The Effect of Gonadotrophins on Follicular Development and Subsequent Oocyte Competence

Alison A Murray

Ph.D

University of Edinburgh

2003

Declaration

I declare that this thesis has been composed by myself and has not been submitted for any previous degree. The work described herein is my own and all work of other authors is duly acknowledged. I also acknowledge all assistance given to me during the course of these studies.

Alison A Murray

Abstract

The growth and development of the ovarian follicle is a long and complex process. Throughout this process the oocyte maintains close contact with the surrounding somatic cells and through bi-directional communication acquires the developmental programs necessary for fertilisation and embryonic development. During the latter phase of follicular development the signals between oocyte and the somatic cells are dependent on stimulation by the gonadotrophin hormones, FSH and LH. The precise and individual role that FSH and LH play in this tightly co-ordinated process has yet to be determined. Despite this, exogenous administration of gonadotrophin preparations is used extensively in clinical and agricultural settings as a means of obtaining a large number of oocytes for use in ART. However, the basic question as to what affects gonadotrophins have on oocyte development within the follicle prior to ovulation remains to be answered. The main aim of this thesis was to use an animal model, the mouse, to begin to address this question.

In an attempt to distinguish between intra follicular and inter follicular/extra ovarian effects, experiments were performed using both *in vitro* and *in vivo* models. The results obtained using the hypogonadal mouse as an *in vivo* model were limited. However they did show that stimulation by both gonadotrophins might be necessary to induce sufficient ovarian oestrogen production to prime the reproductive tract prior to pregnancy. After optimising the *in vitro* system, which gave interesting insights into the nutritional aspects of follicular development, the results obtained indicated that elevation of LH during the follicular growth phase affected the ability of the oocyte to complete pre-implantation development.

Manipulation of gonadotrophins resulted in alterations in the production of the sex steroids. Both androgens and oestrogens are potential mediators of gonadotrophin action. Using a different experimental paradigm this was investigated directly by elevating androgens, oestrogens or both steroids *in vitro*. It was found that the fertilisation rates of the oocytes was adversely affected by elevating oestrogens but positively correlated in the presence of elevated androgens.

Whether oestrogen plays an obligatory role within the ovary is controversial. The finding that two receptors exist for this steroid has complicated the issue. Investigations using the ERKO transgenic mouse and *in vitro* techniques have gone some way towards clarifying this situation. Lack of the ERα receptor does not impair follicular development or developmental competence of the oocyte. Observations made during these experiments and those using the hypogonadal mouse suggest that oestrogen may facilitate the ovulatory process.

In conclusion the findings presented in this thesis have demonstrated that androgens, independently of oestrogens, are a necessary component of the signalling system in follicular development and subsequent oocyte maturation. Additionally, alterations in the **pattern** of intra-follicular steroid production either directly, or as a result of manipulating gonadotrophin levels such as occurs during ovulation induction, can influence the viability and developmental potential of the oocyte.

Publications arising, or containing work from this thesis

Published papers.

Rosenfeld C.S., Murray A.A., Simmer G., Hufford MG., Smith M.F., Spears N., Lubahn D.B.

Gonadotrophin induction of ovulation and corpus luteum formation in young estrogen receptor-alpha knockout mice.

Biol Reprod 2000; 62: 599-605

Murray A., Spears N.

Follicular development in vitro.

Semin Reprod Med 2000; 18:109-122

Murray A A., Moline M D., Baker S J., Kojima F N., Smith M F., Hillier S G., Spears N.

Role of ascorbic acid in promoting follicle integrity and survival in intact mouse ovarian follicles in vitro.

Reproduction 2001; 121 :89-96

In preparation

Murray A A., Edwards D., Smith R., Hillier S G., Spears N.

The role of ER-alpha in follicular development and oocyte developmental competence.

Abstracts of oral presentations

Muttukrishna S., Murray A A., Asselin J., Molinek M., Ledger W L., Groome N P., Spears N.

Production of inhibin A, inhibin B and activin A by mouse follicles in vitro.

Proceedings of the National Ovarian Workshop 1998

Murray A A., Molinek M., Baker S J., Hillier S G., Spears N.

Ascorbic acid promotes follicle integrity and survival in in tact murine ovarian follicles in vitro.

Proceedings of the National Ovarian Workshop 1999

Murray A A., Smith R., Sršeň V., Hillier S G., Spears N.

The effect of elevated androgens and oestrogens on follicular development and oocyte competence in vitro.

J. Reprod. Fert. Abstract Series 2000

Murray AA., Edwards D., Smith., RE., Rosenfeld CS., Lubahn DB., Hillier SG., Spears N.

The effect of alpha oestrogen receptor deletion on ovarian function.

Proceedings of the National Ovarian Workshop 2002

Abstracts of poster presentations

Murray A A., Molinek M., Hillier S G., Spears N.

The effect of gonadotrophins during follicle growth on oocyte viability and subsequent development.

Serono Symposia, Ovulation: Scientific and Clinical Concepts. Salt Lake City USA. 1998.

Assistance given throughout this investigation

All the work detailed throughout this thesis was conducted by myself with the following exceptions:

Chapter 3: Gelatin zymography to examine MMPs and TIMPs was conducted in the laboratory of Dr Mike Smith, Animal Sciences Department, University of Missouri, Columbia, Missouri, USA.

Chapter 4: Dr Shanthi Muttukrishna, UCL, London, carried out the assays for inhibins A and B.

Chapter 6: Dominic Edwards (undergraduate student) under my supervision assisted in the IVF procedure and evaluation of the blastocysts obtained from the ERKO mice.

Histology: Vivian Allison and Grace Grant cut the sections of ovary and follicles shown in Chapters 3, 4 and 6.

TUNEL Staining of Follicles: Dr Vlastimil Sršeň carried out the TUNEL staining of *in vitro* grown follicles shown in Chapters 4 and 5.

Oestradiol and Androstenedione Assays: Ms Martha Urquhart, Reproductive Endocrinology, University of Edinburgh, performed the androstendione assays described in Chapters 4 and 5. Michael Molinek and Rowena Smith carried out some of the oestradiol assays described in Chapters 3, 4 and 5.

Various members of the laboratory assisted me in the setting up of large follicle cultures and also with PCR genotyping. They were Vivian Allison, Stuart Baker, Michael Molinek, Rowena Smith, Vlastimil Sršeň and Derek Thomson.

Acknowledgements

I am indebted for the help and advice that I have received from many people throughout the course of these studies. First and foremost my thanks must go to Norah Spears in whose laboratory I have worked for a number of years. Without her encouragement, patience and kindness this thesis would never have been undertaken or completed. A better boss, mentor and friend would be hard to find. My thanks must go to Steve Hillier who also gave every encouragement, his support and advice. Special thanks go to Michael Molinek whose enthusiasm for the project knew no bounds and who willingly came in to work at all hours and didn't make too much fuss when his chocolate went missing. Special thanks also to Rowena Smith who also shared 'out of hours' duties and whose support and friendship has been much appreciated throughout the writing of this thesis. To Vivian Allison, Stuart Baker, Vlastimil Sršeň, Helen Cameron, Anna Swales and Grace Grant, thank you not only for all the practical help you have given, but the for the many interesting discussions, scientific and otherwise.

Help and advice was given from people outwith the laboratory too. I am grateful to Dr Shanthi Muttukrishna for doing the inhibin assays and Ms Martha Urquhart for the androstenedione assays. I am also grateful to Dr Daniel Brison for taking the time to teach me how to label blastocysts and to Linda Sharp for assistance with the confocal microscopy so that I could examine them. I was fortunate to have the opportunity to spend three weeks working in the laboratory of Dr Mike Smith at the Animal Sciences Center, University of Missouri. My thanks are due not only for

the opportunity, but for the friendship extended to me by Mike and all members of the department during my stay.

Family and friends have lent their support and allowed me to bend their ears. A BIG thanks go to Adam for his patience - maternal duties are now resumed and the computer is all yours. Thanks to my parents for housekeeping and gardening duties, to Monika for comments and cake and to Linda for being there. To the manufacturers of Cadburys' Whole Nut and Bells Whisky- you have my gratitude!

Finally, like so many things, this thesis would never have been accomplished without the unconditional love, encouragement and support of my late husband Brian. Even when I doubted myself, his complete faith in my abilities never wavered. I think he would have been proud. Brian, this is from me to you.

For Brian

Who is just around the corner, just out of sight.

Table of Contents

Chapter Two. General Materials and Methods

Chapter Four. The Effects of Different Gonadotrophin regimes on Ovarian Function

List of Figures

List of Tables

Abbreviations

cAMP	cyclic Adenosine Monophosphate
ART	Assisted Reproductive Technologies
IVF	In Vitro Fertilisation
BSA	Bovine Serum Albumin
CSF	Cytostatic Factor
CSS	Charcoal Stripped Serum
ddH_2O	Double Distilled Water
DNA	Deoxyribonucleic Acid
DES	Diethylstiboestrol
EGF	Epidermal Growth Factor
ER	Oestrogen Receptor
ERα	Oestrogen Receptor alpha
ERβ	Oestrogen Receptor beta
ERKO	Oestrogen Receptor alpha Knock Out Mouse
BERKO	Oestrogen Receptor beta Knock Out Mouse
FBS	Foetal Bovine Serum
FSH	Follicle Stimulating Hormone
GVB	Germinal Vesicle Breakdown
hCG	Human Chorionic Gonadotrophin
hpg$^{-/-}$	Hypogonadal Mutant Mouse
ICM	Inner Cell Mass
IGF	Insulin Growth Factor

IVM	In vitro Maturation
LH	Luteinising Hormone
α-MEM	Minimal Essential Medium, alpha modification
MII	Meiotic Arrest, stage two
MMP	Matrix Metalloproteinase
MPF	Maturation Promoting Factor
OCCs	Oocyte Cumulus Complexes
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PG	Prostaglandin
PGCs	Primordial Germ Cells
PMSG	Pregnant Mare Serum Gonadotrophin
PR	Progesterone Receptor
STWS	Scotch Tap Water Substitute
TBE	Tris Borate Buffer
TE	Trophectoderm
TGF	Transforming growth factor
TIMP	Tissue Inhibitor of Matrix Metalloproteinase
TTLB	Tail Tip Lysis Buffer
TUNEL	Terminal Deoxynucletidyl Transferase Mediated dUTP Nick End Labelling
WT	Wild Type
ZP	Zona Pellucida

Chapter One

General Introduction

1.1 Overview of Follicular Development

By birth, or shortly after, the mammalian ovary is endowed with its lifetime supply of oocytes. Oocytes are maintained within follicles that form the basic unit that sustains the oocyte before its release at ovulation. In the human between 1-2 x 10^6 follicles are present in the ovary at birth (Baker, 1972) of which approximately only 400 will sequentially mature and ovulate (Gosden *et al.,* 1993). The remaining 99.9% begin development but never complete it and default to atresia (Gosden *et al.,* 1993).

Follicles are maintained in a primordial pool from which a few leave each day and begin their growth phase (Gosden, 1995; Snow and Monk, 1983). After initiation into growth, the follicles enlarge becoming multi-laminar before forming a fluid filled cavity in preparation for ovulation. Of the cohort of follicles, which begin their growth phase simultaneously, only a species–specific number will release their oocytes for fertilisation (Gougeon, 1996).

Follicles can leave the primordial pool and begin growth at any time from their formation (Gosden, 1995). During pre-pubertal life follicles cannot complete their development and only reach the earliest stage of antral development. In order to mature fully, follicles during the latter part of their development are dependent on adequate gonadotrophin stimulation (Hillier, 1994). It is not until puberty that the gonadotrophins, follicle stimulating hormone (FSH) and luteinising hormone (LH), are released from the anterior pituitary gland in sufficient amounts to ensure full follicular development (Peters *et al.*, 1981). During the final phase of follicular development the ovary secretes the steroid oestrogen and the glycoprotein inhibin into the circulating bloodstream. Although both gonadotrophins act in concert to

promote oestrogen synthesis (Armstrong and Dorrington, 1977), FSH and LH play different roles in the life cycle of the ovarian follicle. FSH is the primary hormone responsible for the latter stages of follicular development, a function that LH alone is unable to fulfil (Spears *et al.*, 1998). The rising concentration in systemic oestrogen and inhibin acts on the hypothalamic –pituitary axis that, through negative feedback, down regulates FSH release (Clarke IJ, 1996). Only those follicles destined to ovulate can overcome this fall in FSH. Following the fall in FSH, LH release increases in both frequency and amplitude resulting in the LH 'surge' (Ling *et al.,* 1986; Zeleznik and Hillier, 1994). This surge in LH concentration results in ovulation and mature oocytes are released from the follicle for fertilisation. The follicle then forms a corpus luteum the function of which is the production of progesterone (Yong *et al.,* 1994), the hormone responsible for the maintenance of any subsequent pregnancy (Rothchild, 1983). Although LH is the primary gonadotrophin released just prior to ovulation there is also a small surge in FSH. However, it has yet to be established precisely what function this rise in FSH performs (Galway *et al.,* 1990). Thus the fall and rise of gonadotrophins govern the cyclic nature of oocyte release. On release the oocyte is picked up from the surface of the ovary and transferred to the fallopian tube where it awaits fertilisation. After this event the developing zygote travels towards the uterus undergoing a programme of cleavage and differentiation in order to establish the cell populations that give rise to a new organism and the tissues necessary to support it.

It is the oocyte that is responsible for much of the programming that results in a new organism being formed (Gosden, 2002; Gandolfi and Gandolfi, 2001). Only oocytes that have grown and matured successfully within the ovarian follicle have

the ability to become fertilised and support the earliest stages of development. The male gamete contributes its genome and mitotic machinery but very little cytoplasmic material (Schatten *et al.,* 1986). Therefore, the oocyte must provide all the necessary cellular components to support the earliest cleavage stages before the new genome becomes active. The follicular oocyte growth phase is therefore of crucial importance if reproduction is to be successful. Endocrine regulation of ovarian function has been extensively studied and there is an increasing body of literature that focuses on aspects of follicular growth and development such as steroidogenesis, follicle selection and oocyte control of somatic cell function. Far less is understood about how the follicular environment affects the quality of the oocyte.

Due to the limitations of obtaining and working with human material, much of our knowledge on the processes that govern ovarian development and function has been obtained from rodent models. These species are readily available and have the advantage of being easily manipulated, with comparatively short ovarian cycles. It is also comparatively easy to alter gene function in the mouse. A number of models have been created to investigate the role of glycoproteins, steroids and hormones in ovarian function, some of which are detailed in Table 1.1. While the results obtained from these models may not be directly extrapolated to the human, they nonetheless offer a means of exploring some of the basic unanswered questions that intrigue reproductive biologists. The focus of much of my work, and the subject of this thesis, has been to examine, using the mouse as a model, how the factors that the oocyte is exposed to while within the follicle affect its ability to be fertilised and support subsequent embryonic development.

Mutant Gene	Fertility	Ovarian Phenotype	Reference
Activin/Inhibin βB subunit	Sub-fertile		Vassalli *et al,* (1994)
Connexin 37	Infertile	Defects in Follicular development and oocyte meiosis	Simon *et al,* (1997)
Cyclin D2	Infertile	Defects in granulosa cell proliferation	Sicinski *et al,* (1996)
Cyp 19 (Aromatase)	Infertile	Block in Follicular development. Ovulation defects	Fisher *et al,* (1998)
ERα receptor	Infertile	Ovary develops haemorrahagic cysts	Lubahn *et al,* (1993)
ERβ receptor	Sub-fertile	Ovulation defects	Krege *et al,* (1998)
ERα + β receptor	Infertile	Ovary develops haemorrahagic cysts	Dupont *et al,* (2000)
FSH β sub-unit	Infertile	Block in follicular development	Kumar *et al,* (1997)
FSH receptor	Infertile	Block in follicular development	Dierich *et al,* (1998)
GDF9	Infertile	Block in follicular development	Dong *et al,* (1996)
GnRH (*hpg)*	Infertile	Immature ovaries	Halpin (1986)
LH receptor	Infertile	Block in follicular development	Zhang *et al,* (2001)

Table 1.1. Mouse models generated to investigate aspects of ovarian function.

1.2 Oogenesis and Folliculogenesis

During foetal development primordial germ cells (the precursors of the oocytes -PGCs) are first recognisable in the posterior yolk sac. From here they migrate in an ameboid fashion through the connective tissue of the hindgut into the gut mesentery before finally congregating in the gonadal ridges which represent the developing gonads. When first recognised (at embryonic day 7 in the mouse and 1 month in the human) the PGCs are low in number. They multiply rapidly both during migration and when they reach the developing gonad (Besmer *et al.,* 1993; Byskov, 1978). The germ cells, now termed oogonia, reach a peak of around 7 million by the 5^{th} month of pregnancy in humans, and in mice this number reaches 1 million by embryonic day 17. Numbers then fall equally dramatically so that by birth approximately 1million remains in the human ovary and 5,000 in the mouse ovary (Figure 1.1). This loss of cells is due to apoptosis, migration from the gonad and possibly an inability to associate with supporting somatic cells (Austin, 1995; Baker and Neal 1973; Baker 1963).

Shortly before mitosis of oogonia ceases, oogonia leave the mitotic cycle and enter meiosis to become arrested at the diplotene stage of the first prophase of meiotic division. The mechanisms that halt meiotic progression are not yet fully understood. These oogonia are now referred to as primary oocytes. Oocytes are held in meiotic arrest throughout the growth phase, of the resulting follicles, until shortly before ovulation.

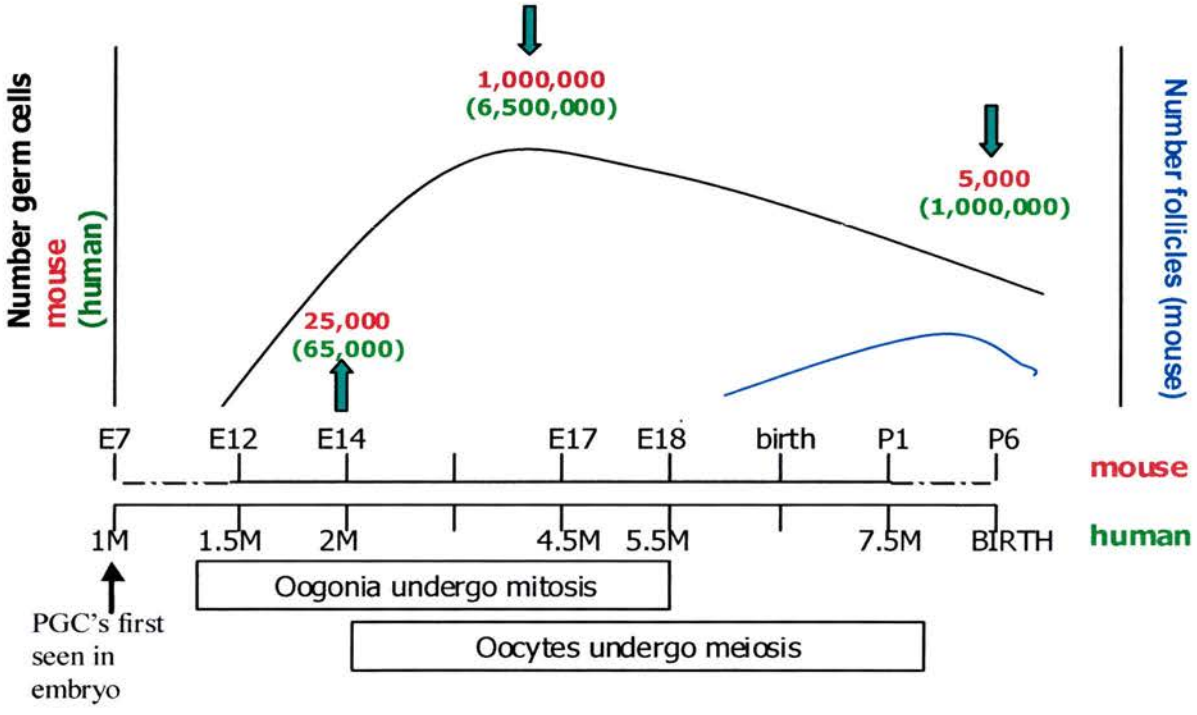


Figure 1.1 Comparison between the mouse and humans of the numbers of oogonia forming in the developing gonad. In both species there is a dramatic loss of oocytes by the time follicles have completed formation

1.3 Initiation of Primordial Follicles into the Growth Phase

In order to form follicles, the primary oocytes need to associate with somatic cells (Gondas, 1970). The gonadal ridge is initially composed of undifferentiated mesenchymal cells. In the mouse the first differentiated cells appear at 12 days post coitum. The three main types of differentiated cells in the gonads are the gamete-associated supporting cells, the steroidogenic cells and connective tissue cells. In females the supporting cells lack the *Sry* (testes determining) gene and become granulosa cells (Albrecht and Eicher, 2001). At around the time of birth the somatic cells invade clusters of germ cells so that a layer of flattened granulosa cells surround each surviving oocyte forming primordial follicles. By birth, or shortly after, the ovary is endowed with a lifetime supply of gametes, contained within primordial follicles, where they remain until initiated into growth.

Depletion of the primordial pool is either through atresia or entry into the growth phase (Gougeon, 1996). Some primordial follicles begin growth shortly after formation while others, depending on species, may wait almost 50 years before beginning growth. The mechanisms and control systems that propel the quiescent primordial follicle into the active growth phase are poorly understood. It has yet to be determined whether follicles grow due to the removal of inhibitory factors or due to their response to stimulatory factors. *In vitro* experiments using pieces of new-born ovary, containing mostly primordial follicles, have indicated that systemic factors may prevent initiation (Wandji *et al.*, 1996) while similar experiments using whole ovaries indicate that intra-ovarian factors may be involved in promoting initiation into the growth phase (Eppig and O'Brien, 1996). In addition, oocyte-derived factors may be involved (Gougeon 1996; Parrot and Skinner, 1999; Vendola *et al.,* 1999). It

seems most likely that a combination of factors controls initiation of follicle growth. Mathematical modelling has suggested that the rate of initiation into the growth phase is dependent on the number left within the pool, with the numbers leaving the quiescent state accelerating with age (Faddy and Gosden, 1995). The first signs of growth are an increase in oocyte volume and proliferation of the surrounding granulosa cells (Lintern-Moore and Moore, 1979). Once follicles have started to grow follicular development can be considered as a two-phase process; a slow growing phase with no absolute requirement for gonadotrophin stimulation and a faster growing phase where responsiveness to FSH, and subsequently LH, is obligatory if the follicle is to proceed to the ovulatory stage. The transition between these two phases is characterised by the formation of a fluid-filled antral cavity and the production of steroid hormones.

Primordial follicles that are initiated into the growth phase at the same time can be considered as a growing cohort. Pre-antral follicular growth proceeds slowly taking weeks in rodents and many months in larger species. As this process requires no systematic pattern of gonadotrophin stimulation, this phase of development is often deemed gonadotrophin independent. In animals naturally or surgically rendered incapable of producing pituitary gonadotrophins follicles are capable of reaching the early stages of antral development (Halpin *et al.,* 1986). Although the actions of gonadotrophins that are associated with later stages of follicular development do not appear to be obligatory during the early growth phase, they may affect pre-antral follicles. It is possible that they may act as survival factors (McGee *et al.,* 1997) or be involved with the acquisition of the steroid producing cells associated with subsequent follicular development (Wu *et al.,* 2000).

1.4 Pre-antral Follicular Development

By the early pre-antral phase stage the follicle has a distinct multi-laminar avascular granulosa cell layer and has also acquired vascularised and distinct thecal cell layers. It has been suggested that thecal precursors may be present at the outset of follicular growth (Hirshfield, 1991) and more recently, growth differentiation factor –9 has been implicated in this process (Soloveva *et al.,* 2000). As the follicle grows, two layers of thecal cell associate with the basement membrane; the theca interna and externa. These cells are derived from the surrounding interstitial tissue. The mechanisms that initiate their differentiation and organisation are as yet unknown although kit-ligand, produced by the granulosa cells, has been implicated (Parrot and Skinner, 1999). Likewise it has been suggested that thecal cells contribute to the development of the granulosa cell layers during early follicular development (Nilsson and Skinner, 2001; Parrott and Skinner, 1998). During the latter faster growing phase of follicle development they are necessary for steroidogenesis (Hillier *et al.,* 1994; Hedin *et al.,* 1987).

In all mammalian species studied so far, most follicles that simultaneously begin growth reach the late pre-antral stage of development and few are lost to atresia (Richards, 1994). This may be because granulosa cells are pre-programmed to replicate a set number of times before reaching the end of their natural life span (Hirshfield, 1991). In order to further proliferate and differentiate the cells must be capable of responding to the cyclic rising levels of FSH. Only a proportion of follicles within the growing cohort, are at a stage of development capable of

responding and most of them will die at the early antral stage of development. In humans, approximately 20 follicles from the growing cohort continue through to the pre-ovulatory stages of development (Gougeon, 1996; Hillier, 1994). The growth rate of the selected follicles must now accelerate as it is from this population that some will attain dominance and release their oocytes for fertilisation, a process that takes ~4 days in the rodent and ~28 days in the human.

1.5 Antral Formation and Gonadotrophin Dependence

While follicles are not dependent on gonadotrophin stimulation to form small antral cavities, those selected to develop further now become increasingly dependent upon stimulation by the gonadotrophins if they are to form large antral cavities and reach the pre-ovulatory stage (Kumar *et al.*, 1997; Halpin *et al.,* 1986). Antrum formation occurs when 2-3,000 granulosa cells are present regardless of species and the final size of the follicle (Gosden *et al.*, 1988). Fluid begins to accumulate within the small spaces between the granulosa cell layers. These eventually coalesce to form a large antral cavity. Follicular fluid is filtered from the blood supply that penetrates the thecal layers of the follicle. Proteins of high molecular weight are excluded and the concentrations of glucose, lipids and amino acids differ to those found in plasma. During later stages of development fluid is accumulated rapidly, accounting largely for the marked expansion in follicular size (Hirshfield, 1991). A variety of molecules including steroids, growth hormone binding proteins and proteoglycans accumulate within the fluid (Driancourt *et al* 2001; Driancourt and Theul, 1998). While the precise purpose of antral fluid has yet to be elucidated, it may act as a 'sink', diluting or concentrating metabolites from

different cell types of the follicle. For example, the actions of oocyte derived factors may only be allowed to exert an influence over their immediate surrounding cells before being 'diluted out' within the follicular fluid, hence limiting their action on cells furthest from them.

During this period of differentiation and antral formation, follicles become increasingly dependent upon stimulation by the gonadotrophins. In response to FSH granulosa cells start to rapidly proliferate and differentiate into two populations, the mural granulosa cells which are adjacent to the basement membrane and the cumulus cells which surround the oocyte (Amsterdam *et al.,* 1975). The antral cavity rapidly enlarges separating the two cell types and eventually the oocyte, surrounded by the cumulus cells, becomes embedded within the follicular fluid connected by a stalk of cells. During the latter stages of follicular growth the mural granulosa cells cease proliferating, however the cumulus cells continue to do so until ovulation. It has been proposed that the granulosa cell complement may be a marker of an ovulatory follicle as mice deficient in the cell cycle activator, cyclin D2, are anovulatory (Richards, 2001, Robker and Richards, 1998; Sicinski *et al.,* 1996).

FSH receptors are found exclusively within the granulosa cells of follicles and first appear when 2-3 layers of granulosa cells are present (O'Shaughnessy *et al.*, 1996). The function of FSH receptors during the early growth stages is unclear, and as growth until follicles are selected for further development is subject to many cyclic fluctuations in FSH concentration, presumably these receptors are not capable of inducing the intracellular pathways associated with later follicular development. LH receptors are initially confined to the thecal layers and appear from the time of thecal cell differentiation (Camp *et al.,* 1991). FSH receptors up-regulate the second

messenger cyclic AMP and protein kinase pathways (Simoni *et al.,* 1997). These are responsible for activating certain genes crucial to growth and steroid production Yong *et al.,* 1992; Langan, 1968). The acquisition of FSH receptors may be crucial for follicles if they are to undergo antral development, as only those able to respond to the cyclic rise in circulating FSH carry on down the developmental pathway. It has been postulated that each follicle has an individual FSH threshold, beyond which it must be stimulated if it is to continue to develop. Failure to respond to FSH pushes the follicle down the atretic pathway (Hillier, 1994; Brown, 1987).

During the latter stage of growth, follicles acquire LH receptors within the mural granulosa cell layers (Camp *et al.,* 1991). These LH receptors act in synergy with FSH to augment steroid production by the follicles (Hillier *et al.,* 1994). It has been proposed that developing follicles have a finite requirement for stimulation by LH, with each follicle having an upper limit which if exceeded, becomes detrimental to follicular and perhaps oocyte development (Loumaye *et al.,* 2003; Hillier, 1994). LH, like FSH, exerts its actions via stimulation of the cyclic adenylase pathway, elevating cyclic adenosine monophosphate (cAMP), which sustains the follicle during the pre-ovulatory drop in systemic FSH concentrations (Yong *et al.,* 1994; Zeleznik anmd Hillier, 1984). Therefore, only follicles with FSH induced granulosa cell LH receptors (Whitelaw *et al.,* 1992; Amsterdam *et al.,* 1975) are mature enough to reach the ovulatory stage

While responses to the endocrine environment can explain many aspects of how follicles are selected for antral development and which of those ultimately reach ovulation, it is likely that interactions between the follicles themselves also play a role in modulating the responses to gonadotrophins (Spears *et al.,* 2002; Baker *et al.,*

2001; Baker and Spears,1999). The whole process of follicular development from the primordial stage through to ovulation is depicted in Figure 1.2.

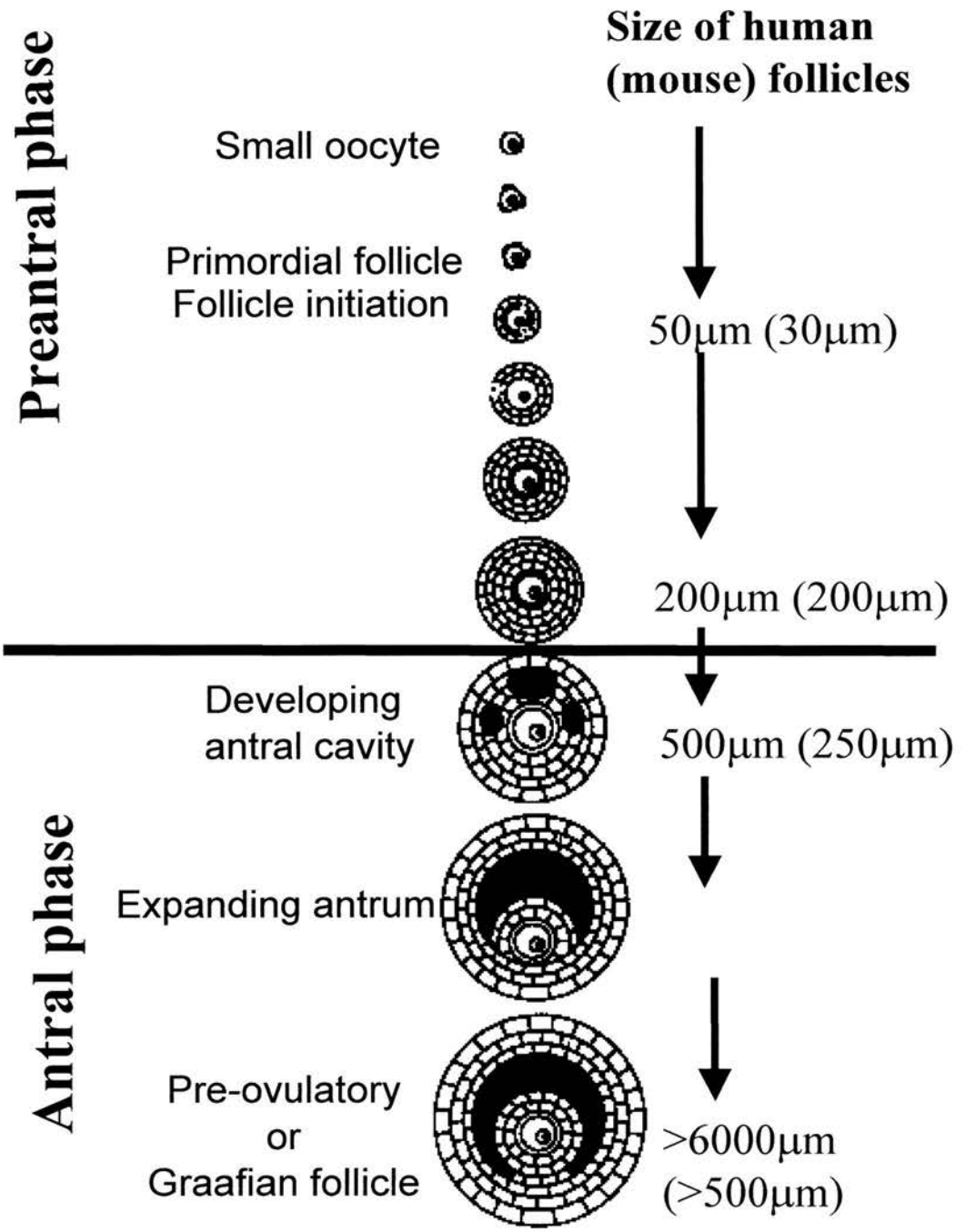


Figure 1.2 Diagrammatic illustration of stages and sizes of developing follicles in the human and mouse.
(*adapted from Gosden et al., 1993)*

1.6 Steroidogenesis

During the antral growth phase, follicles also acquire the ability to synthesise steroid hormones. It has long been recognised that oestrogen biosynthesis requires the co-ordinated actions of both FSH and LH acting on the two somatic cell types (Armstrong and Dorrington, 1977) as shown in Figure 1.3. The key to steroidogenesis is the induction of specific enzymes within each of these two cell types that permit steroid production from circulating cholesterol. Cholesterol is imported into the thecal cells where it is mobilised to the inner mitochondrial membranes mediated by steroid acute regulatory protein (Clark *et al.,* 1995). Once internalised within the mitochondria, the first step in steroidogenesis is the conversion of cholesterol to pregnenolone by the enzyme cytochrome P450 cholesterol side chain cleavage (P450scc). Both the internalisation of cholesterol within the mitochondria by StAR and conversion to pregnenolone by P450scc can be considered rate-limiting steps in steroidogenesis (Clark *et al.,* 1995; Lambeth and Stevens, 1985). Thecal cells express cytochrome P450c17, the enzyme responsible for the conversion of pregnenolone to androgen (Smyth *et al.,* 1993*)*. Binding of LH to its receptors on thecal cells activates the cAMP and protein kinase pathways, which enhances these steroidogenic pathways (Gelety and Magoffin, 1997). Androgens diffuse through the follicular basement membrane where they are available to the granulosa cells for aromatisation to oestrogens. In order for aromatisation to occur, FSH, via the cAMP/kinase A pathway induces cytochrome p450 aromatase (encoded by the CYP19 gene) in the granulosa cells (Yong *et al.,* 1994). This enzyme is responsible for the formation of oestrogens from substrate

androgen. The principal androgen produced is androstenedione while oestradiol is the major oestrogen produced (Hillier *et al.,* 1994).

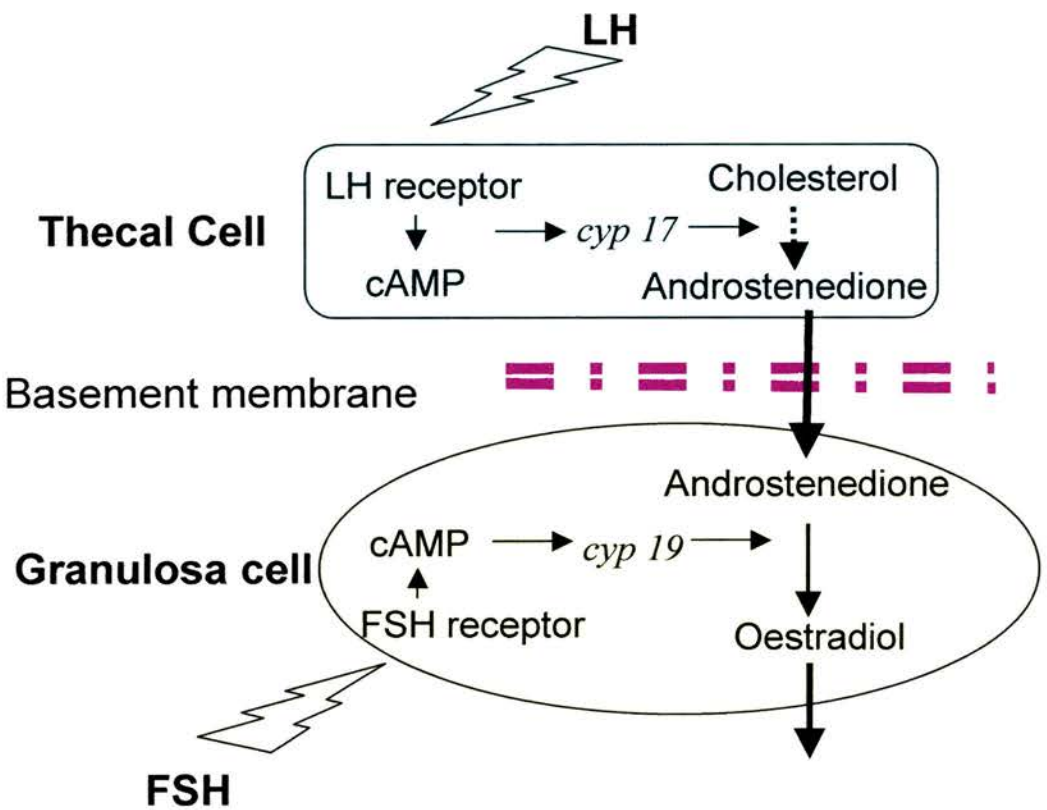


Figure 1.3 The 'two-cell two gonadotrophin' model of steroidogenesis. LH initiates androstendione production in the thecal cells. Under the influence of FSH androgen is aromatised to oestradiol in the granulosa cells.

