School of Biomedical Science

The Mode of Action of Bone Morphogenetic Protein (BMP) in the Regulation of Ovarian Function

Sheena Linnette Patricia Regan

This thesis is presented for the Degree of
Doctor of Philosophy
of
Curtin University

July 2015

Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Signature: Shona Ragan

Date: 30th July 2015

Acknowledgments

My sincerest gratitude goes to the supervisory team that guided and supported me during the PhD thesis and the technical staff of the School of Biomedical Science at Curtin University.

Initially Dr Jim McFarlane (University of New England) and Dr Ghanim Almahbobi (Curtin) provided a stimulating insight into the world of reproduction research. My time at the University of New England, Armidale NSW, in the company of Dr Tim O'Shea was inspiring, and I was privilege to benefit from his experience and his lifetime dedicated to breeding the unique Booroola sheep. Tim sadly passed away soon after I completed my research in Armidale. Professor Garry Allison was great support and mentored me during the supervision transition.

Under the procedural guidance of Professor Arun Dharmarajan (Dharma) I flourished and I am very grateful for the support and intellectual nourishment from him, and also from Frank Arfuso and the rest of the research team. In particular Malini Visweswaran, I thank for passing on to me many techniques in the lab and for her friendship.

I learnt a lot from the anonymous reviewers of my manuscript submissions. I am also very grateful for Dr Phil Knight's (Reading University, UK), mentorship, and review of this thesis. A special thank you to his research team, Claire Glister and Leanne Satchell, for their encouragement and for guiding me through various lab techniques at the University of Reading.

The world of flow cytometry is a labyrinth of techniques, and I thank sincerely Delia Wilson (School of Biomedical Science, Curtin) and her research group for guiding me rigorously, especially through method validation

The time I spent at PIVET Medical Centre (IVF clinic) was special, being witness to the start of new life. The nurses, administration staff, embryologists and clinicians were extremely accommodating and great companions. My exchanges with Professor John Yovich (medical director of PIVET) a pioneer in IVF, were always rich and inspiring, and I hope will continue into the future. The supervisory support and encouragement from Professor John Yovich and Dr Jim Stanger was much appreciated by me.

The nurses, doctors and patients from King Edward Memorial Hospital (KEMH, Perth) the departments of oncology and pathology, were amazing in accommodating the research into their schedule. I thank Professor Yee Leung for his continuing enthusiastic support of the research project.

I also wish to thank the early supporters of my PhD application, Dr Sarah Barrett, Dr Amanda Devine, Dr Alan Needham and Dr Ricardo Mancera which contributed to me receiving the Australian Postgraduate Award and a Curtin University Postgraduate Scholarship.

I was captivated by the research, and was thoroughly engaged for the duration of the PhD. The journey has been an enriching one for me, which would not have been possible without the support that I received from my family and friends. I can't thank my family enough for their encouragement, and banter on the now infamous 'granulosa cell'; my husband Paul Saunders, my parents, Elysia Regan and Andrew Turner and my five children, Paige, Sean, Tom, Luke and Gibson,

"The greatest gift of life, is your imagination"

Sheena Regan

Thesis Abstract

The aim of this thesis was to establish the effect ovarian ageing has on receptor expression on granulosa cells, which surround the oocyte in the ovarian follicle, and to delineate the specific role of bone morphogenetic protein (BMP) in the regulation of ovulation rate and folliculogenesis.

The first major body of work was to determine the direct cause of the mutation-induced, increased ovulation rate in Booroola Merino sheep (BB). Granulosa cells were removed from antral follicles pre and post-ovulation from five BB and 12 wild type (WT) Merino ewes. Immunofluorescent measurement of mature cell surface receptors using flow cytometry demonstrated a significant up-regulation of follicle stimulating hormone receptor (FSHR), transforming growth factor beta (TGFβ) type 1, bone morphogenetic protein receptor (BMPR1B), and luteinising hormone receptor (LHR) in the BB.

The up-regulation of LHR provides evidence of an earlier acquisition of receptors with greater expression density at an earlier follicle size in the BB compared to the WT genotype. The increased density of FSHR and LHR provided novel evidence of a mechanism for increasing the number of follicles that are recruited during dominant follicle selection. The compounding increase of receptors with increasing follicle size maintained the multiple follicles and reduced the apoptosis, which contributed to the high ovulation rate in the BB sheep. In addition, we report a mutation-independent mechanism of down-regulation of receptor density of the leading dominant follicle in sheep. The suppression of receptor density coincided with the cessation of mitogenic growth and steroidogenic differentiation as part of luteinisation of the follicle. The suppression of receptor density coincided with the cessation of mitogenic growth and steroidogenic differentiation as part of luteinisation of the follicle. The BB mutation-induced attenuation of BMPR1B signalling, led to an increased density of the FSHR and LHR, with a concurrent reduction in apoptosis or necrosis, which increased the ovulation rate. The role of BMPs in receptor modulation was implicated in the development of multiple pre-ovulatory follicles.

The second major body of work aimed to profile the expression of BMPR1B, FSHR, LHR and growth hormone receptor (GHR), and the level of apoptosis or necrosis in IVF patients, in a range of ovarian primordial follicle depletion. Reproductive ageing is linked to the declining capacity to regenerate cells, causing irreversible changes to ovarian cellular dynamics, which ultimately reduce the capacity to reproduce. The 415 ovarian follicles collected from 54 IVF patients, in a range of diameter from 4 to 27 mm, and ~8000 granulosa cells, per follicle, were analysed. The density of the granulosa cell surface, mature receptor

protein was measured by immunofluorescent labelling and analysed by flow cytometry. Ovarian reserve was measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm in size that are present on day 2-5 of a cycle.

A decline in BMPR1B density in granulosa cells occurred at the time of cyclic dominant follicle selection, and again during the maturation stage of folliculogenesis in young IVF patients (23-30 years (y)) with a good ovarian reserve. The older patients with poor ovarian reserve (40+ y), experienced a reversal of the biphasic pattern of receptor density. The reversal seen with declining AFC rather than chronological age provides compelling evidence of a fundamental shift in granulosal BMPR1B receptor density with ovarian ageing. High levels of granulosal FSHR density were observed in the smaller antral follicles, and the depletion of the ovarian reserve did not generally disrupt the expression. Conversely, the down-regulation of granulosal FSHR density during the maturation process was disrupted by ovarian reserve depletion. The LHR density was lower and failed to be down-regulated during pre-ovulatory maturation in the older patients with a poor ovarian reserve compared to the younger patients. Ovarian ageing appears to disrupt the prerequisite down-regulation of granulosal FSHR, BMPR1B and LHR density required for maturation of the follicle before ovulation.

The level of GHR density was generally reduced with ovarian ageing, yet a correlation was evident between the increasing level of recombinant FSH (rFSH) treatment and the increase in GHR of the small antral follicles. The effect of exogenous rFSH to increase the GHR expression, diminished in the older age groups with the poorest ovarian reserve. The results demonstrate the disrupting effect that age-induced depletion of the ovarian reserve has on receptor density at the two stage-specific, critical time points of dominant follicle selection and pre-ovulatory maturation. This dysregulation is potentially responsible for the reduction in oocyte quality in older patients.

The level of apoptosis increased during dominant follicle selection and during pre-ovulatory maturation in young IVF patients with an uncompromised ovarian reserve. The granulosal BMPR1B and FSHR density was strongly associated with a reduction in apoptosis; and the reduced level of apoptosis in the poor ovarian reserve patients was also strongly correlated to the level of rFSH drugs administered. The greater level of apoptosis and necrosis of the young uncompromised ovarian reserve patients, compared to the older poor ovarian reserve, may reflect the overall greater mitogenic proliferation in the younger patient cohort.

The third major body of work examined the effect of growth hormone (GH) co-treatment on the changes to granulosal BMPR1B, FSHR, LHR, and GHR density, and the level of apoptosis or necrosis during follicle development. Previously, GH co-treatment during IVF increased the pregnancy rate of infertility patients with a poor ovarian reserve. In an attempt to reverse the poor prognosis of older patients with a poor ovarian reserve, GH was provided to 11 patients as an adjunct to IVF treatment. The 72 ovarian follicles collected from GH co-treated IVF patients ranged in diameter from 4 to 23 mm, and ~8000 granulosa cells, per follicle were analysed. The density of the granulosa cell surface, mature receptor protein, was measured by immunofluorescent labelling and analysed by flow cytometry.

The GH co-treatment increased the receptor density of BMPR1B, FSHR, and LHR, and restored the pre-requisite down-regulation during pre-ovulatory maturation which may improve oocyte quality as observed in GH co-treated patients. The study has revealed several cellular mechanisms that could contribute to an improved oocyte quality in GH co-treated IVF patients with a poor ovarian reserve. The dysregulation of the ovarian micro-environment caused by ovarian depletion was partially reversed, providing an explanation for the positive pregnancy rate associated with GH co-treatment. GH co-treatment of the 40+ y poor ovarian reserve patient group did not significantly change the level of apoptosis or necrosis in the same age group and AFC, when compared to those without GH co-treatment. Preliminary data on recombinant GH (rGH) co-treated older patients, with good ovarian reserve, demonstrated an increased level of apoptosis or necrosis.

In the fourth major body of work, biochemical analysis of the serum and follicular fluid obtained from patients with a range of ovarian reserves from the previous three bodies of work was undertaken. Follicular fluid aspirated from follicles of ~20 mm did not reveal any significance in the level of oestrogen, progesterone, or LH, whereas FSH levels were correlated to the rFSH treatment dose. Serum oestrogen synthesis was reduced as the ovarian reserve depleted, and the level of FSHR density was not changed with reduced ovarian reserve, which indicates that the decrease in oestrogen synthesis capability may be downstream of the FSHR. Progesterone concentrations were consistent for each age class, except for the decrease in the, poor ovarian reserve groups. The reduced oestrogen synthesis could also be associated with a reduced progesterone serum level, and may be related to granulosal receptor density.

The LH serum level, corresponding to the peak oestrogen level of the follicular phase, in an IVF cycle increased as the ovarian reserve became depleted. The LH concentration was significantly greater in the older IVF patients with a poor ovarian reserve compared to the

younger patient group. The level of FSH at the start of the cycle was significantly greater in the older patients with a poor ovarian reserve compared to the younger patients during a natural cycle or during an IVF cycle. The FSH level at the time of peak oestrogen (FSH surge) was not significantly different between the old and young patients during a natural cycle. However, during an IVF cycle, the FSH surge was substantially greater in the older patients with a dose-dependent increase as the ovarian reserve declined.

The GH co-treatment did not alter the oestrogen, progesterone, FSH, or testosterone level in the follicular fluid or serum levels, from older, poor ovarian reserve patients. GH co-treatment increased the granulosal FSHR density, yet had no effect on the serum or follicular fluid oestrogen synthesis. The level of FSHR expression was not significantly reduced in the poor ovarian reserve patients, which indicates that the conversion of androstenedione to oestradiol in the granulosa cell is adversely impacted on, downstream of the hormone-receptor interphase in ovarian ageing.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Abbreviations

3β Hydroxysteroid Dehydrogenase		HSD3β
7-Amino-Actinomycin D		7-AAD
Allophycocyanin		APC
Androgen		A
Androstenedione		A4
Anti-mullerian hormone		AMH
Antral Follicle Count		AFC
Artificial Reproduction Treatment		ART
Bcl-2-Like Protein		BAX
Binding Protein Immunophilin FKBP12		FKBP12
Bone Morphogenetic Protein		BMP
Bone Morphogenetic Protein Receptor		BMPR1B
Booroola Merino Sheep		BB
Corpus Luteum		CL
Cyclic Adenosine 3´,5´-Monophosphate		cAMP
Cyclic Adenosine 3´,5´-Monophosphate – Protein Kinase A		cAMP-PKA
Epidermal Growth Factor		EGF
Extracellular Signal Regulated Kinases 1 And 2		ERK1/2
Fisher's least significant difference		Fisher's LSD
Follicle Stimulating Hormone		FSH
Follicle Stimulating Hormone Receptor		FSHR
Follicular Fluid		FF
Gonadotrophin Releasing Hormone		GnRH
Gonadotrophin Surge Attenuating Factor		GnSAF
Growth Hormone		GH
Human Chorionic Gonadotrophin		hCG
In vitro fertilisation		IVF
In vitro maturation		IVM
Insulin Like Growth Factor 1 Receptor		IGF1R
Insulin Like Growth Factor 2		IGF2
Insulin Like Growth Factor Binding Protein		IGFBP
Janus Nuclease Kinase		JNK
Janus Kinase		JAK
King Edward Memorial Hospital		KEMH
Luteinising Hormone		LH
Luteinizing Hormone Receptor		LHR
Mitogen Activated Protein Kinases		MAPK
Mothers Against Decapentaplegic	MAD	SMAD

C. Elegans Protein	SMA	SMAD
Oestrogen		E2
P450 Aromatase		CYP19A1
P450 Side Chain Cleavage		CYP11A1
Percentage Coefficient Of Variance		CV
Phosphate Buffer Sulphate		PBS
Pregnant Mare's Serum Gonadotrophin		PMSG
Primordial Germ Cell		PGC
Progesterone		P4
Propidium Iodide		PI
Recombinant DNA		r
Recombinant Follicle Stimulating Hormone		rFSH
Recombinant Growth Hormone		rGH
Signal Transducer And Activator Of Transcription		STAT
Steroid Acute Regulatory Protein		StAR
Transforming Growth Factor Beta		TGFβ
Transvaginal Oocyte Aspiration		TVOA
Tumour Necrosis Factor alpha		TNFα
Weeks Post Fertilisation		wpf
Year/s old		y

Published Paper

Regan, SLP, JR McFarlane, T O'Shea, N Andronicos, F Arfuso, A Dharmarajan, and G Almahbobi 2015 Flow cytometric analysis of FSHR, BMPR1B, LHR and apoptosis in granulosa cells and ovulation rate in merino sheep. *Reproduction* **150** 151-163.

Regan, SLP, PG Knight, J Yovich, J Stanger, Y Leung, F Arfuso, A Dharmarajan, and G Almahbobi 2016 Dysregulation of granulosal bone morphogenetic protein receptor 1B density is associated with reduced ovarian reserve and the age-related decline in human fertility. *Molecular and Cellular Endocrinology*. Doi:10.1016/mce.2016.01.016

Published Abstracts

ABSTRACT 1

Regan S, Stanger, Jim, McFarlane, Jim, O'Shea, Tim, Yovich, John, Almahbobi, Ghanim. 2011 Down-regulation of Bone Morphogenetic Protein Receptor (BMPR1B) expression by stimulation with IVF drugs in human and sheep. 2nd World Congress on Reproductive Biology, Brisbane, Australia 2 146.

ABSTRACT 2

Regan S, Stanger, Jim, Yovich, John, Almahbobi, Ghanim. 2012 Growth Hormone increases gonadotropin receptors in poor-prognosis patients. Human Reproduction ESHRE, 2012 Istanbul, Turkey 27 P525

ABSTRACT 3

Regan S, Stanger, Jim, Yovich, John, Almahbobi, Ghanim. 2012 Growth Hormone's mode of action upregulates FSH, LH and BMP receptor expression on granulosa cells from IVF patients. 43rd Society for Reproductive Biology Gold Coast

ABSTRACT 4

Almahbobi G, Regan S.L.P., Al-Samerria S, Al-Ali I & Yovich JL 2013 Down-regulation of BMP action may alleviate the negative impact of age-related menopause. The Australian Society for Medical Research 52nd Annual National Scientific Conference July, Ballarat, Victoria #39.

ABSTRACT 5

Almahbobi G, Regan S, Stanger J & Yovich JL 2014 An indirect mechanism to explain the beneficial effect of growth hormone on human oocyte quality in poor responder patients. ASPIRE November 21-24th 2014 Brisbane FC059.

ABSTRACT 6

Regan S, Stanger J, Yovich J, Arfuso F, Dharmarajan A, Almahbobi G. 2015 Expression profile of reproductive receptors during ageing of the human ovarian follicle. 46th Society for Reproductive Biology, Adelaide South Australia, *27565*.

Thesis Format

A general introduction to ovarian function and fertility is given in Chapter 1. In the second chapter a detailed exposition of previous published research in the field is contained in the literature review, Chapter 2. The general methods, Chapter 3 describes in detail the methodology and procedures used in the collection, isolation, quantification of receptor density, and level of cell death of granulosa cells from sheep and human models. The following chapters are organised into discrete studies, containing an introduction, brief methodology, results, discussion and conclusion. The final concluding chapter links the findings from each body of work, and delivers the scientific rationale behind the proposed change to current best practice principles in IVF. The references are contained after the conclusion of the thesis, followed by supporting appendices.

Animal model:

The animals used for the studies were Australian Merino sheep and Australian Booroola Merino sheep at the University of New England, NSW, Australia, in accordance with NH & MRC Code of Practice for the Care and Use of Animals for Experimental Purposes. All experiments were approved by the University of New England Animal Ethics Committee.

Human model:

A total of 487 follicles were collected from 68 patients undergoing standard fertility treatment at PIVET fertility clinic Perth, Australia. One patient out of three patients undergoing risk reduction removal of the uterus and ovaries was selected to represent an unstimulated natural healthy cycle prior to the LH surge, and was recruited from King Edward Memorial Hospital (KEMH) Perth, Australia. Approval by the Human Research Ethics Committee of Curtin University of Technology and KEMH, Women and Newborn Health Service ethics committee (WNHS) was obtained for this study.

Abbreviations:

These are shown in parenthesis when first mentioned, and are listed in a table above on page xx.

Referencing:

The style of referencing is based on the journal, Reproduction format, and first author and year of publication are displayed within the paragraph.

Published abstracts and paper:

Published works are detailed above on page xxii

INTRODUCTION

CHAPTER 1 INTRODUCTION

The ovarian process of recruitment and folliculogenesis involves a complicated paradigm of dynamic fluctuations of steroid hormones, and cell proliferation and differentiation. The process culminates in the production of a mature ovarian follicle, which ruptures and releases the oocyte with the potential to be fertilised and form a new life.

Primordial germ cells migrate to the gonads and proliferate during gestation which generates a vast sum of oocytes surrounded by epithelium pre-granulosa cells that forms the primordial follicle. The primordial follicle's growth is arrested at the diplotene stage of meiosis. Activation of the primordial follicle to grow beyond this stage is of particular interest because, once activated, the primordial follicle develops into a primary follicle where it is subject to the regulatory mechanisms involved in growth, selection, maturation, and ultimately, ovulation. The vast majority of follicles succumb to atresia, and are permanently lost from the primordial pool of follicles.

The primordial follicles are continuously being activated, and the number of small antral follicles at the beginning of the menstrual cycle is an indirect measurement of the ovarian reserve of primordial follicles. At the onset of puberty, high levels of gonadotrophins are released from the pituitary, and the menstrual cycle begins. Folliculogenesis during the menstrual cycle is driven by follicle stimulating hormone (FSH) and luteinising hormone (LH) in conjunction with many growth factors and inhibitors interrelated in a complex web of regulatory balance.

The bone morphogenetic protein (BMP) signalling system has a major role to play in many species, including the human, in the generation of transcription factors that influence proliferation, steroidogenesis, cell differentiation and maturation prior to ovulation of the oocyte. BMPs are also involved in stimulation of the anterior pituitary gonadotrophin cells' generation of FSH.

LITERATURE REVIEW

CHAPTER 2 LITERATURE REVIEW

2.1 Overview of folliculogenesis

The underlining mechanics of reproduction for the survival of the species are similar in humans and higher order animals. The oocyte or egg in the female is encapsulated by layers of follicular cells that proliferate, and later differentiate and mature in the ovary. The mature follicle wall ruptures and the oocyte is expelled from the follicle and is potentially destined for fertilisation. The recruitment of the follicles, their growth, and the expulsion of the oocyte are dependent on a complex signalling mechanism involving the hypothalamic-pituitary-gonadal axis. Neurotransmitters from the hypothalamus stimulate the release of gonadotrophin releasing hormone (GnRH) into the hypophyseal portal system, which in turn, stimulates the anterior pituitary to release gonadotrophic hormones that act on the ovary to promote follicular growth. Several growth factors which regulate the transcription of genes and control the recruitment and selection of the dominant follicles, belong to the transforming growth factor beta (TGFβ) superfamily including the BMPs, inhibins and activins (Knight and Glister 2006).

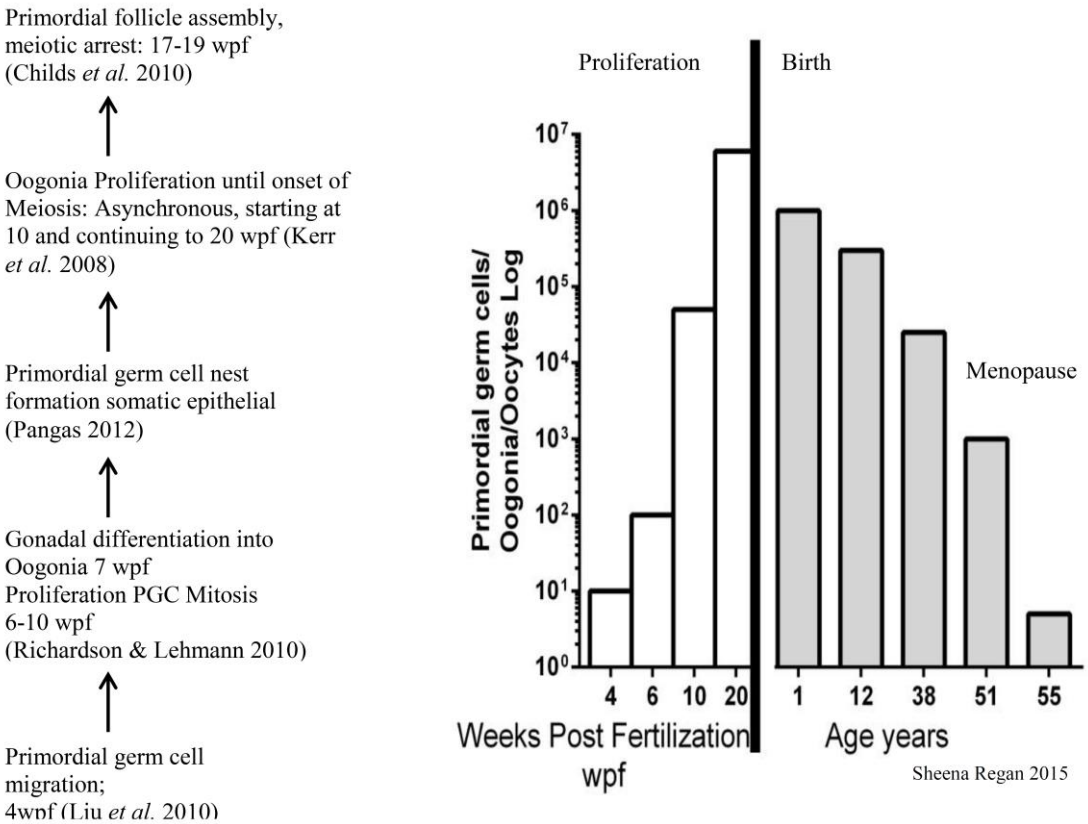


Figure 2.1.1 Primordial germ cell proliferation (PGC) and oogenesis before birth, and the loss of primordial follicles from birth to menopause.
Based on (Baerwald, et al. 2012a, Fabre, et al. 2006, Knight and Glister 2006, Matsuda, et al. 2012, Skinner 2005, Webb 2007)

The sperm and the oocyte come together at the time of conception to form the embryo. At approximately 26 weeks of gestation in humans, the reproductive potential of the foetus is initiated (Childs, et al. 2010). It is at this time that the primordial follicles are fully formed, and begin a lifelong process of initial activation followed by eventual demise (Figure 2.1.1) (Pangas 2012). The primordial follicles grow and differentiate into pre-antral follicles (Figure 2.1.2). With further development pre-antral follicles mature into antral follicles with the formation of a fluid filled central compartment (Rodgers and Irving-Rodgers 2010b). At the onset of puberty, the level of gonadotrophin secreted from the anterior pituitary increases FSH to a threshold point sufficient to rescue a growing cohort of small antral follicles and initiate cyclic folliculogenesis (Figure 2.1.2) (Gougeon 1986, Richards 1994).

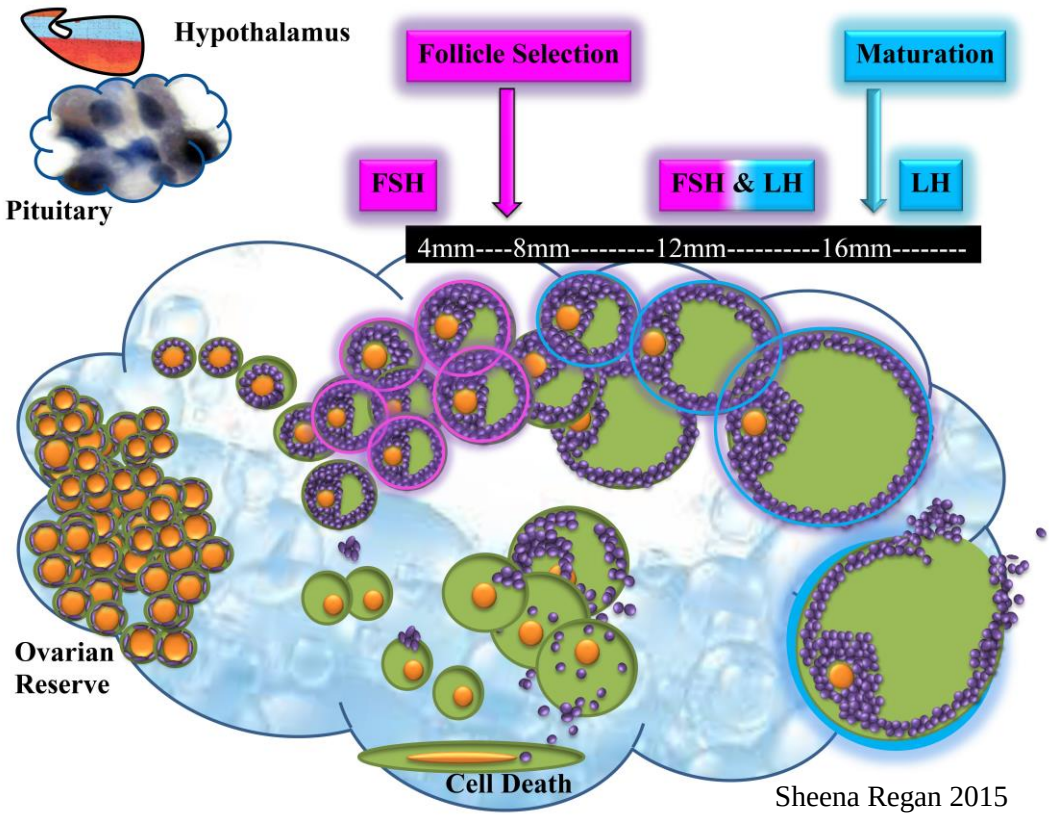


Figure 2.1.2 Folliculogenesis: Activation of the primordial follicle, dominant follicle selection, growth and maturation before ovulation

.

Antral follicles contain an oocyte, surrounded by cumulus granulosa cells suspended in fluid, and anchored to the follicle wall. The follicle wall is composed of granulosa and theca cells separated by the basal lamina membrane. Stromal cells within a connective tissue matrix are encapsulated by epithelial cells at the follicle surface (Erickson and Shimasaki 2003, Rodgers and Irving-Rodgers 2010b).

The process of folliculogenesis involves the stage-dependent expression of growth factor receptors that regulate the process of follicular growth (Erickson and Shimasaki 2003). The

follicle undergoes proliferation of the granulosa and thecal cells, followed by differentiation of the role of the follicular cells, as the follicle matures (Gougeon 1986). The mature follicle or pre-ovulatory follicle completes differentiation and becomes luteinised (morphological and steroidogenic capacity changes) prior to ovulation, and then ruptures, releasing the oocyte into the fallopian tube cavity (Ainsworth, et al. 1980, Rodgers and Irving-Rodgers 2010b).

The number of pre-ovulatory follicles selected for dominance and ovulation is dependent on the regulation by the gonadotrophins follicle stimulating hormone (FSH) and luteinising hormone (LH), and the interaction with intraovarian growth factors that are involved in folliculogenesis (Ginther, et al. 2005, Gougeon 1986).

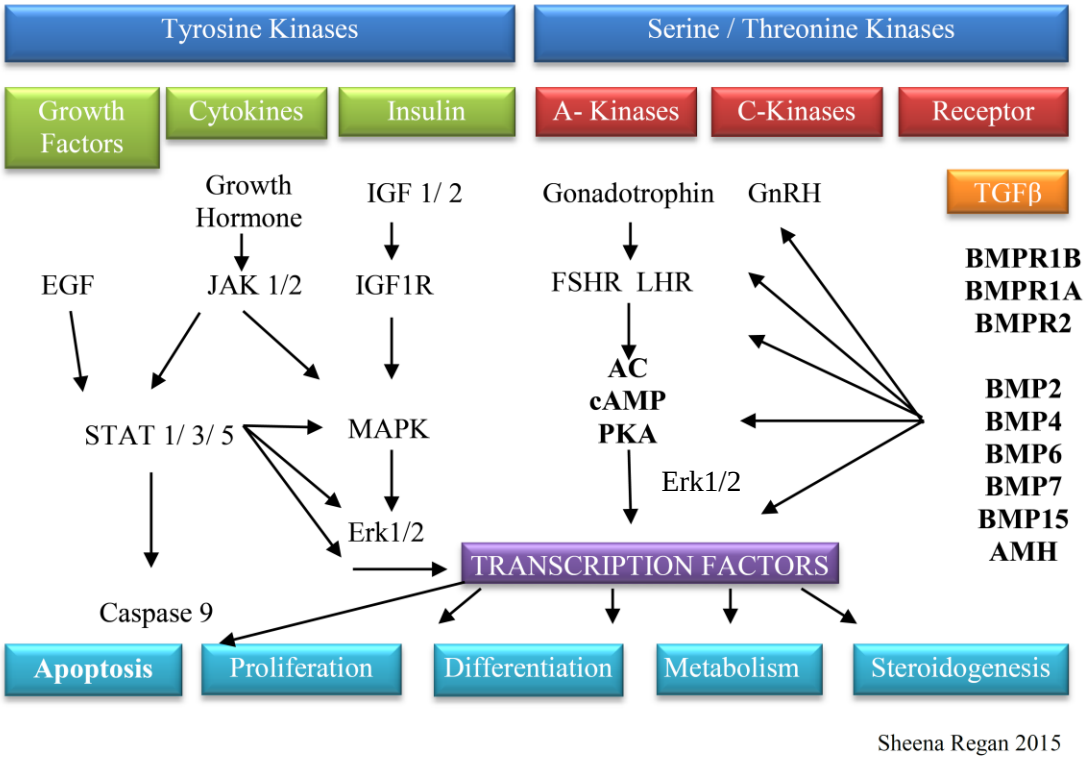


Figure 2.1.3 Overview of the TGFβ and the growth hormone kinase signalling interaction. Based on (Amsterdam, et al. 2003, Fan, et al. 2009, Manna, et al. 2002, Miyazono, et al. 2010, Moore, et al. 2001b, Rice, et al. 2007, Tajima, et al. 2003).

As depicted in Figure 2.1.3, the TGFβ family members, bone morphogenetic proteins (BMP)s, have been shown to play a major role in the recruitment and growth of the ovarian follicle (Knight and Glister 2006). The type 1 TGFβ receptor bone morphogenetic protein receptor (BMPR1B), binds to the BMP ligands 2, 4, 6, 7, and 15,which culminates in gene transcription (Miyazono, et al. 2005). A point mutation of the BMPR1B gene in the Booroola Merino (BB) sheep results in the partial attenuation of the receptor function, and increases

the ovulation rate (Mulsant, et al. 2001, Souza, et al. 2001, Wilson, et al. 2001). In the human model, the ovulation rate is increased during *in vitro* fertilisation (IVF) treatment by the administration of FSH to stimulate the growth of multiple follicles (Edwards, et al. 1996, Edwards and Steptoe 1983). The mode of action of BMPR1B and its interaction with the signalling of follicle stimulating hormone receptor (FSHR) and luteinising hormone receptor (LHR), are therefore central to this thesis (Figure 2.1.3). In addition, programmed cell death or apoptosis forms another mode of regulation of ovulation rate; and the BMPs have also been implicated in the regulation of apoptosis (Haÿ, et al. 2004, Hussein, et al. 2005, Kayamori, et al. 2009, Zhu, et al. 2013).

The effectiveness of the exogenous recombinant DNA follicle stimulating hormone (rFSH) treatment to increase the ovarian response diminishes with age, and declines as the primordial follicle reserve is depleted in the ovary (Yovich, et al. 2012). Patients showing a poor ovarian response to rFSH have previously been shown to have an improved pregnancy rate (Tesarik, et al. 2005, Yovich and Stanger 2010). In the ovary growth hormone receptors (GHR) are found on the theca, granulosa cells, and the oocyte (Abir, et al. 2008b, Carlsson, et al. 1992, Kölle, et al. 1998, Sharara and Nieman 1994, Tamura, et al. 1994). The effect of growth hormone (GH) treatment on the expression of the BMPR1B, FSHR, and LHR is elaborated upon in this thesis.

2.1.1 The ovarian reserve

Oogonia proliferate in the ovary before undergoing meiosis at approximately week 9-11 of gestation in humans (Figure 2.1.1). The oogonia are infiltrated by the somatic cells, forming primordial follicles with a single layer of cells surrounding the oocyte (Pangas 2012). The somatic cells differentiate into granulosa cells, and the oocyte resides in the dictyate-stage of meiotic prophase 1 until the mid-cycle LH surge triggers further development (Edwards, et al. 1996). The decline of the ovarian reserve is well documented, and is related to chronological age (Figure 2.1.1) (Almog, et al. 2011, Hansen, et al. 2011).

The oocyte number increases from 100 to 6,000,000 just before birth, and at birth the number of oocytes has already fallen by 83% (Monniaux, et al. 2014). At puberty the levels of gonadotrophins increase sufficiently to promote primary to tertiary follicles to continue growth, and to resist apoptosis (Figure 2.1.1and Figure 2.1.2) (Matsuda, et al. 2012). Estimates of the number of small antral follicles growing (AFC) or the level of anti-mullerian hormone (AMH) secretion by the small antral follicles is strongly correlated to the ovarian reserve (Hansen, et al. 2011, van Rooij, et al. 2005).

2.1.2 Intra-ovarian regulators of folliculogenesis

Inducement of FSHR and LHR expression appears to be under the control of various intraovarian growth and development regulators as depicted in Figure 2.1.2 and Figure 2.1.3 (Erickson, et al. 1979). BMP ligands, inhibins, activins, and BMP binding proteins have been implicated directly or indirectly, *in vivo*, by experiments that involve treatments, such as ligand infusion and active or passive immunisation (Al-Samerria, et al. 2015, Campbell, et al. 2009, Juengel, et al. 2004, Knight, et al. 2012), and by natural mutations, and knockout gene models in several species (Araújo 2010, Di Pasquale, et al. 2006, Feary, et al. 2007, McNatty, et al. 2007). *In vitro* culture of granulosa and theca cells have provided data on the influence of these growth factors on steroidogenesis and cell proliferation (Brankin, et al. 2005, Campbell, et al. 2006, Glister, et al. 2004b, McNatty, et al. 2009).

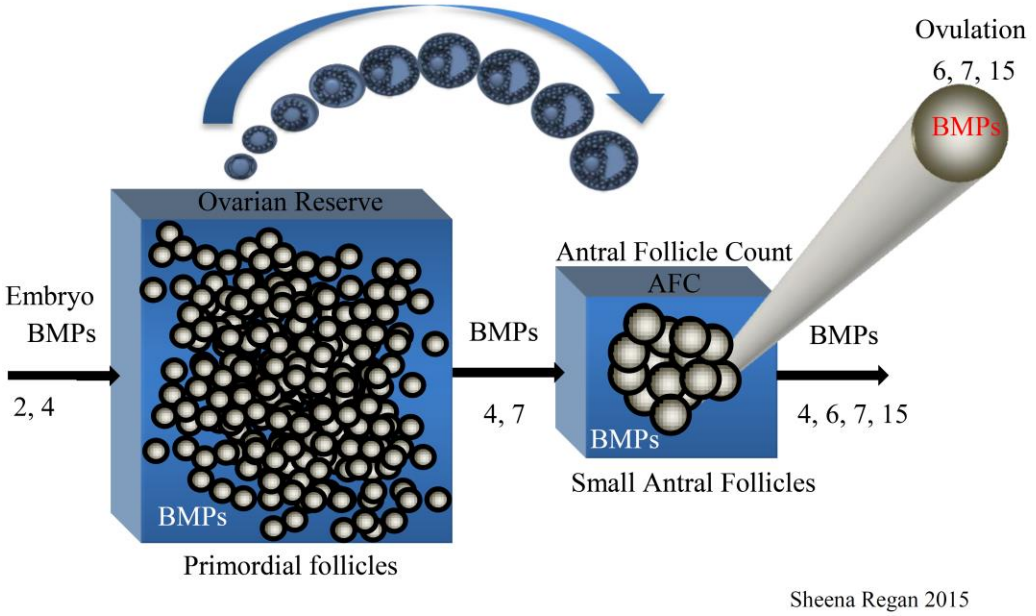


Figure 2.1.4 BMP signalling and the formation of the ovarian reserve through to ovulation.

The involvement of BMP signalling in embryonic ovarian formation of primordial follicles; activation of the primordial to primary follicle; antral follicle formation and recruitment into cyclic folliculogenesis to ovulation.

The ligands that bind with BMPR1B are BMP2, 4, 6, 7, 15, and AMH (Figure 2.1.3) (Mazerbourg and Hsueh 2006). Numerous studies have provided strong evidence that the action of the BMP ligands associated with BMPR1B inhibit progesterone synthesis and promote oestrogen production (Figure 2.1.5 and Figure 2.2.1), and supress early luteinisation of the follicular cells in the rat (Shimasaki, et al. 1999), cow (Glister, et al. 2004b), hamster, mice and sheep (Fabre, et al. 2003, Kayamori, et al. 2009, Young, et al. 2008), and humans

(Shi, et al. 2012, Shi, et al. 2009b, Shi, et al. 2010). In addition, the BMPR1B knockout mice exhibit an increase in the granulosal FSHR expression levels (Edson, et al. 2010).

2.1.3 Hypothalamic-pituitary-ovarian axis

Androgen receptors are first expressed in the transitional follicle between primordial and primary stage, and are early regulators of ovarian development, particularly the inducement of FSHR on the granulosa cell (Figure 2.1.5) (Erickson, et al. 1979, Rice, et al. 2007, Sen and Hammes 2010, Sen, et al. 2014). Granulosa cells are unique because they express FSHRs which are required for the synthesis of oestrogen (Figure 2.2.1) (Miller 2011). Theca cells express LHRs and synthesise androgens which are used by the granulosa cells as substrate for oestrogen synthesis.

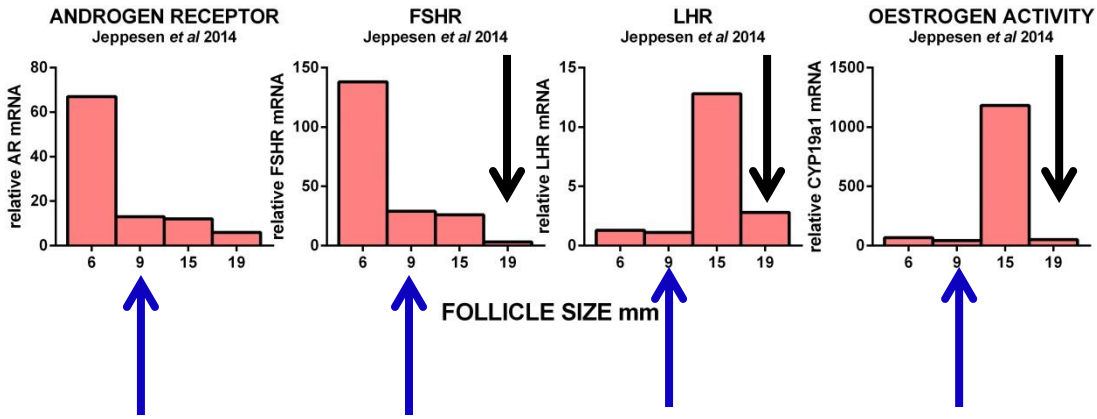


Figure 2.1.5 The stage-specific relationship between granulosal receptor expression and oestrogen activity during folliculogenesis.
Dominant follicle selection takes place when the androgen receptor and FSHR expression decreases, and LHR expression increase (indicated by the blue upwards-arrow). Down-regulation of FSHRs, LHRs and the cessation of proliferation occurs in the pre-ovulatory follicles in humans and animals, (indicated by the black downwards-arrow). CYP19A1 is the gene that encodes aromatase, essential for the production of oestrogen. Based on (Gasperin, et al. 2014, Jeppesen, et al. 2012).

In the small pre-antral follicle, theca cells express LHRs, CYP11A1, and HSD3B1, and have the capacity to produce androstenedione and other androgens prior to granulosal FSHR expression (Sen, et al. 2014). Androstenedione accumulates in the theca cells in preparation for the commencement of steroidogenesis in the granulosa cell (Figure 2.2.1) (Rice, et al. 2007, Sen, et al. 2014). The androstenedione diffuses out of the theca to the adjacent granulosa cells where FSH interaction with FSHR acts via the adenylate cyclase, cyclic adenosine 3´,5´-monophosphate, protein kinase (AC-cAMP-PKA) pathway to stimulate the conversion via CYP19A1 to oestradiol (Figure 2.2.1) (Manna, et al. 2002). Mid-follicular phase (~ day 7) is the time of divergence which results in dominant follicle selection (Figure

2.1.5) (Mihm and Evans 2008a). Granulosal FSHRs induce LHRs on small antral recruited follicles (Mihm, et al. 2006, Minegishi, et al. 1997, Yung, et al. 2014).

As pituitary FSH secretion is reduced, the follicles with granulosal LHRs have the capacity to supplement the FSH-dependent synthesis of oestrogen. The follicle(s) with granulosal-expressed LHR continue to grow and become the selected dominant follicle(s) (Figure 2.1.2). The extent of androstenedione conversion to oestrogen continues to increase, which creates a positive oestrogen feedback loop to the hypothalamic-pituitary complex leading to further GnRH and LH release, (Figure 2.2.1) (Faure, et al. 2005). Proliferation of the granulosa and theca cells continues as the rise in oestrogen level promotes proliferation until a threshold level is reached which culminates in the generation of the ovulation-induced LH surge (Figure 2.2.1) (Austin, et al. 2001, Ginther, et al. 2005). In the event that reduced conversion of androstenedione to oestrogen occurs, androgen levels rise creating an androgen dominant follicle. Greater androstenedione to oestrogen ratios have been shown to result in an elevated level of granulosa cell apoptosis and follicle demise (Yuan and Giudice 1997).

2.2 Ovulation rate

The ability of the follicle to reach the FSH-oestrogen threshold and establish sufficient granulosal LHR mid-cycle, appears to be of paramount importance to the survival of the selected dominant follicle; alternatively, follicular regression proceeds, followed by atresia, (Figure 2.1.2) (Campbell, et al. 1999, Ginther, et al. 2012, Luo, et al. 2011, Picton and McNeilly 1991). The BMP ligands are well established as regulators of granulosa cell proliferation in sheep (Chen, et al. 2008, Fabre, et al. 2003, Kayamori, et al. 2009, Mulsant, et al. 2001, Young, et al. 2008), cows (Austin, et al. 2001, Glister, et al. 2004b, Kayamori, et al. 2009, Kayani, et al. 2009, Knight and Glister 2003), and in humans (Di Pasquale, et al. 2006, Dooley, et al. 2000, Shi, et al. 2012, Shi, et al. 2009b, Shi, et al. 2010, Shimasaki, et al. 1999).

The time of follicle divergence occurs when pituitary FSH secretion reduces and LH secretion increases (Figure 2.1.2) (Austin, et al. 2001, Edwards, et al. 1996). During a natural cycle, the follicles with a higher density of receptors are presumed to be more responsive to the gonadotrophins, and continue to increase in size (Bächler, et al. 2014, Gougeon 1986, LaPolt, et al. 1992). The number of pre-ovulatory follicles stimulated can be artificially enhanced by exogenous rFSH stimulation or by a naturally occurring mutation-induced increase in responsiveness, such as that seen in the Booroola Merino sheep, (Mulsant, et al. 2001, Souza, et al. 2001, Wilson, et al. 2001).

The suppression of granulosal progesterone synthesis in favour of oestrogen, appears to be governed by the action of the BMP growth factors from the TGFβ superfamily (Knight and Glister 2006, Moore, et al. 2001b). The BMP ligands which strongly activate the BMPR1B (Miyazono, et al. 2010), and the role of BMPs in the regulation of gonadotrophin receptor expression has been previously reported (Figure 2.1.4) (Shi, et al. 2012, Shi, et al. 2011, Shi, et al. 2010, Zhu 2013).

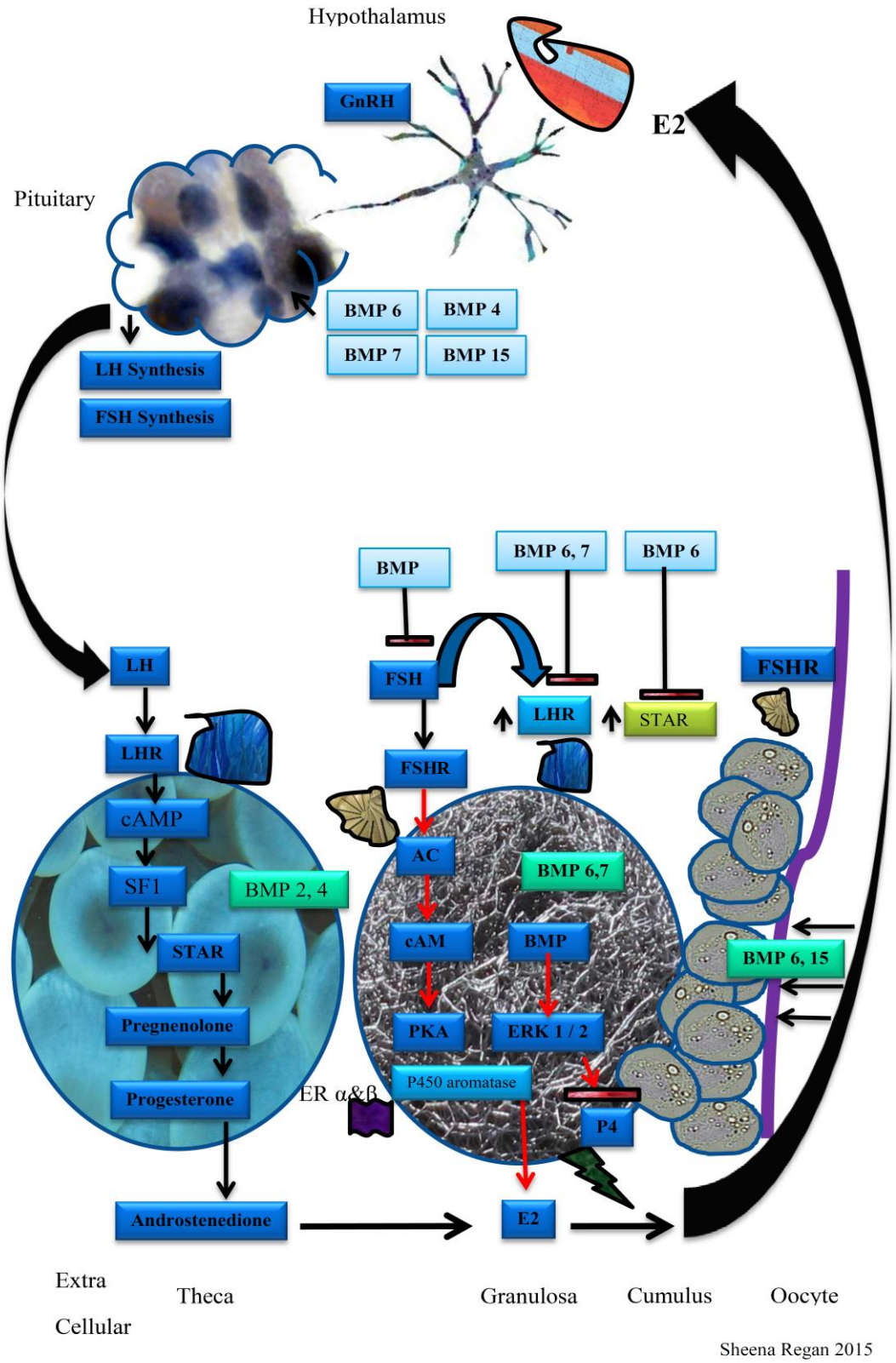


Figure 2.2.1 Hypothalamic-pituitary ovarian axis of regulation, and the granulosa and theca cell interaction.

Based on (Bao, et al. 1997, Chen, et al. 2008, Dijke, et al. 2003, Feary, et al. 2007, Fitzpatrick, et al. 1997, Hillier, et al. 1994a, Hussein, et al. 2005, Kayani, et al. 2009, Miller and Bose 2011, Miyazono, et al. 2005, Miyoshi, et al. 2007, Moore, et al. 2001b, Nicol, et al. 2008, Pierre, et al. 2004, Rice, et al. 2007, Seger, et al. 2001, Sugawara, et al. 2000, Sullivan, et al. 1999, Takeda, et al. 2012, ten Dijke, et al. 2003, Yamamoto, et al. 2002, Yuan 1998)

2.2.1 LH surge and the role of the BMPs

The BMP ligands, 2, 4, 6, 7, and 15 form a receptor-ligand complex with the BMPR1B, and recruit the type 2 TGFβ receptor BMPR2 (Miyazono, et al. 2010). The complex initiates phosphorylation of the intracellular substrate molecules, receptor-regulated c. elegans protein mothers against decapentaplegic (Smad)s. The Smad forms a complex with a common mediator, Smad 4, and translocates to the nucleus where transcription of genes required by the cell takes place (Moore, et al. 2003). Smad signalling is modulated by repressor and activator molecules in the nucleus, cytoplasm and in the extracellular matrix. Alternatively, BMPs can activate the non-Smad pathway, mitogen activated protein kinases (MAPK) such as ERK 1 and ERK 2 (Inagaki, et al. 2009). The cross-talk between the Smads and the extracellular signal regulated kinases 1 and 2 (ERK 1/2) signalling, adds another dimension to the complex signalling which regulates folliculogenesis (Tajima, et al. 2003). Specifically BMP15 increases proliferation of granulosa cells and cytodifferentiation, independent of FSH, yet BMP15 supresses FSH-induced synthesis by reducing steroid acute regulatory protein (StAR) and P450 side chain cleavage enzyme (CYP11A1) in the rat (Otsuka, et al. 2001b). BMP6 also inhibited progesterone synthesis; however, the biological activity in granulosa cells was directed downstream of the FSHR without affecting the synthesis of oestrogen in the rat (Otsuka, et al. 2001a).

In the ovary, granulosa cell signalling induced by the gonadotrophins, FSH and LH, activate the FSHR or LHR and stimulate cAMP-PKA which increases the P450 aromatase (CYP191A) aromatase to facilitate oestrogen synthesis (Figure 2.1.3 and Figure 2.2.1). Progesterone synthesis is inhibited by BMP4 the suppression of StAR (Pierre, et al. 2004, Tajima, et al. 2003, Val, et al. 2003) which is essential for progesterone synthesis in the granulosa cell (Figure 2.2.1) (Moore, et al. 2001b). Alternatively, or in addition, BMPs inhibit ERK 1/2 signalling providing inhibitory control over the balance of progesterone and oestrogen (Figure 2.2.1) (Miyoshi, et al. 2007, Nakamura, et al. 2012, Ogura Nose, et al. 2012).

BMP signalling activity has been established not only in the ovary but also in the pituitary where ERK and janus nuclease kinase (JNK) signalling have been shown to increase LHβ (Harris, et al. 2002), and BMP15 via ERK to increase FSHβ (Figure 2.2.1) (Otsuka and Shimasaki 2002). In addition, BMP6 and 7 were shown to regulate FSHβ expression (Takeda, et al. 2012). Given the above regulatory evidence, it is not surprising that the spatial and temporal relationship of BMP expression patterns change with an increase in follicular size (Otsuka 2010).

There are few previously published studies that cover the changing follicular gene expression pattern during folliculogenesis as the ovarian reserve is depleted. In particular, the common practice is to pool the follicles into size ranges, a practice which fails to recognise the sometimes subtle change over time, or the dramatic fall in gene expression as the follicle matures. The LH surge is characterised by a dramatic shift from proliferation to differentiation and maturation of the follicle prior to ovulation (Peng, et al. 1991b, Richards, et al. 1998). The LH surge initiates irreversible morphological changes to the granulosa cells in preparation for the shift from oestrogen production to progesterone production (Byrne, et al. 1995, Micevych, et al. 2003). The BMPs have been described as locally produced inhibitors of follicle luteinisation, however the exact characterisation of the biological component that reduces pituitary responsiveness to GnRH has not been identified (Dimitraki, et al. 2014, Kita, et al. 1994, Messinis, et al. 1991). The gonadotrophin surge attenuating factor (GnSAF) is one possible candidate. GnSAF bioactivity is measured indirectly by its ability to attenuate the LH secretory response of pituitary cells to GnRH (Fowler, et al. 2001). Evidently, the GnSAF is characterised as being produced by the cohort of recruited small antral follicles in the ovary. It has been proposed that these small follicles regulate folliculogenesis by desensitising the pituitary to suppress LH secretion until mid-cycle (Dimitraki, et al. 2014, Fowler, et al. 2001).

GnSAF bioactivity in follicular fluid has been shown to be greater in patients receiving IVF treatment than during natural cycles (Fowler, et al. 2001); and the effect of a declining ovarian reserve with age, results in reduced GnSAF bioactivity (Martinez, et al. 2002). Previously, an inverse relationship between follicle size and GnSAF was demonstrated (Figure 2.1.2) (Fowler, et al. 2001). In the largest human follicles of 25 mm, GnRH-induced LH secretion was severely reduced compared to the 5-8 mm follicles (Fowler, et al. 2001).

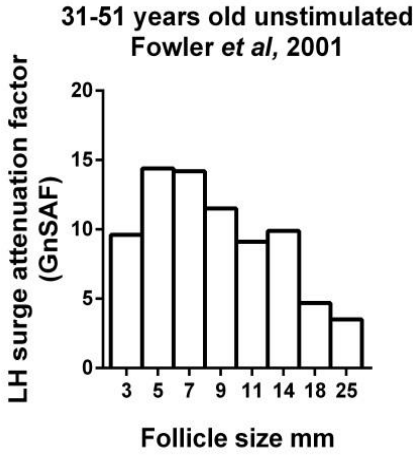


Figure 2.2.2 LH surge attenuation factor (GnSAF) during folliculogenesis in a natural cycle. Modified

In a later study, the level of GnSAF was measured at two time points corresponding to the time of dominant follicle selection and at the time of human chorionic gonadotrophin (hCG) injection or the equivalent of the largest follicle,19-24 mm (Martinez, et al. 2002). The good ovarian response younger patients had high levels of GnSAF at the time of dominant follicle selection that reduced dramatically at the end of folliculogenesis (Figure 2.2.3). The opposite was true for the poor ovarian response older patients of the study (Figure 2.2.3). The comparatively higher level of GnSAF would be accounted for by the increase in the number of follicles producing the attenuating substance, which was greater in the younger patients compared to the older.

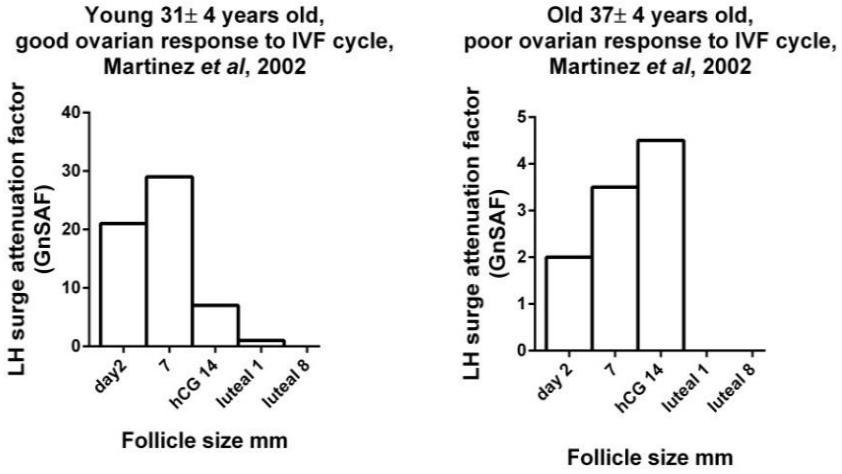


Figure 2.2.3 A comparison of the LH surge attenuation factor (GnSAF) during folliculogenesis in young and older IVF patients.
The ovarian response to rFSH was determined by the number of growing large antral follicles.Modified

It was concluded that the bioactivity of the GnSAF has an endocrine role to play in preventing the premature onset of the LH surge; which would accord with the local intraovarian role of BMPs as an inhibitor of luteinisation (Otsuka 2010, Shimasaki, et al. 1999). The dysregulation of GnSAF or BMP signalling would impede the process of luteinisation and prevent maturation of the follicle (Gordon, et al. 2008).

2.3 LHR expression and the LH surge

LHRs are acquired at the time of dominant follicle selection in granulosa cells, in response to the increased density of FSHRs and the stimulation of oestrogen production (Xu, et al. 1995, Yung, et al. 2014). The level of granulosal LHR in a natural cycle peaks as the estrogen level reaches a threshold mid-cycle before the LH surge (Peng, et al. 1991b). The LH surge induced by the positive feedback action of oestradiol, causes degradation of the *LHR* mRNA, and the expression of the LHRs is reduced dramatically (Lu, et al. 1993, Minegishi, et al.

1997, Nair, et al. 2006). The granulosal LHR expression returns to normal shortly after the end of the LH surge, and rises in the luteal phase (Micevych, et al. 2003).

Clinical administration of hCG or the natural cycle LH surge, induces cytoskeletal reorganisation of the granulosa cell, cessation of mitogenic proliferation, cumulus expansion, gap junction closure, resumption of meiosis, and maturation of the oocyte (Fan, et al. 2009, Izadyar, et al. 1998b). These changes have an effect on the *LHR* mRNA or mature LHR protein density (LaPolt and Lu 2001, Lapolt, et al. 1990, Peng, et al. 1991b). The down-regulation of granulosal LHRs in the ovulating follicle has been previously reported in the bovine and human (Jeppesen, et al. 2012, Ndiaye, et al. 2005, Ophir, et al. 2014, Xu, et al. 1995); however, conflicting reports exist (Irving-Rodgers, et al. 2009, Jakimiuk, et al. 2001, Maman, et al. 2012). When comparing reported levels of receptor density, it is important to consider whether or not exogenous IVF stimulation was administered, and if the follicles had been exposed to a LH/hCG surge. In addition, pooling of follicles would potentially mask the down-regulation by averaging out the LHR density, and thereby reducing the accuracy of the data.

2.3.1 The LH surge and the cumulus granulosa cells

Cumulus cells experience high levels of oocyte secreted BMPs in a concentration gradient radiating outwards from the oocyte, supressing luteinisation and apoptosis, and promoting cell proliferation (Hussein, et al. 2005). At the time of antral formation the granulosa cells closest to the oocyte differentiate into cumulus cells. The cumulus cells have very low levels of LHR, progesterone receptor, and have low EGF concentrations in the follicular phase (Shimada, et al. 2003, Shimada and Terada 2002, Shimada, et al. 2004). Cumulus cell gap junctions are disrupted, severing the influence of the oocyte secreted growth factors such as BMP15 and 6 (Sutovský, et al. 1993). Just before the LH surge, changes in EGF and progesterone receptor expression increase (Prochazka, et al. 1998), resulting in numerous changes to the cumulus cells; one of which is the expression of LHR, and results in the cessation of mitogenic proliferation (Shimada, et al. 2003, Shimada and Terada 2002).

2.3.2 BMP signalling and reproduction.

The body of work investigating the role of BMPs is extensive, and ranges from studies on primordial germ cell migration to the inducement of ovulation and corpus luteum formation (Erickson and Shimasaki 2001, 2003, Knight and Glister 2006, Miyazono, et al. 2010, Otsuka 2013, Pangas 2012, Shimasaki, et al. 2004). Several research models exist; the lower order species; Drosophila and zebra fish; poly-ovulatory rodents; mono-ovulatory animals, and human models. The ability to create gene knockout models in mice and to use natural and created mutations or specific cell lines add to the research tool box. Furthermore, the *in vivo*

and *in vitro* infusion of growth factors and the blocking of receptors and signalling pathways provide other methods of examination available to researchers. In human studies, *in vivo* and *in vitro* research has progressed substantially with the rise of ART centres, providing an unobtrusive source of follicular material. Non-pathogenic human ovarian tissue, free from exogenous gonadotrophin stimulation, is rare, and is therefore infrequently used in research (Bomsel Helmreich, et al. 1979, Fowler, et al. 2001, Garcia, et al. 1981, Gougeon 1986, Klein, et al. 2000, MacNaughton, et al. 1992). Given the stage-specific nature of ovarian regulation and species peculiarities, caution should be used when interpreting results (Erickson and Shimasaki 2003, Otsuka 2010). In addition, the complex interaction between growth factors and the hypothalamic-ovarian axis of control, impedes research of specific growth factors or the cellular regulatory mechanisms involved in ovarian function (Zeleznik 2001).

2.4 BMP and BMP receptor expression patterns within the ovary

The TGFβ type 1 BMPR1B has been localised on sheep granulosa cells from the primordial follicle onwards (Al-Samerria and Almahbobi 2014 , Chen, et al. 2008, Erickson and Shimasaki 2003, Estienne, et al. 2015, Gasperin, et al. 2014). The level of expression increased sequentially from primordial to antral follicles in sheep. BMPR1B is expressed mainly on granulosa cells and the oocyte in bovines, and to a lesser degree in their theca cells (Glister, et al. 2004a). It has also been demonstrated that BMP ligands are produced in a stage-specific manner by follicular cells in animals and humans (Gasperin, et al. 2014, Glister, et al. 2004a, Regan, et al. 2015).

BMP2 is mainly produced by the granulosa cells in the first half of follicle development (Pangas 2012). BMP2 has a low affinity with BMPR1B, and binds preferentially with another type 1 TGFβ receptor, BMPR1A (Miyazono, et al. 2010). BMP2 is involved in oocyte endowment, primordial pool assembly, and activation of primordial follicles (Lawson, et al. 1999, Ying and Zhao 2001).

BMP4 is produced by the theca and granulosa cells in the bovine and human model (Glister, et al. 2004b, Khalaf, et al. 2013). BMP4 has been shown to bind precociously (Fabre, et al. 2006) with BMPR1A, BMPR1B, ACVR1, and ACVR1B in sheep, signalling via the Smad intermediate molecules to modulate ovarian function. In addition, BMP4 is reported to mediate cell function via alternative signalling pathways involving MARK family, in particular ERK1/2. In the absence and presence of FSH, BMP4 inhibits progesterone production in granulosa cells by negatively influencing the downstream gene synthesis from FSH to cAMP, StAR, and CYP11A1 in small antral follicles (Fabre, et al. 2003, Mulsant, et

al. 2001). In contrast, large antral follicles are not responsive to BMP4 ligand-induced progesterone secretion or mitogenic growth, which indicates that BMP4 may be more involved in the regulation up to dominant follicle selection, (Fabre, et al. 2006, Tanwar and McFarlane 2011).

FSH-induced progesterone biosynthesis was inhibited by BMP4, while it was shown to enhance FSH-induced oestrogen production in the rat (Shimasaki, et al. 1999), and in sheep (Fabre, et al. 2003, Mulsant, et al. 2001). In addition, in sheep it was revealed that BMP4 via BMPR1B signalling activated Smad 1 to inhibit *StAR* and *CYP11A1* gene expression to inhibit progesterone synthesis. BMP4 also inhibited granulosa cell luteinisation by reducing SF-1 transcriptional activity on LHβ promoter (Pierre, et al. 2004). SF-1 is a key activator of steroidogenic endocrine function, and BMP4 and SF-1 are also found in gonadotrope cells that produce LH in the anterior pituitary (Ingraham, et al. 1994, Val, et al. 2003). The direct link between BMP4 and LH synthesis via the BMPR1B signalling phosphorylation of Smad 1, provides an explanation for the increase in LHR as the BMPs decline during folliculogenesis that releases the inhibitory effect (Figure 2.1.5) (Nicol, et al. 2008). BMP4 inhibits ovine pituitary *FSHβ* expression and the concentration of FSH (Faure, et al. 2005). BMP4 is also involved in oocyte endowment, primordial pool assembly, and activation to primary follicles (Lawson, et al. 1999, Ying and Zhao 2001).

BMP7 is consistently produced in bovine theca cells, with a small amount being produced in human granulosa cells (Glister, et al. 2004a, Khalaf, et al. 2013). BMP7 has a similar biological effect on the granulosal proliferation and suppression of FSH-induced progesterone biosynthesis as BMP4 ,6 and 15 in the rat by increasing FSH-FSHR induced *CYP19A1* expression (Lee, et al. 2001, Shimasaki, et al. 1999). In the monovulatory species the regulation was inconsistent; in the bovine the FSH-induced hormone secretion was not altered by BMP7; yet, in the goat BMP7 increased *FSHR* and decreased *LHR* mRNA expression; whereas, BMP7 increased *FSHR* expression in humans (Shi, et al. 2010, Zhu 2013). Differences in response are likely to be due to granulosa cells either being luteinised, caused by the addition of serum during culture, or the granulosa cells being collected after the LH surge in humans.

BMP6 is produced in both granulosa cells and the oocyte in humans and other animals (Glister, et al. 2004b, Khalaf, et al. 2013, Wu, et al. 2007). BMP6 is involved in proliferation, steroidogenesis and cytodifferentiation of granulosa cells in a species specific manner in the ovary. In addition, BMP6 inhibits pituitary secreted FSHβ and the concentration of FSH in sheep (Faure, et al. 2005). In the goat, BMP6 did not increase granulosal *FSHR*, yet it had a

dramatic increasing effect on *LHR* (Zhu 2013). In humans, BMP6 increased *FSHR* mRNA whereas, in the rat its effect was downstream of the FSHR (Ogura Nose, et al. 2012, Otsuka, et al. 2001a). BMP6 has been shown to increase proliferation of granulosa cells, compared to BMP2 or BMP4, in cultured sheep granulosa cells. In the rat, however BMP6 had no effect on granulosa cell proliferation (Campbell, et al. 2006, Otsuka, et al. 2001a).

BMP15 is exclusively secreted by the oocyte and has a strong affinity for BMPR1B in sheep and humans, yet bind precociously in mice, indicating that substantial species differences exist (Inagaki and Shimasaki 2010, Pulkki, et al. 2012). In primary follicles, the expression of *BMP15* mRNA progressively increased from primary follicles, peaking in sheep early antral follicles, followed by a sequential decrease in the antral follicle (Feary, et al. 2007). In humans, BMP15 is secreted across a concentration gradient from the oocyte via the cumulus cells to the granulosa cells and has been shown to suppress apoptosis (Hussein, et al. 2005). Oocytes surrounded by cumulus cells with greater levels of *BMP15* mRNA were shown to have an increased pregnancy rate after IVF (Li, et al. 2014) and reduced apoptosis (Hussein, et al. 2005). Moreover, an association between high levels of BMP15 in the follicular fluid and oocyte quality has been reported (Li, et al. 2014, Wu, et al. 2007).

Ovulation rate may be associated with a reported increase in BMP15 in the Booroola mutation (Crawford, et al. 2011, Feary, et al. 2007). Furthermore, in the pituitary, BMP15 has been associated with increasing FSHβ expression (Otsuka and Shimasaki 2002). It has also been shown that BMP15 decreased FSH-induced progesterone biosynthesis by decreasing *FSHR* mRNA, ultimately inhibiting luteinisation. (Moore, et al. 2003, Otsuka and Shimasaki 2002). In parallel, BMP15 promoted granulosa cell mitosis independent of FSHR-Smad 1,5,8 pathway via activation of the ERK 1/2 pathway (Lee, et al. 2001, Moore, et al. 2003, Otsuka, et al. 2001b, Shimasaki, et al. 1999).

AMH and BMPs belong to the TGFβ family, and have an affinity to bind to both BMPR1B and BMPR1A. AMH therefore, may be involved in the complex interaction of cellular regulation of folliculogenesis in the Booroola. AMH peaks in the small antral follicles, followed by a decrease at the time of dominant follicle selection (Rice, et al. 2007). In sheep and humans, AMH is not present in granulosa cells from large antral follicles; however, it is present in the cumulus cells (Campbell, et al. 2012, Weenen, et al. 2004). AMH has been shown to be inhibitory to primordial follicle activation, and declines with a decrease in activin and FSH after dominant follicle selection (Rice, et al. 2007).

When AMH is blocked directly, the ovulation rate was shown to increase, whereas mitogenic activity of granulosa cells remained the same (Campbell, et al. 2012). AMH attenuation has, therefore, been identified as a possible contributor to the observed increase in ovulation rate of the mutation-affected Booroola sheep. When AMH binds to a specific type 2 TGFβ receptor, AMHR2 and the type 1 receptor, BMPR1B, phosphorylation of the Smad 1,5,8 pathway takes place and results in gene transcription which regulates folliculogenesis (Josso and Clemente 2003). AMHR2 has been identified in the transitional follicles between primary and secondary follicles, but not in primordial follicles (Rice, et al. 2007). Recently, the link between the production of AMH and BMP has been made in sheep follicles, whereby BMP4, 6, and 15 increased the transcriptional activity of the AMH promotor activity via SF-1 (Estienne, et al. 2015). In addition, human granulosa cells cultured with BMP6 showed increased *AMH* mRNA (Shi, et al. 2009b); and AMH induced the BMP-specific inhibitory Smad 6, and induced the Smad 1,5,8 pathway to activate BMP specific reporter genes (Josso and Clemente 2003).

The interaction between BMP and AMH indicates a strong connection between the role of the BMPs and AMH, and the gonadotrophin-dependent regulation of ovulation rate. It still remains unclear as to why the immunisation against AMH increased ovulation rate but did not reduce proliferation of granulosa cells, as seen with the Booroola mutation. A possible explanation may be related to the stage-specific down-regulation of AMH after dominant follicle selection. The greatest mitogenic activity of granulosa cells occurs after divergence when AMH is low, whereas dominant follicle selection occurs when AMH is normally peaking (Austin, et al. 2001). Immunisation against AMH alone would therefore, increase ovulation rate but not granulosa cell proliferation. Immunisation against BMPR1B or an attenuating mutation such as the Booroola, would affect both proliferation and ovulation rate.

2.4.1 Conclusion: BMP

Substantial research has provided evidence of the contribution several BMP ligands provide in the cellular regulation of folliculogenesis in a variety of models. The overwhelming position is that the BMPs have a significant role in folliculogenesis to inhibit the onset of the LH surge from the anterior pituitary, and to promote proliferation of the follicular cells. In addition, BMPs inhibit cytomorphological changes, and steroidogenic differentiation of the granulosa cells into granulosa-lutein cells, thus acting as luteinisation inhibitors.

2.5 Booroola Merino Sheep and Ovulation Rate

The Booroola Merino (Figure 2.5.1) with a naturally occurring point mutation of the BMPR1B mutation has an increased ovulation rate (Fabre, et al. 2006, Mulsant, et al. 2001, Souza, et al. 2001, Wilson, et al. 2001). The response of both the ovary and the pituitary to

gonadotrophic stimulation appears responsible for the increased ovulation rate of the Booroola mutation (Campbell, et al. 2003, Fry, et al. 1988, Hampton, et al. 2004, Hudson, et al. 1999). In the past, it has been recognised that the increase in ovulation rate in the Booroola is due to the follicles being more sensitive to FSH at an earlier follicle size (Baird and Campbell 1998, McNatty, et al. 1985). BMPR1B is the common receptor for several BMP ligands that form a receptor ligand complex and activate either the Smad signalling pathway or an alternative non-Smad pathway such as the MAPK pathways (Kang 2001, Miyazono, et al. 2010).

2.5.1 BMPs ligands and the Booroola mutation

In support of the dynamic role of BMPs, it has also been shown that BMP6 reduces post-dominant follicle selection in the rat, and is reduced completely in the ovulating dominant follicle (Erickson and Shimasaki 2003). Similarly, BMP15 and BMPR1B expression in the oocyte were down-regulated prior to ovulation in sheep (Feary, et al. 2007). In cultured rat granulosa cells, BMP15 reduced the expression of *FSHR* mRNA (Otsuka, et al. 2001b). In the goat and human, BMP2, 4, and 7 ligands have been shown to increase the level of *FSHR* mRNA and BMP6 increased *LHR* mRNA, which indicates a direct role in the regulation of receptor density (Ogura Nose, et al. 2012, Zhu, et al. 2013). It is possible that the attenuating mutation of the BMPR1B signal may therefore, reduce the suppression, and lead to an up-regulation of FSHR in the Booroola, increasing the ovulation rate (Otsuka, et al. 2001c, Regan, et al. 2015).

Figure 2.5.1 Wild type Merino sheep, Armidale NSW.

2.5.2 Booroola Mutation and the LHR

The process of luteinisation appears to rely on LHR down-regulation in the leading dominant follicles, (Figure 2.7.1) (Fan, et al. 2009, Izadyar, et al. 1998b, Regan, et al. 2015). Furthermore, LHR density peaks in the pre-ovulatory follicle in the wild type (WT) and the Booroola, followed by a significant reduction in the leading dominant follicle during the LH surge which was also observe in human and rodent studies (Jeppesen, et al. 2012, LaPolt, et al. 1992, Ophir, et al. 2014, Regan, et al. 2015).

2.5.3 Booroola mutation and follicle size and granulosa cell number

The Booroola sheep follicles contained significantly fewer granulosa cells than the normal wild type (Campbell, et al. 2006, McNatty, et al. 1985). Studies conducted on the granulosa cells show that when stimulated *in vitro* with LH or FSH they produced more cAMP and oestrogen, from the same number of cells from the large antral follicle (Campbell, et al. 2006). An increased capacity to deliver oestrogen would compensate for the reduced number of granulosa cells; however, the cellular mechanism has not been identified. In addition, the Booroola granulosa cells were reported to be more responsive to the BMP ligands than the wild type (Campbell, et al. 2006). Taken together, it is apparent that the multiple follicles are more responsive to gonadotrophin stimulation, yet they have reduced follicle cell number, which is attributed to the attenuated signalling of BMPR1B.

2.5.4 Immunisation Sheep and Ovulation Rate

In sheep, short term immunisation against BMP15 increased the ovulation rate from 1-2 to ≥3 without affecting plasma progesterone concentration. (Juengel, et al. 2004, Juengel, et al. 2011). In another study, complete neutralisation of BMP15 prevented exogenous FSH induced rescue that resulted in anovulation, which indicates that BMP15 is required for *FSHR* transcription (McNatty, et al. 2009). Sheep infused with BMP6 showed a reduced cycle length and size of the pre-ovulatory follicles (Campbell, et al. 2009). Although the effect of the infusion was short-lived the oestrogen and androstenedione increased with no change to the ovulation rate. In the Booroola, a combination of BMP15 and BMP6 attenuation may be responsible for the reduced mitogenic activity and the increased ovulation rate.

2.5.5 A BMP15-specific Mutation, Inverdale

BMP15 has been associated with an increase in ovulation rate in sheep with specific mutations (Hanrahan, et al. 2004, McNatty, et al. 2009). Heterozygous Inverdale sheep with an inactivation mutation for BMP15 exhibit an increase in ovulation rate, whereas, the homozygous did not progress past primary follicle growth (Braw-Tal, et al. 1993, McNatty, et al. 2009).

2.6 Ovarian Ageing

In human subjects a decline in the ovarian reserve of primordial follicles leads to a relative dysregulation of folliculogenesis and ovulation. The change to the micro-environment of the ovary and circulating hormone levels are responsible for the reduced fertility and a reduction in the quality of the ovulating oocyte (Klein N A, et al. 1996). Older patients typically have increased circulating FSH and reduced inhibin B, which gives rise to accelerated early follicle development and results in a shorter cycle length (MacNaughton, et al. 1992, Robertson 2009, Santoro, et al. 2003, Seifer, et al. 1999). In poor ovarian reserve patients, the later stages of follicular growth have a reduced rate of growth which reduces maturation of the follicle and oocyte (Santoro, et al. 2003). The granulosa cell proliferation was found to be reduced and the size of the follicle to be smaller in older patients (Bomsel Helmreich, et al. 1979, Robertson 2009, Seifer 1996 , Seifer, et al. 1993).

The follicle increases its capacity to produce oestrogen by increasing the number of granulosa cells, by increasing the size of the follicle and not the thickness of the granulosa layer (Bächler, et al. 2014). A shorter cycle and a reduction in the follicle size due to ovarian ageing would reduce the capacity of the follicle to produce sufficient oestrogen to down-regulate pituitary FSH during dominant follicle selection. Potentially, reduced oestradiol output could also disrupt the positive feedback mechanism responsible for generating the pre-ovulatory LH surge. Over thickening of the granulosa layer may also compromise oocyte quality if the diffusion rate changes the concentration gradient of serum to follicular fluid, FSH and LH (Bächler, et al. 2014). The lower density of the receptors have also been implicated in the reduced fertility, with a decline in ovarian reserve (Bächler, et al. 2014). In addition, it remains to be confirmed if the elevated FSH from the previous cycle causes early growth of the small antral follicles in the following cycle (Bomsel Helmreich, et al. 1979, Robertson 2009).

In several studies in humans the magnitude of the LH surge has not been found to be significantly different (Klein N A, et al. 1996, Leach, et al. 1997, MacNaughton, et al. 1992, Pal, et al. 2010, Santoro, et al. 2003). Several inconsistencies exist in these studies, centring on the classification of old and poor ovarian reserve based on day three FSH level; or when the LH level of the follicular phase was recorded (on day 2 and 7). Convincing data from another study showed that the amplitude of the LH surge in the mare, is reduced with age (Ginther, et al. 2008). In the oldest mares the largest follicle was reduced in size and fewer follicles were present.

2.7 Ovarian Apoptosis

The ovulation rate is generally governed by the number of follicles growing during a cycle, (Figure 2.1.2). Follicle growth is limited by the number of activated primordial follicles and the stimulation to continue growth to ovulation. Gonadotrophins FSH and LH govern the growth rates of the follicles particularly during the later stages of folliculogenesis. The level of gonadotrophin receptor density controls the response of the follicles to the stimulation from the anterior pituitary. Intraovarian growth factors, such as the BMPs, activins and inhibin, impact the receptor expression that ultimately controls the growth rate of the follicle. Reduced growth leads to an accumulation of precursor molecules (e.g. androgens) that can stimulate signalling pathways to induce programmed cell death or apoptosis. Apoptosis of granulosa cells with low responsiveness to FSH (within subordinate follicles) establishes the dominant follicle that continues to grow to ovulation (Yuan and Giudice 1997).

2.7.1 Necrosis and Apoptosis

Necrosis and apoptosis both culminate in cell death of the granulosa cell and nucleic dyes are commonly used to indicate the vitality of the cell (Figure 2.7.1) (Demchenko 2013). Necrosis results from a toxic or destroying exposure that causes swelling and disruption to the cell's organelles that results in an irreversible breakdown of the cell's membranes and the scattering of the cytoplasmic and nucleic contents (Bonfoco, et al. 1995). Apoptosis however, is caused by several distinctive signalling pathways which culminates in shrinkage of the cell, cytoplasmic blebbing, and compartmentalisation of organelles (Figure 2.7.2) (Amsterdam, et al. 1997, Bomsel-Helmreich, et al. 1979, Nottola, et al. 2006, Tilly 1992). The DNA condenses and genomic fragmentation evidenced by DNA laddering is typical (Guraya 1971). Although the cell membranes lose integrity they fold and encapsulate to prevent the contents from affecting neighbouring cells, and reorganisation of lipid/protein storage clustered around the nucleus (Figure 2.7.2) (Nottola, et al. 2006).

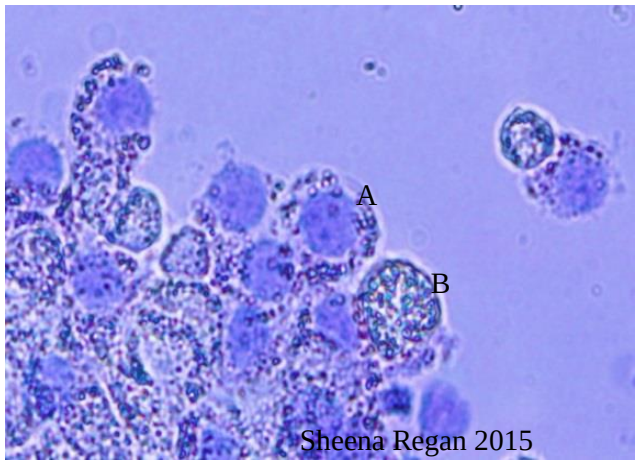


Figure 2.7.1 Isolated granulosa cells and cell membrane integrity.

Trypan Blue indication of membrane breakdown in A, and exclusion of the dye in B.

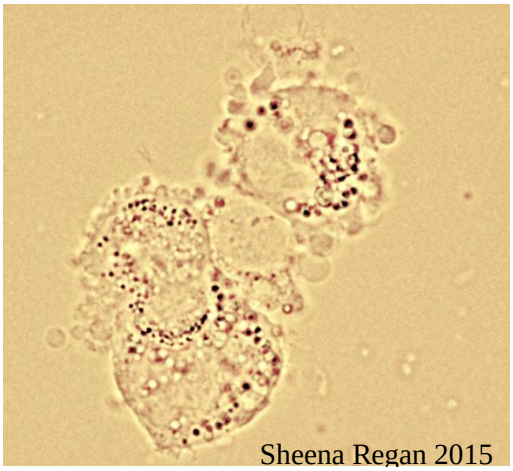


Figure 2.7.2 Granulosa cell, peri-luteal phase, showing extrusion of cytoplasm during apoptosis, and reorganisation of lipid/protein storage clustered around the nucleus.

