MB 752/1 medium (WAY) supplemented with penicillin G (100 U/ml), streptomycin sulphate (100 mg/ml), 5% (vol/vol) fetal calf serum (FCS; Trace Biosciences, Castle Hill, New South Wales, Australia) (WAY/FCS), 50 mIU/ml recombinant human FSH (Puregon; Organon, Oss, The Netherlands) and +/- treatment reagent, overlaid with mineral oil in falcon petri dishes. Ten COCs or OOX complexes were cultured per 50 µl drop. Complexes were incubated for 20 hours at 37°C, 96% humidity in 5% CO_2 in air, followed by morphological assessment of cumulus expansion.

3.3.2 Treatment of OOX complexes

Denuded oocytes

Oocytes were stripped of their surrounding CCs by rapidly agitating COCs using a vortex mixer for ~4 min in 2 ml H-TCM-199/BSA. 40 DOs were cultured per 50 µl drop resulting in a concentration of 0.8 DO/µl

3.3.3 TGFβ superfamily growth factors

OOX complexes were treated with various members of the TGFβ superfamily: recombinant human activin A and recombinant human activin B were obtained from R&D Systems (Minneapolis, MN), whereas recombinant mouse GDF9 was produced in house using a transfected 293 human embryonic kidney cell line (293H) (23). GDF9 and 293H control (conditioned medium from untransfected 293H cells) were partially purified using hydrophobic interaction chromatography (24).

3.3.4 Oocyte-secreted factor antagonists

Attempts were made to antagonise recombinant and oocyte-secreted activin A and B bioactivities using follistatin-288, which was generously donated by S Shimasaki (University of California San Diego, USA). In order to examine the TGFβ superfamily signalling pathway used to enable cumulus expansion oocyte paracrine factors were antagonised by treatment with the small molecule inhibitor, SB-431542 (generously donated by GlaxoSmithKline, Stevenage, UK). SB-431542 acts as competitive ATP binding site kinase inhibitor, potently antagonising the activities of ALK4, ALK5 and ALK7 (25, 26). SB-431542 has no effect on ALKs 1, 2, 3 and 6 and has very low affinity for any other cellular kinases, and so is a highly specific ALK4/5/7 kinase inhibitor (26). A 10 mM stock solution of SB-431542 was prepared in dimethyl sulfoxide (DMSO) and diluted in culture medium prior to addition to culture drops such that the maximum concentration of DMSO was 0.04% (vol/vol).

3.3.5 Cumulus expansion assessment

Morphological cumulus expansion was recorded after a 20 h culture period. This was a blinded assessment to eliminate bias. Cumulus expansion was assessed according to a well-established subjective scoring system (0 to +4); in brief, score 0 indicates no expansion, and

score +4 indicates maximum expansion (10). A cumulus expansion index (CEI; 0.0 to 4.0) was calculated as previously described (27).

3.3.6 RT-PCR

RNA isolation

COCs were collected from mouse ovaries and examined for mRNA expression of the two activin subunits inhβA and inhβB and myostatin. Mouse ovary and skeletal muscle were collected for positive tissue samples. COCs were collected in H-TCM-199/BSA and denuded of surrounding CCs by mouth pipetting. DOs were then washed three times in H-TCM-199/BSA and transferred to Eppendorf tubes (40 DOs per tube) on ice. The RNA from mouse ovary, skeletal muscle and DOs was extracted using a micro RNA isolation kit (QIAGEN, Victoria, Australia) as described previously (19).

3.3.7 RT-PCR analysis

RNA was reverse transcribed using random primers (Boehringer Mannheim, Germany) and a Superscript II RT kit (Life Technologies, Inc., Grand Island, NY) according to the manufacturer's instructions. A negative RT control substituting water for reverse transcriptase was included in each reaction. Primer pairs for inhβA, inhβB and myostatin were designed using Primer Express software (PE Applied Biosystems, Foster City, CA), and synthesised by Geneworks (Adelaide, Australia). βactin primers were generously donated by S. Robertson (University of Adelaide, Australia). Primer pair sequences are listed in Table 1. Each PCR reaction sample consisted of 2.5 µl of Qiagen 10X buffer, 1 mmol l^{-1} $MgCl_2$, 0.4 mmol l^{-1} of each dATP, dCTP, dGTP and dTTP, 0.5 units of HotStarTaq DNA polymerase, 10 pmol of each primer, 3 µl of diluted cDNA sample (1:5) and made up to a final volume of 25 µl with ultra pure water (Biotech, Bentley, NSW, Australia). Water was substituted for cDNA in the negative control sample included in each reaction. PCR samples were treated at 94°C for 5 min, followed by 40 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min and a final extension step of 72°C for 7min. PCR products were analysed by electrophorsis by running a 2% (wt/vol) agarose gel containing 15 mg of ethidium bromide (Boehringer) in Tris borate EDTA buffer (TBE) and visualised with a Kodak 120 digital camera over an ultraviolet light box. PCR product size was determined by comparison with Hpa II digested pUC19. The identities of the inhβA, inhβB and myostatin PCR products were confirmed by sequencing.

3.3.8 Real-time RT-PCR

Experimental design

OOX complexes were cultured in WAY supplemented with 5% (vol/vol) FCS + FSH (50 mIU/ml) for 6 h in one of the following treatments; 1) no treatment (control), 2) GDF9 (250 ng/ml), 3) GDF9 + SB-431542 (4 μM), 4) activin A (200 ng/ml), 5) activin A + SB-431542 (4 μM), 6) oocytes (0.8/μl), or 7) oocytes + SB-431542 (4 μM). Ten OOX complexes were cultured per treatment group, each treatment group was in quadruplicate and the experiment was replicated on five separate occasions.

3.3.9 Real-time RT-PCR analysis

After the 6 h incubation, OOX complexes were collected for RNA isolation as previously described (19). Primer pairs were designed for mouse COX2, TSG6 and PTX3 using Primer Express software and synthesised by Geneworks. The sequences for L19, HAS2, COX2, TSG6 and PTX3 primer pairs are listed in Table 1. PCR conditions were the same as previously described (19) with exceptions of eighty nanograms of RNA was reverse transcribed and the gene expression was calculated for each sample using the standard curve method. Standard curves were generated by serial dilution of COC cDNA. Critical threshold (C_T) values for each given sample were within the range of the standard curve for each gene of interest. Gene expression was calculated for each sample using the standard curve and was then normalised to the housekeeping gene, L19. Finally, PCR products were ran on a 2% (wt/vol) agarose gel for confirmation of single, correctly sized products and the identity of each PCR product was confirmed by sequencing.

3.3.10 Data analyses

Each experiment was performed 3-5 times (see figure legends). Treatment effects on cumulus expansion were examined using a Kruskal-Wallis one-way ANOVA on ranks and differences between means were detected using Dunn's method *post-hoc* comparisons or *t* tests. Real-time RT-PCR data were log transformed and treatment effects were examined using a one-way ANOVA followed by Tukey comparisons. A P value of <0.05 was considered statistically significant.

Table 1. Primer sets used for RT-PCR and Real-time RT-PCR

Name	Oligonucleotide Sequences	Product Length (bp)	Genbank accession no.
βActin	5'- CGTGGGCCGCCCTAGGCACCA -3' 3'- ACACGCAGCTCATTG -5'	186	X03672
InhβA	5'- CCTCGGAGATCATCACCTTTG -3' 3'- CTGCACGCTCCACTACTGACA -5'	102	NM_008380
InhβB	5'- AGGTAGATGTTCTAAACAATCCTTCGA -3' 3'- GTGTACGGACAGAAAAGACACATCA -5'	107	X69620
Myostatin	5'- CAGCAGTGATGGCTCTTTGG -3' 3'- TGCCATCCGCTTGCATT -5'	101	NM_010834
L19	5'- GAAAGTGCTTCCGATTCCA -3' 3'- TGATCGCTTGATGCAAATCC -5'	116	NM_026490
HAS2	5'- CATTCCCAGAGGACCGCTTAT -3' 3'- AAGACCCTATGGTTGGAGGTGTT -5'	167	U52524
COX2	5'- CCTTCCTCCCGTAGCAGATG -3' 3'- ATGAACTCTCTCTCCGTAGAAGAACCTT -5'	111	NM_011198
TSG6	5'- GATGGTCGTCCTCCTTTGCTT -3' 3'- TATCGTCCAGCCCGAGCTT -5'	141	NM_009398
PTX3	5'- GGACAACGAAATAGACAATGGACTT -3' 3'- CGAGTTCTCCAGCATGATGAAC -5'	109	X83601

3.4 Results

3.4.1 Oocyte expression of activin A and activin B mRNA

There are conflicting reports as to whether mouse oocytes express activin (28, 29), and so we examined the mRNA expression of inhβA- and inhβB-subunits in oocytes using RT-PCR. InhβA and inhβB mRNA transcripts were detected in mouse oocytes (Fig. 1). We also examined oocyte mRNA expression of myostatin (GDF8) as it also activates the common TGFβ/GDF9/activin signalling pathway (30). Myostatin mRNA transcripts were not detected in oocytes (Fig. 1). Oocyte samples were examined for possible CC contamination by collecting the medium in which the oocytes were collected from and screening it for inhβA, inhβB and myostatin mRNA expression. All samples were negative, indicating that the oocyte samples were free from CC contamination (data not shown).

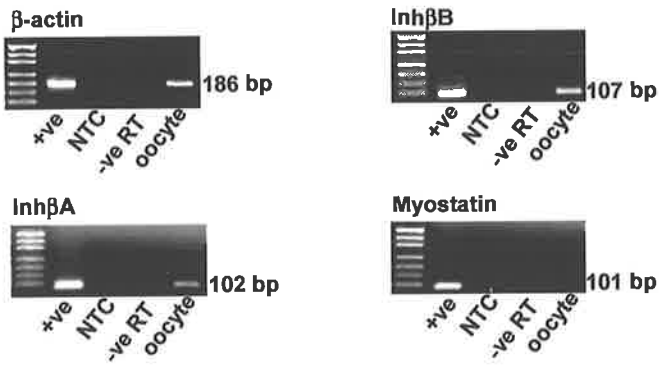

Figure 1. Expression of inhibin subunits and myostatin mRNA in oocytes. COCs were collected and CCs and oocytes were manually separated by careful mouth pipetting. RNA was extracted from oocytes, reverse transcribed and amplified by PCR. +ve, positive tissue sample (mouse ovary for βactin, inhβA, and inhβB; mouse skeletal muscle for myostatin); -ve RT, no reverse transcriptase; NTC, no template control (water substituted for cDNA template). Ladder; Hpa II digested pUC19.

3.4.2 Activin A and activin B enable cumulus expansion

The role of activins in the regulation of cumulus expansion is unknown, however the activins are related to and use a common signalling pathway to TGFβ and GDF9, both of which can enable cumulus expansion (17, 19). To examine the effect of activin A and B on cumulus expansion, OOX complexes were treated with FSH and cultured in the presence of an increasing dose of activin A (Fig. 2A) or activin B (Fig. 2B). OOX complexes underwent expansion ($P<0.05$) induced by activin A (200 ng; 2.8 ± 0.2) and B (200 ng; 3.1 ± 0.1), in a dose-dependent manner. In contrast, OOX complexes cultured without activin failed to expand. Activin A and B mimicked the actions of oocytes in co-culture and GDF9 in enabling CC expansion to a comparable level to in intact COCs.

3.4.3 Neutralisation of activin A and B does not prevent cumulus expansion

Having discovered that exogenous activin A and B enable cumulus expansion, we next assessed whether activin A and/or B are the elusive mouse CEEF. A functional neutralisation experiment was designed using the native activin binding protein follistatin, which is a well-characterised activin antagonist [reviewed in Ref. (21)]. Activin A- and B-induced cumulus expansion was significantly ($P<0.05$) ablated when treated with follistatin-288 or the ALK4/5/7 kinase inhibitor, SB-431542 (Fig. 2C and 2D). In contrast, follistatin-288 had no significant ($P>0.05$) effect on cumulus expansion induced by oocytes (Fig. 2C and 2D). Failure of follistatin-288 to neutralise oocyte-induced cumulus expansion was not due to

insufficient antagonist. Increasing the dose of follistatin to 2 μg/ml also did not prevent oocyte-induced cumulus expansion (3.1 ± 0.1).

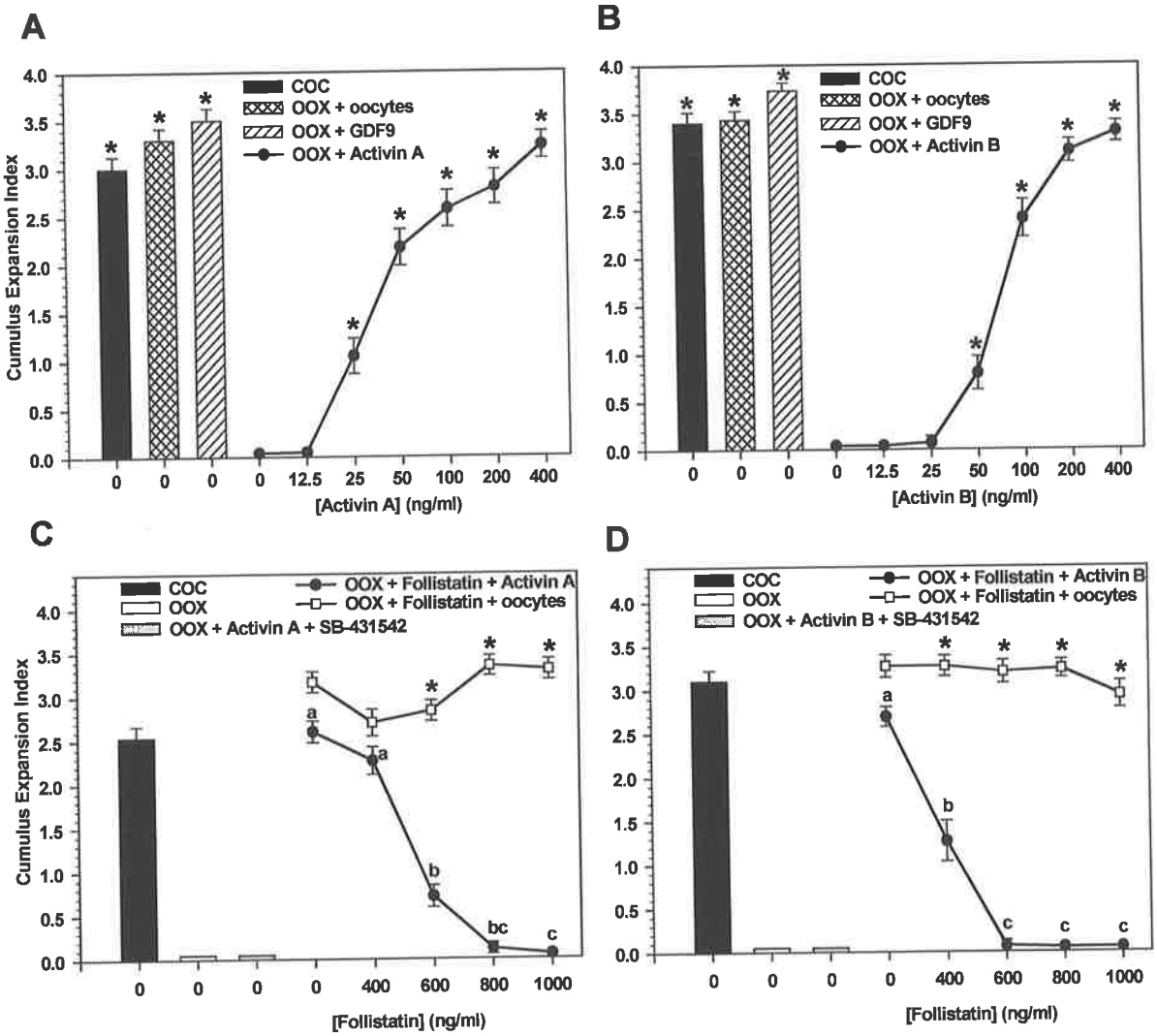

Figure 2. Effect of activin A and B on cumulus expansion and of follistatin on oocyte-induced expansion. COCs and OOX complexes were cultured in media supplemented with 50 mIU/ml rhFSH and 5% FCS, and OOX complexes were treated with denuded oocytes (0.8/μl), GDF9 (145 ng/ml), an increasing dose of activin A (12.5-400 ng/ml) (A), or a activin B (12.5-400 ng/ml) (B). An *asterisk* represents a significant difference to OOX alone ($P<0.05$). OOX complexes co-cultured with oocytes (0.8/μl) (C, D), treated with activin A (200 ng/ml) (C) or activin B (200 ng/ml) (D), were all treated with an increasing dose of follistatin-288 (400-1000 ng/ml). Negative controls included OOX complexes alone or OOX complexes treated with activin A or activin B together with SB-431542 (4 μM). Means within a line graph with *different superscript letters* are significantly different ($P<0.05$). *Asterisks* indicate the two means at that dose of follistatin are significantly different ($P<0.05$). After 20 h of culture, the degree of cumulus expansion was assessed using the subjective scoring system, 0 (no expansion) to +4 (maximal expansion), and the cumulus expansion index calculated. Results show the mean ± SEM of three individual experiments, each with a total of 10 complexes (OOX or COC) per treatment group.