The steroids themselves have been reported to exert paracrine effects within the ovary. High follicular androgen concentration has been reported to have atretogenic effects (Billig *et al.,* 1993). In contrast *in vitro* experiments have shown that androgens have direct stimulatory roles on follicle development (Murray *et al.,* 1998) and that they may increase cAMP signalling with the net result that aromatisation is augmented (Tesuka and Hillier, 1997). Similarly there is conflicting evidence as to the role of oestrogens on the ovary. Some studies have shown that oestrogens have stimulatory effects on follicular development (Hsueh, 1986; Richards, 1980; Williams, 1940), while others have cast doubt on the role of this steroid as a paracrine mediator (Spears *et al.,* 1998; Zelenski-Wooten *et al.,* 1994). Transgenic animals have been generated in order to try and elucidate the role of oestrogen (Krege *et al.,* 1998; Fisher *et al.,* 1998; Lubahn *et al.,* 1993). These animals have deletions in genes that code for oestrogen receptors or the aromatase enzyme. The phenotypes of these animals all indicate ovarian imbalances. However, it is unclear if these effects are due to intraovarian effects or the result of disturbances in the circulating gonadotrophin levels.

1.7 Activins, Inhibins and Follistatin

The inhibins and activins were first isolated from follicular fluid. They were so named as they were found to inhibit (inhibin) or stimulate (activin) FSH release from pituitary cells *in vitro* (Knight, 1996; Muttukrishna *et al.,* 1991; Ling *et al* 1986). These glycoproteins share structural homology with, and are members of, the transforming growth factor (TGF) β super-family of growth factors (Vale *et al.,* 1986). The inhibins are composed of a common α sub-unit that form heterodimers with one of two β subunits (βα and ββ), producing either inhibin A or inhibin B. Activin is a homodimeric form of inhibin β subunits. Different genes encode the three sub-units (Esch *et al.,* 1987; Mason *et al.,* 1986)

Two receptors for activin have been identified in all compartments of the ovarian follicle and these may be differentially expressed, each having its own function in reproduction (Wu *et al.,* 1994). A specific inhibin receptor has yet to be found in the ovary although there is some evidence that a putative membrane bound receptor exists in the testis (Robertson *et al.,* 2000). It has been found that inhibins can bind to the type II activin receptor by binding with elements of the TGF β receptor. The opposing actions of inhibin on activin activity may, therefore, be through competition with activin for its receptor (Lewis *et al.,* 2000).

Follistatin is an activin binding protein that has also been isolated from follicular fluid. However, it is structurally unrelated to inhibin and activin and is encoded by a separate gene (Sugino *et al.,* 1994; Robertson *et al.,* 1987). It shares some similarity with inhibins in that it inhibits FSH release, but with less potency (Ueno *et al.,* 1987). *In vitro* studies of granulosa cells from rats and non-human

primates have shown that production of inhibins and activin is developmentally regulated throughout the ovarian cycle (Miro and Hillier, 1996; Matzuk *et al.,* 1992; Rabinovici *et al.,* 1990). The inhibin β sub-unit is produced more abundantly in early antral follicles, leading to activin being primarily formed (Schwall *et al.,* 1990). As follicles approach the pre-ovulatory stage more inhibin α sub-unit is produced leading to increased inhibin concentration (Knight and Glister, 2001; Magoffin and Jakimuk, 1998; Hillier, 1991). Further to this it has been demonstrated that in *hypogonadal* mice production of the inhibin α sub-unit is gonadotrophin independent while inhibin β production is reliant on gonadotrophin responsiveness of the granulosa cells (O'Shaughnessy and Gray, 1995). Concomitant with the switch between activin and inhibin production, follistatin concentration also rises with follicular development (Nokatani *et al.*, 1991). Thus, not only is inhibin formed preferentially (due to the availability of α sub-unit), but activin action is further dampened by the increase in its binding protein, follistatin. This rise in bioactive inhibin production mirrors the pattern of oestrogen production and both ovarian inhibins and activin have been identified as modulators of pituitary gonadotrophin release (Knight 1996; Rivier and Vale, 1991).

There is also *in vitro* evidence to suggest that the inhibins and activins exert autocrine/paracrine effects within the ovary, modulating follicular growth and steroidogenesis. Activin is associated with proliferation and differentiation of the granulosa cells and in promoting FSH receptor expression (Matzuk *et al.,* 1992; Xiao et al., 1992). It has been hypothesised that activin production by a follicle may confer a developmental advantage at the crucial stage where responsiveness to gonadotrophins becomes obligatory for further development (Knight and Glister,

2001; Miro and Hillier, 1992). Activin also has an effect on steroidogenesis, promoting cytochrome P450 aromatase activity and suppressing progesterone production within the granulosa cells (Miro and Hillier, 1991; Hutchison *et al.*, 1987*)*. Paradoxically, activin has been found to inhibit androgen production through paracrine action on the thecal cells (Smyth *et al.,* 1993). This action of inhibin could be an important regulatory mechanism that ensures pre-ovulatory follicles have an adequate supply of androgen to support increasing oestrogen synthesis. It has been hypothesised that, throughout development, the activin and inhibin 'tone' of the follicle shifts from predominant activin production in the early growth phases to high inhibin concentrations during pre-ovulatory development ensuring that follicles destined to ovulate have an adequate supply of substrate to support oestrogen synthesis, the role of follistatin being to attenuate activin activity (Hillier *et al*, 1991). Further evidence supporting this theory has come from measurements of these glycoproteins in the follicular fluid of normally cycling women (Magoffin and Jakimuk, 1998).

1.8. Oocyte Growth and Maturation

Oocyte growth is commensurate with follicular growth. Primordial follicles that are entering their growth phase can be characterised by morphological changes to the somatic cells and an increase in oocyte size. In the mouse the oocytes in primordial follicles grow from 15 μm to 80 μm over a few weeks while in the human the oocyte grows from 35μm to 120 μm in a few months. This represents an ~300 fold increase in volume (Gougeon, 1996; Gosden and Bownes, 1995). Mammalian oocytes are generally thought to have completed their growth phase by the early

antral stage of follicle growth (Gosden *et al.,* 1997). There are marked changes in the ultra structure of the oocyte, cell organelles become far more abundant and disperse throughout the cytoplasm. One exception is the centrioles, which disappear and are inherited paternally after fertilisation except in the mouse (Schatten *et al.,* 1986; 1985). Lipid, proteins and glycogen granules accumulate to a greater or lesser extent dependent on species. From the outset of growth, the oocyte secretes the extra-cellular matrix components that form the zona pellucida and there is the appearance of some novel organelles such as the cortical granules (Wassarman, 1988). Both these features have an important role to play in the fertilisation process. Another characteristic of growth is the rapid rates of transcription and translation. In rodent oocytes, 0.5ng RNA and 25ng of protein have accumulated by the time the oocyte has reached maturity (Gosden, 2002; Pico and Clegg, 1982). While RNA and proteins are normally subject to rapid turnover, the oocyte has the capacity to package and store these molecules until they are required at points further along the developmental pathway (Gandolfi and Gandolfi, 2000).

At all stages of follicular development bi-directional communication exists between the oocyte and the surrounding somatic cells, facilitated by long processes which penetrate the zona pellucida. The oocyte and somatic cells each contribute half the connexin channel made up of connexon proteins. Each half of the channel can be either the same protein or can be different. It is known that the connexin within the oocyte is connexin 37 but the granulosa cell connexin has yet to be identified (Nicholson and Bruzzone, 1997). The importance of this two-way communication system is illustrated in mice lacking connexin 37. These animals are infertile, have no pre-ovulatory follicles and develop premature corpora lutea (Simon *et al.,* 1997).

Intercommunication between the oocyte and somatic cells has been the subject of much research and, as shown in Figure 1.4, both contribute to the function of the other. Many aspects of follicular development are controlled by factors secreted by the oocyte. The oocyte specific factor GDF9 is an absolute requirement for pre-antral follicle development and alters the expression of granulosa cell genes in the pre-ovulatory follicle (Elvin *et al.,* 2000). It has also been found that during the later phase of follicular growth, oocyte produced factors control the phenotype of the granulosa cells (Eppig *et al.,* 1997), steroid synthesis (Vanderhyden and MacDonald, 1998), prevent premature luteinisation (El-Fouly *et al*., 1970) and stimulate cumulus cell expansion prior to ovulation (Salustri *et al.,*1990).

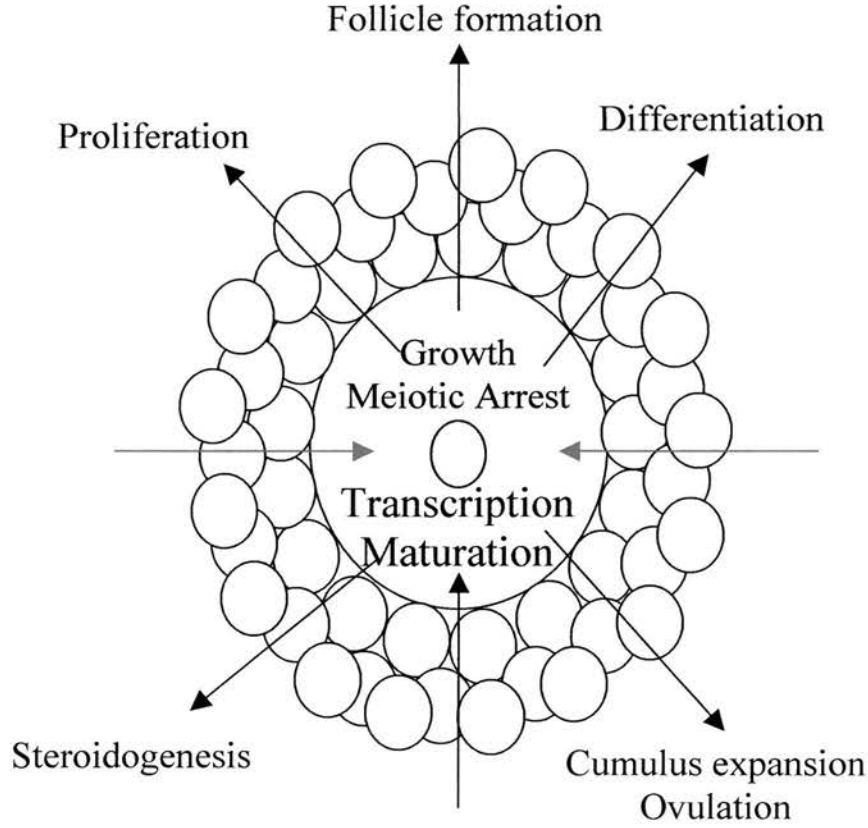


Figure 1.4. Bi-directional communication between the oocyte and the somatic cells showing the influences of the granulosa cells on the oocyte and processes in the granulosa cells controlled by oocytes.
(adapted from Eppig, 2001)

Likewise factors produced by the somatic cells are responsible for many aspects of oocyte development such as nutritional support and transport of metabolites. Another important function of the somatic cells is to prevent oocytes from prematurely resuming meiosis. It is currently thought that high levels of cAMP, produced by the granulosa cells under the influence of gonadotrophin stimulation, are transported into the oocyte via gap junctions. Other mechanisms presumably exist to prevent meiotic resumption during the early gonadotrophin insensitive phase of follicular development (Eppig and Downs, 1988; Downs and Eppig, 1987). Ovulatory events disrupt the transfer of cAMP thus allowing the meiotic mechanisms to activate, but levels of cAMP must rise again ensuring that the oocyte is held in the second meiotic arrest phase, where it remains until fertilisation.

Throughout the follicle-enclosed growth period, the oocyte is maturing in preparation for fertilisation and embryonic development. By the time of release from the follicle, the oocyte will have achieved both nuclear maturation (resumption of meiosis until the first meiotic division and production of the first polar body) and cytoplasmic maturation (the mechanisms that promote monospermic fertilisation and sustain the earliest stages of embryogenesis). The relationship between the follicular stage of development and oocyte competence is highly co-related with the progression of both nuclear and cytoplasmic maturation (Eppig, 1997; 1994). Oocytes from large antral follicles can undergo germinal vesicle breakdown (GVB) and proceed to the second meiotic arrest (MII) whereas oocytes from pre-antral follicles cannot. An intermediate stage exists between the two stages whereby oocytes can undergo GVB but arrest before the first meiotic division is complete (Sorenson and Wasserman, 1976). Similarly, aspects of cytoplasmic maturation are

acquired sequentially, oocytes gradually gaining the ability to firstly cleave to two cells (where they may arrest if not fully mature), and subsequently gain the ability to support pre-implantation development (Eppig and Schroeder, 1989). Although it has been shown that the developmental programmes within oocytes controlling nuclear and cytoplasmic maturation can proceed independently of one another, under normal circumstances these two aspects of maturation are highly co-ordinated. Indeed some aspects of final maturation require mixing of the germinal vesicle contents with those of the cytoplasm (Eppig *et al.,* 1994; Borsuk, 1991).

There is an expanding body of literature from *in vitro* experiments that implicate a wide range of growth factors in the process of oocyte maturation. Some of these have direct effects on the oocyte while others probably exert their actions via the surrounding somatic cells. These include insulin growth factor (IGF) (Adashi, 1998), activin and inhibins (Findlay *et al.*, 2001), epidermal growth factor (EGF) and TGFβ (Driancourt and Theul, 1998). A number of receptors have been found within the oocyte including, oestrogen receptor (ER), IGF receptor, EGF receptor and activin receptors, but very little is known about their regulation (Wu *et al.,* 1993; Hill *et al.,* 1999; Kezele *et al.,* 2002). These data have been obtained from a number of species and it has yet to be investigated whether there are species-specific actions of some of these growth factors.

Another aspect of maturation that needs to be considered is the epigenetic phenomenon of genomic imprinting. Nuclear transplantation studies performed during the 1980s revealed that uniparental embryos that contain two haploid sets of either the maternal or paternal genome are unable to develop beyond the early implantation stage (Surani, 1984). These experiments revealed that the expression of

some genes is differentially controlled and is dependent upon which parent the allele was inherited from. To date, several dozen genes have been found to be imprinted (Reik *et al.*, 2001). Of these it has been found that several play roles in embryo development, tumorigenesis and genetic diseases. The exact nature of the marks that imprint genes has yet to be discovered but a likely candidate is methylation of DNA. Transcription can be silenced either directly by hypermethylation of an allele or through interaction with factors that either promote or suppress transcription (Bartolomei and Tilghman, 1997).

During the formation of the gametes there is a global demethylation of the genome and loss of allele specific imprints. This is the only time that the two gametes have equivalent epigenetic status, therefore, during spermatogenesis and oocyte growth, new sex-specific imprints must be established before fertilisation. Studies using constructed oocytes containing two sets of maternal haploid genomes from non-growing and fully-grown oocytes have shown that maternal epigenetic modifications are introduced during the oocyte growth period (Kono, 1998). Evidence that methylation has a role in either establishing and/or maintaining these marks has come from methyltransferase deficient mice, which die during gestation due to abnormalities in the growth patterns associated with imprinted genes (Li *et al.*, 1992). The nature of the signals that mark certain genes for imprinting either via methylation or by other means has yet to be established.

1.9. Ovulation

The ovulatory surge of gonadotrophins (mainly LH and to a lesser extent FSH) is triggered by the high circulating levels of oestrogen and inhibins produced by the pre-ovulatory follicles. This surge initiates a series of complex cascades that culminates in follicle rupture and the release of a mature fertilisable oocyte or oocytes depending on species (Clarke, 1996).

Within a few hours the ovary becomes red and swollen and there is an increase in angiogenesis and blood volume resulting in the expansion of the thecal capillaries. Ovulation shares many characteristics with that of an inflammatory response. Cells associated with inflammation, such as leukocytes and macrophages invade the tissues and there is an increase in biochemical markers associated with inflammation. These include cytokines, histamine and platelet activating factor. There is also activation of phospholipases and subsequent synthesis of prostaglandins (PG) E and F whose concentrations reach a peak during follicular rupture (Espey, 1994). Recently there has been much interest in the role of 13β hydroxydehydrogenase, the enzyme that controls glucocorticoid synthesis. Commensurate with the rise in prostaglandin synthesis there is a shift in the balance between hydrocortisol and cortisone that promotes wound healing (Hillier and Tesuka, 1999). This whole process of rupture and repair must be completed within a few days in species such as rodents that ovulate on a 4-5 day cycle.

A number of changes occur within the follicle in response to the gonadotrophin surge. The high level of LH inhibits cell cycle regulators resulting in the cessation of granulosa cell proliferation (Robker and Richards, 1998). Lipid droplets accumulate within the cells and there is a change in steroidogenic activity.

An increase in P450side chain cleavage enzyme activity results in increased progesterone and a decrease in oestrogen production. The oocyte is now only attached to the mural granulosa cells by a thin 'stalk' and lies within the follicular fluid surrounded by cumulus cells, which undergo expansion and mucification. 2-3 hours after the elevation in gonadotrophin concentration the cumulus cells begin to excrete hyaluron and extract fluid from the spaces between them. The oocyte itself orchestrates this event but the factors governing the process are as of yet unknown but appear to be mediated by FSH (Buccione *et al.,* 1990). Concurrent with cumulus cell expansion, gap junction communication between the oocyte and its surrounding cells is broken resulting in the oocyte resuming meiosis and reaching the second meiotic block and the production of the first polar body.

Ovulation occurs at the opposite point or apex to the stalk attachment. Within the thecal layers of the apical region the fibroblast cells elongate and the epithelial cells become necrotic (Espey, 1998). The tissue surrounding the apex bulges and the cumulus cells begin to protrude, forming the so-called stigma. Different protease systems are implicated in the breakdown of the follicular basement membrane. The metalloproteinases (MMPs), the serine kinase (tissue plasminogen activator) and the urokinase plasminogen activators all have increased activity around the time of ovulation (Smith *et al.,* 1999; Hagglund *et al.,*1999; Liu and Hsueh, 1987). However, blocking these proteolytic enzymes does not prevent ovulation and, therefore, their actions alone cannot bring about the complete degradation necessary to allow the oocyte to free itself from the follicle. Targeted disruption of the progesterone receptor (PR) gene in mice impairs ovulation suggesting that the actions of this steroid are obligatory for ovulation. Two genes encoding the cathepsin and

ADAMTs protease pathways have been identified as being induced by LH and PR (Robker *et al.,* 2000).

Rupture of the follicular wall results in the extrusion of the oocyte-cumulus complex and antral fluid to the ovary surface. The remaining follicle cells undergo terminal differentiation, the mural granulosa cells luteinise and thecal capillaries and fibroblasts rapidly penetrate the structure to form the corpus luteum. The main function of this gland is to produce progesterone that supports endometrial function. In the absence of pregnancy the corpus luteum undergoes spontaneous regression leaving an avascular scar referred to as the corpus albicans (Adashi, 1994).

1.10. Fertilisation

The ovulated oocyte is picked up from the ovarian surface by fimbria and swept along the fallopian tube until it reaches the ampulla region where it waits to be fertilised. This process is inhibited if the oocyte is denuded of the cumulus cells. Fertilisation has to take place within a short window of time as both male and female gametes have short life spans. Oocytes survive for 6-24 hours in the human, 6-12 hrs in mouse, while the life span of sperm is 24-48 hrs in human and 6-15 hrs in the mouse.

The oocyte-cumulus mass produces chemo-attractants (Eisenbach and Tur-Kaspa, 1999) which have the effect of directing the motility of the sperm, thereby ensuring that some sperm reach the ampulla region. The oviduct undergoes specific changes induced by the hormones released throughout the follicular phase which ensures a favourable environment that optimises the fertilisation process (Leese *et al.,* 2001; Boatman, 1997). In addition components of seminal fluid have been found

to promote the fertilisation process (Fraser, 1999). While ~10^7 sperm are released by the male only about ~100-150 of these will reach the oocyte cumulus mass (Wassarman, 1999). Deposited sperm undergo capacitation within the female tract that allows them to bind to the oocyte and undergo the acrosome reaction. Only capacitated acrosome-intact sperm are capable of fertilisation.

Sperm penetrate the cumulus mass surrounding the oocyte and bind to receptors within the zona pellucida (ZP). The ZP is laid down throughout oocyte growth and the process ceases shortly before ovulation. This extra-cellular matrix, ~7μm thick in the mouse, is made up of three glycoproteins ZP1, ZP2 and ZP3, which are closely related in many mammals (Wasserman, 1988). The zona proteins contain receptors for sperm binding. Binding between sperm and the zona protein receptors hold the key to the prevention of inter-species cross fertilisation. In closely related species or when the zona is removed it is possible to produce hybrids.

Much research has concentrated on sperm-binding interactions and it has been found that, in the mouse, ZP3 is essential to sperm binding and initiation of the acrosome reaction (Rankin *et al.*, 1999). Some attention has also been paid to elucidating whether sperm have reciprocal oocyte binding proteins and over two dozen sperm proteins and glycoproteins have been identified in aiding binding (Wasserman, 2001). Once bound to a binding site on the receptor, capacitated sperm interact with other regions of the ZP3 receptor inducing the acrosome reaction. The acrosome is a large secretory vesicle that lies in the apical region of the sperm head. It contains lytic enzymes which, when released, change the structure of the ZP, digesting away any local ZP3 receptors and so preventing further sperm binding to the oocyte. While ZP3 has been identified as the prime activator of acrosome action,

recently progesterone has been implicated in facilitating the reaction by making the acrosome leaky and allowing hyaluronidase to be released (Calogero *et al.,* 2000*)*, which in turn digests the cumulus matrix from around the oocyte. This allows direct contact between the sperm and oocyte plasma membranes. Once activated, ZP2 receptors bind to the sperm and aid the passage of the sperm to the oocyte plasma membrane where the two plasma membranes fuse (Rankin *et al.,* 2001). Fusion of the membranes occurs at the microvillus surface of the ooplasma but not in the region of the second metaphase plate and first polar body, presumably to prevent aneuploidy. Fusion of the two membranes results in the sperm moving into the cytoplasm of the oocyte and hence contributing the paternal genome and the centrosome (not present in the oocytes of most species, the mouse being the exception) which directs microtubule assembly (Mandahar *et al.*, 1998). This leads to the union of the two nuclei and the signal to initiate the metabolic activation of the oocyte.

Binding of the spermatozoa induces changes within the oocyte known as oocyte activation. In all species studied, sperm binding activates the development of the oocyte by increasing intracellular calcium levels. The exact mechanism by which sperm achieve this has yet to be elucidated. It is unlikely that sperm introduce calcium into the oocyte cytoplasm but, it is thought, they activate the release of oocyte calcium stores through introduction of a soluble sperm factor (Swann, 1996). Further evidence that the oocyte contains all the calcium required for activation comes from experiments that demonstrate that calcium accumulation and mechanisms of free calcium release are acquired throughout oocyte growth and maturation (Carroll *et al.*, 1996). On sperm binding, calcium is released in waves

across the oocyte. In hamsters, the initial wave crosses the oocyte in 6 seconds from the point of sperm-egg fusion (Miyazaki *et al.,* 1986). Subsequent waves also cross the oocyte but originate at a point away from the sperm-egg binding site (Swann, 1996). Release of calcium is responsible for triggering the exocytosis of cortical granules from the oocyte and completion of meiosis and entry into the first embryonic division. Cortical granules accumulate throughout oocyte growth and are found around the periphery of the cytoplasm. The rise in free calcium initiates the release of these granules, which then fuse with the plasma membrane and release their contents into the perivitelline space resulting in a modification of the zona pellucida (Yanagimachi, 1978). Thus polyploidy is prevented by the initial events that occur locally at the sperm-oocyte binding site and with an overall modification to the zona pellucida after cortical granule release.

While the sperm genome is truly haploid the oocyte must still undergo the second meiotic division if it is to contribute only one set of chromosomes to the new organism. In the oocyte, meiotic arrest is dependent upon the activity of maturation promoting factor (MPF) that consists of cdk1 and cyclin B complex stabilised by cytostatic factor (CSF) (Zernicka-Goetz *et al.,* 1995; Eppig *et al.,* 1994). The intracellular rise in calcium associated with fertilisation destroys this complex and therefore, MPF levels drop and the oocyte exits metaphase (Dubicella *et al.,* 2002). This results in the oocyte cleaving, generating a second polar body containing one set of chromosomes and a much larger zygote. Therefore, oocytes can be considered as never truly being haploid.

On entry into the oocyte cytoplasm the sperm nuclear membrane breaks down and the chromatin decondenses, a process induced by oocyte cytoplasmic factors. In

addition the sperm genome needs to be stripped of sperm chromatin packaging proteins i.e. protamines and repackaged with histones (Latham and Schultz, 2001). Approximately 4-7 hours after fusion the two sets of haploid chromosomes are each surrounded by a membrane forming two pro-nuclei. These then move together into a central position each haploid pronucleus synthesising DNA in preparation for the first mitotic division. When they are aligned, the pro nuclear membranes break down and the mitotic metaphase spindle forms along which the chromosomes align Schatten *et al* 1985). The final phase of fertilisation is achieved with the coming together or syngamy, of the gametic chromosomes. Immediately after the first mitotic anaphase and telophase the one cell zygote cleaves becoming a two-cell embryo (Gilbert GS, 2000)

1.11. Pre-Implantation Development

Pre-implantation development is the term given to the 2-cell transition to the blastocyst stage. Throughout this period the conceptus moves down the fallopian tube until it reaches the uterus ready for implantation undergoing many cellular divisions at a rate characteristic to each species. Formation of the blastocyst takes 3-4 days in the mouse (Hogan *et al,* 1994) and 4-5days in the human (Rankin *et al.,* 2000). In all mammalian species cell doubling occurs synchronously until the 8-cell stage followed by an asynchronous stage resulting in a blastocyst with differentiated cell populations. Until the 8-cell stage the cells are called blastomeres and after differentiation the blastocyst consists of an inner cell mass (ICM) eccentrically placed within a fluid filled cavity and the whole being surrounded by the trophectoderm (TE) cells (McLaren, 1982). Throughout the pre-implantation period

the developing blastocyst remains enclosed within the ZP (Bronson and McLaren, 1970). The purpose of this is probably to hold the blastomeres together and it may also prevent chimaeras from forming. Shortly before implantation the blastocyst hatches from the zona.

Stimulation to grow is provided by a number of growth factors, some of which are endogenously produced while others are provided by the oviductal environment. However, it is possible to grow blastocysts *in vitro*, as exploited in assisted reproduction, and much of our information on growth and development has been obtained from *in vitro* produced material. Comparisons between *in vitro* and *in vivo* derived embryos have shown that the rate of growth and development is delayed *in vitro*, indicating that both endogenous and oviductal factors co-ordinate in the development of the blastocyst. Growth factors implicated in the 2-cell to blastocyst transition include insulin and insulin-like growth factors, the TGF family, leukaemia inhibitory factor and gondotropin-releasing hormone factor (Boatman 1997; Kaye 1997; Devreker and Englert 2000; Casan *et al.,* 2000). Receptors for these have been found within the developing blastocyst. Some receptors, such as those for oestrogen receptor alpha and beta (ERα and ERβ), are differentially regulated (Hiroi, 1999), but as of yet their function is unknown. In addition, information on nutritional requirements and energy substrates has been obtained in the same way (Brison and Leese, 1994). It has been found that amino acids improve the rate of the developing blastocyst *in vitro* (Ho *et al.,* 1995) and that energy requirements change throughout blastocyst development (Devreker and Engelhert, 2000).

After fertilisation cell division proceeds under maternal control, the RNA molecules and proteins synthesised in the oocyte cytoplasm sustaining the earliest

stages of development (Latham and Schultz 2001; Schultz, 1993). There is some evidence to suggest that the position of the second polar body and the point of sperm entry determine the first plane of cleavage (Plusa *et al.,* 2002). These markers may pre-determine the two axes of symmetry of the blastocyst, delineating the position of the boundary between the ICM and the TE cells furthest from it (Tam *et al.,* 2001). Other evidence supports the idea that the cell's lineage, either ICM or TE, is determined by very early events and may be under maternal control (Gardner, 2002). The proteins STAT3 and leptin are present in oocyte cytoplasm and may be differentially allocated to daughter cells at the second cleavage. Cells with high levels of these proteins may give rise to the TE lineage where they are implicated in interaction with the uterine wall during implantation (Edwards, 2000; Antzczak M and van Blerkom J, 1997). Therefore, the axis and cellular polarity that the blastocyst exhibits may be programmed during oocyte maturation. Studies in the mouse have also revealed that oocyte factors govern the demethylation of the paternal genome and modify its genome function, which may be relevant to genomic imprinting in the developing organism (Latham and Sapenza, 1998; Oswald *et al.,* 2000). DNA methylation is maintained during the first three cell divisions by maternally inherited methylase enzyme after which activity decreases markedly (Monk *et al.,* 1990).

During the second cleavage division in the mouse and between the second and third division in the human, the embryonic genome becomes activated. In the mouse a small amount of newly synthesised RNA is detected at the late 1-cell stage followed by protein synthesis before the maternal to embryonic transition. Activation of the genome is acquired in a step-wise manner and has been identified as having four major periods a) the one cell stage, b) early two cell c) late two cell and d) eight

cell stage. This progression has been confirmed in other species including humans. These stages are accompanied by changes in histone protein and chromatin structure, which regulate the availability of the genome for transcription. Many of the transcripts at the two cell stage accumulate as development progresses while others are necessary for cell proliferation and the morphological changes that now occur in the developing embryo (Latham and Schultz, 2001).

At the 8-16 cell stage the embryo begins to display morphological changes and undergoes compaction, a feature common to all eutherian mammals (Hardy *et al.,* 1996; Lopata *et al.,* 1983*)*. The blastomeres flatten and form close junctional contacts with each other until it is no longer possible to distinguish individual cells and the bundle of cells are now known as a morula. Associated with compaction is the appearance of tight focal junctions which eventually divide the plasma membranes of the outer blastomeres into apical and basolateral membrane domains. This results in asymmetrical cell contact with the outer cells becoming polarised, which is essential to the formation of the blastocyst (Ziomek and Johnson, 1980). Two cell lineages become distinct i.e the ICM and the polarised outer cells, the TE. The ICM remains apolar and highly adhesive to one another while the TE acquire the characteristics of epithelial cells, becoming flattened and joined together by tight junctional complexes (Schultz, 1999; Hardy *et al.,* 1996)

The developing blastocyst now undergoes cavitation. The TE cells acquire the mechanisms that facilitate the transport and retention of fluid. This is achieved by an active transport mechanism involving a sodium gradient and the upregulation of the aquaporins (Watson and Barcroft, 2001). They then begin to pump fluid into the intracellular spaces forming a fluid filled cavity which expands resulting in a polar

(diametrically opposed to the ICM) and mural region of the TE with the ICM lying eccentrically within the cavity. The different stages of pre-implantation development are shown pictorially in Figure 1.6.

In normally developing embryos very little cell death is seen until the compaction stage however apoptotic cell death has been observed in both *in vitro* and *in vivo* derived blastocysts. It is thought that the role of apoptosis is to eliminate cells with abnormal chromosomes or prevent ICM cells differentiating into TE cells, reducing the risk of inappropriate ectopic trophectoderm expression (Hardy, 1999). It has also been suggested that critical threshold of cells, within the ICM, are required if normal post implantation development is to occur (Brison and Schultz, 1997).

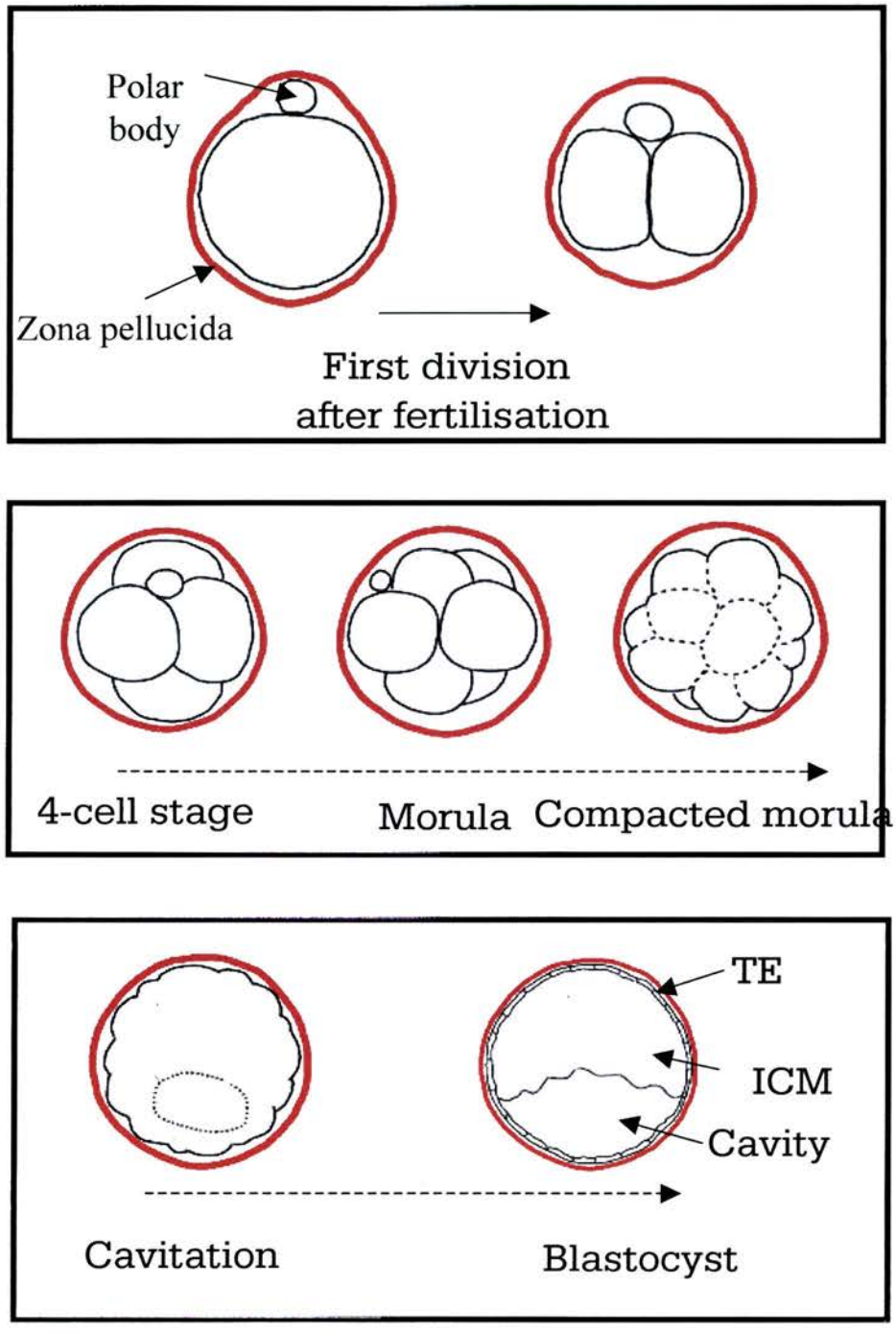


Figure 1.5. Stages of development from fertilisation until the blastocyst stage *adapted from The Mouse Atlas* (http://genex.hgu.mrc.ac.uk)

1.12 Clinical Perspectives

Normal ovarian function requires both FSH and LH. The co-ordinated action of these gonadotrophins brings about follicular development and steroid production, which results in the release of a developmentally competent oocyte and priming of the reproductive tract.

As described earlier, only a species-specific number of follicles are selected to release their oocytes for fertilisation (Gougeon, 1996). While many factors play a role in the selection process (Baker *et al.,* 2001; Spears *et al.*, 2002) the over-riding factor is availability and responsiveness to FSH at the critical developmental time point (Hillier, 1994; Brown, 1987). Exogenous administration of gonadotrophins bypasses the normal selection processes and permits high numbers of follicles to reach the pre-ovulatory stage (Oelsner *et al.,* 1978; Fowler and Edwards, 1957). Within the clinical and agricultural situations, many assisted reproductive technologies (ART) use exogenous gonadotrophin administration routinely as a means of harvesting large numbers of oocytes (Zafeirous *et al.,* 2000). Until recently the preparations used contained both FSH and LH in variable proportions. Original compounds contained equal bioactivities but newer generations of preparations have a much-reduced LH content. Recombinant forms of gonadotrophins have also been used in clinical practice. From the literature there are a number of conflicting reports as to the efficiency of these exogenous gonadotrophin preparations in producing oocytes capable of undergoing development and giving rise to live offspring and there is some concern that oocytes collected in this may be over or under mature (Warne *et al.,* 2000; Fleming *et al.,* 2000). Although clinical *in vitro* fertilisation (IVF) has been practised for over 20 years live birth rates per cycle are still

comparatively low, i.e. ~20%, suggesting that the obtained oocytes give rise to poor quality embryos.

Some attention has been paid to what role LH plays in follicular maturation and subsequent oocyte viability. A transgenic mouse (LuRKO) has been generated that lacks the LH receptor (Zhang *et al.,* 2001). Examination of the ovaries in this mouse has revealed that follicular development arrests at the early antral stage and no preovulatory follicles or corpora lutea are formed. While this may point to a role for LH in the latter stages of follicular development, it is likely that the endocrine system is also disrupted in these animals which may be responsible for the ovarian phenotype. A transgenic mouse has also been created that over-expresses the LHβ subunit (Risma *et al.,* 1995). These animals enter puberty precociously, develop ovarian cysts and granulosa cell tumours resulting in infertility (Risma *et al.,* 1997). Studies in these mice have pointed to a detrimental effect of elevated LH levels. There have also been suggestions from clinical reports that have suggested that high levels of this gonadotrophin (i.e. above systemic basal levels) during ovulation induction may have a detrimental effect on achieving a successful pregnancy (Shoham *et al.,* 1993). Some reports have suggested that high levels of LH administered during the follicular growth phase lead to poor oocyte quality and subsequently poor fertilisation and development rates (Regan *et al.,* 1990; Danforth 1995). However, there are conflicting reports that fail to confirm any correlation between elevated LH levels and pregnancy rate (Thomas 1989; Kovacs *et al.,* 1990). In contrast to the findings in the LuRKO mouse described above, recent studies in rodents and primates have shown that follicles are capable of reaching pre-ovulatory stages in the presence of very low levels or absence of LH (Sills *et al.,* 1999; Spears

et al., 1998; Bergh 1997; Balasch *et al.,* 1995; Zelinski-Wooten 1994; Schoot *et al.,* 1994). In human studies it has also been suggested that the use of recombinant FSH alone results in higher number of oocytes retrieved and improved pregnancy rates (Howles, 2000) but studies in non-human primates have indicated exposure to LH may improve embryo viability and rate of development (Weston *et al.*, 1996).