2.7.2 Primordial Follicle Apoptosis

Initial recruitment or activation of the primordial follicles results in the progression from primary to secondary, to the pre-antral stage, (Figure 2.7.3). The formation of the fluid filled space signifies the development of the small antral follicle (Rodgers and Irving-Rodgers 2010a). The cause of the extreme loss of primordial follicles during gestation to puberty remains controversial (Figure 2.1.1). Furthermore, apoptosis was not found in human primordial, primary and secondary follicles (Figure 2.7.3 12) (Abir, et al. 2002, Albamonte, et al. 2013, Yuan and Giudice 1997). However, secondary follicles just before antral follicle formation were found to exhibit apoptosis, (within the limits of the assay) (Negoescu, et al. 1996, Yuan and Giudice 1997)). In contrast, ovarian foetal germ cells, in mice, underwent BAX-induced apoptosis regulated by aromatic hydrocarbon receptor transcription factor (AHR) (Matikainen, et al. 2002).

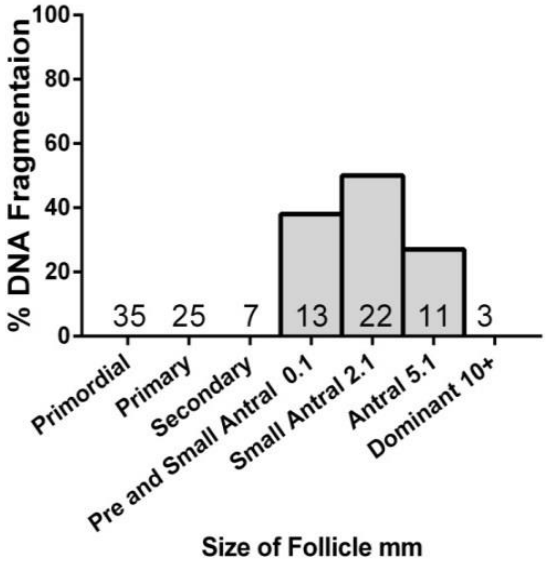


Figure 2.7.3 The level of apoptosis during follicle development
Modified based on Yuan and Giudice, 1997.

Apoptosis in the bovine ovarian follicle can be divided into two phenotypes with potentially different mechanisms of initiation and regulation (Irving-Rodgers, et al. 2003). The first, antral atresia, affects the antral granulosa cells, and progresses from the antrum towards the basal lamina. The second, basal atresia, occurs in the cells closest to the basal lamina membrane of small antral follicles. The cells luteinise and begin producing progesterone; however, they do not complete luteinisation and perish (Irving-Rodgers, et al. 2003).

2.7.3 Hormonal Ratio of Oestrogen and Androgens

The majority of apoptosis occurs in the antral granulosa cells during follicular development. The antral atretic granulosa cells have reduced oestrogen output compared to healthy follicles, and low progesterone in comparison; however, determination of the health status of a follicle, based on the ratio of oestrogen to progesterone or morphological differences are poor indicators of whether or not a follicle is atretic (Grimes, et al. 1987). In particular, a high progesterone level may be indicative of luteinisation of the granulosa cells as opposed to apoptosis (Grimes, et al. 1987). More recent data show that apoptosis increases dramatically around the time of dominant follicle selection when the ratio of androgen was greater than oestrogen (Figure 2.7.4).

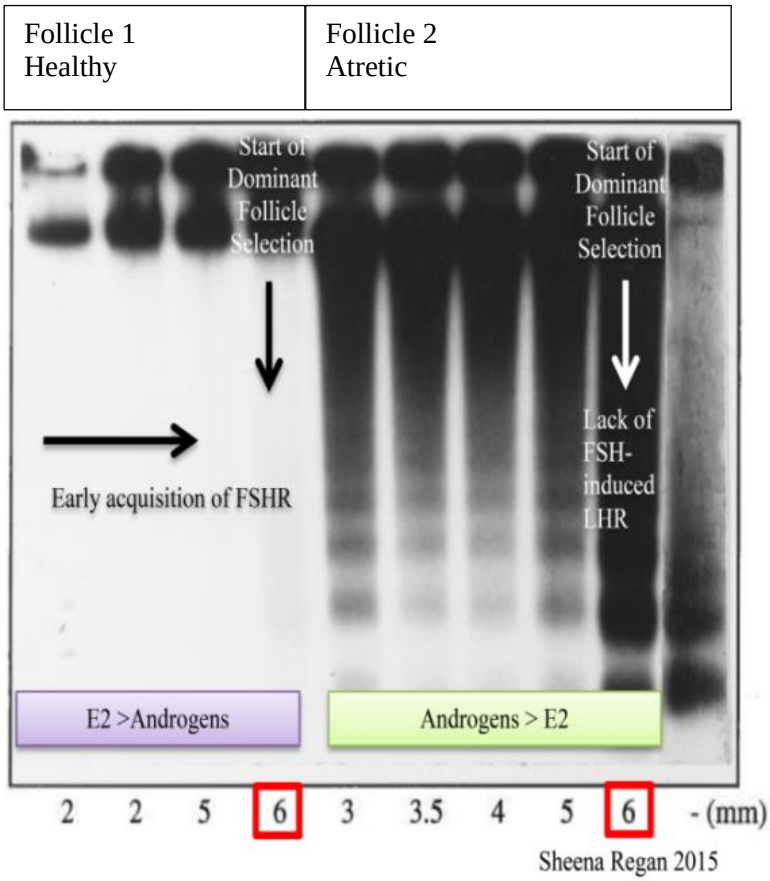


Figure 2.7.4 Apoptosis induced DNA Fragmentation in human small antral follicles.

Follicle 1 is healthy with a greater concentration of oestrogen than androgen due to the early acquisition of FSHRs, at the time of dominant follicle selection and the decline in pituitary FSH. Follicle 2 has insufficient FSHRs to induce LHRs. Based on data from (Yuan & Giudice 1997, Bao & Garverick 1998, Austin et al. 2001, Sen et al. 2014)

The early acquisition of granulosal FSHR and LHR accelerates the dominant follicle growth as the pituitary FSH declines (Figure 2.7.4) (LaPolt, et al. 1992, Sen, et al. 2014). The LHR supplement the FSHR-mediated conversion of androstenedione to oestradiol by CYP19A1, maintaining a positive oestrogen to androgen ratio in the follicle. As the antral follicle increases in size, more oestrogen and anti-apoptotic factors are produced that ensures the survival of the dominant ovulatory follicle (Amsterdam, et al. 2003). With reduced FSHR and LHR density, the granulosa cells of subordinate follicles have a reduced capacity to convert theca derived androgens to oestrogen (Figure 2.7.4), and are destined for atresia (Hillier, et al. 1994a, Xu, et al. 1995).

2.7.4 Factors that Activate Specific Signalling Pathways

Apoptosis can be initiated by a number of factors that activate specific signalling pathways to trigger apoptotic mechanisms (Figure 2.7.5) (Amsterdam, et al. 2003, Matsuda, et al. 2012, Nottola, et al. 2006). Conversely, many growth factors such as FSH, and LH, are anti-apoptotic which creates a micro-environment that ensures survival. During the highly prolific phase of folliculogenesis apoptosis is reduced by the inhibition of apoptotic signalling pathways by various growth factors. The BMP ligands have been shown to inhibit apoptosis signalling in a number of studies (Figure 2.7.5) (Hussein, et al. 2005, Kayamori, et al. 2009). BMP7 activated caspase 9 in granulosa cells, however, BMP4 and 7 had no effect on the levels of mitochondrial apoptotic factors, *BAX* and *Bcl-x1* mRNA (Kayamori, et al. 2009). Cumulus granulosa apoptosis was reduced by BMP6, 7, and 15 (Hussein, et al. 2005).

In vitro culture of caprine (goat) granulosa cells with BMP2, 4, 6, and 7 reduced apoptosis indicated by Hoechst 33342 staining and percentage DNA fragmentation (Zhu 2013). In the same study, FSHR silencing increased apoptosis significantly, whereas, BMP2, 4, and 7 increased *FSHR* (Zhu 2013). The very low incidence of apoptosis in cumulus cells compared to granulosa cells has been reported to be due to the oocyte-secreted BMP6 and BMP15, forming a concentration gradient radiating out from the oocyte (Hussein, et al. 2005). In bone tissue BMP2 signalling via the BMPR1B, induced apoptosis, indicated by an increase in caspase 3, 6, 7, and 9. The action occurred independently of cell proliferation induced by BMP2, highlighting the dual action capability of BMPs (Haÿ, et al. 2004).

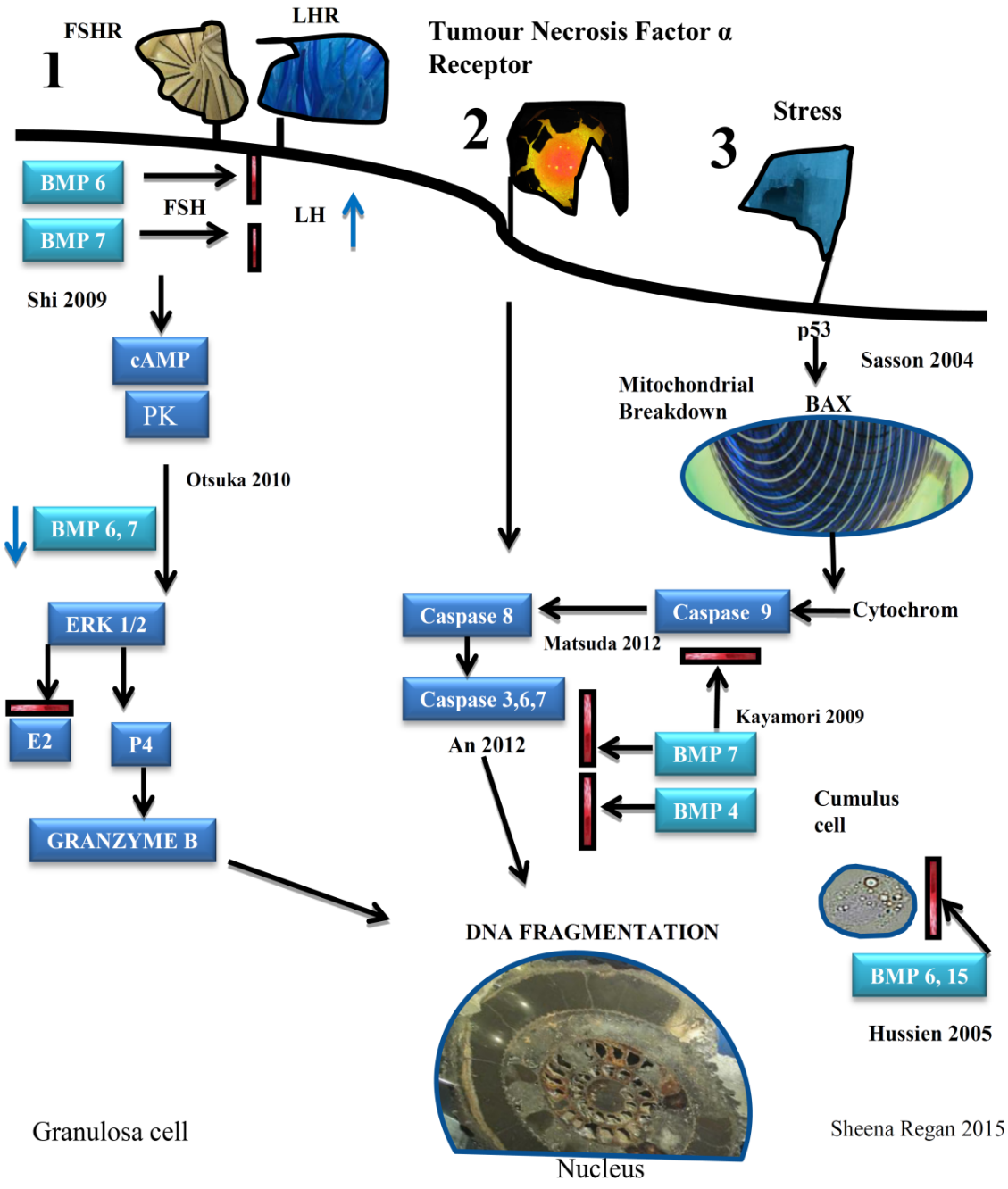


Figure 2.7.5 Diagram of Apoptosis induced by various factors and the involvement of the BMP ligands.

There are two major pathways for the development of apoptosis in granulosa cells (Amsterdam, et al. 1997, Amsterdam, et al. 2003, Austin, et al. 2001). The first affects the mitochondrial function via BAX activation, and the second is via high levels of cAMP through granzyme B or caspases downstream of tumour necrosis factor alpha (TNFα) (Figure 2.7.5). The steroid producing capacity of a follicle is reflected in the hormone levels in serum and follicular fluid from the antral cavity of the follicle (Amsterdam, et al. 2003). In an

apoptotic granulosa cell, DNA fragmentation, cytoplasmic blebbing and compartmentalisation of organelles protects the functional steroid capacity of the cell to ensure the maximum oestrogen synthesis. This protective mechanism involves reorganisation of the actin cytoskeleton to prevent contamination with neighbouring cells, and to centralise the still functioning mitochondria (Figure 2.7.6).

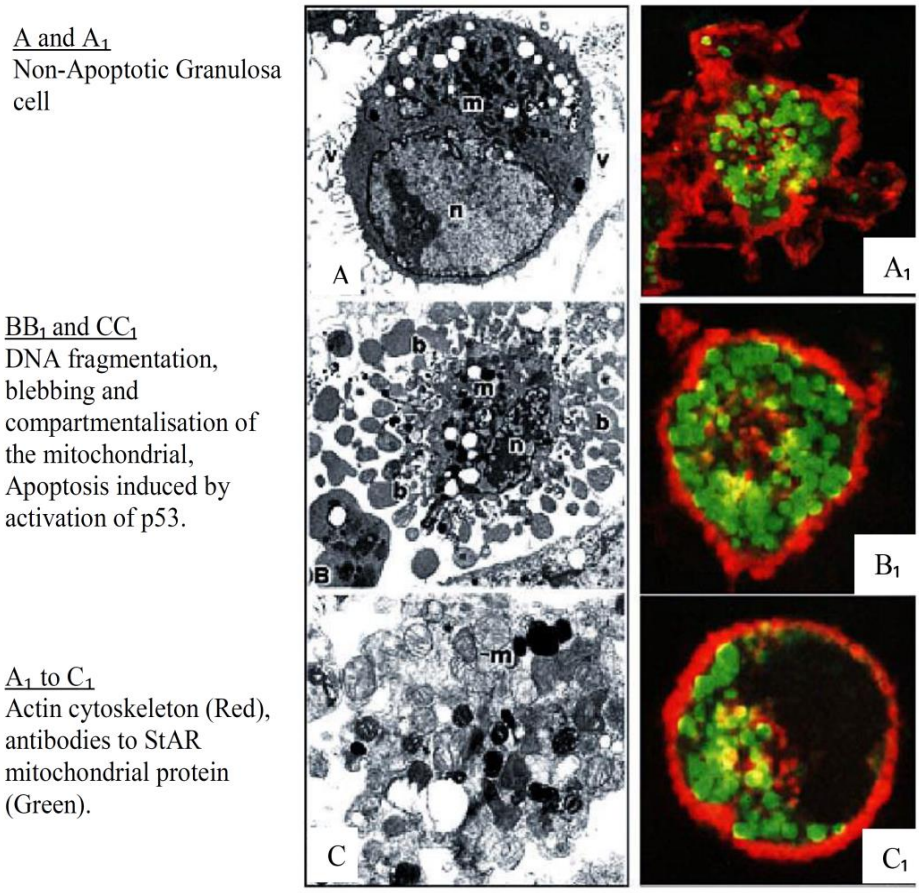


Figure 2.7.6 Granulosa cell steroidogenic function during p53-induced apoptosis.
Immortalised granulosa cells; n = nucleus, b = blebbing, m = mitochondria, v= microvlli. (A) Numerous mitochondria and apoptosis induced cytoplasmic blebbing (10 000 x). (A1) Actin cytoskeloton (red) StAR mitochondrial protein (green). (B) Condensed chromatin in the nucleus and blebbing (10 000 x). (B1) Central reorganisation of the cytoskeloton. (C) Mitochondria with well defined crystae (indicate luteisation) (30 000 x) (Amsterdam, et al. 1999, Hosokawa, et al. 1998).

GENERAL MATERIAL AND METHODS

CHAPTER 3 GENERAL METHODS

3.1 Sheep: Animals, ovaries, and follicles

A total of 17 Merino sheep with an average age of 4 years and 9 months ±2 months were used (Table 3.1:1 and Table 3.2:1). The animals were housed in open paddocks at the University of New England, NSW, Australia, in accordance with NH & MRC Code of Practice for the Care and Use of Animals for Experimental Purposes. All experiments were approved by the University of New England Animal Ethics Committee. Five ewes were genetically confirmed as homozygous Australian Booroola strain (BB); with a flock average ovulation rate of four to six during peak breeding season, (Cummins, et al. 1983) and an ovulation rate of three, based on the corpus luteum (CL) formation in two Booroola sheep at the time of slaughtering, because of miss timed ovulation. Subsequently, the remaining follicles after ovulation were classified as luteinised (Table 3.2:1). The corpora lutea formed in the post-ovulation groups were included in the total number of follicles recruited per genotype, and they were added to the largest follicle size for that genotype (Table 3.2:1). The oestrous cycles of these animals were synchronised using flugesterone acetate sponges (Bioniche Animal Health, Armidale, NSW, Australia). The sponges were removed from the animals 14 days after insertion, and the animals received an intra-musculature injection of 1000 IU of pregnant mare's serum gonadotrophin (PMSG). The animals were euthanised approximately 36 hours after sponge removal, and the ovaries collected (Evans 2003).

Table 3.1:1 The number of individual follicles analysed prior to ovulation and post ovulation.

Genotype	Sheep before ovulation	Follicles	Dominant follicles	Small (1.0-2.1 mm)	Medium (2.2-2.9 mm)	Large (3.0-4.5 mm)	Extra large (4.6-7.0 mm)
WT	8	64	8	25	19	14	6
BB	3	20	3	9	5	6	0
Genotype	**Sheep post-ovulation**	**Luteinized follicles**	**CL per sheep**	**Small (1.0-2.1 mm)**	**Medium (2.2-2.8 mm)**	**Large (3.0-4.5 mm)**	**Extra large (4.6-7.0 mm)**
WT	4	35	1.5	13	14	8	0
BB	2	10	3	7	2	1	0

Corpus Luteum (CL)

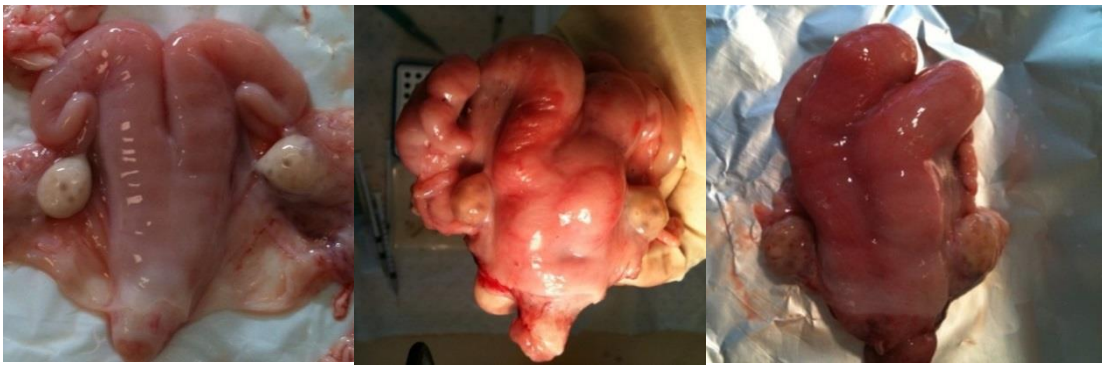
3.2 Sheep: Collection of granulosa cells

Figure 3.2.1 Booroola Merino Sheep raised by Dr Tim O'Shea at University of New England.

Follicle size was established by preparing standard blank volumes of 5µl increments from 5 to 160 µl; these were placed in identical PCR tubes and visually compared to the follicular fluid aspirated from each follicle based on previously published techniques (Andersen, et al. 2010, Jakimiuk, et al. 2002). The blank values were used as a reference to estimate the diameter of the follicle, and cross-referenced with a direct measurement of the diameter of the follicle using a fine ruler gauge before aspiration. The follicular fluid was aspirated from all of the visible follicles using a 25 gauge needle, and placed in PCR tubes. All of the visible surface follicles were harvested, and very small follicles (1-1.8 mm) of the same size were pooled in groups of three or four; whereas, all the medium, large, and extra-large follicles were analysed individually (Table 3.1:1).

On a follicle by follicle basis, an *in situ* incision was made at the exposed surface of the follicle with a scalpel; granulosa cells were gently scraped with the blunt side of the scalpel, flushed repeatedly with phosphate buffered saline (PBS), and re-suspended in PBS. The sheets of granulosa cells were dispersed by mechanical pipetting, and centrifuged at an optimised lower speed of 300 g for 5 min to protect the cells from method-induced apoptosis

and to yield an uncontaminated population of granulosa cells. Collection without excision of the whole follicle prevents contamination with the theca or stroma cells and limits the amount of contamination with blood cells (Figure 3.2.1 and Figure 3.2.2).



Unstimulated abattoir Sheep, 3 years

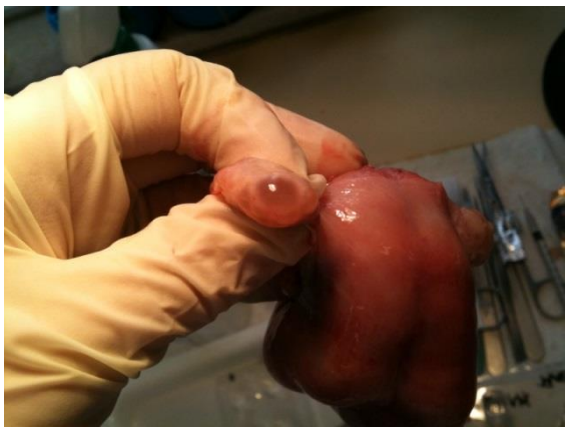
Synchronised Booroola Merino Sheep, 4 years

Synchronised wild type Merino Sheep, 4 years

Sheena Regan 2015

Figure 3.2.2 Collection of sheep ovaries.

Based on the hierarchical down-regulation previously reported, the sheep follicles were divided into subordinate, dominant, and luteinised post ovulation follicles (Driancourt, et al. 1985, Evans 2003). The largest follicle collected from each sheep before ovulation was classified as the dominant follicle, and the remaining follicles were classified as subordinated follicles Figure 3.2.3 (Gasperin, et al. 2014).



Sheena Regan 2015

Figure 3.2.3 An ovary and uterus from a wild type Merino sheep, showing a leading dominant follicle.

Table 3.2:1 Follicle size validation using follicular fluid collected from BB follicles.

BOOROOLA SHEEP		Large 3.0-4.5	mm	Medium 2.2-2.7	mm	Small 1.0-2.1	mm
Total Sheep	5	3CL		55ul	2.7	22 ul	2.1
Sheep ovulated :CL formation	2	3CL		45 ul	2.5	20 ul	2
CL Formation				45 ul		20 ul	
				45 ul		20 ul	
Leading Dominant Follicle				40 ul	2.4	20 ul	
				35 ul	2.3	15 ul	1.8
Granulosa cells collected after ovulation		80ul	3.6	30 ul	2.2	13 ul	1.7
		110ul	4.5	7		13 ul	
Pooled Small follicles		70 ul	3.2			10 ul	1.5
		70 ul	3.2			10 ul	
Granulosa cells collected prior to ovulation		70 ul				10 ul	
TOTAL		65 ul	3.0			10 ul	
		65 ul				10 ul	
		65 ul				10 ul	
		14				10 ul	
						8 ul	1
						16	
					Pooled	12	
					TOTAL	28	

Table 3.2:2 Follicle size validation using follicular fluid collected from WT follicles.

CONTROL SHEEP		Extra Large 4.6-7.0	mm	Large 3.0-4.4	mm	Medium 2.2-2.8	mm	Small 1.0-2.1	mm
Total Sheep	12	2CL		100ul	4.4	60 ul	2.8	25 ul	2.1
Sheep Ovulated	4	2CL		100ul		60 ul		25 ul	
CL Formation		1CL		100ul		60 ul		20 ul	2
		1CL		100 ul		60 ul		20 ul	
Leading Dominant Follicle				100 ul		55 ul	2.7	20 ul	
				100 ul	4.2	55 ul		20 ul	
Granulosa Luteal due to CL formation		160ul	7.0	95 ul	4	50 ul	2.6	20 ul	
		150ul	6.5	90 ul	3.8	50 ul		20 ul	
Pooled Small follicles		130ul	5	85 ul	3.6	50 ul		20 ul	
		120ul	4.8	80 ul	3.4	50 ul		20 ul	
Granulosa cells collected prior to ovulation		120ul		75 ul		45 ul	2.5	20 ul	
TOTAL		120 ul		75 ul		45 ul		15 ul	1.8
		115 ul	4.6	75 ul		45 ul		15 ul	
		115 ul		75ul		45 ul		15 ul	
		14		75 ul	3.2	45 ul		15 ul	
				70 ul		40 ul	2.4	15 ul	
				70 ul		40 ul		15 ul	
				70 ul	3.0	40 ul		15 ul	
				65 ul		40 ul		12 ul	1.7
				65 ul		35 ul	2.3	12 ul	
				65 ul		35 ul		10 ul	1.5
				65 ul		35 ul		10 ul	
				65 ul		35 ul		10 ul	
				23		35 ul		10 ul	
						35 ul		10 ul	
						35 ul		10 ul	
						35 ul		10 ul	
						30 ul	2.2	10 ul	
						30 ul		10 ul	
						30 ul		10 ul	
						30 ul		8 ul	1
						30 ul		8 ul	
						30 ul		8 ul	
						33		8 ul	
								8 ul	
								8 ul	
								8 ul	
								8 ul	
							SUB TOTAL	38	
							Pooled	42	
							TOTAL	80	

3.3 Human: ovaries and follicles

A total of 487 follicles were collected from 68 patients undergoing standard fertility treatment previously reported in accordance with the PIVET Medical Centre Algorithm (Yovich, et al. 2012). Follicles were collected irrespective of previous aetiology, but limited to exclude, unusual medical conditions, hormonal dysfunction, and polycystic ovarian syndrome (PCO); patients were aged between 23 and 45 y (Table 3.3:1). Informed consent was obtained for the use of the follicular fluid samples collected during oocyte retrieval for the purpose of IVF and embryo transfer. Approval by the Human Research Ethics Committee of Curtin University of Technology was obtained for this study (HR RD26-10:2010-2016). One patient out of three patients undergoing risk reduction removal of the uterus and ovaries, was selected to represent an unstimulated natural healthy cycle prior to the LH surge, and was recruited from King Edward Memorial Hospital (KEMH) approved by the Women and Newborn Health Service ethics committee (WNHS).

Table 3.3:1 Patient ovarian reserve, based on antral follicle count (AFC) and the number of follicles collected per group.

AGE	IVF patient	Total follicle	AFC	Major Group	Total Follicle	Sub Group	#	Sub Group	#	Sub Group	#	Sub Group	#
21-30	11	101	20-40	A+ & A	96	A++	14	A+	31	A	64		
31-34	11	86	13-29	A & B	86	A	60	B	26	C	17		
35-39	16	102	9-19	B & C	66	A+	6	B	50	C	16	D&E	30&6
40-45	18	118	3-8	D & E	78	D	59	E	19	B	34	C	6
40-45 GH	11	78	3-8	D & E	47	D	22	E	23	B	16	C	5
40		Natural Cycle Healthy				D	2						

Ovarian reserve measured indirectly by the Antral Follicle Count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle: A+ = 30-39 follicles; A = 20-29; B = 13-19; C = 9-12, D = 5-8; E = ≤4.

3.3.1 Human IVF: Ovarian stimulation, follicular fluid and oocyte.

Patient treatment consisted of one of three types of GnRH-LH suppression in conjunction with rFSH, from cycle day 2 (Puregon or Gonal F). A GnRH antagonist treatment (Cetrotide) (0.25 μg/day) was administered from day seven until ovulation induction. Alternatively, a GnRH flare agonist treatment (Lucrin) (0.25 μg/day) was administered in conjunction with rFSH on day 2, or a long agonist GnRH treatment (Synarel) (0.25 μg/day) on day 21 of the previous cycle, until ovulation induction. Ovulation was triggered with either 10 000 IU hCG derived from a urinary preparation (Pregnyl) or a pituitary derived analogue to LH (Ovidrel). Oocyte retrieval was scheduled for 36 hours post-trigger, by transvaginal oocyte aspiration (TVOA) (Yovich and Stanger 2010).

3.3.2 Human GH co-treated: Ovarian Stimulation

Ovarian cycles were stimulated with fertility drugs (described above), in order to promote follicle development (Yovich, et al. 2012). GH was administered to 11 patients on day 21 of the previous cycle, and on day 2, 6, 8, 10, and 12 of the current cycle. In addition, the criterion for selection was based on women who had generated less than three metaphase II oocytes with maximum rFSH stimulation of 450 IU/day (Yovich and Stanger 2010). Poor ovarian response patients with three or more failed attempts to conceive through IVF treatment with gonadotrophin alone, were co-treated with a total of 10 IU GH, (Saizen, Serono, Australia).

3.4 Antral follicle count

Patients received rFSH based on the patients' profile of age and AFC, to predict the rFSH dose required to stimulate multiple pre-ovulatory follicles (Figure 3.4.1).

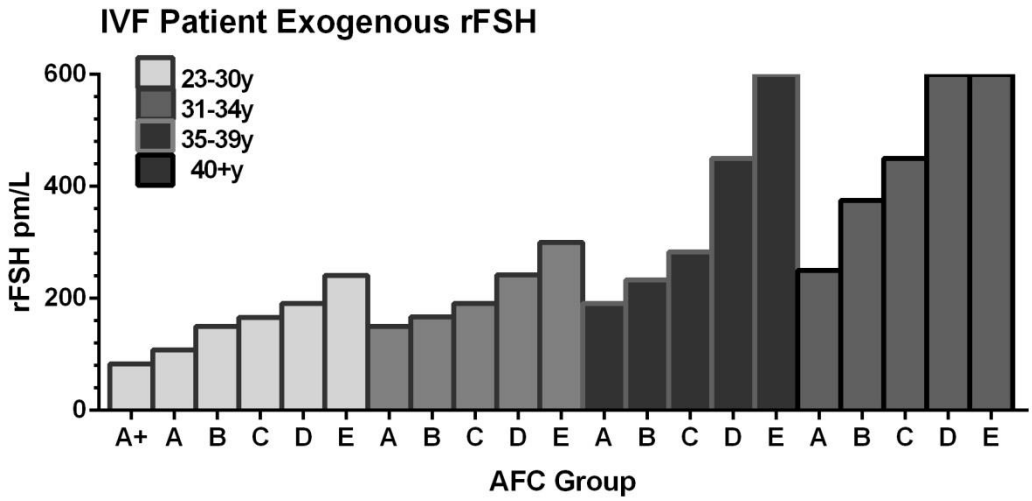


Figure 3.4.1 IVF exogenous rFSH dose with increasing age and declining antral follicle count.
Based on data from Yovich et al, 2010.

Ovarian reserve was measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm in size that are present on day 2-5 of a cycle. Determination of AFC was determined by transvaginal ultrasound and divided into groups based on the number of follicles counted, between 2 and 10 mm; group A++ 40+ group A+ = 30-39 follicles; group A = 20-29; group B = 13-19; group C = 9-12, group D = 5-8; group E = ≤ 4 (Table 3.3:1) (Yovich, et al. 2012)

3.4.1 Human IVF & GH co-treated: Collection of Granulosa Cells

The diameter of the follicle was calculated using ultrasound before the clinical aspiration of individual follicles (Figure 3.4.2). The first aspiration was collected without flush medium into a test tube, and handed to the embryologist to locate the oocyte and attached cumulus cells if present. Further flushing of the follicle (Quinn's Advantage with Hepes, Sage Media, Pasadena, California) removed the loosely attached granulosa layers of cells at~ 1.24-1.72 MPa. Once the oocyte was located and removed, the clinician proceeded to the next follicle and repeated the process (Figure 3.4.2).

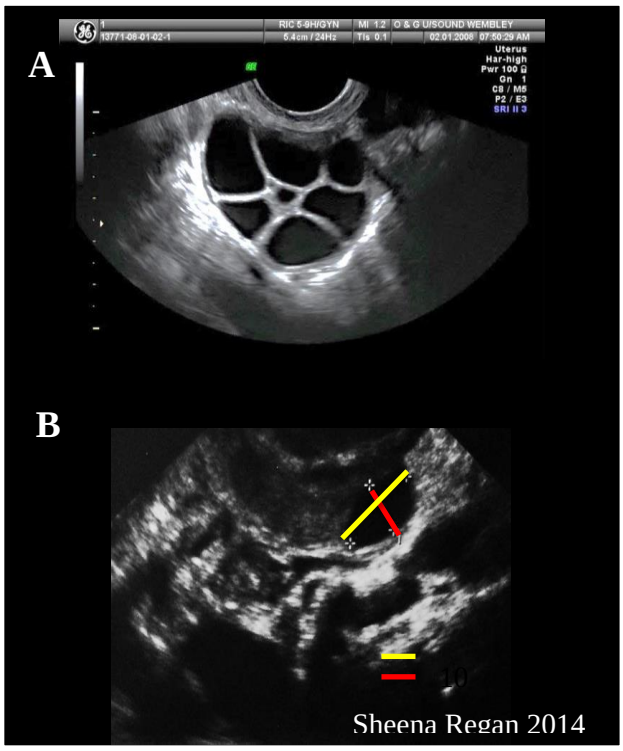


Figure 3.4.2 Ultrasound measurement of human follicles.
(A) Gonadotrophin induced multiple follicles in a human ovary. (B) Ultrasound measurement of a follicle in a human ovary, bar 7.95 mm.

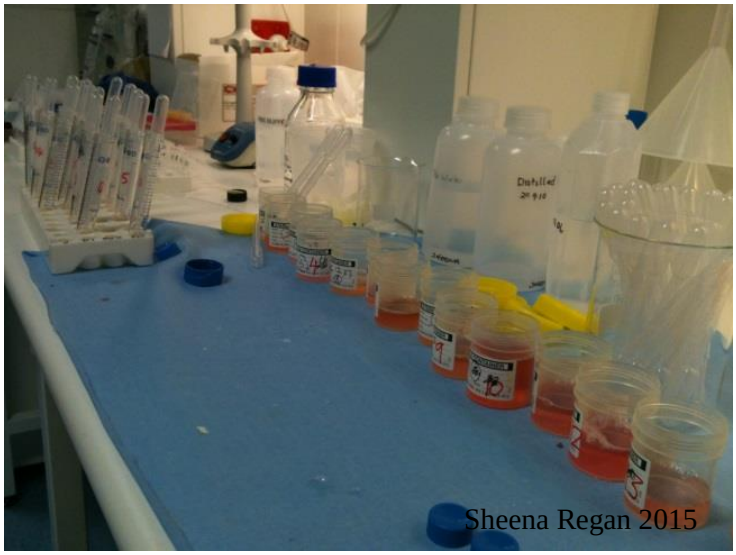


Figure 3.4.3 Follicular fluid and flushing media from individual follicles.
Individual follicle collection, of follicular fluid, and flush media

The sample of medium and granulosa/follicular fluid was then layered onto a histopaque ficoll gradient (Sigma Aldrich: Castle Hill, Australia) and centrifuged at 250 g for 30 min at 25 ºC (Figure 3.4.3). The supernatant was removed and centrifuged at 300 g for 5 min, leaving behind the pellet of granulosa cells and approximately 300 µl of supernatant. The pellet was dispersed with gentle pipetting to manually separate the sheets of granulosa cells. Theca cells are bound by collagen fibers, and vaginal epithelium cells are very large, and if present, would have remained in the blood pellet after the first centrifuge or would be gated out during flow cytometric analysis (Whiteman, et al. 1991)

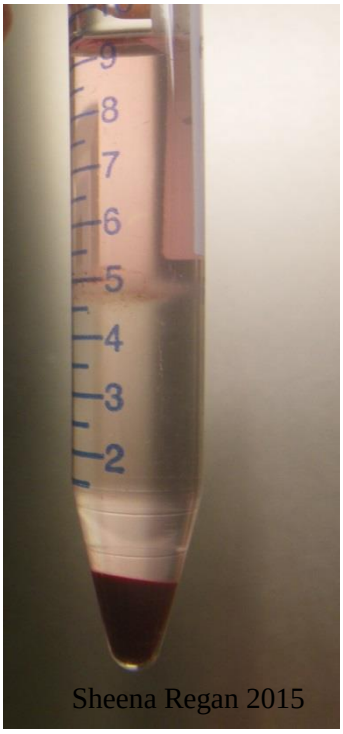


Figure 3.4.4 Isolation of human granulosa cells from individual follicles.
Ficoll gradient used to isolate granulosa cells; pink media at the top, granulosa cells at the interface, (at 5 ml mark) and red blood pellet at the bottom of the test tube.

3.4.2 Natural healthy unstimulated cycle collection

The natural cycle patients scheduled for risk reduction (such as cancer) removal of the reproductive organ was timed to coincide with day 12 of the menstrual cycle. Before removal, ultrasound confirmation of the size of the follicles and the number of follicles present was made. After removal of the uterus and ovaries, the whole follicle was excised, and transported to the laboratory. The collection of follicular fluid, isolation of the granulosa cells and the analysis was performed as described above and below (Figure 3.4.5, Figure 3.4.6 and Figure 3.5.1).

Figure 3.4.5 Human ovaries and uterus removed from an unstimulated healthy patient.

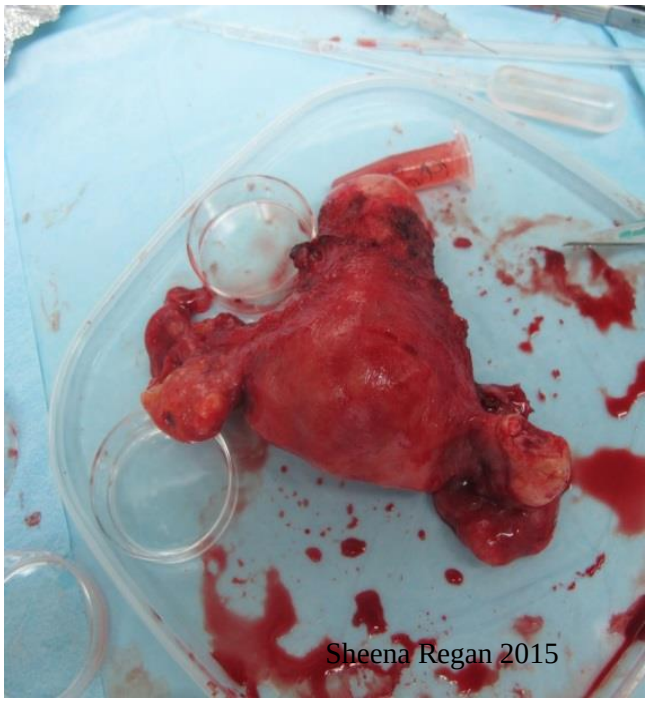


Figure 3.4.6 Human ovaries, uterus and cervix. Recovery of a 10 mm follicle for collection of granulosa cells from KEMH.

3.5 Immunolabelling

3.5.1 Sheep: Immunolabelling sheep

Aliquots of suspended granulosa cells (1x10^{6} cells in 100 µl) were immunolabelled using a double-indirect method as previously described (Abir, et al. 2008a, Cai, et al. 2007, Gao, et al. 2007). The cells were incubated separately with an optimised concentration of 4 µg/ml affinity purified polyclonal antibody to BMPR1B, FSHR or LHR, for 25 min at 5 ˚C (Millennium Science, Surrey Hills, Victoria Australia). Previously, we have established the

specificity in sheep, using immunofluorescence detection and 3D image analysis (Al-Samerria and Almahbobi 2014).

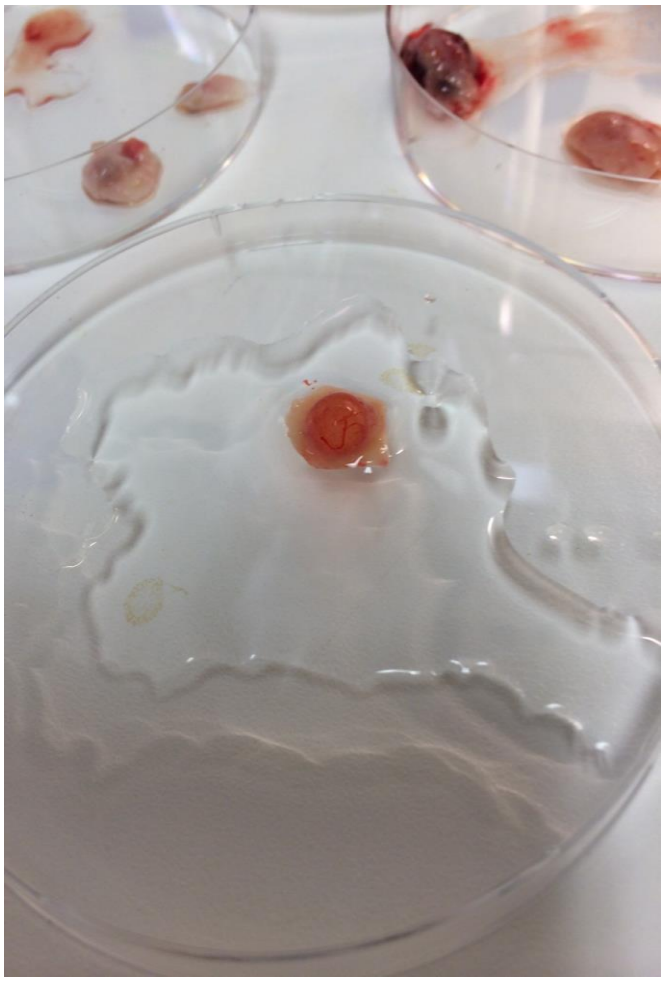


Figure 3.5.1 Natural healthy cycle human ovarian follicle removed from an ovary.

The antibodies were polyclonal goat anti-BMPR1B (sc-5679), goat anti-FSHR (sc-7798) and goat anti-LHR (sc-26341), (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-goat second antibody conjugated with Alexa 488 (Al-Samerria and Almahbobi 2014). In addition, the antibodies have been previously reported in human studies (Abir, et al. 2008a, Cai, et al. 2007, Gao, et al. 2007, Haÿ, et al. 2004, Pidoux, et al. 2007); and using flow cytometry (Gao, et al. 2007). Immunolocalisation of LHR in human placental trophoblast demonstrated positive, strong staining with hCG, and no staining was observed in nonspecific isotypic negative controls (Pidoux, et al. 2007). In the same study, RT-PCR products were sequenced and confirmed to be LHR fragments (Pidoux, et al. 2007). BMPRIB, FSHR, and LHR were raised against a sequence of amino acids of human origin, with 88%, 80%, and 93% sequence identity to the sheep protein, respectively. Donkey anti-goat IgG conjugated with Alexa 488 (Life Technologies Australia, Victoria, Australia), with excitation wave length 495 nm and an emission wave length 519 nm, was applied at an optimised concentration of 4 µg/ml for 25 min and was repeat washed in PBS. Blocking peptides for BMPR1B and FSHR

indicated nonspecific binding applied to human granulosa cells (sc-5679P, sc5679P; Millennium Science, Surrey Hills, Victoria Australia), as previously published (Abir, et al. 2008a, Cai, et al. 2007, Gao, et al. 2007, Haÿ, et al. 2004, Pidoux, et al. 2007).

3.5.2 Human & GH co-treatment: Immunolabelling

Aliquots of suspended granulosa cells (1x10^{6} cells in 100 µl) were immunolabelled using a double-indirect method as previously described (Abir, et al. 2008a, Cai, et al. 2007, Gao, et al. 2007). The cells were incubated separately with an optimised concentration of 4 µg/ml affinity purified polyclonal antibody to BMPR1B, FSHR, LHR or GHR for 25 min at 5 ˚C Previously, we have established the specificity in sheep, using immunofluorescence detection and 3D image analysis (Al-Samerria and Almahbobi 2014). The antibodies were polyclonal goat anti-BMPR1B (sc-5679), goat anti-FSHR (sc-7798) and goat anti-LHR (sc-26341), (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and GHR (AF1210; Life Technologies, Victoria, Australia). In addition, the antibodies have been previously reported in human studies (Abir, et al. 2008a, Bozzola 1998 , Cai, et al. 2007, Gao, et al. 2007, Haÿ, et al. 2004, Pidoux, et al. 2007, Regan, et al. 2015, Weall, et al. 2014); and using flow cytometry (Gao, et al. 2007).

Figure 3.5.2 Isolated granulosa cells from individual follicles, immunolabelled receptors. Sheena Regan Curtin University 2013.

The cells were washed with PBS and centrifuged at 300 g at 5˚C for 5 min. The routinely used monoclonal antibody CD45 was added to BMPR1B, GHR and LHR tubes to enable the subtraction of the positive leukocyte common antigen (~ 3%) not removed during isolation of the granulosa cells with the ficoll gradient, (555485; BD Biosciences, Perth, Australia).

3.6 Flow cytometry

3.6.1 Sheep: Flow cytometry

The samples were prepared as a single cell suspension, stained for flow cytometric analysis, and immediately processed using a LSRII flow cytometer (BD, North Ryde, NSW, Australia). The data were analysed using FlowJo software (Figure 3.5.2) (Tree Star Inc., Ashland, OR, USA). Selective gating of the whole sample to identify a pure granulosa cell population with an average of ~4000 granulosa, was achieved by graphing forward scatter (increasing size) on the y-axis, and fluorescent intensity (Alexa 488) on the x-axis. A large gating box excluded small cellular fragments along the x-axis (letter ‘c’) and cell doublets on the top border (letter ‘d’), (Figure 3.6.1A and B).

The resulting population was graphed on a histogram for the number of granulosa cells and the density of receptors (letters ‘a’ and ‘b’, Figure 3.6.1A and B). Negative control samples were assessed either as unstained samples or by the substitution of primary antibody with pre-immune goat IgG (Figure 3.6.2) (Millennium Science, Surrey Hills, Victoria Australia) at the same concentration as the primary antibody. The fluorescence intensity threshold gate at 10^3 (solid line) excludes emitted autofluorescence, number 4, and nonspecific binding number 1, 3, and 4, (letter ‘a’ combined Figure 3.6.1B).

The resulting population contained a uniform granulosa cell population that revealed positive staining for the FSHR, which is unique to granulosa cells in the follicle (letter ‘b’ Figure 3.6.1A and B) (Gao, et al. 2007, Stilley, et al. 2014). The predetermined gates were established and set for all of the samples. The positive quantitative signal (mean fluorescent intensity (MFI)) for the average granulosa cell receptor density
(letter ‘b’) is indicated in Figure 3.6.1B.

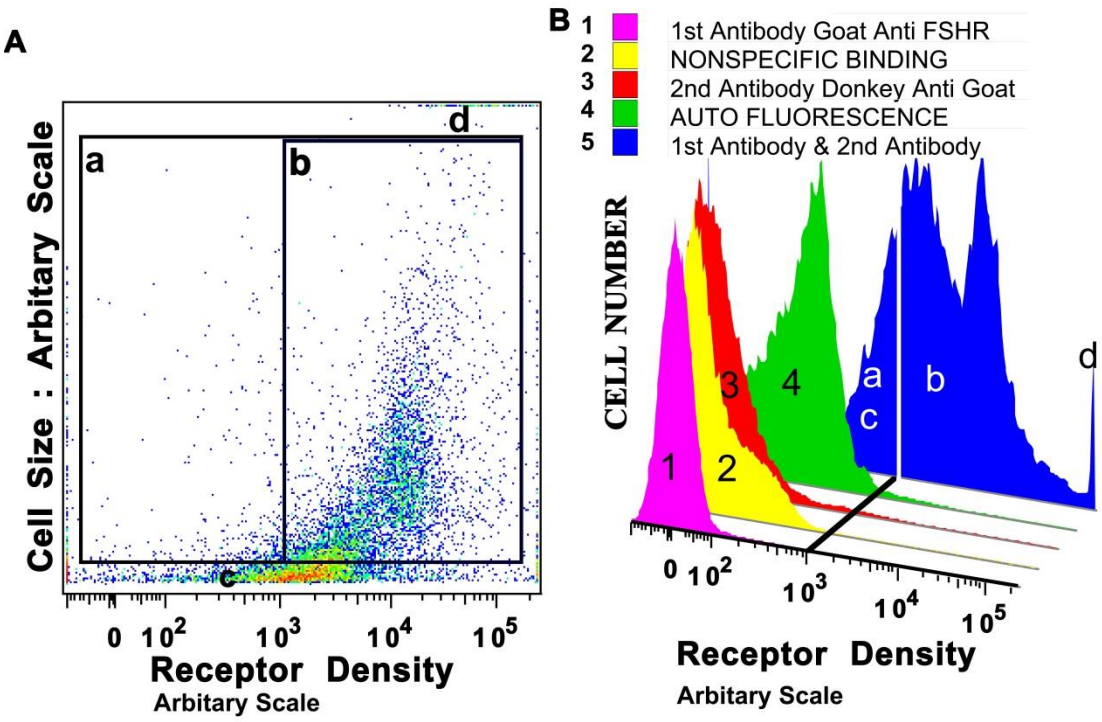


Figure 3.6.1 Validation of the method of subtraction gating; removal of auto-fluorescence and nonspecific binding.

(A) Flow cytometric dot plot, forward scatter representing size of cell, showing positively identified granulosa cells. a; subtracted (gated box) cells due to either auto-fluorescence or nonspecific binding, b; average number of granulosa cells 4971; gating to exclude debris, c; and doublet cells, d. (B) Histogram of cell number and fluorescent intensity of the corresponding a, b, and d population in Figure A. Where b (blue) represents the mean granulosal receptor density; gated between 10^3 and 10^5, to exclude a, c and d combined. Nonspecific binding (2 = yellow); auto-fluorescence (4 = green); 1st antibody goat anti FSHR (1 = pink); and second antibody donkey anti goat (3 = red) combined (a) and gated at 10^3 (c) and doublet cells, gated and $>10^5$ (d) Scale arbitrary units.

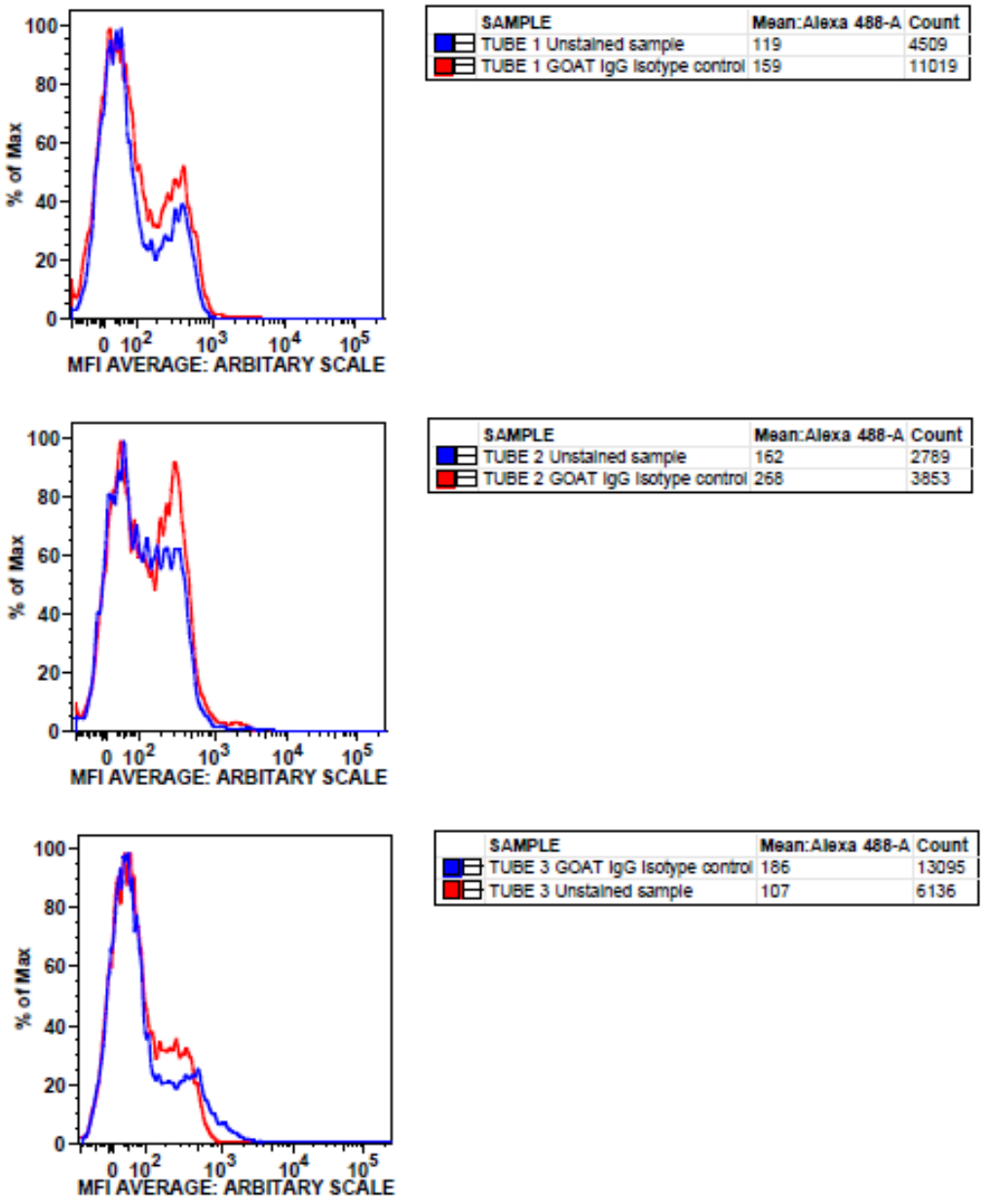


Figure 3.6.2 Unstained control compared to IgG Isotope control for nonspecific binding and auto fluorescence.

3.6.2 Human &GH co-treatment: Flow cytometry

Selective gating of the whole sample to identify a pure granulosa cell population was achieved by graphing forward scatter to remove doublets (FSC-H verses FSC-A). Then Alexa Fluor 488 intensity against APC intensity to identify and gate the granulosa cells positively stained with antibody CD45, which emits in the Allophycocyanin (APC) spectrum. (Figure 3.6.3, and Figure 3.6.5).

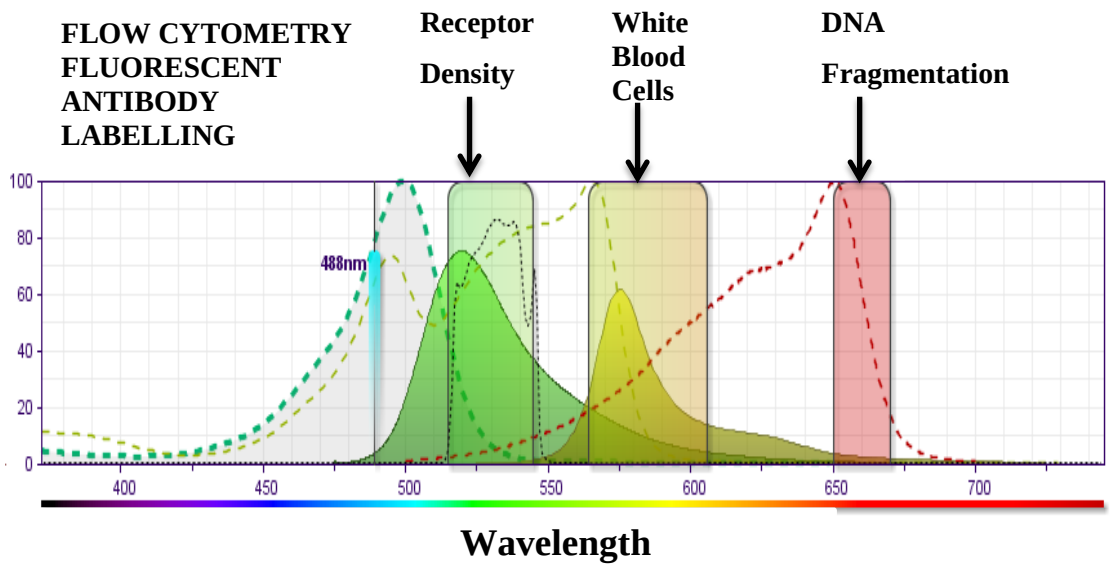


Figure 3.6.3 Validation of spectral overlap exclusion.
The samples were individually stained with antibodies with each follicle having a control unstained sample to increase accuracy and reduce spectral ovalap.

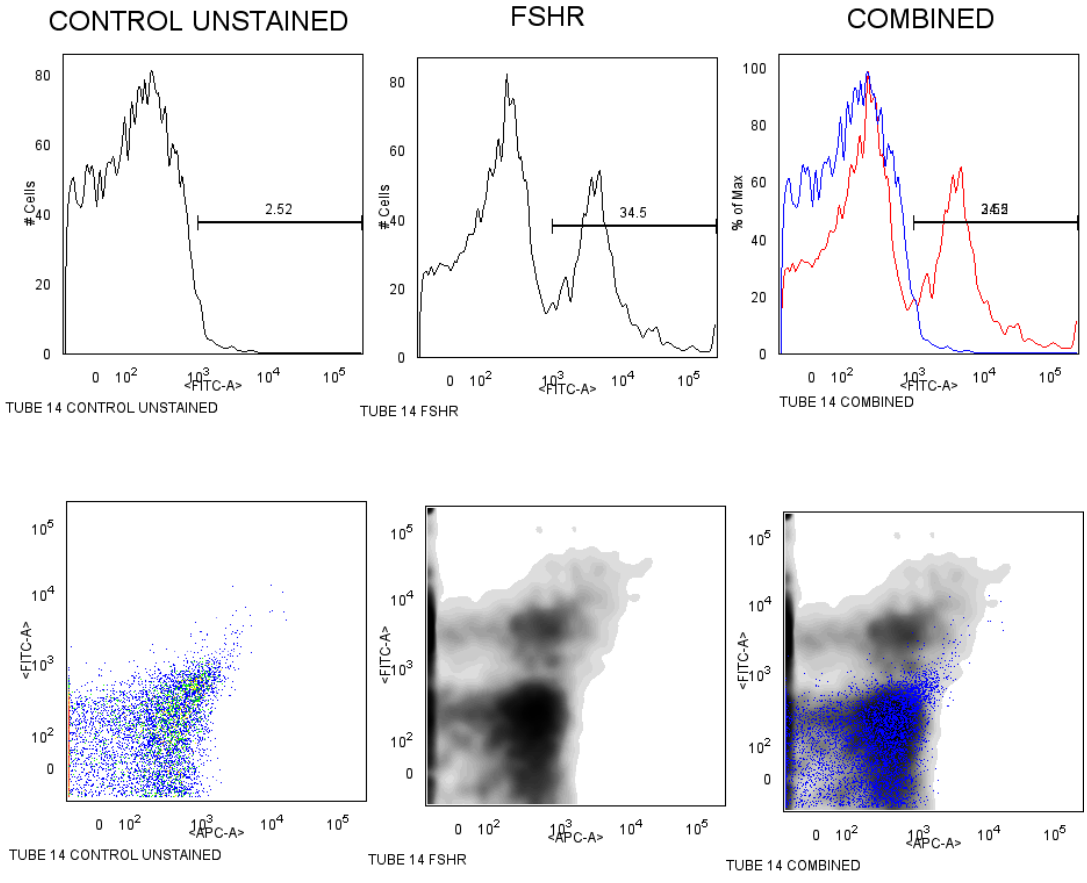


Figure 3.6.4 Validation of gating to measure average receptor density in flow cytometry.
An unstained sample control histogram and dot plot, FSHR expression with two peaks; the first peak is the auto-fluorescence and the second peak the positive population measured. The unstained control and the FSHR was overlayed to validate the gates used in analysis.

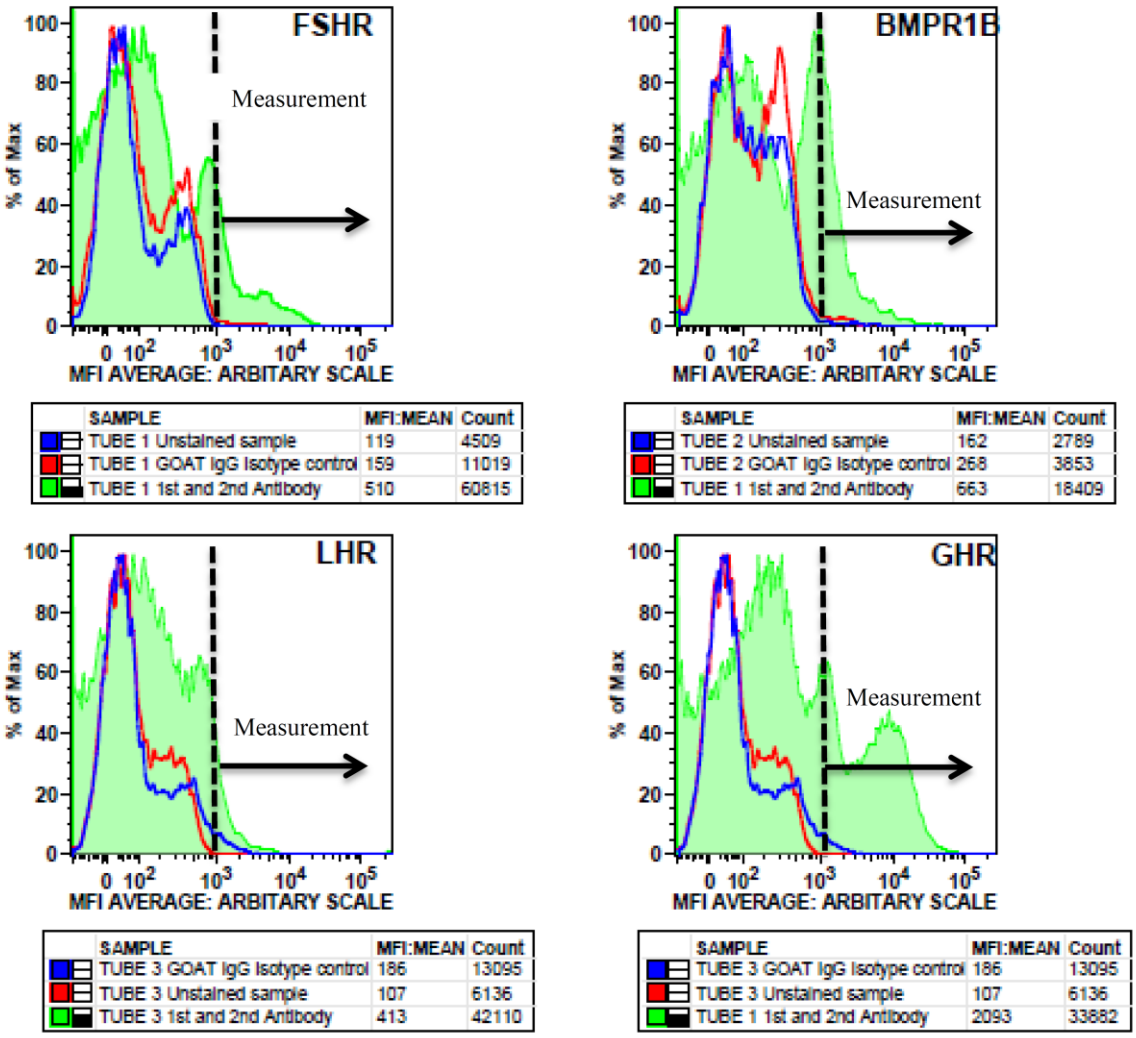


Figure 3.6.5 Measurement of mean receptor density using subtraction gating to remove auto-fluorescence and non- specific binding.
Measurement of receptor density of receptors.

Unstained samples or the substitution of primary antibody with pre-immune goat IgG (Figure 3.6.2) (Millennium Science, Surrey Hills, Victoria Australia) at the same concentration of the primary antibody served as negative controls for auto-fluorescence; and blocking peptides for BMPR1B and FSHR indicating nonspecific binding, (sc-5679P, sc5679P; Millennium Science, Surrey Hills, Victoria Australia) as previously published (Abir, et al. 2008a, Al-Samerria and Almahbobi 2014 , Bozzola 1998 , Cai, et al. 2007, Gao, et al. 2007, Haÿ, et al. 2004, Pidoux, et al. 2007, Weall, et al. 2014). Immunolocalisation of LHR in human placental trophoblast demonstrated positive strong staining with hCG, while no staining was observed in nonspecific isotypic negative controls (Pidoux, et al. 2007). RT-PCR products sequencing confirmed the LHR fragments (Pidoux, et al. 2007). In the current study, the isotype goat IgG and unstained control MFIs were very similar for each individual follicle but different between follicles and patients; therefore, to optimise accuracy the auto-fluorescence and the nonspecific binding was gated and subtracted for each individual

follicle (Figure 3.6.5 and Figure 3.6.6). The resulting population contained a uniform granulosa cell population that revealed positive staining for FSHR, which is unique to granulosa cells (Hermann and Heckert 2007). The data were analysed using FlowJo software (Tree Star Inc., Oregon, USA).

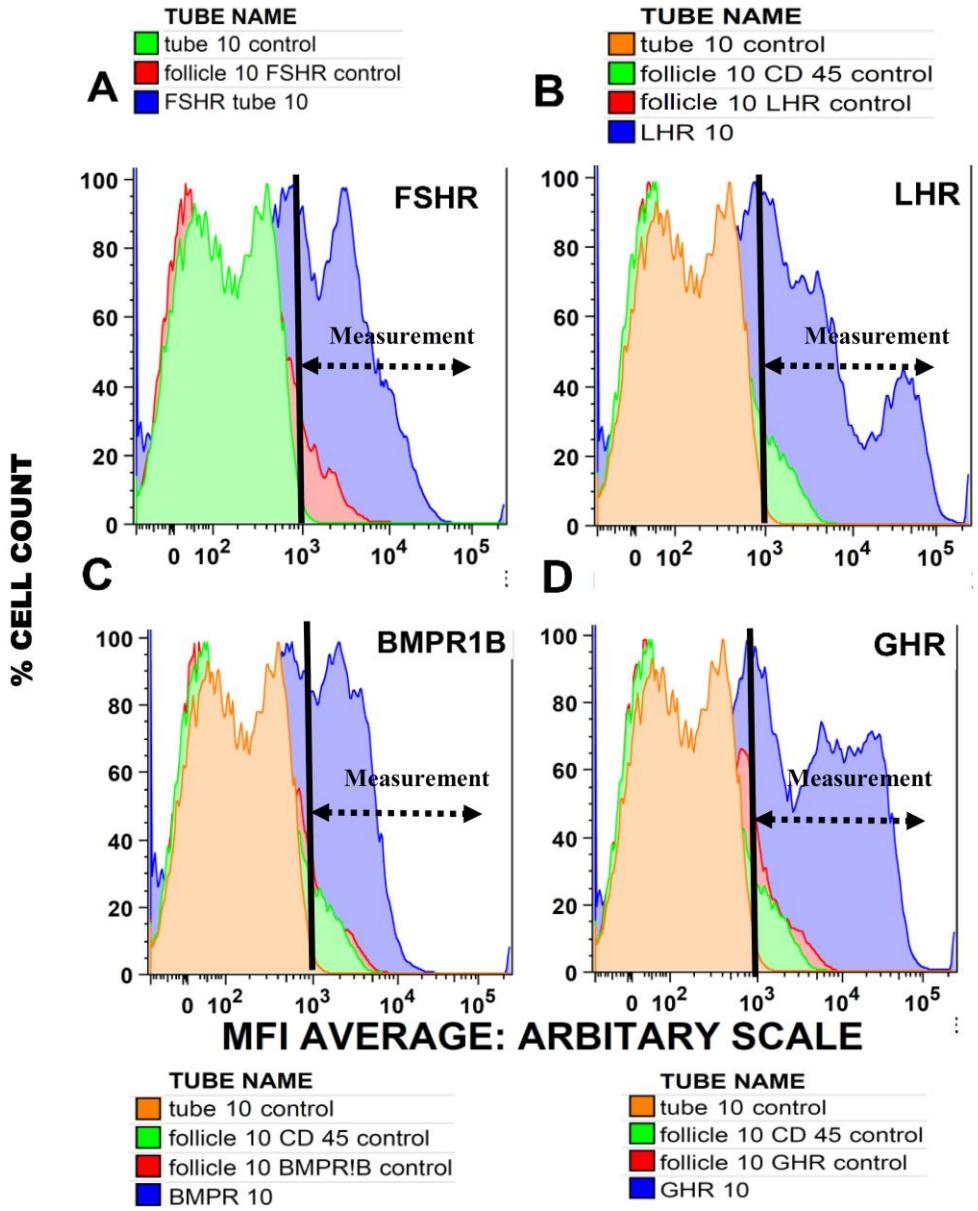


Figure 3.6.6 Measurement of mean receptor density using subtraction gating to remove auto-fluorescence and non- specific binding.

3.6.3 Sheep & Human studies: Flow cytometry, Apoptosis

The 7-AAD marker detects cell membrane disruption in cells undergoing DNA fragmentation apoptosis. However, the marker is limited because it does not differentiate between functionally intact mitochondria with cellular DNA fragmentation and combined mitochondrial and DNA total breakdown (Amsterdam, et al. 1997, Amsterdam, et al. 2003). In hindsight, a more appropriate detection of steroid capacity of a granulosa cell would be CYP19 (P450aromatase). The results however, are still valid because the level of DNA breakdown is a measure of cellular health, and further research is yet to determine how long the apoptotic luteal cells continue to contribute to steroidogenic activity in the corpus luteum, protected by the high levels of progesterone.

Arrival of sheep uterus and ovaries. Collection was spread over a two week period of time.

Separately immunolabelled granulosa cells from individual follicles.

Transported to CSIRO, Armidale, NSW.

Flow cytometry CSIRO, Armidale, NSW.

Figure 3.6.7 Armidale NSW Collection, harvest and analyses of sheep samples.

For the detection of apoptosis and necrosis, the assessment was applied on the same aliquots that were used for the immunolabelling of FSHR with a double-direct method, as previously described (Figure 3.6.7) (Demchenko 2013, Riccardi and Nicoletti 2006). Briefly, after washing the cells with the prescribed annexin V phospholipid-binding, calcium-dependent

buffer, a monoclonal antibody to annexin V conjugated to the fluorochrome phycoerythrin (PE) and the nucleic acid dye, 7-amino-actinomycin (7-AAD) (BD Biosciences, Perth, WA, Australia), were added at for a final concentration of 5 µl/1x10^6 cells. In addition, 7-AAD is a nucleic acid dye used in place of propidium iodide (PI) to further reduce spectral overlap, and is excluded from cells with an intact cell membrane. The solution was incubated in the dark for 15 min at room temperature, as previously reported, and validated against propidium iodine (Schmid 1992, Vermes, et al. 1995). A combination of unstained cells, cells stained with only PE annexin V, and cells only stained with 7-AAD, were used as positive and negative controls, and to establish gating limits (Figure 3.6.8).

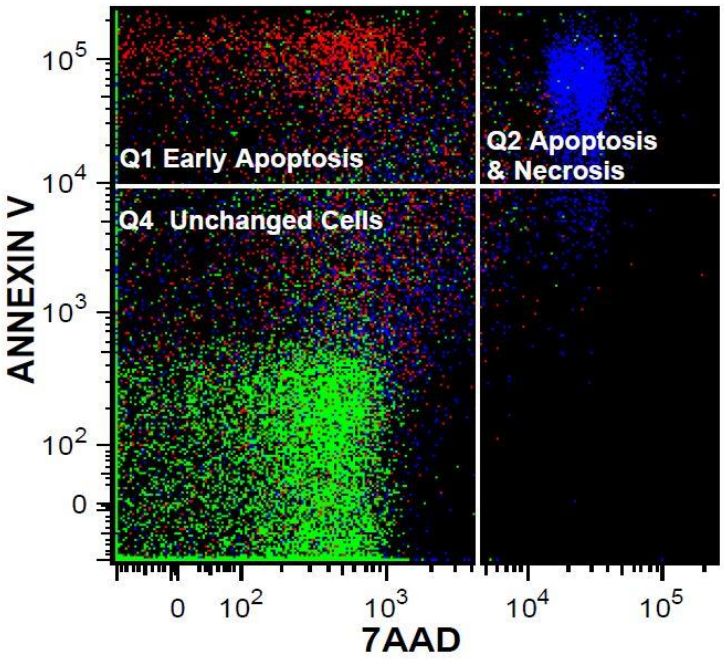


Figure 3.6.8 Apoptosis and necrosis quantification by immunolabelling and flow cytometry. Flow cytometric analysis of granulosa cells stained with fluorescent antibodies for annexin V and 7-AAD to depict the viability of the cells. Quadrant 1 (Q1); positive for annexin V and negative for 7-AAD, which indicates only early apoptosis with an intact cell membrane. Q2; positive for annexin V and 7-AAD, which indicates apoptosis and necrosis. Q4; negative for annexin V and 7-AAD which indicates an intact cell membrane with no externalisation of the phospholipid phosphatidylserine and no apoptosis and necrosis. The frequency of granulosa cell apoptosis or unspecified cell death was graphed Q2.

PE annexin V and 7-AAD were graphed into quadrants (Figure 8). Quadrant 1 (Q1) events were positive for PE annexin V and represent the very early stage of apoptotic granulosa cells. Q2 was positive for both PE annexin V and 7-AAD and therefore represents a later stage of apoptosis and necrosis. Cell membrane integrity breakdown in Q2 allowed 7-AAD to penetrate, whereas in Q4, unchanged cells showed no externalisation of the phospholipid, and were receptive to the annexin stain, yet were negative to 7-AAD penetration (Figure

3.6.8). All of the graphs for apoptosis and necrosis were based on Q2 that indicates a combination of apoptosis and necrosis. Q1 was not reliable because the positive stain for early apoptosis may have been induced during cell preparation, and therefore would not be a true indication of cell health status (Amsterdam, et al. 2003, Demchenko 2013).

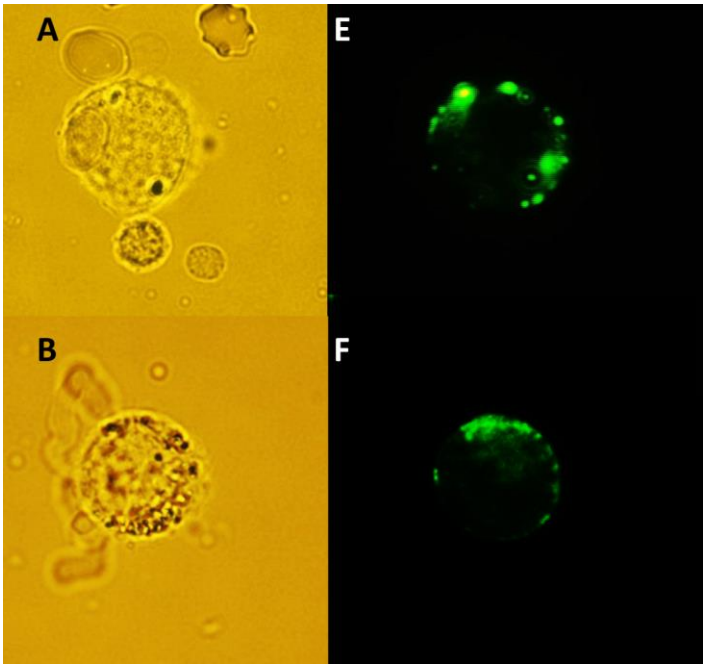
3.6.4 Human & GH co-treated: Flow cytometry, apoptosis

For the detection of early DNA fragmentation, the assessment was applied on the same aliquots used for the immunolabelling of FSHR. The antibody CD45 was required because of the method of collection and blood contamination in the human study. CD45 was not used on the FSHR sample because of the uniqueness of FSHR on the granulosa cells; hence, there would be minimal spectral overlap.

3.7 Fluorescent microscopy

3.7.1 Sheep: Fluorescent microscopy

Re-suspended 10 µl aliquots of FSHR, BMPR1B, and LHR immunolabelled, live granulosa cells were placed on slides and visualised to characterise and confirm receptor expression (Figure 3.7.1) using an Olympus DP 70 camera fitted to a Olympus BX-51 upright fluorescent microscope with a 40x UPlan N 0.4 NA objective (Olympus Imaging Australia, Macquarie Park, Australia). Pelleted aliquots of PE annexin V immunolabelled samples were similarly visualized for apoptosis. Fluorescent microscopy revealed a positive staining of the cell membrane-bound FSHR, BMPR1B, and LHR (Figure 3.7.2 E to G) as an intermittent, bright, ring-like pattern around the cells. Positive staining for early apoptosis was revealed by labelling of annexin V (Figure 3.7.2 H).



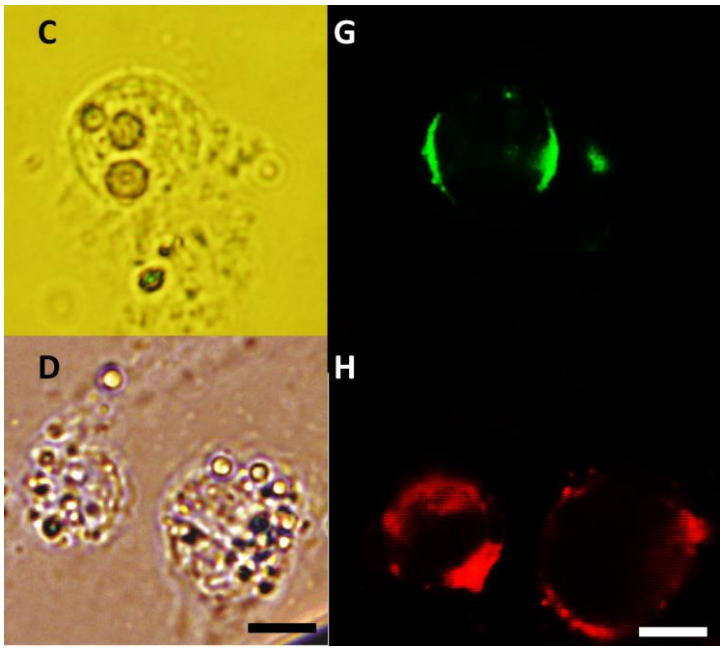
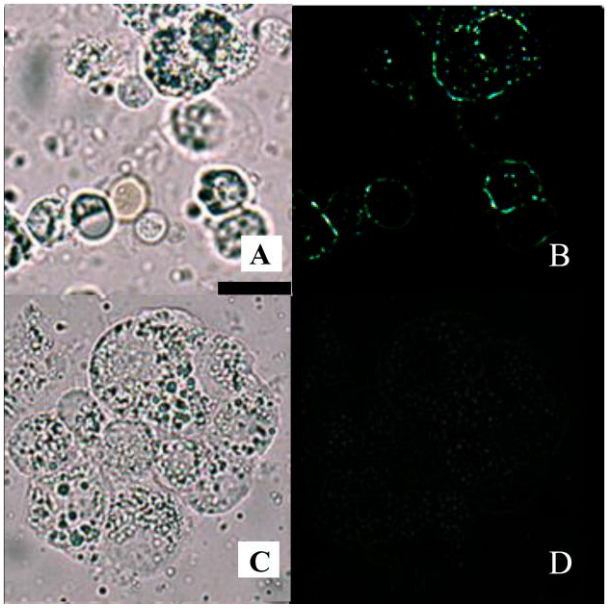


Figure 3.7.1 Characterisation of the presence of the receptors at the cell surface and early apoptosis.
(A) Living un-luteinised granulosa cell (centre), red blood cell (top), and white blood cell (bottom) (E) polyclonal goat antibody FSHR anti-goat donkey IgG conjugated with Alexa 488, which demonstrates specific fluorescence for cell membrane expressed FSHR. (B and F) A granulosa cell labelled with anti-BMPR1B. (C and G) Labelled with anti-LHR. All showing cellular membrane receptor expression viewed under a fluorescent microscope and light microscope. (D and H) Granulosa cells labelled with annexin V conjugated to PE, showing cellular membrane phospholipid externalisation, indicative of early apoptosis. Viewed under a fluorescent microscope. Scale bar equal to 7.5 µm.