3.4.4 Requirement of SMAD 2/3 signalling for cumulus cell expansion

MAPK signalling has been implicated in the regulation of cumulus expansion (16, 31). To characterise TGFβ superfamily signalling pathways involved in the regulation of cumulus expansion, the ALK4/5/7 kinase inhibitor, SB-431542, was used to determine the involvement of SMAD 2/3. Oocytes and GDF9 both stimulated cumulus expansion of OOX complexes (3.9 ± 0.1, 3.3 ± 0.1, respectively; Fig. 3A). However, SB-431542 caused a dose-dependent neutralisation of both GDF9- and oocyte-induced cumulus expansion (Fig. 3A). Oocyte-induced cumulus expansion was completely abolished at 2 μM SB-431542. The SB-431542 carrier DMSO, at a vol/vol dose equivalent to 4 μM SB-431542, had no significant (P>0.05) effect on GDF9- or ooctye-induced cumulus expansion (Fig. 3A and 3B). Accordingly, treatment of intact COCs with an increasing dose of SB-431542 also abolished cumulus expansion (Fig. 3B). To investigate the possibility of toxic effects of SB-431542, a reversibility experiment was conducted where COCs were exposed to SB-431542 for just 6 h, washed free of the inhibitor, and then returned to control culture medium for a further 14 h (Fig. 3C). After 20 h these COCs had undergone cumulus expansion, although the overall degree of expansion was somewhat reduced compared to that of the controls (Fig. 3C).

Using real-time RT-PCR, CC HAS2, TSG6, COX2 and PTX3 mRNA expression were analysed to provide further support for the cumulus expansion-inhibiting effects of SB-431542, assessed above by morphological criteria. FSH-treated OOX complexes were cultured alone or treated with GDF9, activin A or co-cultured with oocytes, in the presence or absence of 4 μM SB-431542 for 6 h. CC HAS2, TSG6, COX2 and PTX3 were all up-regulated notably by GDF9 and oocytes, and to a lesser extent by activin A, compared to the control (Fig. 4). The ALK4/5/7 kinase inhibitor, SB-431542, significantly (P<0.05) antagonised activin A- and oocyte-induced OOX mRNA expression of all four genes. SB-431542 also significantly (P<0.05) antagonised GDF9-induced OOX HAS2 and COX2 mRNA expression. These results verify that the dramatic morphological changes in cumulus expansion observed after treatment with SB-431542 correlate with functional changes in the expression of key genes regulating expansion.

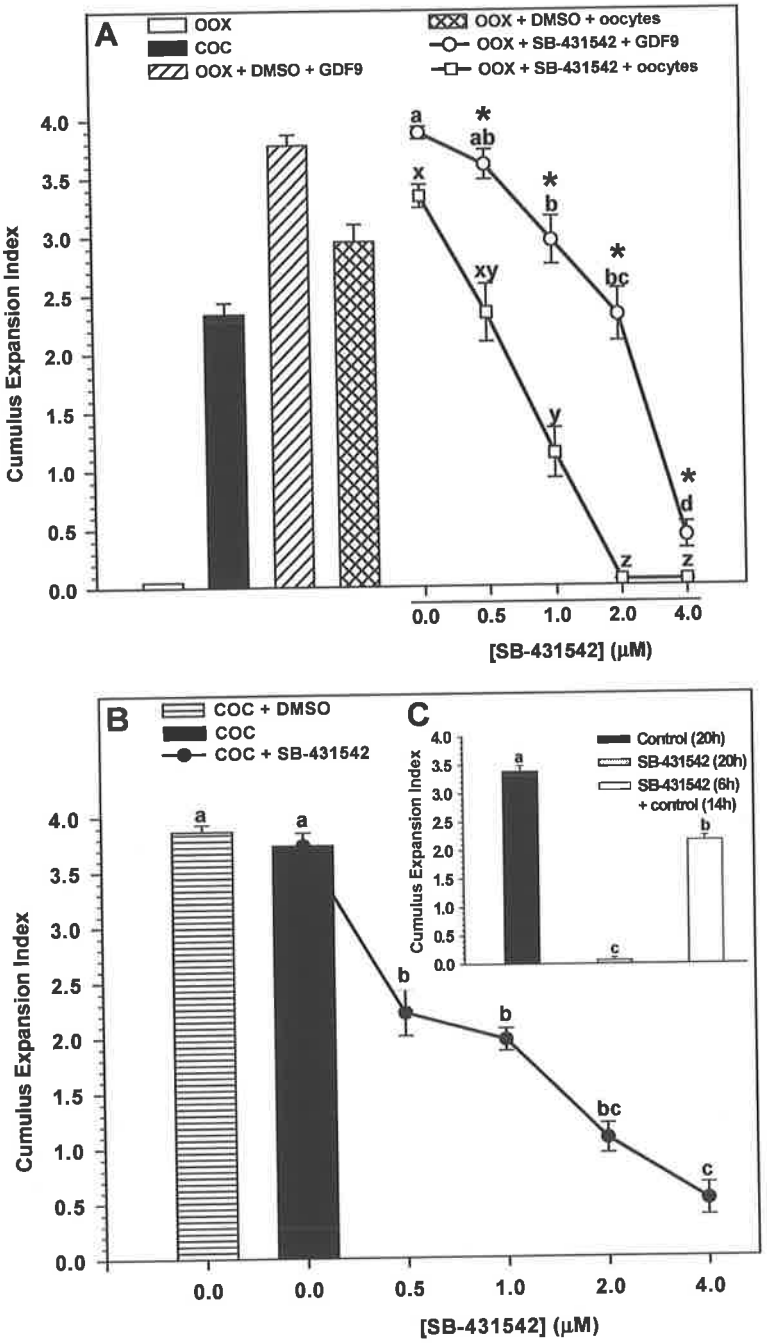

Figure 3. Effect of SB-431542 on cumulus expansion. (A) FSH-treated OOX complexes were cultured with either oocytes (0.8/μl) or GDF9 (145 ng/ml), and treated with an increasing dose of SB-431542 (0.5-4 μM). (B) COCs were cultured alone or treated with an increasing dose of SB-431542 (0.5-4 μM). The degree of cumulus expansion was determined after 20 h of culture using a scale of 0 (no expansion) to +4 (maximal expansion). Means within a line graph with *different superscript letters* are significantly different ($P<0.05$). *Asterisks* indicate the two means at that dose of antagonist are significantly different ($P<0.05$). The SB-431542 carrier, DMSO, at a vol/vol dose equivalent to 4 μM SB-431542 did not effect cumulus expansion. (C) COCs were cultured alone or treated with SB-431542 for 20 h, or exposed to SB-431542 for just 6 h, then washed and returned to control medium for 14 h. Cumulus expansion was assessed 20 h after culture. *Bars with different superscript letters* are significantly different ($P<0.05$).

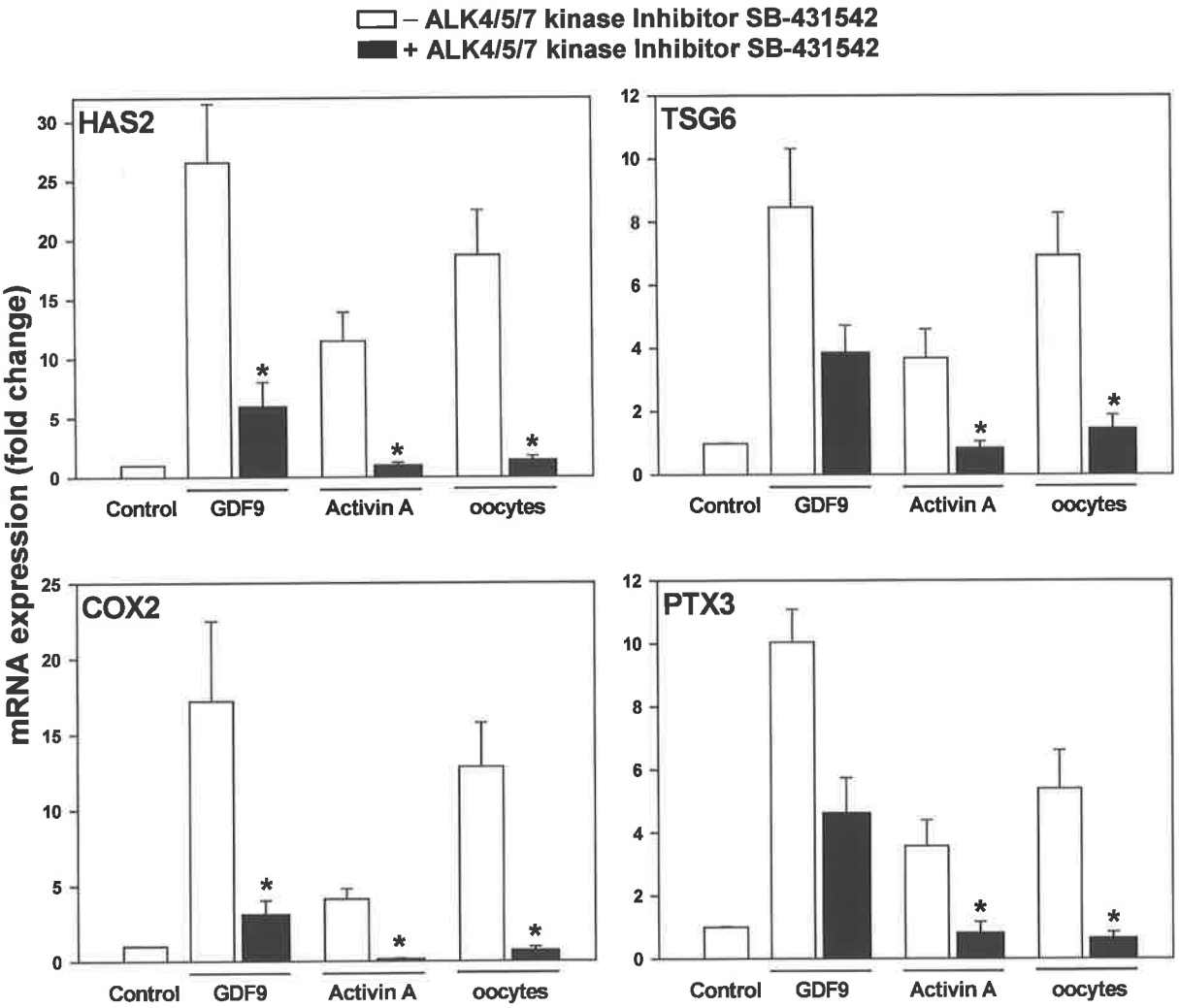

Figure 4. Effect of SB-431542 on CC HAS2, PTX3, TSG6 and COX2 mRNA expression. OOX complexes were cultured for 6 h in media supplemented with 50 mIU/ml rhFSH and 5% FCS. Real-time RT-PCR analysis was performed using primer sets for HAS2, PTX3, TSG6, COX2 and L19 using RNA from CC (OOX) cultured alone, with GDF9 (145 ng/ml), activin A (200 ng/ml) or co-cultured with denuded oocytes (0.8/μl), either in the presence or absence of 4 μM SB-431542. The levels of HAS2, PTX3, TSG6 and COX2 mRNA were normalised to L19. Columns are means ± SEM of five independent experiments and are expressed as a fold change from mRNA levels in OOX complexes cultured alone (control; set to a value of 1). *Asterisks* denote a significant effect of SB-431542 for that treatment ($P<0.05$).

3.5 Discussion

The release of a fertilisable oocyte from the ovary is dependent upon expansion of the CCs. Formation of the ECM surrounding the oocyte requires both gonadotrophin stimuli and unidentified OSFs, the CEEF, which is most likely a combination of growth factors belonging to the TGFβ superfamily. The signalling cascades regulating cumulus expansion are complex and have yet to be entirely elucidated. The gonadotrophin signal appears to be mediated by EGF-like peptides (13), such that activation of the MAPK pathway in the CCs is required for

cumulus expansion (16, 31). *In vitro*, stimulation of COC expansion via the MAPK pathway is mostly commonly achieved by treatment with FSH. However, it is well known that a gonadotrophin/EGF signal alone is not adequate for launching the pathway that leads to cumulus expansion, and that an oocyte paracrine signal is required to enable gonadotrophin/EGF-stimulated expansion. We have previously demonstrated that both TGFβ1 and GDF9 can mimic the oocyte CEEF and enable FSH-induced cumulus expansion, but neither alone nor the two together account for the mouse CEEF (19). More recently we have shown that OSFs primarily activate the SMAD 2/3 signalling cascade to regulate granulosa cell (GC) proliferation (22). This led us to hypothesise that TGFβ superfamily signalling via the SMAD 2/3 pathway is responsible for mediating the CEEF paracrine signal from the oocyte to CCs enabling expansion.

Results from this study provide evidence that the CEEF signals through the SMAD 2/3 pathway and that this is essential for the induction of cumulus expansion. We utilised an ALK4/5/7 kinase inhibitor, SB-431542, to completely neutralise GDF9-, activin A-, activin B- and oocyte-induced cumulus expansion. In addition, CC genes involved in the formation of the ECM, including HAS2, TSG6, PTX3 and COX2, were all attenuated by treatment with SB-431542. Our results also demonstrate that the activin subunits are expressed in the oocyte and that activin A and B can mimic the actions of the oocyte CEEF and enable FSH-induced cumulus expansion. However, as for GDF9 and TGFβ1, neither activin A nor activin B in isolation account for the mouse CEEF.

Intracellular SMAD proteins mediate the signal transduction of TGFβ superfamily ligands, which control cell proliferation, differentiation and apoptosis. Activation of specific SMAD proteins is dependent on which type-I and type-II receptors the ligands utilise. SMAD 2/3 is activated by ligands that signal through ALK4, 5 or 7, whereas SMAD 1/5/8 is activated by molecules (mainly BMPs) that utilise ALK2, 3 or 6 (32). We have very recently demonstrated using SMAD-reporter constructs and Western blotting that OSFs activate GC SMAD 2/3 (22), however, prior to the current study, it was unknown whether the SMAD signalling pathways play a role in the regulation of cumulus expansion. In this study, we have utilised the small molecule inhibitor, SB-431542, in the cumulus expansion assay. SB-431542 acts as competitive ATP binding site kinase inhibitor that is highly specific for ALK4, 5 and 7; the kinases that activate SMAD 2/3 (26). Accordingly, SB-431542 prevents oocyte-induced

activation of GC SMAD 2/3 phosphorylation and oocyte-stimulated GC proliferation (22). Our current results demonstrate that FSH-stimulated COC expansion was eliminated by the kinase inhibitor. In addition, SB-431542 effectively inhibited recombinant GDF9-, activin A-, activin B, as well as oocyte-induced, cumulus expansion. These data demonstrate that oocyte paracrine signalling through the SMAD 2/3 pathway is required for mouse cumulus expansion.