A main function of gonadotrophin action within the ovary is to stimulate oestrogen production. Apart from being a major steroid in controlling the reproductive cycle via the hypothalmic-pituitary-ovarian axis, the effect of oestrogen in priming the reproductive tract in preparation for pregnancy has also been well documented. Evidence from the LuRKO and *hypogonadal* mouse models has clearly demonstrated the effects of oestradiol on uterine weight (Zhang *et al.,* 2001; Halpin *et al.,* 1986). Although oestradiol does not seem to effect follicular maturation (Zelinski-Wooten 1994; Spears *et al.,* 1998), McNatty *et al* (1979) demonstrated that the intra-follicular concentration ratio of androgen to oestrogen is highly correlated to oocyte status. The presence of oestrogen receptors (Hiroi *et al.*, 1999; Wu *et al.,* 1992; 1993) suggests that this and other steroids may be necessary for optimal viability. Other studies have shown that lack of oestrogen may result in poor fertilisation rates and compromised early embryonic development (Zelinski- Wooten *et al.,* 1994; Wang and Greenwald 1993; Paesi, 1952). Presumably gonadotrophin administration in ovulation induction can alter both intra-follicular steroid concentrations as well as systemic levels. Therefore, the actions of inappropriate stimulation may alter either, or both, oocyte developmental competence and the synchronisation of events within the reproductive tract that lead to implantation and successful maintenance of pregnancy.

1.13 Research Aims

The development of the follicle and the maturation of the oocyte is a long process involving a highly complex set of regulatory factors. The communication that exists between the oocyte and the somatic cells must be correctly programmed if it is to acquire full developmental potential. Many aspects of oocyte maturation occur during the antral growth phase when the follicle has become increasingly dependent on gonadotrophin action, but until recently it has been difficult to elucidate the precise individual roles that FSH and LH play in this process. From the literature there are a number of conflicting reports on the effects of exogenous gonadotrophin stimulation, in particular how LH affects follicular development and oocyte viability. With recombinant forms of FSH and LH both now available it should become possible to design more efficient therapies for assisted reproductive technologies. However, the basic question remains as to what effect gonadotrophins have on oocyte development within the follicle prior to ovulation and how do these effect the quality of the embryo after fertilisation? The aim of this thesis was to begin to address this question.

Specifically my aims were to:

1 Optimise a follicular culture system that would yield a high number of follicles able to grow *in vitro* to the antral stage and yield fertilisable oocytes

2 Use this culture system to investigate the effects of gonadotrophins on both follicular development and oocyte viability.

3 Use an *in vivo* model, the *hypogonadal* mouse, to investigate whether any *in vitro* effects are affected by inter-follicular and extra ovarian

factors.

4 Investigate whether any effects noted, by manipulating gonadotrophins, are a result of aberrant steroid synthesis.

5 Investigate the role of the α oestrogen receptor on follicular development oocyte developmental competence.

Chapter Two

General Materials and Methods

2.1. Follicle Isolation

2.1.1 Dissection medium

Liebovitz L-15 medium (Invitrogen) was used for all bench top manipulations of ovarian material. Each 100ml bottle was adjusted to 285-293 mOsm/kg H_2O with sterile water (Phoenix Pharmaceuticals) using an osmometer (Roebling). Each bottle of media was kept for a period of 5 days before being discarded. Aliquots of the medium were supplemented with 0.3% Bovine serum albumin (BSA ; Fraction V, tissue culture grade, Sigma) and filter sterilised before use (syringes: Becton Dickinson; 0.22μm cellulose acetate filters:Iwaki). Medium was made 1 day in advance of the culture day and kept at 4°C. Prior to use the medium was transferred to embryo dishes (1ml aliquots) and warmed to 37°C

2.1.2. Gross Dissection

Three-week old female mice were killed by cervical dislocation, the ovaries removed and transferred to dishes containing dissection media. Under magnification and using insulin needles (Sherwood), the ovaries were dissected free of non-ovarian tissue, such as oviduct and fat. Each ovary was bisected and each piece placed into a fresh dish of dissection media. From the initial dissection and throughout all the following procedures the material was handled within a laminar flow hood (Astecair) to ensure sterility. Once the ovaries had been removed from the animals, all subsequent manipulations were performed on a heated stage (Lincam).

2.1.3 Micro-dissection.

Follicles were dissected from each piece of ovary using an inverted dissecting microscope (Zeiss or Nikon) fitted with an ocular micrometer and heated stage. Each piece of ovary was teased apart with insulin needles (Sherwood) and acupuncture needles (Acumedic) mounted into steel holders. Individual follicles of appropriate sizes were released. Once dissected, follicles were transferred to a fresh dish of dissection media before being transferred to culture dishes. Tissue was worked on for a maximum time of 30 minutes *post mortem*, with isolated follicles being placed in culture within a 45minute period.

2.2. Standard Follicle Culture

2.2.1 Standard culture media.

Bottles of α-Minimum Essential Media (α-MEM; Invitrogen) were checked and adjusted for osmolarity in the same way as the dissection media (see 2.1.1). Each bottle of media was used for a period of 5 days. Supplements (see below) were added to aliquots of media prior to filter sterilisation (see 2.1.1).

On the day prior to dissection, 30 μl of culture media was placed into the 'U' wells of 96 well non tissue culture treated plates. Two rows of media were prepared in advance. Each well of media was overlaid with 75 μl of filter sterilised silicon fluid (0.45mm filters:Iwaki, silcon fluid: Merck) to prevent evaporation. The plates were placed into a 37°C, 5% CO_2, humidified incubator (Forma Scientific) and allowed to equilibrate. Fresh media was prepared every second day.

Media Supplements

Recombinant gonadotrophins.

During the development of the follicle culture system, recombinant human FSH (rhFSH) was not available and a pituitary source of FSH was used. Using this preparation it was found that follicles could grow from ~180μm in diameter to over 400μm in a period of 5 days. When rhFSH became available to the laboratory we found that this preparation could stimulate follicle growth and give similar growth profiles to the pituitary preparation (Spears *et al.*, 1998). However we have found that after reconstituting vials of rhFSH with the same IU content, different batches did not induce the same degree of growth when used in the follicle culture system. For each section of work described in this thesis it was necessary to utilise different batches of rhFSH. Therefore each batch was titrated to ensure that follicles reached sizes of > 400 μm over a 5-6 day period. The IU ml^{-1} concentrations stated in each experimental chapter can be considered arbitrary.

Serum

Media was supplemented with 5% serum. The type of serum used is detailed within each experimental chapter. Where mouse serum was used this was prepared by withdrawing blood from anaesthetised animals via cardiac puncture. The blood from each animal was collected into an eppendorf tube and allowed to clot for at least 10 minutes before centrifugation (Eppendorf centrifuge/ 13,000rpm/10 minutes). The resulting serum was pooled, aliquoted and stored at –70°C.

Ascorbic acid

While initially experiments were carried out in media supplemented with gonadotrophins and serum alone, during the course of this research project it was

found that the addition of ascorbic acid was beneficial when added to the culture media. This formed the subject of a separate study detailed in Chapter 3.

2.2.2. Follicle incubation.

Following isolation, healthy follicles were transferred into the wells of the equilibrated culture plates. Care was taken to choose follicles with a centrally placed oocyte, some thecal cells attached and with no gaps in the granulosa cell layer. Using finely drawn pasteur pipettes that had been coated with BSA to prevent follicles adhering to the glass, the isolated follicles were allocated randomly between experimental groups. Once the follicles were allocated to the wells, the trays were returned to the incubator. Care was taken to minimise the length of time that the trays were out of the incubator.

2.2.3. Media changes and assessment of follicular growth and morphology.

Follicles were moved into a new well of media daily by transferring them with glass pipettes. The length of the culture period is described in each experimental chapter. Each day follicles were measured using the calibrated eyepiece graticule fitted in the dissecting microscope. Antral development was noted and classified. Follicles that lost their 3D morphology within the first 48 hours of culture were excluded, as these were most likely damaged during dissection. Any follicles that showed signs of atresia or oocyte damage were also excluded.

The culture system is outlined schematically in Figure 2.1

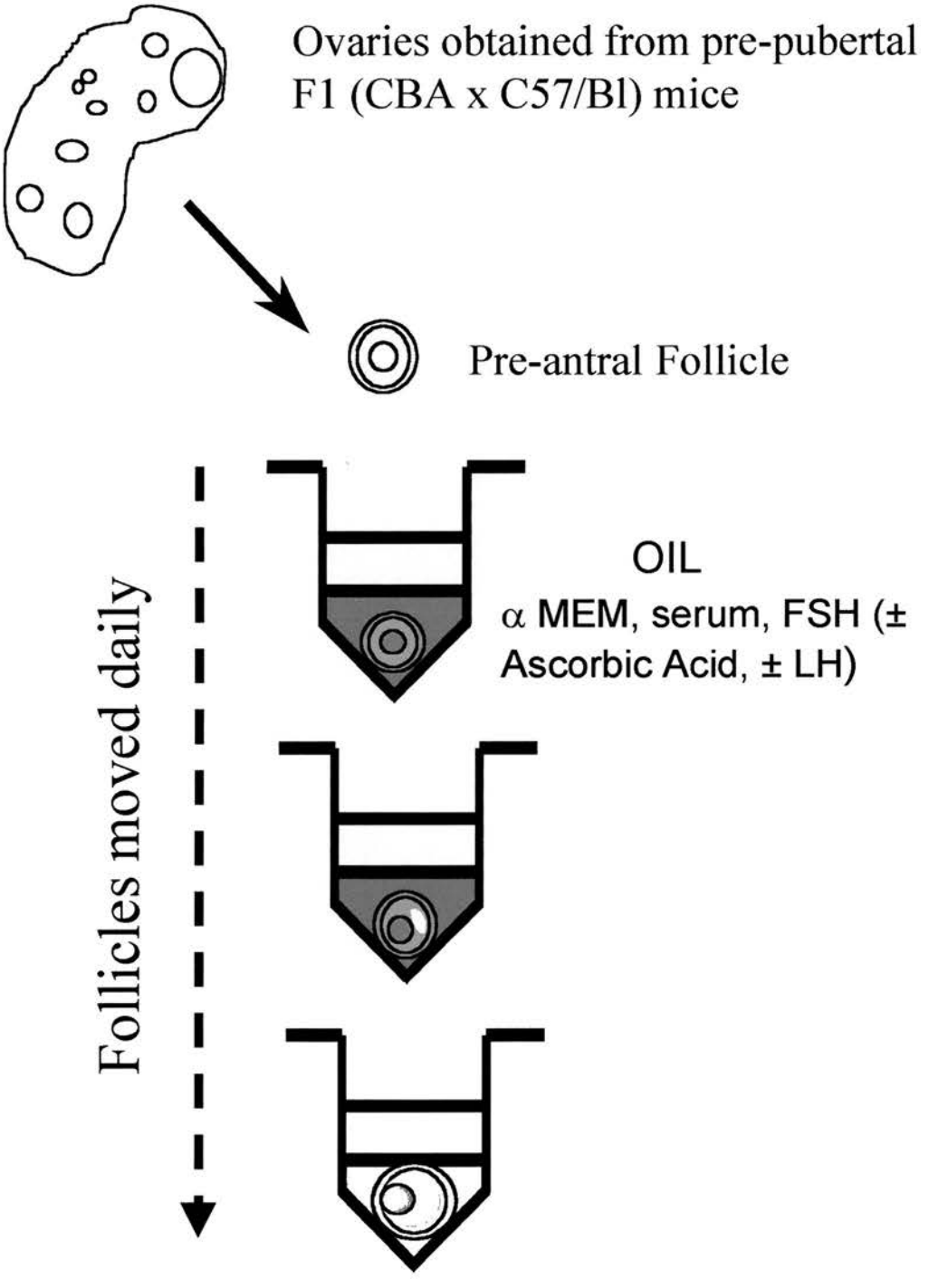


Figure 2.1 Schematic representation of the follicle culture system

2.3. In vitro fertilisation (IVF)

2.3.1. Preparation of T6 (fertilisation medium).

Pre-prepared aliquots of each of the four components required to make T6 media (Quinn *et al.,* 1982) were stored in the –70°C freezer. The composition of the media is detailed in Appendix B. The aliquots were defrosted and mixed before being added to 7.8ml sterile water (Phoenix). The total volume of media prepared was 10ml. The osmolarity of the medium was checked to 287 +/- 5 mmOsm/kg H_2O and then supplemented with 10mg.ml^{-1} BSA (Fraction V, TC grade, Sigma). The medium was then filter sterilised using a 0.22μm cellulose acetate syringe filter (syringes-Becton Dickinson, filters-Iwaki). 0.5ml aliquot drops were placed gently onto the bottom of 6 well tissue culture plates (Iwaki) and completely covered by silicon fluid (Merck). Enough drops were prepared for sperm collection and oocyte fertilisation. Additional drops were set up as washes. The plates were placed into a 37°C, 5% CO_2 humidified incubator on the day prior to the IVF being carried out to ensure that they were fully equilibrated.

2.3.2 Preparation of KSOM (embryo development medium).

Medium for the development of resultant fertilised oocytes was prepared in a similar manner to the fertilisation medium. Aliquots of each of the four components of the KSOM media (Devreker and Hardy, 1997) were stored frozen in a –70°C freezer and defrosted as required. The components are detailed in Appendix B. The four components were added to 7.8ml of sterile water and the osmolarity checked to 254 ± 5mOsm/kg H_2O. The final volume of 10ml was supplemented with 1mg.ml^{-1}

BSA (Fraction V, fatty acid free TC grade-Sigma). 100 µl drops were carefully laid onto the bottom of 6 well plates (Iwaki) and completely covered by silicone fluid (Merck). Enough drops were prepared for washing and holding fertilised oocytes from each experimental group overnight. All plates were prepared one day prior to the day of IVF and equilibrated in an incubator (conditions as 2.3.1). The prepared KSOM and silicon fluid were also placed in to an incubator and allowed to equilibrate so that fresh drops of media could be prepared for two-cell embryos (detailed below).

2.3.3 Preparation of *in vitro* maturation medium for oocytes recovered from cultured follicles.

1ml of α-MEM media (which had been adjusted to 285 mOsm/kg H_2O) was supplemented with 5% serum (as used in the medium for follicle culture) and 10ng. ml^{-1} Epidermal Growth Factor (EGF-Boehringer or Sigma) was added. The medium was filter sterilised as above. 100 µl drops were carefully laid onto the base of 6 well culture plates and covered with silicon fluid. Sufficient drops were prepared for each experimental group plus wash drops. The plates were placed into the incubator one-day prior to use.

2.3.4. *In vitro* maturation of oocytes collected from cultured follicles.

24 hours prior to fertilisation, cultured follicles were transferred into embryo dishes containing 1ml aliquots of warmed dissection media. The follicles were teased apart using insulin needles (Sherwood) to release the oocyte and the surrounding cumulus cell complexes (OCCs). These complexes were then transferred through two

wash drops of pre-equilibrated IVM medium (2.3.2) using fine drawn, BSA coated pipettes before being placed into a final drop medium. The complexes were placed into the incubator for 24 hours before fertilisation. After 24 hours the OCCs were washed through two drops of fertilisation media before being placed into a final drop prior to fertilisation.

2.3.5 Sperm Preparation

F1 (CBA male x C57Bl female) males between 6-10 weeks of age were used for sperm collection. Two males were used on each occasion. The animals were killed by cervical dislocation. All dissections were carried out within a laminar flow hood (Astecair). After incision and exposing the reproductive organs, the testis and vas deferens were removed to dishes containing dissection media (2.1.1.above). From each animal one testis and vas deferens was placed into an embryo dish with one testes and vas deferens of the other animal. Further dissection was conducted under an inverted dissecting microscope fitted with a heated stage (Nikon/Zeiss: Linkam). Each testis was removed and discarded leaving the vas deferens and caudis epididemis. These were carefully cleaned up, using irridectomy scissors and watchmaker forceps, removing adherent fat and blood vessels. After cleaning, both vas deferens and caudis were placed into a 0.5ml pre-equilibrated drop of fertilisation media (see 2.3.1). Under the microscope the caudis was teased apart and the contents of the vas deferens squeezed out releasing the sperm into the media. The tissue was then removed and discarded. Each dish contained sperm from both animals. The sperm preparation was placed into the incubator (Forma Scientific) and left for 2 hours before use.

2.3.6 Superovulation of female animals

For each run of IVF, oocytes were collected from superovulated adult F1 animals and used as a control for the IVF system. 68 hours before the day of IVF at least two adult females between the ages of 6-8 weeks were injected into the peritoneum with 5IU Pregnant Mare Serum Gonadotrophin (PMSG) contained wthin 100µl of phosphate buffered saline (PBS) (Intervet). Followed by 5IU Human Chorionic Gonadotrophin (hCG) /100µl PBS (Intervet) 54 hours later.

2.3.7 Collection of super ovulated oocytes

The animals were killed by cervical dislocation and transferred to a laminar flow hood where the ovaries and oviducts were removed to embryo dishes containing 1ml warm dissection media (2.1.1). Under an inverted dissecting microscope (Nikon/Zeiss) and using a heated stage (Linkam), the oocyte-cumulus complexes (OCCs) were released from the ampulla region of the oviduct using insulin needles (Sherwood). Using fine drawn, BSA coated glass pipettes, the OCCs were transferred through two wash drops of pre-equilibrated T6 fertilisation media (2.3.1) before being placed into a final drop of media to await fertilisation.

2.3.8 Assessment of sperm

20 µl of the sperm preparation was added to an equal volume of 4% paraformaldehyde and mixed. 10 µl of fixed sperm was counted using a haemocytometer chamber and the total number of sperm per ml calculated.

2.3.9 Fertilisation of oocytes

All the oocytes, both control and experimental, had sperm added to them at the same time. Based on the assessment of the sperm preparation (above), sperm was added to the 0.5ml fertilisation drops at an approximate concentration of 1-2 $x10^6$ per ml. The plates were then placed back into the incubator and left undisturbed for 4-5hours.

2.3.10. Embryo development

At the end of the fertilisation period, the oocytes were transferred by glass pipette to the pre-equilibrated drops of KSOM (2.3.2). The oocytes were washed twice before being placed into final drops of medium. Counts were made of the total number of oocytes within each group. The plates were returned to the incubator and left undisturbed for 17 hours. After this period, fertilised oocytes that had cleaved to two-cells were counted.

2.3.11 Development to blastocyst stage

Using the pre-equilibrated media and silicon fluid from the incubator, fresh drops of KSOM media were made. The sizes of the droplets were calculated based on the number of two-cell embryos counted with 10 embryos being placed into 20 µl droplets. A strict 1 embryo to 2 µl of medium ratio was adhered to. For example if 17 embryos were counted two drops of medium (1x20 µl and 1x14 µl) were made. This was to insure that the effects of any endogenous products, produced by the embryos, were present at the same concentration throughout the culture period. The droplets of medium were covered by the silicone fluid and returned to the incubator for 2-3

hours. The two-cell embryos were transferred into the fresh droplets of KSOM, 24-26 hours after the beginning of the fertilisation period. The plates were returned to the incubator where they remained until the end of the culture period. Figure 2.2 is a flow diagram outlining the IVF procedure.

2.3.12 Assessment of developing blastocysts.

The embryos were examined on a daily basis using an inverted microscope (Nikon) fitted with a heated stage (Linkam). Within each treatment group the number of embryos were examined and a note of their developmental stage made. Practically this was at the 4-cell, morula and blastocyst stage of development as many intermediate stages progressed overnight.

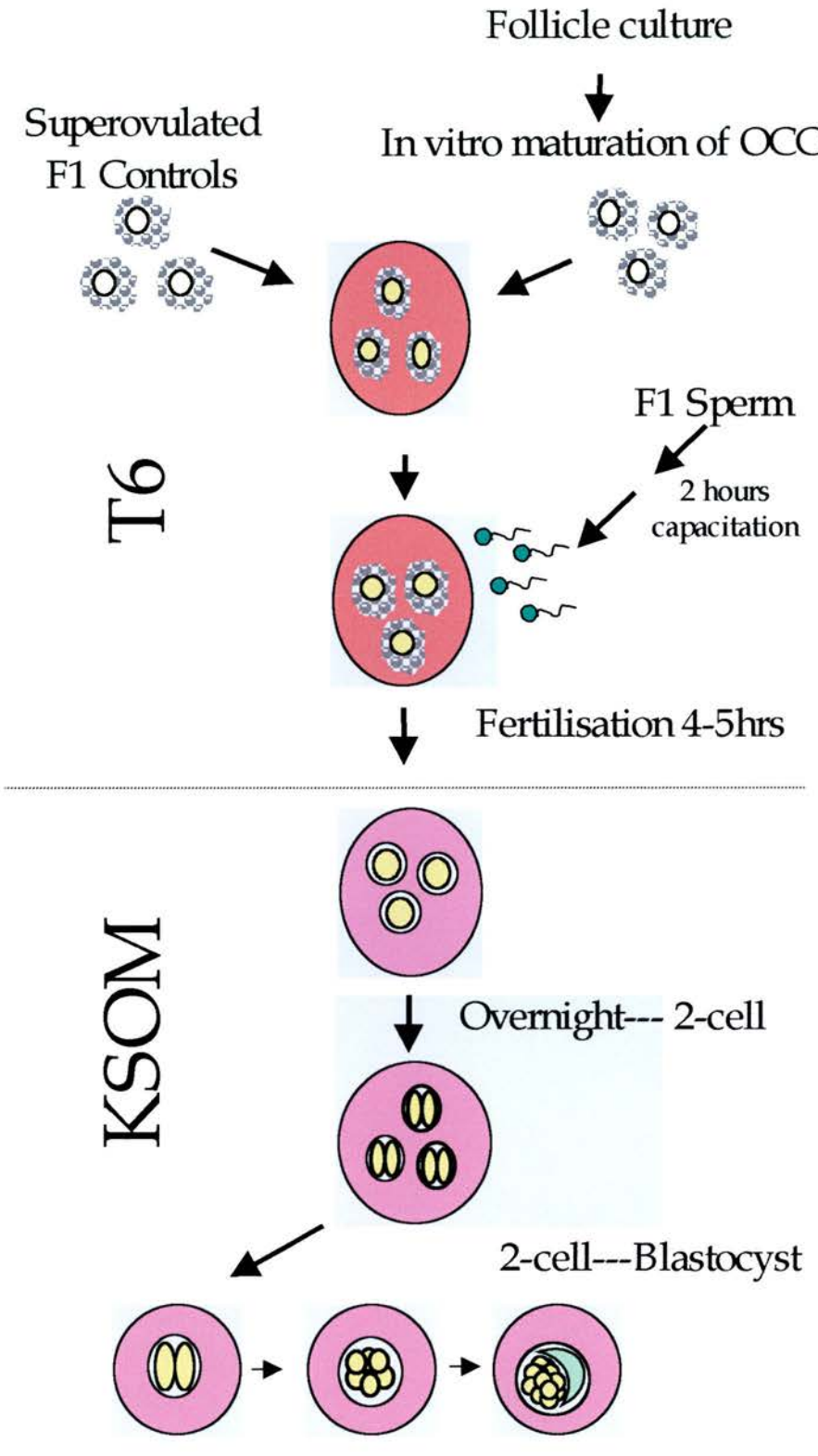


Figure 2.2 Flow diagram illustrating the IVF procedure

2.4. Detection of apoptosis by TUNEL labelling

2.4.1 TUNEL Staining of cultured follicles.

All steps were carried out in the wells of 96 well plates as in 2.4.1. Follicles were washed in PBS at 37°C for 10minutes before being transferred into 0.5% Triton X-100 and 0.25% paraformaldehyde in PBS (37°C, 40 minutes). They were then fixed in 4% paraformaldehyde (30 minutes) and washed twice in PBS (2 x 10 minutes). At this point follicles could be stored in PBS with 0.02 % sodium azide at 4°C. After removal from storage, follicles were washed in PBS (10 minutes); transferred into 17.1 mg ml^{-1} Proteinase K at 37°C (30 minutes); washed in PBS with 0.01% Triton X-100 (10 minutes); fixed in 3% paraformaldehyde (30 minutes), then washed twice in PBS (2 x 20 minutes). Each follicle was placed in a commercially available TUNEL reaction mixture (Roche) for 2.5 hours. Follicles were then washed in PBS (10 minutes) and moved into Rnase buffer (as 2.4.1) containing 2.5 mg ml of propidium iodide for 1 hour. They were then washed in PBS containing 0.01% Triton X-100 (20 minutes) followed by two washes in PBS (2 x 20 minutes). As before, to preserve fluorescence the follicles were equilibrated in 50% Vectashield (Vector). They were then stored overnight at 4°C at this stage or transferred into 100% Vectashield on a concave microscope slide (TAAB) cover-slipped and sealed with nail polish for microscopic analysis.

2.4.2 Confocal Analysis

Follicles were analysed by confocal microscopy (Leica) using the 63x water corrected PL APO lens. Follicles were examined by taking a single scan through the

centre of each follicle as determined by central positioning of the propidium iodide stained germinal vesicle in the oocyte. Propidium iodide staining was red in colour while TUNEL labelled cells were detected as green therefore simultaneous scans at 488 nm and 568 nm were taken to produce an amalgamated true colour RGB image. Each channel could also be viewed separately.

2.5 Hormone assays

Hormone assays were performed on the spent media from follicle cultures. All the culture plates were stored frozen at –20°C until the assays could be performed.

2.5.1 Oestradiol

Media from the follicle cultures were analysed for oestradiol immunoenzymatically using an ELISA method supplied as a kit (Biostat). Samples were prepared as per the kit instructions and analysed colourmetrically using a Serozyme I analyser (Intersept). Oestradiol was measured from individual follicles. The inter-and intra assay coefficients of variation were ≤ 5% and the sensitivity was ≤ 0.48nmol l^{-1}

2.5.2 Androstenedione

Media from the follicle cultures were analysed for androstenedione by radioimmunoassay, which has been validated for this purpose (Hillier *et al.,* 1991*).*

2.6. Genotyping of mutant animals.

2.6.1 Collection of material

In order to obtain DNA for analysis, either a piece of tail tip or an ear clipping was used. The animal house staff collected the material when the animals were between 2-3weeks of age. The clippings were placed into an eppendorf tube and stored at –20°C until extraction.

2.6.2. Extraction of DNA

Tail Tip Lysis buffer (TTLB see Appendix C) was supplemented with proteinase K (Boehringer) at a ratio of 2.5 µl per 0.5 ml. 0.5ml of this was added to each eppendorf containing either a tail tip or ear clipping. This was digested overnight at 55°C in a shaking water bath. The digested material was then vortexed (Rotamixer) and centrifuged at 14K (Eppendorf centrifuge) for 15 mins. While the material was being spun down, fresh eppendorfs containing 500 µl isopropanol were prepared. The supernatant obtained was mixed with the isopropanol. The tube was then mixed by gently inverting until a 'cotton-wool' like precipitate was seen. The tubes were then centrifuged at 14K for 15minutes and the supernatant discarded. 1ml of 70% ethanol was added to the pellet and the tubes spun down (30 seconds @14K). The supernatant was discarded and the tubes re-spun to ensure that all the ethanol was removed. The pellet was dried in a 37°C oven for 30 minutes. 300 µl double distilled water (ddH_2O) was added the tubes vortexed and then placed in a 55°C shaking water bath for 1hour. In a fume hood (Astecair) 300 µl of PCIA (Sigma) was added to the tubes, mixed and allowed to stand for 5 minutes before spinning at 14K

for 10mins. 200µl of the aqueous layer was transferred to fresh eppendorf tubes to which 20 µl sodium acetate (pH5.5) and 400ml 100% ethanol was added to precipitate DNA. The mixture was mixed by inverting the tube and then centrifuged at 14K for 5mins. The pellet was washed twice in 1ml 100% ethanol before being dried in a 37°C oven for 30mins.The DNA was re-constituted in ddH_2O (200 µl for tail tips and 50 µl for ear clippings) and stored frozen.

2.6.3. Polymerase Chain Reaction

The DNA obtained as above was subjected the polymerase chain reaction (PCR) before identification of the genotyping of the animals was determined. 1µl of the DNA extracted was added to 24µl of a bulk mix containing PCR buffer, primers and polymerase enzyme. The details used for each protocol used are given in Appendix C. All reactions were set up in 0.5ml thin wall PCR tubes and the reaction was performed using a thermal cycler (MJ Research). The PCR programme used was dependent on the genotype being analysed (see Appendix C).

2.6.4 Agarose gel analysis of PCR products.

5 µl of loading buffer (see Appendix C) was added to each of the PCR tubes after they had been reacted. Agarose gels were prepared by adding 1.6 grams of agarose (Flowgen) to 40 ml of Tris borate buffer (TBE –see Appendix C). The agarose was dissolved by heating the mix in a microwave oven on medium power for 2 minutes. The gel was cooled and 1µl of ethidium bromide solution (see Appendix C) was added to it. The gel was then poured into the chamber of a mini-gel tank (BioRad), a well comb inserted and allowed to set for 30 minutes. The comb was

then removed and 60ml TBE added to the chamber. The PCR samples were then carefully pipetted into the wells and the gel was connected to a power pack (BioRad) at 40 volts for 1 and half-hours. A 100 base pair molecular weight marker was also run along with the samples (Boehringer)

2.6.5. Detection of DNA products.

The gel was visualised using a UV transilluminator (BioRad) linked to Alpha Imaging Software. The PCR products were compared with the molecular weight markers to confirm the genotypes of the animals.

2.7 Histology

2.7.1.1 Fixation of ovaries

Whole ovaries were placed into Bouins fixative for a period of 24 hours. Ovaries were then transferred to 70% ethanol for storage until processing. The ovaries were dehydrated by passing them through a series of increasing strength alcohol solutions (70%, 90%, 100%x2) for 1 hour in each. The ovaries were then placed into toluene for ~2 hours for clearing before being placed into plastic moulds filled with molten paraffin wax. The tissue was allowed to impregnate with wax for 3-5 hours before being orientated and the wax allowed to solidify.

2.7.1.2 Sectioning and mounting.

The wax blocks were removed from the plastic moulds and set onto the top of metal chucks. The blocks were then positioned onto a microtome (Reichart-Jung) and 6μ sections cut. The sections were floated out in a water bath at 50°C, picked up and transferred to gelatin coated slides. The slides were then placed into a 37 °C oven overnight.

2.7.1.3. Staining

The dried sections were dewaxed in xylene, re-hydrated through descending alcohol concentrations to water before staining in haemotoxylin for 5 minutes. Sections were then acidified in acid alcohol, rinsed under running tap water then placed into Scotch Tap Water Substitute (STWS) for 2 minutes. After another wash in tap water the slides were placed into eosin for 2 minutes, fixed with potassium alum before washing and dehydrating through ascending alcohol concentrations. The slides were placed into xylene before coverslips were applied using DPX mountant (BDH).

2.7.2 Processing of cultured follicles.

2.7.2.1 Fixation.

In vitro grown follicles were fixed at the end of the culture period by placing them into 4% paraformaldehyde for 24 hours at 4°C after which they were transferred to 70% ethanol.

2.7.2.2 Processing of samples.

Follicles were then dehydrated through a series of alcohols (70%; 90%; 95%; 100%; 100%, 30 minutes each). They were then embedded into LR White resin (Taab,) contained within gelatin capsules and incubated overnight at 60°C. The gelatin capsule was dissolved in running hot water ~4 hours and the resin block mounted onto a wooden chuck.

2.7.2.3 Sectioning and mounting

2μm sections were cut using a plastic-section microtome (Rechert-Jung), floated out onto a 50°C water bath and transferred to gelatin coated slides. Sections were then dried on a hotplate overnight at 60°C.

2.7.2.4 Staining

The dried sections were stained as described in 2.7.1.3.above.

Chapter Three

Improvement of the Follicle Culture System:

The Role of Ascorbic Acid.

3.1 General Introduction

Over the last decade a number of investigators have developed *in-vitro* culture systems that support the growth and development of follicular units from pre-antral through to pre-ovulatory stages. The oocytes obtained from these follicles are viable and live young have been produced (Eppig and Schroeder 1989, Spears *et al.*, 1994). Essentially two types of system have evolved for the culture of pre-antral follicles; those that maintain the follicle as a 3-D unit and those that consist mainly of the oocyte surrounded by granulosa cells. In the latter system some of the basement membrane may be present but the follicle does not maintain its structure. There are advantages and disadvantages to both systems. The follicular culture system described in Chapter 2 was developed in the laboratory here. Follicles, of approximately the same developmental stage, are isolated as intact units and grown individually. Growth and development mirrors that of follicle *in vivo* to a remarkable degree i.e. the system is highly physiological. As the follicles can be grown in defined conditions any extra-ovarian or even extra follicular effects are negated. As one of the principle aims of this thesis was to investigate how factors arising during the follicular growth phase may affect oocyte developmental competence, many of my studies have employed this *in vitro* follicular culture system.

While the culture system has been used successfully for a number of years, periodically we have found that over the years there have been phases where the follicles would 'burst' and lose their basement membrane integrity. Initially the follicles would start to grow normally but after 72 hours in culture they would assume a disorganised appearance making it difficult to gain information on growth and antral formation.

During follicular development the granulosa cells are separated from the surrounding stromal tissue by a basal lamina. This membrane has to expand rapidly as the granulosa cells proliferate and also during antral formation. In bovine follicles it has been calculated that the surface area doubles nineteen times during follicular development implying that the basal lamina has to undergo constant re-modelling. Studies in ovine and bovine follicles have shown that the basal lamina influences granulosa cell function and morphology while the granulosa cells themselves may produce components necessary for the synthesis of the basal lamina (Rodgers *et al.*, 1999; Huet *et al.*, 2001). The basal lamina probably serves a number of functions; it may regulate the transfer of molecules between the stroma and the somatic compartments during the latter phase in follicular development and serve as a store of growth factors and regulate fluid uptake during antral formation.

As the aim of some of the work was to investigate growth and development, experiments were devised and carried out to improve the number of follicles able to maintain their basement membrane throughout the culture period. Initially experiments investigated the effects of insulin, selenium and ascorbic acid, which are common additions to culture media. From these experiments we found that ascorbic acid alone was responsible for increasing the number of follicles able to maintain their 3 D morphology. This chapter describes a more detailed study of the effects of this vitamin on follicular development.

3.2 Ascorbic acid and the ovary

The actions of ascorbic acid (Vitamin C) are amongst the best understood of all the vitamins. It is a dietary requirement for primates, and a few other mammals, which lack the necessary hepatic enzymes for synthesis. It has been associated with conditions such as ageing, the common cold and cancer and the recommended daily requirement has been the subject of many debates.

The ovary, and other endocrine tissues, accumulates high levels of ascorbic acid. Within the ovary, concentrations accumulate in the granulosa, thecal and luteal cells (Deane, 1952) and it has long been associated with fertility (Luck *et al.*, 1995). Ascorbic acid has been used to treat infertility in cows (Phillips *et al.*, 1941) and has been shown to enhance the effect of clomiphene on ovulation induction in women (Igarashi, 1977). Conversely high dosages have been implicated in spontaneous abortion in both women and rats (Samborskaia and Ferdman, 1966). The ovarian content of ascorbic acid changes throughout the oestrus cycle. In response to the pre-ovulatory surge of LH, ascorbic uptake by the ovary is blocked and the tissue concentration depleted, a phenomenon that formed the basis of an early bioassay (Parlow, 1958). In response to LH the ovary produces increasing concentrations of progesterone. Studies on luteinizing granulosa cells have shown that ascorbic acid stimulates progesterone production (Byrd *et al.,* 1993) and that rising progesterone concentrations block the uptake of ascorbic acid (Stansfield and Flint, 1967). Therefore the action of LH may indirectly control the fluctuations in ascorbic acid concentration throughout the ovarian cycle. In addition ascorbic acid acts as a co-factor in the amidation of some proteins and has been implicated in the regulation of oxytocin secretion by the ovary (Luck and Junglas, 1987).

The role of ascorbic acid in promoting collagen biosynthesis has been extensively studied (Pinnell, 1985). During follicular growth, ovulation and corpus luteum formation, basement membranes and the extra cellular matrix are undergoing constant remodelling and will therefore have a high requirement for collagen. Early studies have implicated ascorbic acid in the regulation of the Graafian follicular basement membrane, lack of the vitamin causing degeneration of the follicle membrane and high doses inhibiting collagenolytic activity in the mature follicle (Kramer *et al.*, 1933; Espey and Coons 1976).

The matrix metalloproteinases (MMPs) and their inhibitors, tissue inhibitors of metalloproteinases (TIMPS), are members of an enzyme family associated with the re-modelling of extra-cellular matrix within the ovary (Smith *et al.*, 1999). Many studies of the actions of MMPs and TIMPs within the ovary have concentrated on their functions during the peri-ovulatory period (Hagglund *et al.*, 1999), but little is known about their expression and control throughout follicular development. Vitamin C deficiency has been associated with the premature rupture of placental membranes. Addition of ascorbic acid to cultured human amnion cells resulted in a decrease in matrix metalloproteinase activity (Pfeffer *et al.*, 1998), suggesting that ascorbic acid may play a role in the control of these enzymes.

While there is much information on the role of ascorbic acid during corpus luteum formation, little is known about its role during follicular growth and development. The aim of this study was to investigate the role of this vitamin during follicular growth.

3.3 Materials and Methods

3.3.1 Animals

Three-week hybrid F1 female mice were used for these experiments. They were housed as described in Chapter Two.

3.3.2 Experiment 1: Effect of Ascorbic acid on basement membrane integrity, follicular growth, morphology and steroidogenesis.