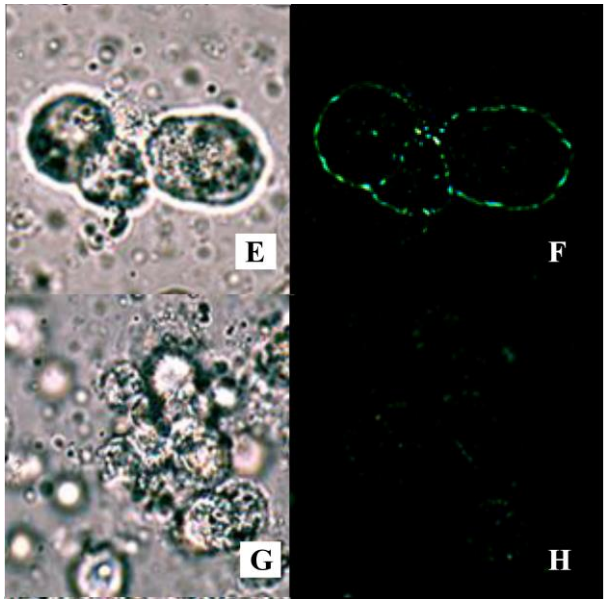
3.7.2 Human & GH co-treated: Fluorescent microscopy

Re-suspended 10µl aliquots of FSHR, BMPR1B, and LHR immunolabelled, live granulosa cells were placed on slides and visualized using an Olympus DP 70 camera fitted to a Olympus BX-51 upright fluorescent microscope with a 40x UPlan N 0.4 N.A. objective; (Olympus Imaging Australia, Macquarie Park, Australia),(Figure 3.7.2). Fluorescent microscopy revealed a positive staining of the cell membrane-bound FSHR, BMPR1B, and LHR as an intermittent, bright, ring-like pattern around the cells. All control samples showed negative staining. Granulosa cells ranged from 8 µm to 25 µm, the average of 15 µm for granulosa cells

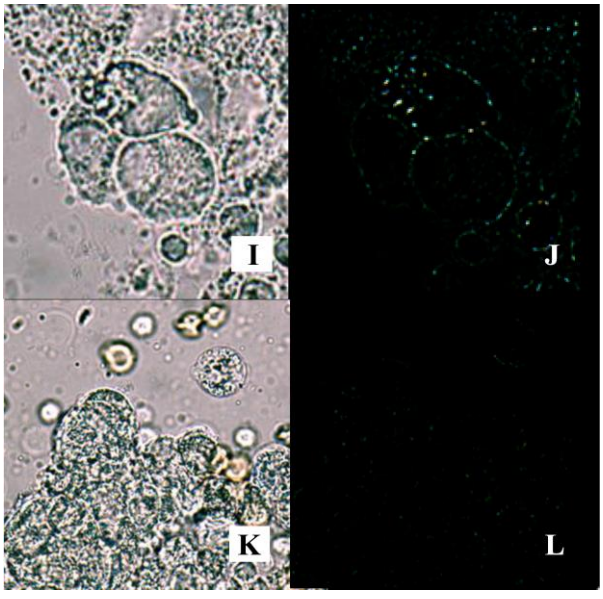
FSHR



BMPR1B



LHR



GHR

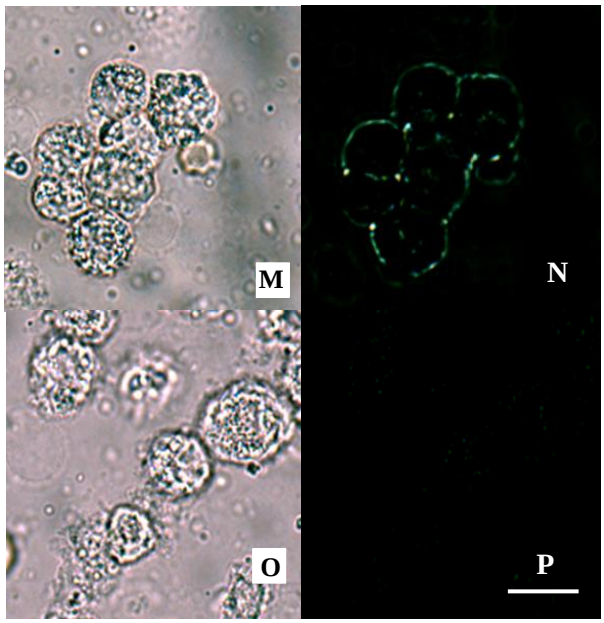


Figure 3.7.2 Live human granulosa-luteal cells immunostained, positive cell surface receptor expression, and negative for nonspecific binding.

Live human granulosa luteal cells with positive and negative (pre-absorbed or blocking agent). (AB) positive FSHR, (CD) negative FSHR (EF) positive BMPR1B (GH) negative BMPR1B (IJ) positive LHR (KL) negative LHR (MN) positive GHR (OP) negative GHR. Bar 10 µm

3.8 Cell culture

3.8.1 Human cell culture

Human IVF granulosa cells, were collected and isolated as described above, and were then assessed for cell vitality using trypan blue exclusion using a Hemacytometer (Baxter Scientific). Trypan blue is excluded from the cell by an intact cell membrane. A cell suspension of 50 µl was mixed with 200 µl of culture medium and 250 µl of trypan blue solution (0.4% v/v). The sample was then diluted to a concentration of 75 000 viable cells/50 µl. Cells were seeded into wells containing 200 µl pre-equilibrated medium to give a final concentration of 3 x 10^5/ml. Plates were incubated in a 5% CO_2 and 95% , air humidified, at 37 ºC (Shi, et al. 2010). Media was gently removed from the wells and replaced with conditioned media (175 µl) after three hours, 24 hours and at 48 hours. The collected media was stored at -80ºC until biochemical analysis. All cell cultures were repeated a minimum of three independent collections.

3.8.2 Preparation of media

Culture medium used throughout granulosa cell culture were based on protocols previously published (Campbell, et al. 1996, Glister, et al. 2004a, Glister, et al. 2001, Gutierrez, et al. 1997, Knight and Glister 2001). The culture medium consisted of McCoy's modified medium supplemented (Sigma, M-8403) supplemented with: Antibiotic/Antimycotic solution 1% v/v (Sigma A9909), insulin 10 ng/ml, bovine pancreas, (Sigma, I-1882), L-Glutamine 2

mM (Sigma, G-7513); HEPES (Sigma, H-4034) ; 10 mM, apo-transferrin 5 µg/ml (Sigma,T-2036) and sodium selenite 5 ng/ml (Sigma, S-9133), BSA 0.1% (Sigma, A-9418), androstenedione 10-7 M. The cells were cultured in complete absence of serum to avoid spontaneous luteinisation.

3.9 Biochemical Analysis

Follicular fluid collected from follicles 17 to 23 mm were analysed for testosterone, FSH, and LH using a random access immunoassay system (Siemens Medical Solutions, Bayswater, Victoria, Australia). Follicular fluid, testosterone, FSH, and LH were analysed undiluted, whereas oestrogen and progesterone were diluted manually 1:1000 with a multi-diluent and, when required, a further manual dilution of progesterone; 10 x and oestrogen; 5 x. Percentage coefficient of variance (CV) for a concentration range 137.4 pmol/L to 3257 pmol/L was oestrogen = 5.2; LH = 3.9; FSH = 2.9; testosterone = 5.9; progesterone = 9.4.

3.10 Statistical analysis

3.10.1 Sheep: Statistical analysis

MFI was obtained using an average of ~4000 granulosa cells/follicle for the direct measurement of mature functional receptor protein density. All of the data were subject to statistical verification by one-way ANOVA with an uncorrected Fisher's least significant difference (LSD) for follicular size and genotype using GraphPad Prism 6 (GraphPad Software, Inc., CA, USA). Values in graphs are means ± S.E.M., and differences were considered significant if *p<0.05, **p<0.01, ***p<0.005, and ****p<0.001. The letter, such as 'a', signifies a statistical difference to the matching letter, and an attached asterisk (a*) indicates the significance level for the size follicle. The results from an analysis of a contingency table by a two tailed, Fisher's exact test were used.

3.10.2 Human IVF & GH co-treated

Mean fluorescent intensity (MFI) was obtained using ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size using GraphPad Prism 6 Values in graphs are means ± S.E.M., and differences were considered significant if *p<0.05, **p<0.01, ***p<0.005, and ****p<0.001. The letter, such as 'a', signifies a statistical difference to the matching letter, and an attached asterisk (a*) indicates the significance level for the size follicle.

CHAPTER 4

Characterisation of granulosa cells and steroidogenesis

CHAPTER 4 Characterisation of granulosa cells & steroidogenesis

4.1 Introduction

The ovarian reserve of primordial follicles has been strongly correlated to the number of small antral follicles (AFC) at the start of a menstrual cycle in humans (Hansen, et al. 2011, van Rooij, et al. 2005). It has also been found that a lower AFC is associated with infertility (Rosen, et al. 2011, Scheffer, et al. 1999). Circulating hormone levels are a reflection of follicle growth and development of the oocyte. The granulosa cells within the follicle convert androgen to oestrogen under the control of the gonadotrophins FSH and LH, secreted by the anterior pituitary. Small antral follicles are stimulated to continue growing based on the density of FSHR and LHR during the time of dominant follicle selection (Mihm, et al. 2006, Zeleznik 2001). The dominant cohort of follicles continues to produce increasing amounts of oestrogen until a threshold for the cycle is reached, and cell differentiation begins. In the large antral follicle, the granulosa population is composed of cells at different stages of differentiation, luteinisation and apoptosis or necrosis (Figure 4.1.1).

Towards the middle of the menstrual cycle, high levels of oestrogen and progesterone receptor activation trigger the LH surge, and many changes take place in the follicle which result in reduced proliferation and altered steroid production (Gordon, et al. 2010). These changes also induce maturation of the oocyte, facilitate rupture of the follicle, and expulsion of the oocyte (Fan, et al. 2009, Gordon, et al. 2008).

The granulosa cell expands and reorganises the content of the cytoplasm, forming new organelles, particularly smooth endoplasmic reticulum (SER) for progesterone production (Figure 4.1.1) (Motta 1969). The SER and mitochondria are assembled in close association with the nucleus (Motta 1969, Nottola, et al. 2006). The mitochondria and large, round, lipid droplets are closely associated with the SER, all of which have substantially increased in number, and cluster around the nucleus to increase efficiency of steroid synthesis (Nottola, et al. 2006).

During luteinisation, granulosa cells form irregular microvilli and tight junctions between the cells, whereas, in an apoptotic granulosa cell, the cell membrane disintegrates and spaces form (Guraya 1971). The apoptotic granulosa cell continues to produced steroids in large antral follicles until complete mitochondrial breakdown (Figure 4.1.1) (Amsterdam, et al. 2003).

The present study aims to characterise the morphological changes during folliculogenesis, and the associated change in steroid synthesis of the granulosa cell.

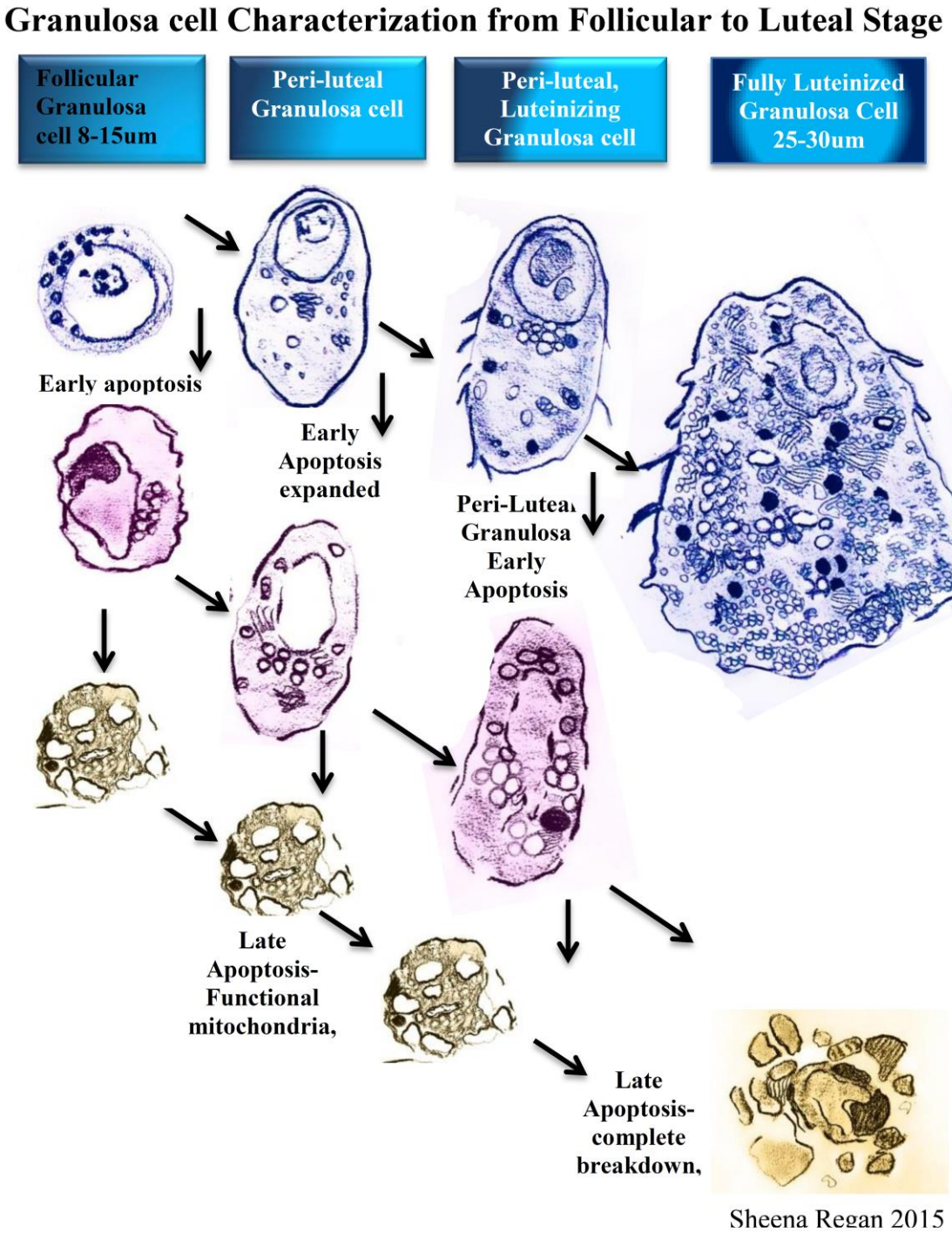


Figure 4.1.1 Ovarian granulosa cell development and steroidogenic differentiation and apoptosis.

The apoptotic granulosa cell continues to synthesise hormones until the mitochondrial membrane is disrupted. The functioning apoptotic granulosa cell undergoes reorganisation of the cell cytoplasm creating blebs of non-cytoplasmic organelles, whereas the luteinising cell creates microvilli and expands the cytoplasm. Based on findings from (Amsterdam, et al. 2003, Guraya 1971 , Motta 1969).

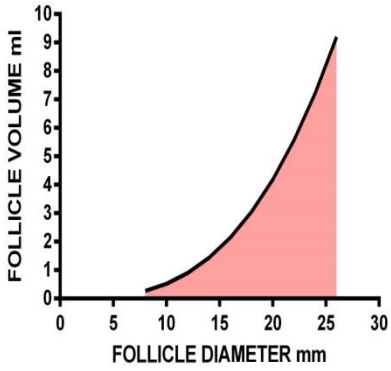
4.2 Collection Method

Methods are described in detail in the General Methods Chapter (page 38). Granulosa cells were collected from hCG/LH induced follicles from patients receiving IVF exogenous gonadotrophins to artificially stimulate multiple follicles. Serum was analysed using biochemical analysis on the days leading up to collection and on the day of collection. Human IVF granulosa cells, were collected and isolated, and were then assessed for cell vitality using trypan blue exclusion using a Hemacytometer (Baxter Scientific). Cells were seeded into wells containing 200 µl pre-equilibrated medium to give a final concentration of 3×10^5/ml (General Methods Chapter, page 53). Plates were incubated in a 5% CO_2 and 95%, air humidified, at 37 ºC (Shi, et al. 2010). Media were gently removed from the wells and replaced with conditioned media (175 µl). The collected media were stored at -80ºC until biochemical analysis. All cell cultures were repeated a minimum of three independent collections.

The peak oestrogen concentration in serum was used to predict the follicular health of the follicle as opposed to the serum levels collected at the time of follicle aspiration. When the largest follicles reached 18 mm, a LH surge trigger injection of 10 000 IU hCG/LH was administered. Then 36 hours later, the follicles were punctured and aspirated by guided ultrasound to collect the fluid, granulosa cells and oocyte, via transvaginal oocyte aspiration (TVOA). Serum was analysed using biochemical analysis on the days leading up to collection, and on the day of collection.

IVF patients undergoing treatment were examined in a natural cycle and during exogenous rFSH stimulated cycles. In addition, a patient with normal fertility (prior naturally conceived births) provided a control sample during a natural cycle. During IVF treatment, the patient is stimulated with rFSH to enhance follicular recruitment and growth, and suppression of LH and FSH release from the anterior pituitary. Sustained high doses of rFSH recruit and develop multiple antral follicles that produce oestrogen and progesterone by the follicular cells.

The granulosa cells that lines the cavity of the follicle were suspended in the follicular fluid and the medium used to flush the follicle. The initial aspiration of the follicle was performed to yield a pure sample of follicular fluid for analysis. At the time of guided ultrasound for collection of the oocyte, measurement of the follicle diameter was made by the measurement of the pure follicular fluid, and cross-referenced with the measurement from the ultrasound (Figure 4.2.1).



Sheena Regan 2015

Figure 4.2.1 Volume of follicular fluid surrounding the oocyte in a follicle.
Measurement of the diameter of the ovarian follicle by ultrasound and by the quantity of follicular fluid collected during trans vaginal aspiration using guided ultrasound (TVOA).

4.3 Results and Discussion

Peri-luteal granulosa cells from different size follicles collected from IVF patients were cultured for 48 hours. The oestrogen and progesterone concentration was measured after one hour in culture, and later at 24 hours and 48 hours (Figure 4.3.1). The capacity of the granulosa-luteal cells from the small and medium follicles to produce oestrogen and progesterone was substantially lower than the large follicle during the one hour culture. All the follicles had been exposed to hCG/LH for a 36 hour period, and it was predicted that luteinisation would be in progress (Figure 4.3.2).

The earliest signs of luteinisation are the appearance of large lipid droplets, which contain cholesterol for progesterone synthesis (Nottola, et al. 2006). In the current study the small follicles in culture produced greater amounts of progesterone than oestrogen, which indicates early luteinisation, and the transition from the follicular to the luteal phase previously documented (Westergaard, et al. 1986). Previously, follicles collected in a natural cycle, produced a greater concentration of oestrogen than progesterone up to 16 mm in diameter, but during pre-ovulatory maturation (17-20 mm), oestrogen reduced and progesterone synthesis increased exponentially. (Westergaard, et al. 1986). Moreover, the oestrogen levels in follicles less than 7 mm were very low.

The acquisition of FSHR (~4-7 mm) gives the granulosa cell the ability to convert theca derived androstenedione to oestradiol. The increase in FSHR induces the expression of LHR on the granulosa cell surface (Figure 4.3.3). As the density of LHRs increase, the progesterone synthesis increases sequentially with follicle size and peaks in the pre-ovulatory follicle (Figure 4.3.1). During the follicular phase, the serum level of LH retains a basal rate

(Santoro, et al. 2003); inferring that, before the LH surge is initiated, the density of the receptor increases, not the level of pituitary LH secreted (Jeppesen, et al. 2012, Peng, et al. 1991a). As the ovarian reserve is depleted, the basal LH rate increases, and has been shown to negatively influence pregnancy rate and fertility (LaPolt and Lu 2001, Rotmensch, et al. 1986, Stanger and Yovich 1985).

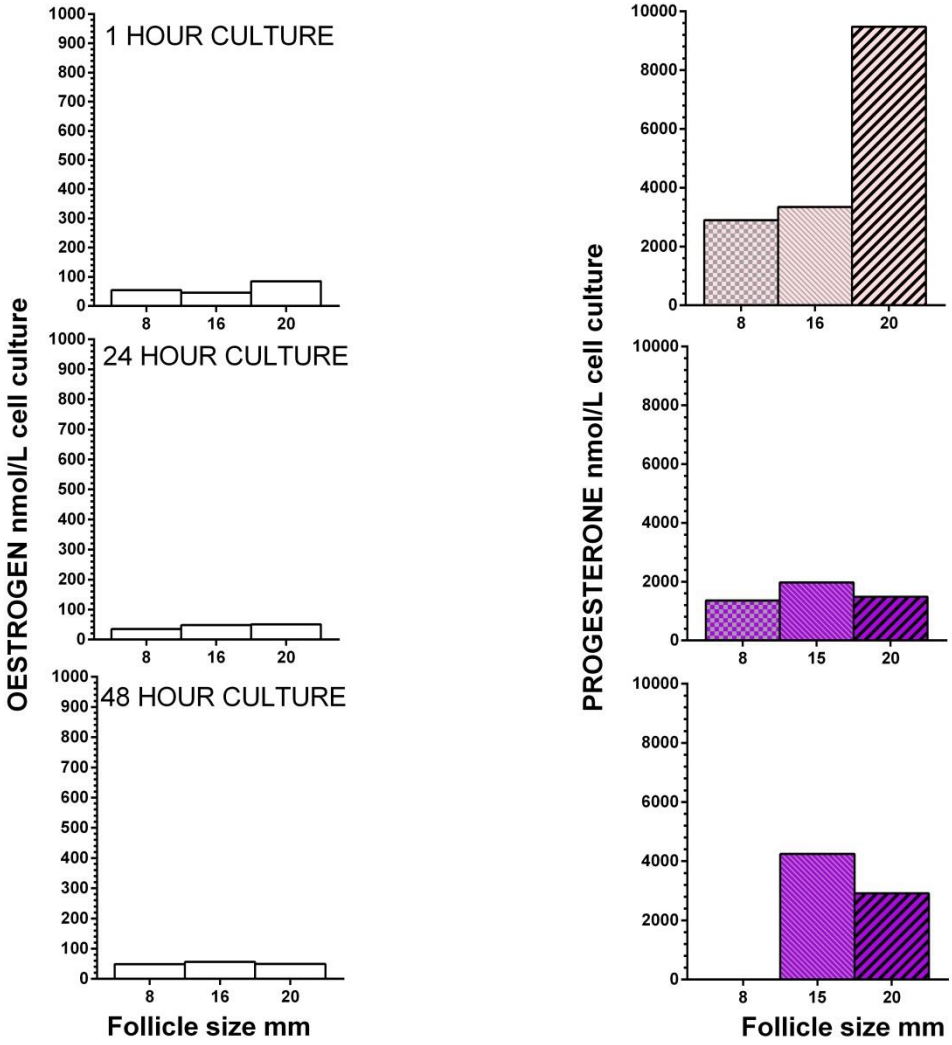


Figure 4.3.1 Oestrogen and progesterone synthesis from cultured granulosa cells collected from IVF patients during the peri-luteal phase.

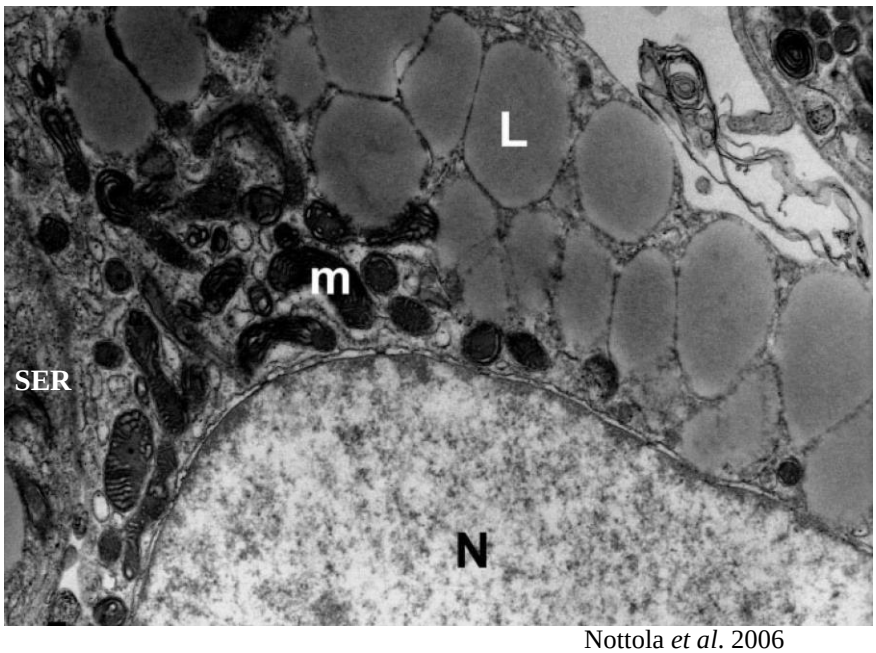
During an IVF cycle, the granulosa cells from small follicles appear more compact and smaller in diameter (Figure 4.3.3). The granulosa cells from large follicles have a heterogeneous group of granulosa cells in different stages of luteinisation (Figure 4.3.3). The luteinised granulosa cell has an expanded cytoplasm with prominent lipid droplets clustered around SER, and are closely associated with numerous mitochondria and well as defined golgi apparatus in the cytoplasm (Figure 4.3.2). The lipid droplets can be clearly observed in granulosa cells as grape like spaces (Figure 4.3.3). In the small follicle, viewed under a light

microscope, the cytoplasm was smaller and compact, with some evidence of expansion (Figure 4.3.3).

The pre-ovulatory follicle granulosa cells obtained from a natural cycle, healthy patient, had compact large granulosa cells, with some cells exhibiting an expanded cytoplasm and lipid droplets (Figure 4.3.4). The LH surge was just starting (LH: 18 IU), and the progesterone surge had not commenced (Progesterone: 4.5 pmol/L) (Figure 4.3.4). In the cell culture, the small follicle progesterone synthesis was initially similar to the medium size follicle; however, the progesterone concentration in the small follicle after 48 hours in serum free culture was reduced, which indicates that the progesterone precursor cholesterol contained within the lipid droplets was probably depleted (Figure 4.3.2). The oestrogen synthesis was maintained at a constant level in all follicle sizes due to the androstenedione fortified medium.

4.3.1 Conclusion

In summary, when exposed to a hCG/LH surge, granulosa cells are stimulated to mature, which results in differentiation of small, medium and large antral follicles. The extent of luteinisation is dependent on the stage of development of the follicle, at the time of the LH surge.

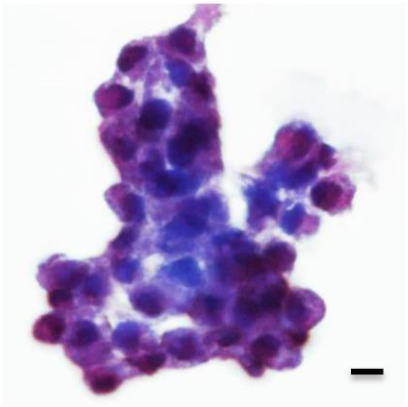


Nottola *et al*. 2006

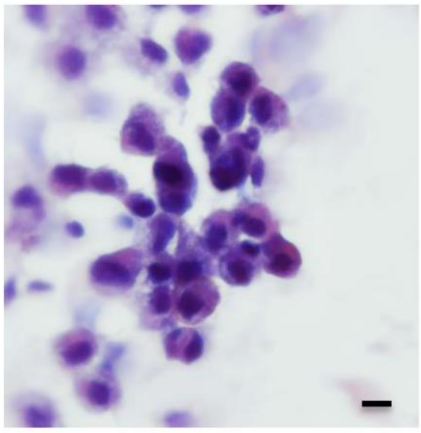
Figure 4.3.2 Granulosa cell undergoing luteinisation viewed by an electron microscope.

Early signs of luteinisation are the accumulation of lipid droplets containing precursor cholesterol for progesterone synthesis in the granulosa cell. The nucleus (N) contains dispersed chromatin, and in the cytoplasm, the mitochondria (m) and smooth endoplasmic reticulum (SER) are in close association with the lipid droplets (L), transmission electron microscopy (TEM: x10000) (Nottola, et al. 2006).

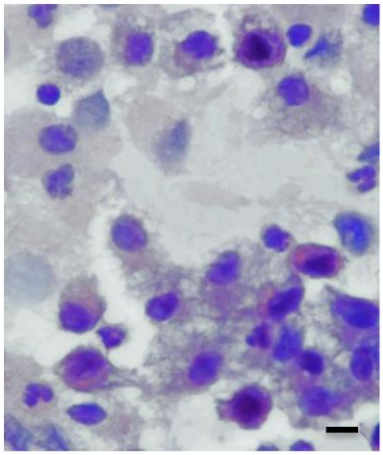
Granulosa cells from a small follicle of 8 mm



Granulosa cells from a medium follicle of 15 mm



Granulosa cells from a large follicle of 23 mm

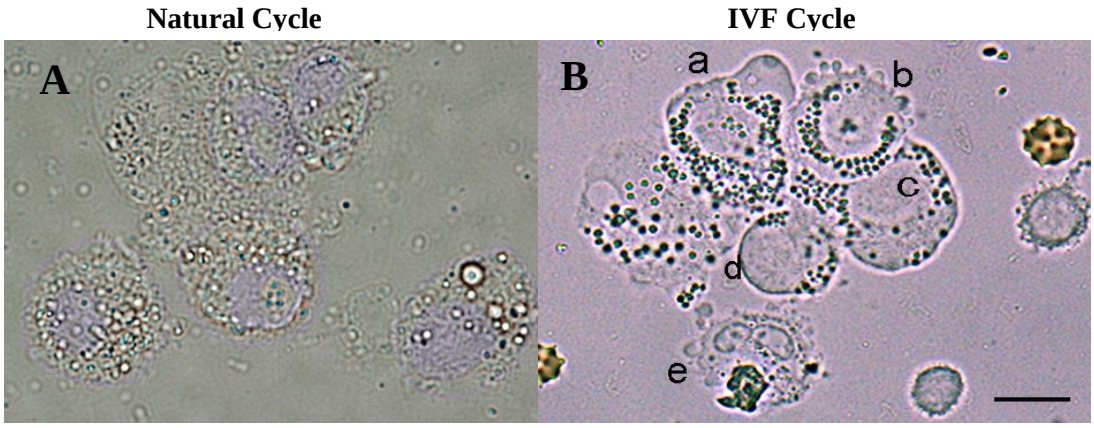


Sheena Regan 2013

Figure 4.3.3 In vivo small, medium, and large human IVF follicles containing granulosa cells at different stages of luteinisation.

Small antral follicle of 8 mm, compact morphology with large nucleus compared to cytoplasm. (B) Medium size antral follicle of 15 mm post dominant follicle selection, showing larger granulosa cells with compact morphology similar to the small antral follicle. (C) Granulosa cells from a large antral

follicle of 23 mm, showing expanded cytoplasm with many lipid droplet spaces. A heterogeneous population with few granulosa cells with a compact morphology, and many with an expanded cytoplasm. Oil red O and Harris stained. Bar = 10 µm. granulosa cell.



Sheena Regan 2014

Figure 4.3.4 Peri-luteal granulosa cells and cell morphology.
Granulosa cells from an 18.5 mm follicle during a natural cycle collected at the start of the LH surge, before the progesterone surge; a normal healthy patient 41 y with an AFC of D. (B) Granulosa-luteal cells from an IVF stimulated patient, post LH surge; 20 mm follicle. An expanded cytoplasm (a) clustering of organelles around nucleus (b), with cytoplasmic extrusions or blebbing indicating apoptosis (b) Expanding cytoplasm (c): Un-expanded small granulosa cell (d) Granulosa cell undergoing mitotic division (e).

CHAPTER 5

Ovulation Rate and Receptor Expression in Sheep

Title: Flow cytometric analysis of FSHR, BMPR1B, LHR and apoptosis in granulosa cells and ovulation rate in merino sheep

CHAPTER 5 Ovulation Rate and Receptor Expression in Sheep

5.1 Introduction

Ovarian follicular maturation relies on the gonadotrophins, FSH and LH to regulate cyclic recruitment of dominant follicle selection that governs the ovulation rate in animals and in humans (Ginther, et al. 2005, McNatty and Henderson 1987b, Mihm and Evans 2008b). It is commonly believed that intra-ovarian growth factors such as the BMPs influence the steroidogenic activity of LH and FSH directly, and indirectly downstream of the receptor (Otsuka 2010). The cellular mechanism responsible for the selection of the dominant follicle cohort remains elusive (Andersen, et al. 1993, Ginther, et al. 2005, Knight and Glister 2006, Mihm and Evans 2008b, Westergaard, et al. 1986).

Previous studies have delineated the initial role of FSH as a stimulatory growth factor for the proliferation of follicular cells and the oocyte, culminating in a mid-follicular peak of FSH (Mihm and Evans 2008b, Thomas, et al. 2005). A sharp decline in FSH causes the follicles still dependent on FSH to succumb to atresia, which results in a dominant follicle cohort. The follicles survive, because they are mature enough to be FSH independent, or they become more responsive to FSH, because of the greater concentration of FSHRs. Manipulation of FSH through the use of exogenous FSH forms the basis of artificial reproduction treatment (ART) in humans. By artificially increasing FSH in ART, the FSH concentration remains high, thereby supporting the smaller FSH-dependent follicles; this results in multiple pre-ovulatory follicles for collection and fertilisation. Treatment with high doses of FSH is a systemic whole body approach to artificial stimulation, and has associated risks such as hyper stimulation, hormonal disruption, and discomfort. An in depth understanding of follicular regulation might lead to improved targeted approaches to infertility treatment.

It has been known for some time that the Booroola Merino genotype (BB), with a naturally occurring point mutation of the superfamily of cytokines TGFβ type 1 receptor, BMPR1B, has an increased ovulation rate (Mulsant, et al. 2001). The cellular mechanism responsible, however, has not been revealed (Campbell, et al. 2003, Crawford, et al. 2011, Mulsant, et al. 2001, Souza, et al. 2001, Wilson, et al. 2001). Evidence from the previous findings on the BB has shown that the attenuation of BMP signalling produces elevated FSH levels, and the initial number of primordial follicles are reduced; yet the rate of recruitment of primary follicles is substantially lower and results in a greater retention of the ovarian reserve in the mature ewe (Ruoss, et al. 2009, Xia, et al. 2003). Given that FSHRs are not expressed in primordial follicles, it is unlikely that FSH plays a role in this initial recruitment (Ruoss, et al. 2009). The BMP ligands have been implicated in the stimulatory regulation of primordial to

primary follicle recruitment, particularly BMP4 and BMP7, which provides some explanation for the reduction reported (Ruoss, et al. 2009).

The mutation in BMPR1B in BB sheep provides a unique opportunity to study the roles of BMPs in ovarian function including follicle development, growth, ovulation, and the clinical treatment of subfertility in humans. The response of both the ovary and the pituitary to gonadotrophic stimulation appears responsible for the increased ovulation rate of the BB mutation (Campbell, et al. 2003, Fry, et al. 1988, Hampton, et al. 2004, Hudson, et al. 1999). The results have led us to initiate further research to delineate the direct cellular mechanism responsible for the increased response of the ovary to gonadotrophins, which leads to an increased ovulation rate, and to provide further insight into the interplay between the intra-ovarian BMP ligands and the regulatory FSH and LH in terminal-end folliculogenesis.

It has been hypothesised that if there is reduced initial primary follicle recruitment, greater survival of antral follicles would be required to deliver a greater ovulation rate. Direct measurement by flow cytometry of the protein expression of FSH, LH, and BMP mature membrane-bound receptors on granulosa cells, and apoptosis and necrosis, was undertaken to fully delineate the natural point mutation effect of the BB responsible for the increased ovulation rate. We therefore, hypothesise that the attenuation of the BMP signal would provide a more direct and accurate intra-ovarian method of increasing the ovulation rate in ART.

5.2 Materials and methods

Methods are described in detail in the General Methods Chapter (page 38).
A total of 17 Merino sheep with an average age of 4 years and 9 months ± 2 months were used (Table 5.2:1and Table 5.3:1). Five ewes were genetically confirmed as homozygous Australian Booroola strain (BB); with a flock average ovulation rate of four to six during peak breeding season, (Cummins, et al. 1983) and an ovulation rate of three, based on the corpus luteum (CL) formation in two Booroola sheep at the time of slaughtering, because of miss timed ovulation. Subsequently, the remaining follicles after ovulation were classified as luteinised (Table 5.2:1). The corpora lutea formed in the post-ovulation groups were included in the total number of follicles recruited per genotype, and they were added to the largest follicle size for that genotype (Table 5.3:1).

Table 5.2:1 The number of sheep and individual follicles analysed prior to ovulation and post ovulation.

Genotype	Sheep before ovulation	Follicles	Dominant follicles	Small (1.0-2.1 mm)	Medium (2.2-2.9 mm)	Large (3.0-4.5 mm)	Extra large (4.6-7.0 mm)
WT	8	64	8	25	19	14	6
BB	3	20	3	9	5	6	0
Genotype	**Sheep post-ovulation**	**Luteinised follicles**	**CL per sheep**	**Small (1.0-2.1 mm)**	**Medium (2.2-2.8 mm)**	**Large (3.0-4.5 mm)**	**Extra large (4.6-7.0 mm)**
WT	4	35	1.5	13	14	8	0
BB	2	10	3	7	2	1	0

Corpus Luteum (CL)

5.3 Results

The largest follicle in BB ovaries was 4.5 mm, whereas the largest follicle in the WT animal was 7 mm (Table 5.3:1). The percentage of small follicles, including the pooled follicles, was similar between genotypes, 53% WT and 57% BB. The medium WT follicles collected contributed to 22% of the adjusted total, whereas, the medium BB follicles had significantly less, at 14%. The large follicle numbers were reversed, with the BB having significantly more large ovulatory follicles compared to the WT extra-large ovulatory, 28.6% and 9.3% respectively, $p<0.011$ (Table 5.3:1). The absolute relative frequency derived from the total number of follicles collected was compared to the number of ovulatory-size follicles (Figure 5.3.1). The BB did not produce any extra-large follicles, and the corpora lutea formed was included in the largest follicle group for the genotype.

Table 5.3:1 Recruitment and loss of follicles during folliculogenesis in WT and BB Merino sheep.

				Small (1.0-2.1mm)			Medium (2.2-2.9mm)		Large (3.0-4.5mm)		Extra Large (4.6-7.0mm)	
Genotype	**Total sheep**	**Total follicles ñ**	**Total per sheep**	**Small follicles pooled**	**Total collected**	**Total (%)**	**Total collected**	**Total (%)**	**Total collected**	**Total (%)**	**Total collected**	**Total (%)**
WT	12	150	12.5	42	80	53	33	22	23	16	14 ñ	9.3a
BB	5	49	9.8	12	28	57	7	14	14 ñ	28.6*	0	0

*Significantly greater number of ovulating follicles: $p<0.011$ ñ Including Corpus Luteum

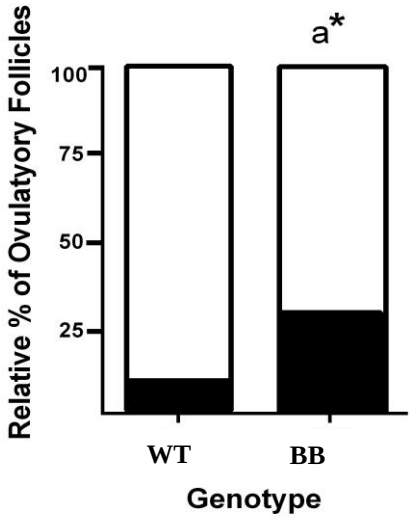
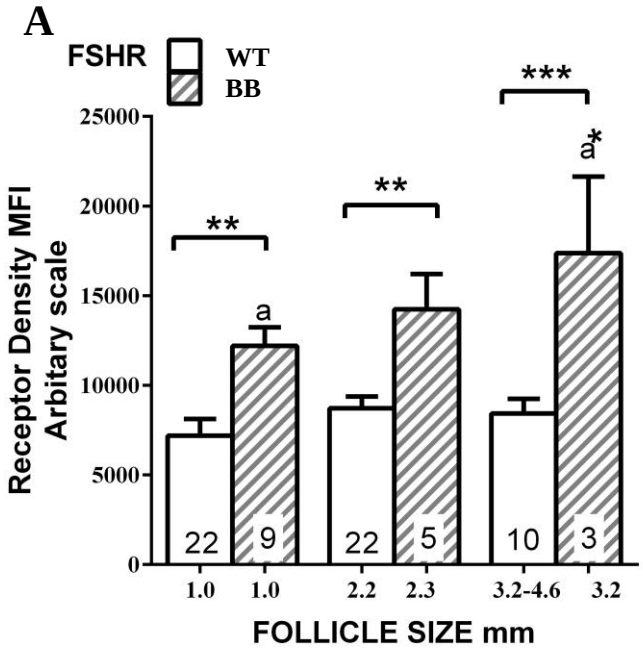


Figure 5.3.1 Relative frequency of ovulatory follicles for genotype.
The total number of follicles (clear section of bar) from 1 mm to terminal-end of folliculogenesis and ovulation including corpus luteum are presented for the BB and WT sheep. The ovulatory follicles (black section of bar) being the largest follicles for the genotype, including CL, provide evidence of the relative survival of recruited follicles for the WT and the BB genotype. The letter 'a' signifies a statistical difference in the retention of ovulatory follicles during folliculogenesis. Analysis of the contingency table using a two tailed, Fisher's exact test was performed with a p value of 0.011.

5.3.1 Flow cytometry quantification of BMPR1B, LHR, and FSHR

The genotype effect on density of BMPR1B expression in granulosa cells from small, medium and large subordinate follicles of BB sheep was significantly higher than that in the WT ($p<0.05$, $p<0.01$ and $p<0.005$, respectively, Figure 5.3.2 B). Similarly, the density of FSHR was significantly increased in the BB sheep from the small antral follicle to the large follicle ($p<0.01$, $p< 0.01$, $p<0.005$, respectively, Figure 5.3.2A); whereas, the LHR density was greater (Figure 5.3.2 C).



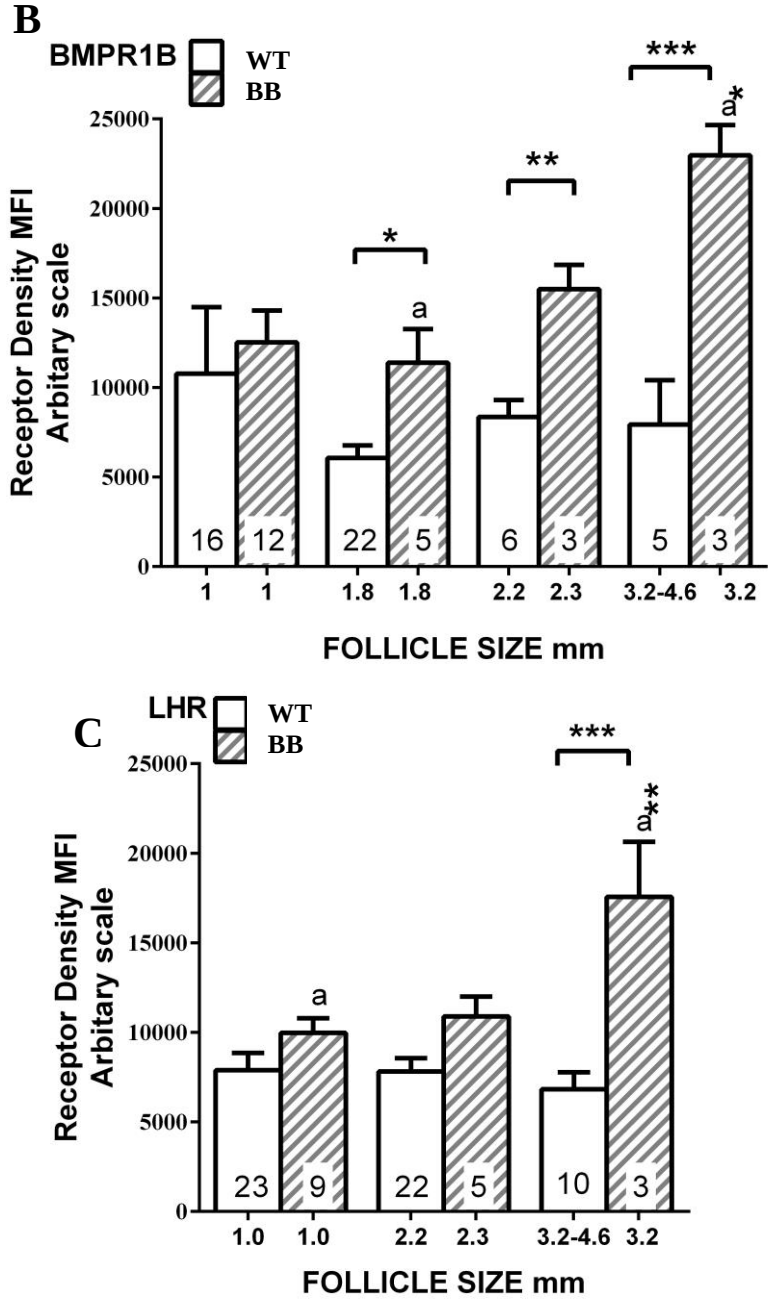
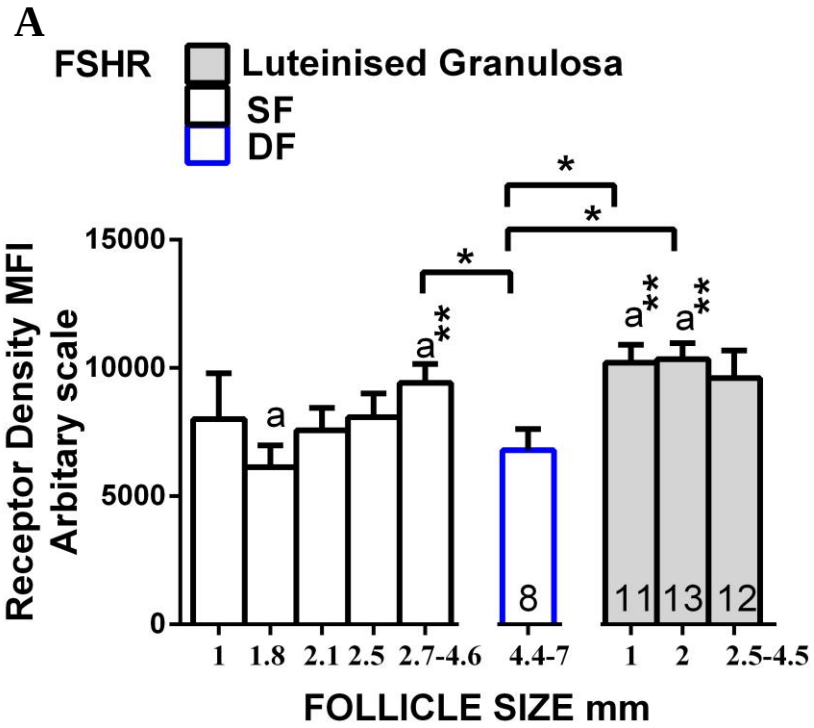


Figure 5.3.2 Genotype comparison of WT and BB receptor density from granulosa cells from subordinate follicles.

All of the follicles are subordinate follicles (i.e. the leading dominant follicle based on size; and the granulosa-luteal follicles were not included because of the mutation-independent down-regulation; see material and methods. (A) The average granulosa cell FSH receptor density, as measured by mean fluorescent intensity (MFI) of ~4000 granulosa cells harvested from individual follicles were analysed by immuno-fluorescent labelled, flow cytometry for BB (striped) and WT (clear) genotypes. Exclusion gating based on positive FSHR signal and comparative size difference produced a uniform granulosa sample. The same gating was applied to all samples, and auto-fluorescence and nonspecific binding were removed by subtraction gating. (B) The average BMPR1B receptor density MFI for small antral follicles was divided into smaller size classes to reflect the down-regulation. (C) The average LHR receptor density MFI for small antral follicles. Statistical verification using one-way ANOVA with an uncorrected Fisher's least significant difference (LSD). Values in graphs are means ± S.E.M., *p<0.05; **p<0.01 and ***p<0.001. The letter, such as, 'a' signifies a statistical difference to the matching letter, with its attached asterisk (a*) which indicates the significance level for the size follicle. The number of subordinate follicles analysed is shown for each follicle size class.

The WT sheep had elevated levels of FSHR in the largest of the subordinate follicles ($p<0.01$), followed by down-regulation in the leading dominant follicles ($p<0.05$, Figure 5.3.3A). The granulosa-luteal cells revealed that the FSHR level after ovulation was significantly higher than the dominant pre-ovulatory follicle was, and this finding was maintained across the follicle sizes ($p<0.05$, Figure 5.3.3A). In the WT sheep, the BMPR1B density was significantly decreased in a pattern of biphasic down-regulation as was seen with the 1.8 mm subordinate follicle and the 4.4-7 mm dominant follicle ($p<0.05$ and $p<0.01$, Figure 5.3.3B). The granulosa-luteal cells from the sheep ovaries that had ovulated, and contained one or more corpora lutea, revealed that the BMPR1B level after ovulation was significantly higher than in the dominant pre-ovulatory follicles, and this finding was maintained across the follicle sizes ($p<0.05$, Figure 5.3.3B). The LHR density was greater in the WT sheep, at 4.2 mm, and down-regulated in the 4.6 mm subordinate follicle and the 4.4 mm dominant follicles ($p<0.05$, Figure 5.3.3C). The largest of the dominant follicles (5-7 mm) had similar levels of LHR density to those of the granulosa-luteal cells, whereas the LHR expression increased and was then down-regulated in the smaller dominant follicles ($p<0.05$, Figure 5.3.3C).



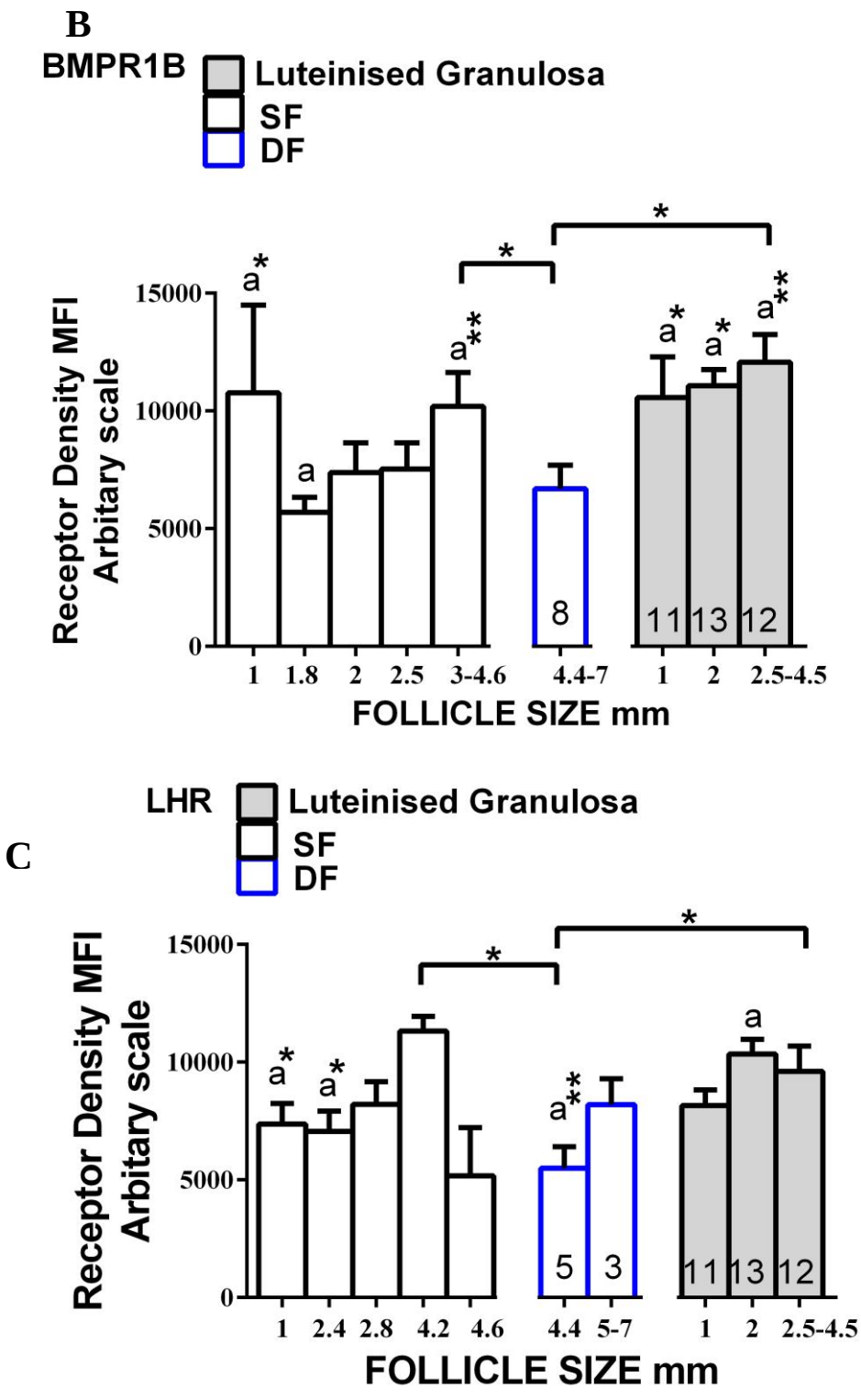
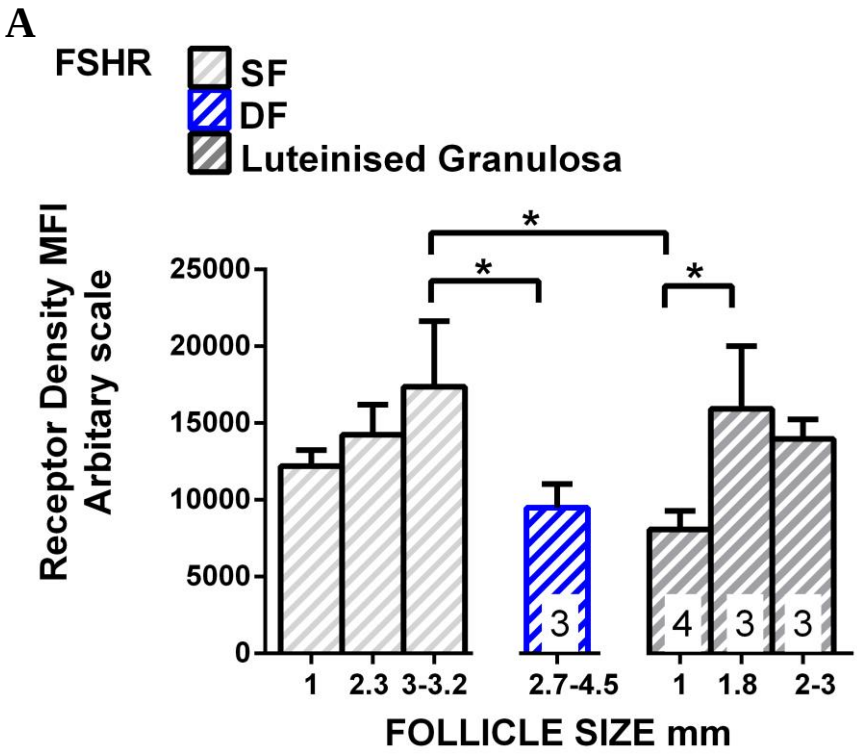


Figure 5.3.3 WT sheep receptor density and follicle size in granulosa cells from dominant compared to subordinate and luteal follicles.

(A-C) Receptor density measured by mean fluorescent intensity (MFI) of subordinate (pre-ovulation) granulosa cells (clear) and (pre-ovulation), dominant follicle (blue); and (post-ovulation), granulosa luteal cells (grey) for different follicle size groups for the WT genotype. (A) FSHR; (B) BMPR1B; (C)

LHR. Statistical verification using one-way ANOVA with an uncorrected Fisher's LSD. Values in graphs are means ± S.E.M., *p<0.05; **p<0.01. The letter, such as, 'a' signifies a statistical difference to the matching letter, with its attached asterisk (a*), which indicates the significance level for the size follicle. The number of dominant follicles and the number of follicles from ovulated sheep are indicated for each follicle size class.

The FSHR demonstrated a trend of increasing FSHR followed by a significant down-regulation of receptors in the dominant follicle and a significant up-regulation in the granulosa-luteal cells ($p<0.05$, Figure 5.3.4A). There was a significant steady increase from 1.0 mm to 3.2 mm in subordinate granulosa cell BMPR1B density with follicle size in BB sheep ($p<0.005$, Figure 5.3.4B). The leading dominant follicles were down-regulated ($p<0.005$), followed by a trend of increasing levels of BMPR1B in the granulosa-luteal cells. The LHR density was significantly increased from 1.0 mm to 3-3.2 mm follicles ($p<0.01$), followed by the down-regulation of LHR density in the dominant pre-ovulatory follicles ($p<0.01$, Figure 5.3.4C). The granulosa-luteal cells demonstrated no significant difference to the down-regulated follicles of the dominant pre-ovulatory follicles in the BB sheep (Figure 5.3.4C).



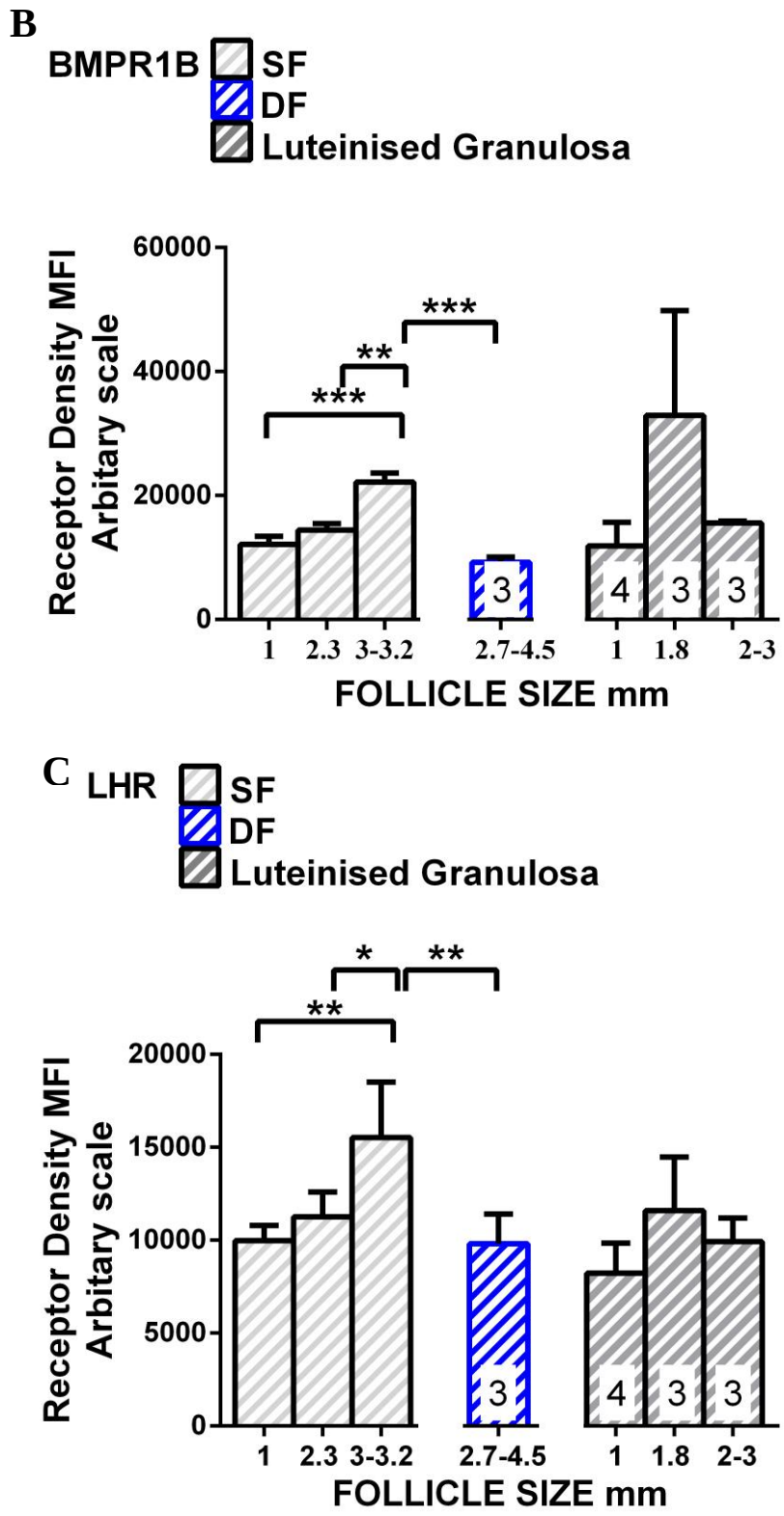


Figure 5.3.4 Booroola receptor density and follicle size in granulosa cells from dominant compared to subordinate and luteal follicles.

(A-C) Receptor density measured by mean fluorescent intensity (MFI) of subordinate (pre-ovulation) granulosa cells (clear) and (pre-ovulation), dominant follicle (blue); and (post-ovulation), granulosa-luteal cells (grey) for different follicle size groups for the WT genotype. (A) FSHR; (B) BMPR1B; (C)

LHR. Statistical verification using one-way ANOVA with an uncorrected Fisher's LSD. Values in graphs are means ± S.E.M., *p<0.05; **p<0.01 and ***p<0.005. The number of dominant follicles and the number of follicles from ovulated sheep are indicated for each follicle size class.

5.3.2 Flow cytometry quantification of apoptosis

The apoptosis and necrosis levels were significantly elevated in the largest of the subordinate follicles of the WT sheep (p<0.01, Figure 5.3.5). The BB genotype did not demonstrate elevated levels in the largest of the subordinate follicles. The WT dominant follicle reported greater apoptosis and necrosis levels as compared to the BB dominant follicle of 3 mm (b, p<0.05, Figure 5.3.6). An unusually large BB follicle was recorded, and the level of its apoptosis and necrosis was similar to the WT largest subordinate follicle of 40%. The level of apoptosis and necrosis in the BB 1 mm follicles was greater post-ovulation as compared to the WT subordinate follicles (c, p<0.05), but not as compared to the WT post ovulation follicles (Figure 5.3.6).

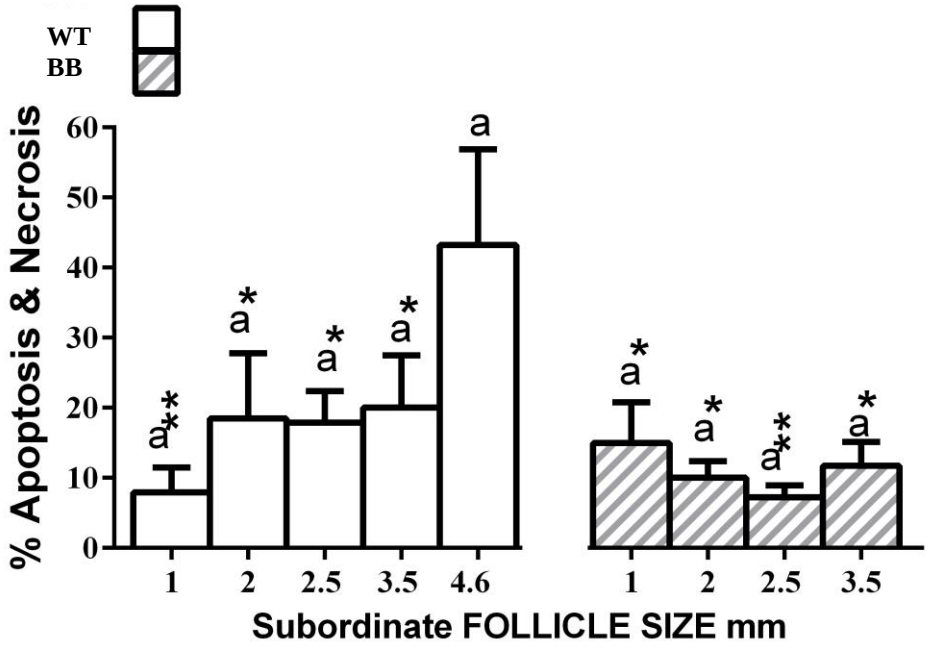


Figure 5.3.5 WT sheep compared to BB genotype: apoptosis and necrosis of granulosa cells from subordinate follicles.

All follicles are subordinate follicles, (i.e. the leading dominant follicle based on size was not included because of the mutation-independent down-regulation). Statistical verification using one-way ANOVA with an uncorrected Fisher's LSD Values in graphs are means ± S.E.M., *p<0.05 and **p<0.01. The letter 'a' signifies a statistical difference to the matching letter, with its attached asterisk (a*), which indicates the significance level for the size follicle.

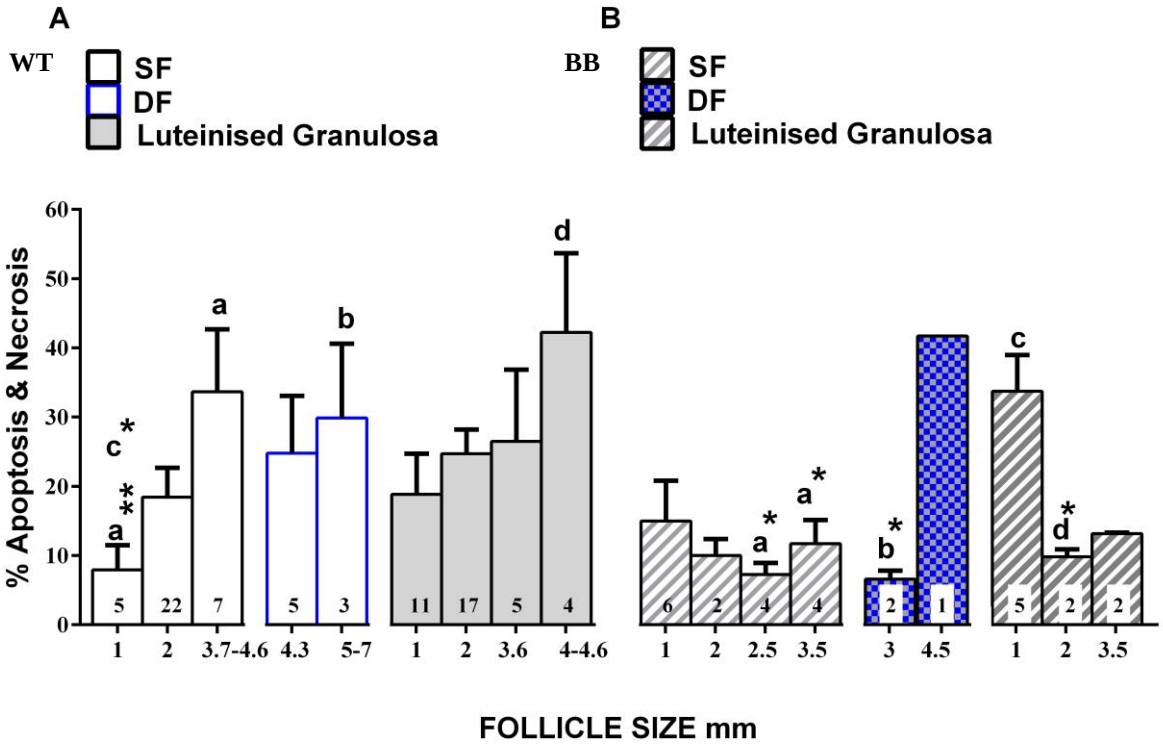


Figure 5.3.6 Comparison of genotype granulosal apoptosis and necrosis during follicle development.

WT sheep comparison of percentage apoptosis and necrosis in granulosa cells from dominant compared to subordinate and luteal follicles. Apoptosis and necrosis of subordinate follicles (pre-ovulation granulosa cells) (clear) and dominant follicles (blue); and post-ovulation granulosa luteal cells (grey) of different follicle size classes for WT genotype. (B) BB sheep comparison of apoptosis and necrosis in granulosa cells from dominant compared to subordinate follicles and granulosa luteal cells. Apoptosis and necrosis of subordinate follicles (pre-ovulation granulosa cells) (clear) and dominant follicle (blue); and post-ovulation granulosa luteal cells (grey) of different follicle size classes for BB genotype. Statistical verification using one-way ANOVA with an uncorrected Fisher's LSD. Values in graphs are means ± S.E.M., *$p<0.05$ and **$p<0.01$. The letter, such as, 'a' signifies a statistical difference to the matching letter, with its attached asterisk (a*), which indicates the significance level for the size follicle. The number of follicles is indicated for each follicle size class.

5.4 Discussion

The BMP signalling axis has been strongly associated with the gonadotrophins, FSH and LH, in the pituitary-ovarian axis of control in regulation of follicle recruitment, growth, and ovulation rate (Miyoshi, et al. 2006, Otsuka, et al. 2001b, Takeda, et al. 2012).The BMP ligands 4, 6, 7, and 15 have a stage dependent, high affinity to bind to the TGFβ type 1 receptor, BMPR1B (Mazerbourg and Hsueh 2006). The Booroola mutation that impacts the BMPR1B has been studied extensively, but the findings mainly focused on the indirect heightened physiological responsiveness of the BB compared to the WT (Campbell, et al. 2006, Fabre, et al. 2006, McNatty, et al. 1985).

In the present study's novel approach, the mature cell surface protein for FSHR, LHR, and BMPR1B were measured by flow cytometric analysis. Immunolabelled granulosa cells were collected from individual antral follicles, and harvested *in situ* to reduce contamination with other ovarian or blood cells. The receptor density was quantified by the average fluorescent intensity of ~4000 isolated granulosa cells per follicle. The selected antibodies have been previously validated by immunolocalisation in sheep by our research group (Al-Samerria and Almahbobi 2014). Positive and negative signals were used to subtract auto-fluorescence and nonspecific binding; whereas, the uniqueness of the FSHR on granulosa cells and the relative size of other cells, positively identified the population of granulosa cells.

5.4.1 Major Findings

The receptor density of BMPR1B, FSHR, and LHR was significantly increased in the granulosa cells of developing antral follicles of BB compared to the WT (Figure 5.3.2). Furthermore, the leading dominant follicle from both genotypes had reduced receptor density in comparison with subordinate follicles, indicating a vital prerequisite down-regulation prior to ovulation (Figure 5.3.3 and Figure 5.3.4).

The direct *in vivo* quantitative analysis between genotype for BMPR1B and LHR expression has been previously reported, with acknowledged limitations of follicle class and sensitivity of the technique (McNatty, et al. 1986b, Mulsant, et al. 2001). Previously, qualitative data reported that the level of *BMPR1B* mRNA in follicles from 1.to 3 mm antral follicles indicated that the *BMPR1B* mRNA was not reduced in the BB (Mulsant, et al. 2001). Recently, quantitative findings have shown that the level of *BMPR1B* mRNA in 1 to 3 mm follicles to be equivalent in the BB, which is not consistent with the findings (Estienne, et al. 2015). In the Estienne et al. (2015) study, the follicles were divided into small (1-3 mm) and large (3-5 mm) groups, preventing an accurate comparison of the genotype effect due to the large range of development within the 1-3 mm range (McNatty, et al. 1985), and the down-regulation of the leading dominant follicle in the 3-5 mm range (Gasperin, et al. 2014). It has been reported previously (McNatty, et al. 1986a) that the BB follicles reach steroidogenic capacity at 2 to 2.5 mm compared to the WT at 4 to 4.5 mm. The pooling of follicles would mask the cellular changes taking place during the follicular development in this range. In addition, in the present study the translated mature FSHR, BMPR1B, and LHR proteins were measured, as opposed to measuring the mRNA for the receptor. Differences may therefore, have resulted, seeing as the mRNA measurement would also include immature and mature proteins, that are potentially not expressed (Ascoli, et al. 2002).

5.4.2 Pattern of biphasic down-regulation in the WT

An unexpected finding of the study was the reduction in density of BMPR1B in the WT at two stages of folliculogenesis (Figure 5.3.3B). The density of BMPR1B was elevated at the time of divergence (1-1.7 mm), followed by down-regulation after dominant follicle selection (1.8 mm), which lead to a steady increase in density to a peak in the largest of the subordinate follicles (3-4.6 mm) (Figure 5.3.3). In both the BB and the WT, the leading dominant follicle (4.4-7 mm) had significantly reduced density of receptors, which demonstrated the presence of a hierarchical organisation of the follicles that has been reported to be present in sheep (Figure 5.3.3 and Figure 5.3.4). (Evans, et al. 2002, Gasperin, et al. 2014).

Chen, et al. (2009) and Estienne, et al. (2015) have also reported that the WT granulosa cells have increasing BMPR1B with follicle size, which is consistent with the present finding of an increased density of BMPR1B in subordinate follicles from 1.8 mm to the 4.6 mm, which were the largest of the subordinate follicles ($p<0.010$, Figure 5.3.2 B).

In the present study, the high BMPR1B in the BB was not present in the 1-1.7 mm follicles, possibly due to the earlier onset of development, which is typical of the BB genotype (McNatty, et al. 1985). The high level of receptor density may have occurred at a smaller BB follicle size of < 1 mm. In the BB, however, the down-regulation of the dominant follicle was comparable to the WT, albeit at a reduced size (Figure 5.3.3 and Figure 5.3.4). It is, therefore, apparent that the down-regulation mechanism is independent of the mutation as it occurred in both genotypes. BMP4, 6, 7, and 15 signal via the BMPR1B, and have been previously shown to play a regulatory role in the suppression of progesterone production before through to ovulation (Ryan, et al. 2008, Takeda, et al. 2012). The up-regulation of extracellular signal regulated kinases 1 and 2 (ERK1/2) signalling (Fan, et al. 2009) occurs coincident with the down-regulation of BMPR1B activity which suppresses progesterone synthesis (Feary, et al. 2007, Miyoshi, et al. 2006). The failure to down-regulate the BMPR1B activity would, therefore impede the process of luteinisation and prevent maturation of the follicle (Gordon, et al. 2008).

5.4.3 Gonadotrophin regulation and FSHR signalling

FSHR and LHR were found to be greater in follicles from another prolific breed (Romanov) compared to the Île-de-France ewes; and in the Chinese prolific Small Tail Han sheep with a mutation of the *BMPR1B* gene (Abdennebi, et al. 1999, Jia, et al. 2007). The quantitative data from the current research confirm and expand these observations, and propose that the mutation-induced changes to the BMPR1B signalling in the BB sheep are responsible for the

significantly up-regulated density of BMPR1B, FSHR, and LHR, and they have a cumulative effect as the follicle increases with size (Figure 5.3.2).

Quantitative genotype data on the FSHR have not been previously reported, although, FSH stimulation and granulosa cell responsiveness in the BB sheep have been previously reported (Henderson, et al. 1985). Moreover, the BMP2, 4, and 7 ligands in the WT goat, and human have been shown to increase the level of *FSHR* mRNA, whereas BMP6 was shown to increase *LHR* mRNA, which indicates a direct role for BMPs in the regulation of receptor density (Ogura Nose, et al. 2012, Zhu, et al. 2013). Furthermore, ovine granulosa cells cultured with both FSH and oestrogen increased the expression of BMPR1B (Chen, et al. 2009), which supports the present findings of an increased BMPR1B density in the growing subordinate follicles, and the reduced BMPR1B activity observed in the down-regulation of FSHR and BMPR1B in preparation for ovulation of the dominant follicle (Chen, et al. 2009). In another study, FSH and BMP ligand-induced production of oestrogen was significantly increased in the BB compared to the WT, which is also supportive of the present findings of an increased density of FSHR and LHR in the BB (Figure 5.3.2) (Campbell, et al. 2006).

In support of the dynamic role of BMPs, it has also been shown that BMP6 reduces post-dominant follicle selection in the rat, and is reduced completely in the ovulating dominant follicle (Erickson and Shimasaki 2003). Similarly, BMP15 and BMPR1B activity in the oocyte has been shown to be down-regulated before ovulation in sheep (Feary, et al. 2007). Furthermore, in cultured rat granulosa cells, BMP15 reduced the expression of *FSHR* mRNA (Otsuka, et al. 2001b). It is therefore possible that the attenuating mutation of the BMPR1B signal may reduce the suppression and lead to the up-regulation of FSHR in the BB (Otsuka, et al. 2001c).

5.4.4 Down-regulation in peri-luteal and corpus luteum

In humans, the BMP2, 6, 7, and 15 were previously, all shown to increase *FSHR* mRNA (Ogura Nose, et al. 2012). The granulosa cells, however, were not cultured in serum free culture and therefore spontaneously luteinised. This was acknowledged by the authors of the paper. They suggested that the cells were not representative of granulosa cells, but were rather granulosa-luteal cells. A reduction in the BMP inhibition therefore seems to be an initiating change towards luteinisation. The brief stage-specific, peri-ovulatory decline in oestrogen production coincides with the cessation of mitogenic growth and steroidogenic differentiation (Komar, et al. 2001). In the present study, the follicles that were not destined to ovulate continued to express a high density of receptors across all sizes in the WT, whereas, in the BB, they were inconsistent (Figure 5.3.3 and Figure 5.3.4). The possible

mechanism involved may be related to the ability of the BB to prepare multiple follicles for ovulation.

5.4.5 Steroidogenic differentiation & LHR earlier acquisition

In the past, it has been recognised that the increase in ovulation rate in BB sheep is a result of the follicles being more sensitive to FSH at an earlier follicle size (Baird and Campbell 1998, McNatty, et al. 1985). The previously reported increased sensitivity to both FSH and LH can now be attributed to the novel findings in this study, of an increased density of the mature FSHR and LHR at the cell surface, which has not previously been reported (Figure 5.3.2A and Figure 5.3.2 C). In particular, the cAMP response to FSH and LH stimulation was previously shown to increase at >3-4 mm, and >2-3 mm respectively, in the BB, which is consistent with the present findings (Figure 5.3.2A and Figure 5.3.2C) (Henderson, et al. 1985).

The up-regulation of LHR provides expanded evidence of an earlier acquisition of receptors with greater expression density at an earlier follicle size of 3 mm in the BB compared to 4.2 mm in the WT genotype (Figure 5.3.2). Furthermore, we demonstrated that the LHRs are present in granulosa cells from antral follicles. This was previously reported to be first expressed in secondary follicles in humans, but it was not observed in the rodent until late antral stage (Camp, et al. 1991, Yung, et al. 2014).

Consistent with other authors, LHR density in the present study was accelerated in the pre-ovulatory follicle in the WT and the BB and it was followed by a significant reduction in the leading dominant follicle of each animal (Figure 5.3.3C and Figure 5.3.4 C) (Jeppesen, et al. 2012, LaPolt, et al. 1992, Ophir, et al. 2014). The ovine granulosa cells in the present study would have been exposed to the LH surge that initiates cytoskeletal reorganisation, the cessation of proliferation, and the alteration of steroidogenic capacity. The process of luteinisation appears to rely on LHR down-regulation of the leading dominant follicles (Figure 5.3.4B) (Fan, et al. 2009, Izadyar, et al. 1998b).

5.4.6 Ovulation rate and apoptosis

There are several ways that a high prolificacy breed increases ovulation rate, and it appears that the BB follicles may mature early because of an increased receptor density and a reduction in atretic follicles or apoptosis, as was reported in the present study and previous studies (Driancourt, et al. 1985, Estienne, et al. 2015, McNatty, et al. 1986a). The increased FSHR and the ability to wait up to two days allows the subordinate follicles to continue to grow, which resulted in the three fold increase in the terminal-end follicles that was reported in the present study (Figure 5.3.6) and by others (Driancourt, et al. 1985, McNatty, et al.

1986a). It was indeed evident that the post-ovulation BB sheep produced three corpus lutea, and that the very large BB pre-ovulatory follicle of 4.5 mm had not ovulated, possibly because the next subordinate follicle was only 2.4 mm, which thereby delayed ovulation (Figure 5.3.5) (Driancourt, et al. 1985, McNatty, et al. 1986a). The prolonged delay may have initiated the exceptionally high level of apoptosis and necrosis that was observed, seeing that the size of the follicle was not typical for the genotype.

The finding of significantly less apoptosis and necrosis in the pre-ovulatory follicles of the BB is consistent with the number of follicles recorded at the ovulatory size in the BB, which was three times the amount remaining in the WT (Table 5.3:1, Figure 5.3.4 and Figure 5.3.5). The high level of apoptosis and necrosis in the largest of the subordinate follicles in the WT is supportive of the increased rate of follicle loss and results in fewer ovulations than the BB (Figure 5.3.6). Moreover, the WT dominant and luteal follicles continued to have high levels of cell death, whereas, in the BB, the levels were generally low pre- and post-ovulation (Figure 5.3.5). It is important to note that, in the present study, we did not excluded any follicles on the basis of oestrogen production or morphological indications of programmed DNA Fragmentation, because previous findings have demonstrated that the LHR binding assays did not reveal any difference between the follicles that were deemed non-atretic in the same size class (McNatty, et al. 1986b). Furthermore, it has also been reported more recently that granulosa cell apoptosis (which is indicated by DNA fragmentation and cytoplasmic blebbing) can be present with functional steroidogenic mitochondria (Amsterdam, et al. 2003). It is, however, acknowledged that the levels of follicle apoptosis and cell DNA fragmentation are lower in antral follicles with greater oestrogen production compared to androgen production (Yuan and Giudice 1997). The reduction in BB apoptosis may be a result of the maintained, elevated levels of FSH after divergence as compared to the declining FSH in the WT (Baird 1987), or due to the increased FSHR density being more responsive to the FSH levels available.

5.4.7 Conclusion

In conclusion, these results provide further insight into the mechanism that governs the interaction between gonadotrophins and intra-ovarian BMPs in the regulation of ovulation rate. These results are significant and central to the development of a new clinical approach to improve human female fertility. Rather than focusing on increasing the patient's response to exogenous gonadotrophins, in order to increase the ovulation rate, a targeted approach could be incorporated by manipulating the signalling pathways of BMPs. A new approach would be particularly beneficial to sub-fertile patients who respond poorly to gonadotrophin stimulation.

CHAPTER 6

Ovarian Ageing and BMPR1B

Title: The decline in ovarian ageing and the effect on the type1 TGFβ receptorBMPR1B in humans

CHAPTER 6 Ovarian Ageing and BMPR1B

6.1 Introduction

Reproductive ageing is linked to the declining capacity to regenerate cells and tissues, causing irreversible changes to ovarian cellular dynamics and ultimately reducing the capacity to reproduce. As the average age of fertility-challenged patients climbs towards 40 years, there is an urgency to characterise the cellular changes that occur in the ovary with time. The response of the ovaries to cyclic recruitment of primordial follicles forms the basis of the clinical documentation of the AFC (Almog, et al. 2011). The AFC and age are highly correlated to histologically determined ovarian primordial reserve (Hansen, et al. 2011, van Rooij, et al. 2005). As the primordial follicle reserve declines, the endocrine, paracrine, and autocrine regulation adapts to a changing environment. It is this changing landscape that requires further investigation to provide alternative treatment to preserve the primordial follicles and to adjust the cellular regulation to achieve oocyte competence and improve fertility rates in older patients.