The CC morphological changes that were observed after treatment with the ALK4/5/7 kinase inhibitor were supported by changes in CC HAS2, TSG6, PTX3 and COX2 gene expression, all necessary for cumulus expansion. Our results demonstrate that GDF9, activin A and oocytes can all up-regulate CC HAS2, TSG6, PTX3 and COX2 mRNA expression. SB-431542 antagonised oocyte-induced expression of all four genes, thereby supporting the morphological observations and demonstrating that oocyte-activation of SMAD 2/3 is required for FSH-stimulated CC HAS2, TSG6, PTX3 and COX2 mRNA expression.

The kinase inhibitor SB-431542 was originally characterised as a specific inhibitor of ALK5 (25). Because the kinase domains of ALK4 and ALK7 are similar to that of ALK5, the inhibitor was later tested and described as a specific inhibitor of ALK4, 5 and 7 (26). The effect of SB-431542 was also tested on the activities of ALKs 1, 2, 3 and 6, along with various other protein kinases. The results clearly demonstrated that 10 μM of SB-431542 had no significant effect on the kinase activities of ALKs 1 or 2, neither did it affect components of the ERK or JNK MAPK signalling pathways (26). ALKs 3 and 6 and the p38α MAPK signalling pathways were weakly effected, but only when using SB-431542 at a concentration of 10 μM (26). Hence SB-431542 is specific for ALK4/5/7 when used at <10 μM, and it is important to note, that in this study the highest concentration used was 4 μM, suggesting that the antagonist effects of SB-431542 on CC expansion are indeed due to antagonism of ALK4/5/7 and not that of other protein kinases. The other concern that may arise when using inhibitors is cell toxicity of the kinase inhibitor itself or the carrier employed; in this case DMSO. To exclude the possibility of CC toxicity, we conducted a wash-out experiment where COCs were exposed to SB-431542 for 6 h, followed by 14 h without the inhibitor. Our real-time RT-PCR results demonstrate that this 6 h exposure to SB-431542 is sufficient to completely prevent expression of the key matrix genes required for expansion. The antagonist effects of SB-431542 on CC expansion were reversible as these COCs underwent cumulus

expansion after 20 h, demonstrating that the kinase inhibitor has no or minimal adverse effect on cell viability.

Growth factors identified to date that can mimic the oocyte and enable cumulus expansion are TGFβ superfamily members, but only those of the family that activate SMAD 2/3 (17-19), consistent with the main finding of this study that the CEEF acts through SMAD 2/3. In this study we examined additional members of the TGFβ superfamily that activate SMAD 2/3 as candidate molecules that might contribute to the CEEF. The superfamily members that activate SMAD 2/3 are TGF-β1, -β2, -β3, activin A, activin B, GDF9, nodal and myostatin (GDF8) [reviewed in Refs. (20, 21)]. Of these growth factors, GDF9 (19) and the TGFβs (17, 18) have been studied in terms of their roles in cumulus expansion, it is unknown whether nodal is expressed in the ovary and using RT-PCR, the current results demonstrate that mouse oocytes express both inhβA and inhβB subunits, but do not express myostatin. Previous reports have been contradictory as to oocyte inhβA and inhβB expression (28, 29). Activins are secreted as homo- or heterodimers of the inhibin βsubunits; activin A (βAβA), activin B (βBβB) and activin AB (βAβB). Activin A and B signal by binding to their respective type-II receptors (ActR-II and ActR-IIB), leading to the recruitment and phosphorylation of the type-I receptor, ALK4. Phosphorylation of ALK4 results in the activation of the SMAD 2/3 signalling pathway (33).

We next went on to investigate the effect of activin A and activin B on cumulus expansion. This study demonstrates for the first time that both activin A and activin B enable FSH-induced cumulus expansion of mouse OOX complexes. The activin binding protein, follistatin, effectively inhibited activin A- and B-induced cumulus expansion, but did not antagonise oocyte-induced cumulus expansion. Hence, activin A and activin B act in the same manner as TGFβ1 and GDF9 (17, 19); all activate SMAD 2/3 and all can mimic the paracrine actions of the oocyte and enable cumulus expansion, but none of these in isolation account for the CEEF. Activin βA knockout mice have craniofacial defects and do not survive any longer than 24 hours after birth (34). Activin βB deficient mice have defects in eyelid closure at the time of birth (35). It has also been reported that activin βB knockout mice are fertile (36), implying that these mice have normal cumulus expansion. This supports the findings of our study that activin alone does not play a pivotal role in the regulation of cumulus expansion.

Given that: 1) OSF-activation of SMAD 2/3 is required for cumulus expansion, 2) TGFβs, GDF9 and activins all signal through SMAD 2/3, 3) all these growth factors enable FSH-stimulated cumulus expansion, and yet 4), none of them account for the CEEF in isolation; then it seems apparent that the CEEF consists of some combination of these factors cooperatively enabling CC expansion. This hypothesis might seem inconsistent with the notion that oocyte-secreted BMP15 plays an important role in CC expansion (37), as BMP15 signals through the SMAD 1/5/8 pathway and probably not through SMAD 2/3 (38). BMP15 null mice are subfertile, displaying decreased ovulation and fertilisation rates as well as a decrease in the expression of CC HAS2 and this is compounded on a GDF9 heterozygous background (37). These characteristics imply some sort of GDF9/BMP15 interaction in the regulation of cumulus expansion. This could include the involvement of a putative GDF9/BMP15 heterodimer, which modelling has predicted could activate SMAD 2/3 and SMAD 1/5/8 (39). If and how a BMP15 homodimer might contribute to the process of cumulus expansion is unclear at this stage, although it is of interest that ALK6 deficient female mice are infertile, primarily due to defects in cumulus expansion (40). The only BMP15 antagonist described so far is follistatin (41), and the results from the current study clearly demonstrate that follistatin has no antagonistic effects on oocyte-induced cumulus expansion.

Our findings present clear evidence that the SMAD 2/3 signalling pathway is required for mouse cumulus expansion and hence for fertility. A recent study using Smad 3 deficient mice demonstrate that these mice are infertile, due to defects in folliculogenesis, associated with altered expression of genes involved in cell cycle, differentiation and survival (42). In addition, ovaries from these mice have no corpora lutea, indicative that these mice fail to ovulate. Impaired cumulus expansion may be contributing to the reproductive defect in these mice.

In conclusion, the results of this study provide evidence to support the hypothesis that the oocyte-secreted CEEF signals foremost is mediated through the SMAD 2/3 signalling pathway. This notion is supported by the fact that only ligands of the TGFβ superfamily that activate SMAD 2/3 can enable cumulus expansion. Oocyte-activation of SMAD 2/3 does not stimulate CC expansion in itself, but instead enables FSH or EGF-like peptides to do so. FSH/EGF stimulate cumulus expansion through MAPK and it has been postulated that an

oocyte-secreted paracrine factor is required to enable FSH-activation of CC MAPK (16, 31). The results of the current study support and extend this hypothesis, whereby the oocyte-secreted CEEF activates SMAD 2/3 signalling in the CCs, and this then initiates an obligatory signal to the MAPK pathway that enables FSH/EGF to activate MAPK and eventually stimulate cumulus expansion. Further studies will determine the validity of these hypotheses.

3.6 Acknowledgements

RA Dragovic was supported by Faculty of Health Sciences, Adelaide University and The Queen Elizabeth Hospital Research Foundation. This project was supported by a National Health and Medical Research Council (NHMRC) of Australia Program Grant and the Research Centre for Reproductive Health.

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Chapter 4

Final Discussion

Final Discussion

Oocyte paracrine factors are essential for the expansion of the CC layer surrounding the oocyte. A multitude of studies have demonstrated that cumulus expansion is crucial for normal fertility, as it is required for ovulation, facilitating pick up of the expanded COC by the oviductal fimbria, sperm capacitation and fertilisation. In 1990, the pioneering studies of Buccione *et al.*, (2) Salustri *et al.*, (5) and Vanderhyden *et al.*, (19) illustrated that the oocyte actively participates in the expansion of the CCs, by secreting one or more paracrine factors, termed the CEEF. In addition, mouse cumulus expansion is also dependent upon stimulation by gonadotrophins/EGF-like peptides. Most recently, Park *et al.*, (8) discovered the precise mechanism by which the gonadotrophin surge facilitates cumulus expansion. The *in vivo* LH surge induces the expression of three EGF family members; AR, EPI and BC in the MGCs of the ovarian follicle. These EGF-like peptides then signal to CCs via the EGF-receptor, activating the MAPK pathway, stimulating expansion of the COC thus illustrating that these molecules are the paracrine mediators propagating the LH signal to the COC in the follicle (8) (Fig. 1). As FSH also activates MAPK, FSH is extensively used to stimulate cumulus expansion in oocyte maturation *in vitro*.

For more than a decade now, researchers have faced the challenge of trying to identify the OSF(s) that account for the elusive mouse CEEF. Much of the research has focused on members of the TGFβ superfamily due to their ability to mimic many of the effects of OSFs on GC and CC processes *in vitro*, including that of cumulus expansion (60). Prior to the commencement of this study, both TGFβ and GDF9 had been examined as potential candidates for the mouse CEEF. A TGFβ neutralising antibody failed to antagonise the expansion actions of the oocyte (69), thereby implicating GDF9 as the prime candidate molecule for the mouse CEEF. Furthermore, because GDF9 can mimic the oocyte and promote expansion *in vitro*, as well as the expression of various CC genes regulating matrix expression, it was concluded that GDF9 was the sole OSF regulating cumulus expansion (103). This conclusion was quite premature, considering that there was no evidence at that time to advocate that GDF9 was behaving any differently to that of TGFβ.

One of the aims of this thesis was to elucidate the nature of the CEEF, by further investigating the role of GDF9 in CC expansion (Chapter 2). It had previously been acknowledged that the development of a GDF9 neutralising antibody would greatly facilitate further research in this

area (60). The development and characterisation of two important experimental tools in our laboratory, recombinant mouse GDF9 and a specific GDF9 monoclonal neutralising antibody, ultimately provided the foundations for this study. The research presented in this thesis clearly demonstrates that exploitation of such experimental reagents/tools is a powerful approach to characterising the role of GDF9 in the regulation of cumulus expansion. Results from this study provide evidence that GDF9 alone is insufficient to enable mouse cumulus expansion and suggest that the mouse CEEF is comprised of multiple TGFβ superfamily members, probably including GDF9. During the course of this study, a paper was published also examining the role of GDF9 as the mouse CEEF. By using a double-stranded RNA interference (RNAi) approach, Gui and Joyce (106) showed that cumulus expansion was significantly reduced when mouse oocytes were injected with GDF9 double stranded RNA. Thus, it was concluded that GDF9 was indeed the sole OSF regulating cumulus expansion in the mouse (106).

These contrasting findings, from the current study (Chapter 2; Dragovic *et al* 2005) and Gui *et al*., (106), noticeably sparked the interest of researchers within the field and were the focus of a recent minireview published by Pangas and Matzuk (148). Both approaches were discussed in detail, in addition to the variations in recombinant GDF9 activity between the Gilchrist and Matzuk laboratories, neither of which are pure. Recombinant mouse GDF9 from our laboratory, just like the OSF, requires the presence of FSH to induce cumulus expansion *in vitro*, and the expression of CC HAS2, COX2, PTX3 and TSG6 (Chapters 2 and 3). However, preparations of GDF9 from the Matzuk laboratory, which have been used in numerous studies to examine the role of GDF9, can induce expansion and the expression of downstream target genes in the absence of FSH (24, 44, 53, 103-105). Moreover, Su *et al*., (53) demonstrated that GDF9 (produced by the Matzuk laboratory) in the absence of FSH could also activate CC MAPK activity, a process that is dependent upon gonadotrophin stimuli and the presence of one or more OSFs (58). It is perhaps unjustified to make direct comparisons between the results obtained from GDF9 produced by Matzuk and that in the current study, due to differences in production and purification protocols. This raises the possibility that additional plasma proteins, such as EGF remain in the recombinant protein preparation produced by the Matzuk laboratory. This may provide an explanation as to why GDF9-induced expansion, gene expression and CC MAPK activation can occur in the absence of FSH using this entirely unpurified preparation. Hence, it is questionable as to whether the recombinant GDF9 produced by the Matzuk laboratory truly reflects that of the naturally occurring protein. The

recombinant GDF9 produced by our laboratory is partially purified by HIC and appears to behave more like the OSF, as both require FSH to exert their cumulus-expanding actions on the CCs.

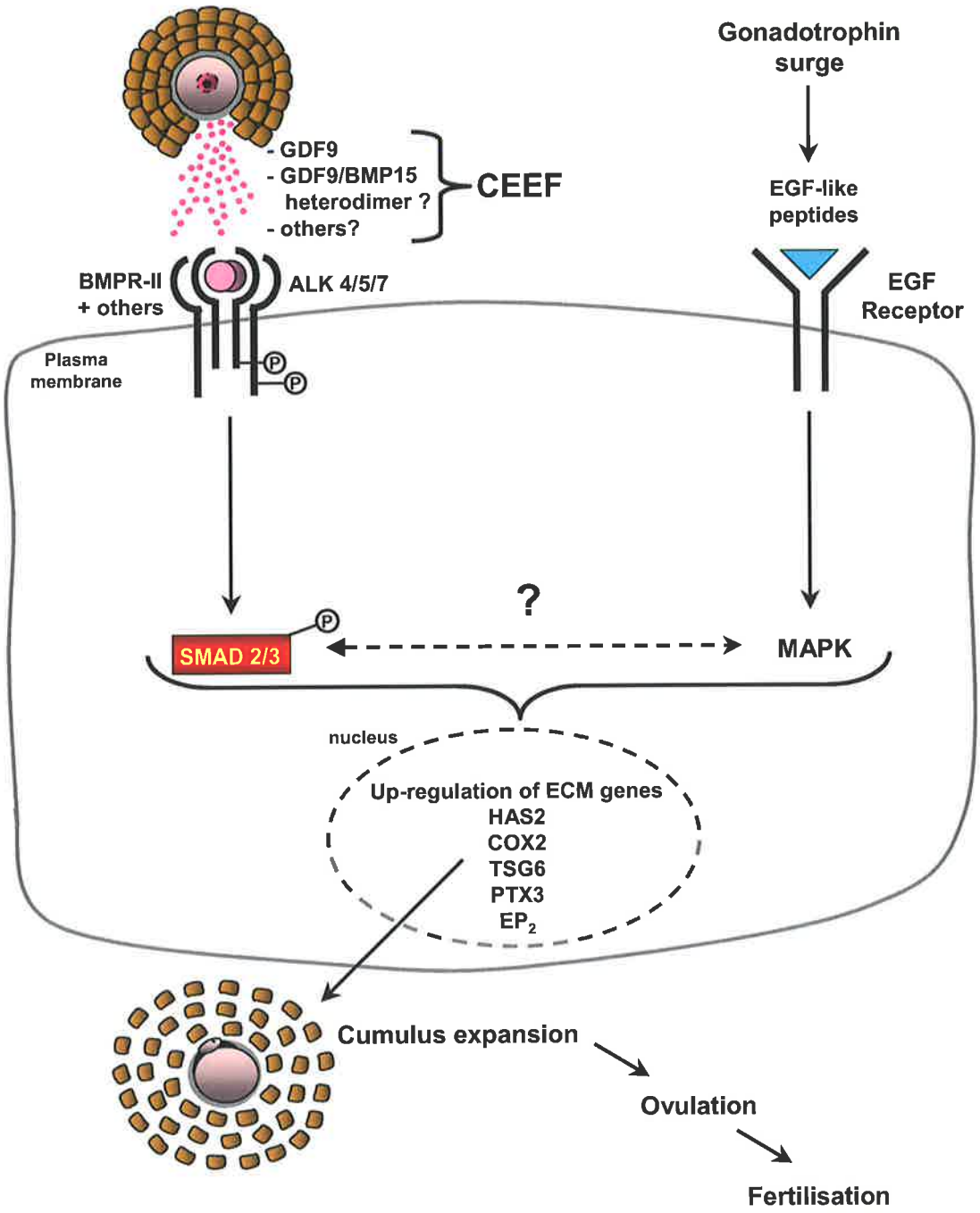

Figure 1. Model showing signalling pathways controlling cumulus expansion. Mouse cumulus expansion requires oocyte-secreted activation of the SMAD 2/3 signalling cascade as well as activation of the MAPK signalling cascade, mediated by EGF-like peptides in response to gonadotrophin stimuli. Both signalling pathways up-regulate the expression of various important CC ECM genes, leading to cumulus expansion, ovulation and fertilisation. SMAD 2/3 and MAPK signalling pathways may crosstalk to regulate cumulus expansion.