Follicles were isolated and placed into culture as outlined in the protocols described in Chapter two. Culture media was supplemented with rhFSH at 1 IU ml^{-1} and 5% mouse serum obtained from adult F1 mice. This was the control media. Ascorbic acid was added to the media as described below. Media was freshly prepared every second day. The α MEM media was supplied as 1X liquid. The formulation includes ascorbic acid at a concentration of 50mg L^{-1}, however, liquid media is batch prepared and supplied with a minimum 3 months shelf life. As ascorbic acid rapidly oxidises in solution, it is therefore likely that the ascorbic acid component would have decayed in the media used. As no measurements of ascorbic acid in the media were made it could not be determined to what degree this had occurred.

Follicles were cultured in control media (as above) or with the addition of either 5μg ml^{-1} or 50μg ml^{-1} L-Ascorbic acid, sodium salt, (Sigma) to the culture media (28μM and 280μM respectively). Ascorbic acid stock solution (5mg ml^{-1}) was prepared in α MEM and aliquots were stored at –70°C for a period of one month. Follicles were examined daily, the basement membrane was defined as intact where

follicles maintained their 3D morphology. Follicular diameter was measured using a pre-calibrated ocular micrometer. Data on follicular growth was obtained from follicles that remained intact on the final day of culture. During the first two days of culture, all follicles were moved to fresh wells of media. Follicles that had lost basement membrane integrity within the first two days of the culture period were discarded from the experiment (as this may have been caused by damage during dissection). Between Days 3 and 6 of culture, intact follicles were moved daily, as before. Ruptured follicles were not moved, but instead 15 μl of fresh media was exchanged for 15 μl of spent media each day. In each run of the experiment 36 follicles were allocated to each treatment group. The experiment was repeated twice, giving a total of 72 follicles per treatment.

At the end of culture, representative follicles from all treatments were fixed, embedded and sections cut for histological examination as described in Chapter 2.

Hormone Assays

Spent culture media from days 4 and 5 of the culture period were analysed for oestradiol and progesterone. Both hormones were analysed using commercially available kits (Biorad) and the Serozyme I spectrophotometer as detailed for oestradiol in Chapter 2. Oestradiol was analysed from the media of individual follicles while progesterone was analysed from the combined media of days 4 and 5 of culture.

3.3.3 Experiment 2: Detection of metalloproteinase and tissue inhibitor of metalloproteinases.

Gelatin zymography and reverse zymographic analyses were conducted for detection of MMP and tissue inhibitor of metalloproteinase TIMP activity in cultured mouse follicles respectively. Freshly isolated follicles, corresponding to the diameters of follicles on each day of culture, were also analysed. These results indicated that MMP activity was detectable in follicles of a similar size to those cultured for a two-day period. On the basis of these initial experiments follicles were cultured for 2 days in the same concentrations of ascorbic acid as Experiment 1 (3.3.1) after which they were analysed for MMP and TIMP activity.

Gelatin Zymographic and Reverse Zymographic Analyses

Gelatin zymography was conducted with 7.5% SDS-polyacrylamide (SDS-PAGE) gels containing 1 mg ml^{-1} gelatin (Sigma), under non-reducing conditions as described previously (Hibbs *et al.*, 1985; Morodomi *et al.,* 1992). To avoid the possible loss of MMP and TIMP activity associated with tissue extraction from small samples, follicles (10 follicles per sample) were dissolved directly in 20 µl of SDS-PAGE loading buffer (Laemmlli, 1970) without a reducing agent. The samples were then subjected to electrophoresis at room temperature (10 µl per lane). Following electrophoresis, SDS was eluted from gels in four changes of buffer containing 50mM Tris-CL (pH 7.5), 5mM $CaCl_2$, 5µM Zn Cl_2, 0.02%NaN_3 and 2.5% Triton X-100 (Sigma) for a total of 60-90 minutes at room temperature. Gels were then incubated in the same buffer without Triton-X at 37°C for 20 hours, stained with Coomassie brilliant blue R250 and destained in 30% methanol and 1% formic acid.

Areas of MMP activity were identified as clear bands of digested gelatin. Molecular weight markers (Invitrogen) and purified human pro-matrix metalloproteinase 2 (MMP 2, a generous gift from Dr. Hideaki Nagase, University of Kansas) were used to determine molecular size of pro- and active forms of gelatinases. Intensity of bands was determined by use of a ChemiImager 4000 Low Light Imaging System (Alpha Innotech Corp.). All gelatinase activity was inhibited by including 10mM EDTA or 10 mM phenanthroline in the incubation buffer.

Reverse gelatin zymography was conducted with 12% SDS-PAGE gels containing gelatin (1mg ml^{-1}) under non-reducing conditions (Reverse Zymography Kit, University Technologies International Inc.). The same samples as described above (10 µl per lane) were subjected to electrophoretic analysis at room temperature. Following electrophoresis, SDS was eluted from the gels and incubated at 37°C for 30 hours as described above. Gels were stained with GelCode® Blue Stain Reagent (Pierce) and washed in water. Metalloproteinase inhibitor activity was identified as dark bands of undigested gelatin. Molecular weight markers and purified recombinant ovine tissue inhibitor of metalloproteinase 1 (McIntush and Smith, unpublished data) were used to determine molecular sizes of TIMPs. Intensity of bands was determined as described above.

In each run of the experiment, 10 follicles were analysed per treatment group. The experiment was repeated 3 times giving a total of 30 follicles per treatment.

3.3.4 Experiment 3: Effect of Ascorbic acid on apoptosis.

In order to test whether ascorbic acid has an effect on preventing apoptosis, follicles were isolated as in Chapter 2 and randomly assigned into three groups. Each group was cultured in 5% CO_2 at 37 °C for 24 hours in the following media:

1). α-MEM supplemented with 1 IU ml^{-1} rhFSH. This was the control group.

2). α-MEM supplemented 1 IU ml^{-1} rhFSH plus 280μM ml^{-1} ascorbic acid. This was the experimental group.

3). α-MEM supplemented with 5% serum, 280μM ml^{-1} ascorbic acid, 0.5ng ml^{-1} sodium selenite (Sigma), and 1 IU ml^{-1} rhFSH. This group was included as a positive control, since the inclusion of serum in culture media inhibits follicular degeneration through apoptosis.

DNA extraction and labelling

Genomic DNA was extracted from the follicles using a commercial kit and according to the manufacturers' instructions (QIAmp Tissue Kit, Quiagen). Extracted DNA was eluted in a final volume of 150 μl (to maximise yield) then ethanol precipitated (45 μl 8M Potassium acetate, 400 μl Ethanol) and re-suspended in 20 μl ddH_2O. Extracted DNA fragments were 3' end labelled with digoxigenin using a 3' end labelling oligonucleotide kit (Boehringer Mannheim) prior to being separated by electrophoresis on a 2% agarose gel. Gels were Southern blotted overnight onto positively charged nylon membranes (Boehringer Mannheim), baked at 120 °C for 30 minutes and labelled fragments detected colourmetrically using the *DIG* detection kit (Boehringer Mannheim) according to manufacturers' instructions. Developed membranes were scanned using an Imaging Densitometer (Bio-Rad, Model GS-670,)

and analysed with the *Molecular Analyst* program (Bio-Rad**).** Density readings were recorded for bands corresponding to ~185, 370 and 555 base pairs on each lane run and an average calculated.

In each run of the experiment, 8 follicles were analysed in each treatment group. The experiment was repeated 3 times giving a total of 24 follicles / treatment.

3.3.5 Statistical Analysis.

The number of follicles remaining intact throughout the culture period in each treatment was compared using chi-square analysis. Oestradiol values, degree of apoptosis and concentrations of MMPs and TIMP were compared by analysis of variance. Where appropriate comparison was made using Student's *t* test.

3.4 Results

3.4.1 Experiment 1: Effect of Ascorbic Acid on basement membrane integrity, follicular growth, morphology and steroidogenesis.

In the absence of ascorbic acid, 33% of follicles maintained integrity of the basement membrane throughout the 6-day culture period. Follicles cultured in 28μM ascorbic acid exhibited a non-significant increase in the percentage remaining intact (47%). However when follicles were cultured in 280μM ascorbic acid there was a highly significant increase in the percentage of follicles remaining intact (88%; $p<0.001$) when compared to controls (Fig 3.1).

Follicular growth rate was unaffected by ascorbic acid concentration. Follicles that remained intact in the control and ascorbic acid treated groups, reached similar sizes by day 6 of culture (Fig 3.2). Histological examination of follicles at the end of the culture period showed that intact follicles were of similar size and had large antral cavities regardless of treatment (Fig 3.3).

Oestradiol analysis was performed on media collected on days 4 and 5 of the culture period. Media was collected from both intact follicles and from those that had lost their basement membrane integrity. The results show that oestradiol production increased between day 4 and day 5 in all treatments. Ascorbic acid addition to the media reduced the production of oestradiol regardless of whether follicles had maintained basement membrane integrity or not. The decrease was, however, not significant (Fig 3.4). Progesterone production was not detected in any group.

3.4.3 Experiment 2: Detection of metalloproteinase and tissue inhibitor of metalloproteinase

The primary gelatinolytic bands (Mr 62,000 and 72,000) corresponded to the pro- and active forms of matrix metalloproteinase-2 (MMP-2 gelatinase A) and co-migrated with the pro- and active forms of recombinant human matrix metalloproteinase-2. The larger gelatinolytic band (pro-MMP-2) was the predominant form present within murine follicles. Addition of ascorbic acid at the higher but not the lower concentration resulted in a small but significant increase ($p<0.01$) in TIMP-1 activity. MMP2 activity increased at the higher concentration but the increase here was below the level of significance ($p<0.08$) (Fig 3.5).

3.4.4 Experiment 3: Effect of Ascorbic acid on apoptosis.

Control follicles grown in the absence of serum showed a high level of apoptosis (measured as the degree of nuclear DNA laddering). Addition of ascorbic acid to the serum-free media (experimental group) significantly reduced the incidence of DNA fragmentation ($p<0.05$) to nearer the values of the positive control group, which also contained serum and selenium (Fig 3.6).

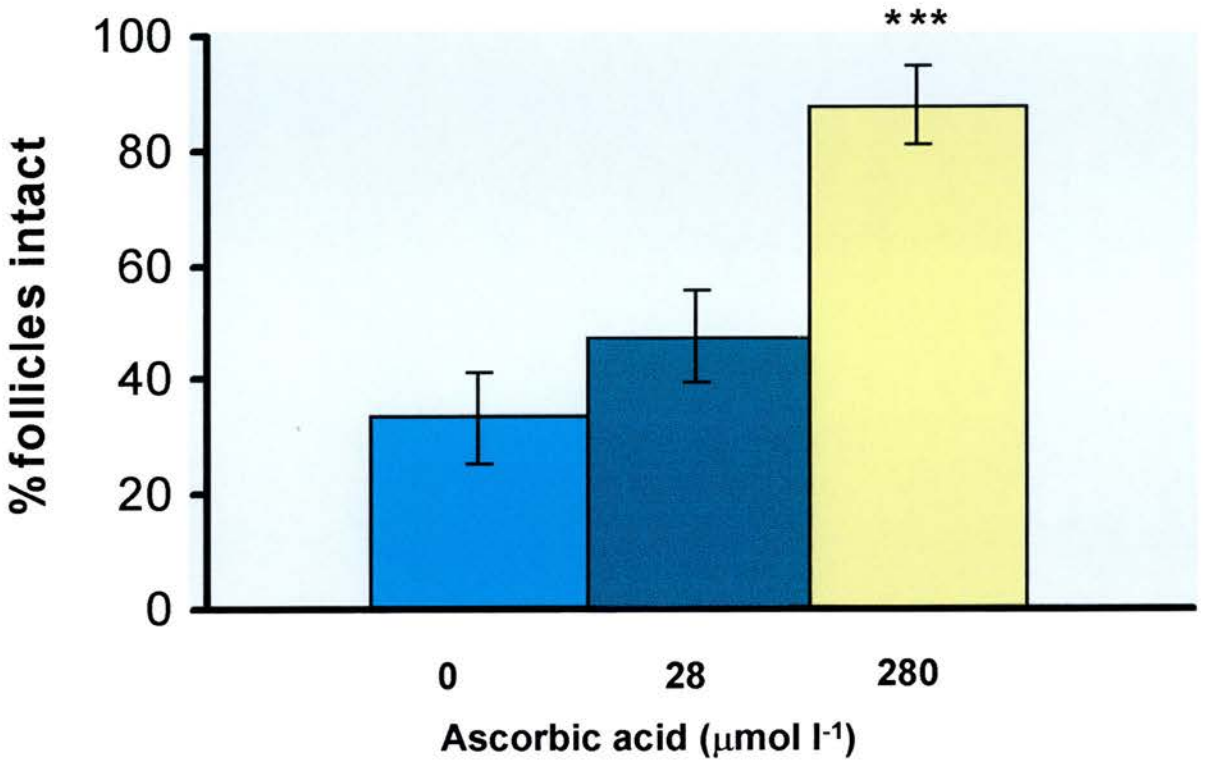


Figure 3.1 Percentage of follicles remaining intact at the end of the culture period. Follicles were cultured in either control media or in media supplemented with 28 or 280μmol ascorbic acid. *** = p<0.001compared to the control. Values are mean ± SEM (n=72)

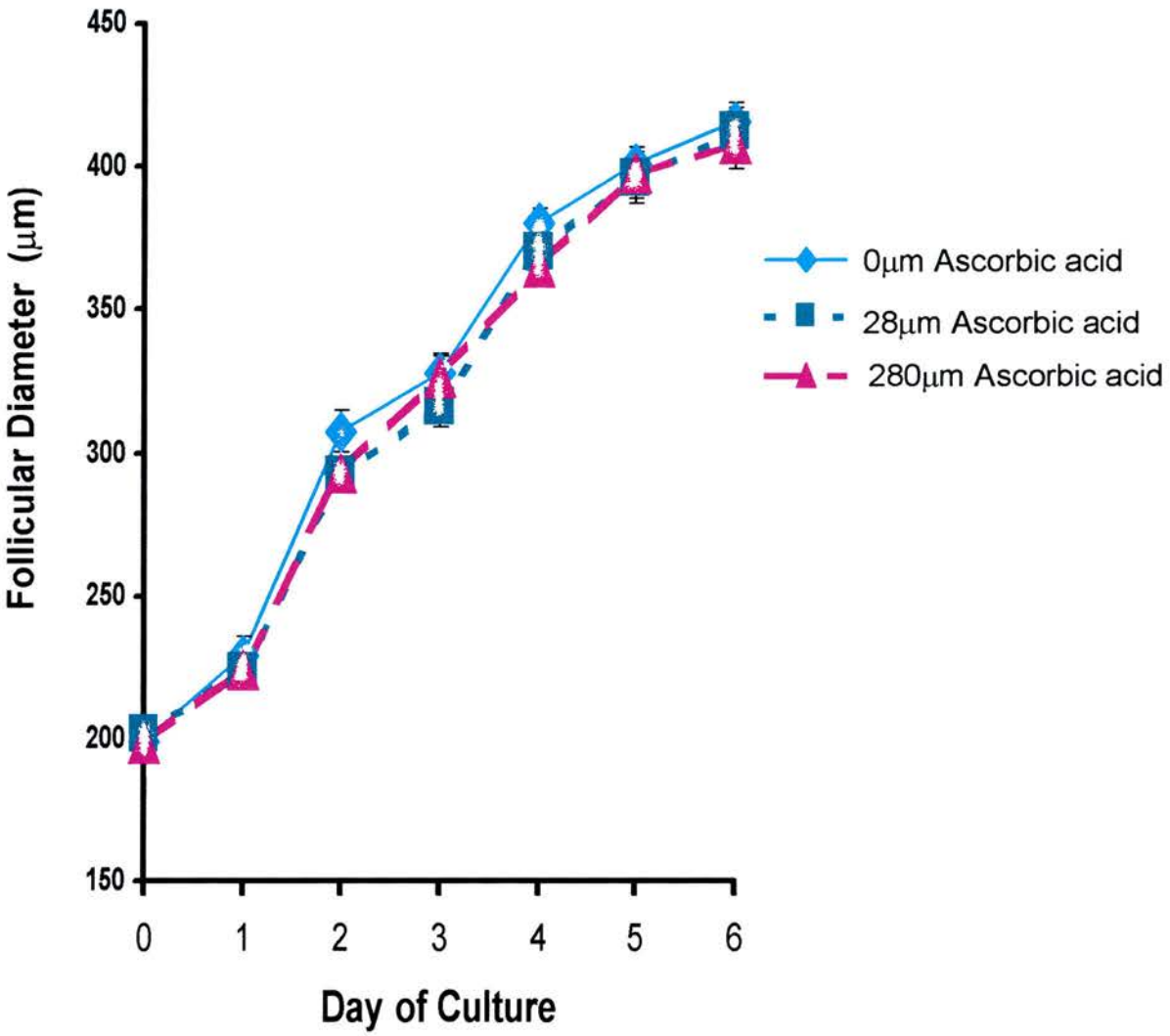


Figure 3.2 Growth rate of follicles grown in control media or with 28 or 280μm ascorbic acid. Values are mean ± SEM (n=72)

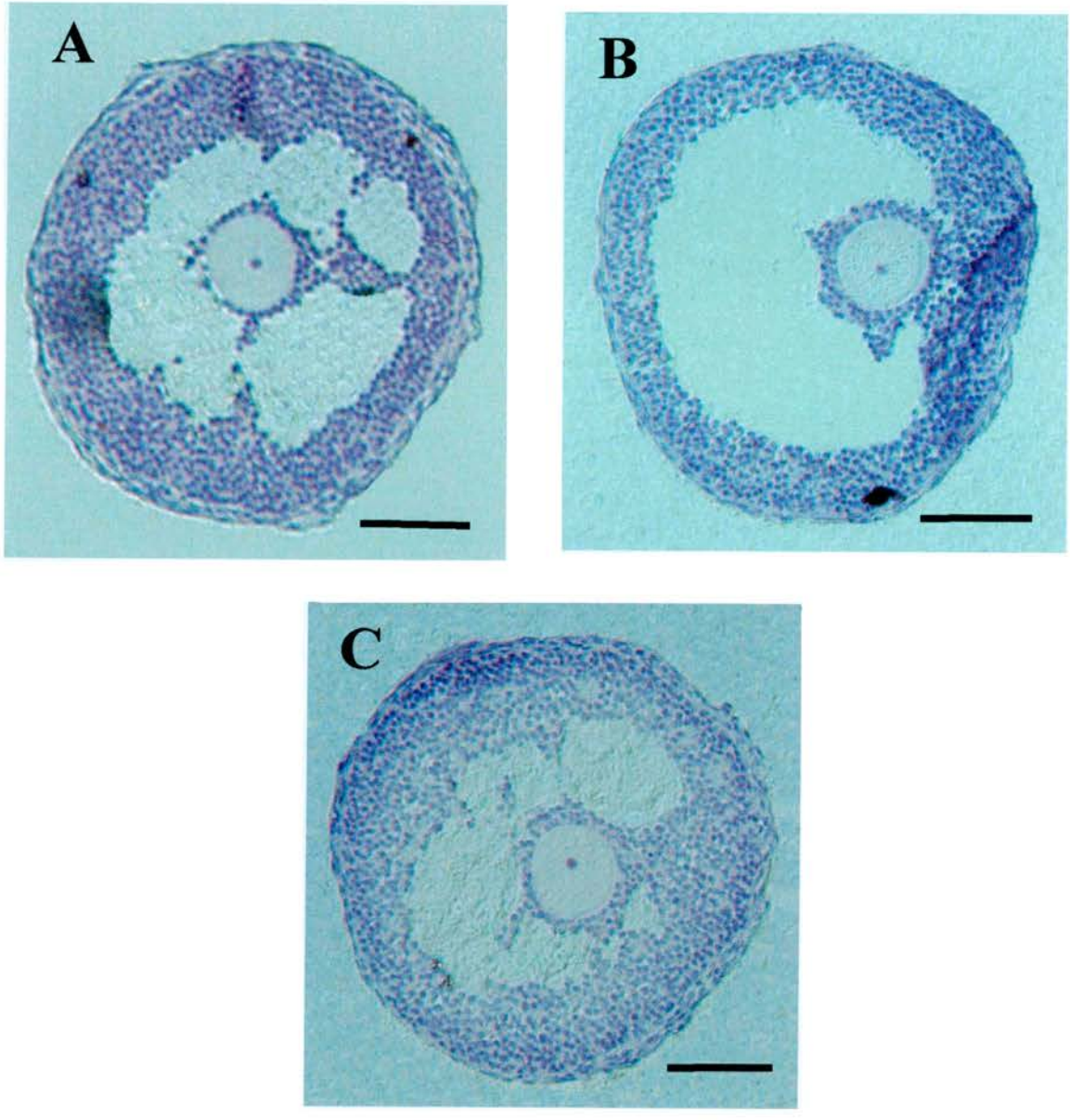


Figure 3.3 Haematoxylin and eosin stained plastic sections of follicles grown in 0μm **(A),** 28μm **(B)**and 280μm **(C)** Ascorbic acid for 6 days *in vitro.* Scale Bar = 80μm

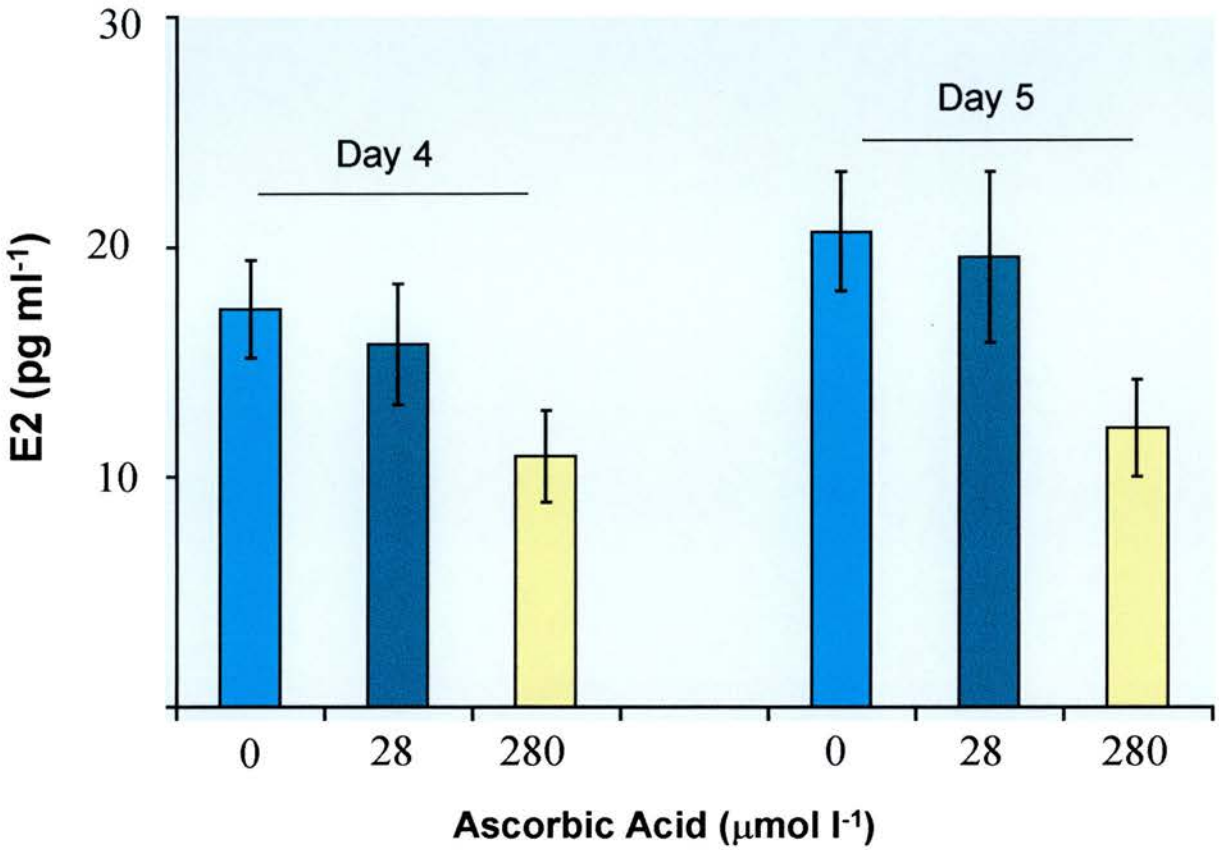


Figure 3.4 Production of oestradiol by follicles on days 4 and 5 of culture. Follicles were cultured in control media or containing 28μm and 280μm ascorbic acid l^{-1}. Data are mean ± SEM ($n \geq 25$)

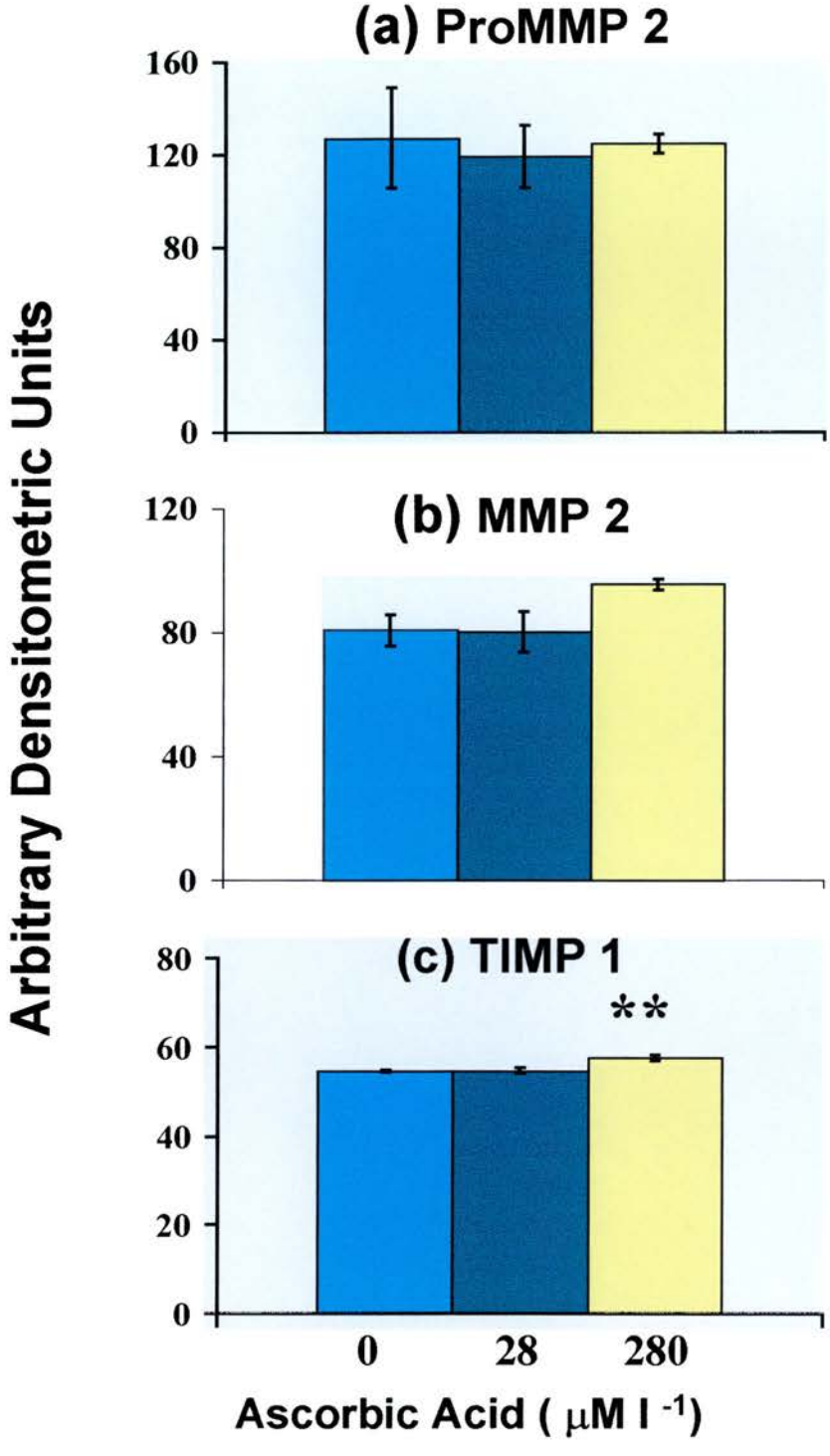


Figure 3.5. Effect of ascorbic acid concentration on (a) pro-matrix metalloproteinase 2 (MMP-2), (b) activated MMP-2 and (c) tissue inhibitor of metalloproteinase 1 activities in mouse follicles. Values were assigned arbitary densitrometric units.
Values are mean ± SEM (n = 30). ** = $p < 0.01$ compared to control.

A

1 2 3

Figure 3.6 A
Representative gel of mouse follicles showing 'laddering' of nuclear DNA into ~185 base pair multiples. Lane 1 shows the positive control group (cultured with serum, 5 μg selenium $ml^{-1}$and 280μm ascorbic acid l^{-1}). Lane 2 is the control group (cultured without serum). Lane 3 is the experimental group (cultured in the absence of serum but with with 280μm ascorbic acid l^{-1}).

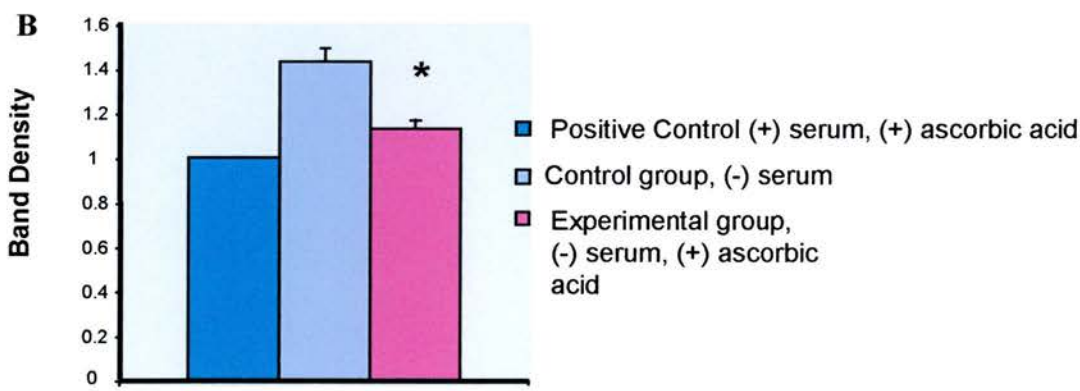


Figure 3.6 B.
Relative densities of apoptotic bands between culture groups calculated relative to the control group containing serum which was assigned an arbitrary value of 1.
Values are mean ± SEM (n=24)* P<0.05 when compared to the control group.

3.5 Discussion

In common with other endocrine tissues, the ovary is a site of ascorbic acid accumulation, which fluctuates in response to stimuli such as luteinising hormone, cyclic AMP and prostaglandins. Most studies have concentrated on the well-known effects of ascorbic acid on steroidogenesis in response to these factors (Sanyal and Datta, 1979). However it has been suggested that the high concentrations of ascorbic acid measured within ovarian tissue are in excess of those required solely for steroidogenesis. As the ovary is a site of intensive tissue re-modelling, ascorbic acid is probably required as a co-factor in collagen production (Luck *et al.*, 1995). Recently much attention has been paid to the ability of ascorbic acid to act as an anti-oxidant (Padh, 1991): as both tissue re-modelling and steroidogenesis are processes that produce reactive oxygen species, it would seem likely that ascorbic acid serves this function within the ovary.

Ascorbic acid is an unstable vitamin and is easily oxidised to its reduced form dehydroascorbic acid by heat, oxygen, pH and temperature. Dehydroascorbic acid also has physiological activity however, it is even more readily degraded to diketoglutonic acid which has no activity (Padh, 1985). The formula of the culture media used in these experiments contains ascorbic acid that may have confounded the results obtained. However, it is likely that much of the vitamin would have degraded in the media. In order to assess to what degree this had occurred, the media would have needed to be titrated for ascorbic acid activity. Nonetheless, the study presented here indicates that high levels of ascorbic acid are needed to contribute to the multi-functional activities of this vitamin in follicular development.

The hormonal influences that affect follicular growth and development have been well documented but little is known about the physical processes that occur throughout the growth phase. During follicular growth and expansion there is a rapid production of the basal lamina that separates the thecal and granulosa compartments. In the mouse this has been estimated as a 30 x 10^3 fold increase (Gosden *et al.*, 1993). In the in-vitro system described here, murine follicles grew from ~200μm to ~415μm over a culture period of 6 days resulting in a 4.3 fold increase in follicular surface area. While ascorbic acid had no effect on the growth rate or morphology of the follicles, there was a marked increase in the percentage of follicles able to maintain basement membrane integrity. Without ascorbic acid only 33% of the follicles had retained an intact membrane after 6 days in culture compared with 88% when media was supplemented with 280μM ascorbic acid. Although this is a higher level than commonly found in human serum, it correlates reasonably well with the high levels of ascorbic acid found in human follicular fluid (Luck *et al.*,1995). These results are in good agreement with a recent report by Rose *et al.* (1999). In that study, the addition of selenium and ascorbic acid also increased the percentage of follicles able to maintain their spherical morphology when cultured *in vitro*. In the culture system used here, no selenium was added to the media and therefore the effect seen can be attributed solely to the addition of ascorbic acid. A very early study with scorbutic guinea pigs noted that these animals were anovulatory and exhibited a marked degeneration of the follicular wall (Kramer *et al.*, 1933). A major component of the follicular basal lamina is collagen IV, which both granulosa and theca cells can produce *in vitro* (Rodgers *et al.*, 1995; Zhao and Luck, 1995). Ascorbic acid has a well-known role in promoting collagen synthesis, both at the level of the gene, and as

a co-factor in the secretion and stabilisation of the protein (Pinnell, 1985). Therefore it could be assumed that the growing follicle would have a high requirement for ascorbic acid if it is to produce sufficient basal lamina components to maintain expansion of this membrane during development. The results presented here support this idea. We also have unpublished data (Sršeň and Spears) showing that follicles grown in the presence of ascorbic acid require a more stringent permeabilisation treatment before fixation, providing further evidence for a role of this vitamin in basal lamina formation and stabilisation. Rodgers *et al.,* (1998) have recently reported that in bovine follicles the distribution of the collagen IV chains within the basal lamina change composition during follicular development. As ascorbic acid was present in the media throughout the culture period, it was not possible to determine whether it was required at all stages of development or, for example, only during pre- or post-antrum formation.

Extra cellular matrix components are constantly remodelled by the action of extra cellular proteases, mainly MMPs and their inhibitors TIMPs. As such, MMPs and TIMPs are obvious candidates for modulating the ovarian follicle basement membrane. The collagenous component of the ovarian stroma includes interstitial collagen type I and III whereas collagen type IV is the collagenous component of the basement membrane. Collagens can be degraded by MMP2, which is activated during structural luteolysis, concurrent with a marked depletion in ascorbic acid (Endo *et al.*, 1993). In humans, a vitamin C deficiency has been associated with increased rupture of placental membranes and addition of ascorbic acid to cultured human amnion cells decreased MMP-2 expression and activity (Pfeffer *et al.*, 1998). These previous reports suggest that ascorbic acid influence the actions of the

metalloproteinases. In the present study the pro- and active forms of MMP2 (gelatinase A) were detected within cultured murine follicles collected on day 2, just prior to the period during which most cultured follicles frequently began to rupture. While similar levels of MMP2 and TIMP1 were present in control follicles and in those cultured with the lower concentration of ascorbic acid, a significant increase in TIMP1 ($p<0.01$) was observed at the higher concentration of ascorbic acid used: this is the same treatment in which a significantly higher percentage of follicles have the ability to maintain basement membrane integrity. TIMPs and MMPs frequently increase simultaneously (Murphy *et al.*, 1985); in this instance there was a concomitant increase in the active form of MMP2 although this was below the level of significance ($p<0.08$). While our result adds further evidence for a role of ascorbic acid in mediating metalloproteinase activity within the growing follicle, it seems most likely to be acting through a mechanism which allows both formation and expansion of the basement membrane, and hence requires both MMPs and TIMPs.

Absence of serum from the culture media induced apoptosis in the pre-antral follicles within a short period of time (24 hours of culture), as detected by DNA laddering. As shown here, addition of ascorbic acid to the serum-free media reduces the degree of apoptosis within these follicles. Tilly and Tilly (1998) have described a similar effect of anti-oxidants (including ascorbic acid) in short term cultures of rat antral follicles. From their experiments they concluded that oxidative stress could play a role in follicular atresia through inducing an apoptotic mechanism. Ascorbic acid accumulation has been described within follicles at all stages of development; in the small follicles of the buffalo (Meur *et al.*, 1999), Graafian follicles of humans (Paszkowski and Clarke, 1999) and from the current study, by late pre-antral follicles

of the mouse. Granulosa cells, under the influence of FSH, actively uptake ascorbic acid (Berhman *et al.*, 1996) and while this is consistent with the idea of ascorbic acid being required as a co-factor in collagen synthesis and hence basal lamina expansion, it is likely that ascorbic acid also functions as an anti-oxidant preventing cell death. At the late pre-antral stage of development follicles acquire responsiveness to gonadotrophins and develop rapidly, therefore it is feasible that an ability to accumulate ascorbic acid confers an advantage to the follicle and promotes survival.

No effect of ascorbic acid was found on oestradiol production. This is in contrast with other systems, where it has been suggested that the addition of ascorbic acid *in vitro* adversely affects aromatase activity through alteration of pH (Milewich *et al.*, 1981). Increasing levels of ascorbic acid are inhibitory to cholesterol side chain cleavage (Pintauro and Bergan, 1982) which subsequently affects androgen production and decreases aromatase activity directly (Sanyal and Datta, 1979, Tsuji *et al.*,1989). As progesterone was not measurable in any media, no effect of ascorbic acid on this steroid could be determined. However the lack of progesterone production shows that the granulosa cells in follicles unable to maintain their basement membrane had not undergone premature luteinisation. The relationship between ascorbic acid and oestradiol appears to be complex. In woman using oral contraceptives circulating ascorbic acid levels are low, perhaps through an inhibitory effect of oestradiol on ascorbic acid uptake by the intestine (Kuo and Lin, 1998). When high doses of ascorbic acid were administered exogenously to pregnant women, oestradiol production increased, resulting in abortion (Samborskaia and Ferdman, 1966). However, the Graafian follicle, which has high levels of oestradiol in the follicular fluid, is the site of ascorbic acid accumulation (Paszkowski and

Clarke, 1999). High concentrations of oestradiol are required to inhibit the oxidation of low-density lipoproteins (LDL) *in vitro*. However, in the presence of ascorbic acid, concentrations of oestradiol close to physiological levels can also protect LDL from oxidation (Huang *et al.*, 1999). While the effect of ascorbic acid on oestradiol may be tissue specific, further investigations are needed on the effect of ascorbic acid on oestradiol production within the follicle.