The earlier research has highlighted the role BMP signalling has in regulating ovulation rate in sheep, and has led us to further investigate the molecular regulation of folliculogenesis by the BMPs (Regan, et al. 2015, Ruoss, et al. 2009). During a natural cycle small antral follicles with sufficient FSHR are recruited in response to the intercycle rise in FSH and one of these is subsequently selected to become the dominant follicle. Follicles with reduced FSHR and LHR become less responsive as the dependence from pituitary FSH stimulation shifts to LH, and circulating FSH concentrations decline (Lapolt, et al. 1990, Xu, et al. 1995, Zeleznik, et al. 1974). These subordinate follicles are destined for atresia. The selected dominant follicle is the one with greatest gonadotrophin responsiveness, and is dependent on the acquisition of FSHR-induced LHR by granulosa cells; and this follicle continues growing to the ovulatory stage.

Previous research has shown that at the time of declining FSH levels, a reduction in BMP 6 (Erickson and Shimasaki 2003), BMP 15 (Feary, et al. 2007) and the type 1 TGFβ receptor BMPR1B (Feary, et al. 2007) occurs after dominant follicle selection. Once selected, follicle growth and cellular proliferation continues, leading to an increased oestrogen and inhibin production by the granulosa cells. Together, these hormones suppress pituitary FSH output further, ensuring the demise of subordinate follicles. When the threshold for oestrogen's positive feedback action on the hypothalamus-pituitary axis is met, preparation for the ovulation phase begins with a cessation of cell proliferation and early luteinisation changes taking place.

During cellular and steroidogenic differentiation activin (Young, et al. 2012), insulin-like peptide 3 (INSL3) (Anand-Ivell, et al. 2013), AMH (Andersen, et al. 2010, Ogura Nose, et al. 2012, Weenen, et al. 2004), and gonadotrophin surge attenuating factor (GnSAF) activity declines from dominant follicle selection to the termination of folliculogenesis at ovulation (Martinez, et al. 2002).

The BMP ligands, 2, 4, 6, 7, and 15 form a receptor-ligand complex with the type 1 TGFβ receptor BMPR1B, and recruit the type 2 TGFβ receptor BMPR2. The complex initiates phosphorylation of the intracellular substrate molecules, receptor-regulated Smads. The Smad forms a complex with a common mediator, Smad 4, and translocates to the nucleus where transcription of genes required by the cell takes place. Smad signalling is modulated by repressor and activator molecules in the nucleus, cytoplasm and in the extracellular matrix. Alternatively, BMPs activate the non-Smad pathway MAPK such as ERK 1/2. (Inagaki, et al. 2009).

In the ovary, granulosa cell signalling induced by the gonadotrophins, FSH and LH, activate the FSHR or LHR and stimulate cAMP-PKA, which increases the CYP19A1 aromatase to facilitate oestrogen synthesis. Progesterone synthesis is inhibited by the suppression of StAR (Pierre, et al. 2004, Tajima, et al. 2003, Val, et al. 2003), which is essential for progesterone synthesis in the granulosa cell (Moore, et al. 2001b). Alternatively, or in addition, BMPs inhibit ERK 1/2 signalling, which provides inhibitory control over the balance of progesterone and oestrogen (Miyoshi, et al. 2007, Nakamura, et al. 2012, Ogura Nose, et al. 2012). BMP signalling activity has been established not only in the ovary but in the pituitary, where ERK and JNK signalling has been shown to increase LHβ (Harris, et al. 2002), and BMP 15 via ERK, to increase FSHβ (Otsuka and Shimasaki 2002). In addition, BMP 6 and 7 were shown to regulate FSHβ (Takeda, et al. 2012). Given the above regulatory evidence, it is not surprising that the spatial and temporal relationship of BMP expression patterns change with an increase in follicular size (Otsuka 2010).

The current study aimed to comprehensively profile the expression of BMPR1B in a range of patients, of different ages and stages of ovarian primordial follicle depletion, who were receiving treatment for infertility. Previous reports documenting ovarian BMPR1B expression have evaluated expression at the mRNA level in pooled follicles from different size classes. mRNA expression does not necessarily reflect expression of translated functional BMPR1B protein on the cell surface (Chen, et al. 2009, Estienne, et al. 2015). In contrast, we collected an average of ~8000 granulosa cells from individual follicles over a

comprehensive range of follicle diameters from 4 mm to 38. In addition, we have measured the granulosa cell surface-expressed mature receptor protein density for BMPR1B by immunofluorescent labelling via flow cytometry.

6.2 Methods

Methods are described in detail in the General Methods Chapter (page 38).
Granulosa cells were collected from hCG/LH induced follicles from patients receiving IVF exogenous gonadotrophins to artificially stimulate multiple follicles (Table 6.2:1). The granulosa cells were isolated and incubated with antibodies to detect the level of BMPR1B, mature protein. In addition, apoptosis and necrosis were also determined by immunolabelling via flow cytometric analyses (page 39).

Table 6.2:1 Patient ovarian reserve, based on antral follicle count (AFC) and the number of follicles collected per group.

AGE Year	**IVF Patient**	**Total Follicle**	**AFC**	**Major Group**	**Number of Follicles Collected Per Group**							
					Sub Group	**#**	**Sub Group**	**#**	**Sub Group**	**#**	**Sub Group**	**#**
21-30	8	95	20-40	A+ &A	A+	31	A	64	C	6		
31-34	11	86	13-29	A & B	A	60	B	26	C	17		
35-39	16	102	9-19	B & C	A+	6	B	50	C	16	D	30 & E 6
40-45	18	118	3-8	D & E	D	59	E	19	B	34		
40	1	Natural Cycle Healthy			D	2						

Ovarian reserve measured indirectly by the Antral Follicle Count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle: A+ = 30-39 follicles; A = 20-29; B = 13-19; C = 9-12, D = 5-8; E = ≤4.

6.3 Results

The level of granulosal BMPR1B expression was down-regulated in the 23-30 y IVF patients with a combined AFC of A+ & A in a biphasic receptor density pattern (Figure 6.3.1). The receptor density of BMPR1B initially decreased from 8 mm to 10 mm ($p<0.05$), followed by an up-regulation in the follicles to 16 mm ($p<0.01$); followed by a significant decline in follicles to the terminal-end of folliculogenesis of 24-26 mm ($p<0.05$), (Figure 6.3.1). In marked contrast, in the 40+ y group the BMPR1B density increased with follicular size ($p<0.01$) in a monophasic manner, (Figure 6.3.1). In a natural health cycle the receptor density of granulosa cells, were collected from a healthy 40+ y with an AFC of D. The two follicles of 10 mm and 18.5 mm when combined had a significantly lower density of BMPR1B compared to the largest follicles of the 40+ y IVF patients with an AFC D & E (Figure 6.3.1).

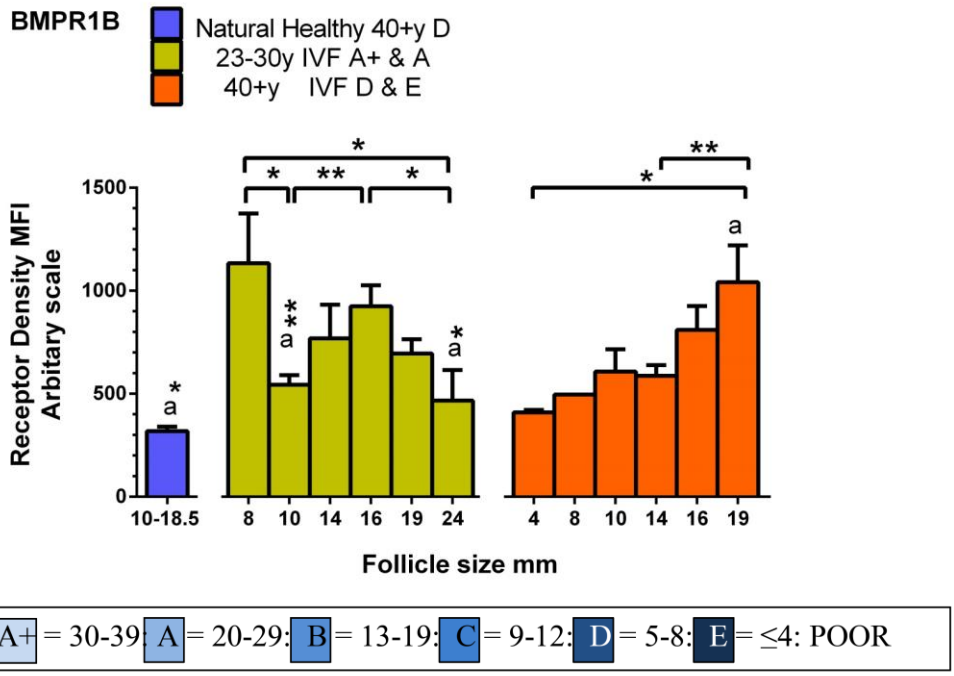


Figure 6.3.1 Granulosal BMPR1B density from follicles of different sizes collected from young and older IVF patients compared to an unstimulated natural healthy cycle.

Granulosal BMPR1B protein density and follicle size profile of a natural healthy unstimulated patient of 41y with an AFC of D before the LH surge, (blue). Patients, 23-30y stimulated, IVF cycle with an AFC of A+ & A, (olive green). Patients, 40+y stimulated IVF cycle with an AFC of D & E, (orange). Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *$p<0.05$ and **$p<0.01$. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) indicates the significance level for the size follicle.

In the youngest age group 23-30 y, the majority of the patients had an AFC of A+ & A (Figure 6.3.2). There was no significant difference between the A+ group and the A; whereas, when the ovarian reserve declined there appears to be a wide range of expression of receptors (Figure 6.3.2). The follicles from the C group patients had increased BMPR1B expression compared to the similar size follicles in the A+ & A group ($p<0.05$ to $p<0.001$, Figure 6.3.2)

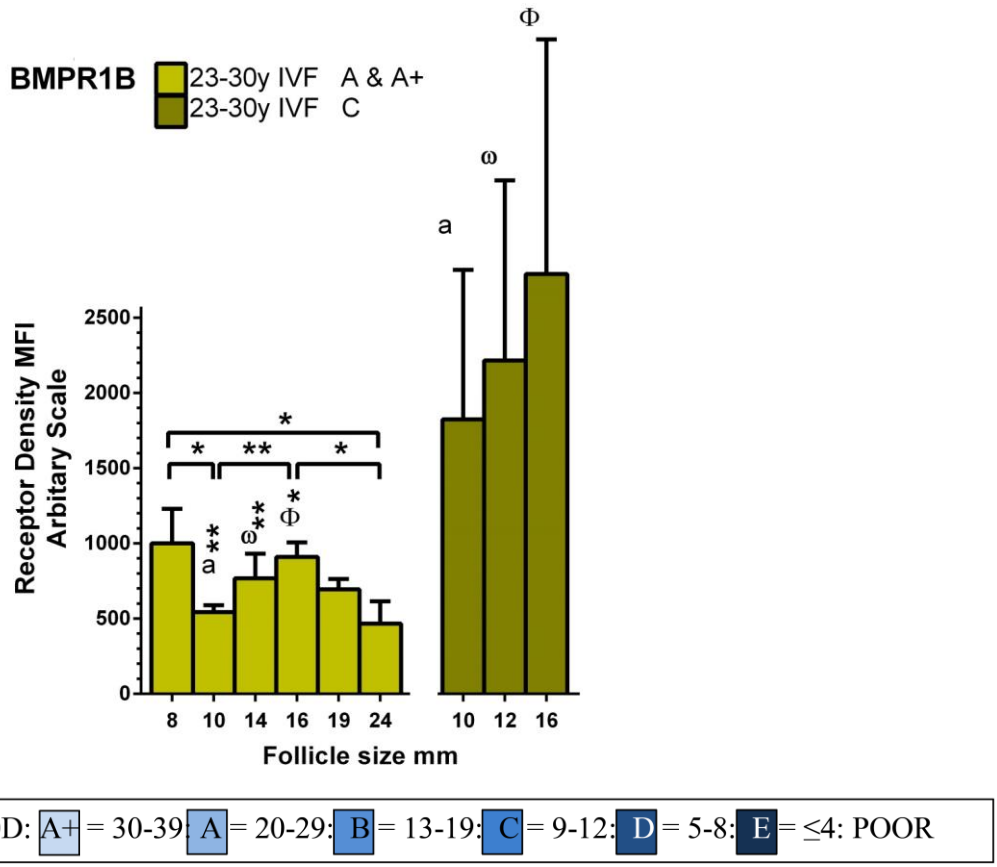
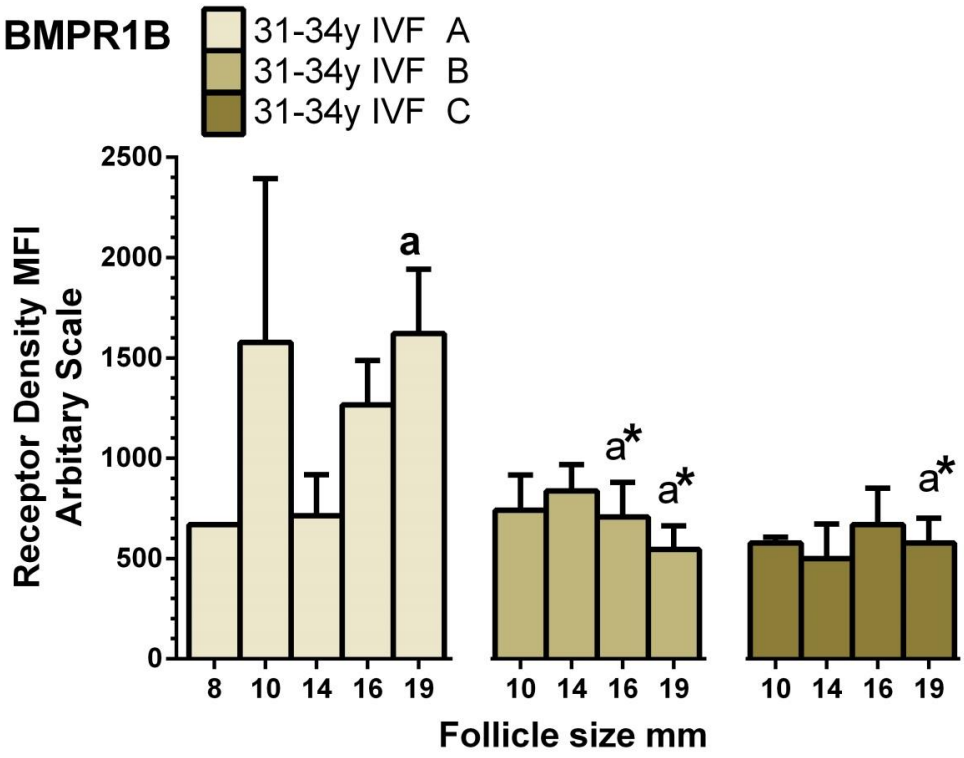


Figure 6.3.2 Granulosal BMPR1B density and ovarian reserve depletion in 23-30 y.
Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *$p<0.05$ and **$p<0.01$. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level for the size follicle.

In the 31-34 y age group the decline in ovarian reserve was associated with a loss of receptor density of BMPR1B in the granulosa cells from a peak in the 19 mm follicles in the A group to a significantly lower number in the B and C groups ($p<0.05$, Figure 6.3.3).



.

GOOD: A+ = 30-39: A = 20-29: B = 13-19: C = 9-12: D = 5-8: E = ≤4: POOR

Figure 6.3.3 Granulosal BMPR1B density and ovarian reserve depletion in 31-34 y.
Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *$p<0.05$. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) indicates the significance level for the size follicle.

The 35-39 y B & C group demonstrated a significant down-regulation in the 14 mm to 16 mm follicles ($p<0.05$), similar to the youngest age group. With a further decline of the ovarian reserve (D & E) the receptor density in the smaller follicles was reduced, ($p<0.05$), followed by a steady increase with increasing follicular size (Figure 6.3.4).

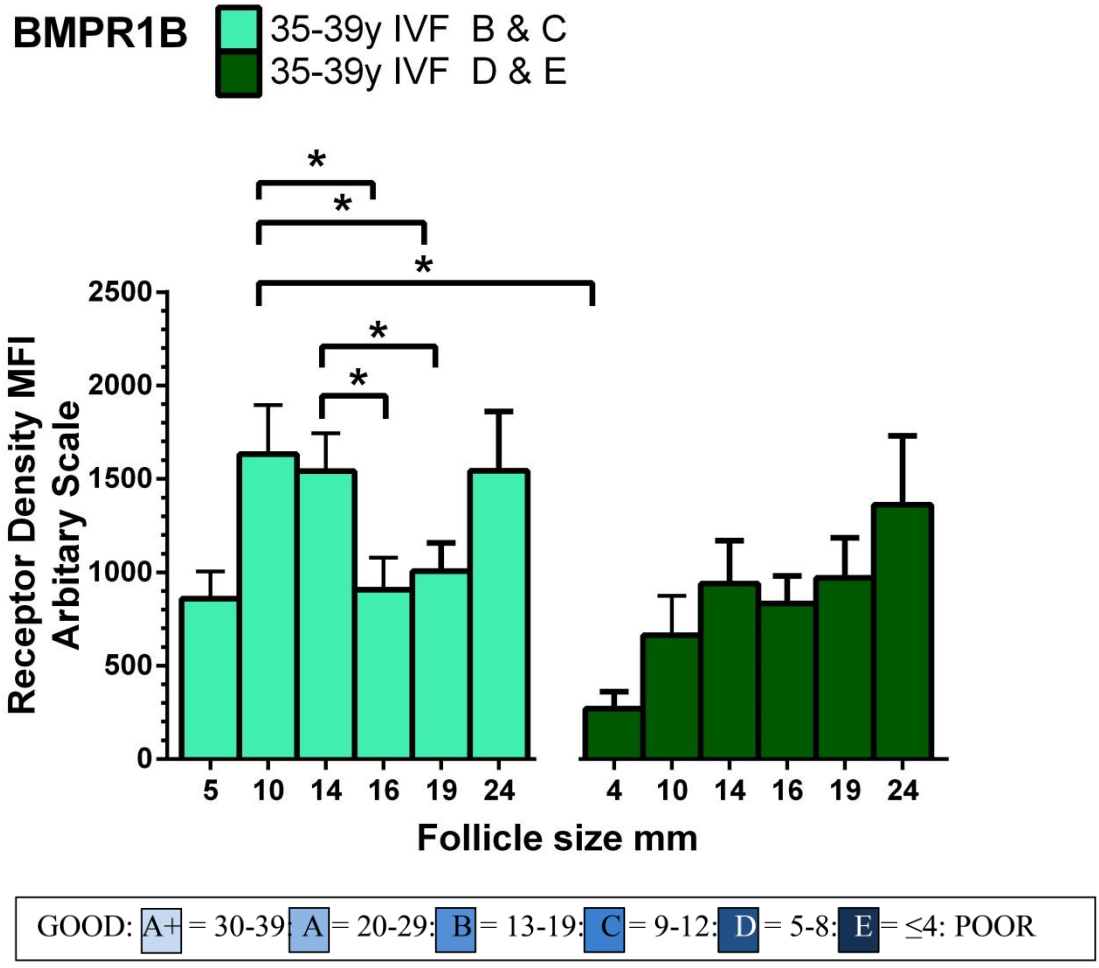


Figure 6.3.4 Granulosal BMPR1B density and ovarian reserve depletion in 35-39 y.
Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *$p<0.05$.

The 40+ y patients ranged in AFC from B to E, and were between 40-45 y, (Figure 6.3.5). The B & C patients combined demonstrate a higher BMPR1B receptor density in the small follicles followed by significant down-regulation of receptors as follicle size increased. With a decline in the ovarian reserve to D, the receptor density in the smaller follicles was reduced, compared to B. With a further decline of ovarian reserve to E ($p<0.05$, Figure 6.3.5) the receptor density significantly increased with follicle size similar to the ageing effect observed in the poor ovarian reserve youngest C patients (Figure 6.3.2).

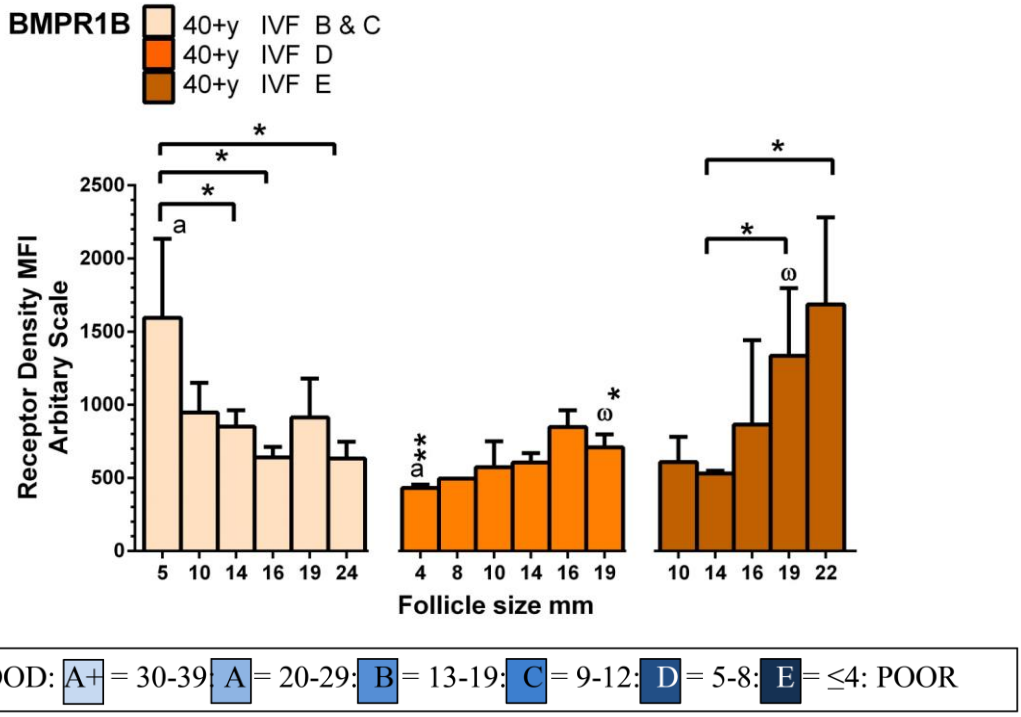


Figure 6.3.5 Granulosal BMPR1B density and ovarian reserve depletion in 40+ y.
Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *$p<0.05$ and **$p<0.01$. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level for the size follicle.

6.4 Discussion

A continuous process of activation of primordial follicles in the ovary leads to the inevitable depletion of the ovarian reserve in women (Almog, et al. 2011). The gradual decline in ovarian reserve can be indirectly measured by the number of small antral follicles at the beginning of a cycle termed the AFC (Hansen, et al. 2011). The response of the ovary to exogenous gonadotrophins used to treat infertility declines with age, which is strongly correlated to the ovarian reserve (Hansen, et al. 2011). Patients with a poor ovarian reserve are treated with increasing doses of rFSH in an attempt to increase the number of small antral follicles with sufficient FSHR to develop into pre-ovulatory follicles.

In sheep, the ovulation rate was increased when BMPR1B signalling was attenuated by the Booroola natural mutation (Chapter 5) (Regan, et al. 2015). Therefore, the role of BMPR1B, and the ovarian ageing effect on ovulation rate are under investigation. In an alternative approach used in the present study, the density of expression of mature cell surface protein for BMPR1B was measured by flow cytometric analysis. In the study a reduction in the number of growing follicles was linked to the sequential disruption to BMPR1B density during both dominant follicle selection, and pre-ovulatory maturation.

6.4.1 Major Findings

The major finding of this study was that a decline in granulosal BMPR1B receptor density occurs at the time of cyclic dominant follicle selection, and again during the terminal stage of folliculogenesis in the best prognosis IVF patients aged 23-30 years. The older, 40+ y poor ovarian reserve patients experience a reversal of this pattern (Figure 6.3.1).

The biphasic down-regulation of the density of the TGFβ type I receptor, BMPR1B protein during folliculogenesis was similar to the finding in young adult normal sheep, presented earlier in Chapter 5 and below Figure 6.4.1. During dominant follicle selection in sheep and in humans granulosal expression of BMPR1B was reduced followed by a sequential increase with follicle size, peaking, and then reducing in the pre-ovulatory follicle in humans or in the leading dominant follicle in the wild-type sheep (Figure 6.4.1). In other studies granulosa cell expression of BMPR1B has been shown to increase with follicle size which is consistent with the findings (Chen, et al. 2009, Estienne, et al. 2015). However, the pre-ovulatory, leading dominant follicle in sheep was pooled with smaller follicles in these studies, which would mask the down-regulation. The down-regulation of granulosa cell BMPR1B expression in the study was consistent with findings for sheep dominant follicles compared to the subordinate follicles reported in another recent study (Gasperin, et al. 2014). The interrelationship between FSH and BMP regulation has been previously reported (Miyoshi, et al. 2006, Shi, et

al. 2009a, Shi, et al. 2010), and the drop in pituitary FSH secretion initiating the dominant follicle selection process would, therefore, be closely related to the decline in BMPR1B expression on the granulosa cell surface.

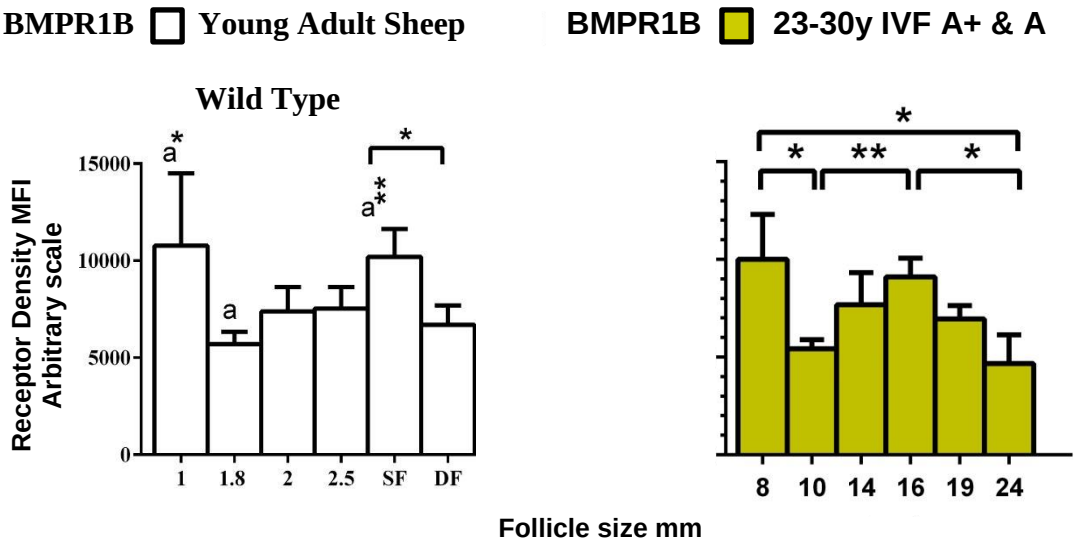


Figure 6.4.1 Sheep WT BMPR1B density with increasing follicular size.
SF subordinate follicle 3.2-4.6 mm, and DF Dominant Follicle, 4.4-7 mm. Mean fluorescent Intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *$p<0.05$ and **$p<0.01$. The letter, such as 'a', signifies a statistical difference to the matching letter and an attached asterisk (a*) indicates the significance level for the size follicle.

In the current study, granulosal BMPR1B density provides an indication of possible BMP ligand receptor signalling. The signalling potential activity of BMPR1B is measured by the density of the receptor which is common to several TGFβ superfamily members. The BMPs 4, 6, 7, and 15 signal via the TGFβ type 1 receptor BMPR1B, and have been previously shown to have a regulatory role in the suppression of progesterone production prior to ovulation (Ryan, et al. 2008, Takeda, et al. 2012).

The degenerative ageing of granulosal BMPR1B density is highlighted by the observation that 40+ y patients with a favourable AFC of B & C exhibit a pattern of declining receptor density with follicle size, whereas the pattern in the 40+ y AFC D & E patient group is reversed (Figure 6.3.5). Further evidence of ovarian ageing is apparent in the down-regulation of BMPR1B density in the 35-39 y in the 14 to 16 mm follicle size class in the B & C group; whereas, in the D & E group, a steady increasing density was observed (Figure 6.3.4). In addition, evidence of ovarian ageing also occurs in the youngest patients of 23-30 y with an AFC of C, demonstrating a compensatory over-expression of BMPR1B, destabilising

the biphasic expression pattern previously found in young adult Merino sheep, and young patients with ample ovarian reserve (Figure 6.4.1) The reversal seen with declining AFC rather than chronological age provides compelling evidence of a fundamental shift in granulosal BMPR1B receptor density with ovarian ageing.

In the literature there are few studies covering the changing landscape of the follicular gene expression with increasing follicular size. In particular, the common practice is to pool the follicles into size ranges which have failed to recognise the sometimes subtle change over time or the dramatic fall in gene expression as the follicle prepares for ovulation. The comprehensive individual nature of the collection has provided compelling evidence of the regulatory nature of signal activation by the receptors with changing follicular development indicated by size.

The LH surge is characterised by a dramatic shift from proliferation to differentiation and maturation of the follicle prior to ovulation. The BMPs have been described as inhibitors of the LH surge and luteinisation, however the characterisation of the ovarian molecule that reduces pituitary LH secretion has not been identified (Dimitraki, et al. 2014). GnSAF is characterised as being produced by the cohort of recruited small antral follicles, which regulate folliculogenesis by their desensitising effect on the pituitary to supress LH release until the mid-cycle (Dimitraki, et al. 2014). The GnSAF bioactivity is measured indirectly by applying steroid free follicular fluid to rat or ovine cultured anterior pituitary cells.

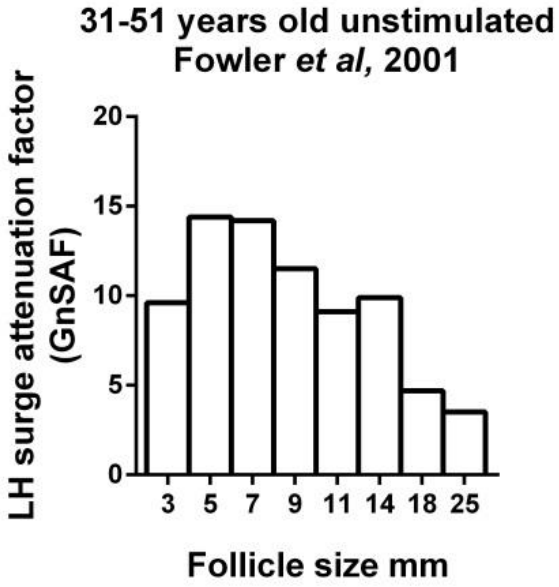


Figure 6.4.2 Relationship between follicle size and the LH surge attenuating factor GnSAF
Based on data from (Fowler, et al. 2001)

The relationship between follicle size and GnSAF has been demonstrated as an inverse relationship with follicle size (Figure 6.4.2) (Fowler, et al. 2001). The pituitary responsiveness to secrete LH has also been shown to be greater in patients receiving IVF than during natural cycles which is consistent with the greater level of BMPR1B reported in the current study (Figure 6.3.1) (Fowler, et al. 2001). In addition, the effect of a declining ovarian reserve with age resulted in reduced GnSAF bioactivity (Martinez, et al. 2002). The comparatively higher level of GnSAF would be accounted for by the greater number of follicles producing the attenuating substance in the younger patients compared to the older patients (Figure 6.4.3).

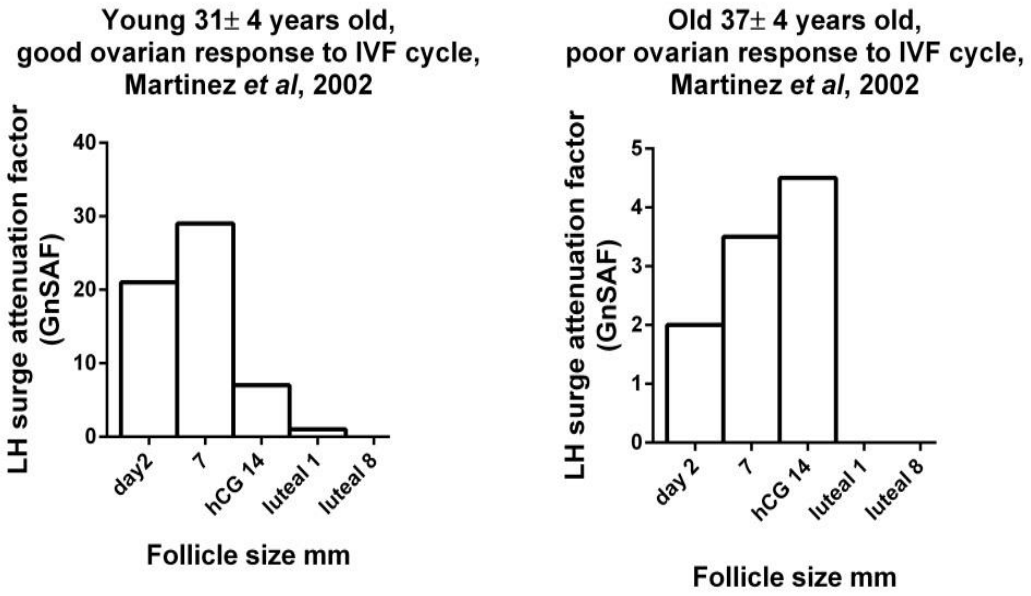


Figure 6.4.3 Ovarian reserve and the relationship between follicle size and the LH surge attenuating factor GnSAF.
Based on data from (Martinez, et al. 2002)

The level of GnSAF was measured at two time points matching the current study's follicle size of 8 mm, (dominant follicle selection), and 19-24 mm (hCG injection or the equivalent of the largest follicle), (Martinez, et al. 2002). The younger patients with good ovarian response, had high levels of GnSAF at the time of dominant follicle selection which reduced dramatically by the end of folliculogenesis (Figure 6.4.3). The opposite was true for the poor ovarian response older patients of the study (Figure 6.4.3).

In the current study the granulosal BMPR1B density in the young and the old mimic, in reverse, the effect of GnSAF described by Martinez et al. (2002). (Figure 6.4.3). Martinez et al. (2002), concluded that the bioactivity of the GnSAF prevented the premature onset of the LH surge, which is not dissimilar to the role of BMPs as an inhibitor of luteinisation described by others (Otsuka 2010, Shimasaki, et al. 1999). From a clinical perspective,

reduced fertilisation rates are common in older patients, due to a higher serum level of FSH, LH and inhibin B (Hale 2007, Stanger and Yovich 1985). In the older patient the reduced BMPR1B could have the potential to limit the suppression of LH in the pituitary and progesterone in the ovary (granulosal) at the time of dominant follicle selection (Chapter 7).

6.4.2 Conclusion

Taken together, the results demonstrate the disrupting effect that ageing-induced depletion of the ovarian reserve has on granulosal BMPR1B receptor density in antral follicles. Likewise, ageing-induced depletion is associated with a biphasic down-regulation of granulosal BMPR1B density at the two stage-specific, critical time points of dominant follicle selection and pre-ovulatory maturation. The findings extend previous research by the comprehensive nature of the range of follicle sizes and age groups studied, together with measurement of the translated mature, BMPR1B protein as opposed to measurement of receptor expression at the mRNA level (Ascoli, et al. 2002). Further work is needed to confirm the identity of the locally-produced TGFβ family ligand(s) (BMP 4, BMP6, BMP 7, and BMP15) whose signalling may be impacted by this change in BMPR1B receptor density on the granulosa cell surface, and also to explore the consequences of altered signalling.

CHAPTER 7

Ovarian Ageing and FSHR

Title: The decline in ovarian ageing and the effect on the FSHR in humans

CHAPTER 7 Ovarian Ageing and FSHR

7.1 Introduction

At the onset of puberty, the levels of gonadotrophin FSH secreted from the anterior pituitary increase to a threshold point sufficient to rescue a cohort of the primordial follicles and initiate cyclic folliculogenesis. The primordial follicles grow and differentiate into pre-antral follicles, and with further development and the formation of a fluid filled central compartment, mature to become antral follicles (Rodgers and Irving-Rodgers 2010b). The oocyte is suspended in a fluid filled cavity, which is surrounded by specialised cumulus granulosa cells which anchor the oocyte to layers of mural granulosa cells. The follicle wall is composed of mural granulosa cells enclosed in a membrane that is wrapped in a layer of theca cells. The follicle undergoes proliferation of the granulosa and thecal follicular cells, followed by differentiation of the role of the follicular cells as the follicle matures. The number of antral follicles selected for dominance and ovulation is dependent on the regulation of the gonadotrophins FSH and LH produced in the anterior pituitary, and the density of FSHR and LHR on the granulosa cells (Ginther, et al. 2005, Rice, et al. 2007, Sen and Hammes 2010, Sen, et al. 2014).

As the FSH level falls, the growth of the smaller subordinate follicles is reduced, and only the follicles with sufficient FSHR and LHR continue to develop further because of the capacity to convert androstenedione to oestrogen for growth (Loumaye, et al. 2003). Pre-ovulatory follicles reach an oestrogen threshold promoting a change from a proliferation to differentiation of the granulosa cells in preparation for ovulation. Substantial reorganisation of the cytoplasm occurs during this maturation stage, reflected by a change to receptor density. The relative expression of FSHR and the stage of follicle development have been shown to have a negative effect on the ovarian response to gonadotrophin stimulation (Cai, et al. 2007) and diminished LHR in the oocyte quality (Maman, et al. 2012).

Artificially increasing the level and the duration of circulating FSH extends the window of growth, producing multiple ovulatory follicles. As the ovarian primordial follicle reserve declines, the rate of recruitment to primary follicles is reduced, which leads to a diminished recruitment of follicles into the cycle. The number of small antral follicles at the beginning of each cycle is representative of the ovarian reserve of primordial follicles remaining in the ovary (Almog, et al. 2011, Baerwald, et al. 2012b, Hadlow, et al. 2013, Hansen, et al. 2011, van Rooij, et al. 2005). AMH and chronological age also have a strong correlation to ovarian reserve; however, AFC demonstrates a more sensitive test over a time course (van Rooij, et al. 2005) and, in the case of AMH, in the older patients (i.e. the lower range of 0-1ng/ml)

(Hansen, et al. 2011). During an IVF cycle, the concentration of exogenous FSH required to maintain a cohort of follicles to grow to ovulatory size increases sequentially with a reduction of ovarian reserve or AFC. Clinicians have developed protocols based on past patient ovarian responses to rFSH stimulation, which can be highly predictable (Yovich, et al. 2012). Superovulation syndrome has become a rare complication using best practice principles. With the increase in the average age of patients seeking assistance, to 39 years in Australia, the number of patients that respond poorly to gonadotrophin stimulation has risen.

We aim to comprehensively profile the receptor expression of FSHR in a range of patients of different ages and stages of ovarian primordial depletion, who are receiving treatment for infertility. Previous accounts of receptor expression have utilised the mRNA of the receptors, which would include a large proportion of non-functional immature FSHR (Ascoli, et al. 2002, Jeppesen, et al. 2012, Pidoux, et al. 2007). However, in contrast, for the current study an average of ~8000 granulosa cells from an individual follicle was collected from a comprehensive range of follicle sizes from 4 mm to 27 mm. In addition, we measured the granulosa cell surface-expressed mature receptor protein density for FSHR by immunofluorescent labelling via flow cytometry.

7.2 Method

Methods are described in detail in the General Methods Chapter (page 38).

Granulosa cells were collected from hCG/LH induced follicles from patients receiving IVF exogenous gonadotrophins to artificially stimulate multiple follicles (Table 7.2:1). The granulosa cells were isolated and incubated with antibodies to detect the level of FSHR mature protein. In addition, apoptosis and necrosis were also determined by immunolabelling via flow cytometric analyses (page 39).

Table 7.2:1 Patient ovarian reserve, based on antral follicle count (AFC) and the number of follicles collected per group.

AGE Year	IVF Patient	Total Follicle	AFC	Major Group	Number of Follicles Collected Per Group							
					Sub Group	#	Sub Group	#	Sub Group	#	Sub Group	#
21-30	8	95	20-40	A+ &A	A+	31	A	64	C	6		
31-34	11	86	13-29	A & B	A	60	B	26	C	17		
35-39	16	102	9-19	B & C	A+	6	B	50	C	16	D	30 & E 6
40-45	18	118	3-8	D & E	D	59	E	19	B	34		
40	1	Natural Cycle Healthy			D	2						

Ovarian reserve measured indirectly by the Antral Follicle Count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle: A+ = 30-39 follicles; A = 20-29; B = 13-19; C = 9-12, D = 5-8; E = ≤4.

7.3 Results

In the 23-30 y patient group, with an AFC of A+ & A combined, the FSHR was significantly down-regulated after dominant follicle selection in follicles of 8 mm to 10 mm, and again from follicles of 16 to 24 mm ($p<0.05$ and $p<0.05$, respectively, Figure 7.3.1). In the oldest group of 40+ y AFC D & E combined, there was significant elevation of density in the 4 mm follicle in keeping with the 23-30 y density pattern, but not in the terminal-end 19 mm follicles, where no down-regulation occurred compared to the young patients ($p<0.0001$, Figure 7.3.1). In addition, the natural healthy cycle follicles appeared to have a lower density than the IVF patients; however, this did not reach significance (Figure 7.3.1).

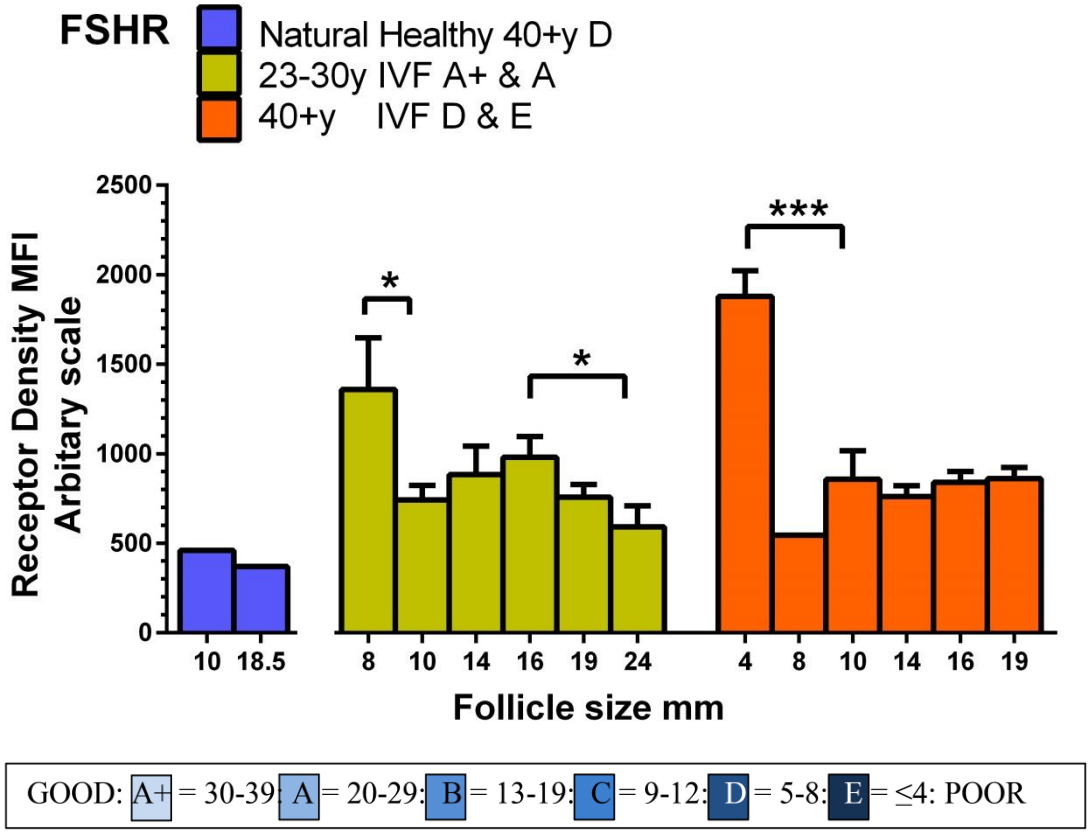
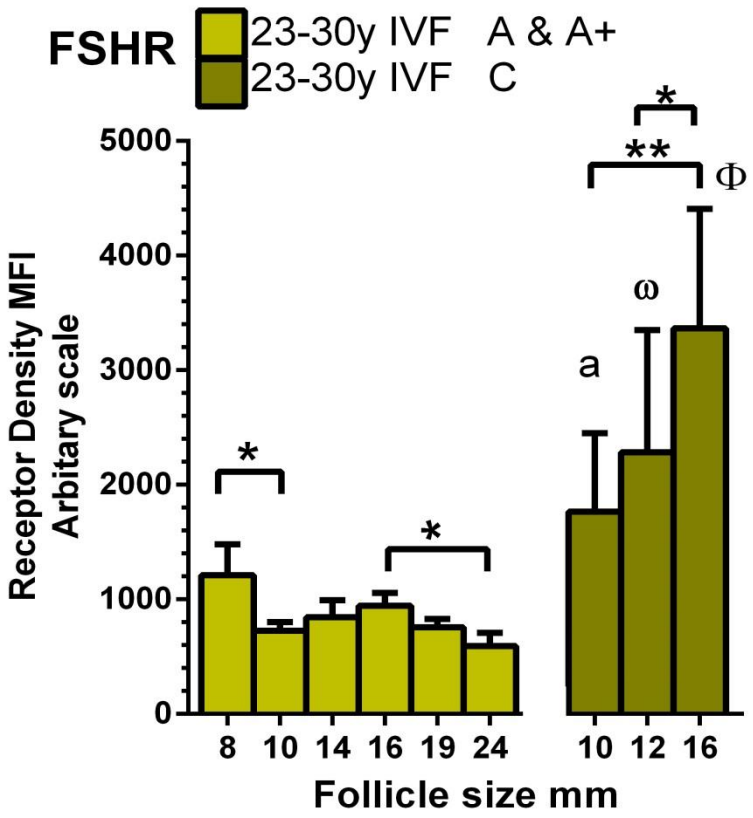


Figure 7.3.1 Granulosal FSHR density from follicles of different sizes collected from young and older IVF patients compared to an unstimulated natural healthy cycle.
Granulosal FSHR density and follicle size profile of a natural healthy unstimulated patient of 41y with an AFC of D before the LH surge, (blue). Patients, 23-30y stimulated, IVF cycle with an AFC of A+ & A, (olive green). Patients, 40+y stimulated IVF cycle with an AFC of D & E, (orange). Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *$p<0.05$ and **$p<0.01$.

In the young patient group of 23-30 y with a good ovarian reserve down regulation of FSHR was observed during dominant follicle selection and during the pre-ovulatory maturation phase in the largest follicles($p<0.05$, Figure 7.3.2). The young patient with a poor ovarian reserve of C produced elevated levels of FSHR density which increased with an increase in follicle size ($p<0.01$, Figure 7.3.2).



GOOD: A+ = 30-39: A = 20-29: B = 13-19: C = 9-12: D = 5-8: E = ≤4: POOR

Figure 7.3.2 Granulosal FSHR density and ovarian reserve depletion in 23-30 y.
Granulosal FSHR density and follicle size of the young 23-30 y patients with declining ovarian reserve from A+ & A to C. Mean fluorescent Intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *$p<0.05$ and **$p<0.01$. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level for the size follicle.

In the 31-34 y patient group, the follicles had increasing levels of granulosal FSHR density with increasing follicle size, whereas the follicles from the 35-39 y B & C patient group partially mimicked the younger pattern of density, whereby the peak reached at 14 mm was followed by a significant down-regulation in the 19 mm follicles ($p<0.05$, Figure 7.3.3 and Figure 7.3.4). The FSHR density was significantly up-regulated in the terminal-end follicles (19-24 mm) from both the 31-34 y and 35-39 y patients compared to the 23-30 y patient group ($p<0.01$ and $p<0.001$ respectively, Figure 7.3.3and Figure 7.3.4, compared to Figure 7.3.2).

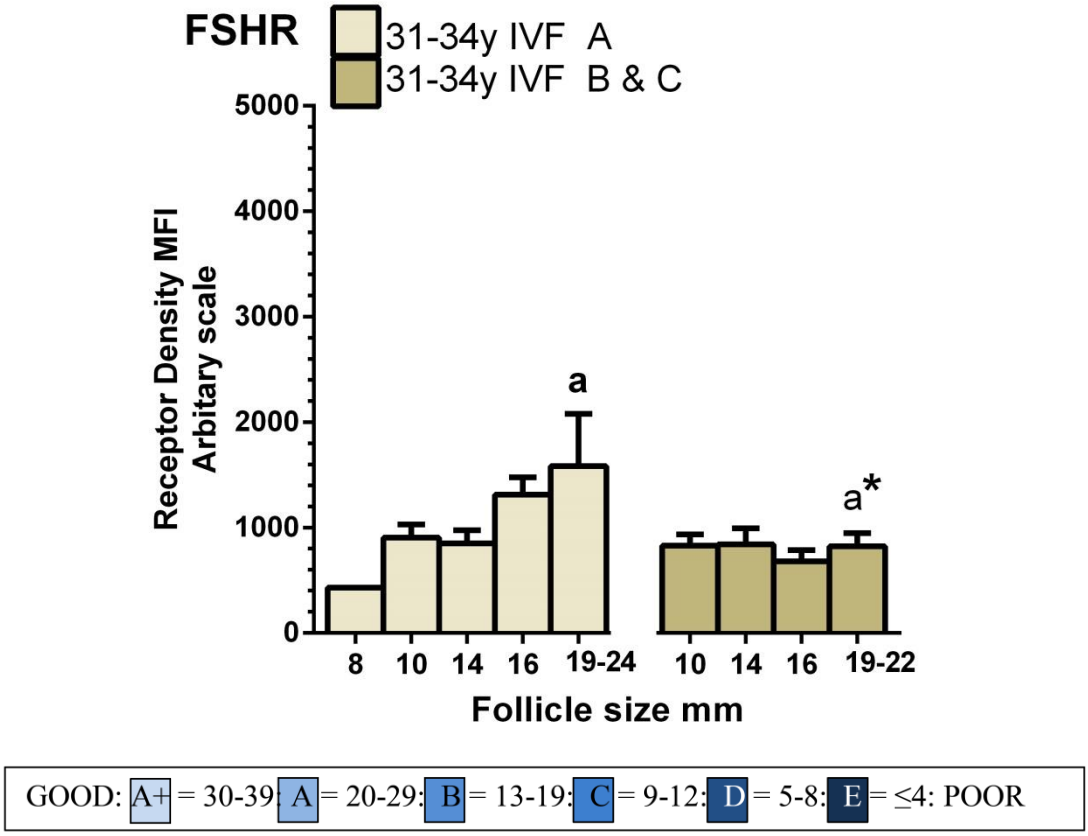


Figure 7.3.3 Granulosal FSHR density and ovarian reserve depletion in 31-34 y.
Granulosal FSHR density and follicle size of the 31-34 y patients with declining AFC from A to B & C. Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *$p<0.05$. The letter 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level for the size follicle.

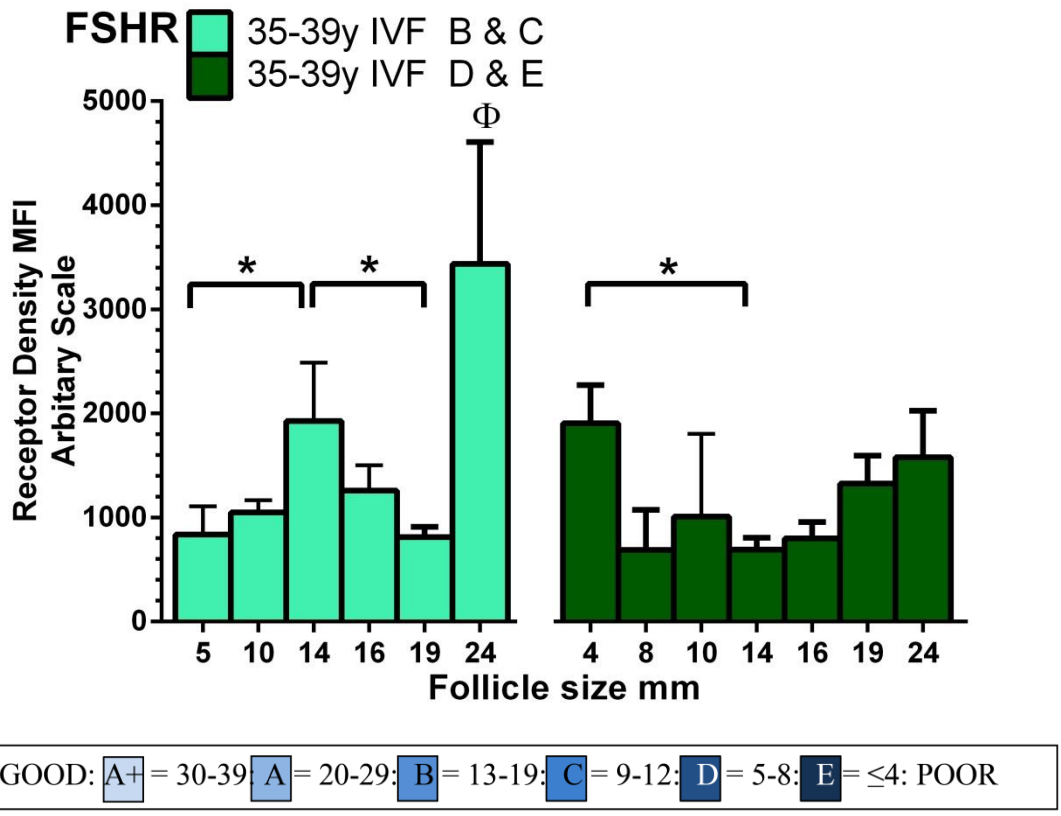


Figure 7.3.4 Granulosal FSHR density and ovarian reserve depletion in 35-39 y.
Granulosal FSHR density and follicle size of the 35-39 y patients with declining AFC from B & C combined to D&E combined. Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent Intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *p<0.05. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level for the size follicle.

The granulosal FSHR density of 40+ y patient group, with an ovarian reserve of B & C, mimicked the 23-30 y ovarian reserve A pattern shown in Figure 7.3.2, with an initial significant down-regulation at 10 mm followed by an up-regulation at 16 mm ($p<0.001$ and $p<0.05$, respectively, Figure 7.3.5). As the ovarian reserve declined, the granulosal FSHR density of the 40+ y, D & E groups was not down-regulated. The granulosal FSHR density of the 40+ y ovarian reserve, D group was not significantly different to the E group (Figure 7.3.5).

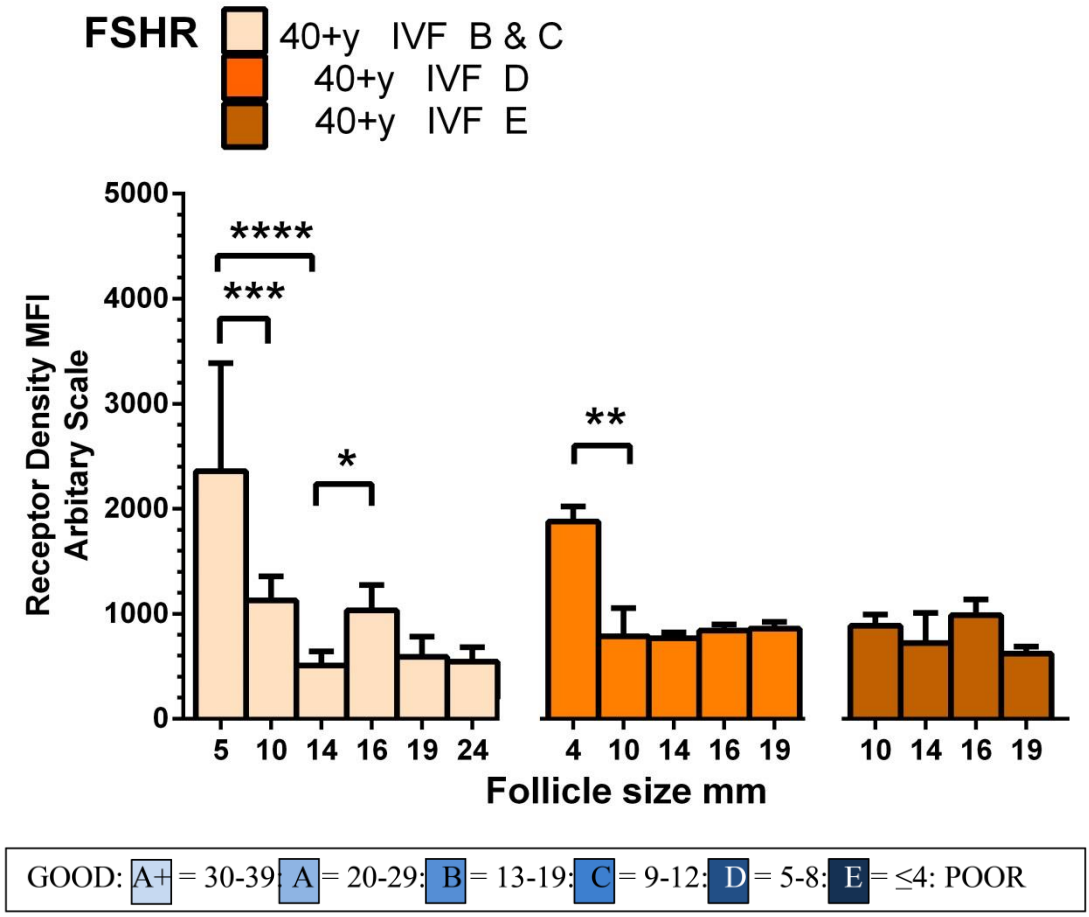


Figure 7.3.5 Granulosal FSHR density and ovarian reserve depletion in 40+ y.
Granulosal FSHR density and follicle size of the 40+ y patient group with declining AFC from B & C combined to D and E. Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *$p<0.05$, **$p<0.01$, ***$p<0.005$, and ****$p<0.001$. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level for the size follicle.

7.4 Discussion

The gonadotrophin FSH has been strongly associated with the regulatory control of cyclic folliculogenesis (Yong, et al. 1992). The level of granulosal FSHR density has been previously shown to fluctuate during folliculogenesis, at the time of dominant follicle selection and at the terminal-end prior to ovulation (Cai, et al. 2007, Jeppesen, et al. 2012, Ophir, et al. 2014). In a novel approach, the mature cell surface receptor protein for FSHR was measured in the current study by flow cytometric analysis. Immunolabelled granulosa cells were collected from individual antral follicles during the peri-ovulatory stage, from patients receiving IVF treatment for infertility. The receptor density was quantified by the average fluorescent intensity of ~8000 isolated granulosa cells per follicle from a range of follicle sizes.

The continuous process of ovarian ageing reduces the primordial follicle reserve, and can be indirectly measured by the number of small antral follicles developing (Almog, et al. 2011, Baerwald, et al. 2012b, Hansen, et al. 2011, van Rooij, et al. 2005). A depleted reserve generates a natural state of menopause, and results in the loss of ovarian function. The decline in the AFC predominantly coincides with an increase in chronological age; however, at any given age there is an overlap in the range of AFC. Premature ovarian failure does occur in younger patients; however, it is an uncommon form of infertility (1-2%) (Shelling 2010). Rather, a general reduction of fertility occurs with the decline of the ovarian reserve that results in subtle changes to ovarian regulation with time. The present study provides a novel description of the change to the granulosal FSHR density that occurs with ovarian ageing during the defining stages of follicular development, dominant follicle selection and pre-ovulatory follicle maturation.

7.4.1 Major Findings

The major findings of the current study were the high level of granulosal FSHR density in the smaller antral follicles, and that the depletion of the ovarian reserve did not generally disrupt the expression. Conversely, the down-regulation of granulosal FSHR density during the maturation process was disrupted by ovarian reserve depletion. In addition, a rise in granulosal FSHR density was evident between dominant follicle selection and pre-ovulatory maturation.

7.4.2 Dominant follicle selection

The dominant follicle selection (8 mm) and terminal-end maturation (19 mm) down-regulation in the granulosal FSHR density observed in the current study, was similarly observed in WT Merino sheep (Chapter 5) (Regan, et al. 2015).

As part of the dominant follicle selection process, follicles with a greater density of FSHR would induce more LHRs (Rice, et al. 2007). As the level of pituitary FSH is reduced, follicles with LHRs retain the capacity to produce oestrogen from androgens, whereas those follicles without sufficient LHR succumb to atresia (Ginther, et al. 2001, Mihm, et al. 2006). In the present study, granulosal FSHR density was high in the smaller antral follicles and was consistent with a change from FSH dependency before dominant follicle selection, and pituitary FSH secretion decline during dominant follicle selection.

The finding of a high density of granulosal FSHR density in the 8 mm follicles from the 23-30 y patient group (A+ & A) appears not to be disrupted by ovarian reserve depletion in the older patient groups. However, in the 35-39 y B & C group the 5 mm follicles did not have high levels of granulosal FSHR density which indicates a possible inadequacy in receptor density during dominant follicle selection (Figure 7.3.4). In several patient groups the smaller follicle size was not collected, and therefore, the high granulosal FSHR density would not have been observed.

Consistent with the current study, Jeppesen et al., (2012) have previously reported a high expression of *FSHR* mRNA in granulosa cells from small antral follicles of ~ 6 mm collected from a wide range of patients (7-38 y). The elevated *FSHR* mRNA levels were followed by a constant, lower level of expression from ~ 9 to 15 mm, which is also consistent with the present study's findings (Jeppesen, et al. 2012). The current study confirms and expands their data by reporting that, after dominant follicle selection, the high level of granulosal FSHR density was dramatically reduced in the 10 mm follicles, followed by an increase in FSHR in the 16 mm follicles from the 23-30 y patient group A & A+ (Figure 7.3.2).

7.4.3 Maturation of the pre-ovulatory follicle

At the terminal-end of folliculogenesis, proliferation of the granulosa cell is reduced and divergence of steroidogenic capacity to predominantly synthesis progesterone commences during the maturation process before ovulation. Ovarian ageing appears to disrupt the prerequisite down-regulation of granulosal FSHR density observed in the 23-30 y patient group (A+ & A) (Figure 7.3.1). In the older patient groups granulosal FSHR density increases or remains constant as the ovarian reserve depletes (Figure 7.3.1- Figure 7.3.5)

In the sheep study, the down-regulation of granulosal FSHR density during the pre-ovulatory follicle maturation was evident in both the WT and BB sheep (Chapter 5) (Regan, et al. 2015). The sheep were young (four years old) hence their ovarian reserve would be

uncompromised, and therefore comparable to the young IVF patients with a good ovarian reserve in the current study.

7.4.4 Natural cycle compared to an IVF stimulated cycle

The Jeppesen's et al. (2012), study was conducted primarily on patients that were not receiving rFSH stimulation, whereas only one patient in the current study was recruited without IVF stimulation, and produced two follicles (Figure 7.3.1). The natural cycle follicles provided a benchmark for the 40+ y AFC D patient group, and indicated that either the levels of granulosal FSHR density was lower in a natural cycle compared to an IVF cycle or the biphasic down-regulation had occurred in the natural cycle. In addition, rFSH has been previously shown to steadily increase FSHR in granulosa cells of the rat, followed by a decrease in FSHR caused by the hCG/LH bolus (LaPolt, et al. 1992). Therefore, it is probable that the level of FSHR in an IVF stimulated patient appears higher than in an unstimulated cycle (Figure 7.3.1).

7.4.5 Conclusion

In conclusion, it is apparent that ovarian ageing, as measured by the reduction in antral follicle count, may disrupt the down-regulation of the granulosal FSHR density required for maturation of the follicle before ovulation.

CHAPTER 8

Ovarian Ageing and LHR

Title: The decline in ovarian ageing and the effect on the LHR in humans

CHAPTER 8 Ovarian Ageing and LHR

8.1 Introduction

The process of folliculogenesis involves the stage dependent expression of growth factor receptors that regulate follicular growth (Erickson and Shimasaki 2001). The oocyte is surrounded by specialised cumulus granulosa cells in a fluid filled antral cavity, which is anchored to the follicle wall, composed of granulosa and theca cells separated by the basal lamina membrane (Baerwald, et al. 2012b, Foxcroft and Hunter 1985, Guraya 1971). The follicle undergoes proliferation of granulosa and theca cells, followed by morphological differentiation as the follicle matures (Fricke, et al. 1996). The mature pre-ovulatory follicle begins to luteinise prior to ovulation, when the follicle wall ruptures, releasing the oocyte for capture by the fallopian tube (Eppig 2001, Mihm, et al. 2006, Rodgers and Irving-Rodgers 2010b, Zeleznik 2001). The number of small antral follicles recruited for dominance and ovulation is dependent on the regulation of the gonadotrophins FSH and LH, and the interaction with other growth factors during folliculogenesis (Ginther, et al. 2005, Hillier 2001).

LHRs are induced by FSHRs, and are localised on granulosa cells from small antral follicles onwards (Minegishi, et al. 1997, Yung, et al. 2014). Once LHRs are expressed on granulosa cells, progesterone produced by the granulosa cells diffuses into the thecal cell, and can be converted from pregnenolone to androstenedione in the theca cell. Androstenedione from the theca cell can then be converted by the granulosa cell to oestrogen, which is essential for growth of the follicle and the oocyte (Miller 2011, Yuan 1998). As the oestrogen levels reach a threshold point, the pituitary produced FSH is reduced. Follicles with insufficient FSHR and LHRs mid-cycle do not sustain growth, and follicular regression proceeds, followed by atresia (Campbell, et al. 1999, Ginther, et al. 2012, Luo, et al. 2011, Picton and McNeilly 1991).

The suppression of progesterone synthesis in favour of oestrogen appears to be governed by the action of the growth factor BMP from the TGFβ family of cytokines (Knight and Glister 2006, Moore, et al. 2001b). The BMP ligands signal via the type 1 TGFβ receptor BMPR1B (Miyazono, et al. 2010), and the role of BMP in the regulation of LHR expression has been previously reported (Shi, et al. 2012, Shi, et al. 2011, Shi, et al. 2010, Zhu 2013). The down-regulation of BMPR1B and FSHR density appears to be fundamental to the process of luteinisation of the follicle, presented earlier (Chapter 5 and 6), and previously supported by data from Gasperin, et al. (2014) and Regan et al. (2015).

With the reduction in BMPR1B density, the LH surge (including the simultaneous progesterone and FSH surge) stimulate a myriad of changes to completely reorganise and differentiate the granulosa and thecal cells for corpus luteum function (Micevych, et al. 2003, Nottola, et al. 2006). Therefore, the density of ovarian LHR during folliculogenesis would impact the growth and maturation of the follicle and the oocyte.

A continuous process of activation of primordial follicles reduces the ovarian reserve over time (Hansen, et al. 2011). Depletion of the ovarian reserve reduces the number of growing follicles available for recruitment into the menstrual cycle. The ovarian reserve can be indirectly measured by the number of small antral follicles growing, and is referred to in a clinical setting as the AFC (Almog, et al. 2011). Ageing depletes the ovarian reserve, creating a state of menopause and results in the loss of ovarian function (Nelson, et al. 2013). Chronological age correlates well with the rate of depletion of the ovarian reserve; however, for any age group, there is an overlap of the AFC (Almog, et al. 2011, van Rooij, et al. 2005). Given the average age of women seeking assistance in Australia is 39 years, the need exists to investigate the age related changes occurring in the ovary as the ovarian reserve diminishes.

The current study aimed to comprehensively profile the ovarian granulosal LHR density in a range of patients of different ages and stages of ovarian primordial depletion, who were receiving treatment for infertility. Previous reports documenting ovarian LHR expression have evaluated expression at the mRNA level in pooled follicles from different size classes. mRNA expression does not necessarily reflect expression of translated functional *LHR* protein on the cell surface (Ascoli, et al. 2002, Jeppesen, et al. 2012, Pidoux, et al. 2007). In contrast, a much larger number of granulosa cells (~8000) were collected in the current study, from individual follicles over a comprehensive range of follicle diameters from 4 to 27 mm.

8.2 Methods

Methods are described in detail in the General Methods Chapter (page 38).
Granulosa cells were collected from hCG/LH induced follicles from patients receiving IVF exogenous gonadotrophins to artificially stimulate multiple follicles (Table 8.2:1). The granulosa cells were isolated and incubated with antibodies to detect the level of BMPR1B, FSHR, LHR, and GHR mature protein. In addition, apoptosis and necrosis were also determined by immunolabelling via flow cytometric analyses (page 39).

Table 8.2:1 **Patient ovarian reserve, based on antral follicle count (AFC) and the number of follicles collected per group.**

AGE Year	IVF Patient	Total Follicle	AFC	Major Group	Number of Follicles Collected Per Group							
					Sub Group	#	Sub Group	#	Sub Group	#	Sub Group	#
21-30	8	95	20-40	A+ &A	A+	31	A	64	C	6		
31-34	11	86	13-29	A & B	A	60	B	26	C	17		
35-39	16	102	9-19	B & C	A+	6	B	50	C	16	D30 &E6	
40-45	18	118	3-8	D & E	D	59	E	19	B	34		
40	1	Natural Cycle Healthy			D	2						

Patient ovarian reserve, based on antral follicle count (AFC) and the number of follicles collected per group. Ovarian reserve measured indirectly by the Antral Follicle Count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle: A+ = 30-39 follicles; A = 20-29; B = 13-19; C = 9-12, D = 5-8; E = ≤4.

8.3 Results

In the youngest patients of 23-30 y with an AFC of A+ & A, the level of LHR density was consistent from the 8 mm follicle to the 19 mm follicle whereas, in the largest follicles of 24 mm, a significant down-regulation was apparent, ($p<0.05$, Figure 8.3.1). The pre-ovulatory follicle density of LHR in the natural, healthy, unstimulated patient of 40+ y, with an AFC of D was comparable to the level reached in both the young or older IVF patients whereas, the 10 mm follicle in the natural cycle was substantially lower (Figure 8.3.1).

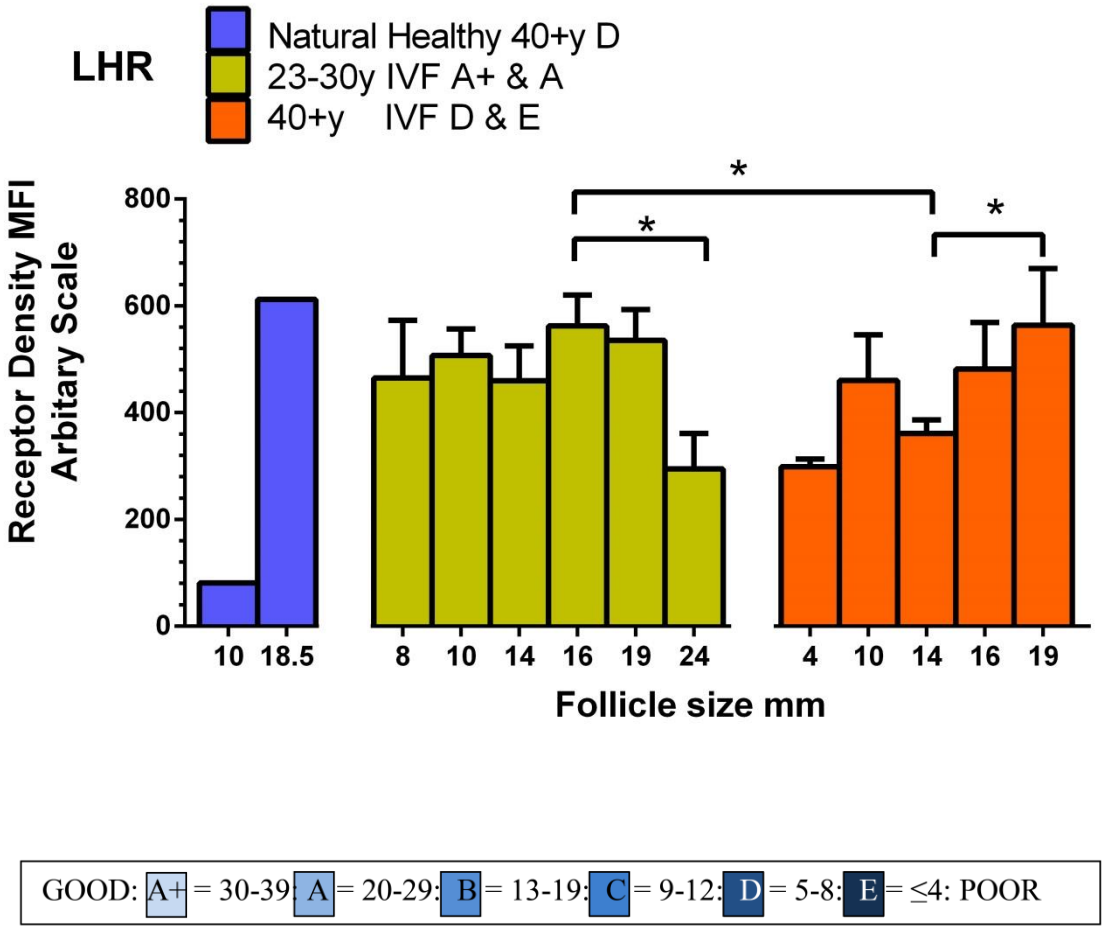


Figure 8.3.1 Granulosal LHR density from follicles of different sizes collected from young and older IVF patients compared to an unstimulated natural healthy cycle.

Granulosal LHR protein density and follicle size profile of a natural healthy unstimulated patient of 41y with an AFC of D before the LH surge, (blue). Patients, 23-30y stimulated, IVF cycle with an AFC of A+ & A, (olive green). Patients, 40+y stimulated IVF cycle with an AFC of D & E, (orange). Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *$p<0.05$.

In addition, the follicles from patients with an AFC of C within the 23-30 y group expressed more LHR in the 10 mm range ($p<0.01$ and $p<0.01$, respectively), which was significantly down-regulated with increasing follicular size ($p<0.05$,Figure 8.3.2). The level of expression in the C AFC was much greater than that of the uncompromised A+ & A group, however there were only two follicles analysed per follicle size in the C patient group, hence the significance was weak.

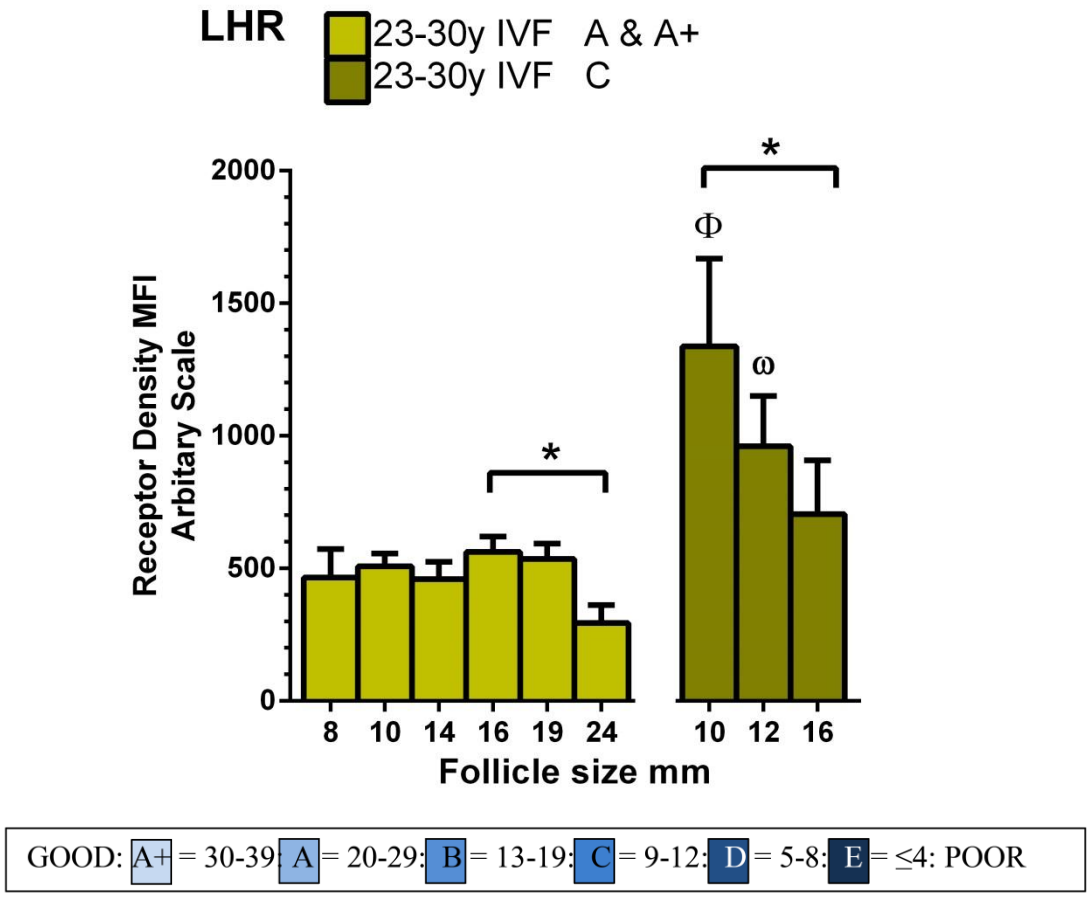


Figure 8.3.2 Granulosal LHR density and ovarian reserve depletion in 23-30 y.
Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent Intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *$p<0.05$. The letter 'ω or Φ' signifies a statistical difference to all other follicles of the A+ & A patient group.

.

In the follicles from the 31-34 y AFC group A, there was a significant up-regulation from the 14 mm to the 19 mm follicles, ($p<0.01$, Figure 8.3.3). The density of LHR was significantly reduced in the B and C AFC groups in the largest follicles of 19 mm ($p<0.01$, $p<0.005$, respectively, (Figure 8.3.3); and with a decline in AFC, the overall density of LHR declined (Figure 8.3.3).

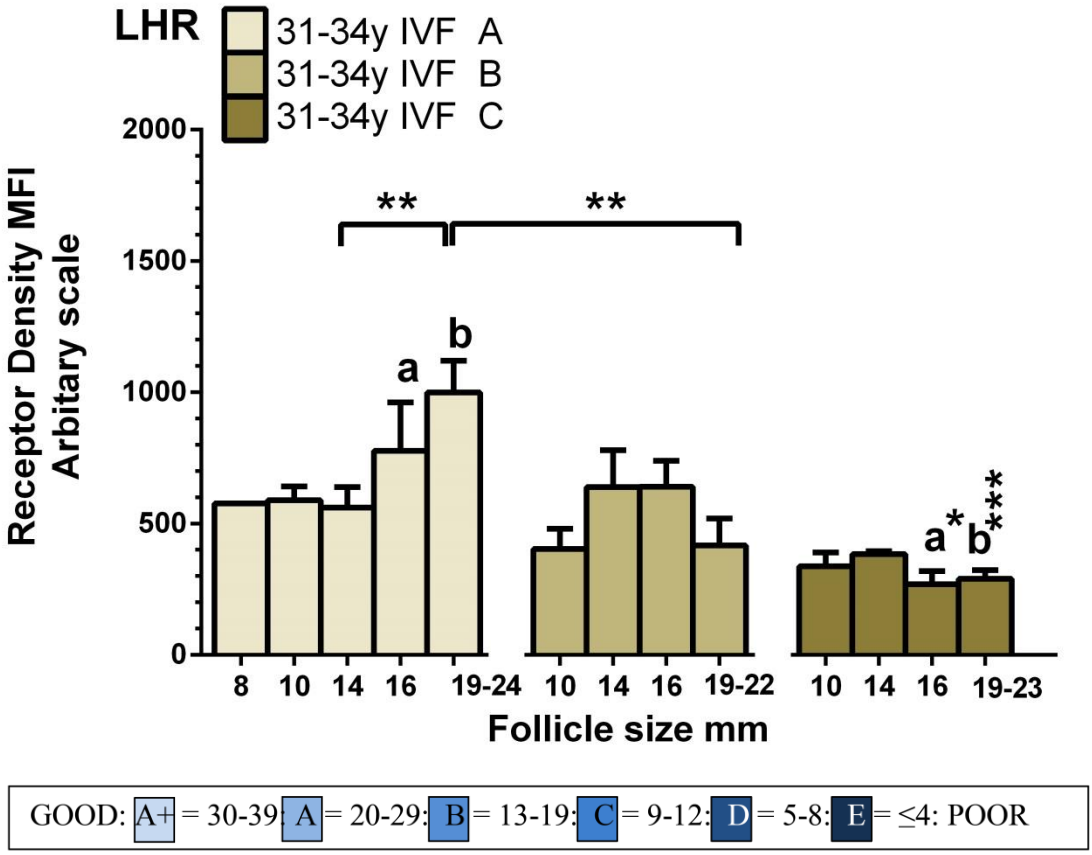


Figure 8.3.3 Granulosal LHR density and ovarian reserve depletion in 31-34 y.
Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *$p<0.05$, **$p<0.01$ and ***$p<0.005$. The letter, such as 'a' signifies a statistical difference to the matching letter. and an attached asterisk (a*) which indicates the significance level for the size follicle.

In the 35-39 y B & C group, the LHR density was significantly down-regulated from 10 mm towards the terminal-end follicles of 24 mm; whereas the small 4 mm follicles from patients with a D & E AFC patient group had significantly reduced granulosal LHR density ($p<0.05$, Figure 8.3.4). In further support of a sequential ageing effect, the changes in receptor density can be observed in the 35-39 y patient groups as the ovarian reserve declines (Figure 8.3.4). The B & C group is down-regulated to the terminal-end of folliculogenesis; whereas the poorest AFC group of D & E have a general lower density in the smaller follicles, and an up-regulation with follicle size which was similar to the 40+ y E group (Figure 8.3.5).