The results presented in this thesis have challenged the current popular notion that GDF9 is the sole OSF that accounts for the mouse CEEF, as well as extending the current knowledge of the signalling pathways involved in mediating the CEEF paracrine signal from the oocyte to the CCs. Results from this thesis have illustrated that in addition to the MAPK signalling pathway, the SMAD 2/3 signalling cascade is essential in the regulation of mouse cumulus expansion (Fig. 1). Moreover, these results have provided an extension of the current hypothesis, whereby the oocyte-secreted CEEF activates CC SMAD 2/3 signalling, which then instigates a requisite signal to the MAPK pathway that enables FSH/EGF to activate MAPK and in turn promote cumulus expansion.

The outcomes of the research presented in this thesis, have, and will continue to make a substantial contribution to our understanding of cumulus expansion and the regulation of fertility. Ultimately, the identification of the OSFs regulating cumulus expansion may assist in the challenge of developing new therapeutic agents for managing female fertility.

Proposed future work

As part of future work, the resolution of the OSFs responsible for regulating cumulus expansion in the mouse should be further investigated. In particular, it would be of interest to pursue interactions between GDF9 and BMP15, including the role of a putative GDF9/BMP15 heterodimer. Characterisation of the signalling cascade that the heterodimer uses would be extremely advantageous to the future of this work. The progression of such work will of course require the production of additional recombinant proteins, antibodies and other antagonists. Clearly, this is not a simple task, yet making headway will ultimately depend on the development of such experimental tools.

Following the work examining the role of the SMAD 2/3 signalling cascade presented in Chapter 3, additional work is required to examine the crosstalk between the SMAD 2/3 signalling pathway and the MAPK pathway in the regulation of cumulus expansion. Western blot analysis of ERK 1/2 phosphorylation following treatment of mouse COCs with the ALK 4/5/7 kinase inhibitor, SB-431542, would not only determine whether these two signalling pathways interact, but would also indicate whether SMAD 2/3 is working up-stream of the MAPK signalling pathway. Future work such as this would help validate the current hypothesis described above.

Following the *in vitro* cumulus expansion experiments carried out with the ALK4/5/7 kinase inhibitor, SB-431542, it would be most insightful to examine whether this inhibitor can also block cumulus expansion and ovulation in the mouse *in vivo*. An ovarian intra-bursal injection of SB-431542, in conjunction with an ovulatory dose of hCG would determine whether or not this inhibitor prevents expansion and ovulation of the COC *in vivo*.

Extensive research has been carried out in this study and others examining the process of cumulus expansion in the mouse. Only in rodents is cumulus expansion dependent on the secretion of oocyte-derived paracrine factors. Although ruminants (porcine and bovine) secrete a CEEF, it is actually not required for cumulus expansion (9-12). These results clearly demonstrate that there are distinct species differences for the requirement of the CEEF. At present, it is unclear whether or not human COCs require a CEEF. Therefore, it would be of particular interest to investigate the role of the CEEF firstly in a non-human primate model, such as the common marmoset, and then if possible, the human. Such an investigation would not be an easy task, in part due to the limited number of oocytes obtainable. However, it is important to establish whether or not the CEEF is required for cumulus expansion in humans as the CEEF has the potential to be targeted for the development of a non-steroidal based contraceptive. Furthermore, it would also be of interest to examine whether cumulus expansion in ruminants also requires interaction between the SMAD and MAPK signalling pathways, considering expansion in these species is independent of the CEEF.

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Appendices

Appendix 1: Additional Experiments

1.1 Comparison of IVM media on cumulus expansion

This experiment was designed to compare two different types of *in vitro* maturation (IVM) media on cumulus expansion. COCs and OOX complexes were cultured in treatment drops of 50 µl of either: (1) Waymouth MB 752/1 medium (WAY) supplemented with penicillin G (100 U/ml), streptomycin sulphate (100 mg/ml), 5% (vol/vol) FCS and 50 mIU/ml rhFSH; (2) bicarbonate-buffered MEM-alpha with Earle's salts (αMEM) supplemented with 75 mg/l penicillin G, 50 mg/l streptomycin sulphate, 0.23 mM pyruvate, 5% FCS and 50 mIU/ml rhFSH. Treatment drops were overlayed with mineral oil. All morphological cumulus expansion experiments were assessed after 20 h of culture according to the subjective scoring system as described in Appendix 3.

COCs and OOX complexes were cultured alone, or OOX complexes were treated with GDF9 (175 ng/ml) or co-cultured with oocytes (0.8/µl) in both types of IVM media. As expected, OOX complexes failed to expand, whereas COCs and OOX complexes co-cultured with oocytes or treated with GDF9 underwent expansion. No obvious difference in cumulus expansion was detected when comparing the two IVM culture media (Fig. 1). All subsequent experiments were cultured in treatment drops of 50 µl using WAY IVM medium and overlayed with mineral oil. Unless otherwise stated, 10 COCs and OOX complexes were cultured per group.

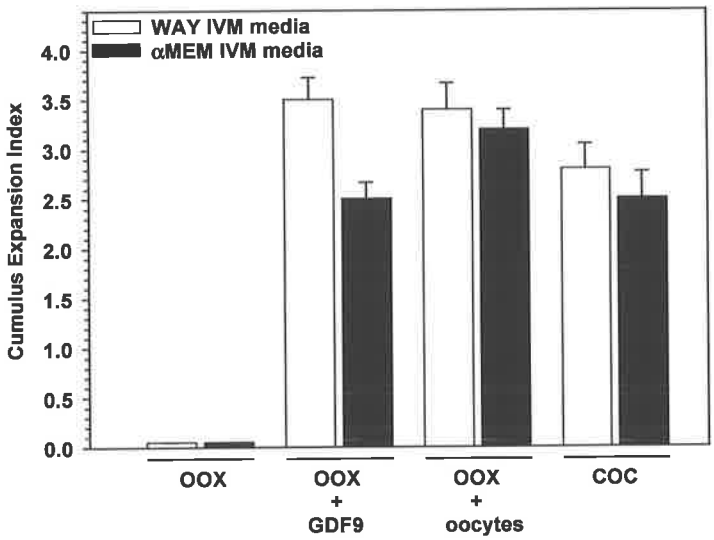

Figure 1. Comparison of WAY vs. αMEM IVM media on cumulus expansion. OOX complexes and COCs were cultured in WAY or αMEM IVM media. COCs and OOX complexes were cultured alone, OOX complexes were treated with GDF9 (175 ng/ml) or co-cultured with oocytes (0.8/µl). Results are the mean ± SEM from one experiment.

1.2 Effect of scattered vs. clustered OOX complexes in co-culture with oocytes on cumulus expansion

At the beginning of the current study, the degree of cumulus expansion observed was very low in treatment groups of COCs or OOX complexes co-cultured with oocytes. In an attempt to increase the overall level of cumulus expansion, an experiment was designed whereby OOX complexes were co-cultured with oocytes either clustered together, or scattered apart. COCs were also cultured clustered together (Fig. 2).

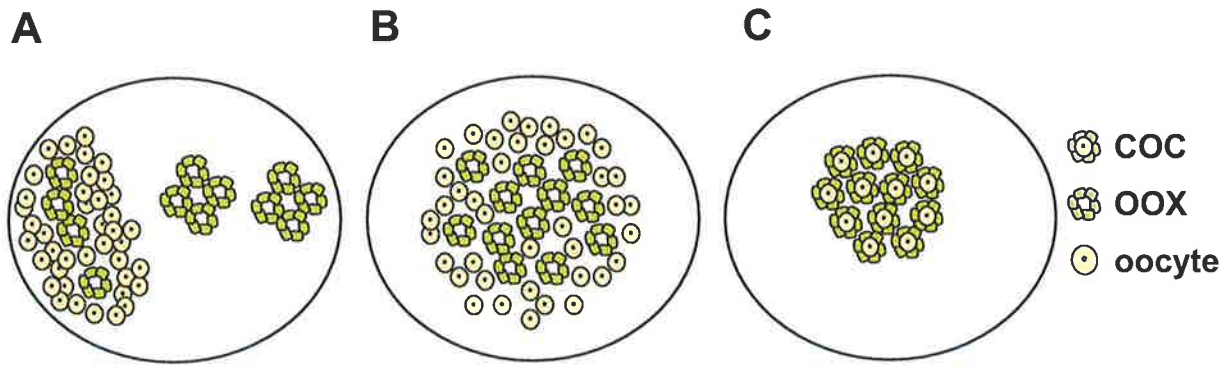

Figure 2. Schematic representation of 50 µl culture drops. (A) OOX complexes co-cultured with oocytes clustered together and scattered apart. (B) OOX complexes co-cultured with oocytes clustered together. (C) COCs cultured clustered together.

Twelve OOX complexes were co-cultured with oocytes (0.8µl) whereby, 4 of the OOX complexes were clustered together with the oocytes and the remaining 8 OOX complexes were scattered apart from the oocytes (Fig. 2A). Twelve OOX complexes were co-cultured with oocytes (0.8/µl) clustered together (Fig. 2B) and 12 COCs were cultured clustered together (Fig. 2C). The 4 OOX complexes clustered together with the oocytes all underwent cumulus expansion, whereas the 8 OOX complexes scattered apart from the oocytes failed to undergo expansion (1 ± 0.4; Fig. 3). All OOX complexes co-cultured with oocytes clustered together and COCs cultured clustered together underwent cumulus expansion (3 ± 0.2, 2.9 ± 0.2 respectively; Fig. 3).

OOX expansion is dependent on the ratio of oocytes cultured per µl and this experiment suggests that cumulus expansion is also dependent on the proximity of the oocyte to the OOX complex. For all subsequent experiments COCs and OOX complexes + oocytes were cultured in close proximity of each other.

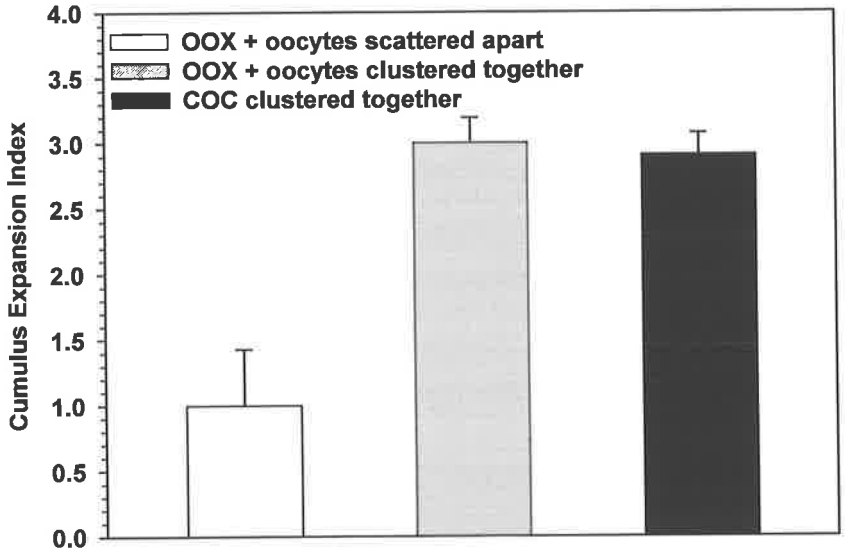

Figure 3. Effect of scattered vs. clustered OOX complexes in co-culture with oocytes on cumulus expansion. OOX complexes were co-cultured with oocytes (0.8/µl) scattered apart or clustered together. COC complexes were cultured clustered together. Results are the mean ± SEM of one experiment each with a total of 12 complexes (OOX or COC) per treatment group.

1.3 Dose response of 293H GDF9 negative control conditioned medium and BMP15 on cumulus expansion

Recombinant mouse GDF9 and ovine BMP15 were produced in-house as previously described (125, 149) using transfected 293 human embryonic kidney cell lines (293H), kindly supplied by O. Ritvos (University of Helsenki, Helsenki, Finland). GDF9 and BMP15 underwent partial purification using hydrophobic interaction chromatography (HIC), as previously described (77) (see Appendix 6). Using Western blot analysis, the concentrations of GDF9 and BMP15 were estimated (125, 149). In addition, control conditioned medium (293H) was produced by culturing untransfected 293H cells and partially purified using HIC. Dose responses of 293H control-conditioned medium and BMP15 were conducted to examine their effects on cumulus expansion.

293H dose response

COCs and OOX complexes were cultured alone as positive and negative controls, respectively, or OOX complexes were treated with GDF9 (175 ng/ml) or an increasing dose (0-20% vol/vol) of 293H, which operates as GDF9-negative control conditioned medium (equivalent to 0-250 ng/ml GDF9). Cumulus expansion was induced by intact COCs (2.6 ± 0.2) and OOX complexes treated with GDF9 (3.6 ± 0.2), whereas OOX complexes treated

with an increasing dose of 293H failed to undergo expansion (Fig. 4). This data demonstrates that it is the GDF9 that is enabling cumulus expansion and is not due to other factors in the 293H control conditioned medium.

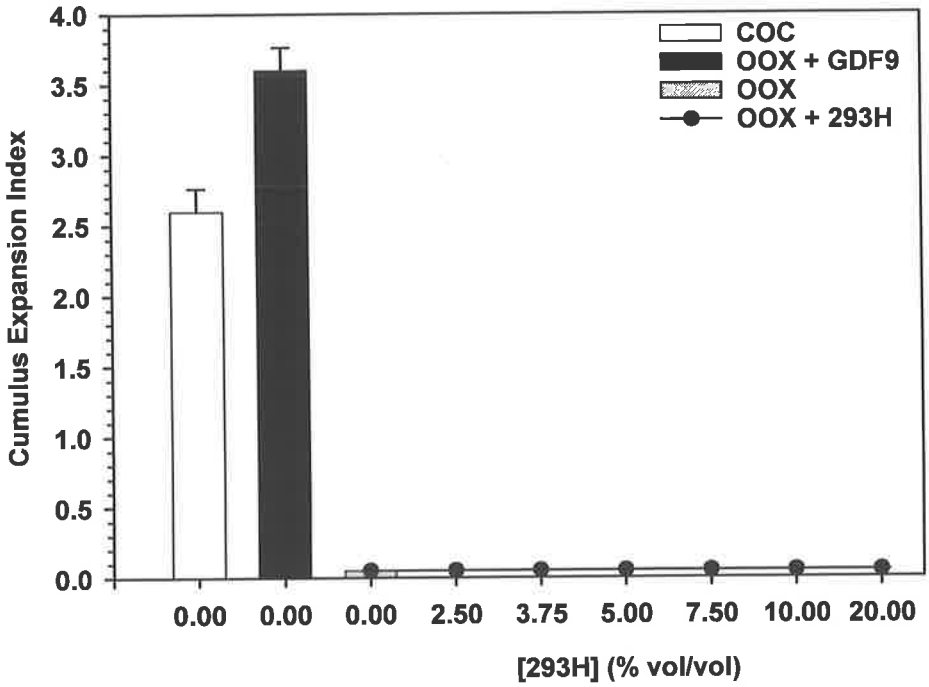

Figure 4. Effect of GDF9 control conditioned medium (293H) on cumulus expansion. OOX complexes and COCs were cultured alone as negative and positive controls. OOX complexes were treated with GDF9 (175 ng/ml) to induce cumulus expansion or an increasing dose of 293H (0-20% vol/vol). Results are the mean ± SEM of one experiment.