The conclusions of this study show that ascorbic acid greatly increased the percentage of follicles able to maintain their basement membrane *in vitro* with affect growth rate. Ascorbic acid also increased the production of MMP2 and TIMP1 by the cultured follicles, which implicates this vitamin in modulating matrixmetalloproteinase re-modelling of the basement membrane. Ascorbic acid reduced the degree of apoptosis within the follicles when subjected to oxidative stress (serum-free cultures) suggesting that accumulation of this vitamin would be advantageous for survival. Any effect of ascorbic acid on oestradiol production requires further investigation.

Chapter Four

The Effects of Different Gonadotrophin Regimes on Ovarian Function

4.1 Introduction

The gonadotrophin hormones FSH and LH were first isolated over 50 years ago (Greep *et al.,* 1942) and since then their roles in the female reproductive cycle have been intensively studied. It is now well established that the cycle of ovarian follicular development and ovulation are dependent upon the actions of these gonadotrophin hormones. In common with the other members of the gonadotrophin family, both hormones share a common α subunit non-covalently joined to a β subunit to form the biologically active hormone. The hormones bind to specific receptors through their β sub-unit and it is this unit that confers hormone specificity (Huhtaniemi and Aittomaki 1998; Layman and McDonough, 2000).

Within the ovarian follicle, FSH receptors are only found in granulosa cells. LH receptors are initially confined to the thecal layers but are also found in granulosa cells as the follicle proceeds towards pre-ovulatory development. Both FSH and LH receptors belong to the G protein coupled family which, when activated, results in the generation of cAMP. Downstream events result in the production of protein kinase A which mediates intracellular signalling events including gene transcription (Catt, 1996).

Follicles are capable of reaching the earliest stages of antral formation in the absence of gonadotrophin stimulation (Kumar *et al.,* 1997; Halpin *et al.,* 1986). However, during the transition between the slow growing pre-antral phase and the faster pre-ovulatory stage, follicles become increasingly sensitive to and are finally dependent on gonadotrophin stimulation. The classic 'two cell-two gonadotrophin' theory advocates that both FSH and LH are necessary for follicular maturation and production of steroids (Armstrong and Dorrington, 1977). FSH has been attributed

with stimulating granulosa cell proliferation and aromatase expression and antral formation (Yong *et al.,* 1992, Gosden *et al.,* 1988). Stimulation of the thecal cells by LH provides androgen substrate for subsequent oestrogen production within the granulosa cells (Hillier *et al.,*1994). FSH also promotes functional LH receptors within the granulosa cells during pre-ovulatory follicular development which act in synergy with FSH to augment steroidogenesis by paracrine signalling (Whitelaw *et al,* 1992; Hillier, 2001) as well as preparing the follicle for the ovulatory surge of gonadotrophins (Toledo *et al.,* 1996; Chappel and Howles, 1991).

The ability of a follicle to respond to the available levels of gonadotrophins at a particular developmental time-point will ultimately decide its fate. Of a growing cohort of follicles, only those that can take advantage of the rising mid-cycle rise in systemic FSH concentration proceed through to the pre-ovulatory stage of development (Zeleznik and Hillier 1996). From this growing cohort only those follicles that have matured to the point of having functional LH receptors can survive the decline in FSH brought about as a result of oestrogen and inhibin feedback on the hypothalamic-pituitary axis (Zeleznik and Hillier 1996). While this process is the result of a complex set of factors (Spears *et al.*, 2002;Baker and Spears, 1999) the administration of exogenous gonadotrophins can over-ride the selection processes resulting in the superovulation of a larger than normal number of oocytes (Fowler and Edwards, 1957). This technique has been widely used in animal experimentation and also in agricultural and clinical settings (Oelsner *et al.,* 1978).

The roles that FSH and LH play in follicular development have been extensively studied for many years. Until recently it has been difficult to ascribe an observed response to either one or other of the gonadotrophins, as preparations of

one devoid of the other had not been available. However, recent advances in recombinant DNA technology has made available recombinant forms of FSH and LH (Recombinant Human FSH Product Development Group 1998) making it possible to define more exactly their individual roles. From the growing number of reports that have utilised these recombinant forms of gonadotrophins it is now becoming clear that FSH alone is capable of promoting follicular growth and development. This has been demonstrated in mice (Spears *et al.*, 1998), rats (Mannaerts *et al.,* 1994, van Cappellen *et al.,* 1995), primates (Weston *et al.,* 1996) and humans (Balasch *et al.,* 1995). Although LH augments steroidogenesis and may play a critical role in the ovulatory process (Zhang *et al.,* 2001; Chappel and Howles, 1991), follicular growth and development are not dependent on its actions.

Controlled hyper-stimulation of the ovaries has been used in assisted reproductive practice for many years as a means of obtaining a large number of oocytes for techniques such as *in vitro* fertilisation (Oelsner *et al.,* 1978). Historically, the preparations used were derived either from pituitary or urinary sources which contain both gonadotrophins. Originally, compounds contained equal bioactivities of FSH and LH, but over recent years newer generations of these preparations have evolved with a much- reduced LH content.

A number of studies have utilised recombinant forms of gonadotrophins to investigate their separate effects. Recombinant FSH alone has been used to treat women with Kallmans syndrome and those with hypogonadotrophic disorders (Levy *et al.*, 2000). In these studies, stimulation produced multiple pre-ovulatory follicles but resulted in inadequate oestrogen synthesis resulting in poor quality oocytes and lack of endometrial development (Shoham *et al.,* 1994; Balasch *et al.,* 1995). This

would suggest that some LH stimulation may be necessary to optimise the follicular and reproductive environments. However, the concentration of LH required to optimise stimulation protocols has been the subject of many debates. Reports from clinical studies have suggested that elevation of LH levels (i.e. above basal rates) during ovarian stimulation protocols may adversely affect the achievement of pregnancy but the nature of the defect caused in the reproductive process is unclear. There is some evidence to suggest that excess administration of LH impacts directly on oocyte quality, giving rise to poor rates of fertilisation and development (Regan *et al.,* 1990; Danforth 1995). Other reports have indicated that high levels of exogenous LH affects endometrial function leading to increased incidences of miscarriage (Balen *et al.,* 1993; Shoham *et al.*, 1993). However, other data have failed to confirm any correlation between elevated LH levels and pregnancy rates (Thomas *et al.,* 1989; Kovacs *et al.,* 1990). From these conflicting reports it is still unclear what effect LH has on oocyte development within the follicle prior to ovulation and how this affects the quality of the embryo after fertilisation.

In order to begin to investigate the influence that gonadotrophin stimulation has on oocyte quality in humans, the setting up of a large multi-centre trial would be required. Such a study would be difficult to achieve for a number of reasons. The protocols used to induce ovarian stimulation vary widely between clinics and there may be a reluctance to change these protocols as any alteration may jeopardise the pregnancy rates that are already achieved. As infertility can occur as a result of many medical conditions and the responsiveness to treatment varies widely between patients, some difficulties could be encountered in comparing results. Another consideration would be the cost of such a study, which in many instances is borne by

the patient, recombinant drugs being more expensive than those derived from other sources. Rodent models have been used extensively to further our knowledge of many reproductive processes and offer certain advantages. Manipulation of the ovary is relatively easy in these animals and there is less variation between individuals so that the starting material for experimentation is more homologous. The use of an animal model would, therefore, seem an appropriate way in which to begin to investigate the effects of exogenous gonadotrophin stimulation on oocyte quality. This study aimed to do this using the mouse as a model.

Specifically the aims of this research were to:

1. Determine if optimally viable oocytes can develop in follicles matured in the absence of LH.
2. Determine if there is an optimal ratio of FSH: LH (should LH be a requirement).
3. Determine whether administration of high levels of LH is detrimental to the oocyte.
4. Determine the effects of LH on potential mediators of follicle development.

In order to achieve these aims, experiments were designed that utilised both an *in vitro* and *in vivo* model. By comparing the results of the two models it should be possible to differentiate between the direct and indirect effects that FSH and LH have on follicular development and subsequent embryonic development.

In Vivo Model

Mutant *hypogonadal* mice are infertile and have no measurable circulating gonadotrophins. Ovarian function can be induced by administration of exogenous gonadotrophins (Halpin *et al.*, 1988) and mating and pregnancy can occur (Gibson *et al.*, 1994). By administering recombinant forms of FSH and LH to these animals I aimed to examine their effects on follicular growth and oocyte viability in the presence of other *in vivo* mediators. Any effects found could be due to either intra and/or extra ovarian effects of the gonadotrophins.

In Vitro Model

In order to investigate the precise effects of LH and FSH on follicular growth and oocyte viability directly, without any extra-ovarian or inter-follicular effects, a follicular culture system was employed. This culture system has been used extensively within our laboratory.

Comparison of the two models

In vitro, the follicles (and their oocytes) are exposed only to the gonadotrophin regimes administered and are isolated from any other gonadotrophin influences and mediators that may arise as a result of any follicular interaction within the ovary as well as any extra-ovarian effects. The results obtained from these experiments will demonstrate only direct effects of gonadotrophin administration. *In vivo*, the results may not only indicate these direct effects but also will indicate how gonadotrophin actions influence other aspects of reproductive function. By

comparison of these results it should be possible to decipher more precisely how LH and FSH act during follicular development.

4.2 Materials and Methods

4.2.1 The effects of differing gonadotrophin stimulation regimes *in vivo.*

Animals

The colony of *hypogonadal* mice was housed under the same conditions as described in Chapter Two. Mating heterozygote parents produced *hypogonadal* mutants (*hpg* $^{-/-}$).

Identification of *hpg* mutants

Analysing DNA extracted from a tail tip by PCR as described in Chapter Two identified female mice homozyous for the *hpg* mutation

Vaginal smears

Vaginal smears, for determination of cycle stage, were performed by inserting the tip of a plastic pipette into the vaginal opening of the animals and flushing distilled water into the vagina. The resultant mixture was then smeared onto a glass slide and allowed to dry. The slides were fixed in 96% alcohol before being briefly dipped into 0.5% toluidine blue stain (w/v H_2O). The slides were then washed in distilled water and allowed to dry before being examined under the microscope. A note was made of the prevalent cell type present in each smear (as described by Bloom and Fawcett in A Textbook of Histology)

4.2.1.1 Experiment 1: Optimisation of the superovulatory regime

Previous trials suggested that *hpg*$^{-/-}$ females did not respond to the standard doses of superovulatory gonadotrophins (5IU PMSG; 5IU hCG) used routinely in the laboratory. To optimise the superovulatory regimes required to induce a response in

mutant animals, different gonadotrophin preparations were used to superovulate both wild type (WT) and mutant animals. The protocols used were as follows;

Nine WT animals were used and allocated to one of three groups and injected with

Group 1 5IU PMSG

Group 2 10IU PMSG

Group 3 10IU rhFSH

Twelve *hpg*$^{-/-}$ animals were split into two groups and administered either

Group 1 10IU PMSG

Group 2 10rhFSH

All animals were subsequently injected with 5IU hCG 54 hours after the initial stimulatory injection. Animals were sacrificed 13-15 hours after hCG administration and their ovaries and oviducts removed to warm L-15 media (see Chapter Two). At the time of sacrifice, a piece of tail tip was taken to confirm genotype. DNA extraction and the PCR protocol are described in Chapter Two.

On re-checking the genotypes it was found that three of the animals were not mutants. These were then excluded from the experiment and data was collected from 3 animals administered 10IU PMSG rather than 6 animals.

In vitro fertilisation

Oocytes obtained from the superovulated animals were transferred to drops of T6 media and IVF was carried out. As a control for the IVF system two F1 animals were superovulated at the same time. The protocols used are described in Chapter Two.

4.2.1.2 Experiment 2: The effect of gonadotrophin stimulation on ovarian function.

Based on the results from Experiment One and from the previous trials carried out, this experiment set out to examine the effects of administering differing gonadotrophin regimes on ovarian function.

Gonadotrophin administration

Sixteen female mutant mice between the ages of 6-20 weeks were allocated into one of four groups. Each group of animals was administered different treatments via intra-peritoneal injection. Recombinant gonadotrophins were reconstituted in 0.9% saline and administered twice daily. As a control, one group of animals was administered with the same volume of 0.9% saline. The treatment groups were as follows and represent the total daily dosage given;

Group 1 1 IU rhFSH **(rhFSH only)**

Group 2 1 IU rhFSH + 0.01IU rhLH **(low rhLH)**

Group 3 1 IU rhFSH + 0.5IUrhLH **(high rhLH)**

These treatments were administered daily for a period of 12 days excluding day 11 when 10IU rhFSH was given as a single bolus. On the 13th day after the start of treatment 5IU hCG was administered as a single bolus. During the course of the experiment one animal from the low LH group (group 2) died, therefore, data was collected from only 3 animals for this treatment.

Vaginal Smears

Throughout the period of gonadotrophin administration animals were examined daily for vaginal opening. Once the vagina was open, smears were taken using the method described in 4.2.1 above.

In vitro fertilisation

After the final bolus injections of rhFSH and hCG, the animals were sacrificed and the reproductive tracts removed into warm dissection medium (as described in Chapter Two). The oviducts were examined and any oocytes present were removed for IVF. The protocol used was exactly as described in Chapter Two.

Uterine Weight.

In order to determine whether the ovaries had been stimulated sufficiently to induce a systemic rise in oestrogen, the uteri from the treated *hpg* animals were weighed. The uteri were trimmed free of adherent tissue and blotted dry before weighing.

Histology.

The ovaries from the animals were processed for histological examination as described in Chapter Two.

4.2.2 The effects of differing gonadotrophin stimulation regimes on follicular development and oocyte competence *in vitro*.

Animals

21-25 day old F1 hybrid female mice were used for these experiments. The animals were bred and maintained as described in Chapter Two.

Follicle isolation

Ovaries were removed from the animals, cleaned and pre-antral follicles isolated as described in Chapter Two.

Follicle Culture

α– MEM media was prepared and the cultures maintained as described in Chapter Two. To minimise any effects of gonadotrophin contamination introduced in serum, the serum used in these experiments was obtained from both male and female *hpg* $^{-/-}$ animals. Isolated follicles (175 ± 10 μm) were randomly allocated to one of three treatment groups. A further group of follicles were allocated to media without the addition of any gonadotrophins. These follicles were the controls. The treatment groups were as follows;

Group 1	5 IU ml^{-1} rhFSH	**(rhFSH only)**
Group 2	5 IU ml^{-1} rhFSH + 0.01 IU rhLH ml^{-1}	**(Low rhLH group)**
Group 3	5 IU ml^{-1} rhFSH + 0.05 IU rhLH ml^{-1}	**(High rhLH group)**

4.2.2.1 Experiment 1: The effect of gonadotrophins on follicular growth, morphology and hormone production.

In order to obtain information on growth, morphology and hormone production, follicles were allocated to the treatment and control groups described above and cultured for a period of six days. As described in Chapter Two any damaged follicles were discarded within the first two days of the culture period. Follicles were examined daily and measured using an ocular micrometer.

At the end of the culture period representative follicles were fixed for histology. The remaining follicles were discarded from the trays. The culture trays, containing the spent media, were then frozen at –20°C so that the media could be analysed for hormones.

Histology.

Follicles were fixed in 4% paraformaldehyde, embedded in LR White resin and 3μm sections were cut. The sections were stained with haemotoxylin and eosin. These methods are given in Chapter Two.

Hormone assays.

Media from throughout the culture period was analysed for androstendione, oestradiol and inhibins A and B. Androstenedione measurements were made by radioimmunoassay as described in Chapter Two. Data was obtained for days 4 and 5 of culture. Oestradiol measurements were made using the technique given in Chapter Two. Data was obtained from days 4,5 and 6 of culture. Inhibin A and B were measured using by enzyme immunoassays described in Muttukrishna *et al.,* 1997. The minimum detection limit of the assay for human recombinant inhibin A was 1 pg./ml and the minimum detection limit of the assay for human recombinant inhibin B was 15pg/ml. Data was obtained for days 4,5, and 6 of culture.

4.2.2.2 Experiment 2: Assessment of oocyte meiotic competence.

To assess whether exposure to different gonadotrophin regimes during the follicular growth period affected the oocytes' ability to undergo germinal breakdown and resume meiosis, follicles were cultured in the treatment groups (1,2 + 3) as outlined above. After four days in culture the follicles were teased apart and the

oocyte-cumulus complexes were removed to droplets of pre-warmed maturation media (as described in Chapter Two). The cumulus cells were removed from the oocytes by gently pipetting the complexes up and down through fine-bore glass pipettes. To compare the timing of maturation *in vitro* with the timing of maturation *in vivo* relative to hormone exposure, oocytes were released from follicles at 4pm. This corresponds to the same time as PMSG was administered to animals as part of the superovulatory regimes used. The oocytes were then transferred to fresh droplets of maturation media and examined every two hours. Within each group a note was made of the number of oocytes with a polar body at each time point.

4.2.2.3 Experiment 3: The effect of gonadotrophins on fertilisation and subsequent embryo development.

To examine the effects of the different gonadotrophin regimes on fertilisation and subsequent embryo development, follicle cultures were set up under control conditions and in the presence of the different gonadotrophin regimes (groups 1,2 and 3) as outlined above. In these experiments the cultures were maintained for a period of 4 days before the oocyte-cumulus complexes were released from the follicles.

Oocyte maturation.

At the end of the culture period, the follicles were teased apart to release the oocyte-cumulus complexes. These were then transferred into pre-warmed maturation media (prepared as described in Chapter Two) and placed into the incubator overnight. On the following morning the complexes were transferred into pre-warmed droplets of T6 media before fertilisation.

In vitro fertilisation

The IVF procedure was carried out exactly as described in Chapter Two. Two F1 animals were superovulated prior to the day of IVF. The oocytes obtained from these animals were a control for the IVF system.

4.2.2.4 Experiment 4: TUNEL labelling of cultured follicles

In light of the results obtained from Experiment 3, TUNEL labelling was performed on follicles exposed to the different gonadotrophin regimes. Follicles were cultured as described in Experiment 3. Typical follicles from each treatment group and the gonadotrophin free control group were TUNEL labelled to detect apoptotic cells. The method used to carry out the staining is given in Chapter Two.

4.2.3 Statistical Analysis

Uterine weights and hormones assays were determined by ANOVA. Where appropriate, comparisons were made by students *t* test or by Tukeys Multiple comparison. The results from the IVF experiments were analysed by chi-square.

4.3 Results

4.3.1 The effects of differing gonadotrophin stimulation regimes in vivo

4.3.1.1 Experiment One: Optimisation of the superovulatory regime.

Groups of wild type and *hpg* -/- animals were administered with PMSG or rhFSH followed by hCG in an attempt to optimise a superovulatory regime that could be subsequently used in experiments using the mutant animals. The results of these treatments and the number of oocytes obtained from each treatment group are given in Tables 4.1A. and 4.1B.

It was found that while wild type animals responded to the standard PMSG preparation routinely used in the laboratory, the mutant animals did not. However, a high dose (10IU) of rhFSH did induce a response in the mutants and this preparation was then used in Experiment Two.

4.3.1.2 Experiment Two: The effect of gonadotrophin stimulation on ovarian function.

A further set of *hpg* mutant females was administered with different gonadotrophin regimes for 10 days prior to superovulation. Superovulation was induced using 10IU rhFSH followed by 5IU hCG.

Throughout the period of gonadotrophin administration vaginal smears were taken. By examining the predominant cell types in these smears it was possible to obtain an indication if any of the treatments had induced oestrus cycles. In all treatment groups vaginal opening occurred by day 5 of treatment in all the animals. In the group of animals administered with rhFSH only, the prevalent cell types seen

in these smears were leukocytes and epithelial cells, with the latter becoming the dominant type during the last 3 days of treatment. This would suggest that these animals had rapidly growing follicles and were in pro-oestrus. No cornified cells (associated with oestrus) were easily distinguishable. However in both the groups treated with rhFSH and rhLH cornified cells were found in the smears. In the group treated with the rhFSH and low rhLH these cell types did not appear until days 9 and 10 of treatment while in the group that had received the higher rhLH dose cornified cells appeared between days 7 and 8. This would suggest that oestrogen production had been initiated at an earlier stage in the latter group. In both instances the smears taken prior to these days consisted of epithelial cells. From these results it would seem that only animals that had been treated with both gonadotrophins exhibited any sign of approaching oestrus.

After the final superovulatory doses of gonadotrophins the animals were sacrificed and the reproductive tracts examined. In the first instance the oviducts were removed and checked for any ovulated oocytes. It was found that three animals from the rhFSH and high rhLH group had done so, each having ovulated once so three oocyte-cumulus complexes were collected in total.

The uterine weights of all the animals were taken and the results are given in Figure 4.1. Although the sample sizes were low, analysis of the data showed that only animals treated with rhLH has significantly greater uterine weights than the other groups (low rhLH = $p<0.5$ and high rhLH = $p<0.001$ compared to the saline and rhFSH only groups). The high rhLH treated group had also significantly higher uterine weights that the low rhLH group ($p<0.05$).

Ovaries from treatment and control group of animals were examined histologically. Figure 4.2 shows photographs of representative sections. Pre-ovulatory follicles can bee seen in each of the groups. Closer examination of the sections showed that in all the groups follicles had matured sufficiently to the ovulatory stage with the oocytes surrounded by expanded cumulus cells. In each group oocytes had resumed meiosis with polar bodies being visible (Figure 4.2e). No differences were noted between the groups. As the saline treated animals responded in the same way as the treatment groups it was concluded that superovulatory regime alone was sufficient to mature the follicles to the ovulatory stage. No conclusion could be reached regarding the prior treatment with gonadotrophins.

***In vitro* fertilisation**

IVF was carried out on the oocytes obtained from both Experiment One and Experiment Two described above. In both cases little fertilisation took place and any resulting two-cells fragmented at the 4-cell stage.

4.3.2 The effects of differing gonadotrophin stimulation regimes on follicular development and oocyte competence *in vitro*.

4.3.2.1 Experiment 1: The effect of gonadotrophins on follicular growth, morphology and hormone production.

The growth rates of the follicles grown in different gonadotrophin regimes are shown in Figure 4.3. The result shows that FSH alone was capable of inducing follicular growth and development and that the addition LH had no effect on the

development of the follicles. There were no significant differences between the growth rates of the gonadotrophin treated groups. In the absence of gonadotrophin stimulation follicles initially began to grow, however, growth was significantly restricted by day three of culture when compared to the groups treated with gonadotrophins ($p<0.001$). Furthermore these follicles began to regress from day 4 onwards.

At the end of the 6 day culture period follicles representative of those in each treatment group were fixed and processed for histological examination as shown in Figures 4.4 The photographs show that there was little morphological difference between the treatment groups. All were similar in size and had developed large antral cavities. Follicles that had been placed in a gonadotrophin free environment did not develop antral cavities and appeared atretic (Figure 4.4).

Media from these cultures were analysed for androstendione, oestradiol and inhibins A and B. While all the groups of follicles were tested, analysis of the media from the gonadotrophin free group was below the assay detection limits for each substance tested. Results for days 4, 5 and 6 of culture were obtained for oestradiol and the inhibins while data on androstendione production was collected from days 4 and 5. The results for these assays are shown in Figures 4.5, 4.6 and 4.7.

In general, hormone production rose significantly in all the treatment groups between days 4 and 5. On day 4 of the culture period, both groups of follicles treated with rhLH were producing androstendione, whereas none was detected in the media from follicles treated with rhFSH alone (Figure 4.5). This was reflected in the oestradiol results, as both groups of rhLH supplemented follicles produced significantly higher concentrations of oestradiol compared to the rhFSH only group

($p<0.001$, Figure 4.6). Androstendione production increased dramatically by day 5 in the low rhLH group (Figure 4.5), however this had little effect on oestradiol production as all groups of follicles produced similar levels of this hormone (Figure 4.6). With time treatment with rhLH reduced oestradiol production which was maintained at a high level by follicles treated with rhFSH alone (Figure 4.6).

While supplementation with rhLH increased androstendione and oestradiol production earlier in the culture period, this was not the case for inhibin A. The results show that significantly higher amounts of this hormone were produced in follicles treated with rhFSH alone compared to the rhLH supplemented groups ($p<0.05$, Figure 4.7A). This difference was maintained until day 6 when all follicles were producing similar concentrations of inhibin A regardless of treatment (Figure 4.7A). Inhibin B was produced by all groups on each day tested. There was no difference between treatments on any day of culture however, only those follicles supplemented with LH showed an increase with time. Both LH groups had produced significantly more inhibin B on day 5 when compared to day 4 ($p<0.05$) but there was no further increase on day 6 (Figure 4.7B).

4.3.2.2 Experiment 2: Assessment of oocyte meiotic competence.

Oocytes from each group of follicles were examined for their ability to resume meiosis and a note made of the time that this event occurred. Oocytes obtained from the gonadotrophin free group of follicles are not included in the graph. It was found that 50% (6/12) of these had already undergone GVB before they were released from the follicle. The majority of these did not complete meiosis and did not have a polar body. Of the remaining oocytes only 2 had a polar body at the end of the

experimental period. The percentage of oocytes in each treatment group with a visible polar body is given in Figure 4.8. In all treatment groups the majority of the oocytes had a polar body visible approximately 9 hours after removal from the follicle. At the end of the experiment it was noted that 25% (3/12) of the oocytes obtained from the group exposed to the higher LH concentration had fragmented.

4.3.2.3 Experiment 3: The effect of gonadotrophins on fertilisation and subsequent embryo development.

Figures 4.9A and 4.9B show the results of the *in vitro* fertilisation experiments. The figures give the combined data from four separate experiments used. The total numbers of oocytes fertilised and reaching blastocyst in each group are presented in Table 4.2. Figure 4.9A represents the percentage of the total oocytes in each group that underwent fertilisation and shows that there was little difference in fertilisation rate regardless of treatment. In all groups 50% (or less) of the oocytes obtained from the cultured follicles fertilised and cleaved to the 2-cell stage. Figure 4.9B shows the percentage of two-cell embryos that reached the blastocyst stage of development. Over 20% of the two-cell embryos obtained from the gonadotrophin free group were able to develop to the blastocyst stage. There was little difference between the groups that had been exposed to FSH only and FSH + 0.01IU LH (50% and 44.5% of the two-cell embryos reaching blastocyst respectively). However only 20% of the two-cell embryos derived form follicles exposed to the higher LH group developed into blastocysts. In both figures (4.9A and B) the results from the superovulated F1 mice are given for comparison. The data from these experiments

was analysed using chi-square analysis but it was found that no statistical difference existed between the experimental groups.

4.3.2.4 Experiment 4: TUNEL labelling of cultured follicles

Figure 4.10 shows a representative section of each follicle labelled with TUNEL and propidium iodide. With the exception of the gonadotrophin free follicle, which had a high level of apoptotic cells, very similar levels of TUNEL labelling were observed in all gonadotrophin treated follicles. In these follicles, TUNEL labelled cells were invariably seen around the antral cavity, as described in Baker *et al.,* 2001. This is likely to be an indication of a healthy antral follicle, as the antral cavity enlarges due to cells lining the cavity becoming apoptotic and their contents then being released into the antral fluid.

TREAMENT (no. of mice)	TOTAL NUMBER OF OOCYTES OVULATED
5IU PMSG (3)	16
10IU PMSG (3)	17
10IU rhFSH (3)	22

Table 4.1A. Number of oocytes obtained from Wild Type mice treated with different superovulatory regimes

TREAMENT (no. of mice)	TOTAL NUMBER OF OOCYTES OVULATED
10IU PMSG (3)	0
10 IU rhFSH (6)	34

Table 4.1B. Number of oocytes obtained from *hpg*$^{-/-}$ mice treated with different superovulatory regimes

	Total number of oocytes	2-Cells (%)	Blastocyst (%)
Gonadotrophin free	114	42 (36)	8 (19)
rhFSH only	123	51 (41.5)	17 (33)
+ 0.01IU rhLH	128	68 (51)	21 (31)
+ 0.05IU rhLH	98	41 (42)	7 (17)
F1 Controls	184	141 (76)	106 (87)

Table 4.2 Total number of oocytes for IVF taken from follicles grown in different gonadotrophin treatments. The figures given are the totals from 4 experiments. Percentages are given in brackets

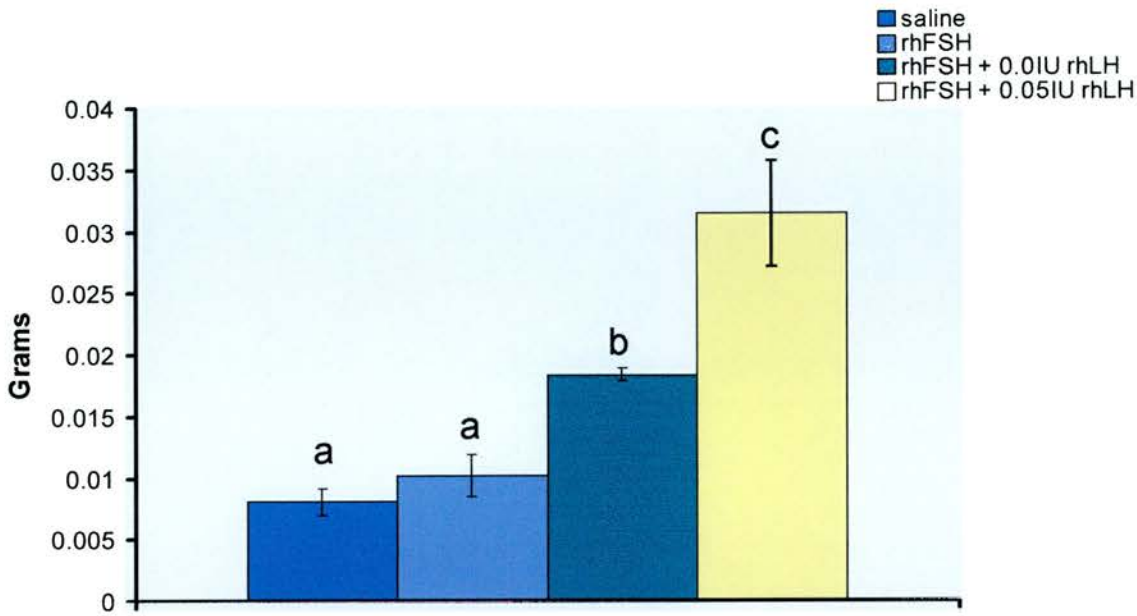


Figure 4.1 Effect of gonadotrophin treatment on uterine weight. Values are mean ± SEM ($n \geq 3$).
Different letters indicate significant differences. Both low and high rhLH treated groups had increased uterine weights compared to the saline and rhFSH only group (b compared with a = $p<0.05$, c compared to a = $p<0.05$) High rhLH was significantly higher than low rhLH (c compared to b = $p<0.05$)

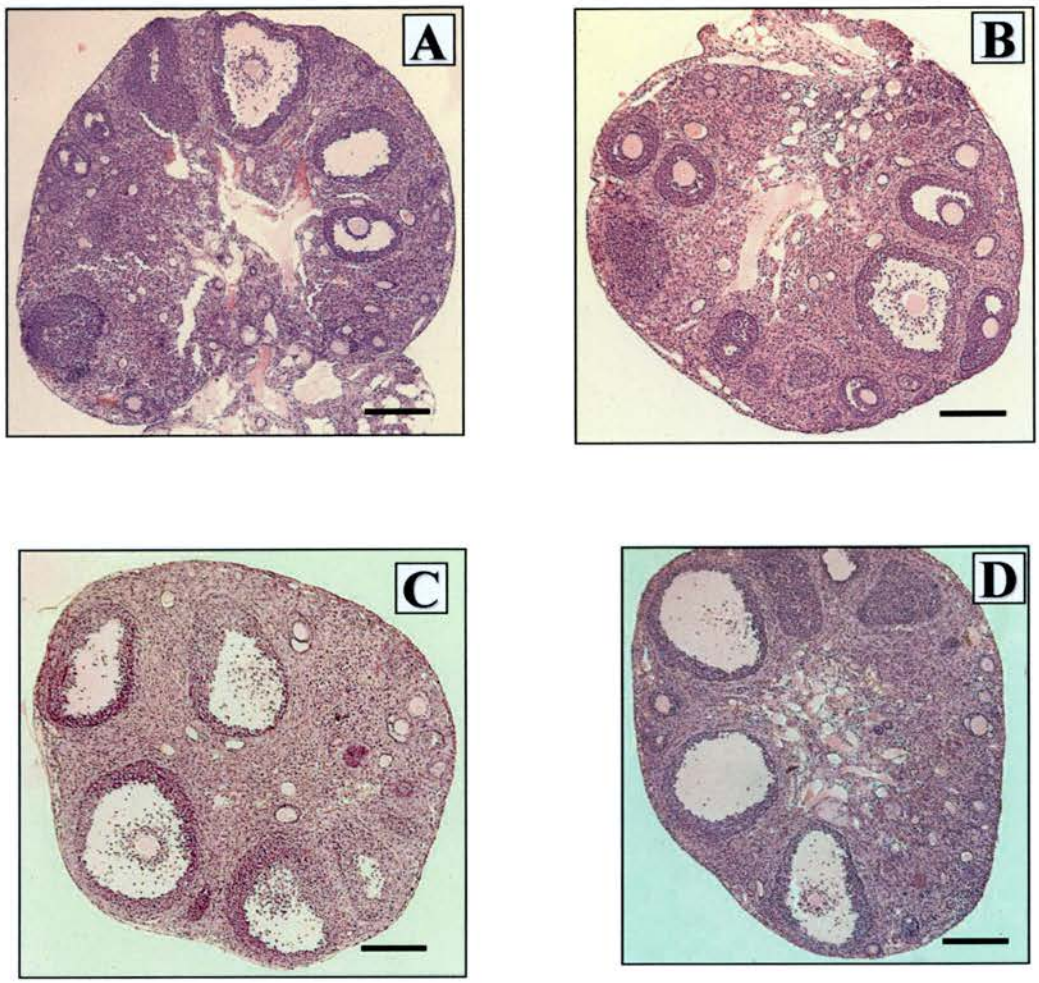
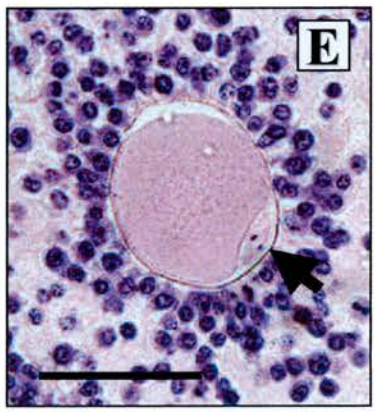


Figure 4.2 Haematoxylin + eosin stained representative sections of *hpg* $^{-/-}$ ovaries after administration of rhFSH (**A**), rhFSH+low rhLH (**B**), rhFSH +high rhLH (**C**) and saline (**D**). Large ovulatory follicles were seen in all groups. Oocytes in all groups had undergone resumption of meiosis. The arrow points to the polar body (**E**). Scale Bar = 80μm



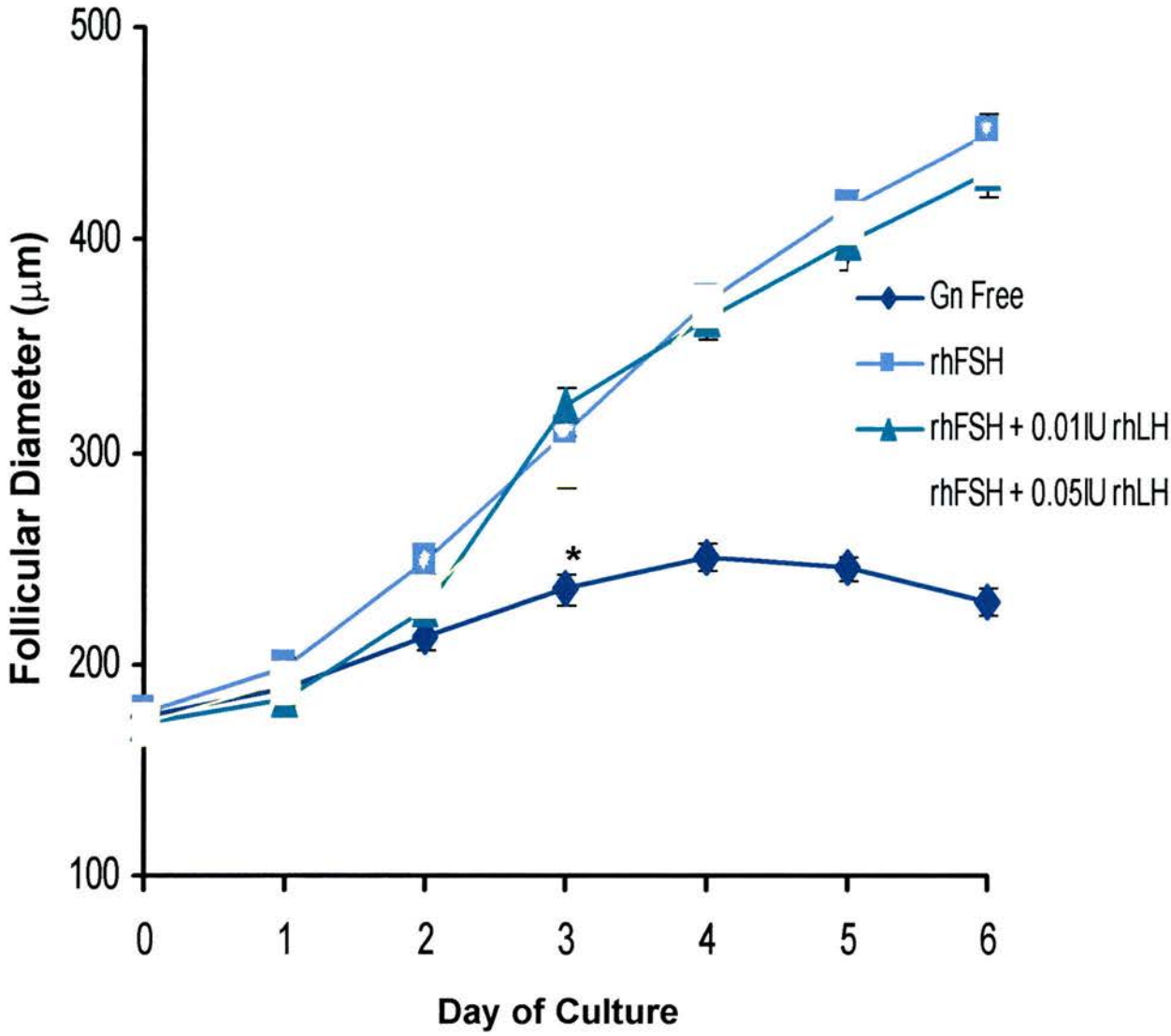


Figure 4.3. Follicular growth rates of follicles exposed to different gonadotrophin environments. Values are Mean ± SEM (n ≥16)
By day three of culture follicles cultured in gonadotrophin free conditions had grown significantly less than the treatment groups (* = $p<0.001$).