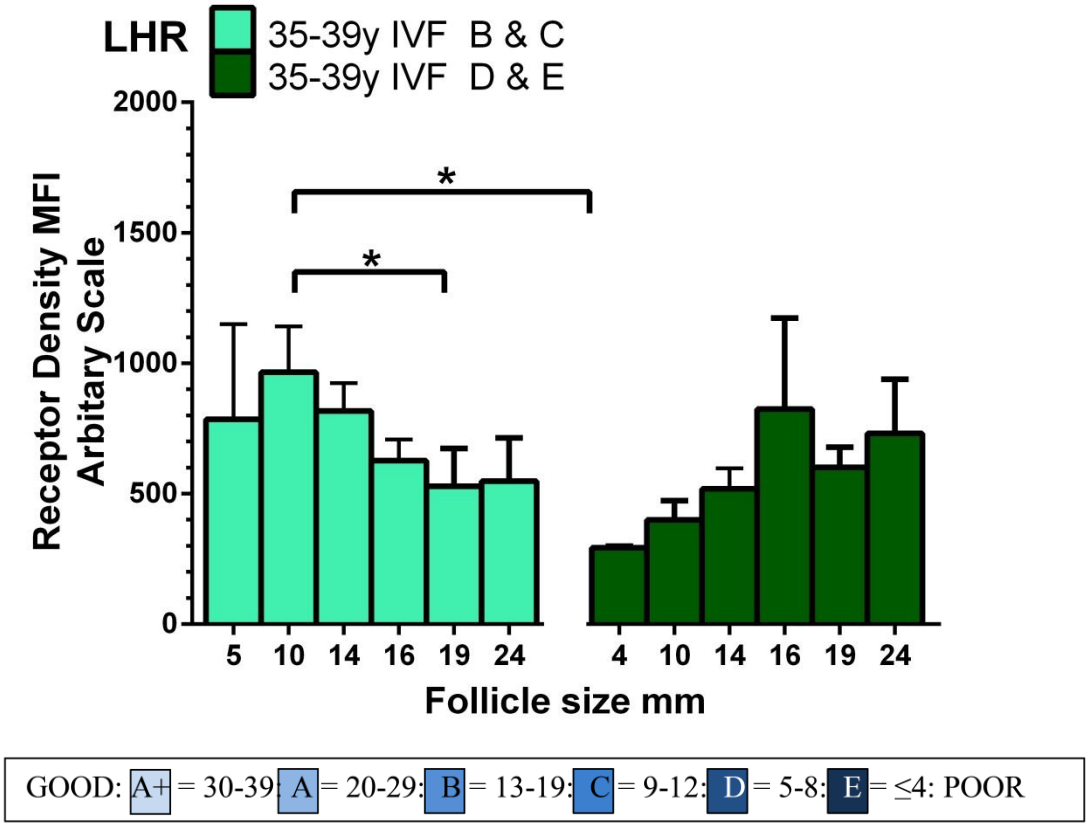


Figure 8.3.4 Granulosal LHR density and ovarian reserve depletion in 35-39 y.
Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *$p<0.05$.

In the 40+ y age group a significant increase in LHR was observed in the poor ovarian patient group, AFC group E compared to the B and D ($p<0.005$ and $p<0.001$, Figure 8.3.5). Furthermore, as the ovarian reserve was depleted (40+ y ovarian reserve E), the LHR protein level was initially reduced, but then increased with follicle size (Figure 8.3.5).

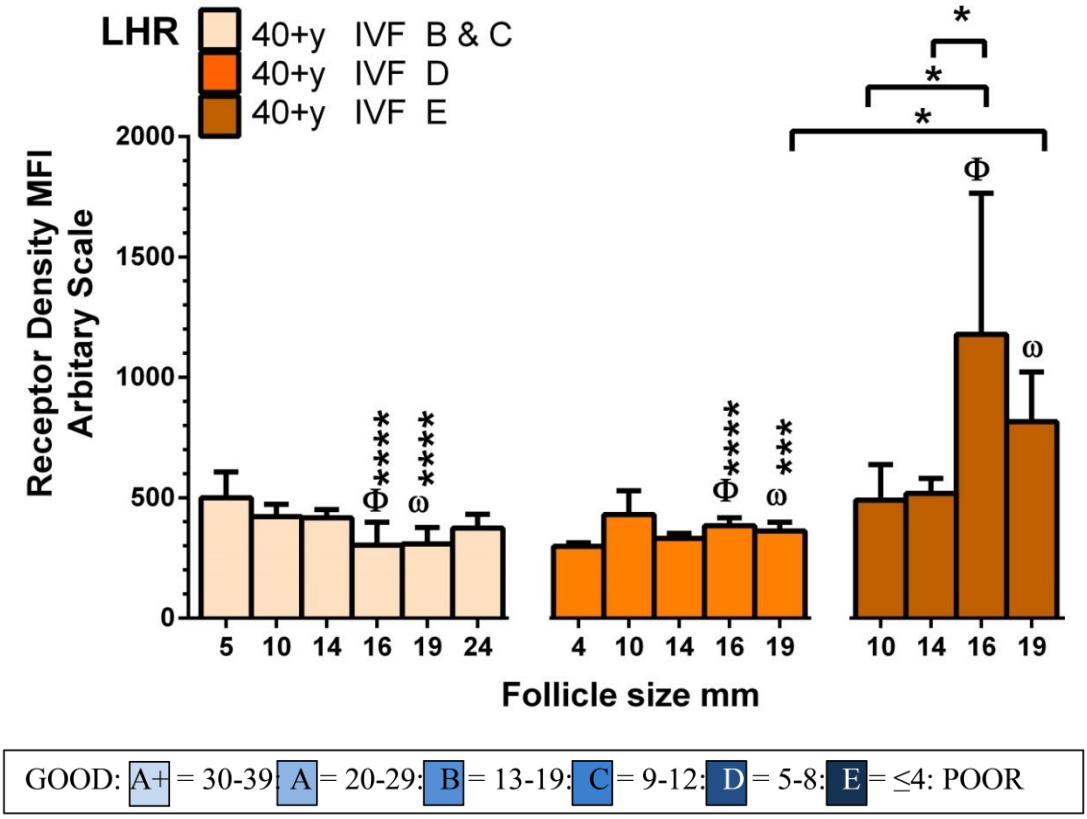


Figure 8.3.5 Granulosal LHR density and ovarian reserve depletion in 40+ y.
Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *$p<0.05$, ***$p<0.005$, and ****$p<0.001$. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level for the size follicle.

A strong correlation was observed between the LHR density and the corresponding BMPR1B density with follicle size in the D & E 40+ y patients (R squared 0.872) but not observed in the 23-30 y patients (R squared 0.078, Figure 8.3.6). As the patient age increased, and the ovarian reserve declined, the correlation between BMPR1B and LHR density sequentially increased

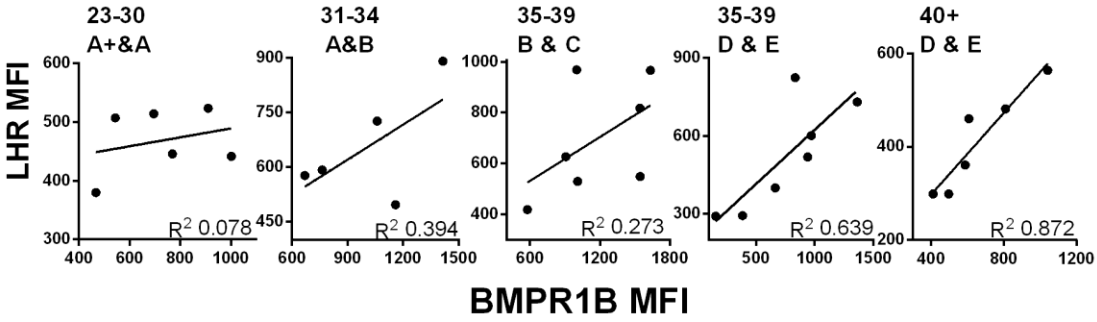


Figure 8.3.6 Correlation of LHR to BMPR1B with ageing and increasing follicular size. Sequential graphs show increasing age and declining ovarian reserve indicated by AFC. Linear regression analysis, R square indicated for each group. The data points were averages of the receptor expression for that follicle size group. Ovarian reserve measured indirectly by the AFC. AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle.

8.4 Discussion

Ovarian ageing caused by the depletion of the ovarian reserve of primordial follicles results in the inevitable loss of fertility (Hansen, et al. 2011). Understanding the changes induced by the ageing process is of paramount importance to effective clinical treatment for infertility. In an alternative approach, the mature cell surface protein for LHR was measured by flow cytometric analysis. Immunolabelled granulosa cells were collected from individual antral follicles during the peri-ovulatory stage from patients receiving IVF treatment for infertility. The receptor density was quantified by the average fluorescent intensity of ~8000 isolated granulosa cells per follicle from a range of follicle sizes.

8.4.1 Major findings

In the present study, the LHR density was reduced significantly with the decline in ovarian reserve (Figure 8.3.2). The combination of a high level of BMPR1B (Chapter 6) and LHR density in the terminal-end follicles appear to give rise to the ovarian ageing effect; which may be associated with reduced fertility as we age.

8.4.2 LHR in early cyclic folliculogenesis

The results of the current study show a constant level of LHR density in IVF treated patients, with a significant drop in granulosal LHR density in the largest follicles collected from the young patient group with a good ovarian reserve (Figure 8.3.2) The follicles from this group were collected from dominant follicle selection (8 mm) to pre-ovulatory terminal-end follicles (26 mm); having all been exposed to a hCG/LH trigger for 36 hours (Figure 8.3.2).

The role of the LHR in folliculogenesis is predominantly involved in the morphological and stereological changes taking place in the follicle in preparation for ovulation and corpus luteum function to support pregnancy (Minegishi, et al. 1997). However, during dominant follicle selection, LHR expression on the granulosa cell influences the rate of growth and development of the follicle and the fertilisation potential of the oocyte (Maman, et al. 2012, Rice, et al. 2007).

Previously published data describe the positive staining of the LHR protein in human granulosa cells from very small antral follicles (0.03 mm), with increasing density and follicular size, in unstimulated natural cycles; which supports an earlier role for LHR (Yung, et al. 2014). In addition, the same study reports that a 5 mm follicle contained granulosa cells with a greater density of LHR protein than the theca equivalent (Yung, et al. 2014). Thecal cell LHR expression appears to peak and then drop at the time of dominant follicle selection, whereas granulosal expression steadily increases. (Xu, et al. 1995). Maman et al. (2012), and Jeppesen et al. (2012) also demonstrated that the early acquisition of LHRs on granulosa cells, at the time of dominant follicle selection which supports a substantial role for LHRs in the selection of the dominant follicle cohort. In addition, during *in vitro* maturation (IVM), small antral follicles with insufficient LHR in the cumulus cells failed to respond to the hCG/LH trigger surge and remained immature (Yerushalmi, et al. 2014).

8.4.3 Natural Healthy cycle and IVF infertility treatment

In the current IVF study, the level of LHR density was sufficient to elicit the changes required prior to ovulation by the clinical confirmation of serum LH and progesterone levels surging, cumulus expansion, and expulsion of the polar body (Fan, et al. 2009, Shimada, et al. 2003). In the rodent *in vivo* studies, (Camp, et al. 1991, Nakamura, et al. 1991) and human *in vitro* culture (Maman, et al. 2012), PMSG and rFSH, respectively, increased the LHR expression on granulosa cells. Given the large dose of rFSH stimulation during IVF, the LHR density appears not to be increased in the pre-ovulatory follicles compared to the natural cycle 18.5 mm follicle, (Figure 8.3.1).

8.4.4 Ovarian ageing and pre-ovulatory maturation

Older patients, typically, have increased circulating FSH and reduced inhibin B, which gives rise to an initial accelerated early follicle development, and results in a shorter cycle length (MacNaughton, et al. 1992, Robertson 2009, Santoro, et al. 2003, Seifer, et al. 1999). The granulosa cell proliferation was found to be reduced and the size of the follicle to be smaller in older patients (Bomsel Helmreich, et al. 1979, Robertson 2009, Seifer 1996 , Seifer, et al. 1993). The reduced rate of follicular growth found in older patients may result in reduced quality of the follicle and oocyte (Santoro, et al. 2003). A shorter cycle and a reduction in the follicle size due to ovarian ageing would reduce the capacity of the follicle to produce sufficient oestrogen to down-regulate pituitary FSH during dominant follicle selection.

In the Booroola sheep, affected by the BMPR1B mutation the follicle size and the amount of oestrogen per follicle is reduced, yet the overall oestrogen reaches the threshold because of the number of follicles developing simultaneously (Gonzalez Bulnes, et al. 2004, McNatty and Henderson 1987a). A reduced follicle size in older patients may cause over thickening of the granulosa layer which would reduce the diffusion concentration gradient of serum to follicular fluid, and lead to reduced follicle and oocyte quality (Bächler, et al. 2014).

In several studies in humans, the magnitude of the LH surge has not been found to be significantly different in older patients. Several inconsistencies exist in those studies, which mainly centred on the classification of ‘old’ and ‘poor’ ovarian reserve, which was based on the day three FSH serum concentration (Klein N A, et al. 1996, Leach, et al. 1997, MacNaughton, et al. 1992, Pal, et al. 2010, Santoro, et al. 2003). However, in Ginther et al. (2008), the LH surge in the mare, is reported to be markedly reduced with declining ovarian reserve, based on chronological age (Ginther, et al. 2008). Regardless, a distinction needs to be made between patients with an uncompromised reproductive cycle, the transitional phase of peri-menopause and the anovulatory post-menopause cycle (Hale 2007).

In this study we are comparing the difference between young and old with a range of ovarian reserves, some of whom would have entered the early peri-menopausal stage of life, equivalent to the patients with an ovarian reserve of E AFC. and/or an AMH of ~1 µg/L (Hale 2007). Depletion of the ovarian reserve is a slow, sequential process, and therefore, the changes to receptor density occur gradually (Hansen, et al. 2011). By comparing the patterns of expression with reducing AFC, the ageing effect can be observed. In the typical older patient with a poor ovarian reserve (40+ y D), compared to the younger patients, the LHR density is reduced (Figure 8.3.1).

The amplitude of the LH surge has been observed previously to reduce with a decline in the ovarian reserve (Anzalone, et al. 2001, LaPolt and Lu 2001).With a decline in the LH surge with ovarian ageing, the corresponding impact on the desensitisation by the degradation of the *LHR* mRNA would be consequently reduced (LaPolt and Lu 2001, Nair, et al. 2006). Therefore, it is probable that the ovarian ageing effect results in a lack of down-regulation of the LHR density in the older poor ovarian reserve patients compared to the younger patients.

8.4.5 Down-regulation and the terminal-end of folliculogenesis

In the present study, the LHR density reduced significantly in the pre-ovulatory follicle of the young, good ovarian reserve group (Figure 8.3.2). The down-regulation of the LHR is consistent with the results from the sheep study (Chapter 5), where the LHR peaked in the largest of the subordinate follicles; yet, the leading dominant follicle had reduced LHR density in both the wild type and mutation affected Booroola Merino sheep (Regan, et al. 2015) . The drop in LHR expression in the WT sheep also indicated that the drop is short lived, as the granulosa cells differentiate into granulosa-lutea cells (Micevych, et al. 2003, Regan, et al. 2015). The down-regulation of the ovulating follicle has also been reported by others in the bovine and human (Jeppesen, et al. 2012, Ndiaye, et al. 2005, Ophir, et al. 2014, Xu, et al. 1995); however, conflicting reports exist (Irving-Rodgers, et al. 2009, Jakimiuk, et al. 2001, Maman, et al. 2012). When comparing reported levels of receptor density, it is important to consider whether or not exogenous IVF stimulation was administered, and if the follicles had been exposed to a hCG/LH surge.

Clinical administration of hCG or the natural cycle LH surge induces cytoskeletal reorganisation of the granulosa cell, cessation of mitogenic proliferation, cumulus expansion, gap junction closure, resumption of meiosis, and maturation of the oocyte (Fan, et al. 2009, Izadyar, et al. 1998b). These changes would have an effect on the *LHR* mRNA and mature LHR protein density (LaPolt and Lu 2001, Lapolt, et al. 1990, Peng, et al. 1991b). Therefore, pooling of follicles would potentially mask the down-regulation by averaging-out the LHR density, and thereby may reduce the accuracy of the data. For example, Jakimiuk et al. (2001), grouped the follicles, and found that the 4.2 to 8.7 mm follicles expressed the same amount of *LHR* mRNA as follicles from 9.1 to 23.2 mm. In the Maman et al. (2012), study, no difference in the *LHR* mRNA expression was found in the small follicles of <10 mm compared to 10 to14 mm; however, it was reported that the pre-ovulatory 17+ mm follicles peaked with no reported down-regulation observed. The standard deviation was much higher in the 17+ mm group, which may be as a result of masking of the down-regulation in the largest follicles.

8.4.6 Correlation of BMP and LHR

It has been reported that the hCG/LH induced surge effect occurs 12 hours later in the theca cells and 24 hours later in the granulosa cells (Chaffin and Stouffer 2000), which indicates that the follicles with sufficient LHR would undergo the pre-luteinisation changes described above. Disruption to this regulatory mechanism was evident with a decline in AFC related to age, and is mirrored in the changing correlation between BMPRIB and the LHR (Figure 8.3.6). The relationship between the high levels of BMPR1B (Chapter 6) and the LHR in the terminal-end follicles appear to give rise to the ageing effect; which may be associated with reduced fertility as we age.

8.4.7 Conclusion

Taken together, the results demonstrate the disrupting effect of age-related depletion of the ovarian reserve has on granulosal LHR. The present study's data extends previous research by the comprehensive nature of the range of follicle size and age groups examined, together with measurement of the translated mature LHR protein as opposed to measurement of receptor expression at the mRNA level (Ascoli, et al. 2002). Further work is needed to confirm the identity of the locally-produced TGFβ family ligand(s) whose signalling may be impacted by the change in LHR density on the granulosa cell surface, and also to explore the consequences of altered signalling.

CHAPTER 9

Ovarian Ageing and GHR

Title: The decline in ovarian ageing and the effect on the GHR in humans

CHAPTER 9 Ovarian Ageing and GHR

9.1 Introduction

It is apparent that GH treatment *in vivo* and *in vitro* in conjunction with recombinant rFSH increases the oocyte survival rate, and increases the pregnancy rate (Barreca, et al. 1993b, Folch, et al. 2001, Izadyar, et al. 1996, Izadyar, et al. 1998b). Tesarik et al. (2005), found convincingly, that the number of oocytes retrieved and conceptions were the same, whereas, the pregnancy rate was increased; which is in line with the previous findings of increased pregnancy rate, enhanced mitochondrial activity and up-regulation of the GHR on the oocyte (Weall, et al. 2014, Yovich and Stanger 2010).

GHR have been reported to be continuously expressed and have a half-life of one hour (Brooks, et al. 2008). GH is activated by changing the conformation of the receptor to form a complex with janus kinase 2 (JAK2), (Lan, et al. 2014). *In vitro* culture with GH has demonstrated its stimulating effect on the synthesis of oestrogen, *BMP2* and *BMP4* mRNA (Karamouti 2008, Li, et al. 1998).

The granulosa, cumulus, and the oocyte of antral follicles express GHR, and are therefore able to react to ovarian or pituitary derived sources of GH (Abir, et al. 2008b, Izadyar, et al. 1999). In addition, granulosa cells and the oocyte have been shown to produce *GH* mRNA (Abir, et al. 2008b, Bevers and Izadyar 2002, Izadyar, et al. 1998a, Izadyar, et al. 1997b, Izadyar, et al. 1999). The GHR forms a complex with JAK2 and initiates phosphorylation of the signal transducer and activator of transcription (STAT1, 3, 5), which leads to other ERK1/2- MAPK pathways. GH activation of the GHR induced transcription activity in the nucleus that leads to an increase in mitogenesis, lipogenesis, and protein synthesis (Bevers and Izadyar 2002, Izadyar, et al. 1997a, Izadyar, et al. 1998b, Moore, et al. 2001a). In addition, GH has been shown to accelerate granulosal luteinisation and to promote cumulus expansion and resumption of meiosis in the oocyte (Hutchinson, et al. 1988, Izadyar, et al. 1997a).

This study reports on the changes to GHR density during the development of the follicle from dominant follicle selection, to the pre-ovulatory maturation before ovulation in IVF patients. Previously, we have presented comprehensive results on the density profiles of patient follicle receptors and ovarian ageing, in young patients through to the older poor response to rFSH stimulation patients (Chapter 6-8). It has been demonstrated that the BMPR1B profile was significantly altered with a decrease in AFC and an increase in age. The biphasic down-regulation of BMPR1B density observed in wild type Merino sheep (Regan, et al. 2015) was

also present in young human patients and sequentially deteriorates with ovarian ageing, culminating in the loss of receptor density and a reversal of the biphasic down-regulation (Chapter 6). The changes to FSHR and LHR density have also demonstrated an ovarian ageing effect, with the lack of pre-ovulatory down-regulation in the largest follicles, previously presented (Chapter 7 and 8) and also present in the sheep model (Chapter 5) (Regan, et al. 2015).

The current study aims, to further explore the changes that take place due to a decline in ovarian reserve, by measuring the granulosa cell surface-expressed mature receptor protein density for GHR by immunofluorescent labelling via flow cytometry. A comprehensive range of follicle sizes from 4 to 27 mm were collected from patients receiving IVF treatment, and an average of ~8000 granulosa cells from individual follicles were used to determine the density of the GHR.

9.2 Methods

Methods are described in detail in the General Methods Chapter (page 38).

Granulosa cells were collected from hCG/LH induced follicles from patients receiving IVF exogenous gonadotrophins to artificially stimulate multiple follicles (Table 9.2:1). The granulosa cells were isolated and incubated with antibodies to detect the level of BMPR1B, FSHR, LHR, and GHR mature protein. In addition, apoptosis and necrosis were also determined by immunolabelling via flow cytometric analyses (page 39).

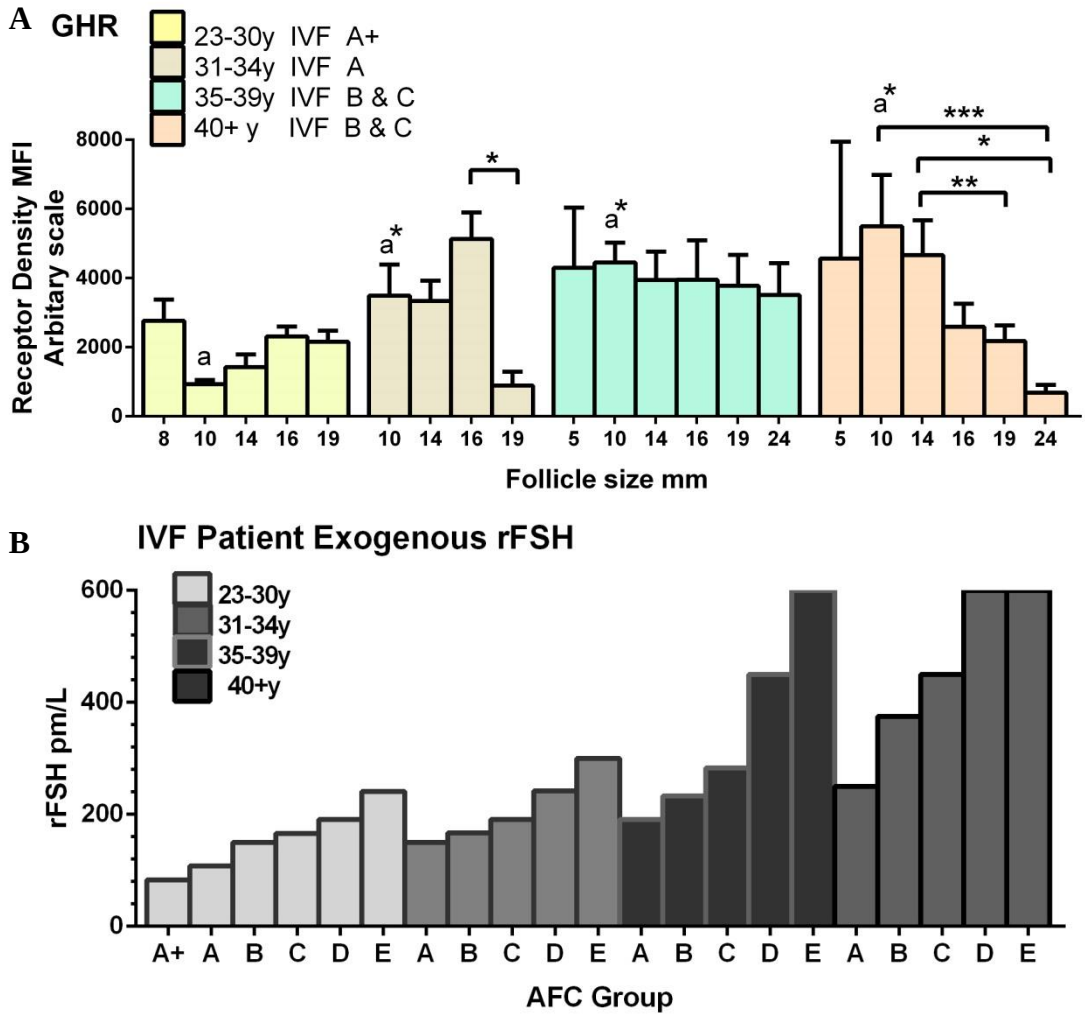
Table 9.2:1 Patient ovarian reserve, based on antral follicle count (AFC) and the number of follicles collected per group

AGE Year	IVF Patient	Total Follicle	AFC	Major Group	Number of Follicles Collected Per Group							
					Sub Group	#	Sub Group	#	Sub Group	#	Sub Group	#
21-30	8	95	20-40	A+ &A	A+	31	A	64	C	6		
31-34	11	86	13-29	A & B	A	60	B	26	C	17		
35-39	16	102	9-19	B & C	A+	6	B	50	C	16	D 30 & E	6
40-45	18	118	3-8	D & E	D	59	E	19	B	34		
40	1	Natural Cycle Healthy			D	2						

Patient ovarian reserve, based on antral follicle count (AFC) and the number of follicles collected per group. Total number of follicles from patients receiving IVF treatment or a natural unstimulated. Follicles are classified into subgroups based on the patients' antral follicle count (AFC) on approximately day 5 of a cycle. Major groups based on AFC are the most common patient profile; and patient subgroup follicles are described. Ovarian reserve measured indirectly by the Antral Follicle Count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle: A+ = 30-39 follicles; A = 20-29; B = 13-19; C = 9-12, D = 5-8; E = ≤4.

9.3 Results

The patients groups were selected to represent the best prognosis patients, based on their ovarian reserves, measured by AFC for the age class (Figure 9.3.1A). GHR density increased, based on the 10 mm follicle size with chronological age from 23-30 y patients to 40+ y, (indicated by 'a', $p<0.05$,Figure 9.3.2A). In the 31-34 y patients, there was a decline in the density towards the terminal-end of folliculogenesis, ($p<0.05$, Figure 9.3.1A). In the 40+ y B & C AFC a sequential significant decline in density of GHR was shown as the follicles increased in size ($p<0.005$, Figure 9.3.3A), even though the gonadotrophin treatment dose was higher (Figure 9.3.3B). The increasing dose of rFSH appears to be ineffective in restoring the younger profile of recptor density of the largest follicles in the oldest patients.



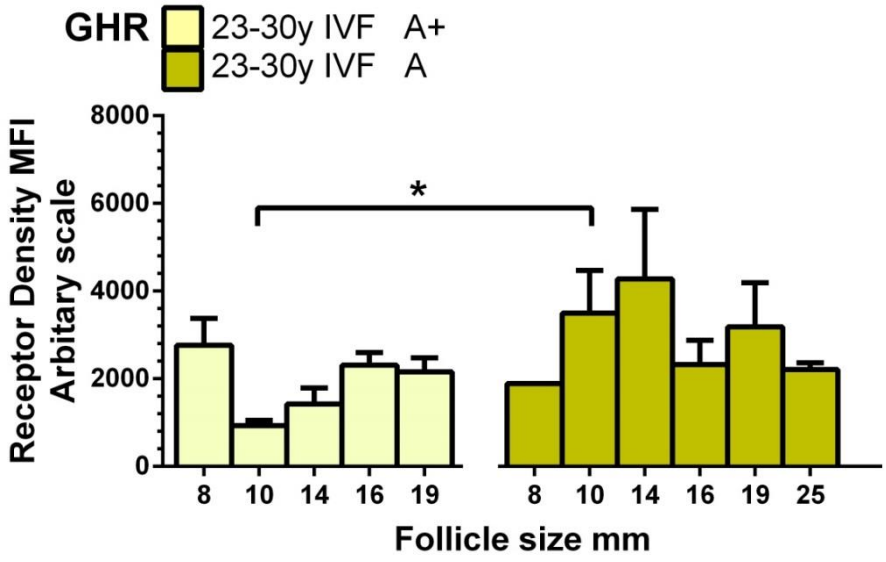
GOOD: A+ = 30-39: A = 20-29: B = 13-19: C = 9-12: D = 5-8: E = ≤4: POOR

Figure 9.3.1 Granulosal GHR density and age of IVF patients with good ovarian reserve and rFSH dose.

GHR expression density with increasing chronological age; from young 23-30 y patients to old patients of 40+ y , in the best ovarian response groups within each age class. Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day

2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *p<0.05, **p<0.01, *** and p<0.005. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level for the size follicle. (B) IVF patient exogenous gonadotrophin treatment dose for rFSH based on a declining AFC and age group; based on data from (Yovich, et al. 2012).

It was evident that the density of GHR in the 23-30y A+ patients, was significantly lower in the small 10 mm follicles compared to the young patients with an AFC of A, (p<0.05, Figure 9.3.2).



GOOD: A+ = 30-39: A = 20-29: B = 13-19: C = 9-12: D = 5-8: E = ≤4: POOR

Figure 9.3.2 Granulosal GHR density and ovarian reserve depletion in 23-30 y.
Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *p<0.05. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level for the size follicle.

In the 31-34 y age class a significant drop in GHR density was evident in the pre-ovulatory follicles of 19 mm ($p<0.005$, Figure 9.3.3). As the ovarian reserve declined the GHR density was reduced significantly in AFC group B and C, $p<0.05$, Figure 9.3.3).

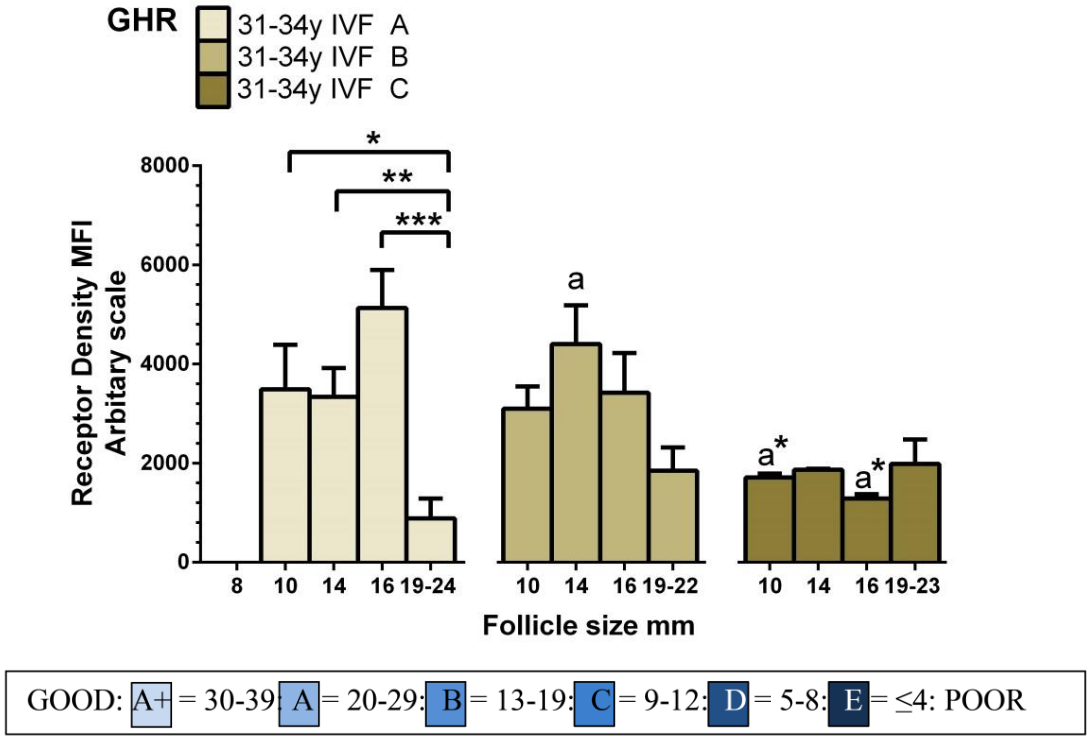


Figure 9.3.3 Granulosal GHR density and ovarian reserve depletion in 31-34 y.
Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *$p<0.05$, **$p<0.01$ and ***$p<0.005$. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level for the size follicle.

The GHR density was significantly greater in the 35-39 y, with an AFC of B & C whereas, with a further decline in ovarian reserve the GHR density failed to response to the FSH treatment producing significantly less GHR particularly in the smaller antral follicles ($p<0.05$, Figure 9.3.4).

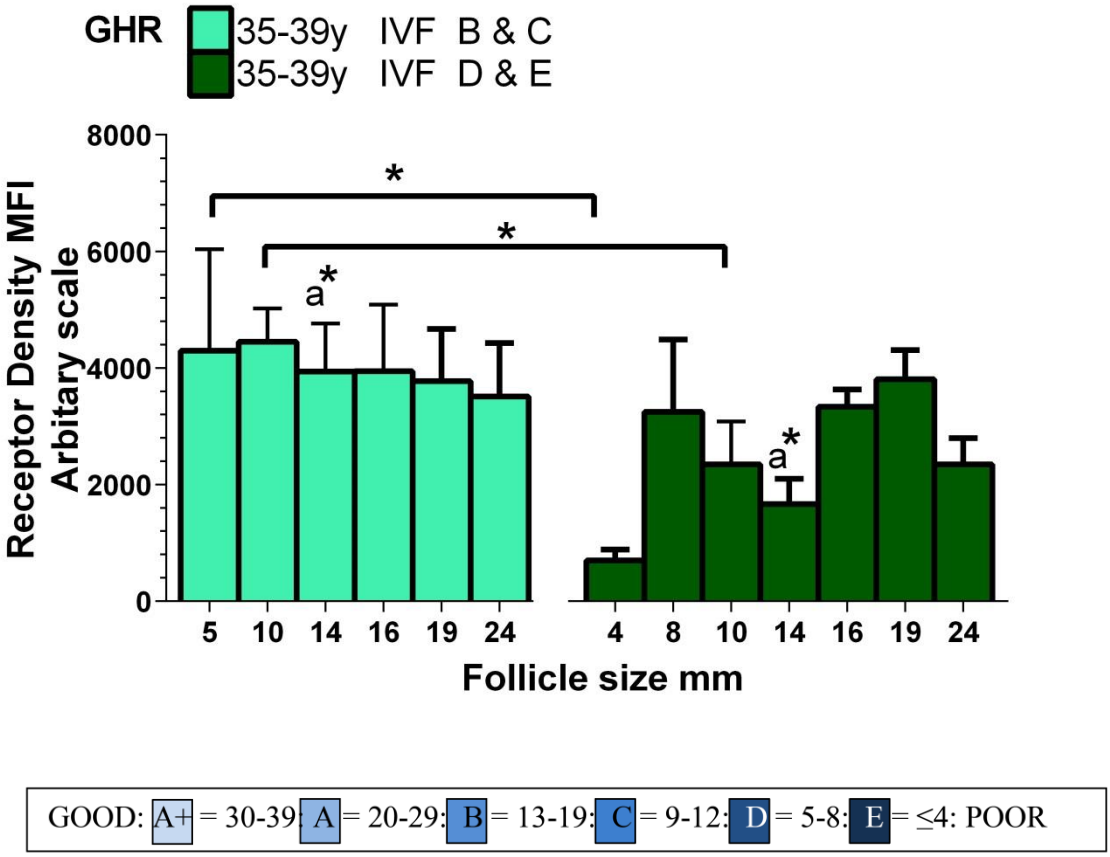


Figure 9.3.4 Granulosal GHR density and ovarian reserve depletion in 35-39 y.
Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *$p<0.05$. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level for the size follicle.

In the 40+ y patients there was a dramatic loss of GHR density in the D & E AFC groups, particularly in the small antral follicles of 8 to14 mm when compared to the 40+ y B & C profile ($p<0.01$, Figure 9.3.5). In the 40+ y B & C group the density is reduced sequentially with increasing follicle size. ($p<0.005$, Figure 9.3.5).

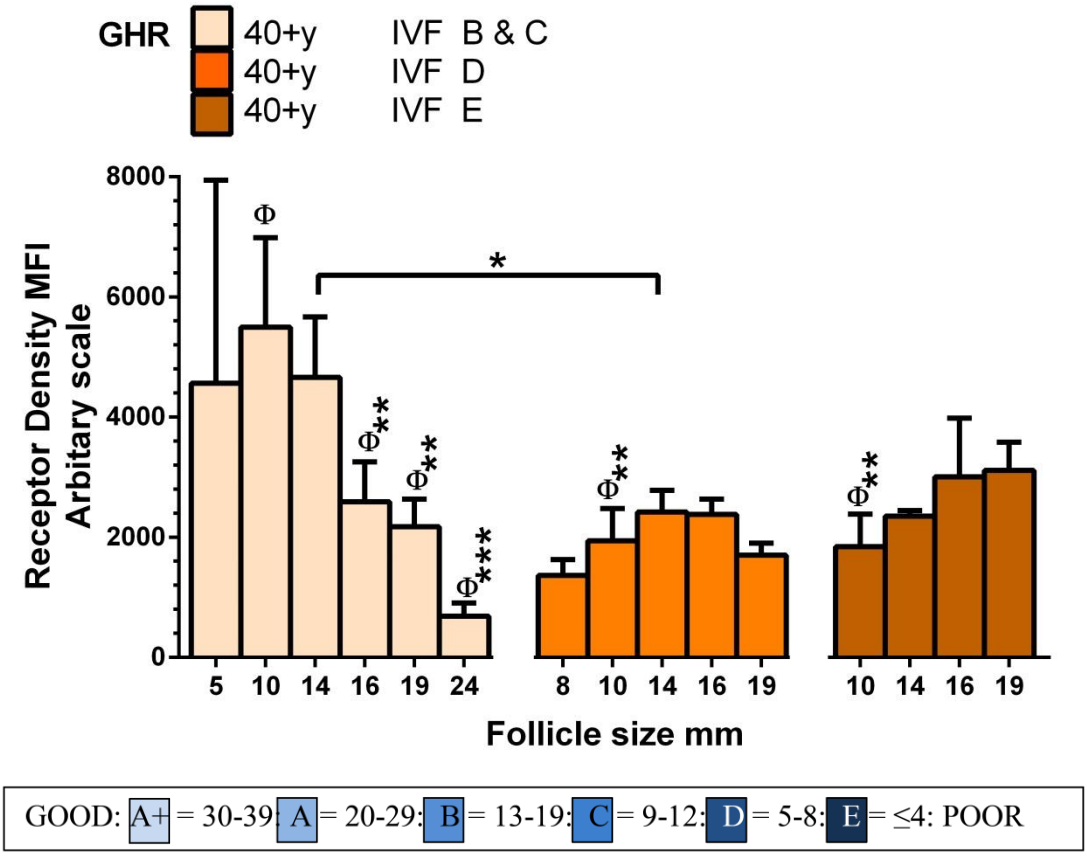


Figure 9.3.5 Granulosal GHR density and ovarian reserve depletion in 40+ y.
Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *$p<0.05$, **$p<0.01$ and ***$p<0.005$. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level for the size follicle.

9.4 Discussion

The oocyte and the granulosa cells produce *GH* mRNA and *GHR* mRNA (Abir, et al. 2008b, Bevers and Izadyar 2002, Izadyar, et al. 1998a, Izadyar, et al. 1997b, Izadyar, et al. 1999) and express the GHR on the cell membrane surface, on the endoplasmic reticulum and on the nuclear membrane (Abir, et al. 2008b, Brooks, et al. 2008, Izadyar, et al. 1999, Weall, et al. 2014, Zhu, et al. 2001). The GHR is regulated by the gonadotrophins and growth factors, such as GH binding proteins, BMPs and somatostatin (Le Roith, et al. 2001, Nakamura, et al. 2012).The granulosal *GHR* mRNA expression during folliculogenesis or the impact of a declining ovarian reserve on the receptor density has not been previously documented.

9.4.1 Major Finding

Chronological ageing is associated with an initial increase in granulosal GHR particularly in the smaller antral follicles selected into the dominant follicle cohort at 10 mm. However, within the same age class, the density of the receptors reduces as the ovarian reserve declines (Figure 9.3.1). A correlation between the increasing level of rFSH treatment and the increase in GHR was evident; however the effect of exogenous rFSH to increase the GHR expression diminished in the older age groups with the poorest ovarian reserve (Figure 9.3.1 to Figure 9.3.5).

9.4.2 The effect of increasing rFSH and the level of GHR density

As the ovarian reserve declines the ovarian response indicated by the number of growing small antral follicles diminishes. An increased dose of rFSH is given to patients to maximise the ovarian response to stimulation during an IVF treatment cycle (Yovich, et al. 2012). The increasing levels of GHR density may be in response to the increasing amounts of rFSH or a compensatory mechanism in response to a declining ovarian reserve (Figure 9.3.1).

The youngest patients with an ovarian reserve of A+ receive the least amount of rFSH, whereas, the 31-34 y A patients receive two times more rFSH, and have an increased GHR density in the 10 to16 mm follicles with no effect on the GHR density of the pre-ovulatory follicle (Figure 9.3.1). Very high doses of rFSH appear to be ineffective in increasing GHR density in the later stages of follicular development compared to the earlier stage of follicular development (Figure 9.3.1). The lack of any effect on the pre-ovulatory follicles may be as a result of the naturally reducing granulosal FSHR during the maturation phase, previously reported in Chapter 7 and in sheep (Regan, et al. 2015). The lower density of FSHR and LHR in patients with a poor ovarian reserve would also reduce the sensitivity to exogenous rFSH stimulation previously described (Chapter 7 and 8) and previously reported in (Cai, et al.

2007). In addition, clinical experience correlates well with the lack of response to rFSH as the AFC diminishes with increasing age (Yovich, et al. 2012).

9.4.3 Conclusion

The reduced ability of the granulosa to express GHR as the ovarian follicle reserve depletes demonstrates a potential mechanism responsible for the reduction in oocyte quality. In addition, the saturation of the FSH-FSHR signalling pathway failed to change the GHR density in the poor ovarian reserve patients. The sensitivity of FSHR and LHR appear to be compromised in poor response patients as abundant levels of rFSH are available in increasing doses. It is therefore, probable that the rFSH treatment reaches an ineffective threshold, via the classic FSH-FSHR-cAMP-PKA signalling pathway as the ovarian reserve declines.

CHAPTER 10

Ovarian Ageing and GH Co-treatment

Title: Growth hormone receptor density regulation with declining ovarian reserve, and the pattern of density of reproductive receptors during IVF co-treatment with GH

CHAPTER 10 Ovarian Ageing and GH Co-treatment

10.1 Introduction

Ovarian depletion of primordial follicles is a continual natural process from gestation to adulthood, which culminates in the loss of ovarian function that produces a state of menopause (Gougeon 1986, Richards 1994). The primordial follicles when activated grow and develop into small antral follicles, and then succumb to apoptosis (Ginther, et al. 2005, Hillier 2001). At puberty sufficient FSH recruits an initial cohort of small antral follicles to the menstrual cycle (Austin, et al. 2001, Ginther, et al. 2005). The follicles grow under the influence of FSH and express FSHR, and LHR. FSH activation of the FSHR stimulates oestrogen synthesis, which stimulates proliferation of the granulosa and thecal cells and development of the oocyte; all of which are contained within the follicle (Campbell, et al. 1999, Ginther, et al. 2012, Luo, et al. 2011, Picton and McNeilly 1991).

The ovulation rate is determined by the stage-specific decrease in pituitary secreted FSH, and results in follicles with insufficient LHRs succumbing to apoptosis (Mihm, et al. 2006, Minegishi, et al. 1997, Yung, et al. 2014).The follicle continues to grow until pre-ovulatory maturation when proliferation ceases and granulosa cell differentiation occurs in preparation for ovulation of the oocyte.

As the ovarian reserve of primordial follicles is depleted, regulation of folliculogenesis is altered, which results in decreased fertility (Klein N A, et al. 1996). Ovarian depletion can be indirectly measured by the number of small antral follicles present at the beginning of a cycle, and is highly correlated to chronological age (Hansen, et al. 2011). During IVF treatment, high doses of rFSH are used to recruit more of the small antral follicles and to maintain the growth during pituitary FSH-down regulation (Yovich and Stanger 2010). Infertility patients with a poor ovarian reserve have fewer small antral follicles available for recruitment, and higher doses of rFSH become ineffective in recruiting more follicles during IVF cycles. In an attempt to improve the pregnancy rate patients have been routinely co-treated with GH and other adjuvant treatments (Tesarik, et al. 2005, Yovich and Stanger 2010).

The patients with a poor response to rFSH treatment represent a large group of patients with critically diminishing ovarian reserve (de Ziegler, et al. 2011, Kyrou, et al. 2009). The challenge was to identify the changes taking place as the ovarian reserve declines and to find alternative stimulation to provide quality oocytes for fertilisation.

Previously, GH co-treatment *in vivo* and *in vitro* in conjunction with rFSH increased the oocyte survival rate, and increased the pregnancy rate (Barreca, et al. 1993b, Folch, et al. 2001, Izadyar, et al. 1996, Izadyar, et al. 1998b). The granulosa, cumulus, and the oocyte of antral follicles express GHR and are therefore able to react to ovarian or pituitary derived sources of GH (Abir, et al. 2008b, Izadyar, et al. 1999). In addition, granulosa cells and the oocyte produce GH, whereas cumulus and theca cells have not been shown to produce *GH* mRNA (Abir, et al. 2008b, Bevers and Izadyar 2002, Izadyar, et al. 1998a, Izadyar, et al. 1997b, Izadyar, et al. 1999). GHRs are activated by GH by changing the conformation of the receptor forming a complex with JAK2, (Lan, et al. 2014). GH induced steroidogenesis was found not to be mediated by the FSH-FSHR-cAMP-PKA signalling pathway (Nakamura, et al. 2012). Alternatively, the GHR-JAK2 complex initiates the subsequent phosphorylation of the signal transducer and activator of transcription STAT1, 3, 5, or a number of other pathways such as the ERK1/2 -MAPK pathway, which results in *GH* transcription activity in the nucleus leading to an increase in mitogenesis, lipogenesis, and protein synthesis (Bevers and Izadyar 2002, Izadyar, et al. 1997a, Izadyar, et al. 1998b, Moore, et al. 2001a). The GHR-JAK2 complex can stimulate over 400 different types of signals, such as cell differentiation and oocyte maturation (Waters, et al. 2006).

The cellular mechanism underpinning the GH induced improvement has not been reported in human studies. However, many attempts have been made to delineate the indirect changes taking place to serum and follicular fluid hormone levels. Previously, we have presented comprehensive results on the receptor density profiles of patients during ovarian ageing (Chapter 5-9). A decline in granulosal BMPR1B and FSHR density occurred at the time of cyclic dominant follicle selection, and again during the terminal stage of folliculogenesis, in the good ovarian reserve IVF patients of 23-30 y. The older poor ovarian reserve patients of 40+ y experienced a reversal of this pattern. In addition, the LHR density failed to be down-regulated during pre-ovulatory maturation in the 40+ y group, and GHR density was reduced with ovarian reserve.

The current study aims, to further explore the effect of GH co-treatment on the changes to the density of BMPR1B, FSHR, LHR and GHR induced by the depletion of the ovarian primordial follicle reserve. An average of ~8000 granulosa cells/follicle were analysed, and the ovarian follicles collected from IVF patients ranged in diameter from 4 to 27 mm. The granulosa cell, surface-expressed mature receptor protein density was measured by immunofluorescent labelling via flow cytometry.

10.2 Methods

Methods are described in detail in the General Methods Chapter (page 38).
Granulosa cells were collected from hCG/LH induced follicles from patients receiving IVF exogenous gonadotrophins to artificially stimulate multiple follicles (Table 10.2:1). The granulosa cells were isolated and incubated with antibodies to detect the level of BMPR1B, FSHR, LHR, and GHR mature protein. In addition, apoptosis and necrosis were also determined by immunolabelling via flow cytometric analyses (page 39).

Table 10.2:1 Patient ovarian reserve, based on antral follicle count (AFC) and the number of follicles collected per group.

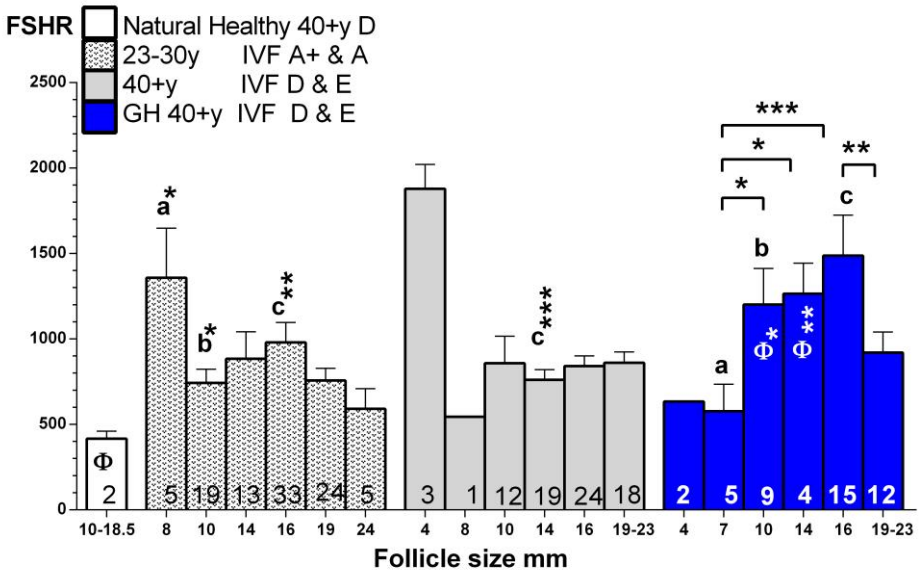
Patient ovarian reserve, based on antral follicle count (AFC) and the number of follicles collected per group. Total number of follicles from patients receiving IVF treatment or a natural unstimulated.

AGE	IVF patient	Total follicle	AFC	Major Group	Total Follicle	Sub Group	#	Sub Group	#	Sub Group	#	Sub Group	#
21-30	11	101	20-40	A+ & A	96	A++	14	A+	31	A	64		
31-34	11	86	13-29	A & B	86	A	60	B	26	C	17		
35-39	16	102	9-19	B & C	66	A+	6	B	50	C	16	D&E	30&6
40-45	18	118	3-8	D & E	78	D	59	E	19	B	34	C	6
40-45 GH	11	78	3-8	D & E	47	D	22	E	23	B	16	C	5
40		Natural Cycle Healthy				D	2						

Follicles are classified into subgroups based on the patients' antral follicle count (AFC) on approximately day 5 of a cycle. Major groups based on AFC are the most common patient profile; and patient subgroup follicles are described. Ovarian reserve measured indirectly by the Antral Follicle Count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle: A+ = 30-39 follicles; A = 20-29; B = 13-19; C = 9-12, D = 5-8; E = ≤4.

10.3 Results

Growth hormone treatment increased the FSHR receptor density in the follicles from 40+ y D & E AFC group patients in the 16 mm follicles ($p<0.005$), with a significant down-regulation of FSHR in the terminal-end 19 mm pre-ovulatory GH co-treated follicles ($p<0.01$, Figure 10.3.1). Granulosal FSHR density significantly increased from 7 to 10 mm, 14 mm, and 16 mm follicles in the GH co-treated patient group ($p<0.05$ and $p<0.005$).



GOOD: A+ =30-39; A = 20-29; B = 13-19; C = 9-12; D = 5-8; E = ≤4: POOR

Figure 10.3.1 Granulosa cell density of FSHR in poor response 40+ y patients co-treated with GH compared to untreated.

Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *$p<0.05$, **$p<0.01$, and ***$p<0.005$. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level for the size follicle. The two natural cycle follicles were combined for statistical comparison as the values were similar.

The BMPR1B density was significantly increased in the GH co-treated 10 mm to 16-18 mm follicles compared to the untreated with GH, and age matched, 40+ y D & E patient group ($p<0.001$ and $p<0.01$, respectively, Figure 10.3.2). A significant down-regulation of the BMPR1B density occurred from 10 mm to 19 mm ($p<0.05$, Figure 10.3.2). The receptor density of the natural cycle was significantly lower than the GH co-treated patients ($p<0.01$, Figure 10.3.2).

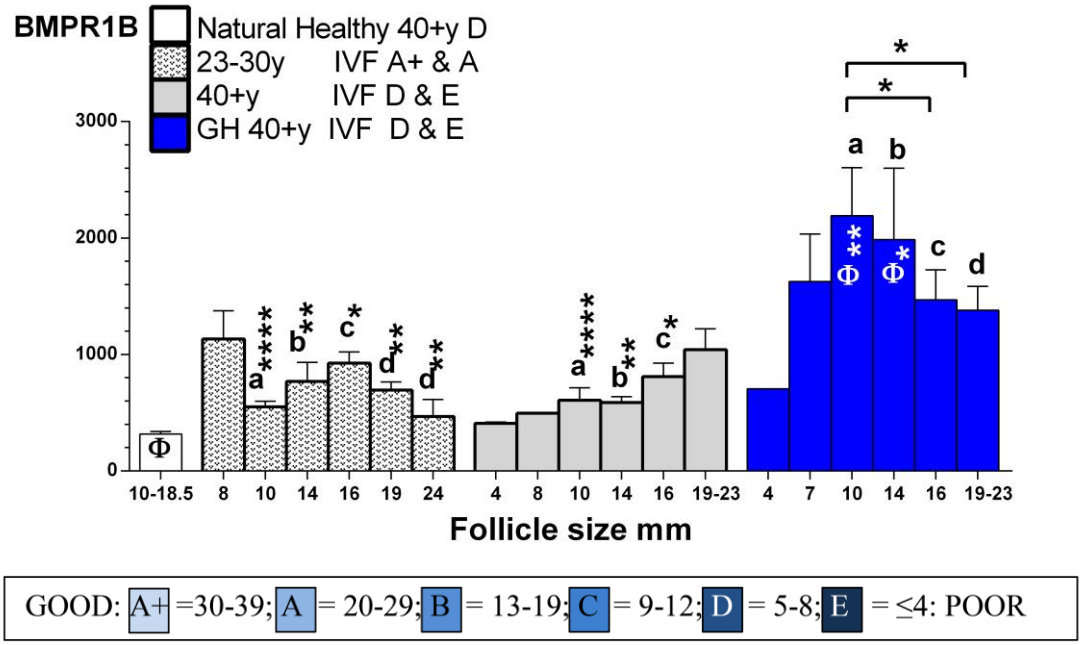


Figure 10.3.2 Granulosa cell density of BMPR1B in poor response 40+ y patients treated with GH compared to untreated.

Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *$p<0.05$, **$p<0.01$, ***$p<0.005$, and ****$p<0.001$. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level for the size follicle.The two natural cycle follicles were combined for statistical comparison as the values were similar.

The LHR density of the granulosa cells collected from patients who received GH co-treatment during an IVF cycle was significantly elevated compared to untreated, age and ovarian reserve matched patient group (40+D and E); particularly in the 16 mm follicles ($p<0.001$, Figure 10.3.3). The GH co-treated terminal-end down-regulation of LHR was significant in the 19-23 mm follicles ($p<0.005$, Figure 10.3.3). The peak receptor density in the 16 mm follicles from patients treated with GH was greater than other younger patient groups ($p<0.001$, Figure 10.3.3). The receptor density of the natural cycle appeared lower in the 10 mm follicle compared to the 10 mm follicle of the age matched GH co-treated IVF patients; whereas the 18.5 mm terminal-end pre-ovulatory follicle appeared similar in density of LHR as significance was not reached due to the small number of follicles.

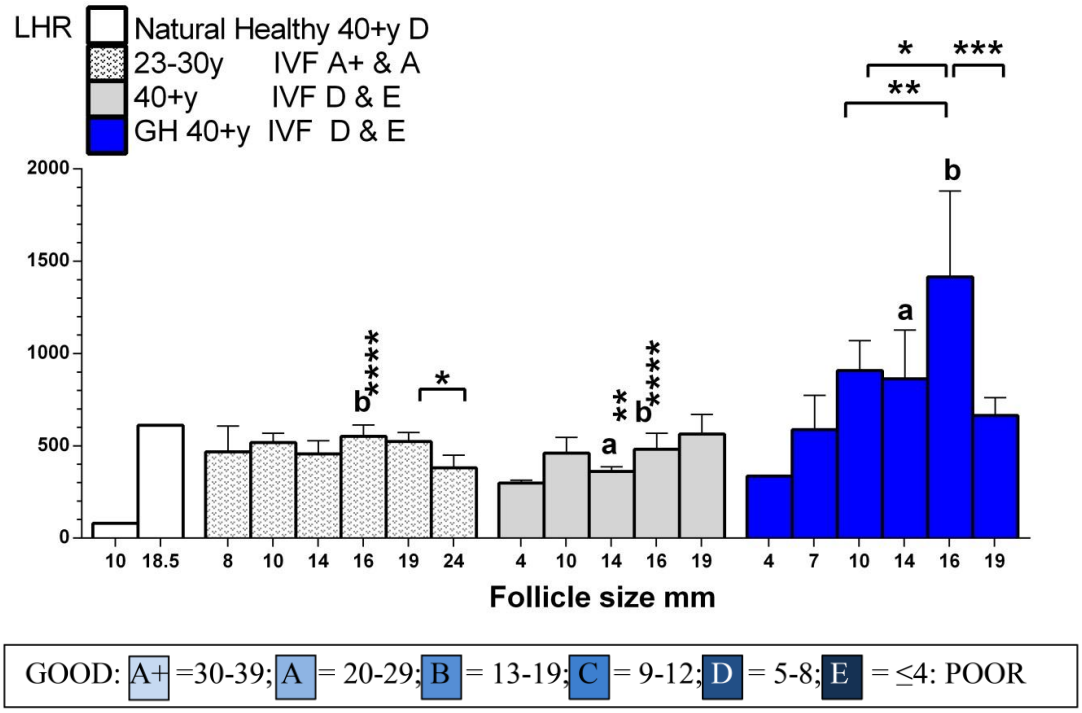


Figure 10.3.3 Granulosa cell density of LHR in poor response 40+ y patients co- treated with GH compared to untreated.

Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *$p<0.05$, **$p<0.01$, ***$p<0.005$, and ****$p<0.001$. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level for the size follicle.

The level of GHR was significantly increased in IVF patients receiving GH co-treatment in follicles from 10 to 23 mm compared to the same age patients of 40+ y with an ovarian reserve of D & E ($p<0.01$ to $p<0.001$, Figure 10.3.4). The receptor density was also greater in the GH co-treated IVF patients compared to the younger patients from 10 mm to 23 mm. The receptor density of the natural cycle healthy patient of 40+ y was significantly lower than the GH co-treated patients ($p<0.05$ and $p<0.01$).

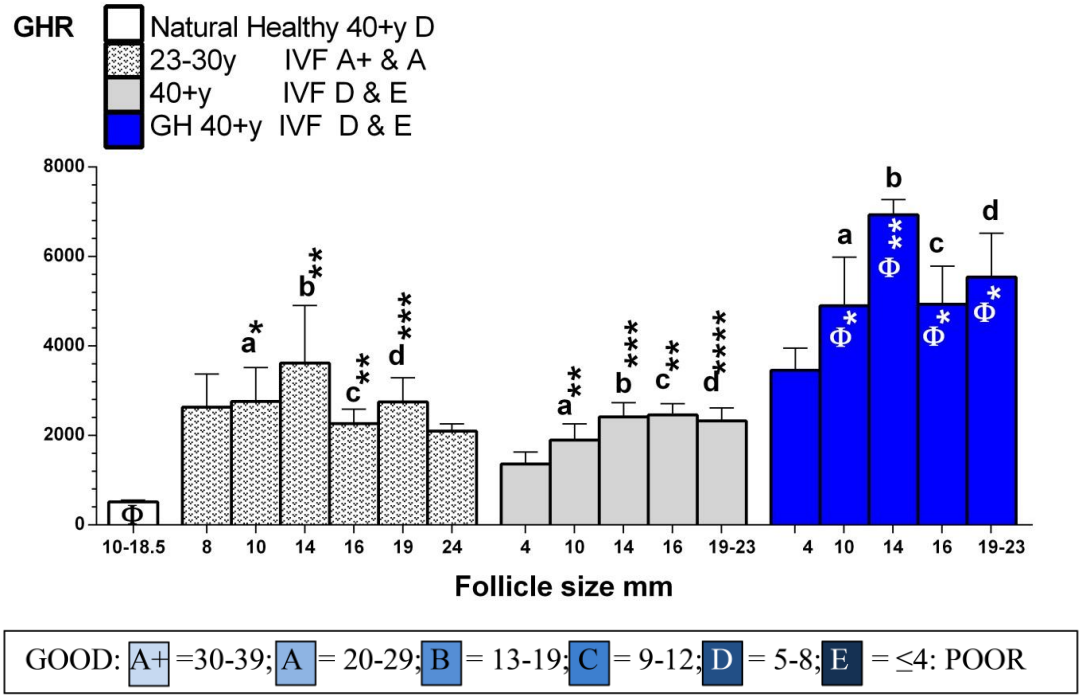


Figure 10.3.4 Granulosa cell density of GHR in poor response 40+ y patients co- treated with GH compared to untreated.

Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *$p<0.05$, **$p<0.01$, ***$p<0.005$, and ****$p<0.001$. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level for the size follicle. The two natural cycle follicles were combined for statistical comparison as the values were similar.

10.4 Discussion

10.4.1 Overview

In this study we report on the changes to granulosal receptor density for FSHR, LHR, GHR, and TGFβ type 1 receptor, BMPR1B in GH co-treated IVF patients. Reduced ovarian reserve patients have a poor response to IVF rFSH treatment, and produce oocytes of poor quality (Cai, et al. 2007). The poor response group of patients also have an associated high risk of

foetal aneuploidy that has been correlated to AFC (Grande, et al. 2014). Recently published data has shown that GH co-treatment increases the pregnancy rate by a suggested improvement in oocyte quality not quantity (Tesarik, et al. 2005, Yovich and Stanger 2010). Overshadowing the GH clinical debate, is the confounding clinical evidence that when young, good response patients and young or old poor response patients are combined, it results in a multivariate clinical patient group, which is difficult to treat uniformly. Furthermore, in the past, many cycles were cancelled that would otherwise still be continued in current times, which may impact the outcome. Several studies have been conducted on IVF patients, classified as having a 'poor response' to IVF stimulation, and were older than 35 y (Kucuk, et al. 2008, Suikkari 1996, Tesarik, et al. 2005, Yovich and Stanger 2010). When these studies are combined, with the studies that report failed cycles or no pregnancies (Eftekhar, et al. 2013, Levy, et al. 1993, Volpe, et al. 1989), a pregnancy rate of 36% for GH co-treated patients and 12% for non-GH treatment can be conservatively calculated. It is, therefore, probable that a cellular regulatory mechanism is affected by GH to produce an increase in pregnancy rate.

10.4.2 Major findings

In an attempt to reverse the poor prognosis of older patients, rGH was provided as an adjunct to IVF rFSH stimulation. While previous research has focused predominantly on clinical and biochemical evidence and basic science studies, using rodents and other species, the current study demonstrates that GH co-treatment increased receptor density in humans. The dysregulation of the ovarian micro-environment caused by ovarian depletion was partially reversed, providing an explanation for the reduction in pregnancy loss, with GH co-treatment. This study provides novel information on the effect exogenous GH has on the density of FSHR, BMPR1B, LHR, and GHR on granulosa cells from a wide range of follicle sizes.

10.4.3 No change to small antral follicles

In this study, GH co-treatment increased the receptor density for FSHR, BMPR1B, LHR and GHR compared to the non-GH treated patients of the same age and ovarian reserve (Figure 10.3.1 to Figure 10.3.4). Given that the driving force of folliculogenesis is FSH, it is apparent that increasing rFSH dose is not effective past a point, in the poor ovarian reserve patients as the pool of small antral follicles available for recruitment is diminished. Previously, poor ovarian response to rFSH stimulation has been associated with reduced granulosal FSHR expression (Cai, et al. 2007). In the current study, the improvement to the FSHR, LHR, and BMPR1B density occurred after the pituitary FSH down-regulation and dominant follicle selection, as this takes place at ~7-10 mm. In addition, in a previous study, GH co-treatment was found not to alter the FSHR density in human small follicles, which is consistent with

the findings for small follicles (Garverick, et al. 2002). Conflicting reports however, show that granulosal FSHR and LHRs from pre-ovulatory follicles from young adult cows were not altered by rGH co-treatment (Gong, et al. 1991). GH co-treatment may not have resulted in any change to the receptor density because the cows were young, with an uncompromised ovarian reserve and sufficient receptor density.

Further research is needed to ascertain the effect of GH co-treatment has on receptor density in good ovarian reserve patients

10.4.4 Oocyte number and LHR

In this study, GH co-treatment increased the level of FSHR in the larger pre-ovulatory follicles of 16-18 mm (Figure 10.3.1). Recruitment of the dominant follicle cohort is initially dependent on FSHR, followed by LHR sufficient to sustain the oestrogen requirements of the follicle as the pituitary FSH declines (Rice, et al. 2007). In the current study, FSHR was high in the 4 mm follicle of the older patients, which suggests that the level of FSHR was sufficient to enable initial growth of the small antral follicles before dominant follicle selection. However, the LHR level in the 4 mm follicle was significantly reduced compared to the level in the younger patients with good ovarian reserve (Figure 10.3.3). The lower density of LHR would reduce the oestrogen producing capacity of the follicle and limit the number of oocytes selected during dominant follicle selection (Yung, et al. 2014).

GH co-treatment did not alter the LHR density of the small follicles, and would therefore not improve the number of follicles recruited during dominant follicle selection. This finding is consistent with the reported increase in the pregnancy rate without an increase in the number of oocytes in GH co-treatment during an IVF cycle (Tesarik, et al. 2005, Yovich and Stanger 2010).

10.4.5 Maturation

The improvement in pregnancy rate could therefore, be attributed to an improvement in the follicle micro-environment, which produces a better quality oocyte. In this study, the GH co-treatment increased the receptor density of BMPR1B, FSHR and LHR, and restored the pre-requisite down-regulation during pre-ovulatory maturation, previously discussed in Chapter 5-9 and reported (Jeppesen, et al. 2012, Ophir, et al. 2014, Regan, et al. 2015).

Specifically, the biphasic down-regulation of the BMPR1B was observed in 4 year old sheep and again in the human 23-30 y AFC group A+ &A (Chapter 5 and 6) (Regan, et al. 2015). The current study provides evidence of a GH-induced increase in BMPR1B density in the 10 mm onwards follicles; which reverses the increasing density pattern of BMPR1B observed in

the patients of 40+ y with an AFC of D & E (Figure 10.3.2). The return to the younger patient biphasic pattern of BMPR1B density may improve oocyte quality, and account for the improvement in pregnancy rates observed in GH co-treated patients with reduced ovarian reserve (Yovich and Stanger 2010).

During the follicular phase, the BMPR1B activity has been shown to supress progesterone synthesis in favour of oestrogen production (Glister, et al. 2004a, Otsuka 2010). Importantly, it is the down-regulation of the BMPR1B density that is reversed in the 40+ y AFC D & E patients (Figure 10.3.2). The increase in LHR density (Chapter 5 and 8) inhibits the luteinisation required for pre-ovulatory maturation of the follicle and oocyte, and progression to the peri-luteal phase of differentiation. In addition, GH co-treatment also produced significantly higher LHR density in the 40+ D & E patients, when the follicle size was 14 to 16 mm (Figure 10.3.3). The increased LHR density would provide an increase in sensitivity during the hCG/LH surge to trigger ovulation and maturation of the oocyte (Donadeu 2005, Greisen, et al. 2001). In agreement, a previous study's examination by electron microscopy revealed that oocytes that did not fertilise had reduced levels of granulosal luteinisation and were less responsive to hCG (Rotmensch, et al. 1986).

In conclusion, the increase in LHR sensitivity and the restored pre-ovulatory maturation provide a possible cellular mechanism responsible for the improved pregnancy rate reported.

10.4.6 The GHR is regulated by the GH binding proteins

The poor ovarian reserve older patients co-treated with GH had increased granulosal GHR density. GHRs are predominantly found on the granulosa cell membrane surface and in the endoplasmic reticulum, and to a lesser degree, but common, in the nuclear membrane of highly prolific cells such as the peri-implantation embryo and in tumours (Brooks, et al. 2008, Zhu, et al. 2001). The GHR is regulated by the GH binding proteins, which are the soluble form of the GHR, and by other growth factors such as FSH, BMPs (Nakamura, et al. 2012), and somatostatin (Le Roith, et al. 2001).

Given that the granulosa cell and the oocyte produce their own *GH* mRNA, it is possible that the increase in FSHR, LHR, and BMPR1B density leads to an increase in intracellular GH production; which potentially improves oocyte quality, as reported and previously reviewed (de Ziegler, et al. 2011, Homburg, et al. 2012, Tesarik, et al. 2005, Yovich, et al. 2012, Zhuang, et al. 1994). Exogenous GH co-treatment via the granulosa *GH* mRNA may have an effect on the GH synthesis in the granulosa cell or oocyte, which may lead to an improvement in oocyte quality.

10.4.7 *In vitro* culture studies

Several studies on the effect of GH on cultured granulosa cells have inadvertently induced spontaneous luteinisation (Karamouti 2008, Ovesen 1998, Tapanainen, et al. 1992), which has produced a result that reflects the effect of GH in the luteal phase, and not the follicular phase of follicle development (Erickson, et al. 1991, Gutierrez, et al. 1997). In natural cycle studies, employing serum-free culture, granulosa cells from the follicular phase in humans, increased oestrogen and decreased progesterone levels when treated with GH combined with rFSH (Carlsson, et al. 1992, Mason, et al. 1990).

These findings support the hypothesis that GH co-treatment increased FSHR activity that increases oestrogen synthesis in the granulosa cell.

10.4.8 GHR signalling and GH induced changes to receptor density

The FSH-BMP activity during folliculogenesis predominantly activates the ERK1/2 pathway, and the BMP ligands 2, 4, 6, 7, and 15 appear to regulate GH by the suppression of *StAR* mRNA, *HSD3B1*, and *CYP11A1* which is required for activation of the ERK1/2 pathway to produce progesterone (Pierre, et al. 2004). *In vitro* culture with GH, without FSH also increased STAT3 signalling, which can be regulated by BMP ligands, and have the effect of reducing STAT3 in rodents (Nakamura, et al. 2012). In the goat, BMP ligands in granulosa cell culture were shown to regulate FSHR and LHR receptor density (Zhu, et al. 2001); and in the human, BMP4, 6, 7, and 15 appeared to increase FSHR and LHR density, which demonstrates the interrelated *in vivo* balance present in the ovarian follicle (Shi, et al. 2012, Shi, et al. 2009b, Shi, et al. 2011, Shi, et al. 2010).

An increase in the density of FSHR observed in the present study would increase the FSH induced AC-cAMP- PKA activity, leading to an increased transcription of intermediate components that would directly up-regulate the receptor density of FSHR itself (Fan 2010, Miyoshi, et al. 2006, Nakamura, et al. 2012), LHR (Jia, et al. 1986), BMPR1B (Shi, et al. 2009b, Shi, et al. 2011) and GHR (Nakamura, et al. 2012).

Given the above evidence, the sensitivity of the FSHR appears to be compromised in poor ovarian reserve patients because abundant levels of rFSH were available. It is, therefore, probable that the GH co-treatment increased the GHR-JAK-STAT activity, and hence increased the intermediate products of transcription, which has led to an overall increase in reproductive receptor density (Figure 10.4.1).

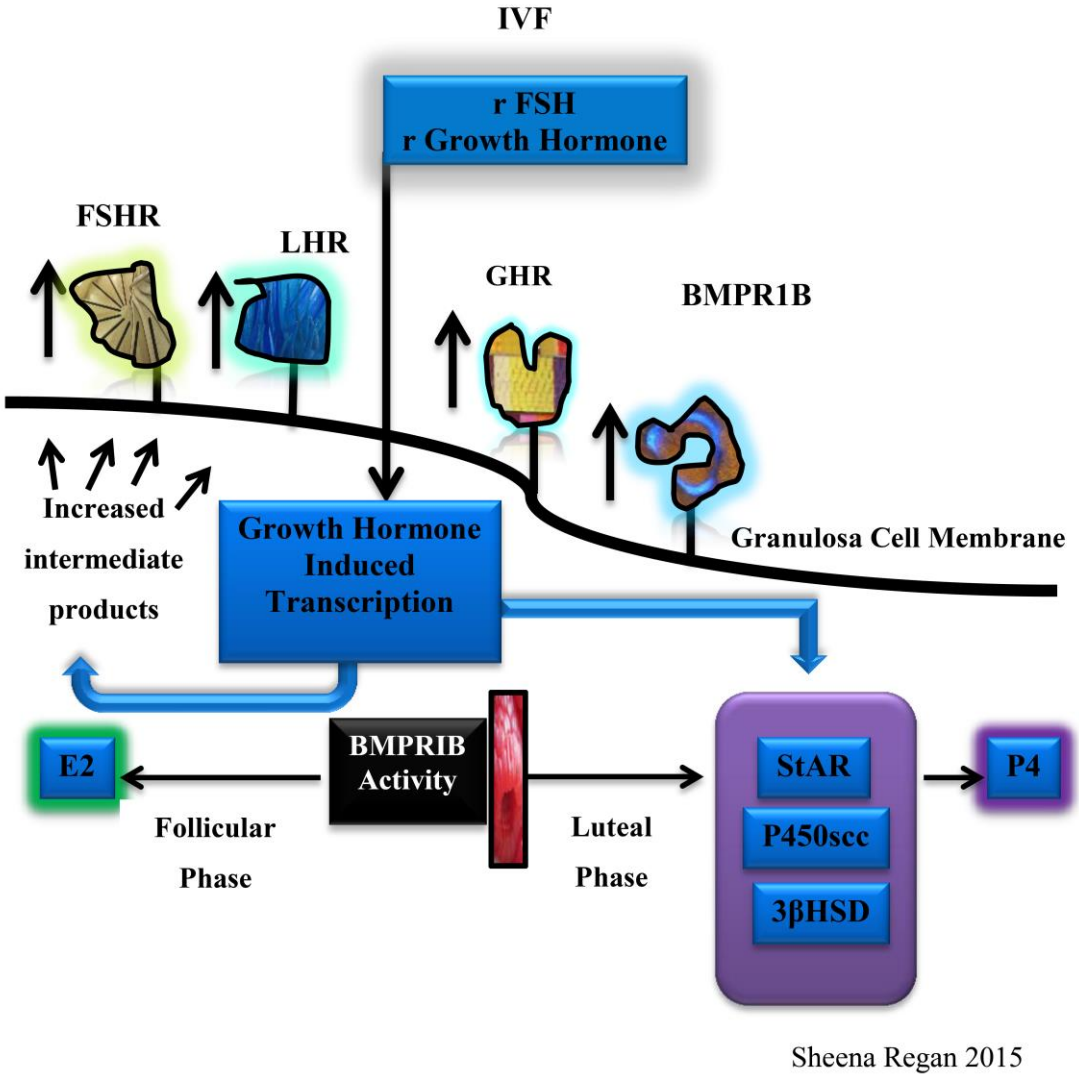


Figure 10.4.1 Diagram of cellular interactions of growth hormone and receptor expression in the granulosa cell.

The GH co-treatment increased the GHR-JAK-STAT activity to increase intermediate products of transcription, which leadsto an overall increase in reproductive receptor density.

10.4.9 Conclusion

The complexity of the *in vivo* balance of gonadotrophins and growth factors regulating follicular growth and differentiation makes it difficult to define the cellular mechanism involved. However, the present study has revealed several cellular mechanisms that could contribute to an improved oocyte quality in GH co-treated IVF patients with a poor ovarian reserve.

GH co-treatment would increase GHR-JAK-STAT activity, and result in an increase in the intermediate products of transcription, which explain the increase in reproductive receptor density. GH co-treatment did not alter the LHR density of the small follicles, and would therefore account for the lack of improvement in the number of follicles recruited during dominant follicle selection. However, the increase in LHR sensitivity of the larger follicles

would restore the pre-ovulatory maturation and provides another possible cellular mechanism responsible for the improved pregnancy rate. In addition, the return to the younger patient biphasic pattern of BMPR1B density may improve oocyte quality observed in GH co-treated patients with reduced ovarian reserve

Further research is needed to ascertain the effect GH co-treatment has on receptor density in good ovarian reserve patients, and the direct effect on the synthesis of granulosa/oocyte GH synthesis.

CHAPTER 11

Apoptosis and Necrosis and Ovarian Ageing

Title: The decline in ovarian ageing and the effect on granulosa cell apoptosis or necrosis in humans

CHAPTER 11 Apoptosis and Necrosis and Ovarian Ageing

11.1 Introduction

In human subjects a decline in the ovarian reserve of follicles leads to a relative dysregulation of folliculogenesis and ovulation. The changes to the micro-environment and hormonal systemic levels is responsible for the reduced fertility and the reduction in quality of the oocyte (Klein N A, et al. 1996). Older patients typically have increased circulating FSH and reduced inhibin B, which gives rise to accelerated early follicle development, and results in a shorter cycle length; yet the growth rate is reduced and the follicles are smaller with fewer granulosa cells (MacNaughton, et al. 1992, Robertson 2009, Santoro, et al. 2003, Seifer, et al. 1999)

Previously, we have presented comprehensive results on the density profiles of patient follicle receptors, correlated with ovarian follicle depletion in young patients through to the older poor ovarian response patients (Chapter 6-9). Reproductive ageing is linked to the decline in capacity to express receptors that cause an irreversible change to ovarian cellular dynamics, and ultimately reduces the capacity to reproduce (Cai, et al. 2007, Nelson, et al. 2013). As the ovarian reserve declines, the health of the follicles appears to decline which results in an increase in pregnancy loss (Hansen, et al. 2005, Lapolt, et al. 1986, Santoro, et al. 2003).

The apoptotic granulosa cell continues to synthesise progesterone until the mitochondrial membrane is disrupted (Amsterdam, et al. 2003). The functioning apoptotic granulosa cell undergoes reorganisation of the cell, which creates blebs of cytoplasmic organelles (Guraya 1971, Motta 1969). The cytoskeleton is reorganised and clusters of lipid droplets, mitochondria, endoplasmic reticulum, and lysosomes are assembled around the nucleus (Aharoni, et al. 1995, Amsterdam, et al. 1997). The condensed chromatin in the nucleus moves to the periphery of the nucleus, and the cell membranes disintegrate, which allow the nucleic contents to be stained (Demchenko 2013).

The heterogeneous population of granulosa cells collected during the peri-luteal stage of folliculogenesis are at different stages of luteinisation and apoptosis (Centurione, et al. 2010, Nottola, et al. 2006). The oocyte potential can be compromised by an increase in the level of granulosa cell death caused by either apoptosis or necrosis, and may result in the demise of the follicle (Oosterhuis, et al. 1998b, Seifer 1996).

The granulosa cells closely regulate the health of the follicle, and ultimately the oocyte; and are susceptible to apoptosis and necrosis in the follicle. However, apoptosis is a normal regulatory process that occurs in the follicle, yet high levels of granulosa cell death could impact follicle development and suppress oocyte growth (Irving-Rodgers, et al. 2003, Sasson and Amsterdam 2002). The response of the ovaries to cyclic recruitment of primordial follicles forms the basis of the clinical measurement of the AFC (Almog, et al. 2011). The AFC and age are strongly correlated with histologically determined ovarian primordial reserve (Hansen, et al. 2011, van Rooij, et al. 2005).

The BMPR1B expression profile is significantly altered with a decrease in AFC and age (Chapter 6). It has been previously reported that a similar biphasic down-regulation of BMPR1B density in wild type Merino sheep occurs, which was also present in young human patients (Chapter 5 and 6) (Regan, et al. 2015). The direct measurement by flow cytometry of the granulosal protein density of FSHR, LHR, and BMPR1B mature membrane-bound receptors was applied. The granulosal receptor expression deteriorates with ovarian ageing, which culminates in the loss of receptor density and the lack of down-regulation of the maturing pre-ovulatory follicle (Chapter 6-9).

The current study aims to further explore the changes to granulosal apoptosis and necrosis that transpire as the ovarian reserve declines. The frequency of granulosa-luteal cell apoptosis or necrosis from individual follicles collected from patients who received treatment for infertility was examined. The change to the relationship between reproductive receptor density and the level of apoptosis or necrosis with ovarian follicle reserve depletion was also examined.

11.2 Methods

Methods are described in detail in the General Methods Chapter (page 50). Granulosa cells were collected from hCG/LH induced follicles from patients receiving IVF exogenous gonadotrophins to artificially stimulate multiple follicles (Table 11.2:1). Flow cytometry provides a rapid and reliable method to quantify viable cells in a cell suspension. 7-AAD is a membrane impermeant dye that is generally excluded from cells with an intact cell membrane. It binds to double stranded DNA. 7-AAD can be excited at 488 nm, emitting at a maximum wavelength of 647 nm.