BMP15 dose response

OOX complexes were treated with GDF9 (175 ng/ml), 293H (20% vol/vol) or with an increasing concentration of BMP15 (0-20% vol/vol). OOX complexes and COCs were also cultured alone as negative and positive controls respectively. As expected, intact COCs expanded to a degree of (2.3 ± 0.2) and the negative control OOX complexes failed to expand. OOX complexes treated with GDF9 (175 ng/ml) underwent expansion (3 ± 0.15), whereas OOX complexes treated with 293H (20% vol/vol) or an increasing dose of BMP15 (0-20% vol/vol) failed to expand (Fig. 5). These results suggest that unlike GDF9, BMP15 does not enable cumulus expansion in the mouse.

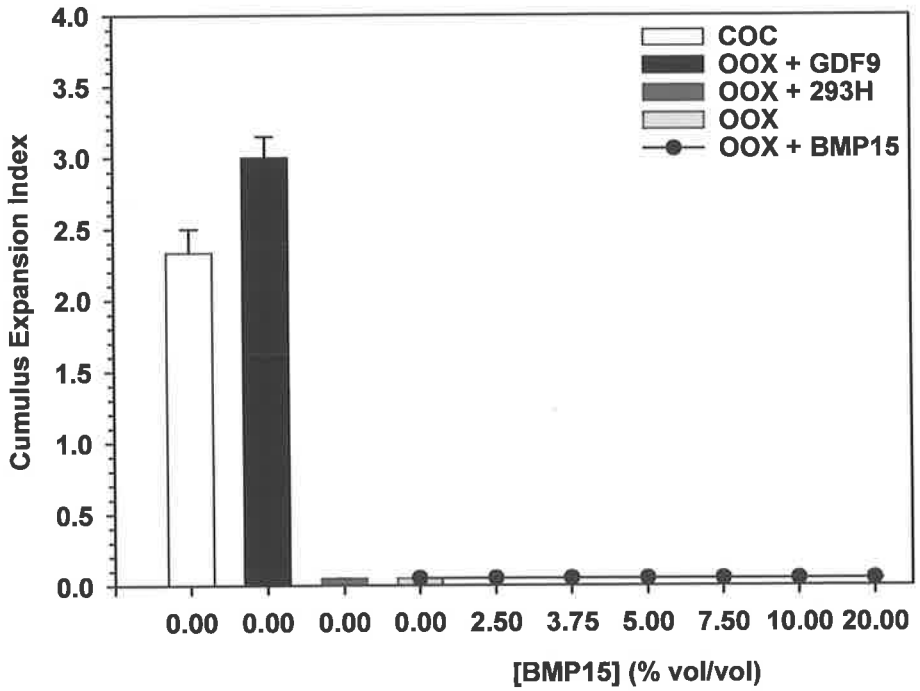

Figure 5. Effect of BMP15 on cumulus expansion. OOX complexes were cultured alone, or treated with GDF9 (175 ng/ml), 293H (20% vol/vol) or an increasing dose of recombinant BMP15 (0-20% vol/vol). Results are the mean ± SEM from one experiment.

1.4 Effect of a combination of GDF9 and BMP15 on cumulus expansion

Su *et al.,* (117) studied cumulus expansion of COCs from BMP15 null and BMP15 $^{-/-}$ GDF9 $^{+/-}$ double mutant mice, making the observation that cumulus expansion was impaired in the double mutant COCs in comparison to those from the BMP15 null mice. From these results it was suggested that GDF9 and BMP15 may operate in a synergistic manner to enable cumulus expansion in the mouse. This experiment was conducted to examine whether GDF9 and BMP15 act cooperatively to enable cumulus expansion in the mouse.

To examine the combinatorial effect of GDF9 and BMP15 on cumulus expansion, OOX complexes were cultured alone or treated with recombinant mouse GDF9 (66 ng/ml), recombinant ovine BMP15 (3.75% vol/vol) and a combination of the two together. COCs were cultured as a positive control. The dose of GDF9 chosen was based on results from Chapter 2 (Fig. 1B). It was expected that this would result in a low level of cumulus expansion and when used in conjunction with BMP15 would demonstrate whether BMP15 had any additive effect on cumulus expansion and hence, whether these two OSFs worked together to enable expansion. OOX complexes treated with BMP15 alone failed to undergo expansion, whereas complexes cultured with GDF9 expanded to a degree of 1 ± 0.3.

Expansion was not increased when OOX complexes were treated with GDF9 and BMP15 together (0.5 ± 0.2; Fig. 6). This result suggests that GDF9 and BMP15 do not work together in a synergistic manner to enable cumulus expansion in the mouse.

The species from which the growth factors were derived must be taken into consideration before any definite conclusions can be made. Studies by McNatty *et al.,* (124, 125) have demonstrated that the effects of GDF9 and BMP15 on inhibin production appear to be dependent upon: (1) species of granulosa cells and (2) species of origin of the growth factor. Perhaps GDF9 and BMP15 are operating in a synergistic manner to enable cumulus expansion in the mouse, but no effect is evident due to the origin of the BMP15 used in this experiment.

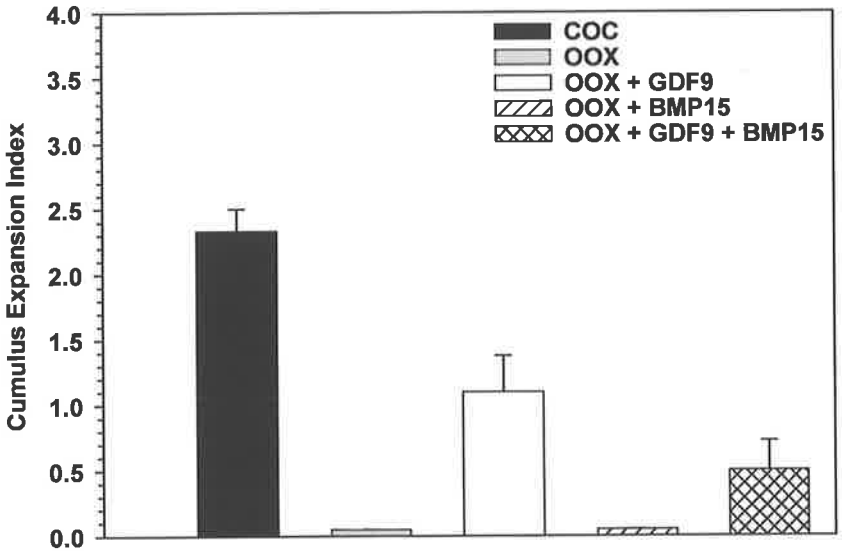

Figure 6. Combinatorial effect of GDF9 and BMP15 on cumulus expansion. OOX complexes were cultured alone or treated with GDF9 (66 ng/ml), BMP15 (3.75% vol/vol) or the two growth factors together. Results are the mean ± SEM from one experiment.

1.5 Effect of IgG on GDF9 and oocyte-induced cumulus expansion

The GDF9 neutralising antibody, mAb-GDF9-53, the TGFβ antagonist, TGFβR-II ECD and the BMP antagonist, BMPRII ECD, were important reagents used to investigate the role of GDF9 in the regulation of cumulus expansion as described in Chapter 2. The aim of this experiment was to illustrate that the neutralising actions of these antagonists were not due to general antagonist actions of the class of immunoglobulins.

OOX complexes were cultured with either oocytes (0.8/μl) or GDF9 (175 ng/ml) and treated with 2 doses of mouse IgG (20 and 40 μg/ml) or BMPRII ECD (20 μg/ml). BMPRII ECD partially neutralised oocyte-induced cumulus expansion, whereas GDF9-induced cumulus expansion was completely ablated. The increasing dose of IgG had no effect on either oocyte- or GDF9-induced expansion (Fig. 7).

Maximum concentrations of 40 μg/ml of mAb-GDF9-53, 200 ng/ml TGFβR-II ECD and 20 μg/ml of BMPRII ECD were used in the experiments carried out in Chapter 2. The results from this experiment demonstrate that the neutralisation of cumulus expansion observed was due to the actions of mAb-GDF9-53, TGFβR-II ECD and BMPRII ECD and not caused by general antagonist actions of the class of immunoglobulins.

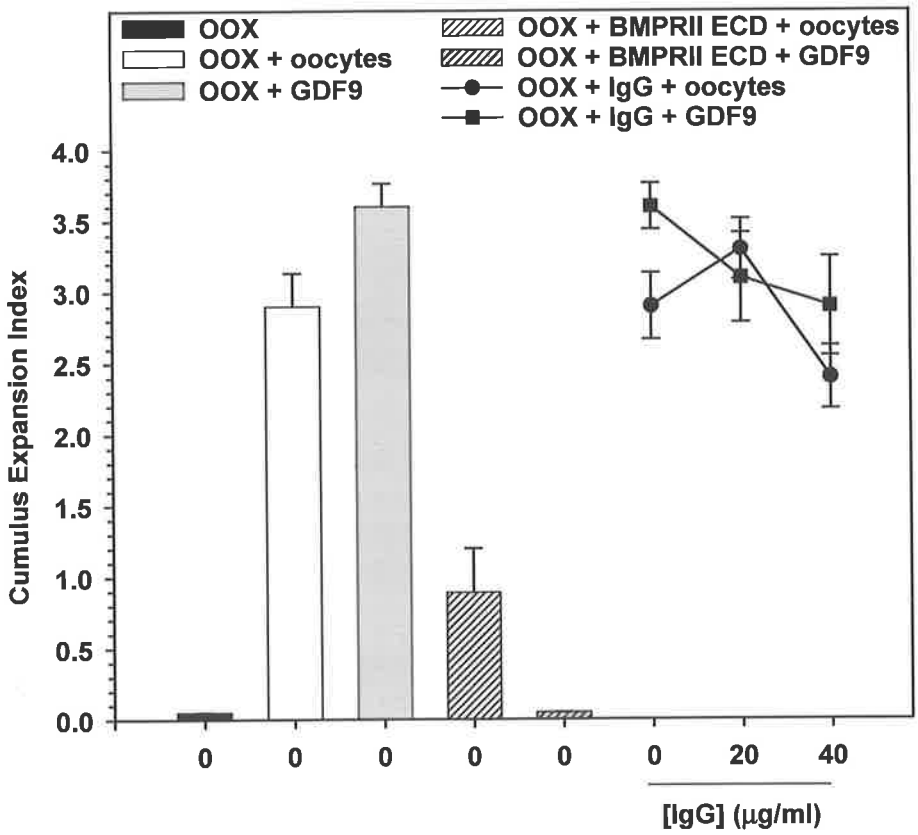

Figure 7. Effect of IgG on GDF9- and oocyte-induced cumulus expansion. OOX complexes were co-cultured with oocytes (0.8/μl) or treated with GDF9 (175 ng/ml) and with a dose of BMPRII ECD (20 μg/ml) or with an increasing dose (20 and 40 μg/ml) of IgG. Results are the mean ± SEM from one experiment.

1.6 Effect of follistatin on GDF9 and oocyte-induced cumulus expansion

The binding protein follistatin binds directly to activin and BMP15 with high affinity, thereby forming inactive complexes and inhibiting their biological functions. There are two isoforms of follistatin that are generated by alternative splicing, follistatin-288 and –315. Follistatin-288 can inhibit activin to a greater degree than that of follistatin-315 (150). Neither of the

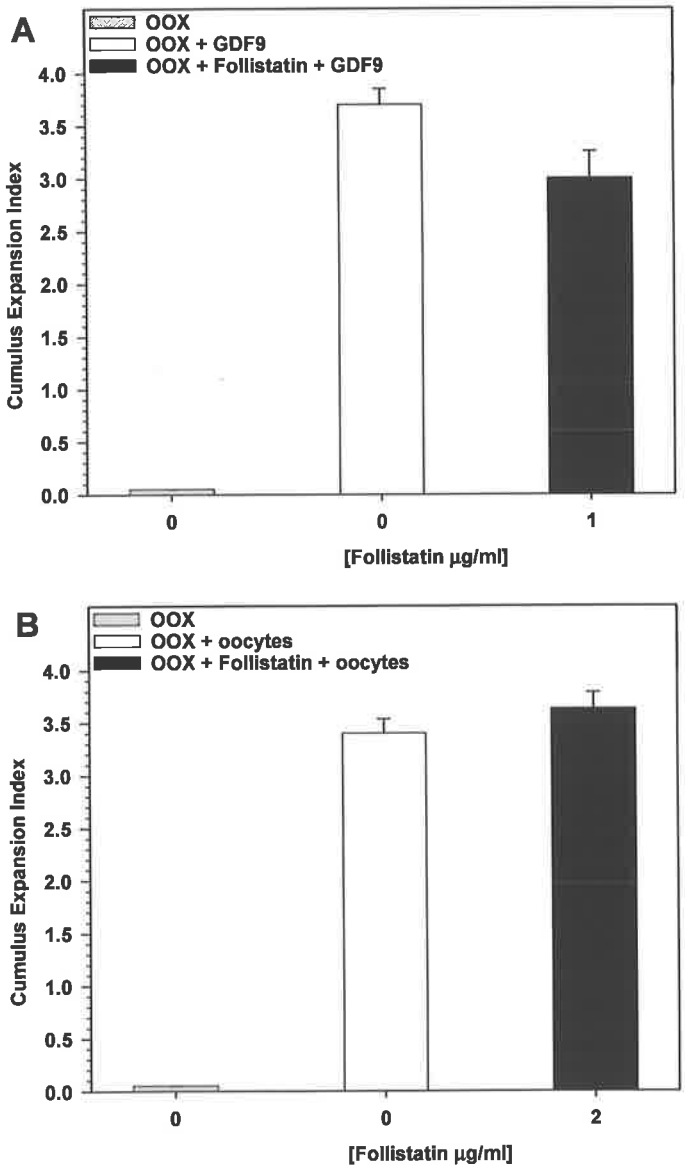

Figure 8. Effect of follistatin on GDF9- and oocyte-induced cumulus expansion. (A) OOX complexes were cultured alone or treated with GDF9 (175 ng/ml) with or without follistatin (1 µg/ml). Each column represents the mean ± SEM from one experiment. (B) OOX complexes were co-cultured with oocytes (0.8/µl) to induce cumulus expansion and treated with follistatin (2 µg/ml). Each column represents the mean ± SEM from two experiments.

follistatin isoforms are commercially available. Follistatin-288 was generously donated by S Shimasaki (University of California San Diego, USA). Otsuka *et al.*, (116) used a concentration of 1 μg/ml of follistatin-288 to inhibit the biological activities of recombinant BMP15. In Chapter 3, it was demonstrated that 1 μg/ml of follistatin is sufficient to neutralise activin-induced expansion, but has no effect on oocyte-induced expansion. The following experiments were conducted to firstly examine whether follistatin could antagonise GDF9-induced cumulus expansion and secondly, to examine the effect of 2 μg/ml of follistatin on oocyte-induced cumulus expansion.

OOX complexes were cultured alone or treated with GDF9 (175 ng/ml) with or without follistatin (1 μg/ml). OOX complexes cultured alone failed to undergo expansion, whereas complexes underwent expansion induced by GDF9 (175 ng/ml; 3.7 ± 0.2). GDF9-induced cumulus expansion was slightly reduced when treated with follistatin (1 μg/ml 3 ± 0.3; Fig. 8A). OOX complexes were cultured with or without denuded oocytes (0.8/μl) and treated with follistatin (2 μg/ml). OOX complexes underwent expansion induced by oocytes (3.4 ± 0.1), whereas OOX complexes cultured without oocytes failed to expand. Oocyte-induced cumulus expansion was not attenuated when treated with follistatin (3.6 ± 0.2; Fig. 8B).