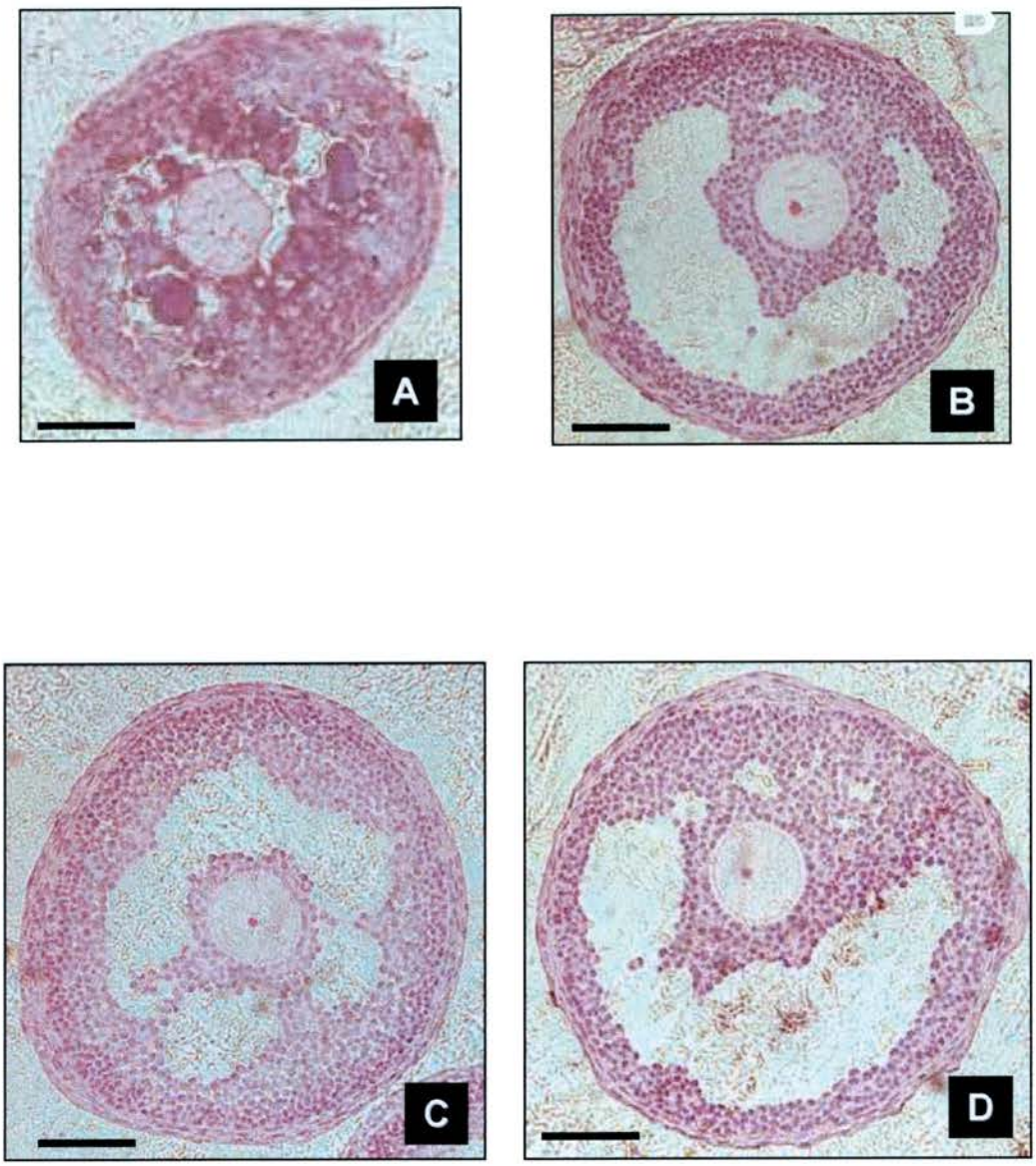


Figure 4.4 Photomicrographs of plastic sections stained with haematoxylin + eosin. Follicles were cultured in gonadotrophin free conditions **(A)**, rhFSH only **(B),** rhFSH + low rhLH **(C)** and rhFSH + high rhLH **(D).** The scale bar = 80μm.

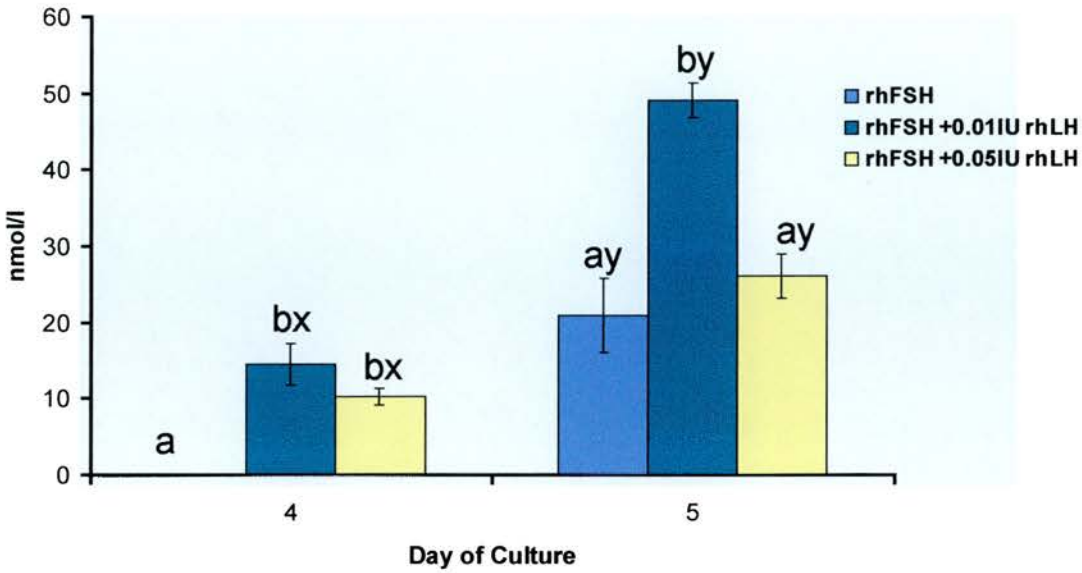


Figure 4.5 Concentration of androstenedione produced by follicles treated with different gonadotrophin regimes on days 4 and 5 of culture. Values are mean ± SEM ($n \geq 9$). Different superscripts indicate significant differences. a,b refer to comparisons between treatments on the same day of culture and x,y refer to comparisons between days within the same treatment. $p<0.05$

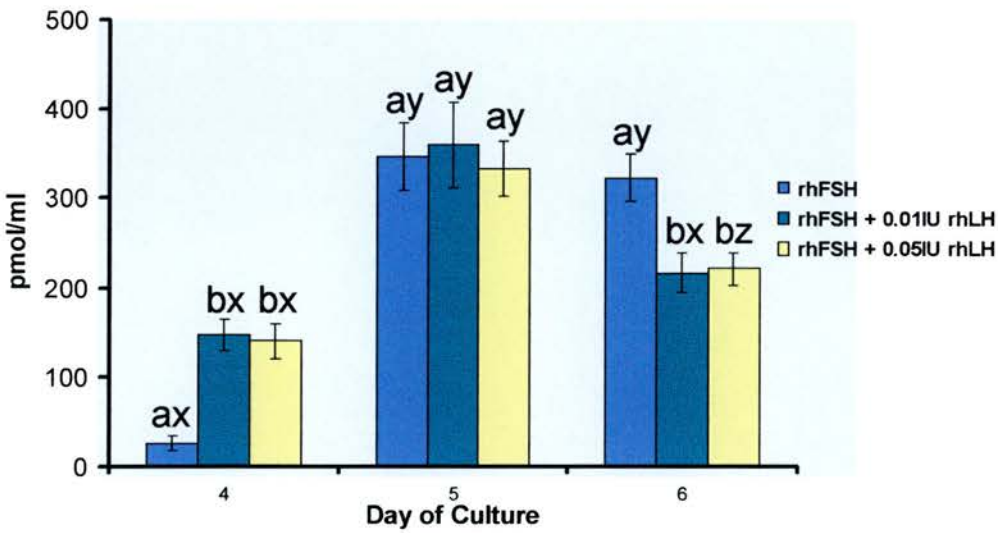


Figure 4.6 Concentration of oestradiol produced by follicles treated with different gonadotrophin regimes on days 4, 5 and 6 of culture. Values are mean ± SEM ($n \geq 16$). Different superscripts indicate significant differences. a,b refer to comparisons between treatments on the same day of culture and x,y refer to comparisons between days within the same treatment. $p<0.05$

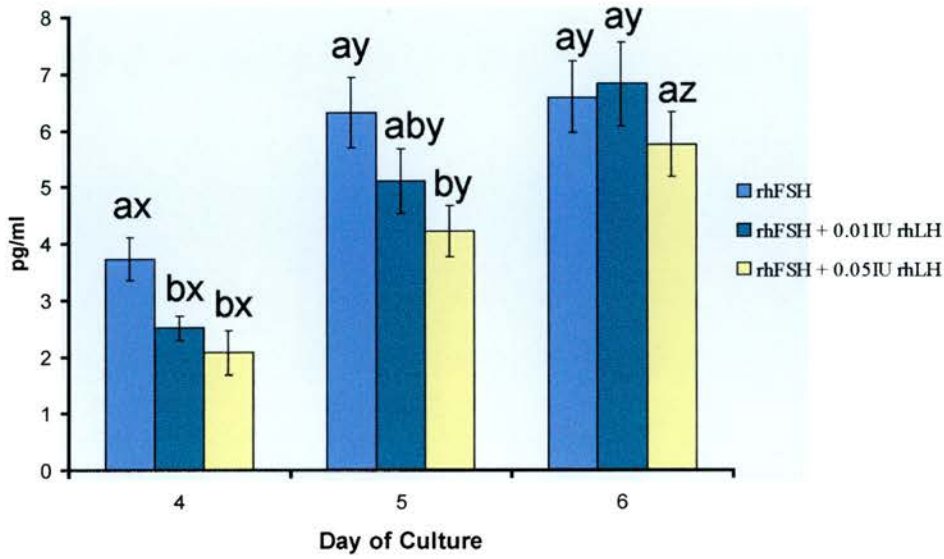


Figure 4.7A Concentration of Inhibin A produced by follicles treated with different gonadotrophin regimes on days 4,5 and 6 of culture. Values are mean ± SEM (n ≥ 16). Different superscripts indicate significant differences. a,b refer to comparisons between treatments on the same day of culture and x,y refer to comparisons between days within the same treatment. $p<0.05$

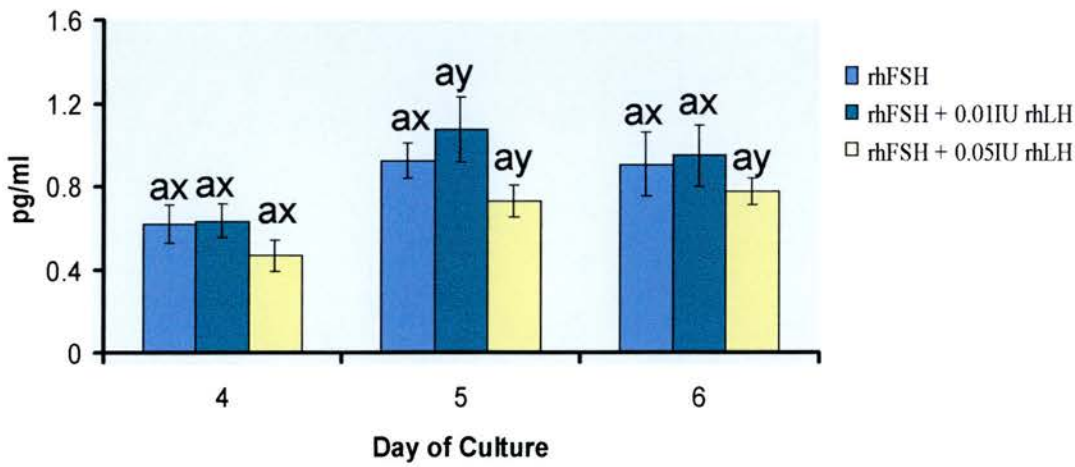


Figure 4.7B Concentration of Inhibin B produced by follicles treated with different gonadotrophin regimes on days 4,5 and 6 of culture. Values are mean ± SEM (n ≥ 16). Different superscripts indicate significant differences. a,b refer to comparisons between treatments on the same day of culture and x,y refer to comparisons between days within the same treatment. $p<0.05$

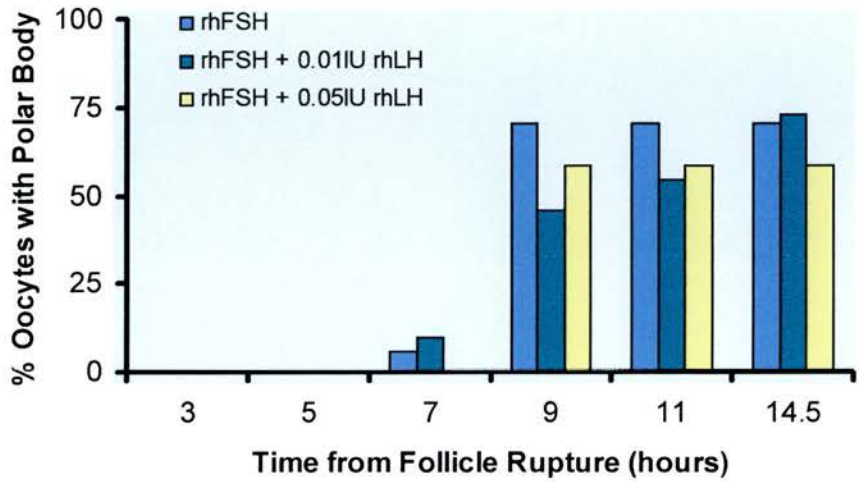


Figure 4.8 Percentage of oocytes with a visible polar body. Oocytes from follicles grown in different gonadotrophin regimes were released and examined every two hours for polar body production (n =12).

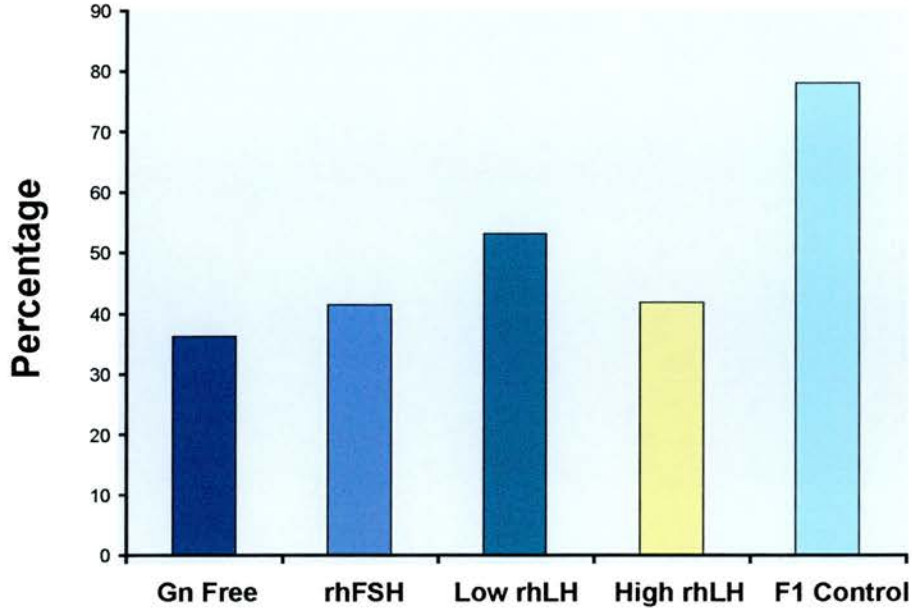


Figure 4.9A. Percentage of oocytes fertilising from *in vitro* grown follicles subjected to different gonadotrophin regimes. There were no significant differences between groups. F1 controls are shown for comparison.

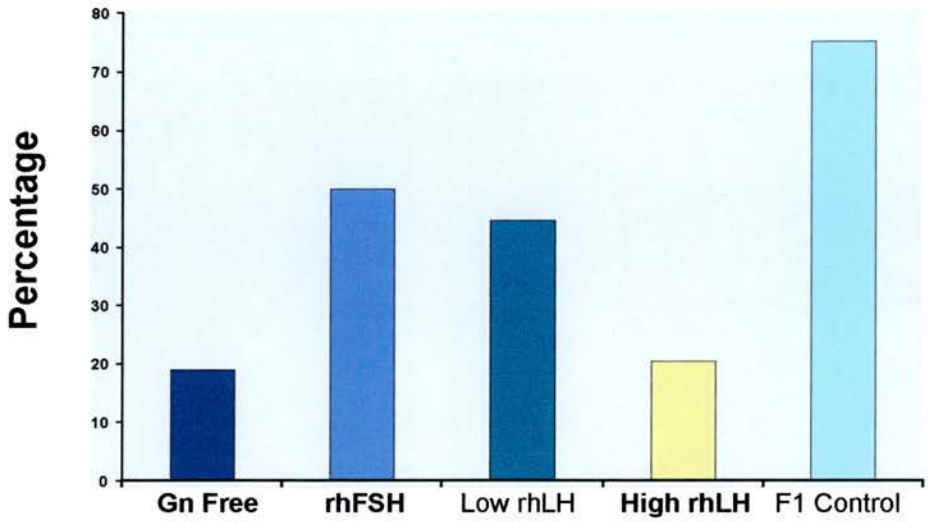


Figure 4.9B. Percentage of 2-cell embryos developing to the blastocyst stage. There were no significant differences between groups. F1 controls are shown for comparison.

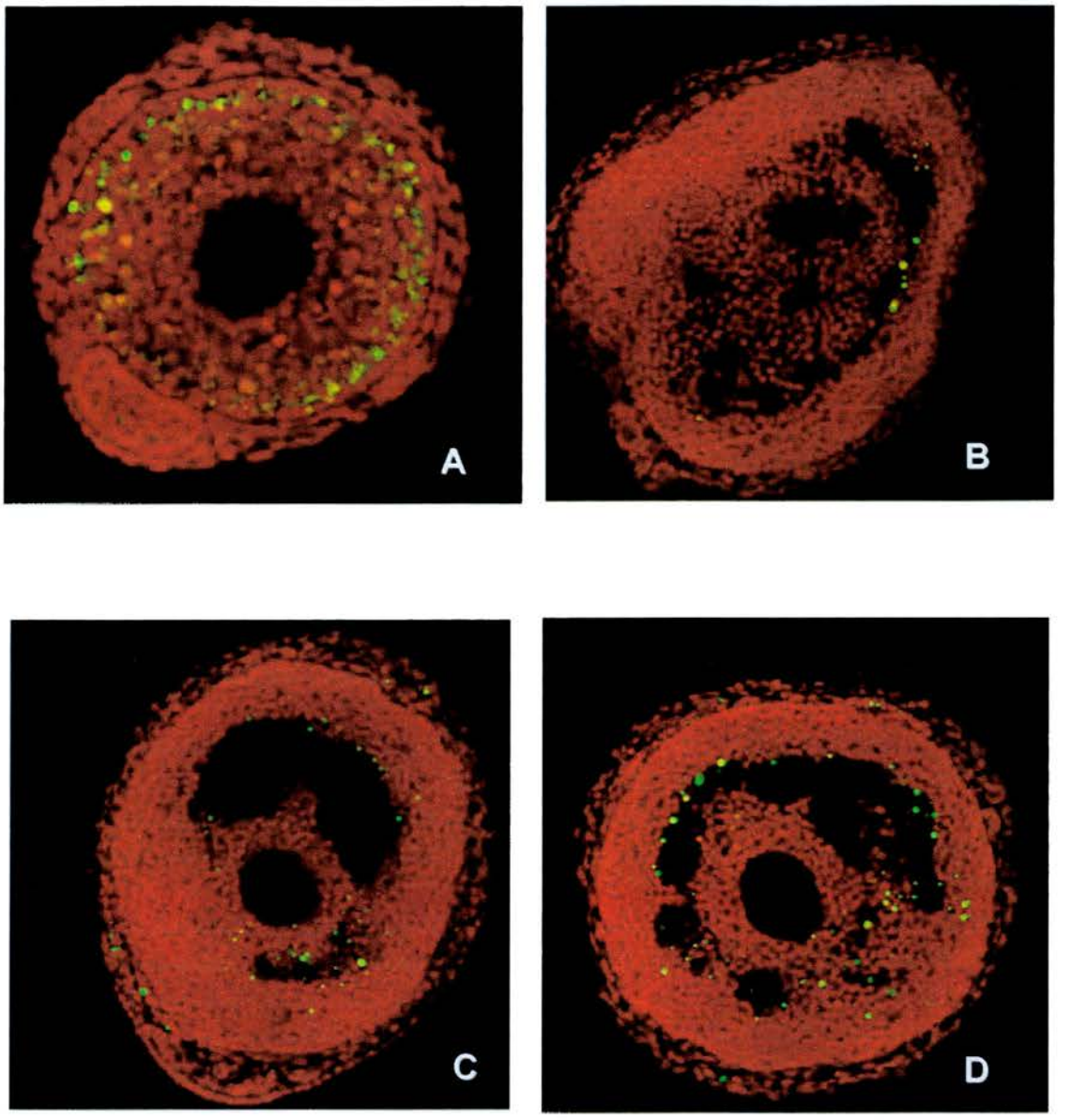


Figure 4.10 Confocal micrographs after labelling with TUNEL and propidium iodide. The green cells indicate apoptosis.

A representative follicle from the gonadotrophin free group
B from the rhFSH only group, **C** from the rhFSH + low rhLH group and
D from the rhFSH + high rhLH group.
There is little difference in TUNEL staining between the treatment follicles. The follicle grown in gonadotrophin free media (A) shows a high degree of TUNEL labelling

4.4 Discussion

The main aim of this study was to examine how altering gonadotrophin stimulation during the follicular growth phase affects oocyte developmental competence. Specifically, the experiments were designed to investigate what effects LH may exert. To distinguish between any extra-ovarian and direct effects seen, experiments were conducted using an *in vivo* and *in vitro* model both of which utilised *hypogonadal* mutant mice.

The results of these investigations were only partially successful in achieving these aims. The results from the *in vivo* work were disappointing due to the difficulties in inducing $hpg^{-/-}$ mice to ovulate and little information was gained on follicular development and oocyte viability. Therefore, no comparisons on this aspect of the study could be drawn between the two models. However, some information was gained on the *in vivo* production of steroids. Examination of vaginal cytology and uterine weight indicated that the inclusion of rhLH was necessary to induce oestrogenic responses in the reproductive tract. Although uterine weights increased three fold in the highest rhLH group this was still well below the normal weight of a uterus from a superovulated F1 animal (~30mgs compared to ~80 mgs) and such a uterus may be insufficiently developed to support pregnancy.

Ultrastructural examination of the *hypogonadal* ovary has shown that these animals have poorly developed interstitial tissue (Halpin *et al.,* 1986) from which the androgen producing thecal layers of the follicle arise. Follicles within the *hypogonadal* ovary may not have a normal attachment of thecal cells and may therefore have a limited capacity to produce oestrogen.

One of the main problems encountered in this particular part of the study was the lack of mutant mice available for experimentation. The available colony was not large, with only $1/8^{th}$ of animals being mutant females. If more *hpg*$^{-/-}$ animals had been available it would have been interesting to perform further studies with rhFSH at higher concentrations and examine whether it is possible to induce oestrogenic responses.

The *in vitro* studies proved more successful. Results were obtained on how follicular growth, development, steroid and hormone production were affected in response to different gonadotrophin regimes. Since recombinant forms of the gonadotrophins have become available a number of studies in different species have established that rhFSH alone can drive follicular development to the pre-ovulatory stage and that LH is not necessary for growth. Similar results were found here with no differences in growth rates being seen in any of the gonadotrophin treatment groups. However, some reports have suggested that the inclusion of rhLH in culture media accelerates the formation of the antral cavity (Liu *et al.,* 2002, Cortvrindt *et al.,* 1998). No differences in antral formation between groups were observed under the conditions used in these experiments. These contrasting observations are possibly the result of the different culture systems used to support follicular development.

Analysis of the culture media showed the pattern of steroid production by the follicles was dependent on the gonadotrophin regime they had been exposed to. The inclusion of rhLH in the culture media augmented androstendione and oestradiol production at an earlier time point in follicular development. This was perhaps not surprising as the effects of LH on follicular steroidogenesis are well documented. However, the results show that the increased oestradiol production could not be

sustained as, with time, both rhLH groups showed a marked reduction in output. Although initially slower to initiate oestradiol synthesis, follicles grown in rhFSH alone were able to maintain production of this steroid throughout the culture period. A possible explanation for these results may lie in the production of inhibin A, as levels of this hormone were also altered dependent on gonadotrophin exposure. Inhibin A production is a function of FSH granulosa stimulation and a marker of follicular development, increasing in concentration as the follicles approach the ovulatory stage of development (Ohshima *et al.*, 2002). The slowing down of oestrogen production and decreased levels of inhibin A in response to rhLH suggest that granulosa cell function could have been altered in the rhLH treated groups. It may be that prolonged exposure to LH results in the premature luteinisation of the granulosa cells when steroid synthesis switches to progesterone production. Further analysis of the media in future experiments would be needed to investigate this possibility.

The ability to produce high levels of oestrogen in response to rhFSH alone *in vitro* is in contrast to the findings of the *in viv*o experiments. Similar *in vivo* studies in humans and primates (Balasch *et al.*, 1995; Zelinski-Wooten *et al.,* 1994) have also reported low oestrogen levels in the absence of LH stimulation. The concentration of rhFSH used in the culture system may well be far in excess than those of the circulating levels found *in vivo.* It is possible that this high level promoted a greater steroidogenic capacity within the follicles.

The *in vitro* model was also used to investigate how different follicular gonadotrophin environments affected subsequent oocyte developmental competence by performing IVF at the end of the culture period. Although many attempts were

made to perform these experiments, there was a great deal of variability in oocyte quality between cultures, in some instances no fertilisation took place while in others the two-cell embryos arrested with no further development. Throughout these experiments serum was used from *hypogonadal* mutant mice to minimise any extraneous source of gonadotrophins and while this serum source supported follicular development it was not optimal in supporting the oocyte. Because of this, it was only possible to obtain consistent data from four separate experiments that yielded fertilisable oocytes for each treatment group. Statistical analysis showed that there were no significant differences between groups. Analysis of a larger sample size may have well given a different result as the figures did give an indication that gonadotrophins may affect oocyte developmental competence.

Surprisingly, it was found that ~20% of the oocytes obtained from the follicles exposed to gonadotrophin free conditions could fertilise and complete pre-implantation. These follicles ceased growth by day three, did not form antral cavities and exhibited quite a high degree of cell death in the granulosa cell layers. In the gonadotrophin- treated groups no evidence of increased cell death was seen in relation to any given treatment. The acquisition of competence has been extensively studied in the mouse and occurs in a step-wise manner with oocytes from pre-antral follicles capable of undergoing GVB but not completing meiosis to the MII stage (Eppig 1997; Sorenson and Wassarman 1976). The ability of the oocyte to complete resumption of meiosis is influenced by non-specific paracrine factors produced by many cell types (Eppig, 1996; Canapari *et al.*, 1994). Furthermore, once oocytes have become competent they may no longer be sensitive to atresia (Mermillod, 1999). Therefore, oocytes cultured under gonadotrophin free conditions could have

acquired competence from growth factors in the serum and have been unaffected by the health of the somatic cells. Whether these blastocysts are capable of supporting full term development is unknown. Further studies using embryo transfer techniques are needed to determine this.

Two of the aims of this study were to investigate whether LH is necessary for optimal oocyte maturation and whether the effects of high LH exposure affect oocyte competence. From the data obtained it would seem that the addition of rhLH to the culture media did not confer any advantage to the oocyte, as similar fertilisation results were seen in all the treatment groups. However, the follicles that had been exposed to the high rhLH concentration yielded fewer oocytes capable of developing into blastocysts. Previous reports have indicated that high intra-follicular LH permits the premature resumption of meiosis resulting in 'aged' oocytes (Regan *et al.,* 1990, Homburg *et al.*, 1988). At the concentrations rhLH used in these experiments, it was found that the majority of oocytes, irrespective of treatment, had completed the second meiotic division within 10 hours of release from the follicle, a time consistent with oocytes ovulated *in vivo* after hCG administration. Although the number of oocytes examined in this experiment was small (and a larger study would be needed to confirm the data) there was no evidence to suggest that high rhLH administration induced premature meiosis. However it was noted that some of these oocytes had begun to fragment after 14.5 hours in culture media. While it is possible that these oocytes were damaged during the procedure, it could be that they were beginning to undergo apoptosis (Liu *et al.*, 2002, Reynaud and Driancourt 2000).

The effects of gonadotrophins on follicular function are complex, interacting with growth factors and cytokines in the control of cellular proliferation,

development and steroidogenesis. Both FSH and LH exert their effects by elevating intracellular cAMP, although it has been shown that LH generates a much higher level of the second messenger compared to FSH resulting in differential regulation of the genes responsible for certain steroidogenic pathways (Yong *et al.,* 1994). It was found here that altered gonadotrophin regimes altered the pattern of steroid production and that may have affected oocyte developmental competence. Altered ratios of the steroids in follicular fluid have been implicated in affecting oocyte quality (Moor *et al.*, 1998). In order to investigate more precisely whether alteration in steroid levels influences follicular and oocyte development, a more direct experimental approach was required and formed the basis of the work detailed in Chapter Five. Recently, mRNA transcripts for both FSH and LH receptors have been found in murine oocytes (Patsoula *et al.*, 2001) and a direct role for gonadotrophins in oocyte maturation cannot be ruled out.

The most disappointing aspect of this study was the inability to gain data from the *hypogonadal in vivo* mouse model. *Hypogonadal* mice have a specific deletion in the gene for gonadotrophin releasing hormone (GnRH) and the majority of studies conducted in these mutants have concentrated on the restoration of GnRH function (Gibson *et al.*, 1997). Fewer studies have investigated the restoration of fertility by gonadotrophin administration. To the best of my knowledge only one report has shown that superovulation can be achieved in *hypogonadal* mice (Hashizume *et al.,* 1995). However approximately 50% of the mice showed little or no response to exogenous gonadotrophin stimulation raising interesting questions about the capability of the ovary to respond. FSH and LH receptors are present in the ovaries of *hypogonadal* mice (O'Shaughnessy *et al.*, 1997) but presumably they

remain inactive. Little is known about gonadotrophin action at the earliest stage of follicular development but it has been proposed that gonadotrophins act as survival factors (Hsueh *et al.,* 2000) and induce the capacity for subsequent growth critical to ovulatory development (Wu *et al.,* 2000). It is possible that the ovaries of *hypogonadal* mice are less sensitive to FSH stimulation as they have never been exposed to normal levels of circulating gonadotrophins.

Oocytes obtained from superovulated *hpg*$^{-/-}$ mice have successfully undergone IVF and live births have been reported, although the rates of fertilisation and blastocyst development were much reduced in the mutants when compared with WT mice (Hasizume *et al.*, 1995). IVF was attempted on all the oocytes obtained from the superovulated *hypogonadal* mice in this study but, although fertilisation took place, none developed to the blastocyst stage. Together these findings suggest that the ovulatory protocols necessary to induce ovulation are, by themselves, detrimental to oocyte quality. It was interesting to note that ovulation was only found in the groups of animals where significant levels of oestrogen were produced. Oestrogen has also been implicated in ovulation and lack of this steroid may limit the ovulatory process. This aspect of oestrogen action is discussed further in Chapter Six.

A transgenic mouse has been engineered to over-express the LHβ sub-unit (Risma *et al.*, 1995). The female mouse has enlarged cystic ovaries, elevated circulating oestrogen to testosterone ratios and is anovulatory. These animals can be induced to ovulate and oocyte quality has been investigated. Experiments have been conducted where fertilised oocytes from transgenic mice have been transplanted to normal animals giving rise to viable offspring (Mann *et al.,* 1999). We found here that the effect of high LH administration did not abolish the ability of oocytes able to

complete pre-implantation development but did reduce the percentage able to achieve it. It would be interesting to see if that was also the case in the transgenic model.

Chapter Five

The Effect of Altering Steroid Levels during Follicular Growth *in vitro.*

5.1 Introduction

As detailed in the Chapter One and elsewhere in this thesis, ovarian follicles produce steroid hormones in response to gonadotrophin stimulation. Both androgens and oestrogens have been proposed as intra-ovarian regulators of follicular growth and factors that influence oocyte growth and maturation.

The exact mechanism as to how steroids exert their effects on oocytes has yet to be elucidated. Oocytes could be affected either indirectly, as a consequence of steroid-mediated actions on the somatic cells within the follicle, or by direct activation of oocyte receptors. Expression of oestrogen receptor transcripts has been found in both human and mouse oocytes (Wu *et al.,* 1992, 1993). Studies in human oocytes undergoing *in vitro* maturation have supported the notion that activation of these receptors contributes to oocyte maturation. The addition of oestrogen to these oocytes directly influenced calcium release, which seemed to be mediated by a non-genomic membrane-bound receptor (Tesarik and Mendoza, 1995). Whether androgens also directly influence the oocyte has yet to be shown as, to date, no androgen receptor has been reported in the oocyte of mammals.

Apart from serving as substrates for oestrogen synthesis, androgens may act as paracrine/autocrine regulators of follicular development. Excess levels of androgen have been associated with promoting follicular atresia (Billig *et al.*, 1993) and are implicated in abnormal follicular development in some conditions such as polycystic ovarian syndrome (Mason, 2000). However, they may also act as growth factors during the earlier stages of growth (Vendola *et al.*, 1999), influence FSH receptor expression (Weil, 1999) and enhance FSH stimulated follicular differentiation (Tetsuka and Hillier, 1997). Studies from our laboratory have shown

that androgens can directly stimulate antral follicle growth *in vitro* (Murray *et al.*, 1998).

The role of oestrogen within the follicle is also controversial. Some reports have indicated that this steroid promotes follicular development (Drummond and Findlay, 1999) and prevents granulosa cell atresia (Billig *et al.,* 1993) while other studies, including ours, have found that this steroid has no effect in promoting ovulatory follicular development (Zelinski-Wooten *et al.,* 1994; Spears and Murray *et al.,* 1998,). However, oocytes obtained from oestrogen deficient follicles failed to undergo fertilisation (Zelinski-Wooten *et al.,* 1994), consistent with a direct role for this steroid in oocyte maturation. It is now known that two oestrogen receptors exist within the mammalian ovary thus complicating the potential role of this steroid in follicular development and oocyte maturation. A potential role for one of these receptors formed the basis of a separate study the results of which are given in Chapter Six.

It has been proposed that the oocyte needs to be exposed to the correct sequence and pattern of steroid secretion in order to acquire the molecular programming required for fertilisation and further development (Osborn and Moor 1983, Moor *et al.,* 1998). Furthermore, the balance of steroids present within the follicle may also influence oocyte quality, as high androgen to oestrogen ratios in follicular fluid have been co-related with poor fertilisation and development rates (McNatty 1979; Andriesz and Trounson 1995; Xia and Younglai 2000).

In the previous chapter, evidence was obtained to indicate that over-stimulation with LH during the follicular growth phase gave rise to poorer quality oocytes. As discussed, one possible explanation for this result could have been that

the follicle and oocyte were exposed to altered steroid levels. Follicles in that study would have been exposed to higher than usual levels of androgens and oestrogens. As mentioned above, lack of an oestrogen stimulus may adversely affect oocyte viability but very little is known about whether there is a point at which over-stimulation is also detrimental. This study aimed to investigate the effects of elevating these steroids individually and in combination, on follicular growth, health and subsequent oocyte competence.

In order to achieve this experiments were designed to manipulate the steroidal environment of *in vitro* grown follicles directly. The oestrogen agonist diethylstilboestrol (DES) was added to the culture media to elevate oestrogen. DES has been widely used experimentally to obtain oestrogen pre-treated granulosa cells to study cellular differentiation (Erickson, 1983; Liu *et al.,* 1999; Picazo *et al.*, 2000). Elevation of androgens was achieved by addition of the aromatase inhibitor Arimidex (Zeneca ZD1033). This compound selectively blocks P450aromatase activity without inhibiting other enzymes responsible for steroid biosynthesis or by itself having steroidal actions (Lonning *et al.*, 1998). By blocking aromatase activity with Arimidex, follicles will be unable to synthesis oestrogen but still be able to produce androgens. This will result in a high androgen, low oestrogen steroidal environment.