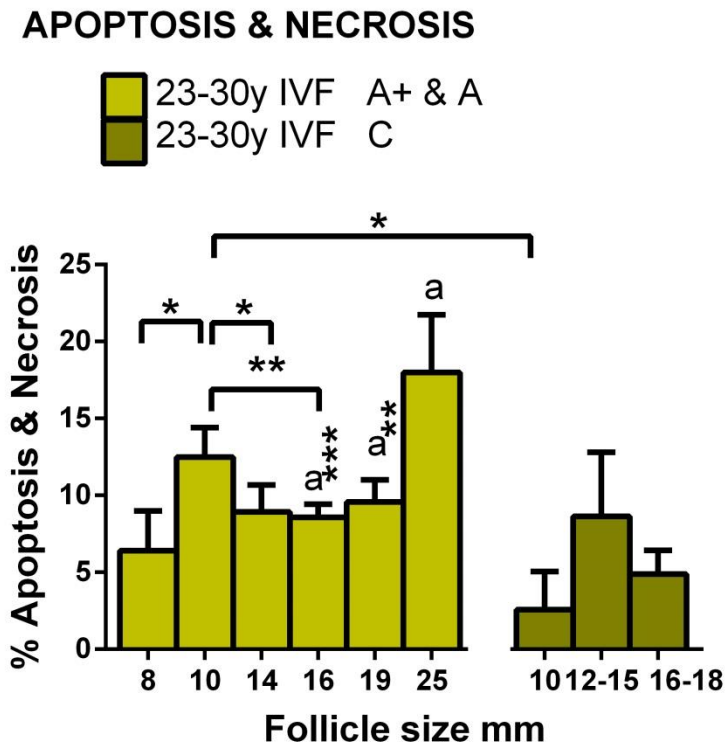
Table 11.2:1Patient ovarian reserve, based on antral follicle count (AFC) and the number of follicles collected per group.

AGE Year	**IVF Patient**	**Total Follicle**	**AFC**	**Major Group**	**Number of Follicles Collected Per Group**							
					Sub Group	**#**	**Sub Group**	**#**	**Sub Group**	**#**	**Sub Group**	**#**
21-30	8	95	20-40	A+ &A	A+	31	A	64	C	6		
31-34	11	86	13-29	A & B	A	60	B	26	C	17		
35-39	16	102	9-19	B & C	A+	6	B	50	C	16	D	30 & E 6
40-45	18	118	3-8	D & E	D	59	E	19	B	34		
40	1	Natural Cycle Healthy			D	2						

Ovarian reserve measured indirectly by the Antral Follicle Count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle: A+ = 30-39 follicles; A = 20-29; B = 13-19; C = 9-12, D = 5-8; E = ≤4.

11.3 Results

In the youngest patient group, with an AFC of A+ & A, the level of apoptosis was increased during the dominant follicle selection and the maturation of the largest follicle ($p<0.05$ and $p<0.01$, Figure 11.3.1). In the patient group A+ & A, the level of apoptosis and necrosis was significantly greater compared to patients of the same age with ovarian reserve depletion in the 10 mm follicle ($p<0.05$, AFC: C).The size corresponds to the time of dominant follicle selection.



GOOD: A+ =30-39; A = 20-29; B = 13-19; C = 9-12; D = 5-8; E = ≤4: POOR

Figure 11.3.1 Granulosal apoptosis and necrosis and ovarian reserve depletion in 23-30 y.

Granulosa cells from individual follicles of different sizes collected from young IVF patients with a good ovarian reserve compared to young patients with a poor ovarian reserve. Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *$p<0.05$, **$p<0.01$ and ***$p<0.005$. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) indicates the significance level for the size follicle.

The level of apoptosis was reduced in the 40+ y age group compared to the youngest patients receiving IVF stimulation at the time of dominant follicle selection ($p<0.005$) and at the maturation of the pre-ovulatory follicle, at 19 mm ($p<0.01$). The granulosa cells from the natural cycle follicle of 18.5 mm had an elevated level of apoptosis and necrosis (23%) similar to the mature pre-ovulatory follicles from the younger patients (18 ± 4%). In the natural cycle the smaller follicle of 10 mm had a low level of apoptosis and necrosis (2.5%). The size of the follicle corresponds to the time of dominant follicle selection, and is similar to the level reported for the 40+ y D & E patient (1.5 ± 0.5%).

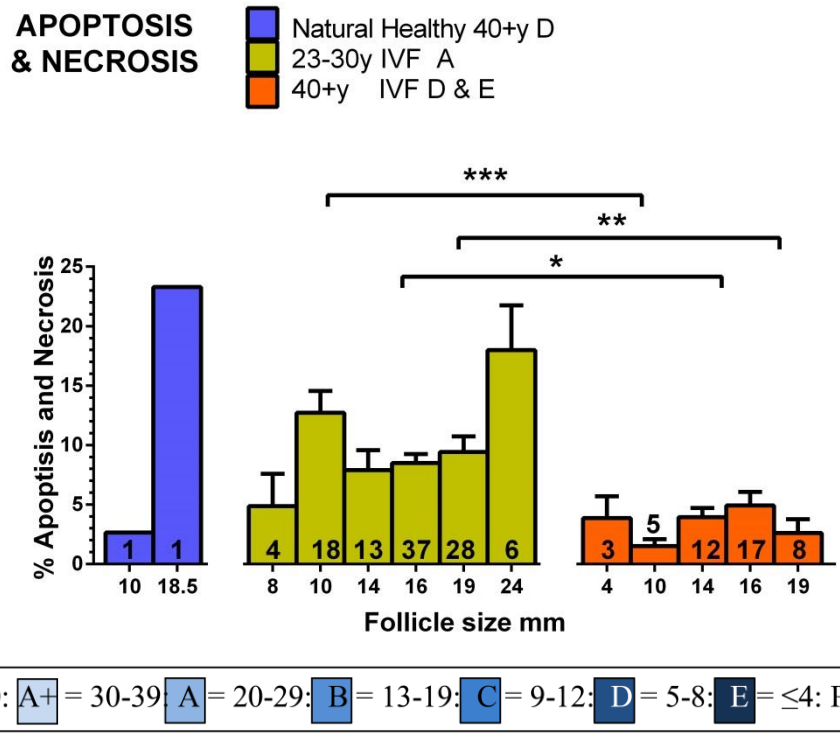


Figure 11.3.2 Granulosal apoptosis and necrosis and ovarian reserve depletion in young and older patients.

Percentage of apoptotic or necrotic granulosa cells and follicle size collected during a natural healthy cycle from an unstimulated patient of 41 y, with an AFC of D, (blue). Patients, 23-30y stimulated, IVF cycle with an AFC of A+ & A, (olive green). Patients, 40+y stimulated IVF cycle with an AFC of D & E,(orange). Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *$p<0.05$, **$p<0.01$ and ***$p<0.005$.

A strong correlation was observed between the granulosal FSHR and BMPR1B density and the corresponding level of apoptosis and necrosis based on follicle size. High levels of FSHR and BMPR1B density were associated with reduced apoptosis and necrosis levels in the youngest patients of 23-30 y with an AFC of A+ & A, R square 0.752 and 0.835, respectively. The correlation was reversed in the next age group of 31-34 y for both FSHR and BMPR1B, and sequentially reduced in association with increasing age and a reducing AFC. In the 40+ y patient the correlation for FSHR and BMPR1B was R square 0.137 and 0.011, respectively.

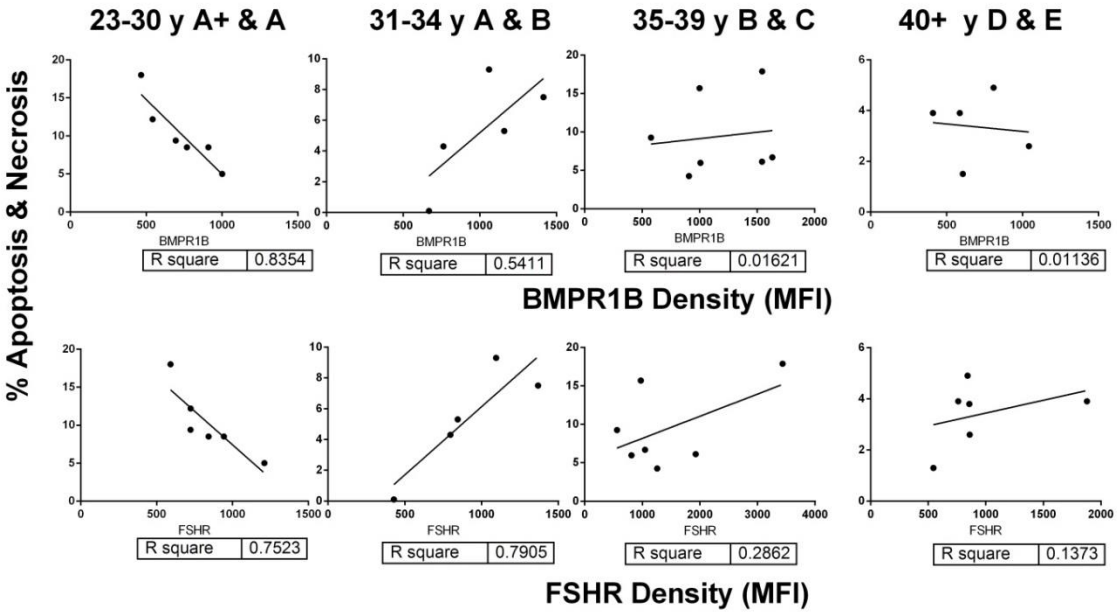
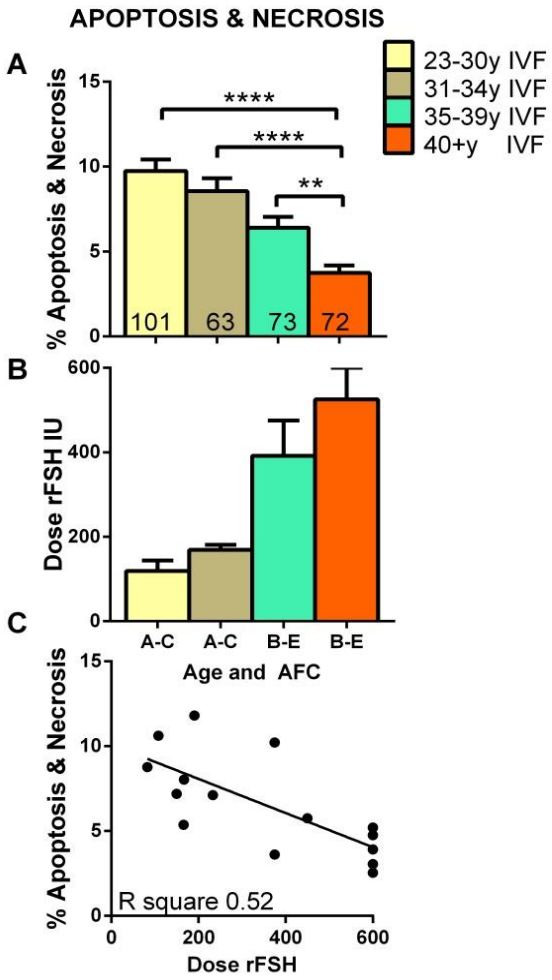


Figure 11.3.3 Correlation of granulosal FSHR and BMPR1B density and apoptosis and necrosis, and depletion of the ovarian reserve during folliculogenesis.

Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Sequential graphs show increasing age and declining ovarian reserve indicated by AFC. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Linear regression analysis; R square is indicated for each group. The data points are the average of the receptor expression for that follicle size group.

The level of apoptosis and necrosis was strongly correlated to the dose of rFSH treatment received by IVF patients. (A). AFC declines with age with considerable overlap of the ovarian response to stimulation. Patients were grouped into age classes and follicles of different sizes were combined. (B). The clinical administration of exogenous FSH during treatment for infertility is dependent on the ovarian reserve (AFC) and age (Yovich, et al. 2012). Dosage was averaged across the range of AFC for each age class. (C). A strong correlation exists between the level of apoptosis and necrosis and the dose of rFSH administered to patients receiving IVF treatment.



GOOD: A+ = 30-39: A = 20-29: B = 13-19: C = 9-12: D = 5-8: E = ≤4: POOR

Figure 11.3.4 Correlation between the dose of rFSH and the level of apoptosis and necrosis.

(A) Age and the level of granulosal apoptosis and necrosis as the ovarian reserve declines; based on major AFC groups per age class. (B) Corresponding dose of exogenous recombinant FSH given to IVF patients to stimulated follicular growth based on antral follicle count and age group. (C) Correlation between the level of apoptosis and necrosis of granulosa cells and the dose of rFSH used during IVF treatment. Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number

of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if **p<0.01 and ****p<0.001. Linear regression analysis, R square is indicated for each group. The data points are the average of the receptor expression for that follicle size group, R square 0.52.

11.4 Discussion

11.4.1 Overview of the study

The ovulation rate is generally governed by the limitation of the number of follicles growing in a stage-specific manner, and follicle growth is limited by the stimulation to continue growth to ovulation. The gonadotrophins FSH and LH govern the growth rates of the follicles during cyclic folliculogenesis, and the level of receptor density controls the response of the follicles to the gonadotrophin stimulation. Intraovarian growth factors, such as the BMPs, impact the receptor expression that ultimately controls the growth rate of the follicle. Reduced growth leads to an accumulation of precursor molecules that can stimulate signalling pathways to induce programmed cell death or apoptosis. Apoptosis of unfavourable granulosa cells and follicles can lead to follicle collapse, and establishes the dominant follicle that continues to grow to ovulation (Yuan and Giudice 1997).

11.4.2 Major Findings

The major findings of this study are that the level of apoptosis increased during dominant follicle selection and during pre-ovulatory maturation in young IVF patients with an uncompromised ovarian reserve. Secondly, that the granulosal BMPR1B and FSHR density was strongly associated with a reduction in apoptosis; and thirdly, that the level of apoptosis was reduced as the ovarian reserve declined with age.

11.4.3 Dominant follicle selection

A significant increase in apoptosis and necrosis occurred at the time of dominant follicle selection (Figure 11.3.1). At the time of dominant follicle selection, the circulating FSH decreases, and the follicles with greater FSHR and LHR density are stimulated to produce oestrogen and proliferate. FSH has been reported to be anti-apoptotic. Therefore it may be expected that the drop in pituitary FSH (mid-follicular phase) may initiate an increase in apoptotic signalling in the granulosa cell. In humans, it has been reported that the majority of intense staining for P450 aromatase is located in the basal mural granulosa cells, and radiates towards the antral cavity of the follicle (Guigon 2003, Sakurada, et al. 2006, Turner, et al. 2002). The link between high levels of aromatase activity and an anti-apoptotic effect appears to exist, as it has been previously reported that the majority of apoptosis occurs in the antral region of the follicle, which is consistent with the reduced aromatase activity

(Matsuda, et al. 2012). During the different stages of follicle development the level of apoptosis changes. Essentially, primordial, primary, secondary, and pre antral follicles <1 mm in size were found to be free of DNA breakdown (Yuan and Giudice 1997). Small antral follicles (1 to 9.9 mm) from natural cycles that were androgen dominant (androgen > oestrogen) were found to have substantial levels of DNA fragmentation (DNA fragmentation, 27% to50 %) whereas, follicles (>10 mm) that were oestrogen dominant (oestrogen > androgen), had a very low rate of cell death (0%) (Yuan and Giudice 1997).

In the current study, the apoptosis peaked at the time of dominant follicle selection and then maintained a consistent level of apoptosis and necrosis in the follicles from 10 to 19 mm. The plateau could signify a base rate of the continuous removal of atretic granulosa cells via phagocytosis as part of the normal clean-up of the decaying debris (Figure 11.3.1, AFC A+ & A) (Amsterdam, et al. 2003, Giampietro, et al. 2006).

11.4.4 Pre-ovulatory maturation

Previous research has shown that when FSH levels decline, a reduction in BMP6 (Erickson and Shimasaki 2003), BMP15 (Feary, et al. 2007), AMH (Andersen, et al. 2010, Ogura Nose, et al. 2012, Weenen, et al. 2004), GnSAF (Martinez, et al. 2002) and BMPR1B (Feary, et al. 2007, Regan, et al. 2015), occurs after dominant follicle selection to the termination of folliculogenesis. Once selected, the dominant follicles' granulosa cells continue to proliferate, which leads to an increase in oestrogen and inhibin B production by the granulosa cells. Together, these hormones suppress pituitary FSH output further, ensuring the demise of subordinate follicles. When the threshold for oestrogen's positive feedback action on the hypothalamus-pituitary axis is met, preparation for the ovulation phase begins with a cessation of cell proliferation and early luteinisation changes taking place.

The decline in gonadotrophin and growth factors possibly induce apoptosis and necrosis in the largest follicle during the pre-ovulatory maturation phase, which culminates in the peri-luteal LH surge. Considerable morphological changes take place to facilitate the rupture of the follicle and expulsion of the egg, which may also contribute to the increase in apoptosis or necrosis observed in the 23-30 y with a good ovarian reserve (Figure 11.3.2) (Fan, et al. 2009).

11.4.5 Ovarian Ageing

The lack of peri-ovulary luteinisation has been previously shown to be associated with reduced fertility as the ovarian reserve diminishes (Seifer 1996). In the present study, the level of apoptosis was reduced as the ovarian reserve declined with age (Figure 11.3.2). A disruption to the density of receptor expression during antral follicular growth was previously

shown to contribute to the reduced fertility experienced as the ovarian reserve became depleted (Chapter 6-9).

The greater level of apoptosis and necrosis of the young uncompromised ovarian reserve patients, compared to the older poor ovarian reserve may reflect the overall greater mitogenic proliferation in the younger patient cohort (Figure 11.3.2) (Seifer 1996 , Seifer, et al. 1993). The base line level of apoptosis and necrosis in the 23-30 y patient group was approximately two fold greater compared to that of the 40+ y patient group (Figure 11.3.2). The oldest patients did not have elevated levels of apoptosis at the time of dominant follicle selection or during the maturation of the largest follicle size (Figure 11.3.2). Previously, in reduced ovarian reserve patients, the antral stages of follicular growth have a reduced rate of growth (Santoro, et al. 2003); that possibly results in reduced maturation of the follicle and oocyte (Seifer 1996). A reduced growth rate and number of growing follicles in the current study limited the size of the largest follicles collected from the older patients to 19-22 mm compared to the younger patients' largest follicles of 23-26 mm (Figure 11.3.2).

In older patients, the granulosa cell proliferation was found to be reduced and the size of the follicle to be smaller (Bomsel Helmreich, et al. 1979, Robertson 2009, Seifer 1996 , Seifer, et al. 1993). Furthermore, a previous study reported that the level of apoptosis was decreased (7%) in patients who fell pregnant compared to those that did not (20%) (Oosterhuis, et al. 1998a). In addition, the study reported that age, rFSH, and the number of oocytes produced and fertilised were similar, yet the lower apoptosis group fell pregnant. The granulosa cells were pooled from each patient and the patient numbers were low, which may have had an impact on the study or the findings were associated with other fertility issues.

In a previous study, the women with a poor ovarian reserve who had greater levels of apoptosis, had fewer granulosa cells undergoing luteinisation (Seifer 1996), yet were of the same age (36 y), received a similar rFSH dose, and produced a corresponding different number of oocytes (Seifer 1996). The level of apoptosis measured via flow cytometry was reported to be, 0.5% from a good ovarian reserve patient and 2% from a poor ovarian reserve. However, it is difficult to compare results between studies, because of the differences in methodology. The current study's findings are novel, because of the range of patient ages and ovarian reserves, and different follicle size analysed on an individual basis within the one study

Previously, the density of FSHR and LHR receptors have been associated with reduced fertility as the ovarian reserve declines (Chapter 7-8) (Cai, et al. 2007, Maman, et al. 2012).

A combination of high BMPR1B and LHR levels in the terminal-end follicles from poor ovarian reserve older patients (Chapter 6 and 8) may explain the low levels of luteinisation of granulosa cells reported (Seifer 1996). Maturation of the pre-ovulatory follicle requires down-regulation of the BMPR1B, FSHR and LHR (Regan, et al. 2015). Furthermore, the lack of maturation of the pre-ovulatory cells observed in (Chapter 6 and 8), would potentially reduce the level of apoptosis in the current study, as demonstrated in the younger patients with a poor ovarian reserve (AFC C, Figure 11.3.1), and with an increase in age and decline in AFC (Figure 11.3.2).

11.4.6 rFSH

Austin et al. (2001), described the reduction in apoptosis in granulosa cells treated with FSH in heifers (young cow) (Austin, et al. 2001). In the current study, the reduced level of apoptosis in the poor ovarian reserve patients was strongly correlated to the level of rFSH drugs administered (Figure 11.3.4). It was also observed that the natural cycle patients' pre-ovulatory follicle had a high level of apoptosis (24%) compared to the same age patient receiving IVF rFSH treatment (Figure 11.3.4). In the present study, the follicles > 10 mm had a greater level of apoptosis than the previously reported 0% in a natural cycle (Yuan and Giudice 1997). However, the sensitivity of the method of analysis may be different. In another study of IVF patients, a similar method of annexin V staining and flow cytometry was applied, and the level of apoptosis of 33 y olds was 7.8-9.8% (Giampietro, et al. 2006). The follicles were pooled, whereas in the current study the follicles were individually analysed and ranged in size from 4 -26 mm. The previous study applied a second method on the same patient group, and the level was found to be much higher (20%). The difference highlights the inaccuracy that may occur when comparing results using different methodologies (Giampietro, et al. 2006).

11.4.7 Granulosal BMPR1B and FSHR density correlation

Previously we presented data which showed that ovarian ageing generates dysregulation of the stage-specific receptor expression of BMPR1B, FSHR, LHR and GHR (Chapter 6-9) (Regan, et al. 2015). The reduced levels of receptor density and lack of pre-requisite down-regulation in older poor ovarian reserve patients results in a lack of correlation with the level of apoptosis and necrosis observed in the current study (Figure 11.3.4) In the young patients with an AFC of A+ & A, the granulosal BMPR1B and FSHR density was strongly correlated with a reduction in apoptosis (Figure 11.3.4). Under normal ovarian conditions (young patients with an AFC of A+ & A) the level of apoptosis and necrosis was lower when BMPR1B and FSHR were up-regulated. The biphasic down-regulation of BMPR1B and FSHR may give rise to the strong anti-apoptotic qualities associated with receptor-induced gene transcription. A reduction in BMP activity reduces the suppression of progesterone

synthesis and facilitates luteinisation of the granulosa cells (Kayamori, et al. 2009). The follicular-luteal phase differentiation causes a shift to progesterone as the anti-apoptotic protector in the luteal phase (Kayamori, et al. 2009). Meanwhile, *in vitro* studies have shown that the BMP ligands 2, 4, 6, and 7 inhibit apoptosis in goat granulosa cells (Zhu, et al. 2013).

11.4.8 Conclusion

In conclusion, apoptosis was reduced with the depletion of the ovarian reserve and may reflect the overall greater mitogenic proliferation in the younger patient cohort.

The ovarian reserve is rapidly depleted with chronological age and was associated with the disruption to the receptor density of FSHR, BMPR, and LHR at the time of dominant follicle selection and during pre-ovulatory maturation. Furthermore, the dysregulation of receptor expression (Chapter 6 and 8) would potentially reduce the level of apoptosis by the suppression of maturation of the pre-ovulatory cells.

Further work is required to distinguish between the associated anti-apoptotic effect that rFSH has on the level of apoptosis and the influence that ovarian ageing has on specific BMP ligand activity in relation to apoptosis.

CHAPTER 12

Apoptosis and Necrosis and GH Treatment

Title: The decline in ovarian ageing and the effect on granulosa cell apoptosis or necrosis in IVF patients co-treated with GH

CHAPTER 12 Apoptosis and Necrosis and GH Treatment

12.1 Introduction

Artificially increasing the level and the duration of circulating FSH extends the window of growth, which produces multiple ovulatory follicles. As the ovarian primordial follicle reserve declines, the rate of recruitment to primary follicles is reduced, which leads to a diminished recruitment of follicles into the cycle. The number of small antral follicles at the beginning of each cycle is representative of the ovarian reserve of primordial follicles remaining in the ovary (Almog, et al. 2011, Baerwald, et al. 2012b, Hadlow, et al. 2013, Hansen, et al. 2011, van Rooij, et al. 2005). AMH and chronological age also have a strong correlation to ovarian reserve; however, AFC demonstrates a more sensitive test over time (van Rooij, et al. 2005); and in the older patients, AMH is less accurate at low levels (i.e. the lower range of 0-1 ng/ml) (Hansen, et al. 2011).

During an IVF cycle, the concentration of exogenous FSH required to recruit a cohort of dominant follicles increases as the ovarian reserve is depleted, indirectly indicated by a lower AFC. As the ovarian reserve depletes, the available number of antral follicles available for recruitment is reduced. Clinicians have developed protocols based on ovarian response to rFSH stimulation to maximise the number of antral follicles recruited; however, the effectiveness is reduced as the potential cohort is diminished because fewer primordial follicles are activated to grow.

The average age of patients seeking pregnancy assistance in Australia is approaching 40 y, increasing the number of patients who respond poorly to gonadotrophin stimulation. Reduced sensitivity to rFSH stimulation has resulted in the development of adjunctive treatments such as GH. In the 1980s, clinical evidence alluded to an increase in birth rate (Homburg, et al. 1988), followed by a more accurate confirmed increase in serum oestrogen and reduced progesterone (Karamouti 2008). Recently, the GHR has been localised on the surface of follicular cells and found bound to the endoplasmic reticulum, golgi apparatus and the nuclear membrane (Brooks, et al. 2008). Moreover, GHRs are predominantly found on the surface of highly prolific cells such as the peri-implantation embryo and in tumours (Brooks, et al. 2008, Zhu, et al. 2001). Other studies have employed large sample groups and have demonstrated an increase in the pregnancy rate after stimulation with GH during IVF treatment (Tesarik, et al. 2005, Yovich and Stanger 2010).

The capacity of the ovarian follicle to generate steroids is governed by FSH and LH stimulation and the density of FSHRs and LHRs on the surface of the follicle cells.

Programmed granulosa cell death can reduce the steroid function of the follicle, and high levels of apoptosis can result in the complete breakdown of the follicle and oocyte. Granulosa cell apoptosis is a normal regulatory process during folliculogenesis. In the Booroola Merino sheep, the attenuation of the BMPR1B has been previously shown to increase the ovulation rate by reducing the level of granulosal apoptosis or necrosis (Regan, et al. 2015). In Chapter 11 it was reported that the level of apoptosis increased during dominant follicle selection and during pre-ovulatory maturation in young IVF patients with an uncompromised ovarian reserve. However, as the ovarian reserve declined with age, the level of apoptosis was reduced (Chapter 11). In the current study, we explore the effect of GH co-treatment with rFSH on the level of apoptosis or necrosis of granulosa cells from IVF patients.

12.2 Methods

Methods are described in detail in the General Methods Chapter (page 38). Granulosa cells were collected from hCG/LH induced follicles from patients receiving IVF exogenous gonadotrophins to artificially stimulate multiple follicles (Table 12.2:1).

For the detection of early DNA fragmentation, the assessment was applied on the same aliquots used for the immunolabelling of FSHR since antibody CD45 was not required because of the uniqueness of FSHR to granulosa cells, hence there would be no spectral overlap. 7-AAD is a nucleic acid dye used in place of propidium iodide to reduce spectral overlap, and is excluded from cells with an intact cell membrane. The 7-AAD positive granulosa cells did not distinguish between necrotic cell death and apoptotic cell death; however, trypan blue exclusion revealed a consistent low level of cell death.

Table 12.2:1 Patient ovarian reserve, based on antral follicle count (AFC) and the number of follicles collected per group.

AGE Year	**IVF Patient**	**Total Follicle**	**AFC**	**Major Group**	**Number of Follicles Collected Per Group**							
					Sub Group	**#**	**Sub Group**	**#**	**Sub Group**	**#**	**Sub Group**	**#**
21-30	8	95	20-40	A+ &A	A+	31	A	64	C	6		
31-34	11	86	13-29	A & B	A	60	B	26	C	17		
35-39	16	102	9-19	B & C	A+	6	B	50	C	16	D 30 & E	6
40-45	18	118	3-8	D & E	D	59	E	19	B	34		
40	1	Natural Cycle Healthy			D	2						

Ovarian reserve measured indirectly by the Antral Follicle Count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle: A+ = 30-39 follicles; A = 20-29; B = 13-19; C = 9-12, D = 5-8; E = ≤4.

12.3 Results

The level of apoptosis was not significantly altered by the addition of GH co-treatment to the 40+ y IVF patients with an AFC of D or E. The younger 23-30 y group demonstrated greater levels of apoptosis and necrosis combined compared to the GH co-treated group, in the 10 mm, and 16 mm to 19 mm follicle sizes ($p<0.05$, Figure 12.3.1). The level of apoptosis was less in the GH treated patient follicles of 16 mm when compared to the 31-34 y patients ($p<0.05$), and 19 mm compared to the same size follicle in the 35-39 y B & C group ($p<0.05$, Figure 12.3.1).

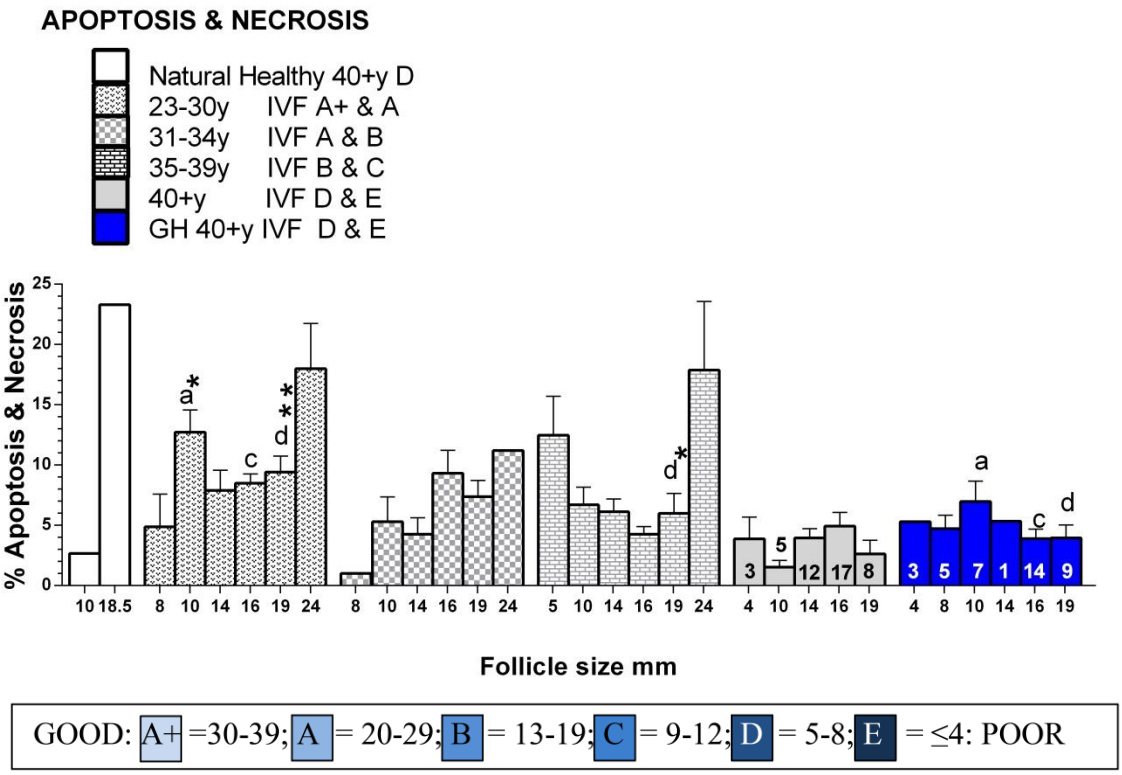


Figure 12.3.1 Granulosa cell apoptosis and necrosis combined in poor response 40+y patients treated with GH compared to untreated.

Ovarian reserve measured indirectly by the Antral Follicle Count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *$p<0.05$, **$p<0.01$ and ***$p<0.005$. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) indicates the significance level for the size follicle.The characters Φ, ω, and b were used in a similar manner to denote a significant difference between the symbol and the same symbol with an asterisk *. The number of follicles analysed are indicated.

Patients with a poor ovarian reserve and 40+ y treated with GH did not have any significant change to apoptosis and necrosis levels of granulosa cells. However, GH co-treatment increased the level of apoptosis and necrosis AFC of B compared to patients without GH treatment of the same age and AFC of B. These results are considered preliminary because the follicles were from a single patient with an AFC of B.

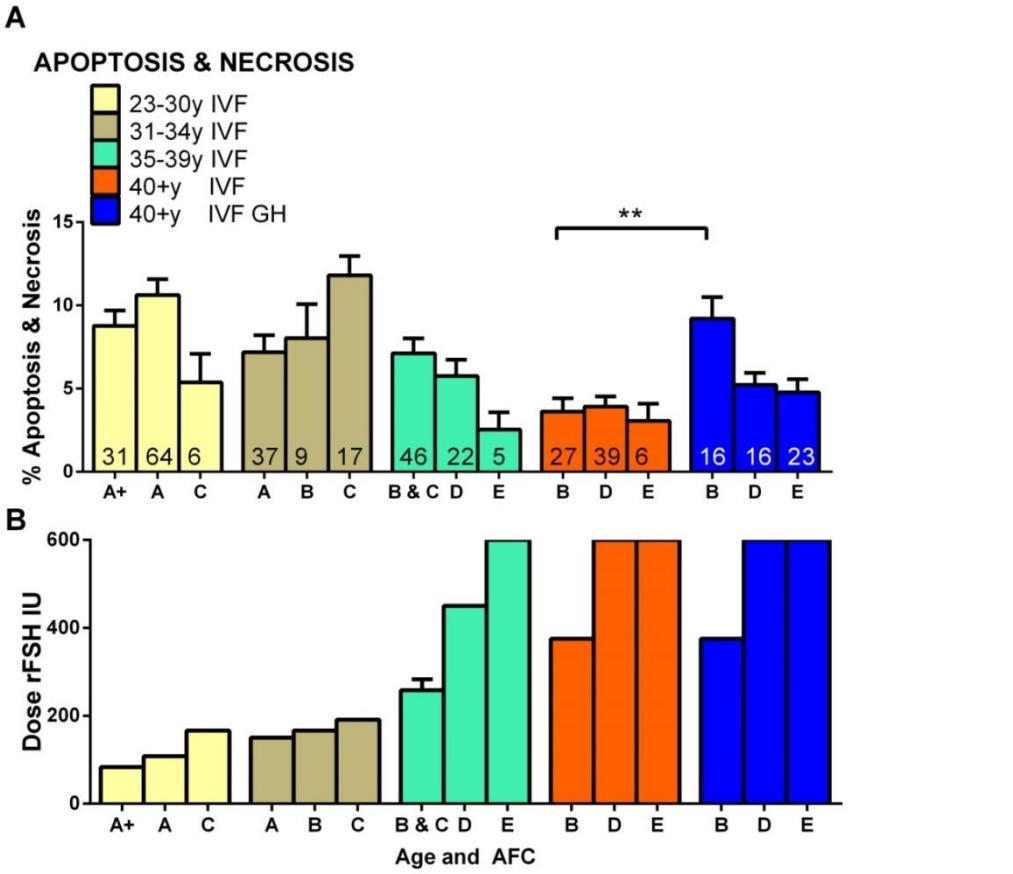


Figure 12.3.2 Granulosa cell apoptosis and necrosis was correlated to the dose of exogenous rFSH treatment.

Apoptosis and necrosis percentage level found in granulosa cells removed from patients with declining ovarian reserve with increasing age. The numbers of follicles are indicated in (A), and the corresponding dose of rFSH administered to IVF patients (B). Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *p<0.05 and**p<0.01. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) indicates the significance level for the size follicle.

The level of apoptosis and necrosis was strongly correlated to the dose of rFSH treatment received by IVF patients (Chapter 11). AFC declines with age with considerable overlap of the ovarian response to stimulation (Yovich, et al. 2012). In the GH treated 40+ y patients with an AFC of B, a significant difference was observed in the level of apoptosis and necrosis compared to the 40+ y IVF patients without GH with an AFC of B ($p<0.05$, Figure 12.3.3). The increase in the 40+ y patients with an AFC of B was similar from 15 mm to 19 mm, and was very low in the one 24 mm follicle.

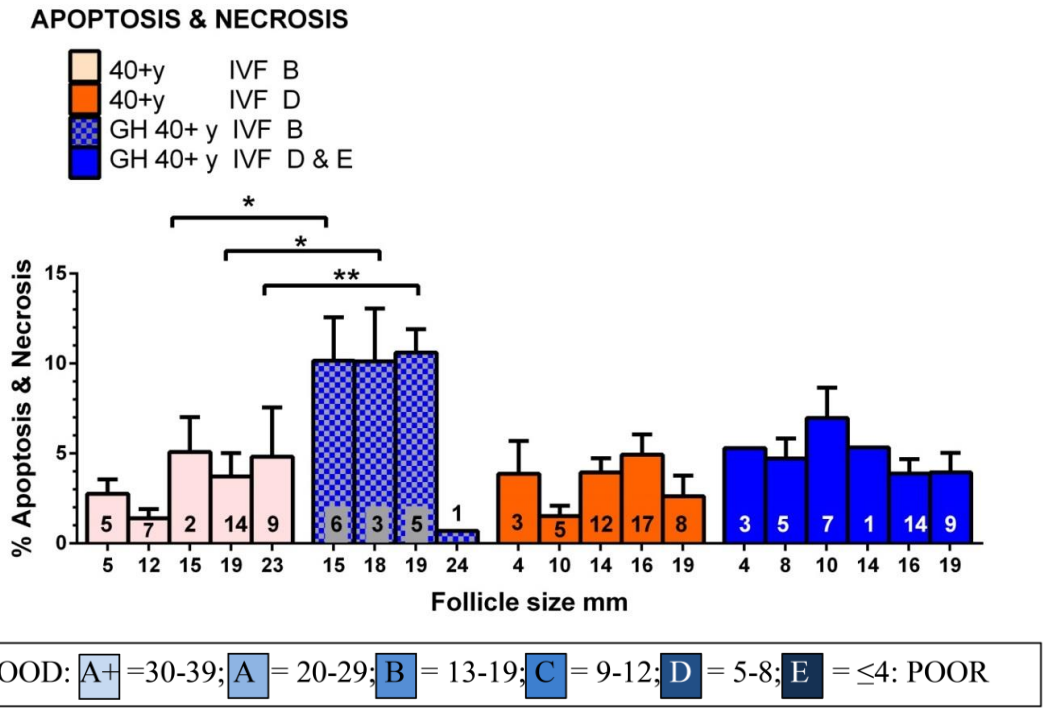


Figure 12.3.3 Growth hormone treatment in 40+ y IVF patients and the level of apoptosis and necrosis in granulosa cells of different size follicles

Ovarian reserve measured indirectly by the AFC. AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Percentage of apoptotic or necrotic granulosa cells and follicle size collected from patients, 40+y in a stimulated IVF cycle with an AFC of either B (pink) or D & E, (orange) compared to 40+y in a stimulated IVF cycle co-treated with GH; with an AFC of either B (blue check) or D & E, (blue). Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *$p<0.05$, ** and $p<0.01$.

FSHR and BMPR1B density reduced apoptosis levels in the youngest patients of 23-30 y with an AFC of A+ & A (R square 0.752 and 0.835, respectively). The level of apoptosis and necrosis declined with ovarian ageing, reversing the relationship between BMPR1B and FSHR, and the level of apoptosis or necrosis. As the ovarian reserve of primordial follicles decreased, no correlation was observed between the receptors and the apoptosis and necrosis. Growth hormone treatment in the poor ovarian reserve patients of 40+ y had a strong effect on the positive relationship with BMPR1B (R square 0.65), but not with FSHR (R square 0.0).

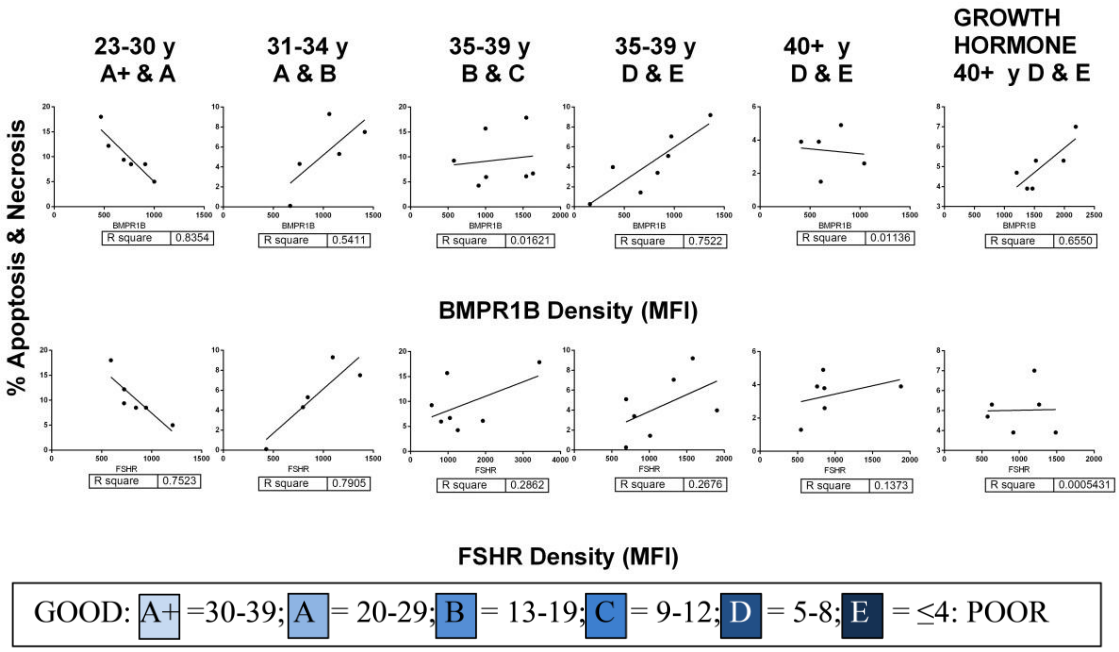


Figure 12.3.4 The relationship between the density of the receptor for FSH and BMPR1B and the level of granulosa cell DNA Fragmentation.

Sequential graphs show increasing age and declining ovarian reserve indicated by AFC. Linear regression analysis, R square is indicated for each group. The data points are the average of the receptor expression for that follicle size group. Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle.

12.4 Discussion

12.4.1 Overview

During the preparation for ovulation, the level of apoptosis and necrosis combined was amplified in the largest follicles of the young age groups, whereas in the older 40+ y patient the apoptosis and necrosis was significantly reduced compared to the youngest patient group (Chapter 11). Furthermore, the elevated granulosal BMPR1B and LHR density previously reported in the older, poor ovarian reserve patient group appears to prevent peri-luteal granulosa cell maturation (Chapter 6 & 8).

12.4.2 Major findings

In the current study, GH co-treatment of the 40+ y poor ovarian reserve (D & E) patient group did not significantly change the level of apoptosis or necrosis in the same age group and AFC, compared to without GH co-treatment (Figure 12.3.1). Whereas GH co-treatment increased the level of apoptosis in the 40+ y group with a good ovarian reserve of B; however although there were 16 follicles in total, from the B group, they were from a single patient, and are therefore preliminary results.

Previously, the correlation of the dose of rFSH to the level of apoptosis indicated that the high levels of rFSH may reduce the apoptotic pathways (Chapter 11). Alternatively, the reduction in apoptosis may be associated with the reduced growth of the follicle and proliferation of the granulosa cells as the ovarian reserve was depleted (Chapter 11).

The 40+ y B patient without GH had the same level of apoptosis and necrosis as the D & E patients who received more rFSH, which indicates that rFSH may not affect apoptosis as the ovarian reserve declines. GH treatment did increase the level of apoptosis in the 40+ y B patients, which suggests an improvement in the growth and development of the follicle as the level of apoptosis is similar to the younger patient levels (Figure 12.3.2). The level of apoptosis in the youngest patient's follicles after dominant follicle selection and before terminal-end maturation of the pre-ovulatory follicle is approx. 10% (14-19 mm ,Figure 12.3.1), which is similar to the level of apoptosis in the GH treated AFC B patients (Figure 12.3.3). Ovarian ageing has been previously shown to reduce the rate of follicle growth, reduce the number of granulosa cells, and reduce the length of the cycle (Seifer 1996). In the oldest patients, very few follicles were greater than 19-22 mm, yet one follicle was collected in the GH treated B patient of 24 mm. The follicle was healthy and the oocyte fertilised which indicates that the level of apoptosis was low in the largest follicle of a GH treated patient. A previous study showed that the pregnancy rate was greater in IVF patients with a lower level of apoptosis from pooled follicles of different sizes (Oosterhuis, et al. 1998b).

The two methods used to determine apoptosis and necrosis were very early apoptotic markers of phospholipid externalisation of the cell membrane, and the 7-AAD marker detects cell membrane disruption in cells undergoing apoptosis and necrosis; however, it is limited to not differentiating between functionally intact mitochondria with apoptosis, and both mitochondrial and DNA total breakdown (Amsterdam, et al. 1997, Amsterdam, et al. 2003). In hindsight, a more appropriate detection of steroid capacity of a granulosa cell would be CYP19A1. The results however, are still valid because the level of DNA breakdown is a

measure of cellular health, and further research is yet to determine how long the apoptotic luteal cells continue to contribute to steroidogenic activity in the corpus luteum.

The stage-specific sequential decline in receptor density observed previously in the different age classes, provides evidence of the loss of function with age and declining ovarian reserve (Chapter 6-9). Apoptosis and necrosis declined with ovarian reserve depletion, therefore it is more likely to be associated with reduced proliferation and not the level of rFSH administered as previously proposed in Chapter 11.

12.4.3 Conclusion

The addition of GH combined with rFSH provides an alternative cellular mechanism to increase the level of receptor density however it did not affect the level of apoptosis and necrosis in the older poor ovarian patient group. Apoptosis and necrosis was more likely to be associated with reduced proliferation and not the level of rFSH administered.

Preliminary work on older patients with good ovarian reserve showed that GH co-treatment increased the level of apoptosis or necrosis. Further work is required to determine if GH co-treatment increases apoptosis in, good ovarian reserve patients, and if the increase is associated with an increase in proliferation and the impact on the oocyte.

CHAPTER 13

Serum and Follicular Fluid and Ovarian Ageing

Title: The decline in ovarian ageing and the effect on the ovarian hormone regulation in humans

CHAPTER 13 Serum and Follicular Fluid and Ovarian Ageing

13.1 Introduction

In human subjects a decline in the ovarian reserve of primordial follicles leads to a relative dysregulation of folliculogenesis and ovulation. The change to the micro-environment of the ovary and circulating hormone levels are responsible for the reduced fertility and a reduction in the quality of the ovulating oocyte (Klein N A, et al. 1996). Older patients typically have increased circulating FSH and reduced inhibin B, that give rise to accelerated early follicle development and results in a shorter cycle length (MacNaughton, et al. 1992, Robertson 2009, Santoro, et al. 2003, Seifer, et al. 1999). In poor ovarian reserve patients, the later stages of follicular growth have a reduced rate of growth which reduces maturation of the follicle and oocyte (Santoro, et al. 2003). The granulosa cell proliferation was found to be reduced and the size of the follicle to be smaller in older patients (Bomsel Helmreich, et al. 1979, Robertson 2009, Seifer 1996 , Seifer, et al. 1993). The follicle increases its capacity to produce oestrogen by increasing the number of granulosa cells by increasing the size of the follicle and not the thickness of the granulosa layer (Bächler, et al. 2014). A shorter cycle and a reduction in the follicle size due to ovarian ageing would reduce the capacity of the follicle to produce sufficient oestrogen to down-regulate pituitary FSH during dominant follicle selection. Potentially, reduced oestradiol output could also disrupt the positive feedback mechanism responsible for generating the pre-ovulatory LH surge. The present study aims to profile the changing levels of oestrogen, progesterone, FSH, LH, and testosterone in IVF patients, and observe the impact ovarian reserve has on the levels of serum and follicular fluid.

13.2 Methods

Methods are described in detail in the General Methods Chapter (page 38). The peak oestrogen concentration in serum was used to predict the follicular health of the follicle as opposed to the serum levels collected at the time of follicle aspiration. When the largest follicles reached 18 mm, a LH surge trigger injection of 10 000 IU hCG/LH was administered. Then 36 hours later, the follicles were punctured and aspirated by guided ultrasound to collect the fluid, granulosa cells, and oocyte via transvaginal oocyte aspiration (TVOA). Serum was analysed using biochemical analysis on the days leading up to collection and on the day of collection. IVF patients undergoing treatment were examined in a natural cycle and during exogenous rFSH stimulated cycles. In addition, a patient with normal fertility (naturally conceived births) provided a control sample during a natural cycle.

13.3 Results and Discussion

13.4 Follicular Fluid

13.4.1 Results

Pure follicular fluid was collected from IVF patients with a range of ovarian depletion, and analysed for hormone content (Figure 13.4.1). The majority of the follicles were 20 mm and ranged from 16 mm to 25 mm. Oestrogen, progesterone, and testosterone were not significantly different as the ovarian reserve declined (Figure 13.4.1). FSH was significantly increased commensurate with an increase in IVF rFSH treatment ($p<0.05$, Figure 13.4.1 and Figure 13.4.2). FSH increased as the ovarian reserve increased, and was correlated to the dose of rFSH administered. Follicular fluid LH data were not shown, as the level was low (less than 0.7 IU/L) for all patients due to GnRH suppression of LH release from the anterior pituitary. LH in the serum was much higher, ranging from 1.7-5.2 IU/L (Figure 13.4.2).

13.4.2 Discussion

Serum and follicular fluid are an indirect reflection of the growth and maturation of the follicle and the oocyte. The hormone profile reaches a peak just before TVOA collection. The administration of the LH trigger changes the hormone profile from the follicular phase to the luteal phase of development. The follicular fluid hormone concentration is therefore, not a reflection of the follicular phase but rather the peri-luteal phase (Rotmensch, et al. 1986, Westergaard, et al. 1986). In the current study, the concentration of follicular fluid, oestrogen, LH, progesterone, and testosterone were constant with age and ovarian reserve (Figure 13.4.1). This is not surprising as the majority of the follicles were at the pre-ovulatory stage (20 mm) producing maximum amounts of oestrogen and progesterone.

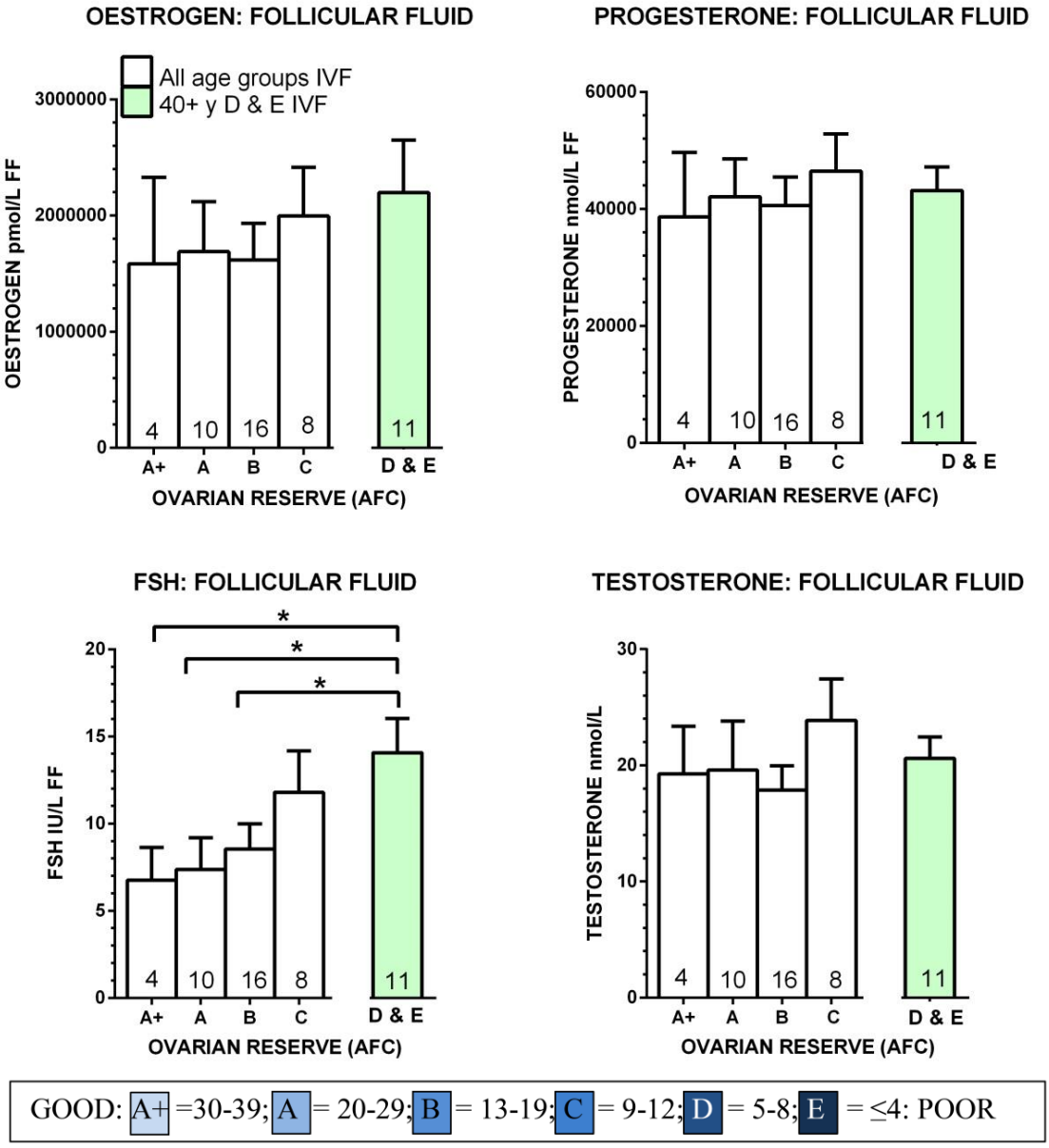


Figure 13.4.1 Follicular fluid levels of IVF patients during stimulated cycles.
Pure follicular fluid was collected 36 hours after the hCG/LH surge trigger by TVOA. The follicles ranged in size from 16-25 mm, with a mean of 20 mm. The number of samples is indicated by the numbers in the bars. Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *p<0.05, **p<0.01, ***p<0.005, and ****p<0.001. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level for the size follicle.

FSH increased significantly with age, which reflected the increase in rFSH dose administered as part of the fertility treatment provided (Figure 13.4.1 and Figure 13.4.2) (Yovich, et al. 2012). The follicle size ranged from 16 to 25 mm, with the majority being ~20 mm; hence,

the high progesterone level compared to the oestrogen level, which indicates luteinisation (Figure 13.4.3) (Westergaard, et al. 1986).

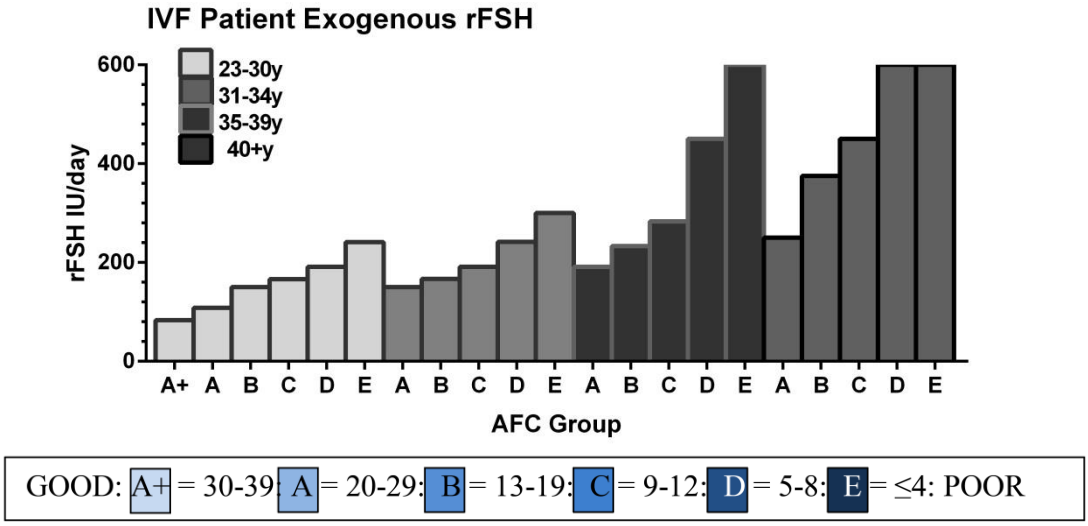


Figure 13.4.2 Exogenous recombinant FSH (rFSH) treatment dose, dependent on age and antral follicle count (AFC).
Based on data from Yovich et al. (2012).

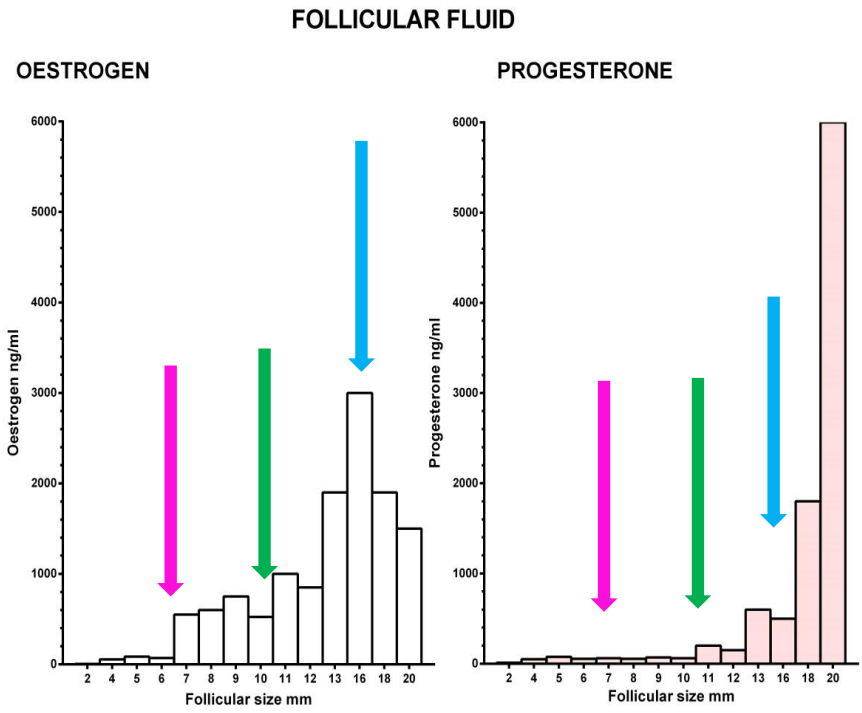


Figure 13.4.3 Changes in follicular fluid concentration of oestrogen and progesterone with follicle sized during dominant follicle selection and maturation.
The acquisition of FSHR (pink arrow) and LHR (green arrow) during the process of dominant follicle selection in a natural cycle. Follicle differentiation and maturation of the granulosa cells increase the progesterone concentration (blue arrows). Modified from previously published data (Westergaard, et al. 1986).

13.5 Oestrogen

During the follicular phase, the oestrogen peaks followed by the hCG/LH surge, and the oestrogen drops at the time of oocyte collection 36 hours post hCG/LH trigger.
Therefore, the earlier peak-oestrogen serum analysis time point reveals distinct information on the follicular development rather than the start of the peri-luteal phase at the time of oocyte collection (Andersen, et al. 1993).

13.5.1 Result: Oestrogen natural cycle compared to IVF young patients

The serum from 23-30 y, natural, unstimulated IVF patients was collected during an assessment cycle and compared to the same age patients during an IVF cycle (Figure 13.5.1). The oestrogen levels were significantly higher in the IVF cycle, except for the D & E patients who had reduced oestrogen production ($p<0.001$, Figure 13.5.1). The level of oestrogen corresponded to the number of large antral follicles, and the 'per follicle rate' was not significantly different during an IVF cycle(Figure 13.5.3).The young patient oestrogen levels decreased significantly as the ovarian reserve declined from an AFC of C to D & E combined ($p<0.05$, Figure 13.5.2).

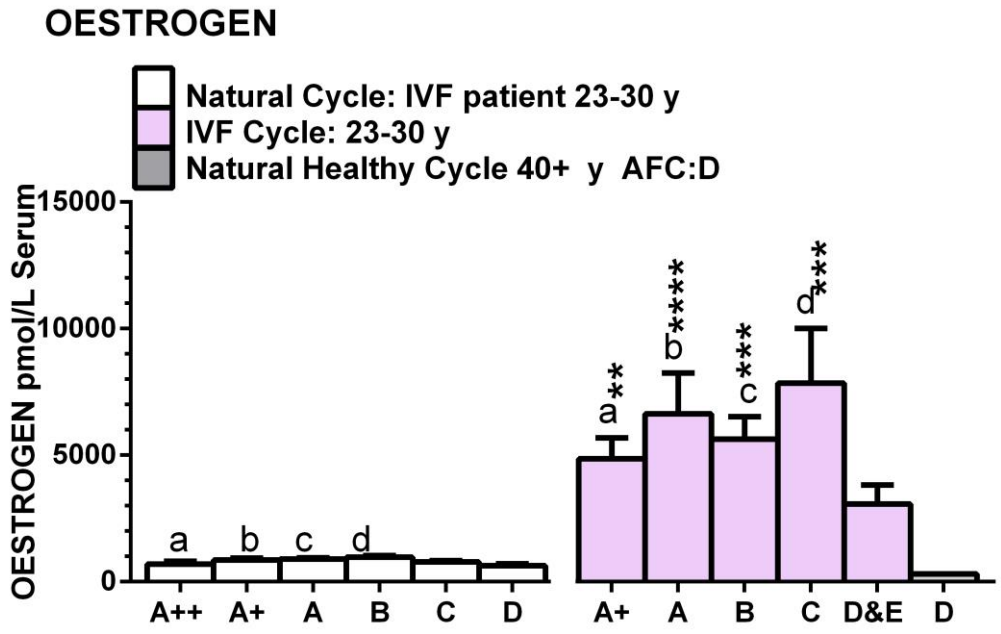


Figure 13.5.1 Serum oestrogen level during a natural cycle compared to a stimulated IVF cycle from young patients.

Peak serum oestrogen measured during a natural cycle, and a stimulated IVF cycle from patients 23-30 y with a range of ovarian depletion. Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *$p<0.05$, **$p<0.01$, ***$p<0.005$, and

****p<0.001. The letter, such as ‘a’ signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level for the size follicle.

In the 31-34 y age group, the oestrogen level was also constant, followed by a significant drop from D to the E patient group (p<0.01, Figure 13.5.2). The 35-39 y age group showed an earlier sequential decline in the oestrogen level from the B level of ovarian reserve to the D & E level (p<0.001, Figure 13.5.2). The change in the 40+ y age group produced a dramatic reduction in oestrogen production by the granulosa cells from the AFC A to the E patient group as the ovarian reserve declined (p<0.001, Figure 13.5.2).

13.5.2 Discussion: Oestrogen and ovarian depletion

The total oestrogen peak was similar between the age groups; however, it is not until the antral follicle count is applied to distinguish the ageing process, that the differences are revealed. Peak oestrogen is consistently produced at approximately 6000 pmol/L for all age groups, but falls away progressively in the C, D, and E AFC groups with increasing chronological age (Figure 13.5.1). Age alone was not predictive of oestrogen levels, demonstrating no difference between age groups (see appendix). The reduction in oestrogen output per follicle as the ovarian reserve is depleted is a reflection of diminished number of follicles and the oestrogen output capacity. In the older 40+ y group, the per number of follicles rate of oestrogen synthesis was reduced as the ovarian reserve depleted (Figure 13.5.3). The reduced follicular capacity to produce oestrogen would compromise oocyte survival. In Chapter 7 the level of FSHR density was similar in the older patients with reduced AFC. This indicates that the decrease in oestrogen synthesis capability is not solely associated with the FSHR density.

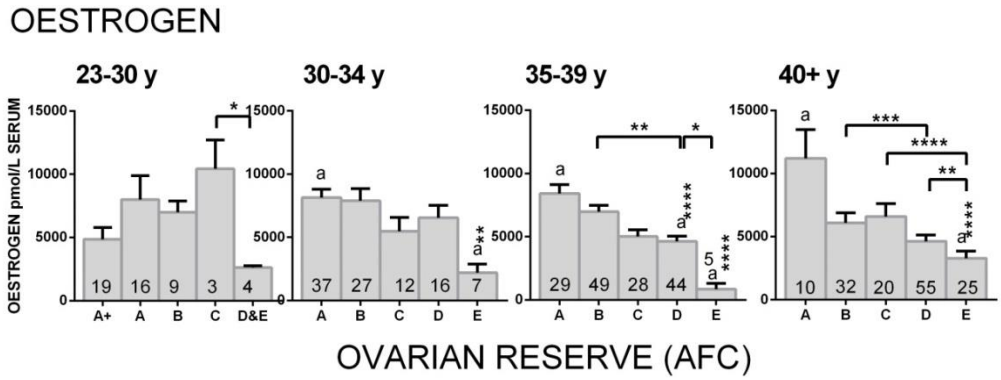


Figure 13.5.2 Peak serum oestrogen levels from IVF patients during stimulated cycles.
Serum oestrogen levels were taken at the time of peak oestrogen during a stimulated IVF cycle. Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher’s LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered

significant if *p<0.05, **p<0.01, ***p<0.005, and ****p<0.001. The letter, such as ‘a’ signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level for the size follicle.

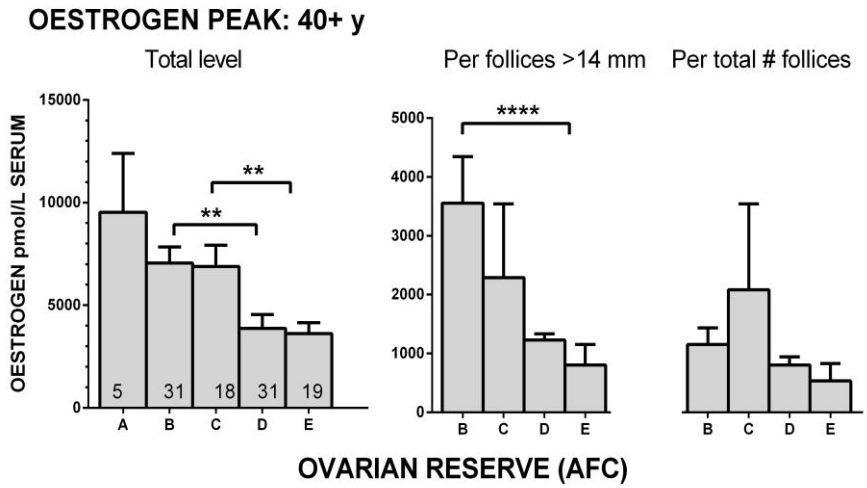


Figure 13.5.3 Peak serum oestrogen levels from IVF patients during stimulated cycles. Serum oestrogen levels were taken at the time of peak oestrogen during a stimulated IVF cycle. Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher’s LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *p<0.05, **p<0.01, ***p<0.005, and ****p<0.001. The letter, such as ‘a’ signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level for the size follicle.

13.6 FSH

13.6.1 Result: FSH natural cycle compared to IVF young patients

The serum from 23-30 y, natural, unstimulated IVF patients was collected during an assessment cycle, and compared to the same age patients during an IVF cycle (Figure 13.6.1). There was no significant difference in the FSH surge levels at the time of peak oestrogen, except for the B group of young IVF cycle patients who had significantly more FSH in serum (p<0.001).

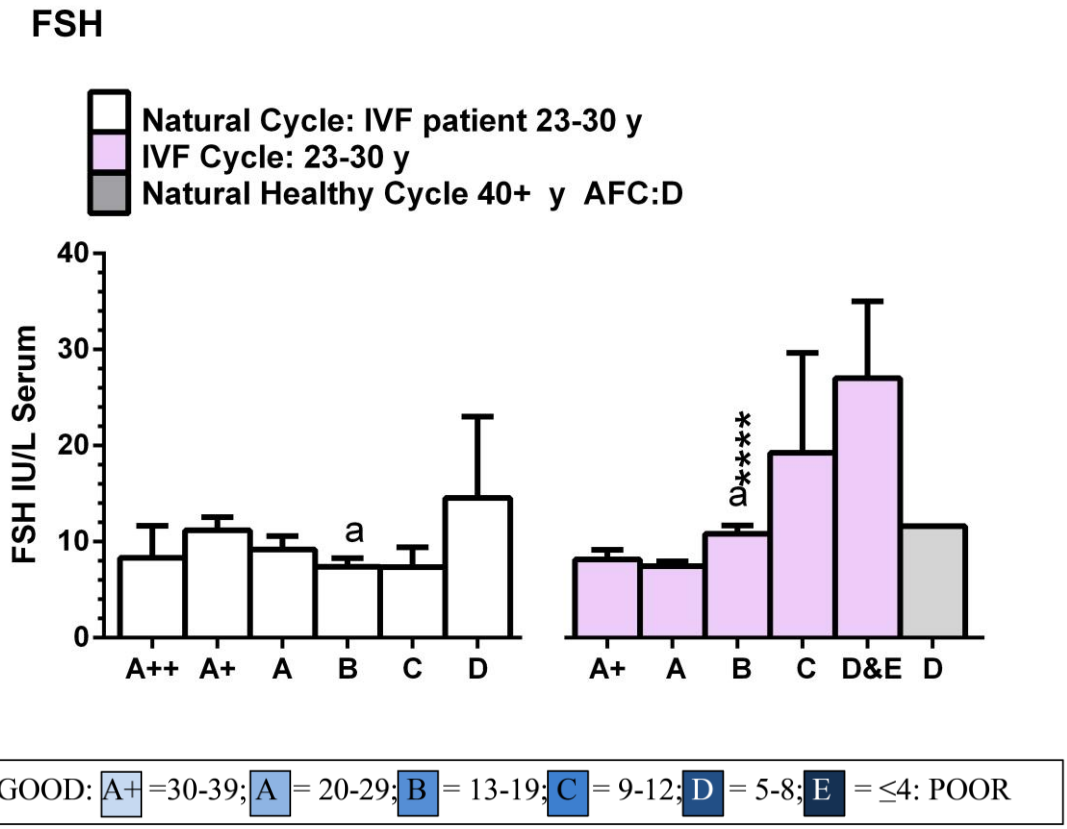


Figure 13.6.1 Serum FSH level during a natural cycle compared to a stimulated IVF cycle from young patients.

Serum FSH levels were taken at the time of peak oestrogen during a natural cycle, and a stimulated IVF cycle from patients 23-30 y with a range of ovarian depletion. Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *p<0.05, **p<0.01, ***p<0.005, and ****p<0.001. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level for the size follicle

.

At the time of the peak oestrogen, FSH serum levels were reflective of the dose of rFSH administered to the patient groups. In the 40+ y age group a low serum level was found in the AFC group A, followed by a commensurate rise in serum FSH in the B to E patient groups ($p<0.005$, Figure 13.6.2). rFSH administered to patients was based on AFC, BMI, AMH, and age (Yovich, et al. 2012). The FSH serum levels were strongly correlated to the exogenous dose of rFSH administered. Age was also significantly correlated to the increasing FSH in serum levels (see appendix).

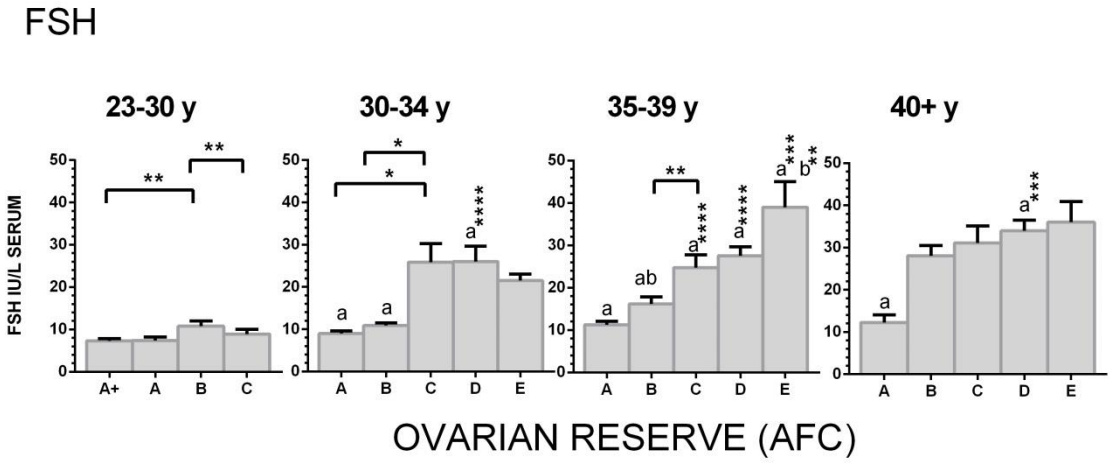


Figure 13.6.2 FSH serum levels at the peak of the follicular phase from IVF patients during stimulated cycles.

Serum FSH levels were taken at the time of peak oestrogen during a stimulated IVF cycle. Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *p<0.05, **p<0.01, ***p<0.005, and ****p<0.001. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level for the size follicle.

In the current study, the level of FSH at the start of the cycle was significantly greater in the older patients with a poor ovarian reserve compared to the younger patients during a natural cycle or during an IVF cycle (Figure 13.6.3A). The FSH level at the time of peak oestrogen (FSH surge) was not significantly different between the old and young patients during a natural cycle (Figure 13.6.3B). However, during an IVF cycle, the FSH surge was substantially greater in the older patients with a dose dependent increase as the ovarian reserve declined ($p<0.001$, Figure 13.6.3D).

13.6.2 Discussion

In a natural cycle, depletion of the ovarian reserve has been associated with an increased FSH serum level at the start of the cycle (Figure 13.6.3A and C) (Seifer, et al. 1999). The elevated FSH at the start of the cycle accelerates growth of the small antral follicles; however, the follicles grow slowly and the length of the cycle is reduced due to early luteinisation with a weaker LH surge (Baerwald, et al. 2009, Klein N A, et al. 1996) The follicles have a reduced number of granulosa cells with a lower percentage of luteinised granulosa cells present at TVOA (Baerwald, et al. 2009, Santoro, et al. 2003, Seifer, et al. 1993).

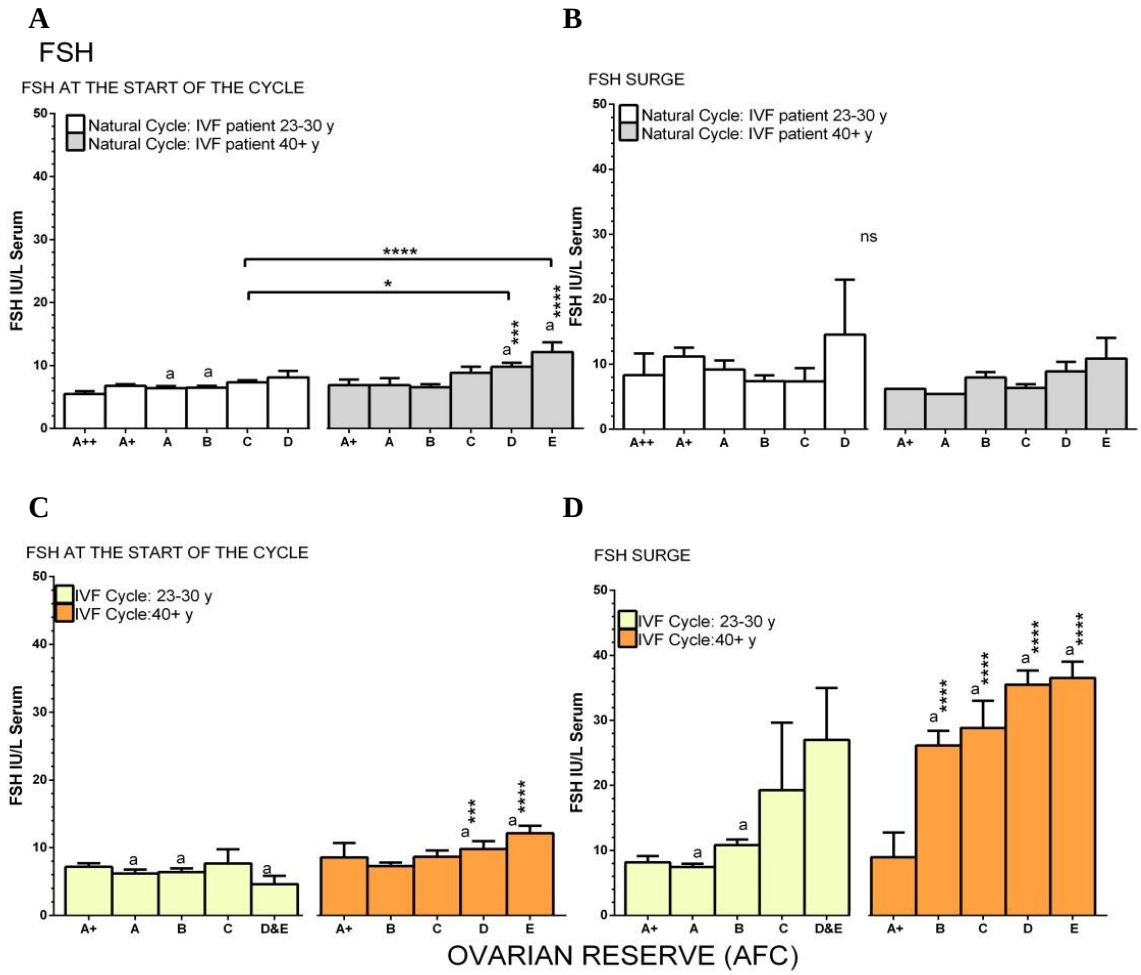


Figure 13.6.3 FSH at the start and during the peak oestrogen phase of a cycle.

Serum FSH level at the start of the cycle and the FSH surge level at the time of peak oestrogen, was collected during a natural cycle and an IVF cycle, from patients of 23-30 y and 40+ y, with a range of ovarian depletion. Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *p<0.05, **p<0.01, ***p<0.005, and ****p<0.001. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level for the size follicle.

13.7 LH

The level of serum LH in a natural cycle surges, followed soon after by the progesterone and the FSH surge. In an IVF stimulated cycle, the LH is supressed and remains very low throughout the cycle to inhibit spontaneous ovulation in order to allow multiple follicles to mature ($p<0.001$, Figure 13.7.1).

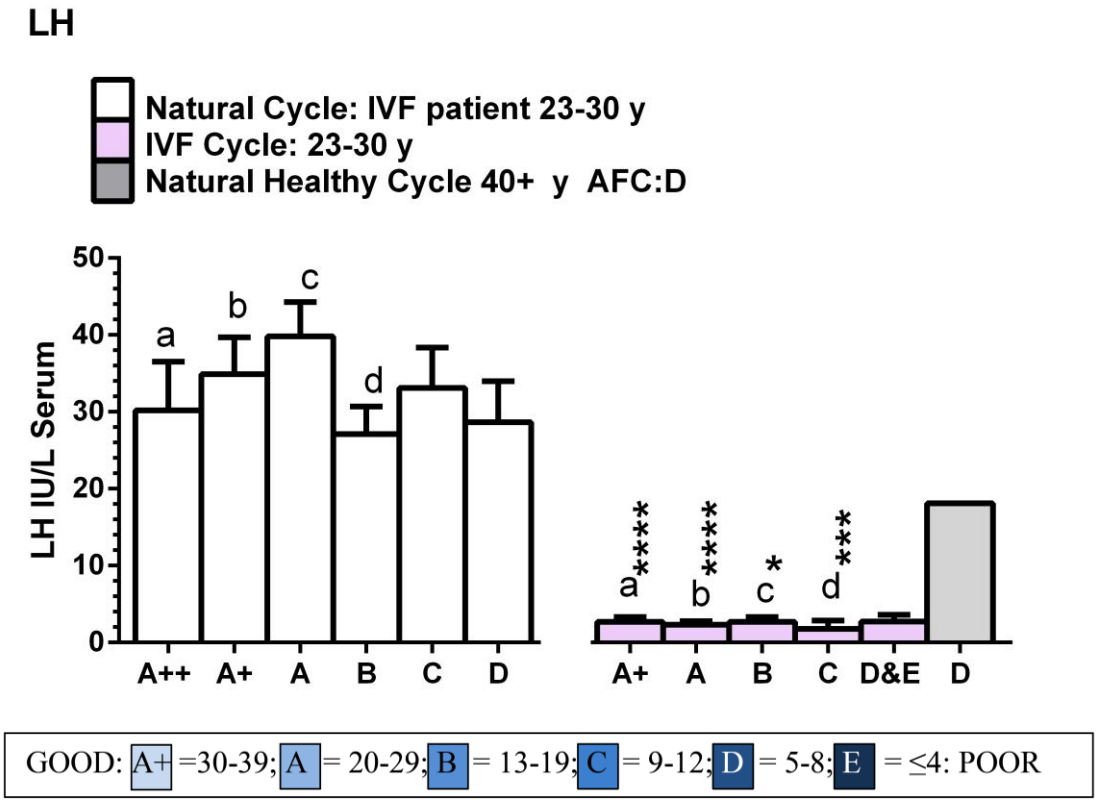


Figure 13.7.1 Serum LH level during a natural cycle compared to a stimulated IVF cycle from young patients.

Serum LH levels were taken at the time of peak oestrogen, during a natural cycle and a stimulated IVF cycle from patients 23-30 years old with a range of ovarian depletion. Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *$p<0.05$, **$p<0.01$, ***$p<0.005$, and ****$p<0.001$. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level for the size follicle.