These results suggest that follistatin has little to no effect on GDF9, although more replicates are needed to validate this. Secondly, these results also demonstrate that increasing the dose of follistatin to 2 μg/ml has no effect on oocyte-induced cumulus expansion.

1.7 Effect of cripto on cumulus expansion

Cripto is a member of the epidermal growth factor-Cripto-FRL-1-Cryptic (EGF-CFC) family of signalling proteins. Gray *et al.*, (151) illustrated that under certain conditions cripto can function either as an antagonist of activin or as an essential cofactor for nodal signalling (Fig. 9). The aim of this experiment was to determine whether cripto could act as an activin antagonist and block activin-induced cumulus expansion. Recombinant human cripto-1 used in these experiments was obtained from R&D Systems, Minneapolis, MN. OOX complexes were cultured alone as a negative control or co-cultured with oocytes (0.8/μl) or treated with activin A (200 ng/ml) and an increasing dose of cripto (1-100 ng/ml). OOX complexes cultured alone failed to undergo expansion, whereas expansion was induced by co-culture

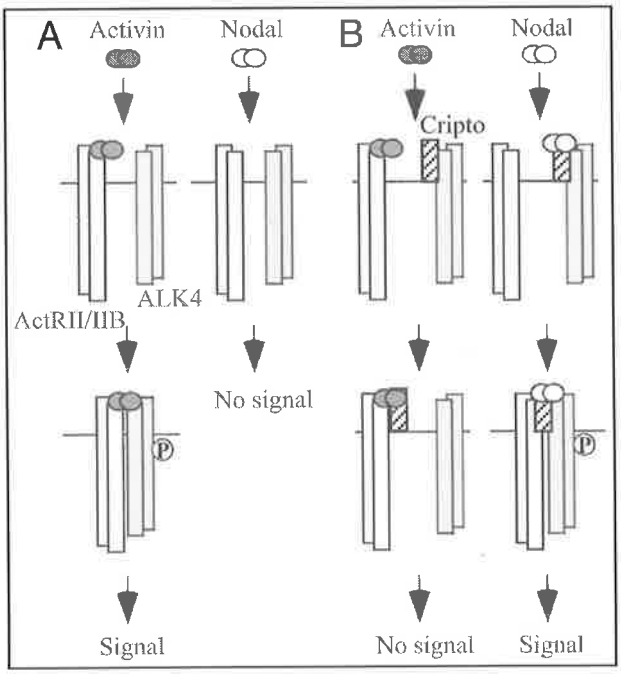

Figure 9. Model of proposed mechanism by which Cripto antagonises activin. (A) ***In the absence of Cripto.*** Activin binds to the type-II receptor ActRII/IIB, followed by recruitment of the type-I receptor ALK4, which is transphosphorylated by the kinase domain of ActRII/IIB and initiates a downstream signalling cascade of events. Nodal is unable to bind to ActRII/IIB, thereby preventing downstream signalling. (B) ***In the presence of Cripto.*** Cripto can antagonise activin signalling by preventing the activin-ActRII/IIB complex from binding ALK4, thereby blocking downstream signalling. Cripto can bind nodal which in turn will bind to ActRII/IIB and recruit and transphosphorylate ALK4 and thus activate downstream signalling. Adapted from Figure 6 in Gray *et al.*, (151).

with oocytes (3.0 ± 0.1) or activin A (3.8 ± 0.1). The activin antagonist cripto, failed to antagonise the expansion of OOX complexes stimulated by activin A or oocytes (Fig. 10A). In a separate experiment the dose of cripto was increased to a top dose of 2 μg/ml, however there was still no antagonistic effect on the expansion of OOX complexes stimulated by oocytes or activin A (Fig. 10B). As a result, no further experiments were carried out with cripto. Alternatively, the activin binding protein, follistatin, was tested and used in all subsequent experiments.

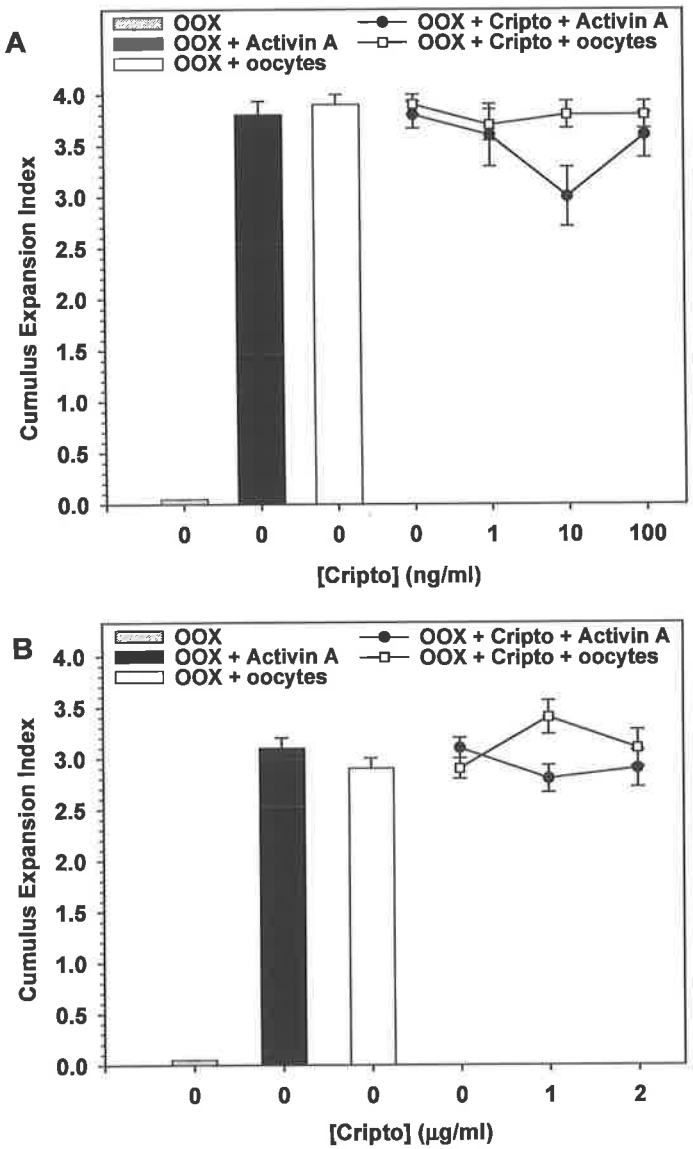

Figure 10. Effect of cripto on activin A- and oocyte-induced cumulus expansion. (A) OOX complexes were co-cultured with oocytes (0.8/µl) or treated with activin A (200 ng/ml) and with an increasing dose of cripto (1-100 ng/ml). (B) OOX complexes were co-cultured with oocytes or treated with activin A and with an increasing dose of cripto (1-2 µg/ml) Results represent the mean ± SEM from one experiment.

1.8 Neutralisation of GDF9, TGFβ and activin does not prevent cumulus expansion

It is well recognised that within the TGFβ superfamily a large amount of redundancy exists. In Chapter 2 (Fig. 5C) it was clearly demonstrated that GDF9 and TGFβ do not operate redundantly to enable cumulus expansion. This experiment was designed to examine whether GDF9, TGFβ and activin operate in a redundant manner to enable cumulus expansion.

Three replicate experiments were performed, with a mean number of 30 complexes in each treatment group. Treatment effects on cumulus expansion were examined using a Kruskal-Wallis one-way ANOVA on ranks and differences between means were detected using Dunns method *post-hoc* comparisons.

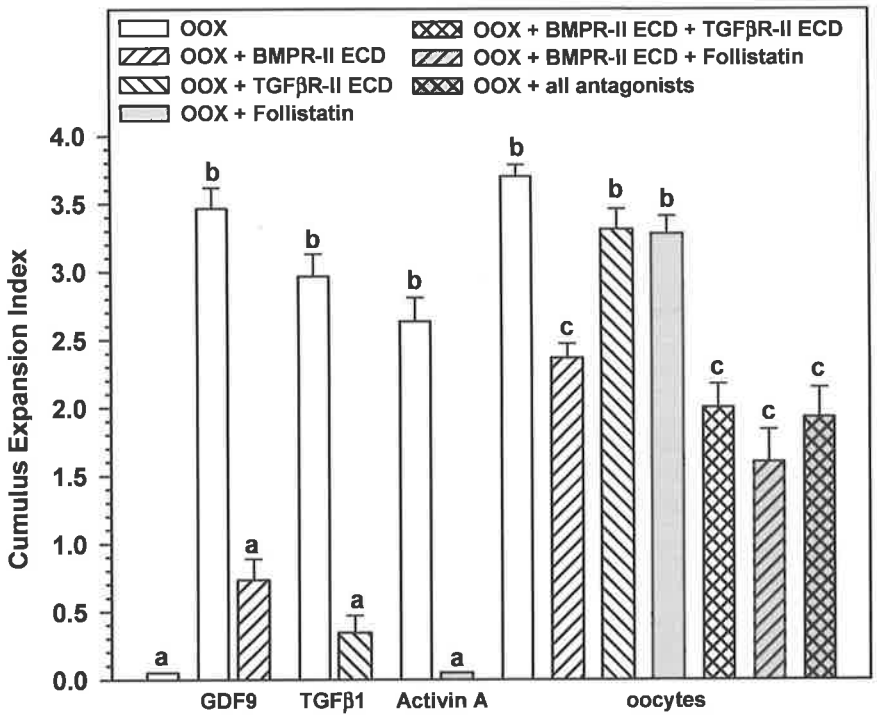

Figure 11. Effect of BMPRII ECD, TGFβRII ECD and follistatin on cumulus expansion. OOX complexes were cultured alone, treated with GDF9 (167 ng/ml), TGFβ1 (10 ng/ml), activin A (200 ng/ml) in the presence or absence of BMPRII ECD (20 μg/ml), TGFβRII ECD (200 ng/ml) or follistatin (1 μg/ml) respectively. OOX complexes were co-cultured with oocytes (0.8/μl) and treated with BMPRII ECD, TGFβRII ECD, follistatin, BMPRII ECD and TGFβRII ECD, BMPRII ECD and follistatin, or the three antagonists together. Results show the mean ± SEM from three individual experiments, and *bars within a graph with different superscript letters* are significantly different ($P<0.05$).

OOX complexes were cultured alone as a negative control. The addition of GDF9 (167 ng/ml), TGFβ1 (10 ng/ml), Activin A (200 ng/ml) and oocytes (0.8/μl) significantly ($P<0.05$) induced OOX complexes to expand to 3.5 ± 0.1, 3 ± 0.2, 2.6 ± 0.2 and 3.7 ± 0.1, respectively. The GDF9 antagonist, BMPRII ECD (20 μg/ml) effectively neutralised GDF9-induced cumulus expansion and caused a partial reduction in oocyte-induced cumulus expansion (2.4 ± 0.1). The TGFβ antagonist, TGFβRII ECD (200 ng/ml) completely impeded the actions of TGFβ-induced expansion, but had no significant effect on the expansion of OOX complexes co-cultured with oocytes. The same pattern was observed using the activin binding protein follistatin (1 μg/ml), which effectively neutralised activin A-induced expansion, but had no

effect on oocyte-induced expansion. OOX complexes co-cultured with oocytes and treated simultaneously with BMPRII ECD and TGFβRII ECD, or BMPRII ECD and follistatin, or all three antagonists together, underwent cumulus expansion to the equivalent level of complexes cultured with BMPRII ECD alone (P>0.05; Fig. 11). This result indicates that GDF9, TGFβ and activin are not operating in a redundant manner to enable cumulus expansion.

BMPRII ECD is not exclusively a GDF9 antagonist, as it antagonises other members of the TGFβ superfamily that utilise BMPRII, such as oocyte-secreted BMP15 and BMP6 (121). In addition, the activin binding protein follistatin has also been shown to antagonise BMP15 (116). Therefore, this result suggests that GDF9, TGFβ, activin, BMP15 and BMP6 are not operating redundantly to control cumulus expansion in the mouse.

1.9 Examination of RT-PCR oocyte samples for possible cumulus cell contamination

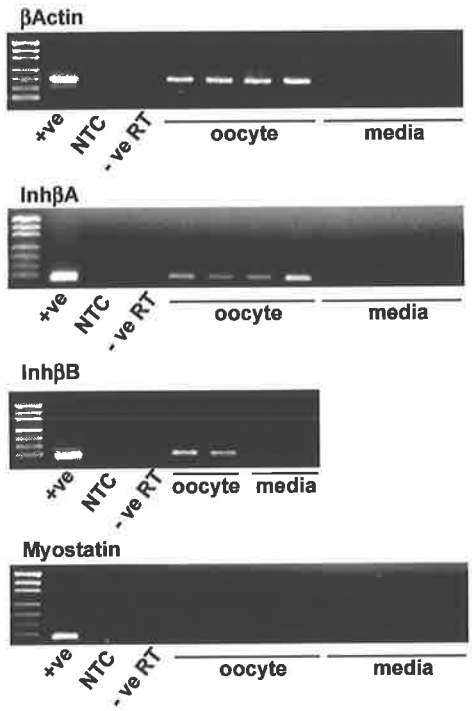

Figure 12. Examination of RT-PCR oocyte samples for possible CC contamination. COCs were collected and oocytes were denuded of surrounding CCs by mouth pipetting. RNA was extracted from oocytes as well as the medium in which the oocytes were collected from. RNA was reverse transcribed into cDNA and amplified by PCR. +ve, positive tissue sample (mouse ovary for β-actin, inhβA and inhβB; mouse skeletal muscle for myostatin); -ve RT, no reverse transcriptase; NTC, no template control (water substituted for cDNA template); Ladder, Hpa II digested pUC19.

In Chapter 3, RT-PCR was used to examine the mRNA expression of the two activin subunits inhβA and inhβB and myostatin in the oocyte. COCs were collected from ovaries and denuded of surrounding cumulus cells (CCs) by careful mouth pipetting. RNA was isolated from the oocytes as well as the medium in which the oocytes were collected from. RNA was reverse transcribed into cDNA and was analysed as described in Chapter 3. The medium in which the oocytes were collected from was screened for β-actin, inhβA, inhβB and myostatin mRNA expression. This would detect any possible CC contamination in the oocyte samples. All of the samples were negative, indicating that the oocyte samples were free of CC contamination (Fig. 12).

Appendix 2: Real-Time RT-PCR Work-Up and Additional Experiments

2.1 Real-time RT-PCR work-up experiments for Chapter 2

The following validation experiments were conducted prior to examining the effects of GDF9 and mAb-GDF9-53 on the expression of HAS2 and TSG6 mRNA.

Primer amplification efficiencies

The housekeeping gene L19 and the target genes HAS2 and TSG6 were examined to ensure that they primed with the same amplification efficiency. Standard curves were generated, consisting of five serial dilutions in water of COC cDNA (0.06 - 65 ng). Samples were run in triplicate for each primer set on an ABI GeneAmp 5700 sequence detection system. Each sample consisted of; 3 µl of diluted cDNA sample (0.06 - 65 ng), 10 µl of 2x SYBR green master mix and 10 pmol of each primer. PCR samples were treated at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of amplification at 95°C for 15 sec and 60°C for 1 min. No template controls, substituting H_2O for cDNA and a negative RT were used in each run. The average slope of each primer set was determined by plotting the C_T values against the log dilutions of the cDNA (Fig. 13). The L19 slope was statistically compared to each target gene by performing t-tests. Target gene slopes were not significantly different to L19 (P>0.05). When using the $\Delta\Delta C_T$ method the amplification efficiency of both housekeeping gene and target gene should be comparable. Average reaction efficiencies were calculated using the equation: Reaction efficiency = $(2^{[\text{std curve slope}]}/2^{3.32}) \times 100$ and the results were as follows; L19 = 120.5%, HAS2 = 120%, TSG6 = 121% (Fig. 13). Applied Biosystems recommends a maximum difference in priming efficiencies of 10% when using the $\Delta\Delta C_T$ method. For reaction efficiencies greater than 10%, a standard curve method must always be used. Reaction efficiencies did not differ more than 10%, therefore the $\Delta\Delta C_T$ method was used.