The experiments carried out in Chapter Four were all conducted using serum obtained from hypogonadal mutant mice. The main advantage in using this source of serum was that it contained negligible quantities of gonadotrophins or steroids. Although this serum was able to support follicular growth and development it was found that, in the majority of cases, the oocytes from the cultured follicles failed to

fertilise or arrested at the two-cell stage. As one of the main objectives of these experiments was to fertilise oocytes and examine their ability to reach the blastocyst stage, an alternative source of serum was sought. Foetal bovine serum (FBS) is available commercially and can be obtained in large quantities making it an attractive alternative to mouse serum which can only be obtained in small quantities. The main problem with using FBS for these experiments was that it contains high levels of conjugated steroids that may be released as free steroids by the enzymatic activities of the cultured follicles. This would then make it difficult to precisely control the level of steroids that the follicles and oocytes were exposed too, which subsequently could have influenced the results. It is possible to strip steroids from FBS using activated charcoal (Reynolds *et al.*, 1982; Huot and Shain 1988) and this technique was employed for these experiments. Initial experiments found that the follicles grown in the presence of charcoal-stripped serum (CSS) were not as successful at maintaining the integrity of their basement membrane. To obtain data on any effects altered steroid levels may have had on follicular growth and morphology experiments were also carried out using serum from *hpg*$^{-/-}$ mice.

5.2 Materials and Methods.

5.2.1 Animals

21-25 day old hybrid F1 female mice were used in these experiments. The strains used to generate these mice and the conditions under which they were kept are as described in Chapter Two.

5.2.2 Follicle culture

Follicles (180μm ± 15μm) were isolated as described in Chapter Two. After isolation they were randomly assigned to one of three treatment groups. A further group of follicles were placed into control media.

The basic media contained 2IU ml^{-1} rhFSH, 140μm ascorbic acid and 5% serum. This media was used in the control group. Treatment groups were supplemented with DES or Arimidex. These compounds were solubilised in ethanol. To minimise any loss of these compounds when the media was passed through sterilisation filters, the media was 'spiked' with steroids just prior to use. The volume of ethanol added to the culture media was adjusted so that each group, including the controls, was exposed to the same concentration. The treatment groups were as follows;

1.	4nmol. DES	**High OE**
2.	0.1μm Arimidex	**High A**
3.	4nmol. DES + 0.1μm Arimidex	**High OE + A**

5.2.3 Experiment 1: Follicular growth

In order to obtain data on growth, morphology and steroids follicles were isolated and allocated to the treatment groups above (5.2.2). In this experiment the serum used in the culture media was obtained from *hpg*$^{-/-}$ animals. Cultures were maintained and the follicles examined as described in Chapter Two. To obtain data on growth and development follicles were cultured for a period of six days. This experiment was performed twice giving a total of 48 follicles in each group. After removing the follicles from the culture trays containing the spent media were frozen at –20°C and kept until they could be assayed for hormones. At the end of one run of the culture, representative follicles were removed for TUNEL labelling.

TUNEL Labelling

Follicles were washed in PBS before being permeabilised and fixed. They were then processed for TUNEL labelling. The method is described in Chapter Two.

Hormone assays

In order to check that the treatments had successfully altered the level of steroids that the follicles were exposed to, media from cultures was analysed for androstenedione and oestradiol by the methods given in Chapter Two.

5.2.4 Experiment 2: Oocyte competence

Follicles were isolated and assigned to control or treatment groups as for Experiment One. In these experiments the serum used was FBS which had been charcoal stripped. 0.5 grams of activated charcoal was added to 50mls of FBS and stirred for 1 hour at 4°C. After this time the serum was centrifuged firstly at 1,700g

for 20 minutes then for 1 hour at 30,000g. Both centrifugation steps were carried out at 4°C. This removed the bulk of the charcoal. Fine charcoal particles were removed by filtering the serum through a 0.45μm cellulose acetate syringe filters. The CSS was then aliquoted and stored at –70°C.

As the majority of follicle could not maintain their basement membrane in this serum, the culture system was adapted. For the first two days of culture follicles were transferred into fresh wells of media and damaged or atretic follicles discarded. On day 3 they were then transferred into 60μl of media overlaid with 75μl of silicon fluid. The follicles were left in this media until day 5 after which the oocytes were removed for IVF. The experiment was repeated twice. In total 72 follicles were allocated to each group.

Oocyte maturation and IVF

Oocyte-cumulus complexes were obtained from the follicles at the end of the culture period. These were placed into maturation media overnight before undergoing IVF. The procedures used were as described in Chapter Two. Two F1 animals were superovulated prior to the day of IVF. The oocytes obtained from these animals were a control for the IVF system.

Hormone assays

To check that charcoal stripping of the FBS had successfully removed any steroids present, an aliquot of the freshly prepared control media was frozen. This was assayed for oestradiol as described in Chapter Two.

5.2.5 Statistical analysis

Hormone assays were analysed by ANOVA followed by Tukey-Kramers multiple comparison tests where appropriate. IVF results were analysed by chi-square.

5.3 Results

5.3.1 Experiment 1. Follicular growth

Figure 5.1 shows the growth rates of control and treated follicles. There was no effect of any treatment and all follicles had reached similar sizes by day 6 of culture. At the end of the culture period some follicle from each group were TUNEL labelled for apoptotic cells. Figure 5.2 shows photomicrographs of a representative follicle from each group. It was found that very little apoptosis was seen in any group.

In order to check that the treatments had successfully altered the level of steroids that the follicles were exposed to, media from day 6 of culture was analysed for androstendione and oestradiol. The results of the analysis are shown in Figure 5.3

Comparison of the levels of androstenedione (Figure 5.3A.) in the media showed that the concentration of Arimidex used, successfully inhibited aromatase activity. In both the High A and High OE +A groups (where Arimidex was included) androstenedione levels were significantly raised when compared to the control group ($p<0.01$).

The oestrogen assay that was used is highly specific for 17-β-oestradiol and does not cross react with any other oestrogen. Therefore, the results shown in Figure 5.3B represent the oestradiol production by the follicles and do not account for the DES addition. The dotted lines on the bars represent the expected level of oestrogens within the culture media. As was expected where Arimidex was included in the treatment (High A and High OE + A), significantly less oestradiol was produced when compared to the control groups ($p<0.001$).

5.3.2 Experiment 2:Oocyte competence

As shown in shown in Figure 5.4A, treatment had an effect on the fertilisation rates of the oocytes obtained. The total numbers of oocytes that fertilised and the numbers of these reaching the blastocyst stage are given in Table 5.1. Compared to the control group, elevating the androgen levels during the follicle culture period had a positive effect on fertilisation (63% compared with 47%). In contrast, when follicles were exposed to High OE fewer oocytes were capable of fertilisation as were those from the High OE + A group (27% and 31% respectively). Statistical analysis by chi-square showed that fertilisation rates differed significantly ($p<0.001$) between groups. There were no statistical differences between the numbers of two-cell embryos able to complete development to the blastocyst stage (Figure 5.4B).

A sample of the freshly prepared control medium was analysed for oestradiol. As no oestradiol was detected in the medium this indicated that the charcoal stripping procedure had successful removed steroids from the FBS.

	Total number of oocytes	2-Cells (%)	Blastocysts (%)
Control	57	27 (47)	19 (70)
High OE	51	14 (27.5)	11 (78.5)
High A	49	31 (63)	22 (71)
High OE + A	51	16 (31.5)	13 (81)
F1 Control	50	40 (80)	33 (82.5)

Table 5.1. Total number of oocytes taken for IVF and reaching the 2-cell and blastocyst stages of development. Percentages are given in brackets. Oocytes were obtained from follicles grown *in vitro* under different steroid environments and with charcoal stripped serum.

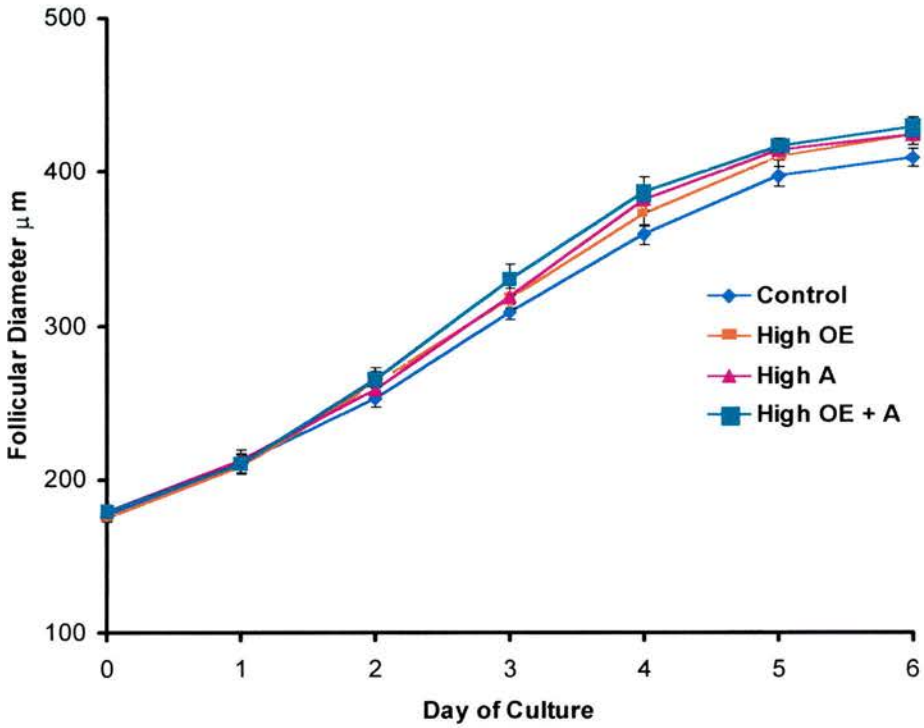


Figure 5.1. Growth rates of follicles grown in control media, elevated oestrogen (High OE), elevated androgen (High A) and both elevated oestrogen and androgen (High OE + A). Values are mean ± SEM (n=30).

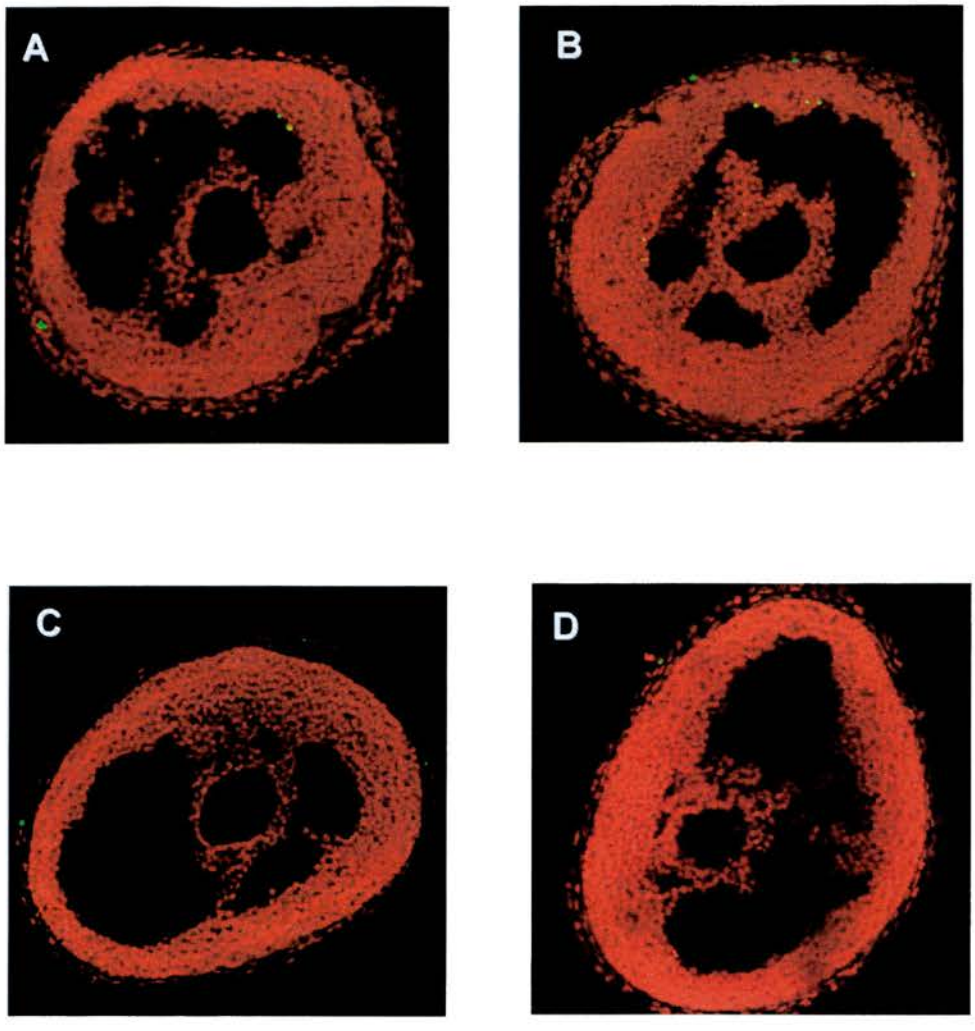


Figure 5.2. Confocal micrographs after labelling with TUNEL and propidium iodide. The green cells indicate apoptosis.
Representative follicle from the control (**A**), elevated oestrogen (**B**),elevated androgen (**C**) and elevated oestrogen and androgen (**D**) groups.
Treatment had no effect on the degree of apoptosis within the follicles.

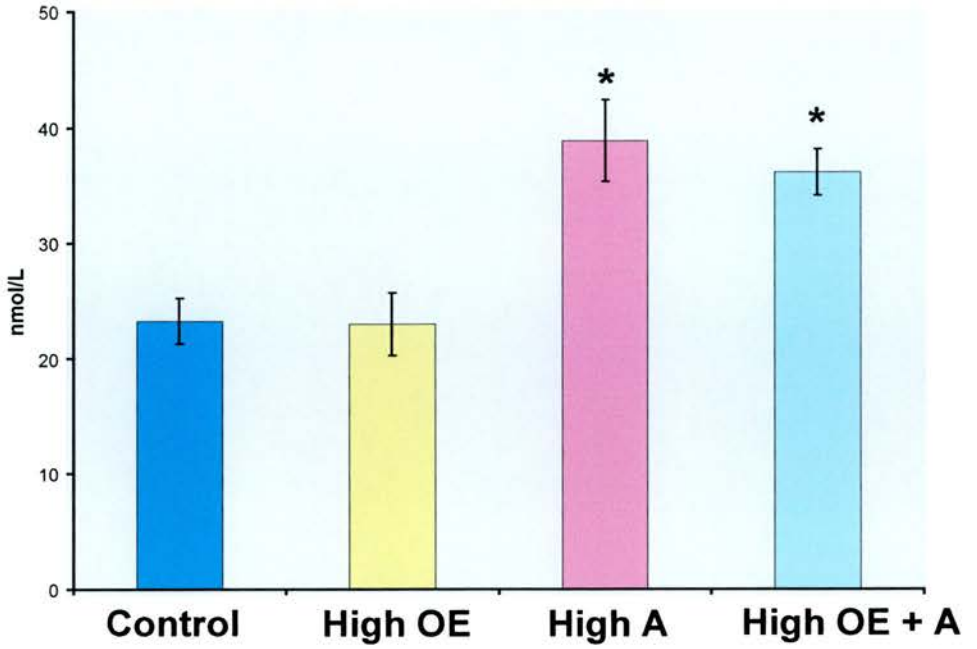


Figure 5.3A. Concentration of androstenedione produced by follicles grown in different steroid environments. Media was analysed on day 6 of culture. Values are mean ± SEM (n ≥ 9). Androstenedione was significantly higher in both Arimidex treated groups (* = p<0.01) when compared to the control group.

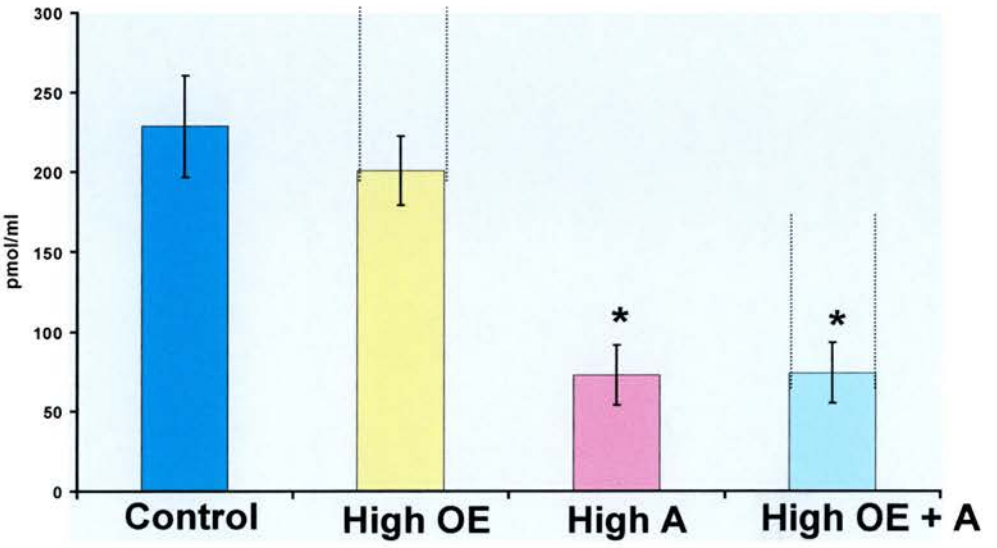


Figure 5.3B. Concentration of oestradiol produced by follicles grown in different steroid environments. Media was analysed from day 6 of culture. Values are mean ± SEM (n ≥ 9). Oestradiol was significantly lower in both Arimedex treated groups (* = p<0.001) when compared to the control group.
The dotted lines indicate the expected values for oestrogens.

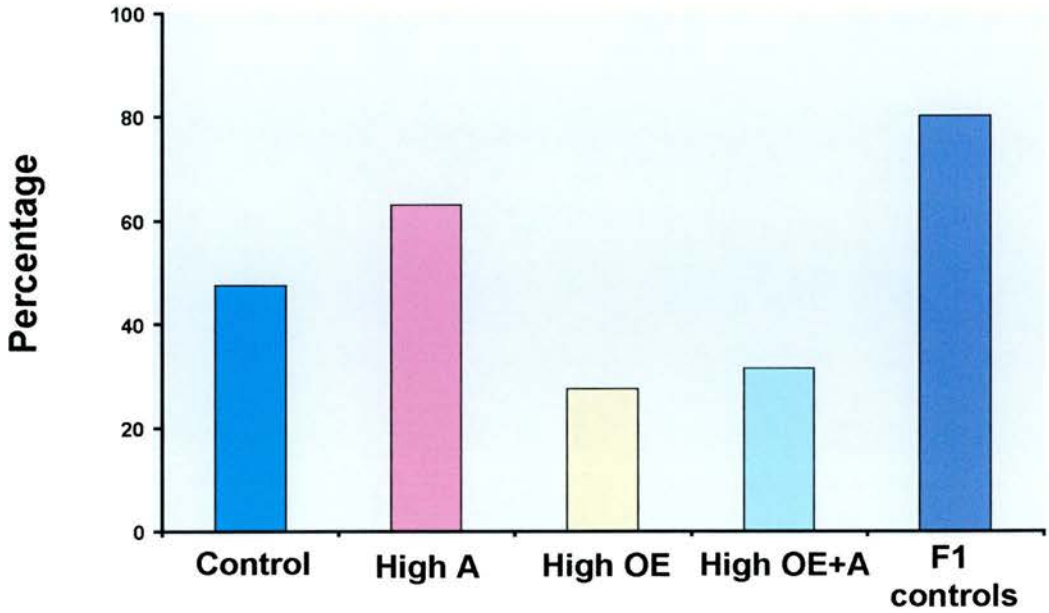


Figure 5.4A. Percentage of oocytes fertilising from follicles grown in different gonadotrophin environments. Fertilisation rates differed significantly ($p<0.001$) between groups.
F1 controls are shown for comparison.

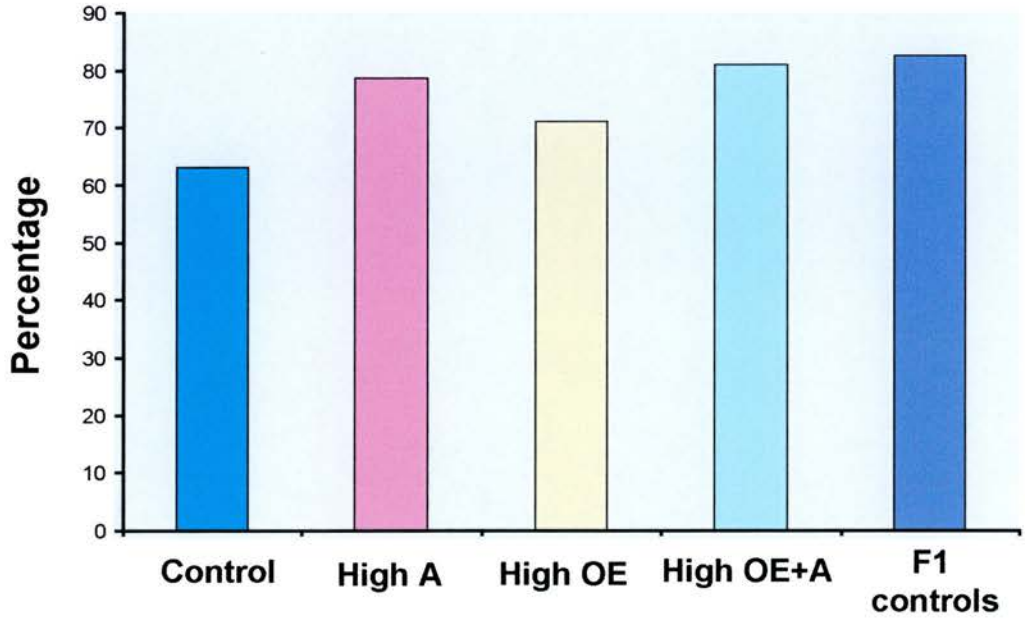


Figure 5.4B. Percentage of 2-cell embryos reaching blastocyst. There were no significant differences between groups. F1 controls are shown for comparison

5.4 Discussion

As indicated by the results in Chapter Four, gonadotrophin manipulation during the follicular growth phase gives rise to oocytes that are not optimally viable. The experimental evidence suggested that a possible reason for this may have been the altered levels and patterns of steroid secretion that arose as result of gonadotrophin manipulation. The aims of this study were to investigate more directly what effect elevation of androgens and oestrogens, both individually and together, have on follicular growth and subsequent oocyte competence *in vitro*.

As outlined in the introduction, two experimental conditions were employed to achieve these aims. Serum is a necessary component of the culture media, as follicles cannot develop in its absence. Although hypogonadal serum supports follicular growth and development, it had proved to be sub-optimal in supporting oocyte maturation. Charcoal stripped serum on the other hand, while more successful in supporting the oocyte, proved to be lacking factors necessary for maintenance of the basement membrane. To obtain data on follicular growth and the degree of apoptosis within treated follicles the culture media was supplemented with hypogonadal serum. Effects on oocyte development were assessed using CSS. The results of these experiments show that, although elevation of steroids did not affect the rate of growth or the health of the follicles, they did have an effect on the quality of the oocyte.

Paracrine and/or autocrine effects of steroids have been described in both *in vivo* and *in vitro* experimental situations but there is no clear consensus from these studies as to the role of either androgens or oestrogens on follicular growth and development. Some studies have reported that androgens inhibit follicular

development both *in vitro* (Jia *et al.,* 1985) and *in vivo* (Farookhi *et al.,* 1985) and increase atresia in pre-antral and early antral follicles (Billig *et al.,* 1993). Other studies have shown that the action of androgens promote follicular growth and development (Murray *et al.,* 1998; Weil *et al.,* 1999). Likewise there are conflicting reports regarding the effects of oestrogens of follicular development. On the one hand these steroids have been implicated in promoting follicular growth (Findlay and Drummond, 1999) and preventing granulosa cell death (Billig *et al.,* 1993), while on the other exogenous administration of oestrogens has also been implicated in inhibiting follicular function by increasing granulosa cell atresia (Dierschke *et al.,* 1994). Under the conditions used for this study no evidence was found to support the hypothesis that either steroid inhibited follicular growth. In all treatment groups follicles grew at the same rate and there was no evidence that any treatment increased the level of apoptosis in the granulosa cell layers. The aforementioned previous studies all employed the rat as a model, which may exhibit species-specific responses to ovarian stimulation by steroids (Hutz, 1989). In these current studies however, the follicles were exposed to a continuos high level of FSH which is a potent survival factor and may have overridden any negative effects of altered steroid levels. Additionally, as described in Chapter Three, the inclusion of ascorbic acid in the culture media may have had a similar effect. Very little apoptosis was seen in any of the follicles irrespective of treatment. While the high level of FSH and inclusion of ascorbic acid may have prevented follicular atresia, it is also possible that the very low numbers of apoptotic cells seen was due to the incomplete labelling by the TUNEL reagents. The inclusion of a positive control for the TUNEL assay would have determined if this were the case.

Although there were no obvious differences in the growth, morphology or degree of atresia between the follicles grown in different steroidal environments, oocyte developmental competence was affected. The results of the experiments (where CSS was used) clearly show that a greater number of oocytes were capable of fertilisation when they had been exposed to high levels of androgens. The opposite effect was seen when oocytes were exposed to high levels of oestrogen with fewer of these oocytes fertilising. No further effects of treatment were seen, as the numbers of 2-cell embryos developing into blastocysts were similar in all groups.

Serum contains a number of proteins and growth factors that potentially can influence oocyte maturation. As charcoal stripping of serum resulted in the removal of some factors affecting maintenance of the basement membrane it was possible that other components that affect the oocyte could also have been removed. The main difference between the two serum experiments was that disruption of the basement membrane occurred in the follicles grown with CSS. The role of the basement membrane is complex, acting as a barrier to the flow of molecules passing from the thecal-interstitial compartment to the granulosa cells within the follicle (Rodgers *et al.*, 1999). It is possible that the differences in fertilisation rates arose as a result of disruption to the follicular structure.

The nature of how steroids influence oocyte maturation has yet to be elucidated. Androgen and oestrogen receptors are found in the granulosa cells of growing follicles and their pattern of expression is developmentally regulated (Tesuka and Hillier 1997; Rosenfeld *et al.*, 2001). This changing pattern of receptor activity could influence the oocyte indirectly by altering the synthesis and transfer of molecules necessary to oocyte growth and maturation. Steroids could also have

direct effects on the oocyte. Oestrogen receptors have been described in mouse and human oocytes (Wu *et al.,* 1993, 1994). In maturing human oocytes Tesarik and Mendoza (1995) have postulated that a membrane bound oestrogen receptor facilitates the calcium response of the oocyte necessary to the fertilisation process. Androgens also have been implicated in directly influencing the oocyte. Androgens have been shown to influence oocyte activation in the primordial follicles of rhesus monkeys (Vendola *et al.,* 1999) however no androgen receptor has yet been described in the mammalian oocyte. Related steroids could act through the same receptor either by acting as antagonists, attenuating responses or inducing discrete downstream events. In *Xenopus laevis,* androgens acting through oestrogen receptors have been found to be the primary steroid in promoting oocyte maturation (Lutz *et al.,* 2001). There is some evidence that this may occur in mammalian oocytes as the calcium response reported by Tesarik and Mendoza (1995) can be directly counteracted by androgens (Tesarik and Mendoza 1997).

The expression patterns of oestrogens and androgens in follicular fluid changes rapidly during the final phase of follicular development in response to gonadotrophin stimulation. Pre-ovulatory follicles have relatively high levels of both steroids in follicular fluid, which change rapidly in response to the LH surge (Osborn and Moor, 1983). Inhibition of follicular steroid synthesis results in an abnormal pattern of proteins synthesised in sheep oocytes (Osborn and Moor 1983). These findings have led to the hypothesis that the oestrogen: androgen ratio is the major determinant in oocyte maturation rather than absolute steroid concentrations. In the experiments described here steroid levels were elevated throughout the culture period. The conclusions drawn from these studies were that elevating androgens

positively influenced oocyte maturation while exposure to high levels of oestrogen had detrimental effects. Recently, a study by Yu *et al* (2002) reported that Arimidex treatment enhanced the percentage of mouse oocyte completing meiosis and concluded that elevation of androgen promoted these aspects of oocyte maturation. Other studies have implicated that prolonged periods of oestrogen exposure *in vivo,* reduces the viability of human oocytes (Russell *et al.,* 1997). Taken together, these findings would indicate that oocyte maturation is influenced by a developmentally regulated pattern of exposure to both androgen and oestrogen. Further studies are currently underway within our laboratory to examine which steroid at which developmental time-point is critical to optimal oocyte maturation.

Chapter Six

The Effect of Oestrogen Receptor alpha Deletion on Ovarian Function

6.1 Introduction

Oestrogens are steroid hormones that have a wide range of physiological effects. In women the main site of oestrogen production during pre-menopausal life is the ovary although other tissues, such as adipose and adrenal, can produce them. The production of oestrogens by the ovary is cyclical with basal circulating concentrations between 70-220 pmol/L in humans. This concentration rises to greater than 740 pmol/L at the time of ovulation. The rise and fall in circulating oestrogens regulates ovarian cyclicity through its action at the hypothalmic-pituitary axis that controls the release of the gonadotrophins. The effects of oestrogen on the reproductive organs have been well documented. Oestrogen has an effect on oviductal transfer of the zygote, uterine receptivity prior to implantation, on breast tissue, cervical and vaginal activity and, in some species, sexual behaviour. It is also now known that oestrogen exerts effects on non-reproductive tissues such as the brain, heart and bones.

It has also been proposed that oestrogens exert direct intraovarian effects. Studies utilising *in vivo* and *in vitro* models have been employed in an attempt to elucidate an exact role for oestrogen in the ovary. Some of these studies have shown that oestrogen facilitates the proliferation of granulosa cells, the actions of FSH and LH (Richards, 1980, Bley *et al.*, 1997), gap junction formation (Merck, 1972) and steroidogenesis (Roberts and Skinner, 1990). Conversely, there are a number of reports demonstrating that any paracrine effects of oestrogen within the ovary are not obligatory and that ovarian function can proceed when the actions of this steroid are blocked (Zelinski-Wooten *et al.*, 1994; Spears and Murray *et al.*, 1998). In hypogonadotrophic women it has been shown that pre-ovulatory follicle

development can occur despite very low oestrogen synthesis (Fauser, 1997; Balasch *et al.,* 1995). As discussed in Chapter Five, many of the studies showing stimulatory effects of oestrogen used the rat as a model and it may be that oestrogen does play an obligatory role in follicle development in this species while it is not mandatory in others (Hutz, 1989). It is known that mRNA for oestrogen receptors exist in the oocyte (Hiroi *et al.,* 1999; Wu *et al.,* 1992;1993) but as yet a direct role for oestrogen in promoting oocyte maturation has yet to be determined. The results obtained from the previous chapter in this thesis would suggest that overexposure to oestrogen is detrimental to oocyte competence.

Oestrogens exert their actions through binding to high affinity receptors. These receptors belong to the nuclear receptor super family of transcription factors that includes glucocorticoids, mineralcorticoids and progesterone (Evans, 1988). Once oestrogen binds to its receptor triggers conformational changes leading to changes in the transcription of oestrogen related genes. These events include receptor dimerization, receptor-DNA interaction and recruitment and interaction with co-regulators (Shibata *et al.,* 1997; Beekman *et al.,* 1993; Beato, 1989). Ultimately, the resulting cascade of events influences the metabolic processes of the cell (Rosselli *et al.*, 2000). While this may be the primary mode of action of activated oestrogen receptors, it is now known that they can modulate gene expression indirectly by physical interaction with other transcription factors (Nillson *et al.*, 2001) or exert so called non-genomic effects via membrane receptors (Levin, 2001). They can also act by ligand independent mechanisms (Ignar-Trowbridge, 1992)

Until recently it was thought that only one oestrogen receptor existed. In 1986 this was cloned and sequenced (Greene *et al.*, 1986) and subsequently mice were

generated with a targeted disruption of the oestrogen receptor gene (Lubahn *et al.,* 1993). However, some tissues in these animals remained oestrogen responsive suggesting the existence of another oestrogen receptor. This second receptor was cloned in 1996 by Kuiper *et al,* and named oestrogen receptor β (ERβ) while the first identified receptor was subsequently referred to as oestrogen receptor α (ERα). Both of these receptors have been identified in many mammalian and non-mammalian species across a variety of tissues and are the products of separate genes. A third oestrogen receptor (ERγ) has been identified in fish (Hawkin *et al.,* 2000*)* but no mammalian homologue has yet been found. It has also been found that there are many alternatively spliced forms of both receptors although variants of ERα have been found mainly in human breast cancers (Rosenfeld *et al.,* 2001).

Oestrogen receptors belong to the nuclear receptor super family. In common with all nuclear steroid receptors the oestrogen receptors consist of three independent but interacting functional domains: the NH_2 –terminal or A/B domain, the DNA-binding domain and the COOH-terminal ligand-binding domain. (Nilsson *et al.,* 2001). The NH_2 –terminal domain encodes a ligand-independent activation function (AF-1) involved in protein-protein interactions and transcriptional activation of target-gene expression (Onate *et al.,* 1998; Tora *et al.,* 1989). The DNA binding domain contains a two zinc finger structure important in receptor dimerization and binding to specific DNA sequences. While the COOH-terminal contains the ligand binding pocket as well as a second activation factor (AF-2) which is critical to the ligand dependent activity of the receptor and is involved in the recruitment of coregulator proteins (Nilsson *et al.,* 2001; Henttu *et al.,* 1997; Daniellian *et al.,*1992). ERα and ERβ are highly homologous in the DNA binding domain, share

~60% homology in their ligand binding domains but only share 18% homology at the A/B domain (Enmark *et al.,* 1997). Both receptors have high affinity binding for oestradiol (Rosselli *et al.*, 2000) but differences in the ligand binding domains and activation function domains of each receptor mediate their responses to oestrogen agonists and antagonists (Katzenellenbogen *et al.,* 2001)

Transgenic mice have been generated with deletions in ERα, ERβ and a combination of both. These have now been termed as ERKO, βERKO and αβERKO respectively. In addition mice lacking the aromatase gene (ArKO) have also been generated. These animals cannot synthesise oestrogen although they can still respond to it (Fisher *et al.,* 1998). Each of these exhibits different phenotypes and have opened up new avenues of research that allow the dissection of the role oestrogens in a wide variety of tissues.

There has been much interest in the role of oestrogen in the reproductive systems in both males and females. In males it has been demonstrated that absence of oestrogen action is detrimental to spermatogenesis, sperm function and mating performance. However, ERβ males are fertile while ERα are not (Krege *et al.*, 1998; Lubahn *et al.,* 1993). Therefore it would seem that the two receptors have differing roles to play in male reproductive physiology. In light of the finding that sperm counts in males have been declining in the last 50years, perhaps in response to environmental oestrogen exposure during foetal development, there has been much interest in investigating the effects of oestrogens on each receptor type (Lombardi *et al.,* 2001). Interestingly the ArKO male mouse is also fertile (Fisher *et al.,* 1998).

Studies of the three ER 'knock-out' mice and the ArKO mouse have revealed differing roles for each of the oestrogen receptors and oestrogen in the female

reproductive system. The table below illustrates the main phenotypic differences between the ArKO mouse and the three ER knockout mice after puberty. The information was drawn from Fisher *et al,* (1998); Couse and Korach (1999), Dupont *et al*, (2000).

TISSUE	**αERKO**	**βERKO**	**αβERKO**	**ArKO**
Ovary	Develop haemorraghic cysts Infertile	Fewer follicles reach ovulatory stages Sub-fertile	Develop haemorraghic cysts Infertile	No corpora lutea
Uterus	Hyperplasia	Exaggerated responses	Hyperplasia	Underdeveloped
Oviduct	No gross phenotypes	No gross phenotypes	No gross phenotypes	?
STEROID PROFILES				
Serum Oestradiol	Elevated	Normal	?	negligible
Serum LH	Elevated	Normal	?	Elevated
Serum FSH	Normal	?	?	?
Serum Androgen	Elevated	?	?	Elevated

In light of the different phenotypes exhibited by these different mice, localisation and relative abundance of each type of oestrogen receptor has been examined in a number of different species. These studies have indicated that ERα is predominant in eliciting hypothalmic –pituitary response to rising oestrogen levels during the oestrus cycle and in priming of the uterus prior to ovulation (Lubahn *et al.,* 1993 ; Couse *et al.,* 1997; Krege *et al.,* 1998).

The expression pattern of each ER has been examined in the ovaries of a number of species. Both ERs have been identified in the ovaries of rodents (Couse *et al.*, 1997; Drummond *et al.*, 1999; Sar and Welch 1999; Yang *et al.,* 2002), domestic species (Schams and Berisha, 2002), humans and marmosets (Saunders *et al.*, 2000). In the mouse, ERα has been localised exclusively to the interstitial tissues of the ovary (Schomberg *et al.,* 1999) and ERβ to the granulosa cells of growing follicles (Cheng *et al.,* 2002). Whereas ERβ expression increases with follicular growth and development (Drummond *et al.,* 1999) and is down regulated in response to LH/hCG regulation (Byers *et al.,* 1997), ERα levels remain at a constant low level and are unresponsive to FSH, testosterone or forskolin stimulation (Sharma *et al.,* 1999). From these studies there is a general consensus that ERβ is the predominant receptor type within the ovary with ERα being expressed at lower levels. Immunocytochemistry studies in primates and humans have suggested that there may be species differences in the expression patterns of the two ER receptors. In baboons, rhesus monkeys, marmosets and in humans ERα has been detected in the granulosa cells of larger follicles (Saunders *et al.,* 2000; Hutz *et al.,* 1993; Billiar *et al.,* 1992).