Serum LH was consistent across the AFC of the 23-30 y patients (Figure 13.7.2). The 31-34 y and 35-39 y age groups with 'good ovarian' reserve had lower levels of LH in serum compared to the poorer E AFC groups ($p<0.05$, Figure 13.7.2). The 40+ y patients had constant LH serum levels, which were ~two times the level of the youngest patients with a good ovarian reserve. LH serum levels were supressed by the IVF treatment (Figure 13.7.2). The GnRH agonist or antagonist IVF treatment induced LH suppression, and produced

consistently low LH serum levels (1.7-5.2 IU/L) across all ages and AFC, yet there was a significant increase in LH in the patients from the middle age groups (31-34y AFC: D-E & 35-39 y AFC: C-E); however, this was not outside the range required in the IVF treatment protocol (follicular phase, natural cycle; 1.9-12 IU/L and IVF cycle; <6 UI/L) (Figure 13.7.2) (Stanger and Yovich 1985)

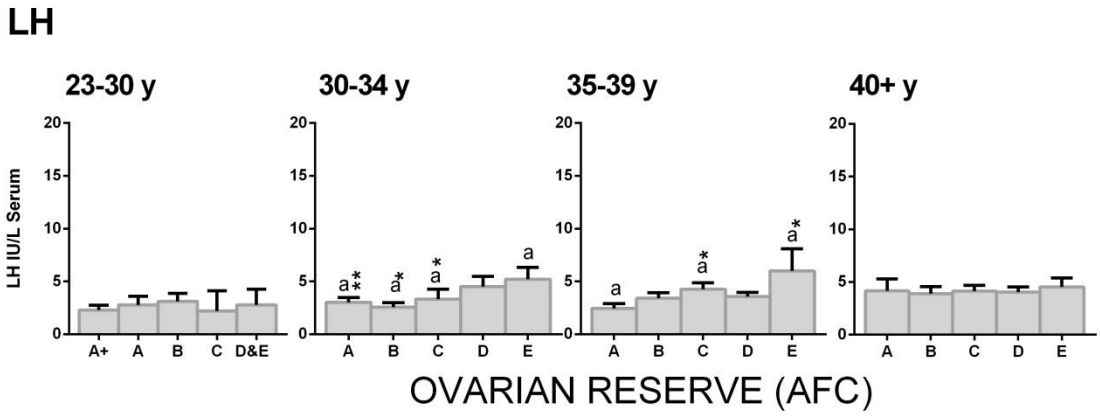


Figure 13.7.2 LH serum levels at the peak of the follicular phase from IVF patients during stimulated cycles.

Serum LH levels were taken at the time of peak oestrogen during a stimulated IVF cycle. Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *p<0.05, **p<0.01, ***p<0.005, and ****p<0.001. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level for the size follicle.

The LH concentration at the peak of the follicular phase was significantly lower in the natural cycle of the older, 40+ y D patient group compared to the younger, natural cycle patient group of 23-30 y A (Figure 13.7.3A). The measurement was taken at the time of peak oestrogen concentration, before the onset of the progesterone surge, and ranged between 20 to 40 IU/L. The LH concentration during an IVF cycle was low and ranged between 1.7 to 5.2 IU/L (Figure 13.7.3B). The LH concentration was significantly greater in the older IVF patients with a poor ovarian reserve of E compared to the younger patient group of A ($p<0.01$, Figure 13.7.3B). The level of LH increased sequentially from A+ to E in the 40+ y IVF patients (Figure 13.7.3B).

13.7.1 Discussion

The data from the current study suggest that the LH serum level, corresponding to the peak oestrogen level of the follicular phase, in an IVF cycle increased as the ovarian reserve depleted, and ranged between ~1.7 and 5.2 IU/L (Figure 13.7.3); whereas, in the young patients during a natural cycle, it ranged from 12 IU/L (AFC: A++) decreasing to 8 IU/L in

the AFC: A & B, which was greater than that seen with the IVF cycles. The elevated LH levels in the natural cycles may be a result of the unexplained infertility previously demonstrated by a similar increase in LH and FSH compared to healthy controls of the same age group (33 y) (Leach, et al. 1997).

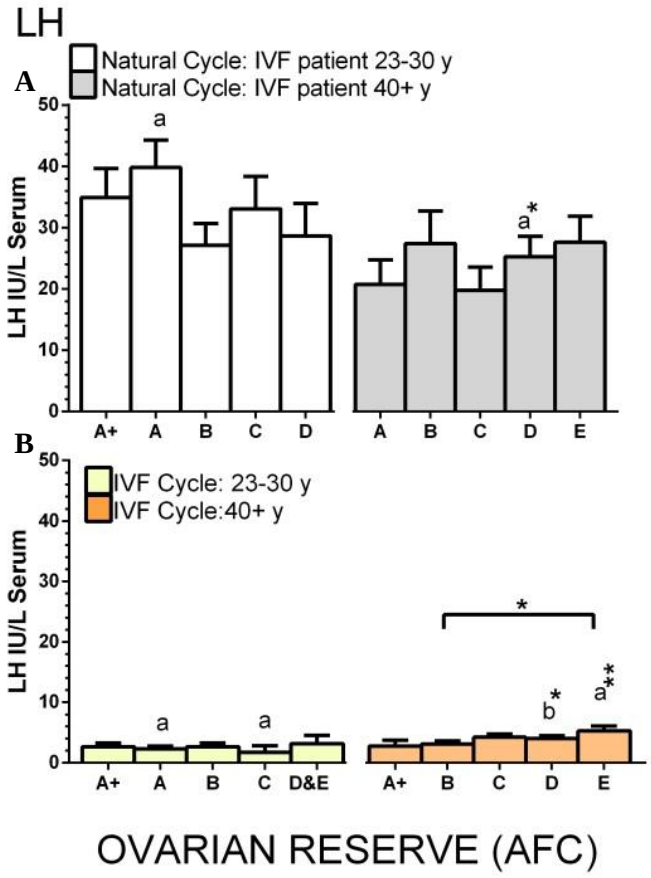


Figure 13.7.3 LH surge in a natural cycle compared to an IVF treatment cycle in old and young patients.

Serum LH levels were taken at the time of peak oestrogen during a natural cycle and a stimulated IVF cycle from patients of 23-30 y and 40+ y with a range of ovarian depletion. Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *p<0.05, **p<0.01, ***p<0.005, and ****p<0.001. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level for the size follicle.

One explanation for the incomplete suppression of GnRH with ovarian ageing is the high levels of BMP activity at the terminal-end of folliculogenesis (Chapter 5), Diminished ovarian response due to an early loss of ovarian reserve amongst young patients (IVF cycle < 3 follicles, 21-35 y) has been previously shown to have significantly extended LH surges with reduced luteal progesterone (Pal, et al. 2010); whereas, in natural cycles, the effect of age reduced the amplitude of the LH surge and reduced oestrogen levels in mares (Ginther, et

al. 2008). The consistent elevated serum LH (4 IU/L) levels recorded in the poor response patient group of 40+ y AFC: A-E, may be a reflection of the lack of effectiveness of the GnRH suppression caused by the lack of BMPR1B down-regulation in ovarian ageing (Chapter 6), or alternatively, may be symptomatic of the increased exogenous rFSH treatment.

13.8 Progesterone

13.8.1 Result

The progesterone level of the young 23-30 y group was similar in a natural cycle compared to an IVF cycle (Figure 13.8.1).

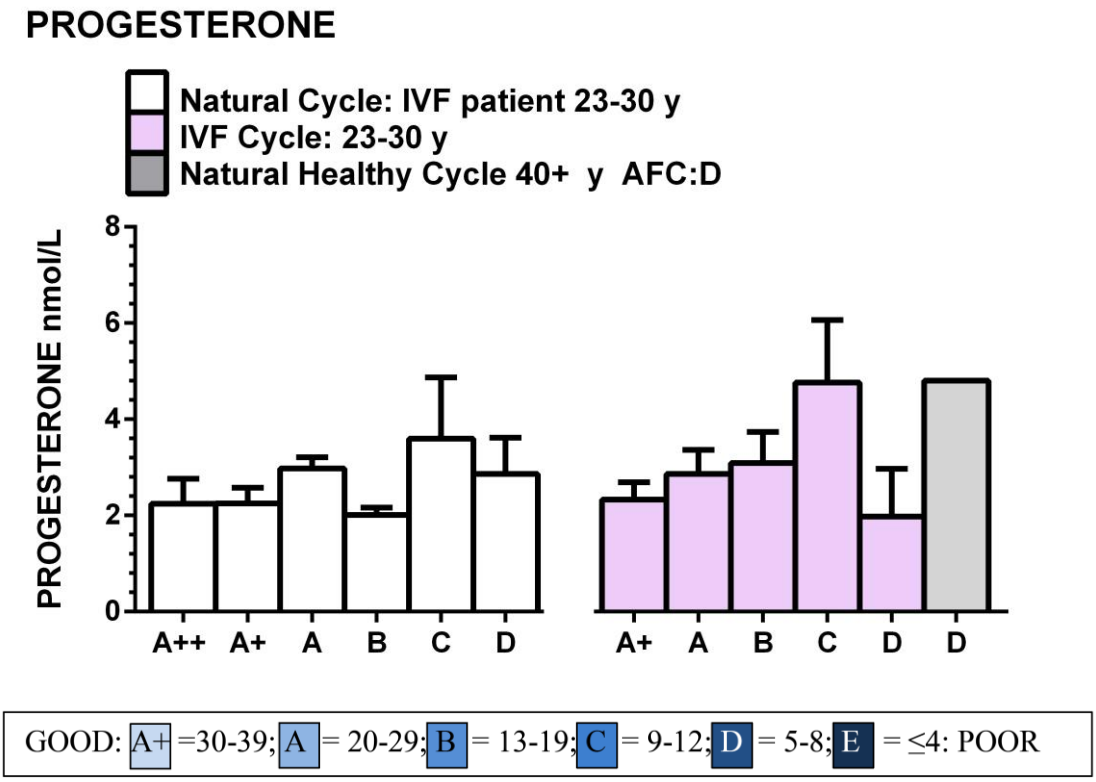


Figure 13.8.1 Serum progesterone level during a natural cycle compared to a stimulated IVF cycle from young patients.

Serum progesterone levels were taken at the time of peak oestrogen during a natural cycle and a stimulated IVF cycle from patients 23-30 years old with a range of ovarian depletion. Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *p<0.05, **p<0.01, ***p<0.005, and ****p<0.001. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level for the size follicle.

Progesterone concentration was constant (1-6 nmol/L) followed by a decrease in the severely depleted patient groups of D and/or E, as the ovarian reserve depleted ($p<0.05$, Figure 13.8.2). In the 40+ y A group, the level of progesterone was high, followed by a constant lower level ($p<0.005$) similar to the younger patients. Progesterone concentration was significantly greater during an IVF cycle compared to a natural cycle of 40+ y patients with the same ovarian reserve of B or D ($p<0.05$, Figure 13.8.3). Progesterone concentration ranged from 1.7 to 5.5 IU/L. The level of progesterone in the natural cycle of a healthy patient, with an ovarian reserve of D, was similar to the IVF cycle patient group with a matching AFC of D.

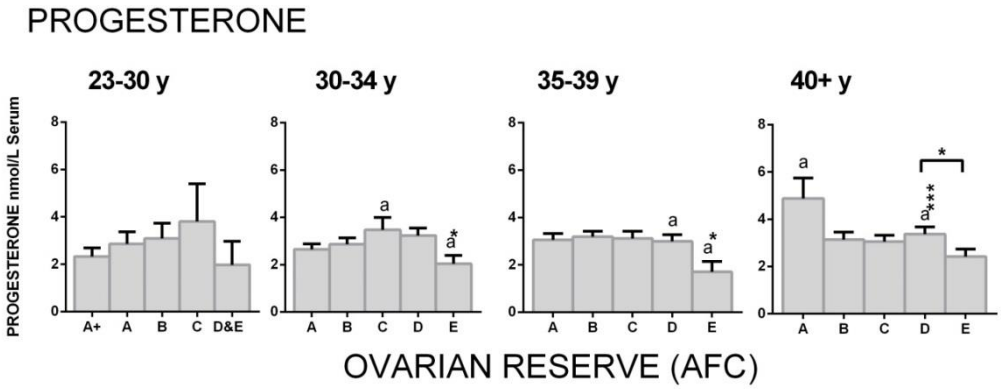


Figure 13.8.2 Progesterone serum levels at the peak of the follicular phase from IVF patients during stimulated cycles.

Serum progesterone levels were taken at the time of peak oestrogen during a stimulated IVF cycle. Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *$p<0.05$, **$p<0.01$, ***$p<0.005$, and ****$p<0.001$. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level for the size follicle.

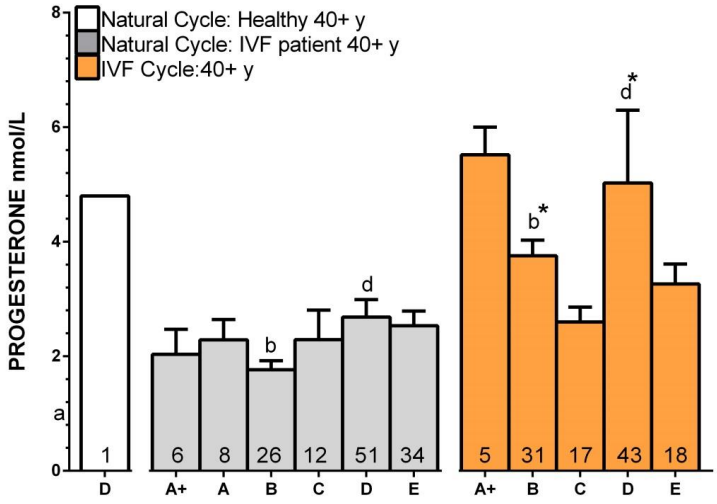


Figure 13.8.3 Progesterone serum levels at the time of peak serum oestrogen during a natural cycle compared to an IVF cycle.

Serum progesterone levels were taken at the time of peak oestrogen during a natural cycle and a stimulated IVF cycle from patients 40+ y with a range of ovarian depletion. Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *$p<0.05$, **$p<0.01$, ***$p<0.005$, and ****$p<0.001$. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level for the size follicle.

13.8.2 Discussion

In an IVF cycle, the hCG/LH trigger initiates cessation of proliferation and a change in steroidogenic capacity from oestrogen to progesterone production in the granulosa cells. Progesterone synthesis during the follicular phase is inhibited, which promotes oestrogen synthesis and follicular growth. The LH surge reduces the inhibition and promotes progesterone production in the granulosa cells. The progesterone initially increases slowly in the first few hours, post LH trigger, followed by a progesterone surge, in preparation for luteinisation of the follicle to form the corpus luteum. Serum progesterone levels in older patients were higher compared to younger patients with an uncompromised ovarian reserve or good ovarian reserve (Figure 13.8.2).

Ovarian ageing was evident in the poor ovarian reserve patient groups for each age class (Figure 13.8.2). Increased progesterone is an indicator of premature luteinisation, which can produce a decreased pregnancy rate (Legro, et al. 1993). The reduced oestrogen synthesis would also be associated with a reduced progesterone serum level, and may be related to granulosal receptor density. The higher progesterone levels in the poor ovarian reserve patient groups were below the concentration that other researchers have deemed to be of

detrimental significance (Legro, et al. 1993). Legro et al. (1993), found that elevated progesterone serum levels that were greater than 3.8 nmol/L (1.2 ng/ml)), were associated with an improved oocyte quality. The higher progesterone levels in the donor patients were, therefore, more likely to be related to endometrial receptivity, and not follicular progesterone concentration and oocyte quality (Legro, et al. 1993).

CHAPTER 14

Serum and Follicular Fluid, GH co-treatment, and Ovarian Ageing

Title: The decline in ovarian ageing and the effect on ovarian hormone regulation in IVF patients co-treated with GH

CHAPTER 14 Serum & Follicular Fluid & GH co-treatment

14.1 Introduction

Ovarian ageing has been associated with an increase in aneuploidy and a diminished recruitment or response to exogenous gonadotrophin stimulation (Hale 2007, Seifer, et al. 2007, Seifer, et al. 1993, Seifer, et al. 2002). GH co-treatment of IVF patients with a poor response to ovarian stimulation with rFSH has been shown to improve the pregnancy and birth rate in an ageing population (Tesarik, et al. 2005, Yovich and Stanger 2010).

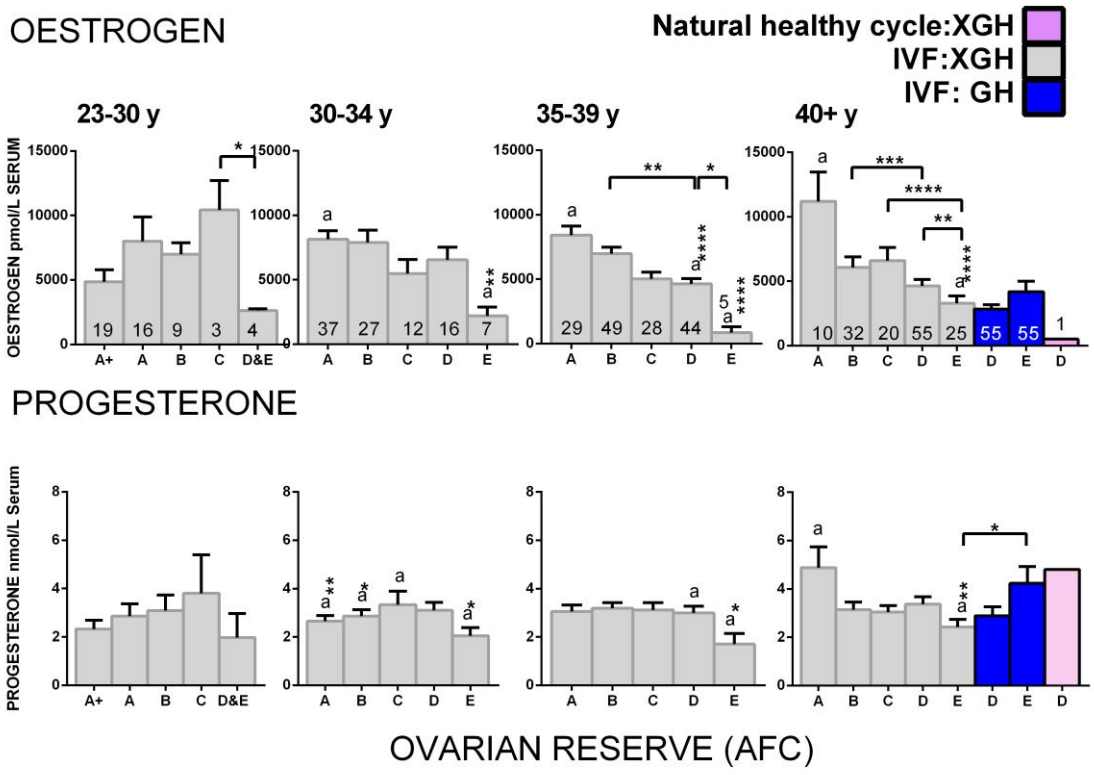
Previously, it has been shown that GH co-treatment increased the density of receptors in the 40+ y poor ovarian response group with an AFC of D or E (Chapter 10). In addition, it has been established that the type 1 TGFβ receptor, BMPR1B was reduced in a biphasic pattern during cyclic folliculogenesis in humans (Chapter 6), and in Merino sheep with an uncompromised ovarian reserve (Regan, et al. 2015). The spatiotemporal regulation occurred at the time of dominant follicle selection and during pre-ovulatory maturation. The results also showed that, as the ovarian reserve declined, the biphasic down-regulation of BMPR1B was disrupted (Chapter 6). In the poor response oldest patients, the BMPR1B failed to be down-regulated towards the end of folliculogenesis. The granulosal receptor density from older poor ovarian reserve patients, co-treated with growth hormone, was reversed. The younger higher receptor level and the pre-ovulatory down-regulation of the BMPR1B, FSHR and LHR were re-established. The aim of this investigation was to determine if GH co-treatment of IVF patients influences the steroidogenic capacity of the follicle.

14.2 Methods

Methods are described in detail in the General Methods Chapter (page 38). The peak oestrogen concentration in serum was used to predict the follicular health of the follicle as opposed to the serum levels collected at the time of follicle aspiration. When the largest follicles reached 18 mm, a LH surge trigger injection of 10 000 IU hCG/LH was administered. Then 36 hours later, the follicles were punctured and aspirated by guided ultrasound to collect the fluid, granulosa cells, and oocyte via transvaginal oocyte aspiration. Serum was analysed using biochemical analysis on the days leading up to collection and on the day of collection. IVF patients undergoing treatment were examined in a natural cycle and during exogenous rFSH stimulated cycles. In addition, a patient with normal fertility (prior naturally conceived births) provided a control sample during a natural cycle.

14.3 Results

The steroidogenic activity of theca and granulosa cells contained within the follicle supports the oocyte during folliculogenesis and pre-ovulatory maturation, has been referred to as the 'two cell' theory (Hillier, et al. 1994b). Androgen produced by the theca is converted to oestrogen in the granulosa cell, and is regulated by a complex mechanism of paracrine and autocrine growth factors driven by a combination of FSH and LH secreted from the anterior pituitary (Miller 2011). The results from the current study indicate that the GH co-treatment did not alter the oestrogen level of the 40+ y with an ovarian reserve of D or E during an IVF cycle (Figure 14.3.1). Furthermore, the ratio of oestrogen was not altered, nor the levels of oestrogen secreted, based on either the total number of follicles or the number of follicles greater than 14 mm present in the ovary at the time of collection which were not significantly different (Figure 14.3.2). In addition, the follicular fluid concentration of oestrogen, progesterone, FSH, or testosterone was not significantly different to the age matched patients with a similar ovarian reserve that were co-treated with GH (Figure 14.3.3).



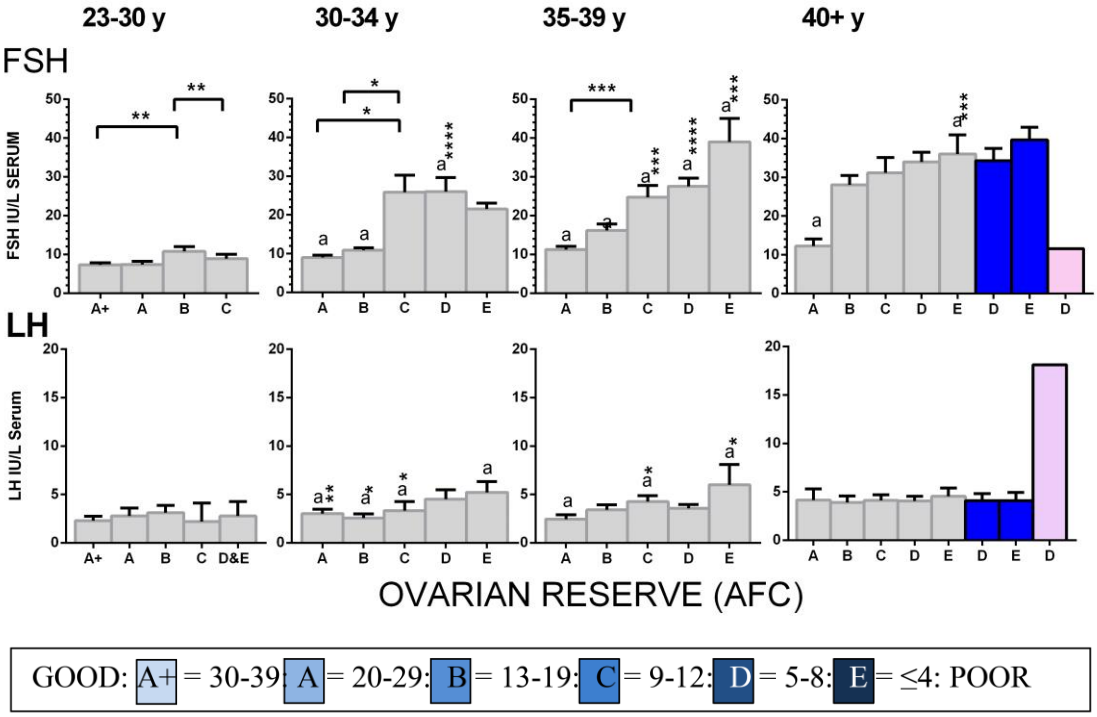


Figure 14.3.1 Serum levels of IVF patients during stimulated cycles, ovarian ageing and the effect of GH co-treatment.

Serum oestrogen, progesterone, FSH, and LH levels were taken at the time of peak oestrogen during a stimulated IVF cycle. Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *p<0.05, **p<0.01, ***p<0.005, and ****p<0.001. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level for the size follicle.

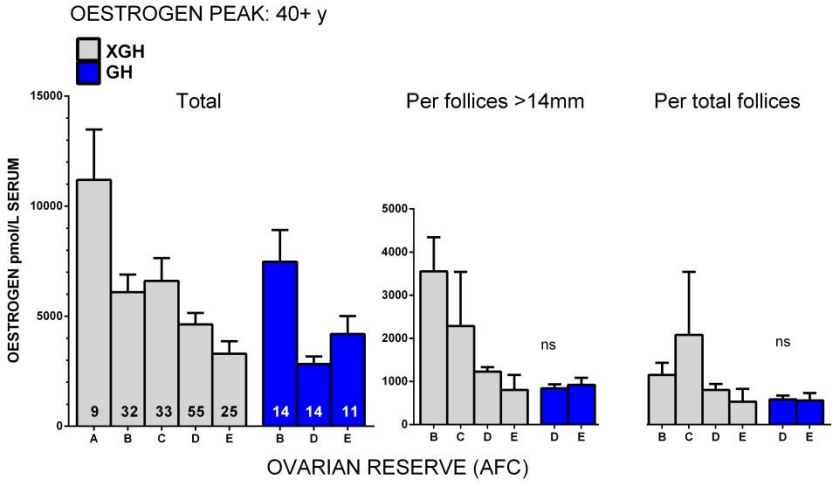
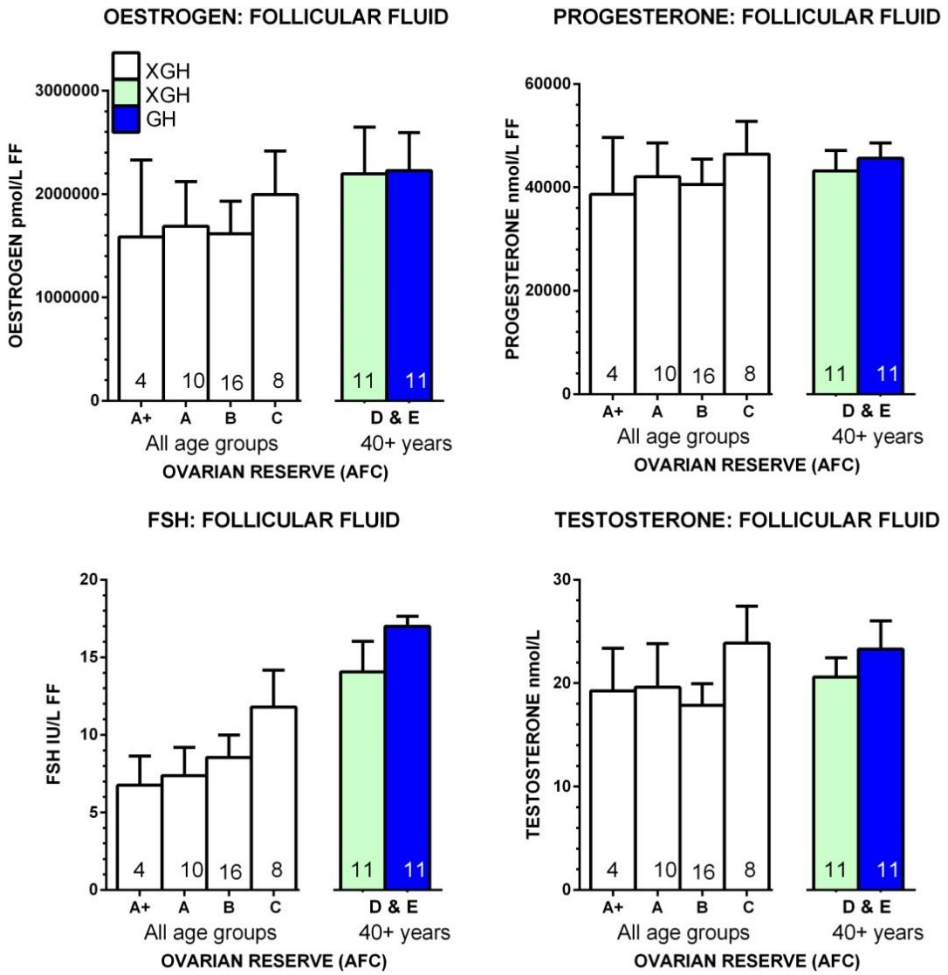


Figure 14.3.2 Serum peak oestrogen level from IVF patients with and without GH co-treatment.

(A) The peak oestrogen level was determined from blood samples from IVF patients during the follicular phase. The blood sample was taken before the hCG /LH trigger injection (B) The peak oestrogen was divided by the number of follicles greater than 14 mm. (C) The peak oestrogen was divided by the total number of follicles present at oocyte collection. Ovarian reserve measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day

2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *p<0.05, and **p<0.01. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level for the size follicle.



GOOD: A+ =30-39; A = 20-29; B = 13-19; C = 9-12; D = 5-8; E = ≤4: POOR

Figure 14.3.3 Follicular fluid levels of hormones from IVF patients during stimulated cycles and co-treatment with GH.

Pure follicular fluid was collected from follicles at the time of oocyte collection for IVF treatment. Follicle size was between 16-23 mm, with an average of 20 mm. Ovarian reserve was measured indirectly by the antral follicle count (AFC). AFC is the number of follicles between 2-10 mm on day 2-5 of a cycle. Mean fluorescent intensity (MFI) was obtained using an average of ~8000 granulosa cells per follicle for the direct measurement of receptor protein expression. The data were subjected to statistical verification using one-way ANOVA with an uncorrected Fisher's LSD for follicular size. Values in graphs are means ± S.E.M., and differences were considered significant if *p<0.05, **p<0.01, ***p<0.005, and ****p<0.001. The letter, such as 'a' signifies a statistical difference to the matching letter, and an attached asterisk (a*) which indicates the significance level for the size follicle.

14.4 Discussion

In a natural cycle, the selection of the dominant follicle is based on the follicle that is first to express the androgen-induced FSHR. The increased granulosal FSHR leads to the induction of LHR at the time of selection (Rice, et al. 2007). The diminishing ovarian reserve of primordial follicles limits the activation of primordial follicles in each cycle. Fewer growing primordial follicles reduce the pool of small antral follicles available for cyclic recruitment. Exogenous rFSH appears to reach a saturation point, beyond which more rFSH fails to alter the number of follicles recruited. Previously, it has been reported that GH increased the receptor density of BMPR1B, FSHR, LHR and GHR (Chapter 10); yet GH did not increase the number of recruited follicles (Yovich and Stanger 2010). The main effect of the GH co-treatment was not during the dominant follicle selection stage of folliculogenesis, and therefore is supportive of an increase in quality, and not the quantity of oocytes. It is therefore probable that the improved pregnancy rate must involve other cellular mechanisms to improve the quality of oocytes.

14.4.1 GH co-treatment and the FSH-FSHR activity

In the present study the oestrogen output from GH co-treated IVF patients was consistent with other *in vivo* studies (Owen, et al. 1991, Tapanainen, et al. 1992). Oestrogen synthesis during an IVF cycle is driven by rFSH and the activation of the FSHR. Gonadotrophin receptor activation induces signalling via the AC-cAMP-PKA cascade, and regulates the ERK1/2 pathways (Tajima 2003). Regulation of folliculogenesis, apoptosis and steroidogenesis has been shown to be via the ERK1/2's vast number of signalling routes, and GH has been shown to have a regulatory effect on ERK1/2 signalling (Caunt, et al. 2006, Nakamura, et al. 2012).

The specificity of the signalling systems relies on compartmentalisation which involves a complex time and spatial gradient. Another possible regulating mechanism involved is the internalisation via arrestins and scaffolding proteins that influence intracellular concentrations, and importantly go on to modulate the intermediate messengers, that control protein synthesis (Caunt, et al. 2006, Tajima 2003).

GH co-treatment increased the granulosal FSHR density, yet had no effect on the serum or follicular fluid oestrogen synthesis (Chapter 10).The level of FSHR expression was not significantly reduced in the poor ovarian reserve patients (Chapter 7), which indicates that the conversion of androstenedione to oestradiol in the granulosa cell is adversely impacted on, downstream of the hormone-receptor interphase in ovarian ageing.

14.4.2 Apoptosis and GH co-treatment

In addition, it is possible that the total oestrogen level is not the cause of the reduced oocyte quality; rather, it is the lack of pre-ovulatory maturation brought about by the reduced density of receptors combined with the lack of down-regulation at the end of folliculogenesis. In particular the 40+ y B AFC patient group when treated with GH had an increase in the level of apoptosis possibly associated with an increase in proliferation commensurate with the increase in serum oestrogen (Chapter 11), whereas the apoptosis and oestrogen levels were not significantly altered in the D & E older patient group (Figure 11.3.2).

14.4.3 BMPR1B and progesterone

The level of progesterone, however, with GH co-treatment was significantly increased in the 40+ y ovarian reserve E patient group (Figure 14.3.1). It has been previously shown that GH co-treatment induced mechanisms that caused an up-regulation and a pre-requisite down-regulation maturation of the mature protein for the granulosal BMPR1B and LHR (Chapter 6 and 8). The levels of BMPR1B were low in the D group compared to the younger patient groups, whereas in the E group the receptor density was initially low, yet increase and this was accentuated in the larger follicles (Figure 14.3.1). The GH induced change to progesterone level is therefore likely to reflect the greater difference in change of the receptor density in this sub-group. In particular, the effect on the GH co-treated E group was to dramatically reduce the BMPR1B density of the largest follicles compared to the effect in the ovarian reserve D patients (Figure 14.4.2). The greater suppression of the BMPR1B at this time would reduce the BMP suppression of progesterone synthesis and significantly increase the progesterone synthesis in the granulosa cell (Figure 14.4.1)

The JAK2-MAPK signalling pathway can activate the ERK1/2 axis, and it is known that the BMPR1B activity involves the suppression of ERK1/2 to inhibit cytoskeletal changes and nuclear translocation activity during folliculogenesis to prevent luteinisation (Harris, et al. 2002, Inagaki, et al. 2009, Le Roith, et al. 2001, Liang 2007, Moore, et al. 2001b, Nakamura, et al. 2012, Seger, et al. 2001, Su 2002). At the time of differentiation during the LH surge, the BMPs are down-regulated and the inhibition of the ERK 1/2 progesterone synthesis pathway is removed, which promotes progesterone synthesis by the granulosa cells (Moore, et al. 2001b). It has been reported that the levels of granulosal luteinisation in follicles from older patients was reduced in line with the lack of maturation down-regulation described in Chapter 6-8, and the reduced pregnancy rate influenced by the lack of luteinisation of granulosa cells (Seifer 1996). The current study provides evidence of an improvement in pre-requisite down-regulation and the corresponding significant increase in progesterone synthesis indicative of greater maturation of the granulosa-luteal cells.

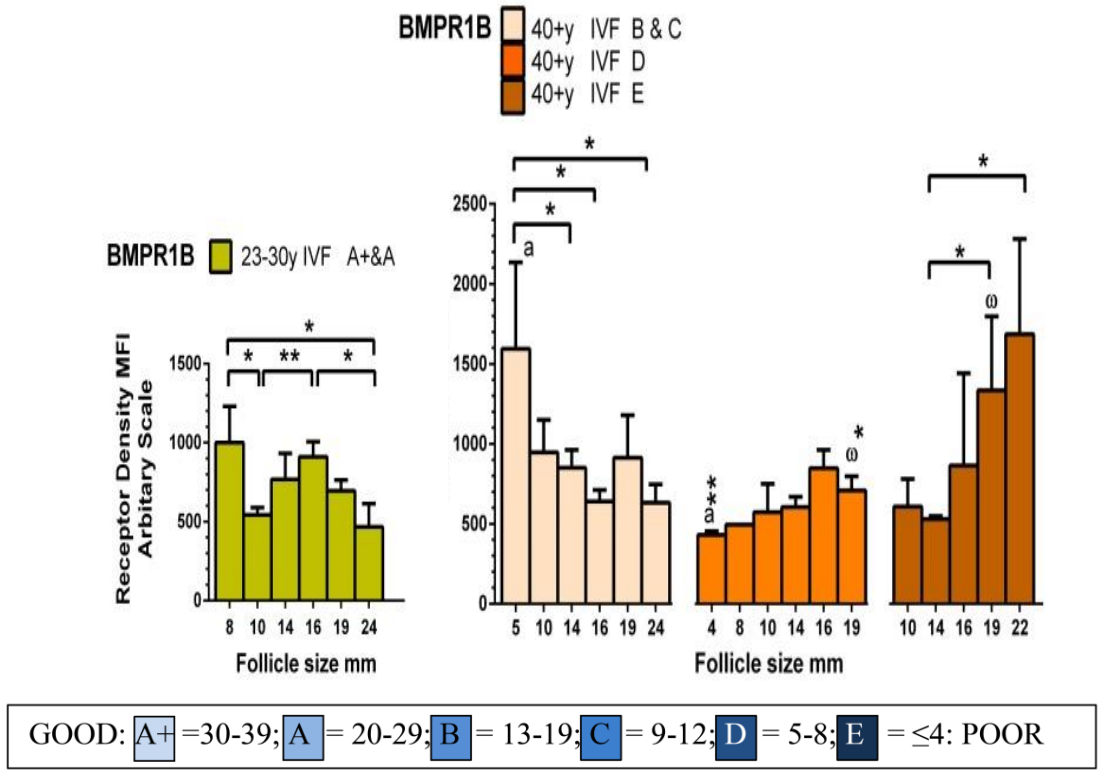


Figure 14.4.1 GH co-treatment and the effect serum progesterone and the granulosal BMPR1B density as the ovarian reserve diminished.
Estracted from Chapter 6.

However, it is noted that IGF and GH signalling mechanisms have also been shown to have common pathways in poly-ovulatory animals (Nakamura, et al. 2012); whereas in the human ovary, GH and IGF pathways have been reported to be independent and therefore are unlikely to influence the current study (Mason, et al. 1990, Peñarrubia, et al. 2000).

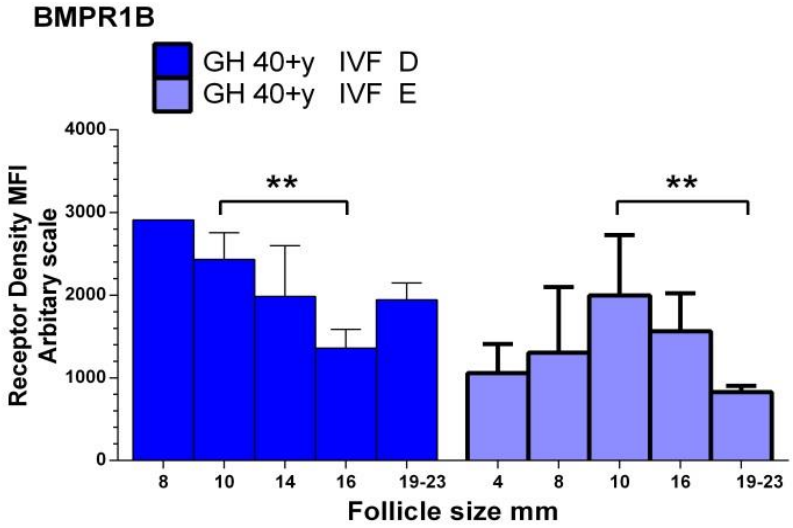


Figure 14.4.2 GH co-treatment on the granulosal BMPR1B density in 40+ y patients.
Extracted from Chapter 10.

14.4.4 IVF patients *in vitro* cell culture

Past research has provided many examples of *in vitro* cultured granulosa cells treated with GH; however, invariably the cells spontaneously luteinise in serum fortified medium or are collected from patients previously exposed to a LH surge (Ahumada-Solórzano, et al. 2012, Barreca, et al. 1993a, Bergh 1996 , Carlsson, et al. 1992, Karamouti 2008, Ovesen 1998). In addition, previously presented results have shown that the granulosa-luteal cells collected after the hCG/LH trigger, from small, medium, and large antral follicles (5-23 mm), when cultured in serum-free media produced a greater concentration of progesterone than oestrogen (Chapter 4). The ratio in favour of progesterone confirms that LH-induced steroidogenic differentiation had taken place after exposure to a LH surge (Baird, et al. 1975, McNatty, et al. 1975, Westergaard, et al. 1986). Notwithstanding, research conducted using granulosa cells collected from the follicular phase and cultured in a serum free environment demonstrated that GH treatment in culture, increased the oestrogen production (Carlsson, et al. 1992, Mason, et al. 1990).

In conclusion, oestrogen and progesterone synthesis was reduced during ovarian reserve depletion, however, the effect of GH co-treatment on the oestrogen synthesis was not evident. The progesterone level, however, was significantly increased in the 40+ y E patients and was consistent with an improvement in the maturation process via the increase in BMPR1B density, and more importantly by promoting down-regulation of receptors during the maturation phase.

CONCLUSION

Conclusion

In the young female adult, several regulatory mechanisms are in place to facilitate folliculogenesis and the maturation of the oocyte for ovulation. As the ovarian pool of primordial follicles diminishes over time in adulthood, the regulatory process is disrupted, which results in reduced fertility.

At the time of cyclic dominant follicle selection, and again during the maturation stage of folliculogenesis, changes to receptor density and increased apoptosis were evident. The change in receptor density seen with declining ovarian reserve provides compelling evidence of a fundamental shift in granulosal receptor density with ovarian ageing. Ovarian ageing resulted in a reversal of this biphasic pattern of receptor expression, disrupting the luteinisation process. The dysregulation also resulted in a reduced level of apoptosis and necrosis, and the declining level of serum oestrogen and progesterone, and the higher LH level.

The BB mutation-induced attenuation of BMPR1B signalling, led to an increased density of the FSHR and LHR, with a concurrent reduction in apoptosis or necrosis, which increased the ovulation rate. When the BMP signalling was attenuated in the BB mutation, the increase in reproductive receptors, together with a reduction in apoptosis, increased the ovulation rate. BMPR1B attenuation reduced apoptosis, which indicates an indirect role for BMPs in apoptosis activity.

The attenuation of BMPR1B activity in sheep increased the receptor expression of BMPR1B, FSHR and LHR, while GH co-treatment in humans, increased these receptors in parallel. Therefore, the GH mode of action may attenuate BMPR1B activity in the human model to cause a similar outcome to that of the BB sheep.

In contrast, in the human model, apoptosis was reduced with ovarian ageing and was not significantly altered by the GH co-treatment in the poor ovarian reserve patients. IVF treatment with rFSH reduced apoptosis, therefore any potential BMP effect on apoptosis was probably masked by the high dose of rFSH.

The potential positive effect on apoptosis may have a greater impact if rGH was used in a stand-alone regime, or on patients with an uncompromised ovarian reserve, as was the case for the young adult sheep, and as was exemplified in preliminary results from good ovarian reserve patients in this thesis.

It is therefore, more probable, that the reduction in apoptosis is associated with the reduced growth of the follicle and proliferation of the granulosa cells, as the ovarian reserve becomes severely depleted. Preliminary data from rGH co-treated older patients, with good ovarian reserve, demonstrated an increased level of apoptosis or necrosis in the stage-specific dominant follicle selection and maturation phase, which was in line with the results from an uncompromised young ovarian reserve. Further work is required to determine if the increase is associated with an increase in proliferation, and the impact this may have on the oocyte.

The corner stone of this thesis was the increase in ovulation rate caused by a naturally occurring point mutation to the BMPR1B. It is possible that, in sheep, proliferation of the granulosa cells is more strongly associated with the BMPR1B ligands; whereas, in humans, the affinity and precocious binding of the ligands may be different. Alternatively, the difference may be explained by the fact that the sheep were young and were not receiving high doses of rFSH.

Further investigation is warranted to determine if a species difference exists in the affinity or precocious signalling of the BMP ligands, and the potential for GH to influence the ovulation rate in humans. The GH treated follicles in humans, were not significantly different in size and produced similar oestrogen serum levels, which leads to the question of an alternative mode of action to increase oocyte quality.

This thesis has revealed several alternative cellular mechanisms that could contribute to an improved oocyte quality in GH co-treated IVF patients with a poor ovarian reserve. An increase in the density of FSHR would increase the FSH-induced AC-cAMP- PKA activity, leading to an increase in transcription of intermediate components that would directly up-regulate the receptor density of FSHR itself, LHR, BMPR1B and GHR. GH co-treatment increased GHR-JAK-STAT activity, and would result in an increase in the intermediate products of transcription, which may explain the increase in reproductive receptor density observed.

GH co-treatment did not alter the LHR or FSHR density of small follicles, and this would therefore account for the lack of improvement in the number of follicles recruited during dominant follicle selection. However, the increase in LHR sensitivity of the larger follicles would restore the pre-ovulatory maturation and provides another possible cellular mechanism responsible for the improved pregnancy rate.

In addition, rGH may attenuate all BMP activity; therefore, the proposed generalised partial attenuation of BMPR1B would most likely also affect BMPR1A receptor activity. The role of BMPR1A was beyond the scope of this thesis; however, it is duly noted that it contributes to ovarian regulation. The realisation that the systemic effect of rGH on the ovary is not isolated to the BMPR1B (activin receptor-like kinase (ALK6)) or even the BMPs alone, leads us to question the effect GH has on other TGFβ type 1 receptors, such as ALK2, 3, 4, and 5. Essentially, the phosphorylation of these pathways results in activation of either the SMAD2/3 pathway or the BMPR1B-induced SMAD1, 5, 8 pathway.

Further research is also needed to delineate the effect of rGH co-treatment on the oocyte that would directly influence its quality, taking into consideration that the oocyte produces GH and expresses the BMPR1B and GHR.

Significance

The clinical significance of these findings is centred on the massive change to the profile of patients requesting assistance with fertility in recent years. The social fabric of our community has changed substantially since the inception of IVF in the 1970s, which was based on much lower doses of gonadotrophins. Since its inception, IVF treatment has been continuously refined to reduce overstimulation, and increase pregnancy rates. The pregnancy rate for frozen embryo transfer is ~50% in Australia, and the birth of children from IVF-conceived parents is only now occurring. It will take another 30 years before the true impact of high rFSH treated patients is realised.

With greater knowledge on the ovarian ageing process delivered in this thesis, and comprehensive quantitative data on the changes of receptor density and hormonal control during folliculogenesis, current practice should be reconsidered. Ovarian reserve challenged patients would benefit from a reduction in the supraphysiological doses of rFSH stimulation during the stage-specific maturation phase of folliculogenesis. A reduction in rFSH would reduce the cost of ART and importantly, reduce the mental and physical stress associated with sustained high doses of rFSH.

The data presented in this thesis have revealed, several mechanisms that are involved in the improved quality of the follicle and the oocyte which has led to an improvement in pregnancy rate, reported in GH co-treated patients. rGH co-treatment increased the receptor expression in a similar way to the BMP attenuation in the BB mutation. The increased density of FSHR and LHR, induced by rGH, may provide a new minimal stimulation treatment which would reduce the reliance on high doses of rFSH in young and older patients.

REFERENCES

REFERENCES

Abdennebi, L, P Monget, C Pisselet, JJ Remy, R Salesse, and D Monniaux 1999 Comparative Expression of Luteinizing Hormone and Follicle-Stimulating Hormone Receptors in Ovarian Follicles from High and Low Prolific Sheep Breeds. *Biology of Reproduction* **60** 845-854.

Abir, R, A Ben-Haroush, N Melamed, C Felz, H Krissi, and B Fisch 2008a Expression of bone morphogenetic proteins 4 and 7 and their receptors IA, IB, and II in human ovaries from fetuses and adults. *Fertility and Sterility* **89** 1430-1440.

Abir, R, R Garor, C Felz, S Nitke, H Krissi, and B Fisch 2008b Growth hormone and its receptor in human ovaries from fetuses and adults. *Fertility and Sterility* **90** 1333-1339.

Abir, R, R Orvieto, D Dicker, Z Zukerman, M Barnett, and B Fisch 2002 Preliminary studies on apoptosis in human fetal ovaries. *Fertility and Sterility* **78** 259-264.

Aharoni, D, A Dantes, M Oren, and A Amsterdam 1995 cAMP-Mediated Signals as Determinants for Apoptosis in Primary Granulosa Cells. *Experimental Cell Research* **218** 271-282.

Ahumada-Solórzano, SM, ME Carranza, E Pedernera, AJ Rodríguez-Méndez, M Luna, and C Arámburo 2012 Local expression and distribution of growth hormone and growth hormone receptor in the chicken ovary: Effects of GH on steroidogenesis in cultured follicular granulosa cells. *General and Comparative Endocrinology* **175** 297-310.

Ainsworth, L, BK Tsang, BR Downey, GJ Marcus, and DT Armstrong 1980 Interrelationships Between Follicular Fluid Steroid Levels, GonadotropiC Stimuli, and Oocyte Maturation During Preovulatory Development of Porcine Follicles. *Biology of Reproduction* **23** 621-627.

Al-Samerria, S, I Al-Ali, JR McFarlane, and G Almahbobi 2015 The impact of passive immunization against BMPR1B and BMP4 on follicle development and ovulation. *Reproduction*.

Al-Samerria, S, and G Almahbobi 2014 Three-Dimensional Image Analysis to Quantify the Temproro-Smacial Expression of Cellular Receptors. *Journal of Medical and Bioengineering* **Vol. 3, No. 3,** 179-182.

Albamonte, MI, MS Albamonte, I Stella, L Zuccardi, and AD Vitullo 2013 The infant and pubertal human ovary: Balbiani's body-associated VASA expression, immunohistochemical detection of apoptosis-related BCL2 and BAX proteins, and DNA fragmentation. *Human Reproduction* **28** 698-706.

Almog, B, F Shehata, E Shalom-Paz, SL Tan, and T Tulandi 2011 Age-related normogram for antral follicle count: McGill reference guide. *Fertility and Sterility* **95** 663-666.

Amsterdam, A, A Dantes, N Selvaraj, and D Aharoni 1997 Apoptosis in steroidogenic cells: structure-function analysis. *Steroids* **62** 207-211.

Amsterdam, A, RS Gold, K Hosokawa, Y Yoshida, R Sasson, Y Jung, and F Kotsuji 1999 Crosstalk Among Multiple Signaling Pathways Controlling Ovarian Cell Death. *Trends in Endocrinology & Metabolism* **10** 255-262.

Amsterdam, A, R Sasson, I Keren Tal, D Aharoni, A Dantes, E Rimon, A Land, T Cohen, Y Dor, and L Hirsh 2003 Alternative pathways of ovarian apoptosis: death for life. *Biochemical pharmacology* **66** 1355-1362.

Anand-Ivell, R, K Tremellen, Y Dai, K Heng, M Yoshida, PG Knight, GE Hale, and R Ivell 2013 Circulating insulin-like factor 3 (INSL3) in healthy and infertile women. *Human Reproduction* **28** 3093-3102.

Andersen, CY, KT Schmidt, SG Kristensen, M Rosendahl, AG Byskov, and E Ernst 2010 Concentrations of AMH and inhibin-B in relation to follicular diameter in normal human small antral follicles. *Human Reproduction* **25** 1282-1287.

Andersen, CY, LG Westergaard, Y Figenschau, K Bertheussen, and F Forsdahl 1993 Endocrine composition of follicular fluid comparing human chorionic gonadotrophin to a gonadotrophin-releasing hormone agonist for ovulation induction. *Human Reproduction* **8** 840-843.

Anzalone, CR, L-S Hong, JKH Lu, and PS LaPolt 2001 Influences of Age and Ovarian Follicular Reserve on Estrous Cycle Patterns, Ovulation, and Hormone Secretion in the Long-Evans Rat. *Biology of Reproduction* **64** 1056-1062.

Araújo, VR, Silva C.M.G., Magalhães D.M., Silva G.M., Báo S.N., Silva J.R.V., Figueiredo J.R. & Rodrigues A.P.R. 2010 Effect of Bone Morphogenetic Protein-7 (BMP-7) on in vitro survival of caprine preantral follicles. *Pesq. Vet. Bras.* **30** 305-310.

Ascoli, M, Fanelli F, and S DL. 2002 The lutropin/choriogonadotropin receptor, a 2002 perspective. *Endocrine reviews.* **Vol.23(2)** 141-174

Austin, EJ, M Mihm, ACO Evans, PG Knight, JLH Ireland, JJ Ireland, and JF Roche 2001 Alterations in Intrafollicular Regulatory Factors and Apoptosis During Selection of Follicles in the First Follicular Wave of the Bovine Estrous Cycle. *Biology of Reproduction* **64** 839-848.

Bächler, M, D Menshykau, C De Geyter, and D Iber 2014 Species-specific differences in follicular antral sizes result from diffusion-based limitations on the thickness of the granulosa cell layer. *Molecular Human Reproduction* **20** 208-221.

Baerwald, A, G Adams, and R Pierson 2012a Ovarian antral folliculogenesis during the human menstrual cycle: a review. *Human Reproduction Update* **18** 73-91.

Baerwald, AR, GP Adams, and RA Pierson 2012b Ovarian antral folliculogenesis during the human menstrual cycle: a review. *Human Reproduction Update* **18** 73-91.

Baerwald, AR, RA Walker, and RA Pierson 2009 Growth rates of ovarian follicles during natural menstrual cycles, oral contraception cycles, and ovarian stimulation cycles. *Fertility and Sterility* **91** 440-449.

Baird, D, and B Campbell 1998 Follicle selection in sheep with breed differences in ovulation rate. *Mol Cell Endocrinol* **145** 89 - 95.

Baird, DT 1987 A model for follicular selection and ovulation: lessons from superovulation. *The Journal of steroid biochemistry* **27** 15-23.

Baird, DT, TG Baker, KP McNatty, and P Neal 1975 Relationship between the secretion of the corpus luteum and the length of the follicular phase of the ovarian cycle *Journal of reproduction and fertility* **45** 611-619.

Bao, B, HA Garverick, GW Smith, MF Smith, BE Salfen, and RS Youngquist 1997 Changes in messenger ribonucleic acid encoding luteinizing

hormone receptor, cytochrome P450-side chain cleavage, and aromatase are associated with recruitment and selection of bovine ovarian follicles. *Biology of Reproduction* **56** 1158-1168.

Barreca, A, PG Artini, P Del Monte, P Ponzani, P Pasquini, G Cariola, A Volpe, AR Genazzani, G Giordano, and F Minuto 1993a In vivo and in vitro effect of growth hormone on estradiol secretion by human granulosa cells. *The Journal of clinical endocrinology and metabolism* **77** 61-67.

Barreca, A, PG Artini, P Del Monte, P Ponzani, P Pasquini, G Cariola, A Volpe, AR Genazzani, G Giordano, and F Minuto 1993b In vivo and in vitro effect of growth hormone on estradiol secretion by human granulosa cells. *Journal of Clinical Endocrinology & Metabolism* **77** 61-67.

Bergh, C, Carlström K, Selleskog U, Hillensjö T. 1996 Effect of growth hormone on follicular fluid androgen levels in patients treated with gonadotropins before in vitro fertilization. *Eur J Endocrinol* **Feb;134(2):190-6.**

Bevers, MM, and F Izadyar 2002 Role of growth hormone and growth hormone receptor in oocyte maturation. *Molecular and Cellular Endocrinology* **197** 173-178.

Bomsel-Helmreich, O, A Gougeon, A Thebault, D Saltarelli, E Milgrom, R Frydman, and E Papiernik 1979 Healthy and atretic human follicles in the preovulatory phase: differences in evolution of follicular morphology and steroid content of follicular fluid. *The Journal of clinical endocrinology and metabolism* **48** 686-694.

Bomsel Helmreich, O, A Gougeon, A Thebault, D Saltarelli, E Milgrom, R Frydman, and E Papiernik 1979 Healthy and atretic human follicles in the preovulatory phase: differences in evolution of follicular morphology and steroid content of follicular fluid. *The Journal of clinical endocrinology and metabolism* **48** 686-694.

Bonfoco, E, D Krainc, M Ankarcrona, P Nicotera, and SA Lipton 1995 Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proceedings of the National Academy of Sciences* **92** 7162-7166.

Bozzola, M, Zecca M, Locatelli F, Radetti G, Pagani S, Autelli M, Tatò L, Chatelain P 1998 Evaluation of growth hormone bioactivity using the Nb2 cell bioassay in children with growth disorders. *J Endocrinol Invest.* **Dec;21(11)** 765-770.

Brankin, V, RL Quinn, R Webb, and MG Hunter 2005 BMP-2 and -6 modulate porcine theca cell function alone and co-cultured with granulosa cells. *Domestic animal endocrinology* **29** 593-604.

Braw-Tal, R, K McNatty, P Smith, D Heath, N Hudson, D Phillips, B McLeod, and G Davis 1993 Ovaries of ewes homozygous for the X-linked Inverdale gene (FecXI) are devoid of secondary and tertiary follicles but contain many abnormal structures. *Biol Reprod* **49** 895 - 907.

Brooks, AJ, JW Wooh, KA Tunny, and MJ Waters 2008 Growth hormone receptor; mechanism of action. *The International Journal of Biochemistry & Cell Biology* **40** 1984-1989.

Byrne, B, PA Fowler, M Fraser, MD Culler, and A Templeton 1995 Gonadotropin surge-attenuating factor bioactivity in serum from

superovulated women is not blocked by inhibin antibody. *Biology of Reproduction* **52** 88-95.

Cai, J, H Lou, M Dong, X Lu, Y Zhu, H Gao, and H Huang 2007 Poor ovarian response to gonadotropin stimulation is associated with low expression of follicle-stimulating hormone receptor in granulosa cells. *Fertility and Sterility* **87** 1350-1356.

Camp, TA, JO Rahal, and KE Mayo 1991 Cellular Localization and Hormonal Regulation of Follicle-Stimulating Hormone and Luteinizing Hormone Receptor Messenger RNAs in the Rat Ovary. *Molecular Endocrinology* **5** 1405-1417.

Campbell, B, M Clinton, and R Webb 2012 The role of anti-mullerian hormone (AMH) during follicle development in a monovulatory species (sheep). *Endocrinology* **153** 4533-4543.

Campbell, B, R Scaramuzzi, and R Webb 1996 Induction and maintenance of oestradiol and immunoreactive inhibin production with FSH by ovine granulosa cells cultured in serum-free media. *J Reprod Fertil* **106** 7 - 16.

Campbell, BK, DT Baird, CJH Souza, and R Webb 2003 The FecB (Booroola) gene acts at the ovary: in vivo evidence. *Reproduction* **126** 101-111.

Campbell, BK, H Dobson, DT Baird, and RJ Scaramuzzi 1999 Examination of the relative role of FSH and LH in the mechanism of ovulatory follicle selection in sheep. *Journal of reproduction and fertility* **117** 355-367.

Campbell, BK, NR Kendall, and DT Baird 2009 Effect of Direct Ovarian Infusion of Bone Morphogenetic Protein 6 (BMP6) on Ovarian Function in Sheep. *Biology of Reproduction* **81** 1016-1023.

Campbell, BK, CJH Souza, AJ Skinner, R Webb, and DT Baird 2006 Enhanced Response of Granulosa and Theca Cells from Sheep Carriers of the FecB Mutation in Vitro to Gonadotropins and Bone Morphogenic Protein-2, -4, and -6. *Endocrinology* **147** 1608-1620.

Carlsson, B, C Bergh, J Bentham, J-H Olsson, MR Nonnan, H Billig, P Roos, and T Hillensjö 1992 Expression of functional growth hormone receptors in human granulosa cells. *Human Reproduction* **7** 1205-1209.

Caunt, CJ, AR Finch, KR Sedgley, and CA McArdle 2006 Seven-transmembrane receptor signalling and ERK compartmentalization. *Trends in Endocrinology & Metabolism* **17** 276-283.

Centurione, L, F Giampietro, S Sancilio, M Piccirilli, L Artese, GM Tiboni, and RD Pietro 2010 Morphometric and ultrastructural analysis of human granulosa cells after gonadotrophin-releasing hormone agonist or antagonist. *Reproductive BioMedicine Online* **20** 625-633.

Chaffin, CL, and RL Stouffer 2000 Role of gonadotrophins and progesterone in the regulation of morphological remodelling and atresia in the monkey peri-ovulatory follicle. *Human Reproduction* **15** 2489-2495.

Chen, AQ, S Yu, Z Wang, Z Xu, and Z Yang 2008 Stage-specific expression of bone morphogenetic protein type I and type II receptor genes: Effects of follicle-stimulating hormone on ovine antral follicles. *Animal Reproduction Science* **111** 391-399.

Chen, AQ, SD Yu, ZG Wang, ZR Xu, and ZG Yang 2009 Stage-specific expression of bone morphogenetic protein type I and type II receptor genes: Effects of follicle-stimulating hormone on ovine antral follicles. *Animal Reproduction Science* **111** 391-399.

Childs, AJ, RAL Bayne, AA Murray, SJMD Silva, CS Collins, N Spears, and RA Anderson 2010 Differential expression and regulation by activin of the neurotrophins BDNF and NT4 during human and mouse ovarian development. *Developmental Dynamics* **9999** NA.

Crawford, J, D Heath, K Reader, L Quirke, N Hudson, J Juengel, and K McNatty 2011 Oocytes in sheep homozygous for a mutation in bone morphogenetic protein receptor 1B express lower mRNA levels of bone morphogenetic protein 15 but not growth differentiation factor 9. *Reproduction* **142** 53-61.

Cummins, LJ, T O'Shea, BM Bindon, VWK Lee, and JK Findlay 1983 Ovarian inhibin content and sensitivity to inhibin in Booroola and control strain Merino ewes. *Journal of reproduction and fertility* **67** 1-7.

de Ziegler, D, I Streuli, D Meldrum, and C Chapron 2011 The value of growth hormone supplements in ART for poor ovarian responders. *Fertility and Sterility* **96** 1069-1076.

Demchenko, A 2013 Beyond annexin V: fluorescence response of cellular membranes to apoptosis. *Cytotechnology* **65** 157-172.

Di Pasquale, E, R Rossetti, A Marozzi, B Bodega, S Borgato, L Cavallo, S Einaudi, G Radetti, G Russo, M Sacco, M Wasniewska, T Cole, P Beck-Peccoz, L Nelson, and L Persani 2006 Identification of new variants of human BMP15 gene in a large cohort of women with premature ovarian failure. *The Journal of clinical endocrinology and metabolism* **91** 1976-1979.

Dijke, P, O Korchynskyi, G Valdimarsdottir, and M-J Goumans 2003 Controlling cell fate by bone morphogenetic protein receptors. *Molecular and Cellular Endocrinology* **211** 105-113.

Dimitraki, M, CI Messini, K Dafopoulos, T Gioka, N Koutlaki, A Garas, P Georgoulias, and IE Messinis 2014 Attenuating activity of the ovary on LH response to GnRH during the follicular phase of the cycle. *Clinical endocrinology* **80** 439-443.

Donadeu, F, Ascoli M. 2005 The Differential Effects of the Gonadotropin Receptors on Aromatase Expression in Primary Cultures of Immature Rat Granulosa Cells Are Highly Dependent on the Density of Receptors Expressed and the Activation of the Inositol Phosphate Cascade. *Endocrinology* **146** 3907-3916.

Dooley, CA, GR Attia, WE Rainey, DR Moore, and BR Carr 2000 Bone morphogenetic protein inhibits ovarian androgen production. *The Journal of clinical endocrinology and metabolism* **85** 3331-3337.

Driancourt, MA, LP Cahill, and BM Bindon 1985 Ovarian follicular populations and preovulatory enlargement in Booroola and control Merino ewes. *Journal of reproduction and fertility* **73** 93-107.

Edson, MA, RL Nalam, C Clementi, HL Franco, FJ DeMayo, KM Lyons, SA Pangas, and MM Matzuk 2010 Granulosa Cell-Expressed BMPR1A and BMPR1B Have Unique Functions in Regulating Fertility but Act Redundantly to Suppress Ovarian Tumor Development. *Molecular Endocrinology* **24** 1251-1266.

Edwards, RG, L R, and B P 1996 Time to revolutionize ovarian stimultion. *Human Reproduction* **11** 917-919.

Edwards, RG, and PC Steptoe 1983 Current status of in-vitro fertilisation and implantation of human embryos. *Lancet (London, England)* **2** 1265-1269.

Eftekhar, M, A Aflatoonian, F Mohammadian, and T Eftekhar 2013 Adjuvant growth hormone therapy in antagonist protocol in poor responders undergoing assisted reproductive technology. *Archives of Gynecology and Obstetrics* **287** 1017-1021.

Eppig, J 2001 Oocyte control of ovarian follicular development and function in mammals. *Reproduction* **122** 829 - 838.

Erickson, G, and S Shimasaki 2001 The physiology of folliculogenesis: the role of novel growth factors. *Fertility and Sterility* **76** 943 - 949.

Erickson, G, and S Shimasaki 2003 The spatiotemporal expression pattern of the bone morphogenetic protein family in rat ovary cell types during the estrous cycle. *Reprod Biol Endocrinol* **1** 9.

Erickson, GF, VG Garzo, and DA Magoffin 1991 Progesterone production by human granulosa cells cultured in serum free medium: effects of gonadotrophins and insulin-like growth factor I (IGF-I). *Human Reproduction* **6** 1074-1081.

Erickson, GF, C Wang, and AJW Hsueh 1979 FSH induction of functional LH receptors in granulosa cells cultured in a chemically defined medium. *Nature* **279** 336-338.

Estienne, A, A Pierre, Nd Clemente, J-Y Picard, P Jarrier, C Mansanet, D Monniaux, and S Fabre 2015 Anti-Müllerian Hormone Regulation by the Bone Morphogenetic Proteins in the Sheep Ovary: Deciphering a Direct Regulatory Pathway. *Endocrinology* **156** 301-313.

Evans, ACO 2003 Ovarian follicle growth and consequences for fertility in sheep. *Animal Reproduction Science* **78** 289-306.

Evans, ACO, JD Flynn, P Duffy, PG Knight, and MP Boland 2002 Effects of ovarian follicle ablation on FSH, oestradiol and inhibin A concentrations and growth of other follicles in sheep. *Reproduction* **123** 59-66.

Fabre, S, A Pierre, P Mulsant, L Bodin, E Di Pasquale, L Persani, P Monget, and D Monniaux 2006 Regulation of ovulation rate in mammals: contribution of sheep genetic models. *Reproductive Biology and Endocrinology* **4** 20.

Fabre, S, A Pierre, C Pisselet, P Mulsant, F Lecerf, J Pohl, P Monget, and D Monniaux 2003 The Booroola mutation in sheep is associated with an alteration of the bone morphogenetic protein receptor-IB functionality. *J Endocrinol* **177** 435 - 444.

Fan, H, Z Liu, M Shimada, E Sterneck, PF Johnson, SM Hedrick, and JS Richards 2009 MAPK3/1 (ERK1/2) in Ovarian Granulosa Cells Are Essential for Female Fertility. *Science,* **324** 938-941.

Fan, H, O'Connor, A, Shitanaka, M, Shimada, M, Liu, Z, Richards, JS 2010 β-Catenin (CTNNB1) Promotes Preovulatory Follicular Development but Represses LH-Mediated Ovulation and Luteinization. *Molecular Endocrinology* **24** 1529-1542.

Faure, MO, L Nicol, S Fabre, J Fontaine, N Mohoric, A McNeilly, and C Taragnat 2005 BMP-4 inhibits follicle-stimulating hormone secretion in ewe pituitary. *The Journal of endocrinology* **186** 109-121.

Feary, E, J Juengel, P Smith, M French, A O'Connell, S Lawrence, S Galloway, G Davis, and K McNatty 2007 Patterns of expression of messenger RNAs encoding GDF9, BMP15, TGFBR1, BMPR1B, and BMPR2 during follicular development and characterization of ovarian follicular populations in

ewes carrying the Woodlands FecX2W mutation. *Biology of Reproduction* **77** 990-998.

Fitzpatrick, SL, DL Carlone, RL Robker, and JS Richards 1997 Expression of aromatase in the ovary: Down-regulation of mRNA by the ovulatory luteinizing hormone surge. *Steroids* **62** 197-206.

Folch, J, JP RamÃ³n, MJ Cocero, JL Alabart, and JF Beckers 2001 Exogenous growth hormone improves the number of transferable embryos in superovulated ewes. *Theriogenology* **55** 1777-1785.

Fowler, PA, T Sorsa, WJ Harris, PG Knight, and HD Mason 2001 Relationship between follicle size and gonadotrophin surge attenuating factor (GnSAF) bioactivity during spontaneous cycles in women. *Human Reproduction* **16** 1353-1358.

Foxcroft, G, and M Hunter 1985 Basic physiology of follicular maturation in the pig. *Journal of reproduction and fertility* **Suppl 33** 1 - 19.

Fricke, P, J Ford, L Reynolds, and D Redmer 1996 Growth and cellular proliferation of antral follicles throughout the follicular phase of the estrous cycle in Meishan gilts. *Biol Reprod* **54** 879 - 887.

Fry, RC, IJ Clarke, JT Cummins, BM Bindon, LR Piper, and LP Cahill 1988 Induction of ovulation in chronically hypophysectomized Booroola ewes. *Journal of reproduction and fertility* **82** 711-715.

Gao, S, C De Geyter, K Kossowska, and H Zhang 2007 FSH stimulates the expression of the ADAMTS-16 protease in mature human ovarian follicles. *Molecular Human Reproduction* **13** 465-471.

Garcia, J, GS Jones, AA Acosta, and GL Wright 1981 Corpus luteum function after follicle aspiration for oocyte retrieval. *Fertility and Sterility* **36** 565-572.

Garverick, H, G Baxter, J Gong, D Armstrong, B Campbell, C Gutierrez, and R Webb 2002 Regulation of expression of ovarian mRNA encoding steroidogenic enzymes and gonadotrophin receptors by FSH and GH in hypogonadotrophic cattle. *Reproduction* **123** 651-661.

Gasperin, BG, R Ferreira, MT Rovani, V Bordignon, R Duggavathi, J Buratini, JFC Oliveira, and PBD Gonçalves 2014 Expression of receptors for BMP15 is differentially regulated in dominant and subordinate follicles during follicle deviation in cattle. *Animal Reproduction Science* **144** 72-78.

Giampietro, F, S Sancilio, GM Tiboni, RA Rana, and R Di Pietro 2006 Levels of apoptosis in human granulosa cells seem to be comparable after therapy with a gonadotropin-releasing hormone agonist or antagonist. *Fertility and Sterility* **85** 412-419.

Ginther, OJ, MA Beg, DR Bergfelt, FX Donadeu, and K Kot 2001 Follicle selection in monovular species. *Biology of Reproduction* **65** 638-647.

Ginther, OJ, MA Beg, EL Gastal, MO Gastal, AR Baerwald, and RA Pierson 2005 Systemic concentrations of hormones during the development of follicular waves in mares and women: a comparative study. *Reproduction* **130** 379-388.

Ginther, OJ, MO Gastal, EL Gastal, JC Jacob, MAR Siddiqui, and MA Beg 2008 Effects of age on follicle and hormone dynamics during the oestrous cycle in mares. *Reproduction, Fertility and Development* **20** 955-963.

Ginther, OJ, FA Khan, MA Hannan, MB Rodriguez, G Pugliesi, and MA Beg 2012 Role of LH in luteolysis and growth of the ovulatory follicle and

estradiol regulation of LH secretion in heifers. *Theriogenology* **77** 1442-1452.

Glister, C, C Kemp, and P Knight 2004a Bone morphogenetic protein (BMP) ligands and receptors in bovine ovarian follicle cells: actions of BMP-4, -6 and 7 on granulosa cells and differential modulation of Smad-1 phosphorylation by follistatin. *Reproduction* **127** 239 - 254.

Glister, C, CF Kemp, and PG Knight 2004b Bone morphogenetic protein (BMP) ligands and receptors in bovine ovarian follicle cells: actions of BMP-4, -6 and -7 on granulosa cells and differential modulation of Smad-1 phosphorylation by follistatin. *Reproduction* **127** 239-254.

Glister, C, DS Tannetta, NP Groome, and PG Knight 2001 Interactions Between Follicle-Stimulating Hormone and Growth Factors in Modulating Secretion of Steroids and Inhibin-Related Peptides by Nonluteinized Bovine Granulosa Cells. *Biology of Reproduction* **65** 1020-1028.

Gong, JG, T Bramley, and R Webb 1991 The effect of recombinant bovine somatotropin on ovarian function in heifers: follicular populations and peripheral hormones. *Biology of Reproduction* **45** 941-949.

Gonzalez Bulnes, A, CJH Souza, B Campbell, and D Baird 2004 Effect of ageing on hormone secretion and follicular dynamics in sheep with and without the Booroola gene. *Endocrinology* **145** 2858-2864.

Gordon, A, R Aguilar, JC Garrido-Gracia, C Bellido, Y Millán, S Guil-Luna, JA García-Velasco, E Bellido-Muñoz, JM de las Mulas, and JE Sánchez-Criado 2010 Human follicular fluid from superovulated women inhibits progesterone receptor-dependent gonadotropin-releasing hormone self-priming in an estrous cycle-dependent manner in the rat. *Journal of Endocrinological Investigation* **33** 564-570.

Gordon, A, JC Garrido-Gracia, R Aguilar, C Bellido, JAG Velasco, Y Millan, M Tena-Sempere, J Martín de las Mulas, and JE Sánchez-Criado 2008 The ovary-mediated FSH attenuation of the LH surge in the rat involves a decreased gonadotroph progesterone receptor (PR) action but not PR expression. *Journal of Endocrinology* **196** 583-592.

Gougeon, A 1986 Dynamics of follicular growth in the human: a model from preliminary results. *Hum. Reprod.* **1** 81-87.

Grande, M, V Borobio, JM Jimenez, M Bennasar, I Stergiotou, J Peñarrubia, and A Borrell 2014 Antral follicle count as a marker of ovarian biological age to reflect the background risk of fetal aneuploidy. *Human Reproduction* **29** 1337-1343.

Greisen, S, T Ledet, and P Ovesen 2001 Effects of androstenedione, insulin and luteinizing hormone on steroidogenesis in human granulosa luteal cells. *Human Reproduction* **16** 2061-2065.

Grimes, RW, P Matton, and JJ Ireland 1987 A comparison of histological and non-histological indices of atresia and follicular function. *Biology of Reproduction* **37** 82-88.

Guigon , C, Mazaud S, Forest MG, Brailly-Tabard S, Coudouel N, Magre S 2003 Unaltered Development of the Initial Follicular Waves and Normal Pubertal Onset in Female Rats after Neonatal Deletion of the Follicular Reserve. *Endocrinology* **144** 3651-3662.

Guraya, S 1971 Morphology, histochemistry, and biochemistry of human ovarian compartments and steroid hormone synthesis. *Physiological Reviews* **4** 786-807.

Guraya, SS 1971 Morphology, histochemistry, and biochemistry of human ovarian compartments and steroid hormone synthesis.

Gutierrez, C, B Campbell, and R Webb 1997 Development of a long-term bovine granulosa cell culture system: induction and maintenance of estradiol production, response to follicle-stimulating hormone, and morphological characteristics. *Biology of Reproduction* **56** 608 - 616.

Haÿ, E, J Lemonnier, O Fromigué, H Guénou, and PJ Marie 2004 Bone Morphogenetic Protein Receptor IB Signaling Mediates Apoptosis Independently of Differentiation in Osteoblastic Cells. *Journal of Biological Chemistry* **279** 1650-1658.

Hadlow, N, K Longhurst, A McClements, J Natalwala, SJ Brown, and PL Matson 2013 Variation in antimüllerian hormone concentration during the menstrual cycle may change the clinical classification of the ovarian response. *Fertility and Sterility* **99** 1791-1797.

Hale, G, Zhao, X, Hughes, CL, BurgeR, HG, Robertson, DM, Fraser, IS 2007 Endocrine Features of Menstrual Cycles in Middle and Late Reproductive Age and the Menopausal Transition Classified According to the Staging of Reproductive Aging Workshop (STRAW) Staging System. *The Journal of Clinical Endocrinology & Metabolism* **92** 3060-3067.

Hampton, JH, JF Bader, WR Lamberson, MF Smith, RS Youngquist, and HA Garverick 2004 Gonadotropin requirements for dominant follicle selection in GnRH agonist-treated cows. *Reproduction* **127** 695–703.

Hanrahan, J, S Gregan, P Mulsant, M Mullen, G Davis, R Powell, and S Galloway 2004 Mutations in the genes for oocyte-derived growth factors GDF9 and BMP15 are associated with both increased ovulation rate and sterility in Cambridge and Belclare sheep (Ovis aries). *Biol Reprod* **70** 900 - 909.

Hansen, KR, GM Hodnett, N Knowlton, and LB Craig 2011 Correlation of ovarian reserve tests with histologically determined primordial follicle number. *Fertility and Sterility* **95** 170-175.

Hansen, KR, AC Thyer, PM Sluss, WJ Bremner, MR Soules, and NA Klein 2005 Reproductive ageing and ovarian function: is the early follicular phase FSH rise necessary to maintain adequate secretory function in older ovulatory women? *Human Reproduction* **20** 89-95.

Harris, D, D Bonfil, D CHuderland, S Kraus, R Seger, and Z Naor 2002 Activation of MAPK Cascades by GnRH: ERK and Jun N-Terminal Kinase Are Involved in Basal and GnRH-Stimulated Activity of the Glycoprotein Hormone LHβ-Subunit Promoter. *Endocrinology* **143** 1018-1025.

Henderson, KM, LE Kieboom, KP McNatty, S Lun, and D Heath 1985 Gonadotrophin-stimulated cyclic AMP production by granulosa cells from Booroola x Romney ewes with and without a fecundity gene. *Journal of reproduction and fertility* **75** 111-120.

Hermann, BP, and LL Heckert 2007 Transcriptional regulation of the FSH receptor: New perspectives. *Molecular and Cellular Endocrinology* **260–262** 100-108.

Hillier, S, P Whitelaw, and C Smyth 1994a Follicular oestrogen synthesis: the 'two-cell, two-gonadotrophin' model revisited. *Mol Cell Endocrinol* **100** 51 - 54.

Hillier, SG 2001 Gonadotropic control of ovarian follicular growth and development. *Molecular and Cellular Endocrinology* **179** 39-46.

Hillier, SG, PF Whitelaw, and CD Smyth 1994b Follicular oestrogen synthesis: the 'two-cell, two-gonadotrophin' model revisited. *Molecular and Cellular Endocrinology* **100** 51-54.

Homburg, R, A Eshel, HI Abdalla, and HS Jacobs 1988 GROWTH HORMONE FACILITATES OVULATION INDUCTION BY GONADOTROPHINS. *Clinical endocrinology* **29** 113-117.

Homburg, R, A Singh, P Bhide, A Shah, and A Gudi 2012 The re-growth of growth hormone in fertility treatment: a critical review. *Human fertility* **15** 190-193.

Hosokawa, K, D Aharoni, A Dantes, E Shaulian, C Schere-Levy, R Atzmon, F Kotsuji, M Oren, I Vlodavsky, and A Amsterdam 1998 Modulation of Mdm2 Expression and p53-Induced Apoptosis in Immortalized Human Ovarian Granulosa Cells. *Endocrinology* **139** 4688-4700.

Hudson, NL, AR O'Connell, L Shaw, IJ Clarke, and KP McNatty 1999 Effect of exogenous FSH on ovulation rate in homozygous carriers or noncarriers of the Booroola FecB gene after hypothalamic-pituitary disconnection or after treatment with a GnRH agonist. *Domestic animal endocrinology* **16** 69-80.

Hussein, T, D Froiland, F Amato, J Thompson, and R Gilchrist 2005 Oocytes prevent cumulus cell apoptosis by maintaining a morphogenic paracrine gradient of bone morphogenetic proteins. *Journal of cell science* **118** 5257-5268.

Hutchinson, LA, JK Findlay, and AC Herington 1988 Growth hormone and insulin-like growth factor-I accelerate PMSG-induced differentiation of granulosa cells. *Molecular and Cellular Endocrinology* **55** 61-69.

Inagaki, K, F Otsuka, T Miyoshi, M Yamashita, M Takahashi, J Goto, J Suzuki, and H Makino 2009 p38-Mitogen-Activated Protein Kinase Stimulated Steroidogenesis in Granulosa Cell-Oocyte Cocultures: Role of Bone Morphogenetic Proteins 2 and 4. *Endocrinology* **150** 1921-1930.

Inagaki, K, and S Shimasaki 2010 Impaired production of BMP-15 and GDF-9 mature proteins derived from proproteins WITH mutations in the proregion. *Molecular and Cellular Endocrinology* **328** 1-7.

Ingraham, HA, DS Lala, Y Ikeda, X Luo, WH Shen, MW Nachtigal, R Abbud, JH Nilson, and KL Parker 1994 The nuclear receptor steroidogenic factor 1 acts at multiple levels of the reproductive axis. *Genes & development* **8** 2302-2312.

Irving-Rodgers, HF, ML Harland, TR Sullivan, and RJ Rodgers 2009 Studies of granulosa cell maturation in dominant and subordinate bovine follicles: novel extracellular matrix focimatrix is co-ordinately regulated with cholesterol side-chain cleavage CYP11A1. *Reproduction* **137** 825-834.

Irving-Rodgers, HF, M Krupa, and RJ Rodgers 2003 Cholesterol Side-Chain Cleavage Cytochrome P450 and 3β-Hydroxysteroid Dehydrogenase Expression and the Concentrations of Steroid Hormones in the Follicular Fluids of Different Phenotypes of Healthy and Atretic Bovine Ovarian Follicles. *Biology of Reproduction* **69** 2022-2028.