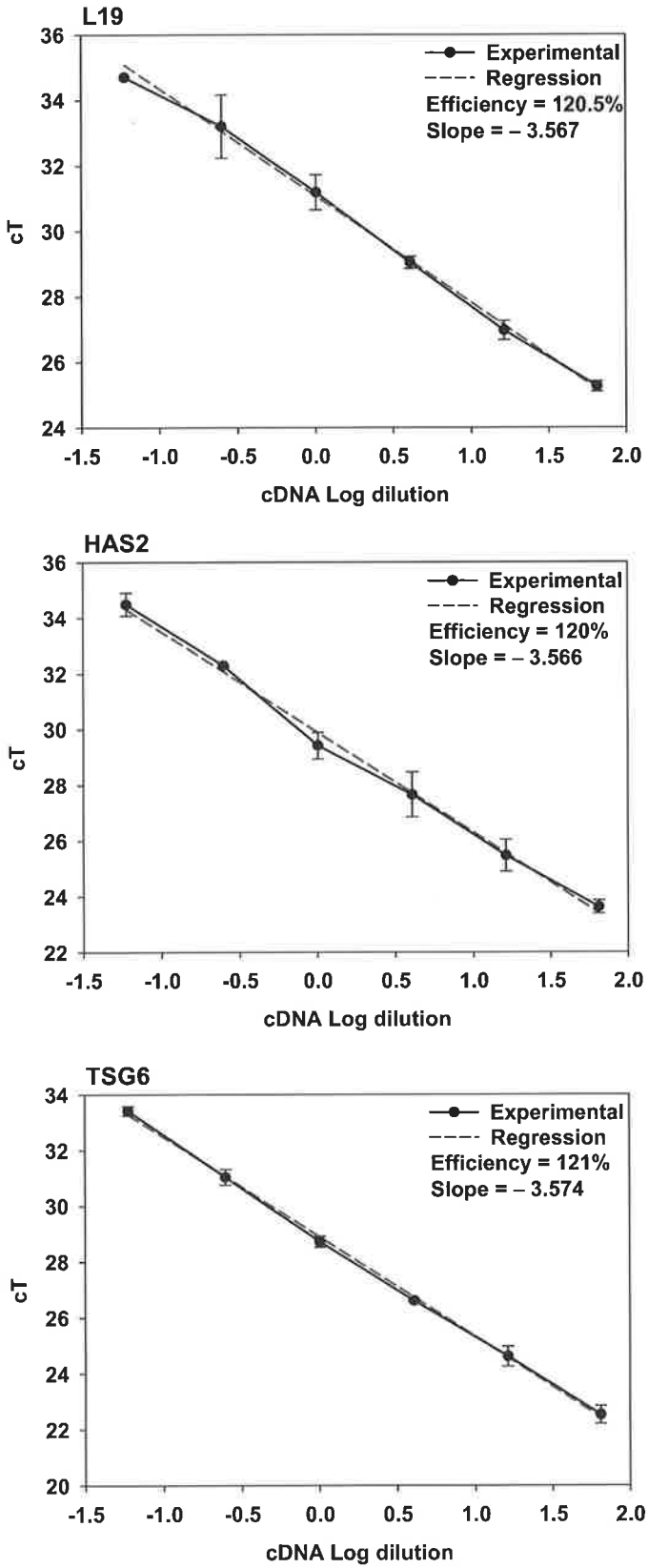

Figure 13. Primer amplification efficiencies. The average slopes of each primer set were determined using the C_T values plotted against the log dilutions of the serially diluted COC cDNA. $P>0.05$ indicates that the amplification efficiencies are not statistically different for the data range examined. Average reaction efficiencies were calculated using data from 3-4 slopes and did not differ more than 10%.

Housekeeping gene validation

To consider L19 as an appropriate housekeeping gene, the ΔC_T value for all samples should not differ significantly across treatment groups. Using an ABI GeneAmp 5700 machine, L19 mRNA levels were measured in triplicate from 5 separate experiments and were then normalised to total RNA measurements, relative to the calibrator, which in this case is the control sample, untreated OOX (Fig. 14). The influence of the treatment groups on L19 mRNA levels was analysed using a one-way ANOVA. There were no significant differences in L19 mRNA levels between treatment groups ($P>0.05$).

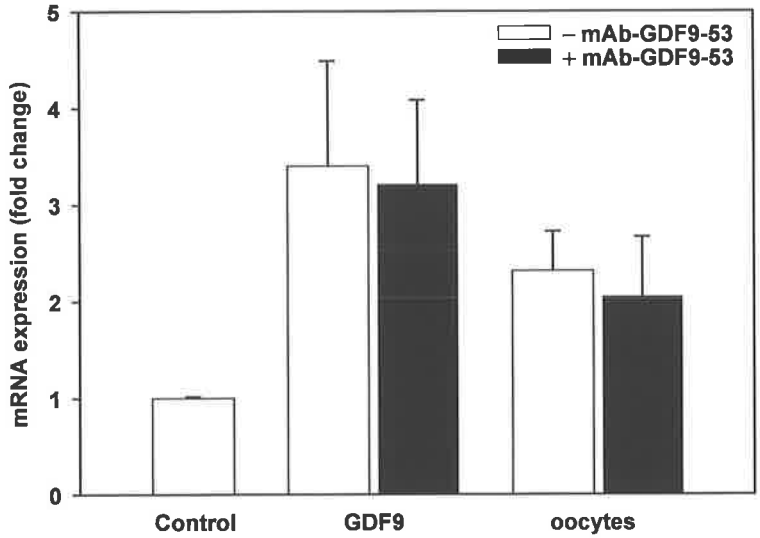

Figure 14. Housekeeping gene validation. L19 mRNA levels were measured in triplicate and were then normalised to total RNA measurements, relative to the calibrator (control; untreated OOX). Results represent the mean ± SEM from five experiments. $P>0.05$ indicates there were no significant differences in L19 mRNA levels between treatment groups.

2.2 Effect of GDF9 and mAb-GDF9-53 on TSG6 mRNA expression

In addition to examining the effect of GDF9 and mAb-GDF9-53 on CC HAS2 mRNA expression (Chapter 2), we also examined mRNA expression of the hyaluronan binding protein, TSG6.

OOX complexes were cultured for 6 h alone, treated with GDF9 (250 ng/ml) or co-cultured with oocytes (0.8/µl), either in the presence or absence of the GDF9 neutralising antibody, mAb-GDF9-53 (40 µg/ml). Complexes were collected and RNA was isolated, quantified, reverse-transcribed and analysed as described in Chapter 2 (*2.3.7 Real-time RT- PCR and 2.3.8 Real-time RT PCR analysis)*. Primer pairs for TSG6 are the same as those described in

Chapter 3 (*3.3.9 Real-time RT- PCR analysis* Table 1). Real-time RT- PCR data was log transformed to satisfy ANOVA criteria and was then subjected to one-way ANOVA followed by Tukey comparisons.

CC TSG6 expression was up-regulated approximately 6-fold by both recombinant GDF9 and oocytes, compared with untreated OOX complexes (Fig. 15). The GDF9 neutralising antibody tended to reduce both GDF9 and oocyte-induced OOX TSG6 expression, however these results were not significant ($P>0.05$). This is probably due to the low number of observations (generally 3-4), as demonstrated by low power ($P>0.05$; one-way ANOVA). This result suggests that GDF9 and oocytes are regulators of CC TSG6, however more replicates are required to confirm this observation.

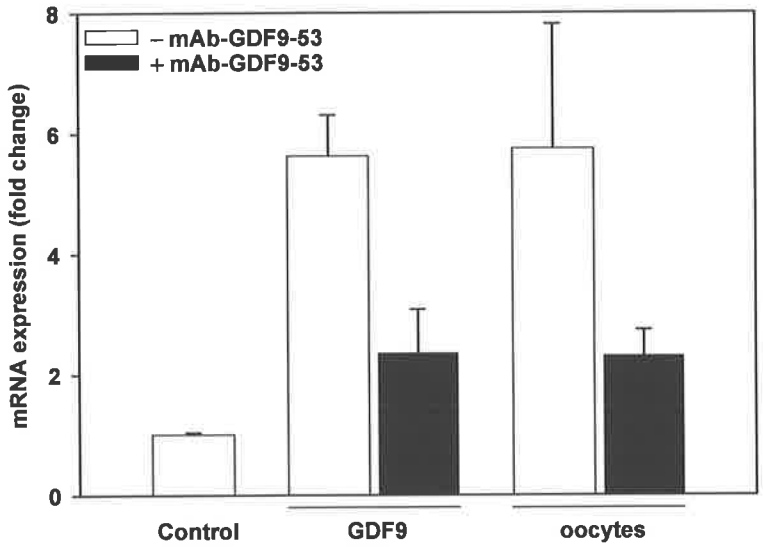

Figure 15. Effect of GDF9 and mAb-GDF9-53 on CC expression of TSG6 mRNA. Real-time RT-PCR analysis was performed using primer sets for TSG6 and L19 using RNA from CCs (OOX) cultured alone, treated with GDF9 (250 ng/ml) or co-cultured with denuded oocytes (0.8/µl), either with or without mAb-GDF9-53 (40 µg/ml). Results are from 4 independent experiments and are expressed as a fold change from mRNA levels in OOX complexes cultured alone (control). All samples have been normalised to L19 mRNA content.

2.3 Real-time RT-PCR work-up experiments for Chapter 3

The following experiments were carried out to examine the effects of the ALK4/5/7 kinase inhibitor, SB-431542 on GDF9-, activin A- and oocyte-induced CC mRNA expression of HAS2, TSG6, COX2 and PTX3.

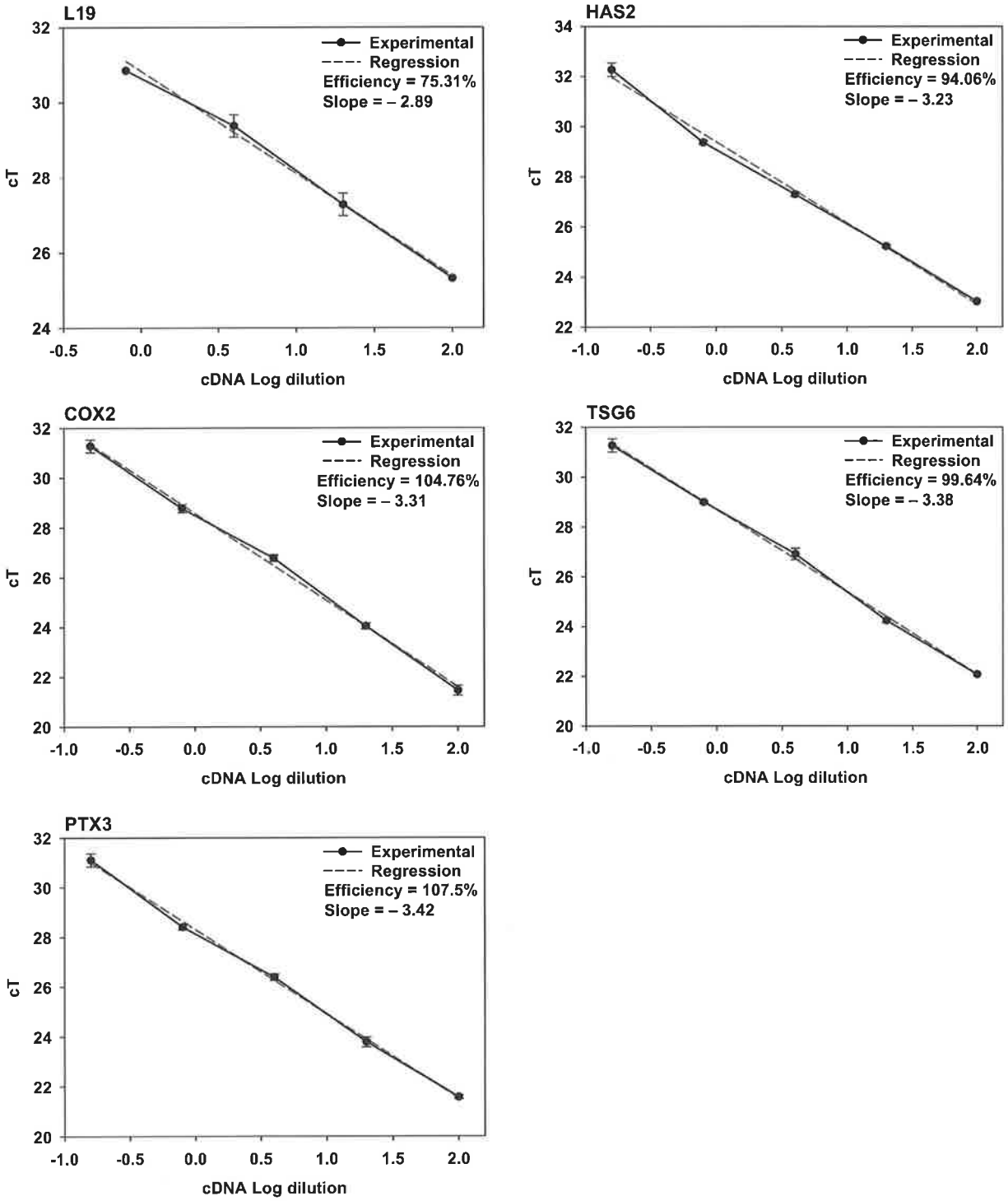

Figure 16. Primer amplification efficiencies. The average slopes of each primer set were determined using the C_T values plotted against the log dilutions of the serially diluted COC cDNA. $P>0.05$ indicates that the amplification efficiencies are not statistically different for the data range examined. Average reaction efficiencies were calculated using data from 2-4 slopes.

Primer amplification efficiencies

The housekeeping gene L19 and the four genes of interest, HAS2, TSG6, COX2 and PTX3, were examined to ensure that they primed with the same amplification efficiency. Standard curves were generated, consisting of five serial dilutions in water of COC cDNA (0.032 - 100

ng). Samples were run in triplicate for each primer set on an ABI GeneAmp 5700 sequence detection system. Each sample consisted of; 3 µl of diluted cDNA sample (0.032 - 100 ng), 10 µl of 2x SYBR green master mix and 10 pmol of each primer. PCR samples were treated at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of amplification at 95°C for 15 sec and 60°C for 1 min. No template controls, substituting H_2O for cDNA and a negative RT were used in each run. The average slope of each primer set was determined using the C_T values plotted against the log dilutions of the cDNA (Fig. 16). The L19 slope was statistically compared to each target gene by performing t-tests. Target gene slopes were not significantly different to L19 ($P > 0.05$). Average reaction efficiencies were calculated and the results were as follows; L19 = 75.31%, HAS2 = 94.06%, TSG6 = 99.64%, COX2 = 104.76% and PTX3 = 107.5% (Fig. 16). In accordance with the recommendations of Applied Biosystems, the standard curve method was used rather than the $\Delta\Delta C_T$ method to quantify mRNA expression, as a difference in priming efficiencies of > 10% was detected.

Housekeeping gene variation

Using an ABI GeneAmp 5700 machine, L19 mRNA levels were measured from 5 separate experiments in triplicate and were then normalised to total RNA measurements, relative to the calibrator, which in this case is the control sample, untreated OOX (Fig. 17). The influence of the treatment groups on L19 mRNA levels was analysed using a one-way ANOVA. There were no significant differences in L19 mRNA levels between treatment groups ($P>0.05$), thereby deeming L19 to be an appropriate housekeeping gene.