Izadyar, F, B Colenbrander, and MM Bevers 1996 In vitro maturation of bovine oocytes in the presence of growth hormone accelerates nuclear maturation and promotes subsequent embryonic development. *Molecular reproduction and development* **45** 372-377.

Izadyar, F, B Colenbrander, and MM Bevers 1997a Stimulatory effect of growth hormone on in vitro maturation of bovine oocytes is exerted through the cyclic adenosine 3',5'-monophosphate signaling pathway. *Biology of Reproduction* **57** 1484-1489.

Izadyar, F, WJ Hage, B Colenbrander, and MM Bevers 1998a The promotory effect of growth hormone on the developmental competence of in vitro matured bovine oocytes is due to improved cytoplasmic maturation. *Molecular reproduction and development* **49** 444-453.

Izadyar, F, HT Van Tol, B Colenbrander, and MM Bevers 1997b Stimulatory effect of growth hormone on in vitro maturation of bovine oocytes is exerted through cumulus cells and not mediated by IGF-I. *Molecular reproduction and development* **47** 175-180.

Izadyar, F, E Zeinstra, and MM Bevers 1998b Follicle-stimulating hormone and growth hormone act differently on nuclear maturation while both enhance developmental competence of in vitro matured bovine oocytes. *Molecular reproduction and development* **51** 339-345.

Izadyar, F, J Zhao, HT Van Tol, B Colenbrander, and MM Bevers 1999 Messenger RNA expression and protein localization of growth hormone in bovine ovarian tissue and in cumulus oocyte complexes (COCs) during in vitro maturation. *Molecular reproduction and development* **53** 398-406.

Jakimiuk, AJ, SR Weitsman, A Navab, and DA Magoffin 2001 Luteinizing Hormone Receptor, Steroidogenesis Acute Regulatory Protein, and Steroidogenic Enzyme Messenger Ribonucleic Acids Are Overexpressed in Thecal and Granulosa Cells from Polycystic Ovaries. *Journal of Clinical Endocrinology & Metabolism* **86** 1318-1323.

Jakimiuk, AJ, SR Weitsman, H-W Yen, M Bogusiewicz, and DA Magoffin 2002 Estrogen Receptor α and β Expression in Theca and Granulosa Cells from Women with Polycystic Ovary Syndrome. *Journal of Clinical Endocrinology & Metabolism* **87** 5532-5538.

Jeppesen, JV, SG Kristensen, ME Nielsen, P Humaidan, M Dal Canto, R Fadini, KT Schmidt, E Ernst, and C Yding Andersen 2012 LH-Receptor Gene Expression in Human Granulosa and Cumulus Cells from Antral and Preovulatory Follicles. *The Journal of Clinical Endocrinology & Metabolism* **97** E1524-E1531.

Jia, C-l, N Li, Z-h Wei, X-p Zhu, H-y Liu, and Z-h Jia 2007 Study on FSHR and LHR mRNA Levels of Different BMPRIB Genotypes of Small Tail Han Sheep During the Oestrum. *Agricultural Sciences in China* **6** 94-99.

Jia, X-C, J Kalmijn, and AJW Hsueh 1986 Growth Hormone Enhances Follicle-Stimulating Hormone-Induced Differentiation of Cultured Rat Granulosa Cells. *Endocrinology* **118** 1401-1409.

Josso, N, and Nd Clemente 2003 Transduction pathway of anti-Müllerian hormone, a sex-specific member of the TGF-β family. *Trends in Endocrinology & Metabolism* **14** 91-97.

Juengel, J, N Hudson, L Whiting, and K McNatty 2004 Effects of immunization against bone morphogenetic protein 15 and growth differentiation factor 9 on ovulation rate, fertilization, and pregnancy in ewes. *Biol Reprod* **70** 557 - 561.

Juengel, J, L Quirke, S Lun, D Heath, P Johnstone, and K McNatty 2011 Effects of immunizing ewes against bone morphogenetic protein 15 on their

responses to exogenous gonadotrophins to induce multiple ovulations. *Reproduction* **142** 565-572.

Kang, S, Tai CJ, Nathwani PS, Choi KC, Leung PC 2001 Stimulation of Mitogen-Activated Protein Kinase by Gonadotropin-Releasing Hormone in Human Granulosa-Luteal Cells. *Endocrinology* **142** 671-679.

Karamouti, M, Kollia, Panagoula, Kallitsaris Athanasios, Vamvakopoulos Nikolaos, Kollios Georgios and. Messinis, Ioannis E 2008 Growth hormone, insulin-like growth factor I, and leptin interaction in human cultured lutein granulosa cells steroidogenesis. *Fertility and Sterility* **Vol. 90, Suppl 2,** 1444-1450.

Kayamori, T, N Kosaka, A Miyamoto, and T Shimizu 2009 The differential pathways of bone morphogenetic protein (BMP)-4 and -7 in the suppression of the bovine granulosa cell apoptosis. *Molecular and cellular biochemistry* **323** 161-168.

Kayani, AR, C Glister, and PG Knight 2009 Evidence for an inhibitory role of bone morphogenetic protein(s) in the follicular–luteal transition in cattle. *Reproduction* **137** 67-78.

Khalaf, M, J Morera, A Bourret, Y Reznik, C Denoual, M Herlicoviez, H Mittre, and A Benhaim 2013 BMP system expression in GCs from polycystic ovary syndrome women and the in vitro effects of BMP4, BMP6, and BMP7 on GC steroidogenesis. *European journal of endocrinology* **168** 437-444.

Kita, M, S Taii, N Kataoka, A Shimatsu, K Nakao, and T Mori 1994 Changes of gonadotrophin surge inhibiting/attenuating factor activity in pig follicular fluid in relation to follicle size. *Journal of reproduction and fertility* **101** 59-66.

Klein N A, BD E, FV Y, DG S, BW J, and SM R 1996 Reproductive aging: accelerated ovarian follicular development associated with a monotropic follicle-stimulating hormone rise in normal older women. *The Journal of Clinical Endocrinology & Metabolism* **81** 1038-1045.

Klein, NA, DE Battaglia, TK Woodruff, V Padmanabhan, LC Giudice, WJ Bremner, and MR Soules 2000 Ovarian Follicular Concentrations of Activin, Follistatin, Inhibin, Insulin-Like Growth Factor I (IGF-I), IGF-II, IGF-Binding Protein-2 (IGFBP-2), IGFBP-3, and Vascular Endothelial Growth Factor in Spontaneous Menstrual Cycles of Normal Women of Advanced Reproductive Age. *The Journal of Clinical Endocrinology & Metabolism* **85** 4520-4525.

Knight, P, and C Glister 2001 Potential local regulatory functions of inhibins, activins and follistatin in the ovary. *Reproduction* **121** 503-512.

Knight, PG, and C Glister 2003 Local roles of TGF-β superfamily members in the control of ovarian follicle development. *Animal Reproduction Science* **78** 165-183.

Knight, PG, and C Glister 2006 TGF-β superfamily members and ovarian follicle development. *Reproduction* **132** 191-206.

Knight, PG, L Satchell, and C Glister 2012 Intra-ovarian roles of activins and inhibins. *Molecular and Cellular Endocrinology* **359** 53-65.

Kölle, S, F Sinowatz, G Boie, and D Lincoln 1998 Developmental Changes in the Expression of the Growth Hormone Receptor Messenger Ribonucleic Acid and Protein in the Bovine Ovary. *Biology of Reproduction* **59** 836-842.

Komar, CM, AK Berndtson, ACO Evans, and JE Fortune 2001 Decline in Circulating Estradiol During the Periovulatory Period Is Correlated with Decreases in Estradiol and Androgen, and in Messenger RNA for P450 Aromatase and P450 17α-Hydroxylase, in Bovine Preovulatory Follicles. *Biology of Reproduction* **64** 1797-1805.

Kucuk, T, H Kozinoglu, and A Kaba 2008 Growth hormone co-treatment within a GnRH agonist long protocol in patients with poor ovarian response: a prospective, randomized, clinical trial. *Journal of Assisted Reproduction and Genetics* **25** 123-127.

Kyrou, D, E Kolibianakis, C Venetis, E Papanikolaou, J Bontis, and B Tarlatzis 2009 How to improve the probability of pregnancy in poor responders undergoing in vitro fertilization: a systematic review and meta-analysis. *Fertility and Sterility* **91** 749-766.

Lan, H, W Li, Z Fu, Y Yang, T Wu, Y Liu, H Zhang, H Cui, Y Li, P Hong, J Liu, and X Zheng 2014 Differential intracellular signalling properties of the growth hormone receptor induced by the activation of an anti-GHR antibody. *Molecular and Cellular Endocrinology* **390** 54-64.

LaPolt, PS, and JKH Lu 2001 Effects of Aging on Luteinizing Hormone Secretion, Ovulation, and Ovarian Tissue-Type Plasminogen Activator Expression. *Experimental biology and medicine* **226** 127-132.

Lapolt, PS, DW Matt, HL Judd, and JK Lu 1986 The relation of ovarian steroid levels in young female rats to subsequent estrous cyclicity and reproductive function during aging. *Biology of Reproduction* **35** 1131-1139.

Lapolt, PS, M Oikawa, X-C Jia, C Dargan, and AJW Hsueh 1990 Gonadotropin-induced up- and down-regulation of rat ovarian LH receptor message levels during follicular growth, ovulation and luteinization. *Endocrinology* **126** 3277-3279.

LaPolt, PS, JL Tilly, T Aihara, K Nishimori, and AJ Hsueh 1992 Gonadotropin-induced up- and down-regulation of ovarian follicle-stimulating hormone (FSH) receptor gene expression in immature rats: effects of pregnant mare's serum gonadotropin, human chorionic gonadotropin, and recombinant FSH. *Endocrinology* **130** 1289-1295.

Lawson, KA, NR Dunn, BA Roelen, LM Zeinstra, AM Davis, CV Wright, JP Korving, and BL Hogan 1999 Bmp4 is required for the generation of primordial germ cells in the mouse embryo. *Genes & development* **13** 424-436.

Le Roith, D, C Bondy, S Yakar, J-L Liu, and A Butler 2001 The Somatomedin Hypothesis: 2001. *Endocrine reviews* **22** 53-74.

Leach, RE, KS Moghissi, JF Randolph, NE Reame, CM Blacker, KA Ginsburg, and MP Diamond 1997 Intensive hormone monitoring in women with unexplained infertility: evidence for subtle abnormalities suggestive of diminished ovarian reserve. *Fertility and Sterility* **68** 413-420.

Lee, W, F Otsuka, R Moore, and S Shimasaki 2001 Effect of bone morphogenetic protein-7 on folliculogenesis and ovulation in the rat. *Biol Reprod* **65** 994 - 999.

Legro, RS, BA Ary, RJ Paulson, FZ Stanczyk, and MV Sauer 1993 Pregnancy: Premature luteinization as detected by elevated serum progesterone is associated with a higher pregnancy rate in donor oocyte in-vitro fertilization. *Human Reproduction* **8** 1506-1511.

Levy, T, R Limor, Y Villa, A Eshel, N Eckstein, I Vagman, A Lidor, and D Ayalon 1993 Another look at co-treatment with growth hormone and human menopausal gonadotrophins in poor ovarian responders. *Human Reproduction* **8** 834-839.

Li, H, PM Bartold, CZ Zhang, RW Clarkson, WG Young, and MJ Waters 1998 Growth Hormone and Insulin-Like Growth Factor I Induce Bone Morphogenetic Proteins 2 and 4: A Mediator Role in Bone and Tooth Formation? *Endocrinology* **139** 3855-3862.

Li, Y, R-Q Li, S-B Ou, N-F Zhang, L Reng, L-N Wei, Q-X Zhang, and D-Z Yang 2014 Increased GDF9 and BMP15 mRNA levels in cumulus granulosa cells correlate with oocyte maturation, fertilization, and embryo quality in humans. *Reproductive Biology and Endocrinology* **12** 81.

Liang, C, Su YQ, Fan HY, Schatten H, Sun QY 2007 Mechanisms Regulating Oocyte Meiotic Resumption: Roles of Mitogen-Activated Protein Kinase. *Molecular Endocrinology* **21** 2037-2055.

Loumaye, E, P Engrand, Z Shoham, SG Hillier, and DT Baird 2003 Clinical evidence for an LH ceiling? *Human Reproduction* **18** 2719-2720.

Lu, DL, H Peegel, SM Mosier, and KM Menon 1993 Loss of lutropin/human choriogonadotropin receptor messenger ribonucleic acid during ligand-induced down-regulation occurs post transcriptionally. *Endocrinology* **132** 235-240.

Luo, W, A Gumen, J Haughian, and M Wiltbank 2011 The role of luteinizing hormone in regulating gene expression during selection of a dominant follicle in cattle. *Biology of Reproduction* **84** 369-378.

MacNaughton, J, M Banah, P McCloud, J Hee, and H Burger 1992 Age related changes in follicle stimulating hormone, luteinizing hormone, oestradiol and immunoreactive inhibin in women of reproductive age. *Clinical endocrinology* **36** 339-345.

Maman, E, Y Yung, A Kedem, GM Yerushalmi, S Konopnicki, B Cohen, J Dor, and A Hourvitz 2012 High expression of luteinizing hormone receptors messenger RNA by human cumulus granulosa cells is in correlation with decreased fertilization. *Fertility and Sterility* **97** 592-598.

Manna, P, M Dyson, D Eubank, B Clark, E Lalli, P Sassone Corsi, A Zeleznik, and D Stocco 2002 Regulation of steroidogenesis and the steroidogenic acute regulatory protein by a member of the cAMP response-element binding protein family. *Molecular Endocrinology* **16** 184-199.

Martinez, F, PN Barri, B Coroleu, R Tur, T Sorsa-Leslie, WJ Harris, NP Groome, PG Knight, and PA Fowler 2002 Women with poor response to IVF have lowered circulating gonadotrophin surge-attenuating factor (GnSAF) bioactivity during spontaneous and stimulated cycles. *Human Reproduction* **17** 634-640.

Mason, HD, H Martikainen, RW Beard, V Anyaoku, and S Franks 1990 Direct gonadotrophic effect of growth hormone on oestradiol production by human granulosa cells in vitro. *Journal of Endocrinology* **126** R1-R4.

Matikainen, TM, T Moriyama, Y Morita, GI Perez, SJ Korsmeyer, DH Sherr, and JL Tilly 2002 Ligand Activation of the Aromatic Hydrocarbon Receptor Transcription Factor Drives Bax-Dependent Apoptosis in Developing Fetal Ovarian Germ Cells. *Endocrinology* **143** 615-620.

Matsuda, F, N Inoue, N Manabe, and S Ohkura 2012 Follicular growth and atresia in mammalian ovaries: regulation by survival and death of granulosa cells. *Journal of Reproduction and Development* **58** 44-50.

Mazerbourg, S, and AJW Hsueh 2006 Genomic analyses facilitate identification of receptors and signalling pathways for growth differentiation factor 9 and related orphan bone morphogenetic protein/growth differentiation factor ligands. *Human Reproduction Update* **12** 373-383.

McNatty, K, D Heath, N Hudson, S Lun, J Juengel, and L Moore 2009 Gonadotrophin-responsiveness of granulosa cells from bone morphogenetic protein 15 heterozygous mutant sheep. *Reproduction* **138** 545-551.

McNatty, K, and K Henderson 1987a Gonadotrophins, fecundity genes and ovarian follicular function. *J Steroid Biochem* **27** 365 - 373.

McNatty, K, S Lun, D Heath, K Ball, P Smith, N Hudson, J McDiarmid, M Gibb, and K Henderson 1986a Differences in ovarian activity between Booroola Merino ewes which were homozygous, heterozygous and non-carriers of a major gene influencing their ovulation rate. *J Reprod Fertil* **77** 193 - 205.

McNatty, KP, and KM Henderson 1987b Gonadotrophins, fecundity genes and ovarian follicular function. *The Journal of steroid biochemistry* **27** 365-373.

McNatty, KP, KM Henderson, S Lun, DA Heath, K Ball, NL Hudson, J Fannin, M Gibb, LE Kieboom, and P Smith 1985 Ovarian activity in Booroola X Romney ewes which have a major gene influencing their ovulation rate. *Journal of reproduction and fertility* **73** 109-120.

McNatty, KP, NL Hudson, L Whiting, KL Reader, S Lun, A Western, DA Heath, P Smith, LG Moore, and JL Juengel 2007 The Effects of Immunizing Sheep with Different BMP15 or GDF9 Peptide Sequences on Ovarian Follicular Activity and Ovulation Rate. *Biology of Reproduction* **76** 552-560.

McNatty, KP, WM Hunter, MA S., and RS Sawers 1975 Changes in the concentration of pituitary and steroid hormones in the follicular fluid of human graafian follicles throughout the menstrual cycle. *Journal of Endocrinology* **64** 555-571.

McNatty, KP, LE O'Keeffe, KM Henderson, DA Heath, and S Lun 1986b 125I-labelled hCG binding characteristics in theca interna and other tissues from Romney ewes and from Booroola × Romney ewes with and without a major gene influencing their ovulation rate. *Journal of reproduction and fertility* **77** 477-488.

Messinis, IE, P Hirsch, and AA Templeton 1991 Follicle stimulating hormone stimulates the production of gonadotrophin surge attenuating factor (GnSAF) in vivo. *Clinical endocrinology* **35** 403-407.

Micevych, P, K Sinchak, RH Mills, L Tao, P LaPolt, and JK Lu 2003 The Luteinizing Hormone Surge Is Preceded by an Estrogen-Induced Increase of Hypothalamic Progesterone in Ovariectomized and Adrenalectomized Rats. *Neuroendocrinology* **78** 29-35.

Mihm, M, PJ Baker, JLH Ireland, GW Smith, PM Coussens, ACO Evans, and JJ Ireland 2006 Molecular Evidence That Growth of Dominant Follicles Involves a Reduction in Follicle-Stimulating Hormone Dependence and an

Increase in Luteinizing Hormone Dependence in Cattle. *Biology of Reproduction* **74** 1051-1059.

Mihm, M, and A Evans 2008a Mechanisms for Dominant Follicle Selection in Monovulatory Species: A Comparison of Morphological, Endocrine and Intraovarian Events in Cows, Mares and Women
Reproduction in domestic animals **43** 48-56.

Mihm, M, and ACO Evans 2008b Mechanisms for Dominant Follicle Selection in Monovulatory Species: A Comparison of Morphological, Endocrine and Intraovarian Events in Cows, Mares and Women. *Reproduction in domestic animals* **43** 48-56.

Miller, W, and H Bose 2011 Early steps in steroidogenesis: intracellular cholesterol trafficking. *Journal of lipid research* **52** 2111-2135.

Miller, WLaA, Richard J 2011 The Molecular Biology, Biochemistry, and Physiology of Human Steroidogenesis and Its Disorders. *Endocrine Reviews,* **32(1):** 81–151.

Minegishi, T, M Tano, Y Abe, K Nakamura, Y Ibuki, and K Miyamoto 1997 Expression of luteinizing hormone/human chorionic gonadotrophin (LH/HCG) receptor mRNA in the human ovary. *Molecular Human Reproduction* **3** 101-107.

Miyazono, K, Y Kamiya, and M Morikawa 2010 Bone morphogenetic protein receptors and signal transduction. *Journal of Biochemistry* **147** 35-51.

Miyazono, K, S Maeda, and T Imamura 2005 BMP receptor signaling: transcriptional targets, regulation of signals, and signaling cross-talk. *Cytokine Growth Factor Rev* **6** 251 - 263.

Miyoshi, T, F Otsuka, K Inagaki, H Otani, M Takeda, J Suzuki, J Goto, T Ogura, and H Makino 2007 Differential Regulation of Steroidogenesis by Bone Morphogenetic Proteins in Granulosa Cells: Involvement of Extracellularly Regulated Kinase Signaling and Oocyte Actions in Follicle-Stimulating Hormone-Induced Estrogen Production. *Endocrinology* **148** 337-345.

Miyoshi, T, F Otsuka, J Suzuki, M Takeda, K Inagaki, Y Kano, H Otani, Y Mimura, T Ogura, and H Makino 2006 Mutual regulation of follicle-stimulating hormone signaling and bone morphogenetic protein system in human granulosa cells. *Biology of Reproduction* **74** 1073-1082.

Monniaux, D, F Clément, R Dalbiès-Tran, A Estienne, S Fabre, C Mansanet, and P Monget 2014 The Ovarian Reserve of Primordial Follicles and the Dynamic Reserve of Antral Growing Follicles: What Is the Link? *Biology of Reproduction* **90** 85, 81-11.

Moore, R, F Otsuka, and S Shimasaki 2001a Role of ERK1/2 in the differential synthesis of progesterone and estradiol by granulosa cells. *Biochem Biophys Res Commun* **289** 796 - 800.

Moore, RK, F Otsuka, and S Shimasaki 2001b Role of ERK1/2 in the differential synthesis of progesterone and estradiol by granulosa cells. *Biochemical and biophysical research communications* **289** 796-800.

Moore, RK, F Otsuka, and S Shimasaki 2003 Molecular basis of bone morphogenetic protein-15 signaling in granulosa cells. *Journal of Biological Chemistry* **278** 304-310.

Motta, PM 1969 Electron microscope study on the human lutein cell with special reference to its secretory activity. *Z Zeltforsch Mikrosk Anat* **98** 233-245.

Mulsant, P, F Lecerf, S Fabre, L Schibler, P Monget, I Lanneluc, C Pisselet, J Riquet, D Monniaux, I Callebaut, E Cribiu, J Thimonier, J Teyssier, L Bodin, Y Cognié, N Chitour, and J-M Elsen 2001 Mutation in bone morphogenetic protein receptor-IB is associated with increased ovulation rate in Booroola Mérino ewes. *Proceedings of the National Academy of Sciences of the United States of America* **98** 5104-5109.

Nair, AK, H Peegel, and KMJ Menon 2006 The Role of Luteinizing Hormone/Human Chorionic Gonadotropin Receptor-Specific mRNA Binding Protein in Regulating Receptor Expression in Human Ovarian Granulosa Cells. *The Journal of Clinical Endocrinology & Metabolism* **91** 2239-2243.

Nakamura, E, F Otsuka, K Inagaki, T Miyoshi, Y Matsumoto, K Ogura, N Tsukamoto, M Takeda, and H Makino 2012 Mutual Regulation of Growth Hormone and Bone Morphogenetic Protein System in Steroidogenesis by Rat Granulosa Cells. *Endocrinology* **153** 469-480.

Nakamura, K, T Minegishi, Y Takakura, K Miyamoto, Y Hasegawa, Y Ibuki, and M Igarashi 1991 Hormonal regulation of gonadotropin receptor mRNA in rat ovary during follicular growth and luteinization. *Molecular and Cellular Endocrinology* **82** 259-263.

Ndiaye, K, T Fayad, DW Silversides, J Sirois, and JG Lussier 2005 Identification of Downregulated Messenger RNAs in Bovine Granulosa Cells of Dominant Follicles Following Stimulation with Human Chorionic Gonadotropin. *Biology of Reproduction* **73** 324-333.

Negoescu, A, Lorimier P, Labat-Moleur F, Drouet C, Robert C, Guillermet C, Brambilla C, and Brambilla E 1996 In Situ Apoptotic Cell Labeling by the TUNEL Method: Improvement and Evaluation on Cell Preparations *The Journal of Histochemistry and Cytochemistry* **44, 9** 919-968.

Nelson, SM, EE Telfer, and RA Anderson 2013 The ageing ovary and uterus: new biological insights. *Human Reproduction Update* **19** 67-83.

Nicol, L, MO Faure, JR McNeilly, J Fontaine, C Taragnat, and AS McNeilly 2008 Bone morphogenetic protein-4 interacts with activin and GnRH to modulate gonadotrophin secretion in LbetaT2 gonadotrophs. *Journal of Endocrinology* **196** 497-507.

Nottola, SA, R Heyn, A Camboni, S Correr, and G Macchiarelli 2006 Ultrastructural characteristics of human granulosa cells in a coculture system for in vitro fertilization. *Microscopy Research and Technique* **69** 508-516.

Ogura Nose, S, O Yoshino, Y Osuga, J Shi, H Hiroi, T Yano, and Y Taketani 2012 Anti-Mullerian hormone (AMH) is induced by bone morphogenetic protein (BMP) cytokines in human granulosa cells. *European journal of obstetrics & gynecology and reproductive biology* **164** 44-47.

Oosterhuis, GJ, HW Michgelsen, CB Lambalk, J Schoemaker, and I Vermes 1998a Apoptotic cell death in human granulosa-lutein cells: a possible indicator of in vitro fertilization outcome. *Fertility and Sterility* **70** 747-749.

Oosterhuis, GJE, HW Michgelsen, CB Lambalk, J Schoemaker, and I Vermes 1998b Apoptotic cell death in human granulosa-lutein cells: a possible indicator of in vitro fertilization outcome. *Fertility and Sterility* **70** 747-749.

Ophir, L, Y Yung, E Maman, N Rubinstein, GM Yerushalmi, J Haas, E Barzilay, and A Hourvitz 2014 Establishment and validation of a model for non-luteinized human mural granulosa cell culture. *Molecular and Cellular Endocrinology* **384** 165-174.

Otsuka, F 2010 Multiple endocrine regulation by bone morphogenetic protein system. *endocrine journal* **57** 3-14.

Otsuka, F 2013 Multifunctional bone morphogenetic protein system in endocrinology. *Acta medica Okayama* **67** 75-86.

Otsuka, F, RK Moore, and S Shimasaki 2001a Biological Function and Cellular Mechanism of Bone Morphogenetic Protein-6 in the Ovary. *Journal of Biological Chemistry* **276** 32889-32895.

Otsuka, F, and S Shimasaki 2002 A novel function of bone morphogenetic protein-15 in the pituitary: selective synthesis and secretion of FSH by gonadotropes. *Endocrinology* **143** 4938-4941.

Otsuka, F, S Yamamoto, G Erickson, and S Shimasaki 2001b Bone morphogenetic protein-15 inhibits follicle-stimulating hormone (FSH) action by suppressing FSH receptor expression. *J Biol Chem* **276** 11387 - 11392.

Otsuka, F, S Yamamoto, GF Erickson, and S Shimasaki 2001c Bone Morphogenetic Protein-15 Inhibits Follicle-stimulating Hormone (FSH) Action by Suppressing FSH Receptor Expression. *Journal of Biological Chemistry* **276** 11387-11392.

Ovesen, P 1998 Synergistic effects of growth hormone and insulin-like growth factor-I on differentiation and replication of cultured human luteinized granulosa cells. *Acta Obstetricia et Gynecologica Scandinavica* **77** 487-491.

Owen, EJ, C West, BA Mason, and HS Jacobs 1991 Co-treatment with growth hormone of sub-optimal responders in IVF-ET. *Human Reproduction* **6** 524-528.

Pal, L, K Zhang, G Zeitlian, and N Santoro 2010 Characterizing the reproductive hormone milieu in infertile women with diminished ovarian reserve. *Fertility and Sterility* **93** 1074-1079.

Pangas, SA 2012 Regulation of the ovarian reserve by members of the transforming growth factor beta family. *Molecular reproduction and development* **79** 666-679.

Peñarrubia, J, J Balasch, M García-Bermúdez, R Casamitjana, JA Vanrell, and ER Hernandez 2000 Growth hormone does not increase the expression of insulin-like growth factors and their receptor genes in the pre-menopausal human ovary. *Human Reproduction* **15** 1241-1246.

Peng, X-R, AJW Hsueh, PS Lapolt, and L Bjersing 1991a Localization of Luteinizing Hormone Receptor Messenger Ribonucleic Acid Expression in Ovarian Cell Types during Follicle Development and Ovulation. *Endocrinology* **129** 3200-3207.

Peng, X-R, AJW Hsueh, PS Lapolt, L Bjersing, and T Ny 1991b Localization of Luteinizing Hormone Receptor Messenger Ribonucleic Acid Expression in Ovarian Cell Types during Follicle Development and Ovulation. *Endocrinology* **129** 3200-3207.

Picton, HM, and AS McNeilly 1991 Evidence to support a follicle-stimulating hormone threshold theory for follicle selection in ewes chronically

treated with gonadotrophin-releasing hormone agonist. *Journal of reproduction and fertility* **93** 43-51.

Pidoux, G, P Gerbaud, V Tsatsaris, O Marpeau, F Ferreira, G Meduri, J Guibourdenche, J Badet, D Evain-Brion, and J-L Frendo 2007 Biochemical characterization and modulation of LH/CG—receptor during human trophoblast differentiation. *Journal of cellular physiology* **212** 26-35.

Pierre, A, C Pisselet, J Dupont, B Mandon-Pepin, D Monniaux, P Monget, and S Fabre 2004 Molecular basis of bone morphogenetic protein-4 inhibitory action on progesterone secretion by ovine granulosa cells. *J Mol Endocrinol* **33** 805 - 814.

Prochazka, R, E Nagyova, G Brem, and K Schellander 1998 Secretion of cumulus expansion-enabling factor (CEEF) in porcine follicles. *Mol Reprod Dev* **49** 141 - 149.

Pulkki, M, D Mottershead, A Pasternack, P Muggalla, H Ludlow, M van Dinther, S Myllymaa, K Koli, P ten Dijke, M Laitinen, and O Ritvos 2012 A covalently dimerized recombinant human bone morphogenetic protein-15 variant identifies bone morphogenetic protein receptor type 1B as a key cell surface receptor on ovarian granulosa cells. *Endocrinology* **153** 1509-1518.

Regan, SL, JR McFarlane, T O'Shea, N Andronicos, F Arfuso, A Dharmarajan, and G Almahbobi 2015 Flow cytometric analysis of FSHR, BMPR1B, LHR and apoptosis in granulosa cells and ovulation rate in merino sheep. *Reproduction* **doi:10.1530/REP-14-0581**

Riccardi, C, and I Nicoletti 2006 Analysis of apoptosis by propidium iodide staining and flow cytometry. *Nature Protocols* **1** 1458-1461.

Rice, S, K Ojha, S Whitehead, and H Mason 2007 Stage-Specific Expression of Androgen Receptor, Follicle-Stimulating Hormone Receptor, and Anti-Müllerian Hormone Type II Receptor in Single, Isolated, Human Preantral Follicles: Relevance to Polycystic Ovaries. *The Journal of Clinical Endocrinology & Metabolism* **92** 1034-1040.

Richards, JS 1994 Hormonal Control of Gene Expression in the OVary. *Endocrine reviews* **15** 725-751.

Richards, JS, DL Russell, RL Robker, M Dajee, and TN Alliston 1998 Molecular mechanisms of ovulation and luteinization. *Molecular and Cellular Endocrinology* **145** 47-54.

Robertson, D, Hale, GE, Jolley, D, Frase,r IS, Hughes, CL, Burger, HG 2009 Interrelationships between Ovarian and Pituitary Hormones in Ovulatory Menstrual Cycles across Reproductive Age. *The Journal of Clinical Endocrinology & Metabolism* **94** 138-144.

Rodgers, R, and H Irving-Rodgers 2010a Formation of the ovarian follicular antrum and follicular fluid. *Biol Reprod* **82** 1021 - 1029.

Rodgers, RJ, and HF Irving-Rodgers 2010b Formation of the Ovarian Follicular Antrum and Follicular Fluid. *Biology of Reproduction* **82** 1021-1029.

Rosen, MP, E Johnstone, C Addauan-Andersen, and MI Cedars 2011 A lower antral follicle count is associated with infertility. *Fertility and Sterility* **95** 1950-1954.e1951.

Rotmensch, S, Dor J, Furman A, Rudak E, Mashiach S, and Amsterdam A 1986 Ultrastructural characterization of human granulosa cells in

stimulated cycles: correlation with oocyte fertilizability. *Fertility and Sterility* **45** 671-679.

Ruoss, C, A Tadros, T O'Shea, J McFarlane, and G Almahbobi 2009 Ovarian follicle development in Booroola sheep exhibiting impaired bone morphogenetic protein signalling pathway. *Reproduction* **138** 689-696.

Ryan, K, C Glister, P Lonergan, F Martin, P Knight, and A Evans 2008 Functional significance of the signal transduction pathways Akt and Erk in ovarian follicles: in vitro and in vivo studies in cattle and sheep. *Journal of Ovarian Research* **1** 2-2.

Sakurada, Y, M Shirota, K Inoue, N Uchida, and K Shirota 2006 New approach to in situ quantification of ovarian gene expression in rat using a laser microdissection technique: relationship between follicle types and regulation of inhibin-α and cytochrome P450aromatase genes in the rat ovary. *Histochemistry and Cell Biology* **126** 735-741.

Santoro, N, B Isaac, G Neal-Perry, T Adel, L Weingart, A Nussbaum, S Thakur, H Jinnai, N Khosla, and D Barad 2003 Impaired Folliculogenesis and Ovulation in Older Reproductive Aged Women. *The Journal of Clinical Endocrinology & Metabolism* **88** 5502-5509.

Sasson, R, and A Amsterdam 2002 Stimulation of apoptosis in human granulosa cells from in vitro fertilization patients and its prevention by dexamethasone: involvement of cell contact and bcl-2 expression. *J Clin Endocrinol Metab* **87** 3441 - 3451.

Scheffer, GJ, FJM Broekmans, M Dorland, JDF Habbema, CWN Looman, and ER te Velde 1999 Antral follicle counts by transvaginal ultrasonography are related to age in women with proven natural fertility. *Fertility and Sterility* **72** 845-851.

Schmid, I, Krall, WJ, Uittenbogaart ,CH, Braun, J, Giorgi, JV. 1992 Dead cell discrimination with 7-amino-actinomycin D in combination with dual color immunofluorescence in single laser flow cytometry. *Cytometry* **13(2):** 204-208.

Seger, R, T Hanoch, R Rosenberg, A Dantes, WE Merz, JF Strauss, and A Amsterdam 2001 The ERK Signaling Cascade Inhibits Gonadotropin-stimulated Steroidogenesis. *Journal of Biological Chemistry* **276** 13957-13964.

Seifer, D, Gardiner, AC, Ferreira, KA, Peluso, JJ. 1996 Apoptosis as a function of ovarian reserve in women undergoing in vitro fertilization. *Fertil Steril.* **66(4)** 593-598.

Seifer, D, E Golub, G Lambert Messerlian, G Springer, S Holman, M Moxley, H Cejtin, N Nathwani, K Anastos, H Minkoff, and R Greenblatt 2007 Biologic markers of ovarian reserve and reproductive aging: application in a cohort study of HIV infection in women. *Fertility and Sterility* **88** 1645-1652.

Seifer, DB, C Charland, D Berlinsky, AS Penzias, RV Haning Jr, F Naftolin, and BE Barker 1993 Proliferative index of human luteinized granulosa cells varies as a function of ovarian reserve. *American Journal of Obstetrics and Gynecology* **169** 1531-1535.

Seifer, DB, V DeJesus, and K Hubbard 2002 Mitochondrial deletions in luteinized granulosa cells as a function of age in women undergoing in vitro fertilization. *Fertility and Sterility* **78** 1046-1048.

Seifer, DB, RT Scott Jr, PA Bergh, LK Abrogast, CI Friedman, CK Mack, and DR Danforth 1999 Women with declining ovarian reserve may demonstrate a decrease in day 3 serum inhibin B before a rise in day 3 follicle-stimulating hormone. *Fertility and Sterility* **72** 63-65.

Sen, A, and S Hammes 2010 Granulosa cell-specific androgen receptors are critical regulators of ovarian development and function. *Molecular Endocrinology* **24** 1393-1403.

Sen, A, H Prizant, A Light, A Biswas, E Hayes, H-J Lee, D Barad, N Gleicher, and SR Hammes 2014 Androgens regulate ovarian follicular development by increasing follicle stimulating hormone receptor and microRNA-125b expression. *Proceedings of the National Academy of Sciences*.

Sharara, FI, and LK Nieman 1994 Identification and cellular localization of growth hormone receptor gene expression in the human ovary. *The Journal of Clinical Endocrinology & Metabolism* **79** 670-672.

Shelling, AN 2010 Premature ovarian failure. *Reproduction* **140** 633-641.

Shi, J, O Yoshino, Y Osuga, I Akiyama, M Harada, K Koga, A Fujimoto, T Yano, and Y Taketani 2012 Growth differentiation factor 3 is induced by bone morphogenetic protein 6 (BMP-6) and BMP-7 and increases luteinizing hormone receptor messenger RNA expression in human granulosa cells. *Fertility and Sterility* **97** 979-983.

Shi, J, O Yoshino, Y Osuga, K Koga, Y Hirota, T Hirata, T Yano, O Nishii, and Y Taketani 2009a Bone morphogenetic protein-6 stimulates gene expression of follicle-stimulating hormone receptor, inhibin/activin beta subunits, and anti-MÃ¼llerian hormone in human granulosa cells. *Fertility and Sterility* **92** 1794-1798.

Shi, J, O Yoshino, Y Osuga, K Koga, Y Hirota, T Hirata, T Yano, O Nishii, and Y Taketani 2009b Bone morphogenetic protein-6 stimulates gene expression of follicle-stimulating hormone receptor, inhibin/activin beta subunits, and anti-Müllerian hormone in human granulosa cells. *Fertility and Sterility* **92** 1794-1798.

Shi, J, O Yoshino, Y Osuga, K Koga, Y Hirota, E Nose, O Nishii, T Yano, and Y Taketani 2011 Bone morphogenetic protein-2 (BMP-2) increases gene expression of FSH receptor and aromatase and decreases gene expression of LH receptor and StAR in human granulosa cells. *American journal of reproductive immunology (1989)* **65** 421-427.

Shi, J, O Yoshino, Y Osuga, O Nishii, T Yano, and Y Taketani 2010 Bone morphogenetic protein 7 (BMP-7) increases the expression of follicle-stimulating hormone (FSH) receptor in human granulosa cells. *Fertility and Sterility* **93** 1273-1279.

Shimada, M, M Nishibori, N Isobe, N Kawano, and T Terada 2003 Luteinizing Hormone Receptor Formation in Cumulus Cells Surrounding Porcine Oocytes and Its Role During Meiotic Maturation of Porcine Oocytes. *Biology of Reproduction* **68** 1142-1149.

Shimada, M, and T Terada 2002 FSH and LH induce progesterone production and progesterone receptor synthesis in cumulus cells: a requirement for meiotic resumption in porcine oocytes. *Molecular Human Reproduction* **8** 612-618.

Shimada, M, Y Yamashita, J Ito, T Okazaki, K Kawahata, and M Nishibori 2004 Expression of two progesterone receptor isoforms in cumulus cells

and their roles during meiotic resumption of porcine oocytes. *Journal of molecular endocrinology* **33** 209-225.

Shimasaki, S, R Moore, F Otsuka, and G Erickson 2004 The bone morphogenetic protein system in mammalian reproduction. *Endocr Rev* **25** 72 - 101.

Shimasaki, S, RJ Zachow, D Li, H Kim, S-i Iemura, N Ueno, K Sampath, RJ Chang, and GF Erickson 1999 A functional bone morphogenetic protein system in the ovary. *Proceedings of the National Academy of Sciences of the United States of America* **96** 7282-7287.

Skinner, MK 2005 Regulation of primordial follicle assembly and development. *Hum Reprod Update* **11** 461-471.

Souza, C, C MacDougall, B Campbell, A McNeilly, and D Baird 2001 The Booroola (FecB) phenotype is associated with a mutation in the bone morphogenetic receptor type 1 B (BMPR1B) gene. *J Endocrinol* **169** R1 - R6.

Stanger, JD, and JL Yovich 1985 Reduced in-vitro fertilization of human oocytes from patients with raised basal luteinizing hormone levels during the follicular phase. *BJOG: An International Journal of Obstetrics & Gynaecology* **92** 385-393.

Stilley, JAW, DE Christensen, KB Dahlem, R Guan, DA Santillan, SK England, A Al-Hendy, PA Kirby, and DL Segaloff 2014 FSH Receptor (FSHR) Expression in Human Extragonadal Reproductive Tissues and the Developing Placenta, and the Impact of Its Deletion on Pregnancy in Mice. *Biology of Reproduction* **91** 74, 71-15.

Su, Y, Wigglesworth K, Pendola FL, O'Brien MJ, Eppig JJ 2002 Mitogen-Activated Protein Kinase Activity in Cumulus Cells Is Essential for Gonadotropin-Induced Oocyte Meiotic Resumption and Cumulus Expansion in the Mouse. *Endocrinology* **143** 2221-2232.

Sugawara, T, M Saito, and S Fujimoto 2000 Sp1 and SF-1 Interact and Cooperate in the Regulation of Human Steroidogenic Acute Regulatory Protein Gene Expression. *Endocrinology* **141** 2895-2903.

Suikkari, A, MacLachlan V, Koistinen R, Seppälä M, Healy D. 1996 Double-blind placebo controlled study: human biosynthetic growth hormone for assisted reproductive technology. *Fertil Steril.* **Apr 65** 800-805.

Sullivan, MW, A Stewart Akers, JS Krasnow, SL Berga, and AJ Zeleznik 1999 Ovarian responses in women to recombinant follicle-stimulating hormone and luteinizing hormone (LH): a role for LH in the final stages of follicular maturation. *The Journal of clinical endocrinology and metabolism* **84** 228-232.

Sutovský, P, JE Fléchon, B Fléchon, J Motlik, N Peynot, P Chesné, and Y Heyman 1993 Dynamic changes of gap junctions and cytoskeleton during in vitro culture of cattle oocyte cumulus complexes. *Biology of Reproduction* **49** 1277-1287.

Tajima, K, A Dantes, Z Yao, K Sorokina, F Kotsuji, R Seger, and A Amsterdam 2003 Down-regulation of steroidogenic response to gonadotropins in human and rat preovulatory granulosa cells involves mitogen-activated protein kinase activation and modulation of DAX-1 and steroidogenic factor-1. *The Journal of clinical endocrinology and metabolism* **88** 2288-2299.

Tajima, K, Dantes A, Yao Z, Sorokina K, Kotsuji F, Seger R, Amsterdam A 2003 Down-Regulation of Steroidogenic Response to Gonadotropins in Human and Rat Preovulatory Granulosa Cells Involves Mitogen-Activated Protein Kinase Activation and Modulation of DAX-1 and Steroidogenic Factor-1. *The Journal of Clinical Endocrinology & Metabolism* **88** 2288-2299.

Takeda, M, F Otsuka, H Takahashi, K Inagaki, T Miyoshi, N Tsukamoto, H Makino, and M Lawson 2012 Interaction between gonadotropin-releasing hormone and bone morphogenetic protein-6 and -7 signaling in LβT2 gonadotrope cells. *Molecular and Cellular Endocrinology* **348** 147-154.

Tamura, M, H Sasano, T Suzuki, T Fukaya, T Watanabe, H Aoki, H Nagura, and A Yajima 1994 Endocrinology:Immunohistochemical localization of growth hormone receptor in cyclic human ovaries. *Human Reproduction* **9** 2259-2262.

Tanwar, P, and J McFarlane 2011 Dynamic expression of bone morphogenetic protein 4 in reproductive organs of female mice. *Reproduction* **142** 573-579.

Tapanainen, J, H Martikainen, R Voutilainen, M Orava, A Ruokonen, and L Rönnberg 1992 Effect of growth hormone administration on human ovarian function and steroidogenic gene expression in granulosa-luteal cells. *Fertil Steril.* **Oct;58(4):** 726-732.

ten Dijke, P, O Korchynskyi, G Valdimarsdottir, and M Goumans 2003 Controlling cell fate by bone morphogenetic protein receptors. *Mol Cell Endocrinol* **211** 105 - 113.

Tesarik, J, A Hazout, and C Mendoza 2005 Improvement of delivery and live birth rates after ICSI in women aged >40 years by ovarian co-stimulation with growth hormone. *Human Reproduction* **20** 2536-2541.

Thomas, FH, J-F Ethier, S Shimasaki, and BC Vanderhyden 2005 Follicle-Stimulating Hormone Regulates Oocyte Growth by Modulation of Expression of Oocyte and Granulosa Cell Factors. *Endocrinology* **146** 941-949.

Tilly, JLK, K I Schomberg,D W and Hsueh A J 1992 Apoptosis in atretic ovarian follicles is associated with selective decreases in messenger ribonucleic acid transcripts for gonadotropin receptors and cytochrome P450 aromatase. *Endocrinology* **131** 1670-1676.

Turner, K, S Macpherson, M Millar, A McNeilly, K Williams, M Cranfield, N Groome, R Sharpe, H Fraser, and P Saunders 2002 Development and validation of a new monoclonal antibody to mammalian aromatase. *Journal of Endocrinology* **172** 21-30.

Val, P, A-M LefranÃois-Martinez, G VeyssiÃre, and A Martinez 2003 SF-1 a key player in the development and differentiation of steroidogenic tissues. *Nuclear receptor* **1** 8-8.

van Rooij, IAJ, FJM Broekmans, GJ Scheffer, CWN Looman, JDF Habbema, FH de Jong, BJCM Fauser, APN Themmen, and ER te Velde 2005 Serum antimüllerian hormone levels best reflect the reproductive decline with age in normal women with proven fertility: A longitudinal study. *Fertility and Sterility* **83** 979-987.

Vermes, I, C Haanen, H Steffens-Nakken, and C Reutellingsperger 1995 A novel assay for apoptosis Flow cytometric detection of

phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *Journal of Immunological Methods* **184** 39-51.

Volpe, A, G Coukos, A Barreca, PG Artini, F Minuto, G Giordano, and AR Genazzani 1989 Ovarian response to combined growth hormone-gonadotropin treatment in patients resistant to induction of superovulation. *Gynecological Endocrinology* **3** 125-133.

Waters, MJ, HN Hoang, DP Fairlie, RA Pelekanos, and RJ Brown 2006 New insights into growth hormone action. *Journal of molecular endocrinology* **36** 1-7.

Weall, BM, S Al-Samerria, J Conceicao, JL Yovich, and G Almahbobi 2014 A direct action for growth hormone in improving oocyte quality in poor responder patients. *Reproduction*.

Webb, R, Campbell BK. 2007 Development of the dominant follicle: mechanisms of selection and maintenance of oocyte quality. *Society of Reproduction and Fertility supplement* **vol:64** 141 -163

Weenen, C, JSE Laven, ARM von Bergh, M Cranfield, NP Groome, JA Visser, P Kramer, BCJM Fauser, and APN Themmen 2004 Anti-Mullerian hormone expression pattern in the human ovary: potential implications for initial and cyclic follicle recruitment. *Mol. Hum. Reprod.* **10** 77-83.

Westergaard, L, IJ Christensen, and KP McNatty 1986 Steroid levels in ovarian follicular fluid related to follicle size and health status during the normal menstrual cycle in women. *Human Reproduction* **1** 227-232.

Whiteman, G, Boldt J, Martinez J, and Pantazis C 1991 Flow cytomentric analysis of induced human graafian follicles.I. Demonstration and sorting of two luteinized cell populations. *Fertility and Sterility* **56** 259-263.

Wilson, T, X-Y Wu, JL Juengel, IK Ross, JM Lumsden, EA Lord, KG Dodds, GA Walling, JC McEwan, AR O'Connell, KP McNatty, and GW Montgomery 2001 Highly Prolific Booroola Sheep Have a Mutation in the Intracellular Kinase Domain of Bone Morphogenetic Protein IB Receptor (ALK-6) That Is Expressed in Both Oocytes and Granulosa Cells. *Biology of Reproduction* **64** 1225-1235.

Wu, Y-T, L Tang, J Cai, X-E Lu, J Xu, X-M Zhu, Q Luo, and H-F Huang 2007 High bone morphogenetic protein-15 level in follicular fluid is associated with high quality oocyte and subsequent embryonic development. *Human Reproduction* **22** 1526-1531.

Xia, Y, T O'Shea, R Murison, and JR McFarlane 2003 Concentrations of Progesterone, Follistatin, and Follicle-Stimulating Hormone in Peripheral Plasma Across the Estrous Cycle and Pregnancy in Merino Ewes That Are Homozygous or Noncarriers of the Booroola Gene. *Biology of Reproduction* **69** 1079-1084.

Xu, Z, HA Garverick, GW Smith, MF Smith, SA Hamilton, and RS Youngquist 1995 Expression of follicle-stimulating hormone and luteinizing hormone receptor messenger ribonucleic acids in bovine follicles during the first follicular wave. *Biology of Reproduction* **53** 951-957.

Yamamoto, T, F Saatcioglu, and T Matsuda 2002 Cross-talk between bone morphogenic proteins and estrogen receptor signaling. *Endocrinology* **143** 2635-2642.

Yerushalmi, GM, M Salmon-Divon, Y Yung, E Maman, A Kedem, L Ophir, O Elemento, G Coticchio, M Dal Canto, M Mignini Renzinu, R Fadini, and A Hourvitz 2014 Characterization of the human cumulus cell

transcriptome during final follicular maturation and ovulation. *Molecular Human Reproduction* **20** 719-735.

Ying, Y, and GQ Zhao 2001 Cooperation of endoderm-derived BMP2 and extraembryonic ectoderm-derived BMP4 in primordial germ cell generation in the mouse. *Developmental biology* **232** 484-492.

Yong, EL, DT Baird, R Yates, LE Reichert, and SG Hillier 1992 Hormonal regulation of the growth and steroidogenic function of human granulosa cells. *The Journal of clinical endocrinology and metabolism* **74** 842-849.

Young, JM, S Henderson, C Souza, H Ludlow, N Groome, and AS McNeilly 2012 Activin B is produced early in antral follicular development and suppresses thecal androgen production. *Reproduction* **143** 637-650.

Young, JM, JL Juengel, KG Dodds, M Laird, PK Dearden, AS McNeilly, KP McNatty, and T Wilson 2008 The activin receptor-like kinase 6 Booroola mutation enhances suppressive effects of bone morphogenetic protein 2 (BMP2), BMP4, BMP6 and growth and differentiation factor-9 on FSH release from ovine primary pituitary cell cultures. *Journal of Endocrinology* **196** 251-261.

Yovich, J, J Stanger, and P Hinchliffe 2012 Targeted gonadotrophin stimulation using the PIVET algorithm markedly reduces the risk of OHSS. *Reproductive BioMedicine Online* **24** 281-292.

Yovich, JL, and JD Stanger 2010 Growth hormone supplementation improves implantation and pregnancy productivity rates for poor-prognosis patients undertaking IVF. *Reproductive BioMedicine Online* **21** 37-49.

Yuan, W, Bao, B, Garverick, HA, Youngquist, RS, Lucy, MC 1998 Follicular dominance in cattle is associated with divergent patterns of ovarian gene expression for insulin-like growth factor (IGF)-I, IGF-II, and IGF binding protein-2 in dominant and subordinate follicles. *Domest Anim Endocrinol.* **15(1) 55-63**

Yuan, W, and L Giudice 1997 Programmed Cell Death in Human Ovary Is a Function of Follicle and Corpus Luteum Status. *The Journal of Clinical Endocrinology & Metabolism* **82** 3148-3155.

Yung, Y, S Aviel-Ronen, E Maman, N Rubinstein, C Avivi, R Orvieto, and A Hourvitz 2014 Localization of luteinizing hormone receptor protein in the human ovary. *Molecular Human Reproduction* **20** 844-849.

Zeleznik, A, A Midgley, and L Reichert 1974 Granulosa Cell Maturation in the Rat: Increased Binding of Human Chorionic Gonadotropin Following Treatment with Follicle-Stimulating Hormone in Vivo1. *Endocrinology* **95** 818-825.

Zeleznik, AJ 2001 Follicle selection in primates: "many are called but few are chosen". *Biology of Reproduction* **65** 655-659.

Zhu, G, Cui Y, Qinglin-Wang, Yonggang-Kang, Yanzhi-Lv, Wang G, Song Y, Cao B 2013 Bone morphogenetic proteins (BMP) 2, 4, 6 and 7 affect ovarian follicular development through regulation of follicle-stimulating hormone receptor (FSHR) and luteinizing hormone receptor (LHR) expression in goat granulosa cells. *Journal of Cell Biology and Genetics* **3(1)** 14-21.

Zhu, G, Y YCui, Q Wang, Y Kang, Y Lv, J Wang, Y Song, and B Cao 2013 Bone morphogenetic proteins (BMP) 2,4,6, and 7 affect ovarian follicular development through regulation of follicle-stimulating hormone receptor

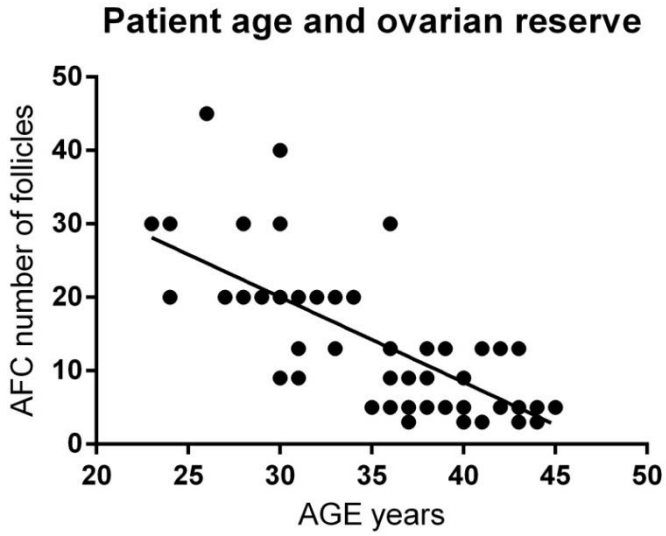
(FSHR) and lutenizing hormone (LHR) expression in goat granulosa cells. *Journal of Cell Biology and Genetics* **Vol 3(1)** 14-21.

Zhu, T, ELK Goh, R Graichen, L Ling, and PE Lobie 2001 Signal transduction via the growth hormone receptor. *Cellular Signalling* **13** 599-616.

Zhuang, G, Wong SX, and Z CQ. 1994 The effect of co-administration of low dosage growth hormone and gonadotropin for ovarian hyperstimulation in vitro fertilization and embryo transfer
Zhonghua Fu Chan Ke Za Zhi **29** 471-474,510.

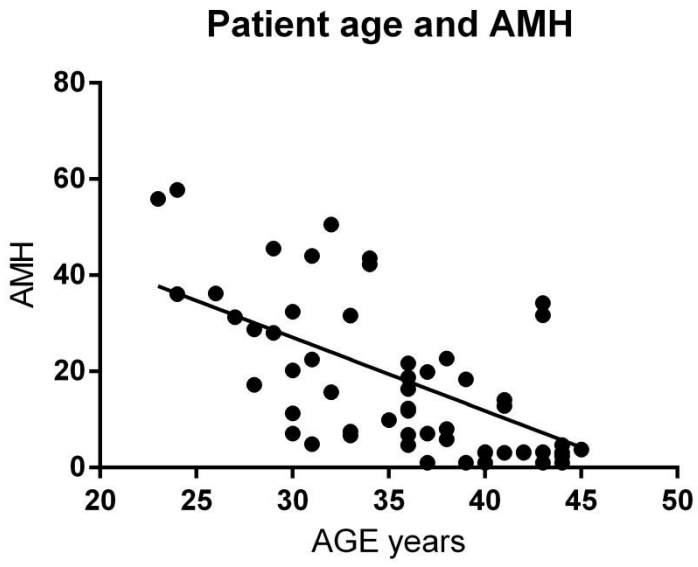
APPENDIX

Additional supporting evidence



R square	0.5213
P value	< 0.0001
Deviation from zero?	Significant
Data	
Number of X values	442

Figure 1 Relationship between AFC and chronological age.



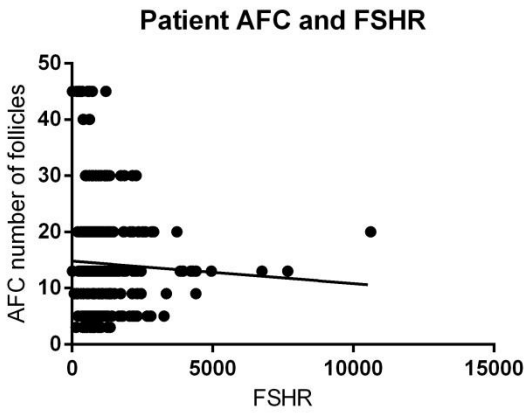
R square	0.3248
P value	< 0.0001
Deviation from zero?	Significant
Data	
Number of X values	415

Figure 2 Relationship between AFC and chronological AMH.

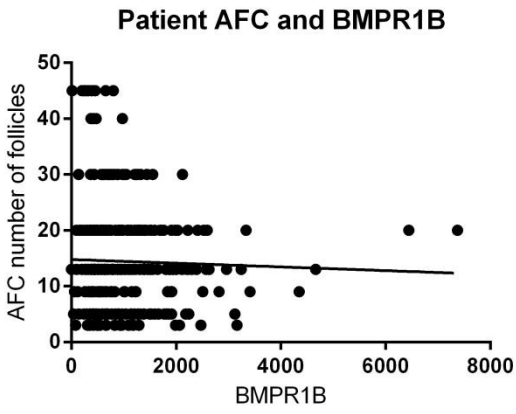
R square	0.003649

P value	0.2044
Deviation from zero?	Not Significant
Data	
Number of X values	443

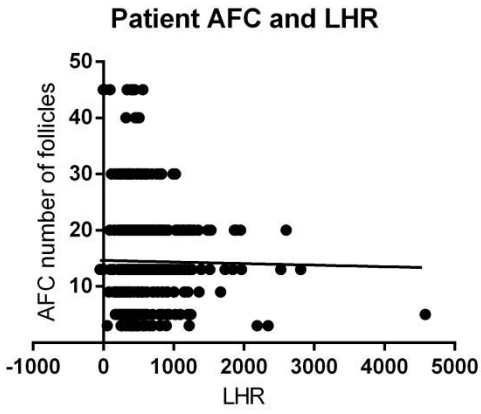
Figure 3 Relationship between follicle size and chronological age.



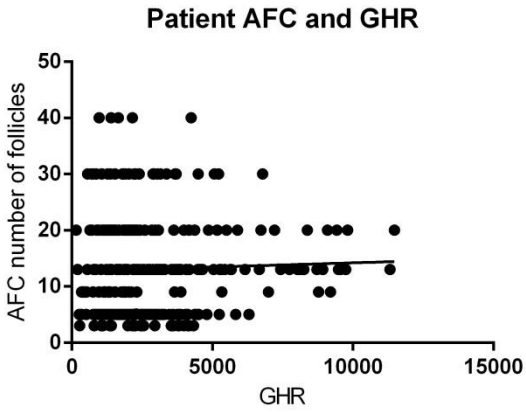
R square	0.001663
P value	0.3957
Deviation from zero?	Not Significant
Data	
Number of X values	436



R square	0.0007784
P value	0.5612
Deviation from zero?	Not Significant
Data	
Number of X values	436



R square	0.0001721
P value	0.7842
Deviation from zero?	Not Significant
Data	
Number of X values	438



R square	0.001435
P value	0.4996
Deviation from zero?	Not Significant
Data	
Number of X values	320

Figure 4 Relationship between the number of follicles AFC and the receptor density for FSHR, BMPR1, LHR and GHR on the surface of human peri-luteal granulosa- cells.

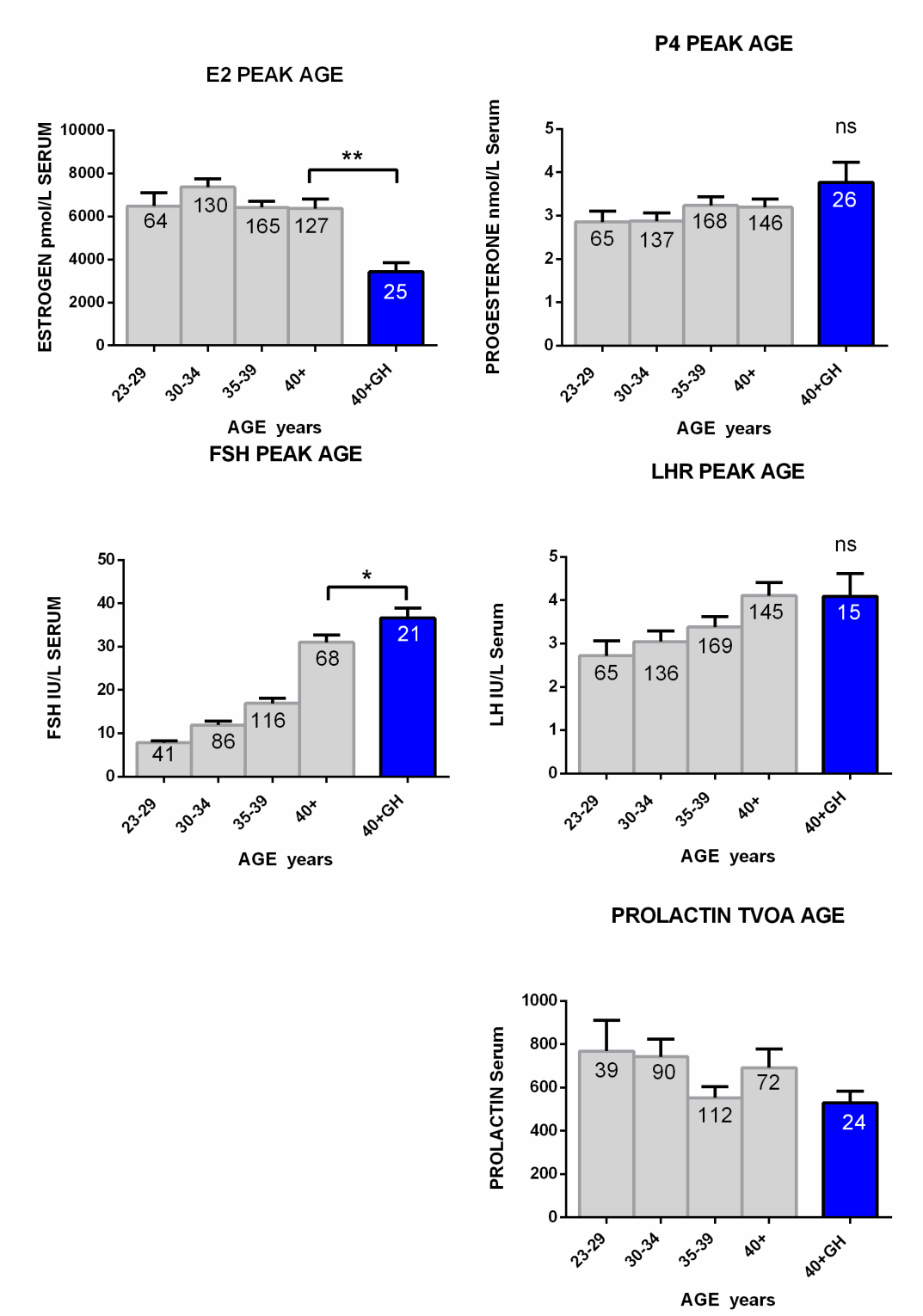


Figure 5 Serum levels of steroid hormones from patients receiving infertility treatment in a stimulated cycle with rFSH; and age compared to patients treated with GH co-treatment.
Note : The significance is brought about because the AFC in the 40+y group is mixed therefore the average is higher compared to the 40+ GH treated which is only AFC D&E.

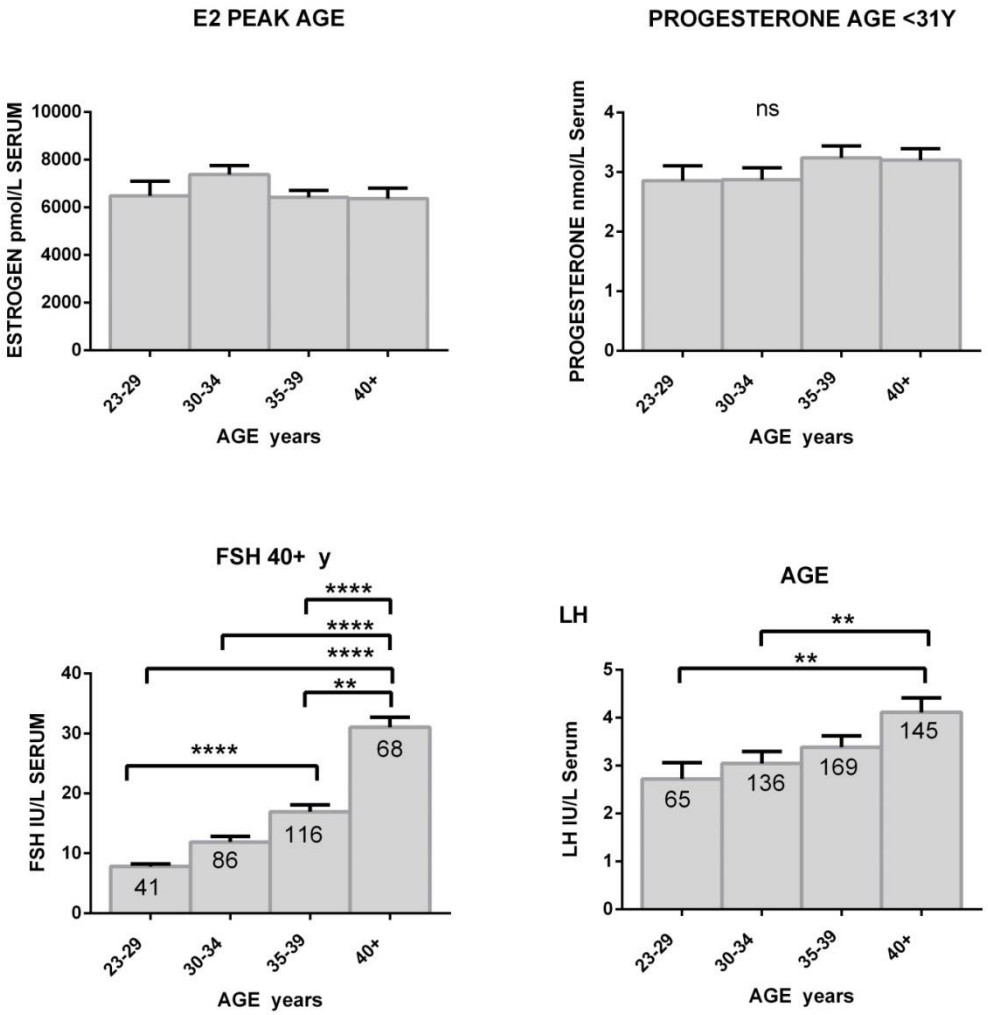


Figure 6 Serum levels of steroid hormones from patients receiving infertility treatment in a stimulated cycle with rFSH; and age.

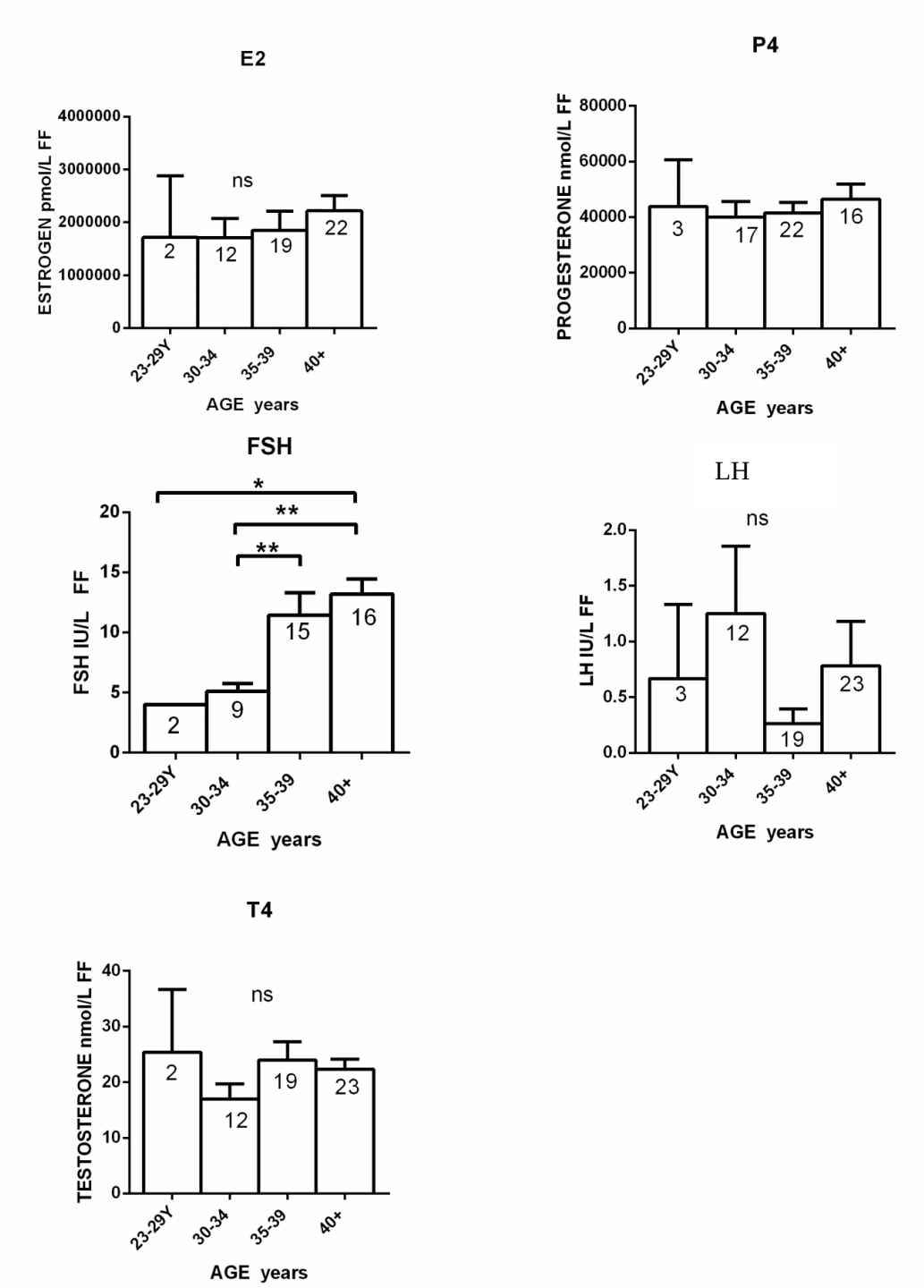


Figure 7 Follicular levels of steroid hormones from patients receiving infertility treatment in a stimulated cycle with rFSH and age.

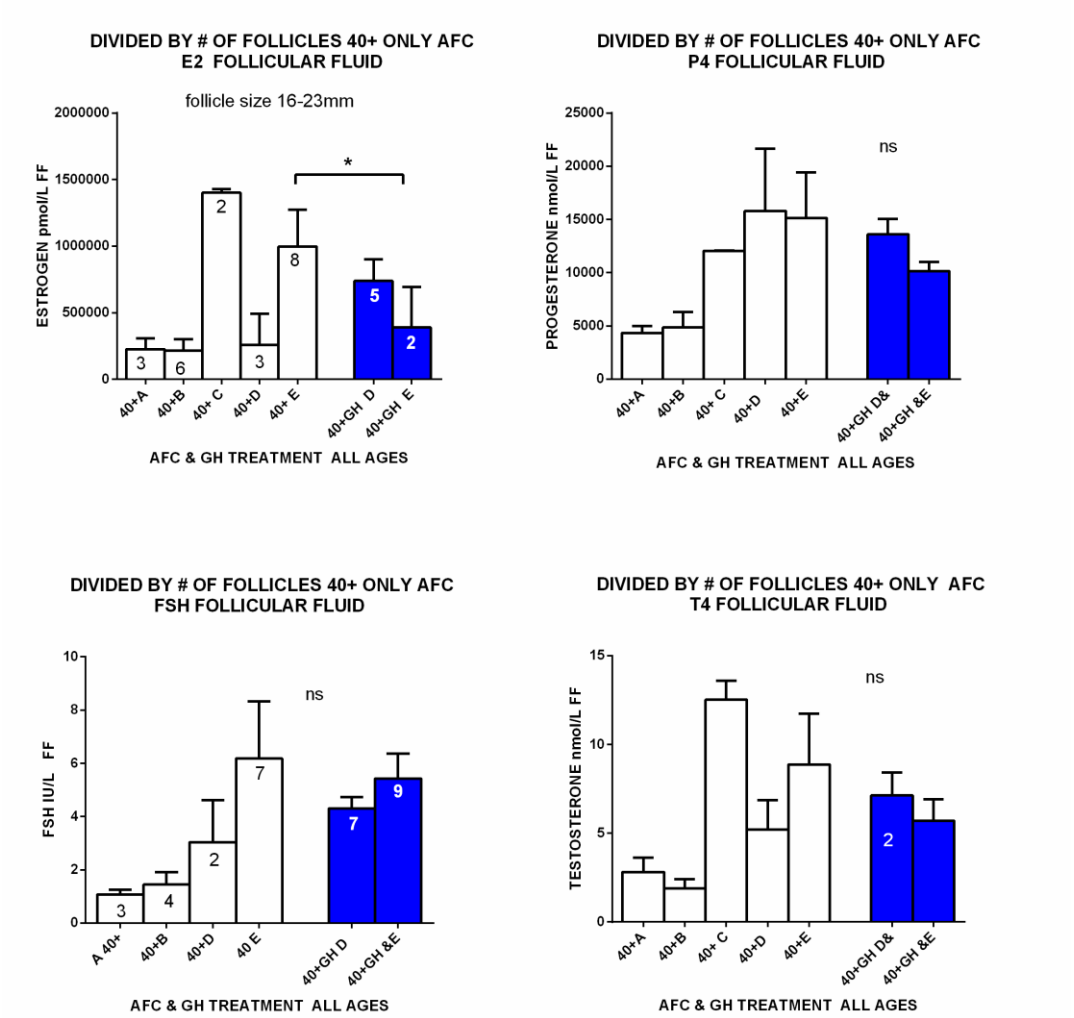


Figure 8 Follicular levels of steroid hormones from patients receiving infertility treatment in a stimulated cycle with rFSH; and AFC, adjusted for the number of follicles between 16-23mm.

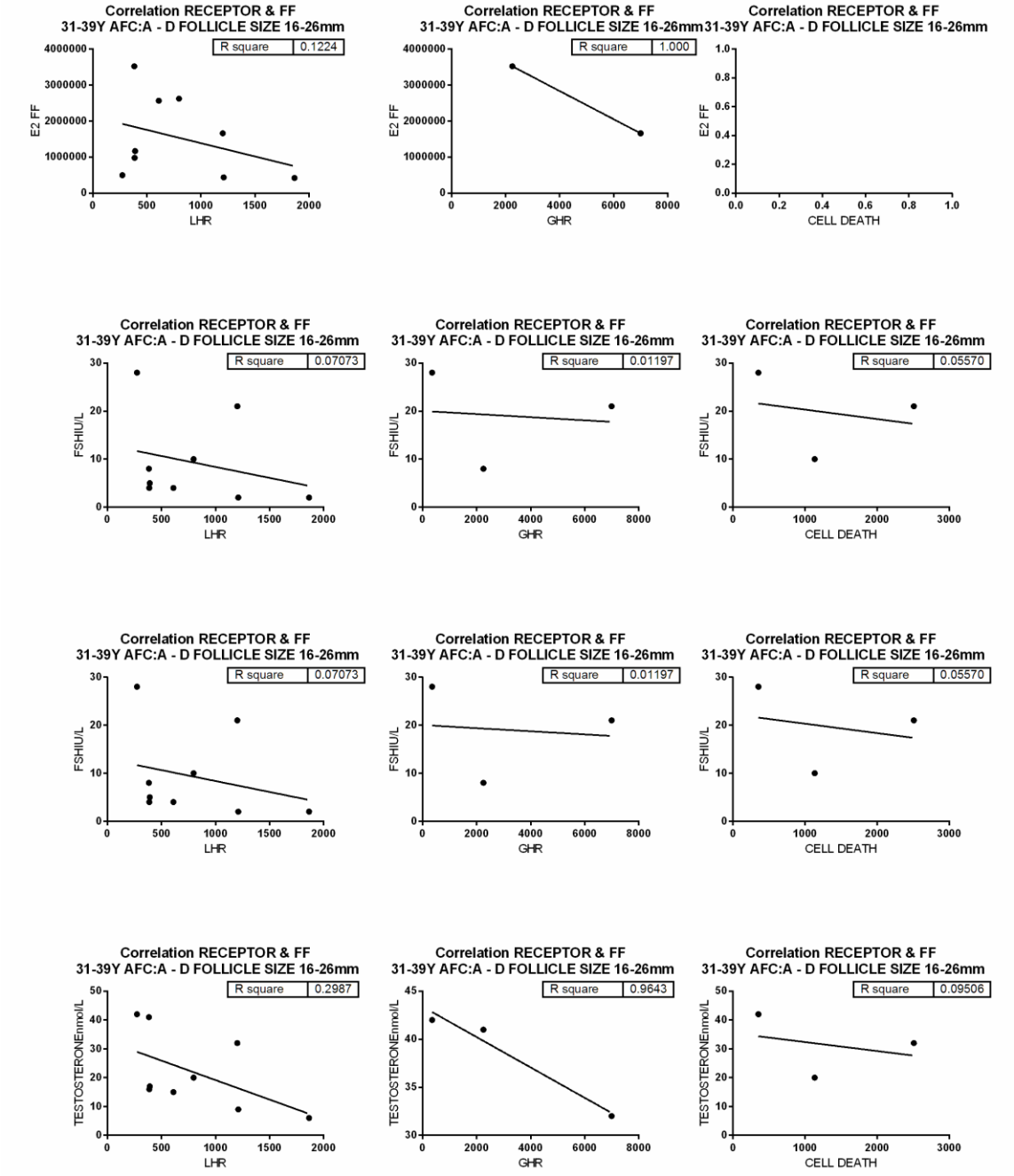


Figure 9 Correlation between the concentration of follicular fluid (FF) steroid hormones and receptor density for LHR, GHR & level (%) of Apoptosis or necrosis of granulosa cells.

Receptor density is a measure of mean fluorescent intensity in arbitrary units. Each point represents the average for that size follicle.

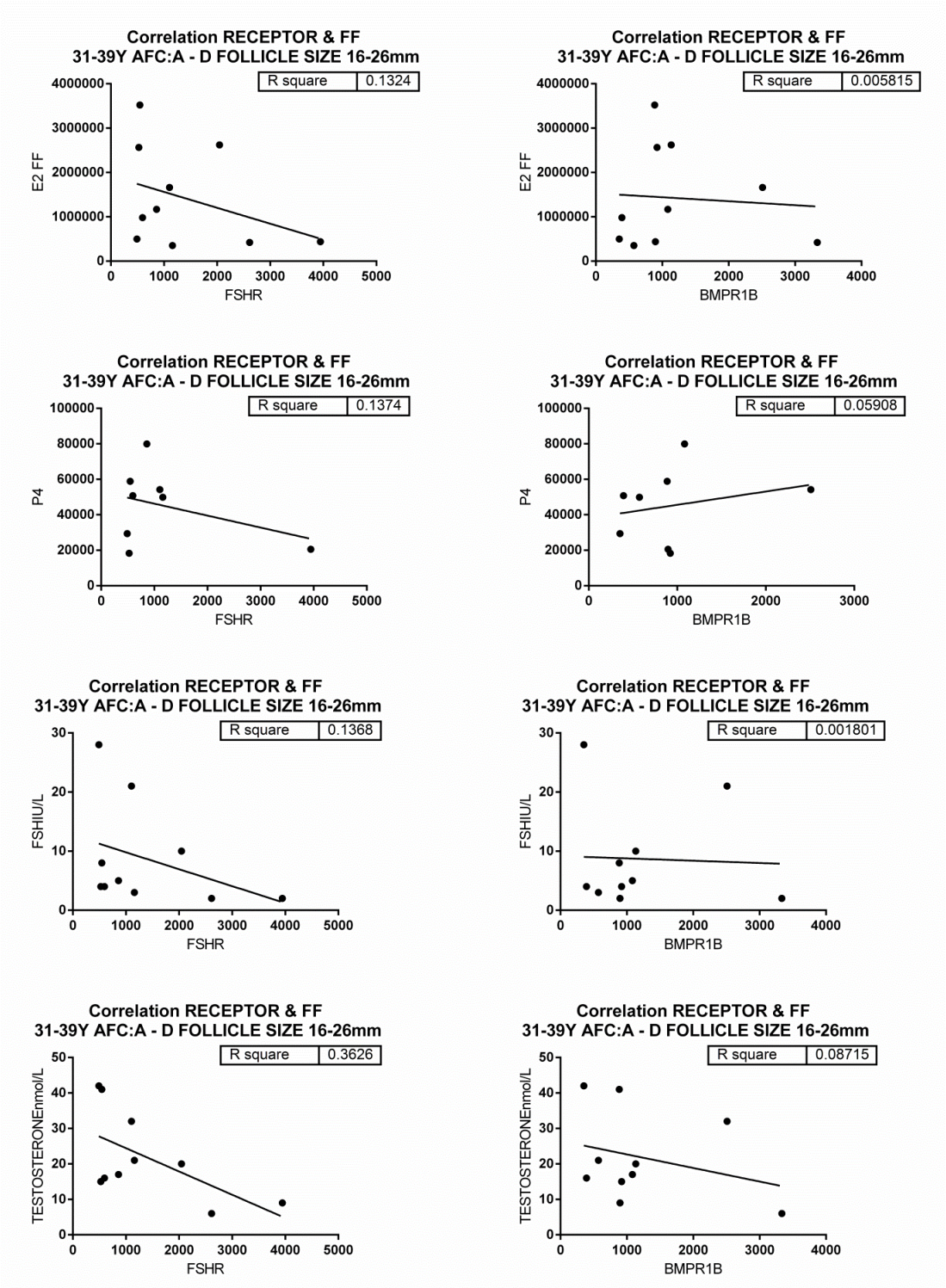


Figure 10 Correlation between the concentration of follicular fluid (FF) steroid hormones and receptor density for FSHR and BMPR1B on granulosa cells.

Receptor density is a measure of mean fluorescent intensity in arbitrary units. Each point represents the average for that size follicle.