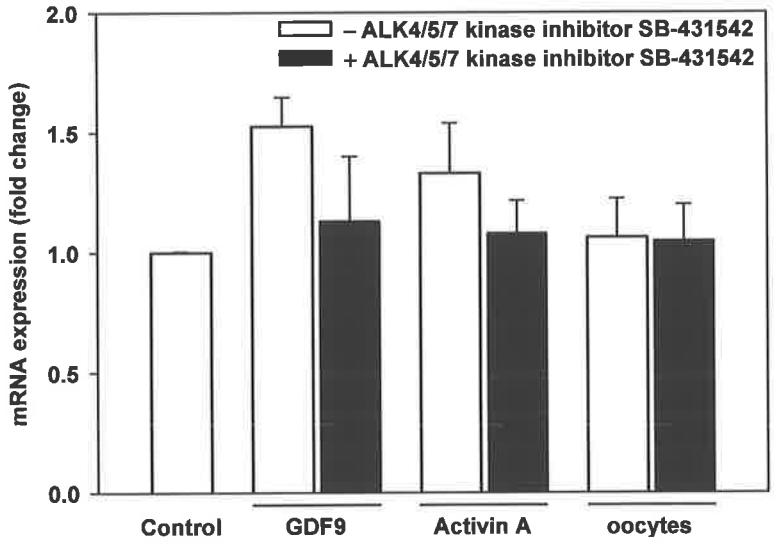

Figure 17. Housekeeping gene validation. L19 mRNA levels were measured from 5 separate experiments in triplicate and were then normalised to total RNA measurements, relative to the calibrator (control; untreated OOX). Results represent the mean ± SEM. $P>0.05$ indicates there were no significant differences in L19 mRNA levels between treatment groups.

Appendix 3: Assessment of Cumulus Expansion

Cumulus Expansion Assessment

Cumulus expansion of OOX complexes and COCs was recorded after 20 h of culture using an inverted microscope. This was a blinded assessment to eliminate any bias. The degree of cumulus expansion was assessed according to a subjective scoring system (0 to +4) originally described by Vanderhyden *et al.*, (19) (Fig. 18). A cumulus expansion index (0.0-4.0) was calculated as previously described (152).

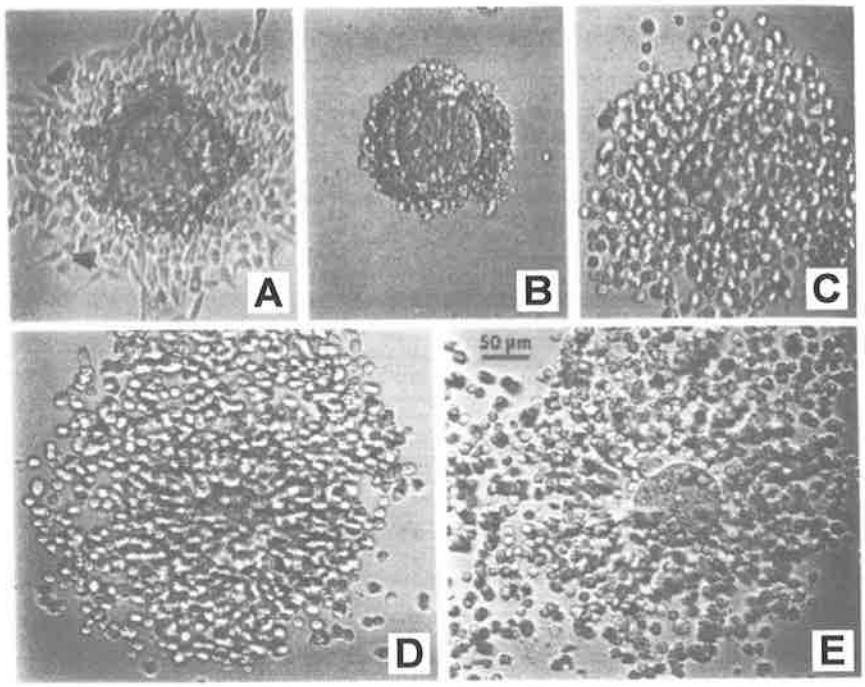

Figure 18. Photomicrographs of COCs depicting the various degrees of cumulus expansion. The degree of cumulus expansion is assessed according to a subjective scoring system (0 to +4). (A) Score 0, indicates no expansion, cells attach to the culture surface and migrate away from the complex to form a monolayer (arrows). (B) Score 1, indicates no expansion, no monolayer, slight attachment, prominent compaction of all CC layers. (C) Score +2, illustrates the outer layers of the complex have started to expand. (D) Score +3, indicates prominent cumulus expansion of all layers with the exception of the corona radiata. (E) Score +4, illustrates prominent cumulus expansion and mucification of all CC layers. Figure adapted and modified from (19).

Appendix 4: Culture Media

All chemicals were purchased from Sigma (St Louis, MO) unless otherwise stated.

Stock Solutions for IVM

2 x Tissue Culture Medium (TCM) 199

TCM 199 powder (ICN Biomedicals) 1 sachet	9.5 g
Milli Q water	500 ml
Kanamycin sulphate	50 mg

Check osmolarity (~ 480)
Filter sterilise and store at 4°C

Stock H

Hepes free acid (125 mM)	6.0 g
Hepes Na salt (125 mM)	6.5 g
Phenol red (100 µg/ml)	20 mg
Milli Q water	200 ml

Filter sterilise and store at 4°C

Stock B

$NaHCO_3$ (125 mM)	2.1 g
Phenol red (100 µg/ml)	20 mg
Milli Q water	200 ml

Filter sterilise and store at 4°C

Hepes buffered TCM-199 (H-TCM-199)

2 x TCM 199	500 ml
Stock H (15 mM)	60 ml
Stock B (2.5mM)	20 ml
Milli Q water	420 ml

Check osmolarity (~ 270)
Filter sterilise and store at 4°C

Waymouth TCM (WAY)

Waymouth MB 752/1 medium	14.0 g
$NaHCO_3$	2.24 g
Milli Q water	1000 ml

Check osmolarity (290-300)
Filter sterilise and store at 4°C

Media for IVM

Hepes buffered TCM-199 (H-TCM-199) + BSA

Hepes-199	100 ml
BSA (Fraction V)	100 mg

Filter sterilise. Equilibrate overnight at 37°C and 96% humidity in an atmosphere of 5% CO_2 in air.

Waymouth TCM (WAY) + FCS

WAY	46.3 ml
FCS (5% vol/vol; Trace Biosciences, Castle Hill, NSW, Australia)	2.5 ml
Penicillin-Streptomycin (100 IU/ml)	1 ml
rhFSH (10 IU/ml; Puregon, Organanon, Oss, The Netherlands)	200 µl

Filter sterilise. Equilibrate overnight at 37°C and 96% humidity in an atmosphere of 5% CO_2 in air. rhFSH added to culture media just prior to commencing experiment.

Appendix 5: Reagents

293 HEK
293H conditioned media (produced in house)
Culturing untransfected 293 human embryonic kidney (HEK) cells (Gibco Life Technologies, Paisley, UK) produced 293H control conditioned media. Partially purified using HIC. Fractions dialysed against PBS and stored at -80°C

Activin A
Recombinant human activin A (R&D systems, Minneapolis, MN)
10 µg/ml stock prepared by dissolving 5 µg in 0.5 ml of sterile saline containing 0.1% (wt/vol) BSA. Stored at -20°C.

Actvin B
Recombinant human activin B (R&D systems, Minneapolis, MN)
10 µg/ml stock prepared by dissolving 5 µg in 0.5 ml of sterile saline containing 0.1% BSA. Stored at -20°C.

BMP6
Recombinant human BMP6 (R&D systems, Minneapolis, MN)
50 µg/ml stock solution prepared by dissolving 20 mg in 400 µl of 4 mM HCl containing 0.1% BSA. Stored at -20°C.

BMP RII/Fc Chimera
Recombinant human BMPRII extracellular domain (ECD) (R&D systems, Minneapolis, MN)
50 µg/ml stock solution prepared by dissolving 100 µg in 2 ml of sterile PBS containing 0.1% BSA. Stored at -20°C.

Cripto
Recombinant human Cripto-1 (R&D systems, Minneapolis, MN)
10 µg/ml stock solution prepared by dissolving 50 µg in 5 ml of sterile PBS containing 0.1% BSA. Stored at -20°C.

eCG
Equine chorionic gonadotrophin (Folligon, Intervet, Castle Hill, Australia)
0.5 IU/ml stock solution prepared by dissolving 1000 IU of pure compound in 20 ml sterile saline containing 0.1% BSA. Stored at -20°C.

Follistatin
Follistatin-288 (kindly donated by S Shimasaki, University of California San Diego, USA).
100 µg/ml stock prepared by ¼ dilution of 400 µg/ml stock in sterile saline containing 0.1% BSA. Stored at -20°C.

GDF9
Bioactive recombinant mouse GDF9 (produced in house)
Recombinant human embryonic kidney-293H cells expressing mGDF-9 were donated by O. Ritvos (Helsinki). Partially purified using HIC. Fractions dialysed against PBS and stored at -80°C.

mAb-GDF9-53
mGDF-9 neutralising monoclonal antibody, mAb-GDF9-53 (generously donated by Nigel Groome, Oxford Brookes University, Headington, UK)
Antibody stored in NaN_3. Antibody was dialysed before use and resuspended in sterile PBS to 750 µg/ml. Stored at 4°C.

Mineral Oil
Embryo grade (Sigma) store at room temperature. Maintain sterility.

rhFSH
Recombinant human follicle stimulating hormone (Puregon; Organon, Oss, The Netherlands)
10 IU/ml stock solution prepared by dissolving 100 IU in 10 ml of sterile saline containing 0.1% BSA. Stored at -20°C.

SB-431542
ALK4/5/7 kinase inhibitor (generously donated by GlaxoSmithKline)
10 mM stock solution prepared by dissolving 5.67 mg in 1.3 ml of DMSO. Stored at -20°C.

TGFβ1
Recombinant human TGFβ1 (R&D systems, Minneapolis, MN)
1 µg/ml stock prepared by dissolving 2 µg in 2 ml of 4 mM HCl containing 0.1% BSA. Stored at -20°C.

TGFβ sRII/Fc Chimera
Recombinant human TGFβRII ECD (R&D systems, Minneapolis, MN)
50 µg/ml stock solution prepared by dissolving 50 µg in 1 ml of sterile PBS containing 0.1% BSA. Stored at -20°C.

Appendix 6: GDF9 Reagents

Production of recombinant mouse GDF9

Full-length mouse GDF9 cDNA was subcloned into pEFIRES-P expression vector. This was then stably transfected into a human embryonic kidney cell line, 293H, using Fugene 6 transfection reagent. The cell line was generously supplied by O. Ritvos (University of Helsinki, Helsinki, Finland). Recombinant mouse GDF9 was then produced in-house by Samantha Schulz. Cells expressing high levels of the recombinant protein were selected at 160 μg/ml of puromycin in Dubelcos Minimal Essential Medium (DMEM) supplemented with 10% (vol/vol) FCS (Trace Biosciences, Castle Hill, NSW, Australia), 2 mM L-glutamine (ICN Biomedicals Inc., CA, USA) and 100 U/ml penicillin G, 100 mg/ml streptomycin sulphate (CSL, Parkville, VIC, Australia). The recombinant protein was produced in production media (DMEM/Ham's F-12 1:1; ICN Biomedicals Inc., CA, USA) supplemented with 2 mM L-glutamine, 100 U/ml penicillin G, 100 mg/ml streptomycin sulphate, 0.01% (wt/vol) BSA and 100 μg/ml heparin (Sigma St Louis, MO, USA). An estimation of recombinant mouse GDF9 concentration in conditioned medium was obtained by Western blot analysis using purified N-tagged recombinant rat GDF9. Control conditioned medium (293H) was produced by culturing untransfected 293H cells (Gibco Life Technologies, Paisley, U.K) under the same conditions used to produce the recombinant mouse GDF9.

Partial purification of recombinant mouse GDF9

GDF9 and 293H control conditioned medium were partially purified in house by Fred Amato, using hydrophobic interaction chromatography (HIC) as recently published (77). In brief, conditioned media was concentrated approximately 20-fold by ultrafiltration, using a YM10 (10 000 MWCO) membrane (Millipore Corporation, Bedford, MA, USA). Ammonium sulphate was added to the media to give a final concentration of 1 M. The ionic strength and pH of the concentrate was adjusted to that of the start buffer (50 mM sodium phosphate, pH 7.0, 1 M ammonium sulphate) and loaded onto a 1 ml phenyl sepharose (low sub) column (Amersham Biosciences, Sydney, Australia), which had been pre-equilibrated with the same buffer. The column was washed with 13 ml of start buffer and eluted with a linear gradient of 100% start buffer to 100% elution buffer (50 mM phosphate buffer, pH 7.0) over 15 ml at a rate of 1 ml/min. 1 ml fractions containing GDF9 were detected by immunoblotting using the monoclonal GDF9 antibody mAb-GDF9-53, and also using a mouse mural granulosa cell

proliferation bioassay, both previously described (102). 1 ml fractions that were both immunoreactive and bioactive were collected, concentrated and dialysed against PBS using a Centriprep concentrator (10 000 MWCO), sterilised by filtration and stored at -80°C.

Generation of monoclonal antibodies for GDF9

The GDF9 monoclonal neutralising antibody, mAb-GDF9-53, was generated by the N. Groom laboratory (Oxford Brookes University, Headington, UK) and kindly donated. The generation of monoclonal antibodies for GDF9 has previously been described (99, 102, 149). In brief, a synthetic peptide corresponding to amino acids 420-450 of the C-terminus of human GDF9 sequence VPAKYSPLSVLTIEPDGSIAYKEYEDMIATKC was produced and coupled to the protein derivative of tuberculin. A four-month immunisation period was carried out using outbred Tyler's Original mice (Southend on Sea, Essex, UK). Spleens were removed and fused to Sp2/0 murine myeloma cells by standard methods. Hybridoma supernatants were screened against recombinant mouse GDF9 and the suitable clones were selected. Each clone was purified on a protein A column using a high salt protocol. The characterisation of the GDF9 neutralising antibody was achieved by epitope mapping using 14-amino acid peptides. Identification of the epitope binding system, involved using a 1-amino acid frameshift covering the entire human GDF9 C-terminal sequence used for immunisation. All four anti-GDF9 monoclonal antibodies were screened, as previously described (102). Based on the results of Gilchrist *et al.*, (102), mAb-GDF9-53 was chosen as the antibody for this study.

Appendix 7: Published Version of Chapter 2

Role of Oocyte-Secreted Growth Differentiation Factor 9 in the Regulation of Mouse Cumulus Expansion

Rebecca A. Dragovic, Lesley J. Ritter, Samantha J. Schulz, Fred Amato, David T. Armstrong, and Robert B. Gilchrist.

Endocrinology; **2005**; 146(6): 2798-2806

STATEMENT OF AUTHORSHIP CONTRIBUTIONS

Rebecca A. Dragovic
Designed and carried out all experiments, interpreted data and wrote the manuscript.

Lesley J. Ritter
Provided technical assistance, assisted with interpretation of real-time RT- PCR data.

Samantha J. Schulz
Provided technical assistance, produced the recombinant GDF9 and assessed all morphological cumulus expansion experiments.

Fred Amato
Purified the recombinant mouse GDF9.

David T. Armstrong
Co-supervised the work, assisted with manuscript preparation.

Robert B. Gilchrist
Overall supervision of the work, assisted with experimental design, data analysis, manuscript preparation and acted as the corresponding author.

Dragovic, Rebecca A. et al. (2005). Role of Oocyte-secreted growth differentiation Factor 9 in the regulation of mouse cumulus expansion *Endocrinology, 146*(6), 2798-2806