Although ERβ has been found to be the most predominant receptor type within rodent ovarian follicles, animals carrying deletions in this gene are still fertile while the ERα females are infertile and develop a more severe ovarian phenotype. ERKO females are anovulatory and develop haemorraghic cysts shortly after reaching puberty (Lubahn *et al.,* 1993). Prior to the work described in this chapter, it had been reported that the ovaries of these animals could not be induced to ovulate when administered with exogenous gonadotrophins. The neo-natal and pre-pubertal mice in this knock-out do not exhibit any gross differences from wild-type mice

(Shomberg *et al.*, 1999) and appear to have similar numbers of primordial, primary follicles and secondary follicles. Another characteristic of the ERKO phenotype is the perturbations in the circulating steroids and gonadotrophins that may influence ovarian function indirectly. It is possible that the ovarian dysfunction seen in the adult ovary may not be a direct consequence of disruption to the ERα signalling pathway but as a consequence of the endocrine disruption. The aims of this study were to examine more directly the effects of ERKO in ovarian function using pre-pupertal mice as a model. Initially experiments using these mice were carried out at the Animal Science Center, University of Missouri, Columbia, Missouri. From the work carried out there it was found that juvenile animals could respond to exogenous gonadotrophin stimulation and oocytes could be collected. This study specifically set out to investigate ovulation rates in these animals, to examine follicular development and to determine whether lack of ERα is detrimental to the oocytes' ability to fertilise and complete pre-implantation development.

6.2 Material and Methods

6.2.1. Animals

ERKO and wild-type (WT) female mice were obtained by breeding heterozygous parents (C57BL/6J/12SV). The colony was housed under the same conditions as described in Chapter Two. At approximately 14 days of age the animals were ear punched as a means of identification and the pieces of tissue frozen.

6.2.2 Genotyping

DNA was extracted from the ear punch tissue and subjected to the PCR reaction as outlined in Chapter 2. Wild type and mutant animals could then be identified before experimental use.

6.2.3 Histological examination of ovaries

Ovaries were obtained from adult and pre-pupertal animals. After gross dissection the ovaries were fixed in Bouins solution, embedded in paraffin wax before being sectioned at 6μm intervals. The sections were stained with haematoxylin and eosin. The methods are detailed in Chapter 2.

6.2.4 Experiment 1: Ovulation rate and oocyte developmental competence in ERKO mice.

In order to ascertain whether the ERKO mice were capable of ovulating at the same rate as WT mice, animals between 21-28days of age were administered with superovulatory doses of gonadotrophins (5IU PMSG followed by 5IU hCG; 48-54

hours later). 12-15 hours post hCG the animals were sacrificed and the ovaries and oviducts removed to warm Liebovitz media. The oviducts were examined carefully for oocyte-cumulus complexes. The number of WT and ERKO mice that ovulated was noted. The ovulated oocytes were released from the oviduct and were then transferred to T6 media for IVF.

IVF of superovulated oocytes

The protocol used to perform IVF was exactly as described in Chapter 2. The sperm used for fertilisation was obtained from F1 (C57BL x CBA) males aged 6-10 weeks. The developing embryos were examined daily and the number of oocytes fertilising and reaching the blastocyst stage of development from the WT and ERKO mice noted.

Light Microscopy of Blastocysts.

Photographs were taken using a Nikon F70 camera and Ektachrome 64T Colour reversal film. The blastocysts were viewed under a Nikon inverted microscope fitted with Hoffman modulation contrast optics and a heated stage (Lincam).

TUNEL staining of blastocysts.

Under the dissecting microscope (Nikon) cavitating blastocysts were initially transferred from the KSOM droplets into the wells of 96 well plates (Iwaki) containing 100 µl PBS supplemented with 3mg ml^{-1} Poly Vinyl Pyrrolidone (PVP; Sigma). Subsequent steps were carried out by transferring the blastocysts through the wells of the 96 well plate using fine-drawn BSA coated pipettes. The blastocysts were washed three times in PBS/PVP before being fixed in 100 µl 3.7%

paraformaldehyde/PBS. Permeabilisation was carried out by transferring the blastocysts to 100 µl of 0.5% Triton/PBS/PVP for 1 hr at room temperature. Cell death detection was performed using a commercially available kit (Roche). The blastocysts were pre-incubated in 15 µl dUTP-FITC labelling mix for 10 min at room temperature before incubation in 15 µl of the TUNEL preparation for one hour at 37°C. They were then washed twice in 100 µl PBS/PVP. Counterstaining, using propidium iodide (PI), was performed by incubation in 20 µl of PI solution (3µl PI in 1ml PBS containing 100mg bovine pancreatic Rnase [Sigma]) for 2 hours at 37°C. Blastocysts were then washed six times in PBS/PVP before being mounted in a drop of PBS/PVP placed on a Coverwell gasket (Molecular Probes). A cover slip was then placed over the gasket. Throughout the TUNEL staining procedure all incubations were carried out in the dark and transfers were carried out as quickly as possible to ensure no loss of fluorescence.

Confocal Analysis

Analysis was performed using a Leica TCS NT confocal microscope using the x63 water corrected PL APO lens. Blastocysts were analysed by scanning sections every 5 µm. TUNEL labelled cells were detected as green and propidium iodide staining was red in colour therefore simultaneous scans at 488 nm and 568 nm were taken. Each could be viewed separately. Images were saved and later analysed on the computer using UTHSCA Image Tool software. Apoptotic and non-apoptotic cells were counted. The combined cell counts gave a total cell number.

6.2.5 Experiment 2: Follicular growth and development *in vitro.*

21-25 day WT and ERKO female mice were sacrificed and the ovaries collected into warm Liebovitz medium. A small piece of tail tissue was collected and the genotypes of the animals confirmed by PCR as described in Chapter Two.

Ovaries were removed and pre-antral follicles were dissected as previously described (Chapter 2). The follicles were cultured in α-MEM media supplemented 1 IU ml^{-1} rhFSH, 140 μm ascorbic acid and 5% serum collected from C57BL mice. The follicles were measured and moved to fresh wells of media daily. The culture period was for 6 days. Three WT and three ERKO animals were used in each run of the experiment. The experiment was performed twice. At the end of one run of the experiment representative follicles were fixed and processed for histological examination as described in Chapter Two

6.2.6 Statistical analysis.

The number of ERKO and WT mice ovulating in response to gonadotrophin was compared using chi-square. The number of oocytes obtained from each genotype was calculated as ± SEM and compared by *t* test. The total cell and apoptotic cells per blastocyst was compared by ANOVA and Bonferroni post test. Follicular growth between the WT and ERKO was compared on each day of culture by a *t* test.

6.3 Results

6.3.1 Histology.

Histological examination of adult ovaries confirmed the presence of haemorrhagic cysts in the ovaries of the adult ERKO females. A representative photograph is shown in Figure 6.1A. In contrast, there is little difference between the ovaries of the pre-pubertal WT and ERKO mice (Figure 6.1B and C).

6.3.2 Experiment 1: Ovulation rate and oocyte developmental competence in ERKO mice.

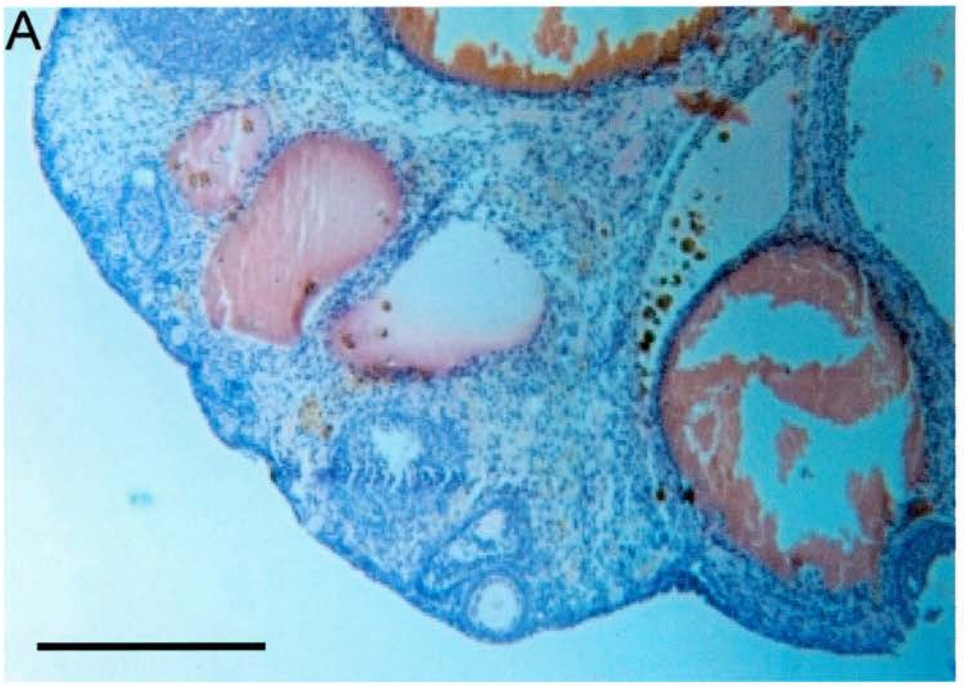
The number of ERKO mice responding to the superovulatory doses of gonadotrophins was compared with WT mice of similar ages. No differences were found in the number of WT and ERKO ovulating (11/14; 78.5% versus 9/16; 56% respectively). However as shown in Figure 6.2 the number of oocytes collected from the animals did differ significantly dependent on genotype (WT; 14.36 ± 3.73 ERKO; 5.417 ± 1.574; $p<0.05$). Oocytes were taken and IVF performed to examine whether the effect of ERα deletion had any effect on oocyte developmental competence. In total 98 WT oocytes and 88 ERKO oocytes were collected for IVF. The results of this experiment are shown in Figure 6.3. The percentage of WT oocytes achieving cleavage to the 2-cell stage was 84% (83/98) and ERKO oocytes 86% (76/88). 75% of the WT (63/83) and 59% (45/76) of the ERKO 2-cell embryos developed to the blastocyst stage. There were no significant differences in the rate of fertilisation and blastocyst development between the two groups. Blastocysts from

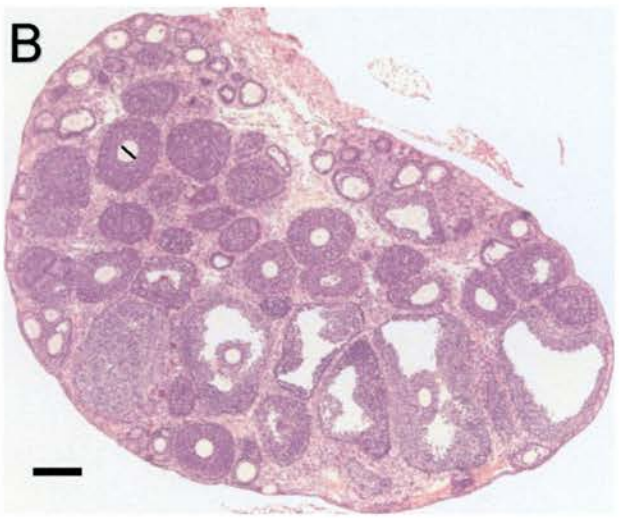
both groups formed cavities and appeared morphologically identical. A typical blastocyst is depicted in Figure 6.4A.

To determine if any differences existed between the WT and ERKO blastocysts, they were analysed using the confocal microscope after TUNEL labelling for apoptosis and counterstaining with propidium iodide. A typical stained blastocyst is shown in Figure 6.4B. From the images total cell counts and the numbers of apoptotic cells in each blastocyst were made. There were no significant differences between the two groups. The mean number of total cells for the WT blastocysts was 43.5 ± 2.82 and 41.35 ± 2.69 for ERKO blastocysts (see Figure 6.5A). The mean number of apoptotic cells in WT blastocysts was 15.45 ± 1.57 (35.5%of the total cells) while ERKO derived blastocysts had a mean number of 15.3 ± 1.25 (37% of the total cells) as shown in Figure 6.5B.

6.3.3 Experiment 2: Follicular growth and development *in vitro*

Follicles isolated from both WT and ERKO mice grew at the same rate and reached the same diameter at the end of the culture period as shown in Figure 6.6. Representative follicles from each group were fixed and processed for histolological examination at the end of the 6 day culture period. As can be seen in Figure 6.7 follicles from the WT type and ERKO mice had antral cavities and there was little morphological difference between them.





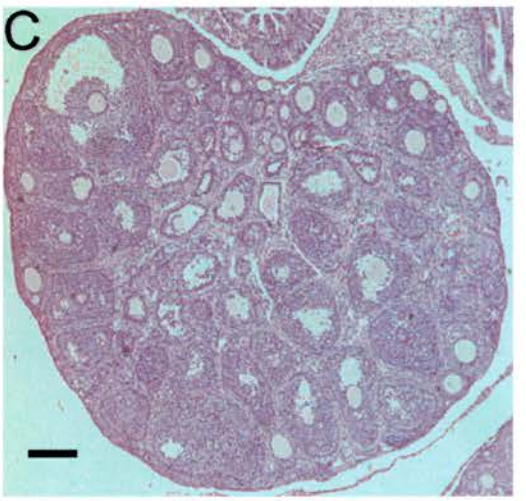


Figure 6.1. Photomicrographs of haematoxylin and eosin stained sections of ERKO and WT ovaries. The adult ovary has large haemorraghic cysts (**A**). There is little morphological difference between the ovaries of ERKO (**B**) and WT(**C**) mice at 3 weeks of age. Scale bar 200 μm

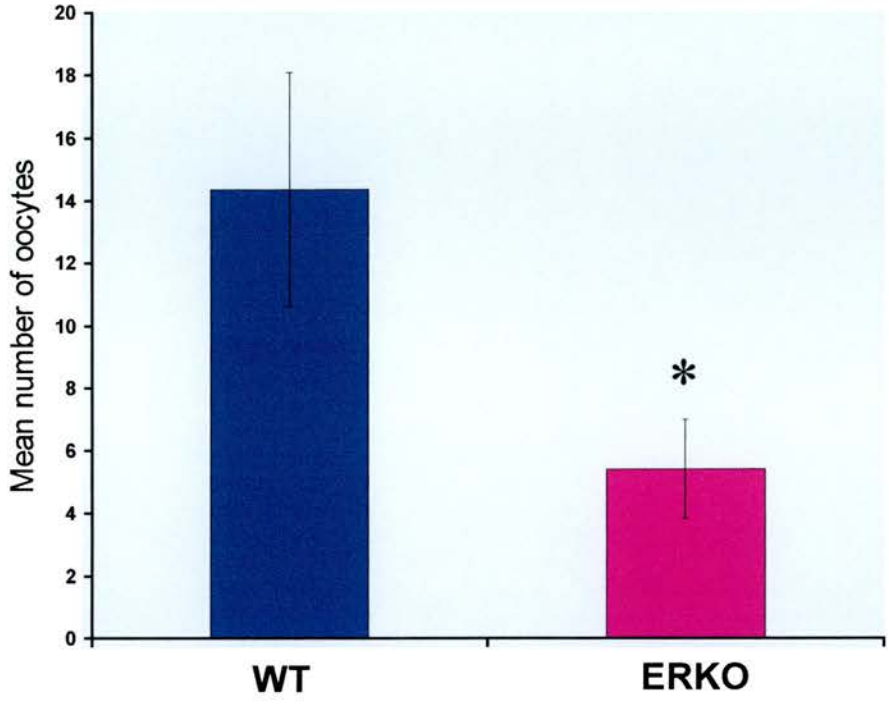


Figure 6.2. Number of oocytes ovulated in WT and ERKO mice in response to exogenous gonadotrophin treatment. Values are Mean ± SEM ($n \geq 11$). Significantly fewer oocytes were retrieved from the ERKO mice.
* = $p<0.05$

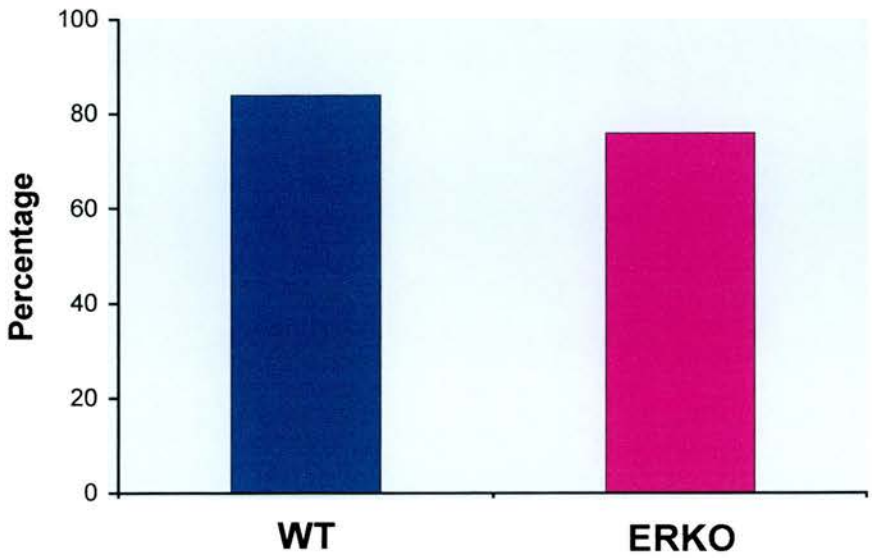


Figure 6.3A. Fertilisation rate of oocytes obtained from WT and ERKO mice. There were no differences between the groups.

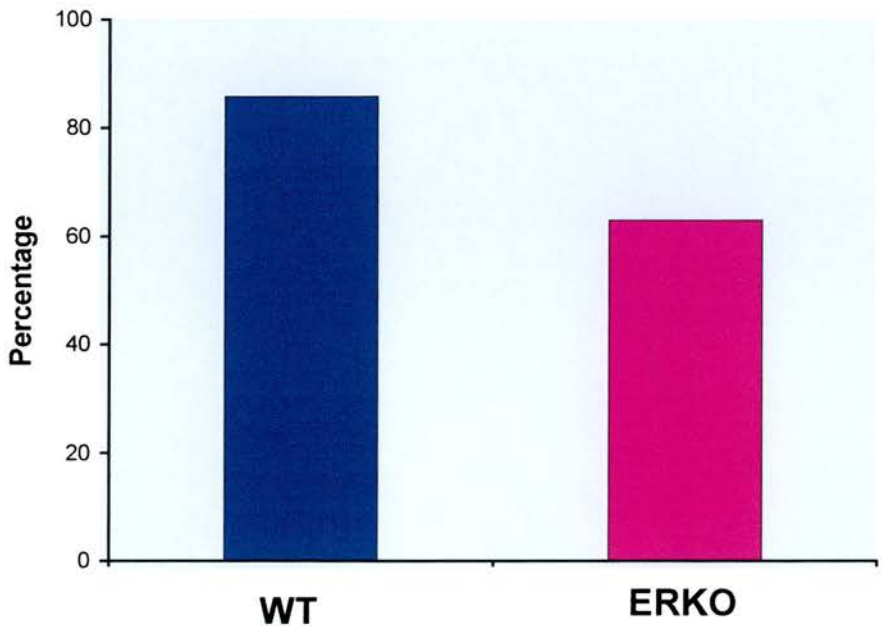


Figure 6.3B. Rate of 2-cell embryos reaching blastocyst in WT and ERKO mice. There were no differences between the groups.

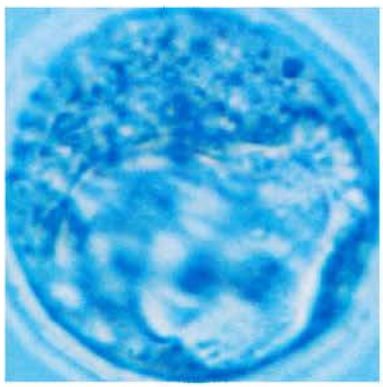


Figure 6.4A. Photograph of a typical blastocyst. Morphologically there was no difference between WT blastocysts and those derived from ERKO animals

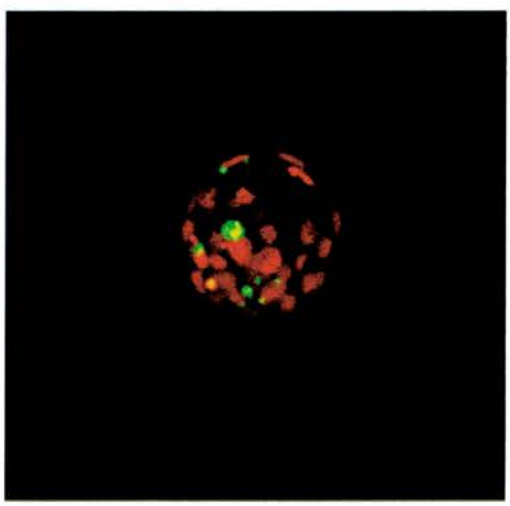


Figure 6.4B. Confocal image of a TUNEL stained blastocyst. Apoptotic cells are green. Blastocysts were counterstained with PI

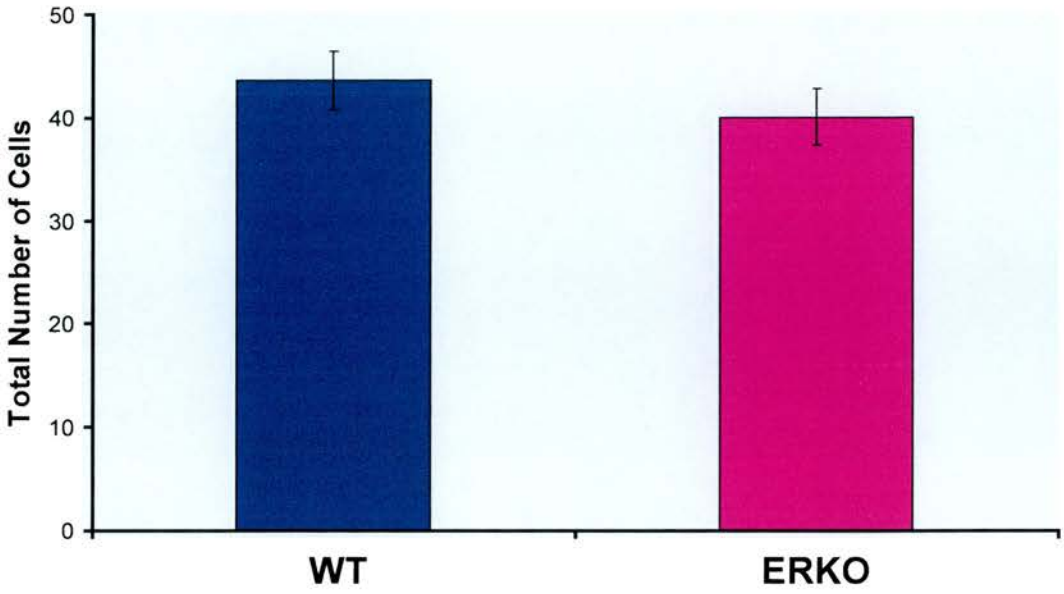


Figure 6.5A Total number of cells in WT and ERKO blastocysts. Values are Mean ± SEM($n \geq 20$). There were no statistical differences between the two groups

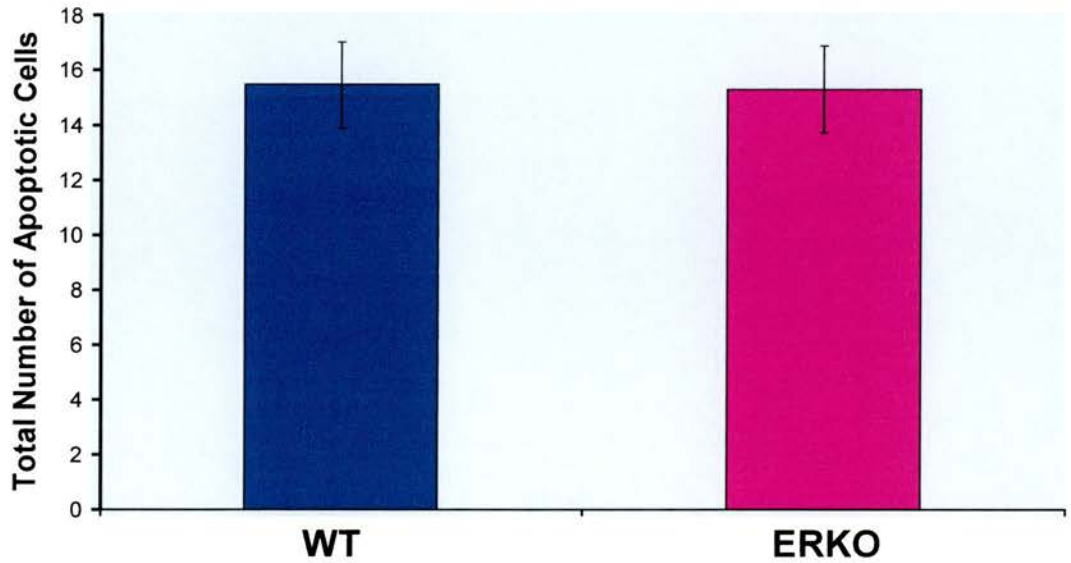


Figure 6.5B Total number of TUNEL labelled cells in WT and ERKO blastocysts. Values are Mean ± SEM($n \geq 20$). There were no statistical differences between the two groups

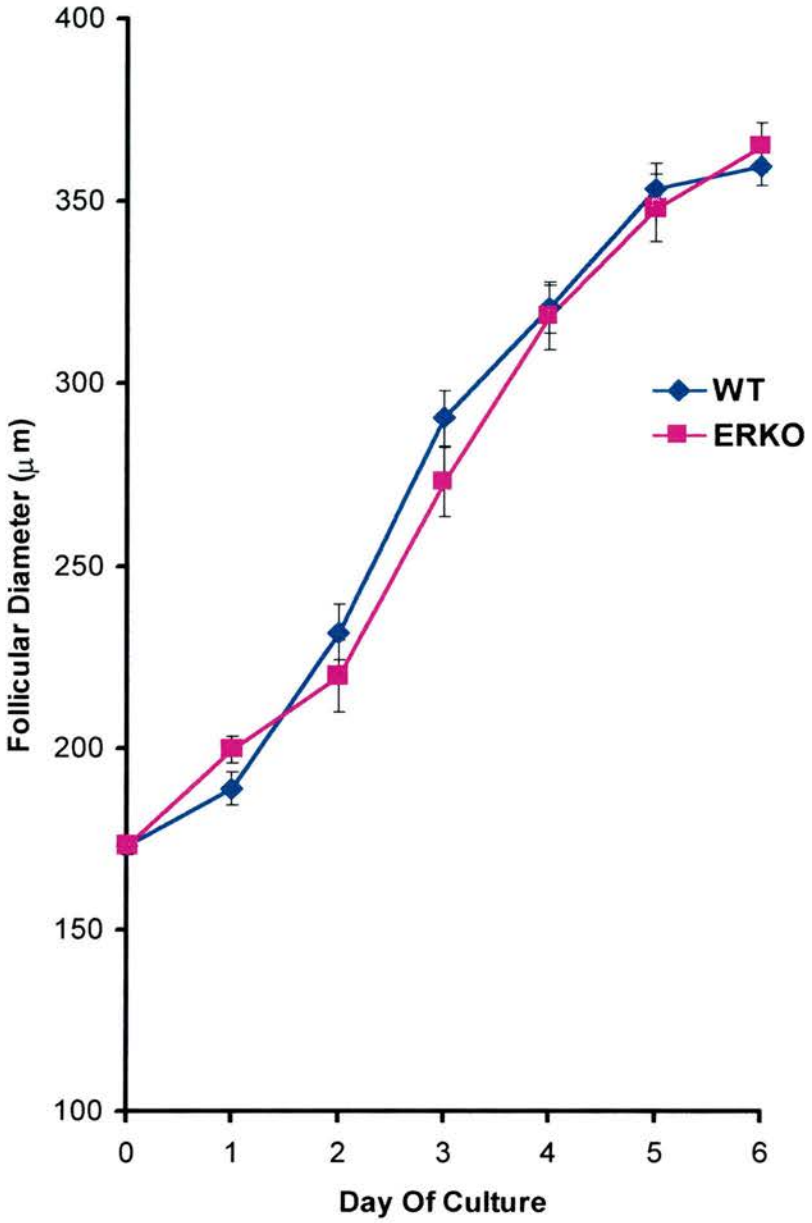


Figure 6.6 Growth rates of follicles from WT and ERKO mice. Values are Mean ± SEM ($n \geq 33$). Follicles from both groups grew at very similar rates.

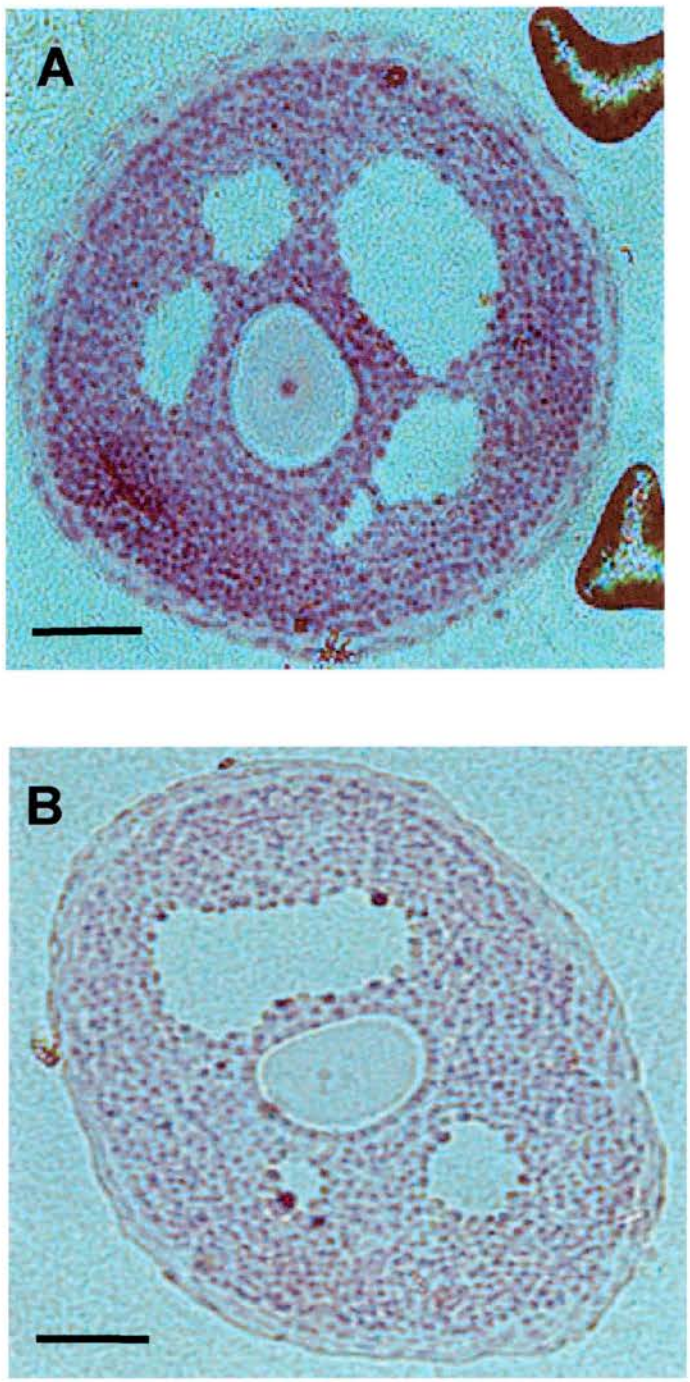


Figure 6.7. Haematoxylin and eosin stained plastic sections of follicles after 6 days in culture. Panel **A** is representative of a WT follicle and panel **B** an ERKO follicle. Scale Bar 80μm

6.4 Discussion

A role for oestrogen within the ovary remains controversial. There is much evidence to suggest that oestrogen acts through autocrine and/or paracrine mechanisms to enhance follicular development (Drummond and Findlay, 1999). However, there are a number of reports that show that oestrogen may not be obligatory for ovarian development. (Coney *et al.*, 1987; Weston *et al.,* 1996; Fauser 1997; Spears and Murray *et al.*, 1998). As oestrogen originates in the ovary and is found at high concentrations there, hypothetically its cognate receptors could be continually activated and result in continual activation of downstream targets. While co-activators and co-repressors of oestrogen act at the receptor level, the finding that two oestrogen receptors exist in mammals adds another layer of complexity to the dissection of the oestrogen response within the ovary. The examination of transgenic mice carrying deletions that knock-out one or both of these receptors are now beginning to reveal that each receptor type plays different roles in the mammalian reproductive cycle.

The distribution of oestrogen receptors within the ovary has yet to be clearly defined. In rodents there are conflicting reports as to the distribution of both types of receptor. Drummond *et al* 1999 have reported detection of mRNA for both ERα and β in the granulosa cells of the follicles while others have failed to detect ERα within these cells (Saunders *et al.,* 1997; Sar and Welch 1999; Schomberg *et al.,* 1999, Pelletier *et al.,* 2000). Apart from differences in localisation, it would also seem that both ERα and β are differentially regulated. Studies in rodents have shown that ERβ increases as follicles grow from the primary to pre-antral stages (Drummond *et al.,* 1999) and that expression is down regulated in response to LH/hCG stimulation

(Byers *et al.,* 1997). In rodents the level of ERα remains at a constant low level (Drummond *et al.,* 1999) showing no response to FSH, testosterone or forskolin stimulation (Sharma *et al.,* 1999). In human and marmoset ovaries ERβ receptor protein has been detected in both pre-antral and antral follicles whereas ERα protein was confined to antral follicles (Saunders *et al.,* 2000) which would suggest there may be species differences in receptor patterns.

The adult female ERKO mouse develops abnormal ovaries shortly after puberty resulting in infertility. Although some antral follicles are present the ovaries contain large haemorrahgic cysts with no corpora lutea being formed. In these animals, high circulating levels of oestradiol, androgen and LH persist, probably as a result of disruption of the feedback mechanisms that regulate ovarian function via the hypothalmic-pituitary axis. Thus the ERKO ovarian phenotype could arise either as a direct lack of ERα action within the ovary and/or as a consequence of altered steroidal and gonadotrophin levels. In order to address whether follicular development is directly affected by the lack of ERα this study examined the *in vitro* growth of follicles obtained from pre-pubertal mice as well as the effects of superovulation. Prior to puberty, the ovaries of the ERKO mice are morphologically very similar to those of wild-type mice with no evidence of reduced follicular development (Couse *et al.*, 2000). The use of a culture system that permits follicular development from the pre-antral through to the antral stages ensured that development was isolated from any potentially altered steroidal and gonadotrophic milieu in the animals. The results of these experiments showed that there were very few morphological differences between the growth of follicles of WT and ERKO animals. The follicles developed at similar rates and reached similar stages of

development by the end of the culture period. These results indicate that follicular development is not dependent on ERα receptor activity. It has been suggested that the ovarian phenotype of the adult animal is most probably due to the elevated circulating steroid and LH levels. A transgenic mouse that has been engineered to hypersecrete LH also develops cystic, annovulatory ovaries (Mann *et al.,* 1999). Further evidence supporting the hypothesis that the ERKO ovarian phenotype arises through an indirect mechanism comes from a report by Couse *et al*, (1999): in this study the authors demonstrated that prolonged treatment with a GnRH antagonist reduced serum LH levels and prevented the cystic ovarian phenotype.

Given that rodent studies have demonstrated that ERα levels are either not detectable or low in the ovary it is perhaps not surprising that follicles obtained from ERKO animals grew and developed at the same rate as their WT counterparts. However, in the experiments described here morphological characteristics were used to examine the follicles and it is possible that differences between the ERKO and WT exist at a cellular molecular level. Additionally these results need to be interpreted with some caution as oestrogen receptors preferentially form heterodimers (ERα/ERβ) rather than homodimers when both are co-expressed. Therefore it is possible that in follicles lacking ERα, ERβ homodimers form and can exert similar actions. It is also possible that other, yet to be identified, oestrogen receptors exist.

The experiments described here have shown that juvenile ERKO animals do respond to gonadotrophin stimulation by ovulating. Initial experiments carried out at the University of Missouri suggested that fewer of the knock-out animals were able to respond to exogenous gonadotrophins than their WT counterparts (Rosenfeld *et*

al., 2000). In the series of experiments described here, it was found that there was no difference in the number of animals able to ovulate. As the two sets of experiments were conducted in different laboratories and each used different gonadotrophin preparations it most likely that this accounts for the disparate results, although perhaps this warrants further study. In both experiments it was found that significantly fewer oocytes were ovulated by the ERKO mice. Couse *et al,* (1999) also reported very similar results. From these results it would seem that ERα while not essential to the ovulatory process (since some oocytes can be ovulated), may have an auxiliary role that facilitates either ovulation or the number of follicles available to ovulate. While similar numbers of follicles would seem to be present in the juvenile ovaries of both WT and ERKO animals, to the best of my knowledge, no study has compared the numbers of follicles present at each stage of development. Therefore it is still unknown whether ERα has a role in determining initiation of follicular growth or selection to the antral stage. Research published by Couse *et al,* (1999) indicated that while fewer ovulations occurred, a large number of follicles had reached the pre-ovulatory stage and these follicles exhibited similar markers of maturity compared to their WT counterparts. The mice used were of an age that approaches sexual maturity and it is possible that although the gross phenotype of the ovary was not apparent, the follicles within them had already been exposed to altered steroidal and hormonal profiles. It is likely that some differences exist as immature ERKO animals have significantly elevated levels of LH receptor mRNA (Couse *et al.,* 1999).

Another possibility for the reduced ovulatory capability could be that ERα directly influences ovulatory events such as extra-cellular matrix re-modelling.

One interesting observation that came from dissection of the knock-out animals was the fragile nature of the interstitial tissue. Far more care was needed to ensure the isolation of intact follicles (personal observation). Two reports have localised ERα exclusively to the interstitial tissues in rodents (Schomberg *et al.,* 1999; Pelletier *et al.,* 2000). Oestrogen has been implicated in the remodelling of extra-cellular matrix, basement membrane (Gianelli *et al* 1999; Keck *et al.,* 2002) and vasculature (Zhang *et al.,* 2000) by exerting effects on the expression of matrix-metalloproteinases which are a necessary component to the ovulatory process (McIntush and Smith, 1998). Therefore it could be speculated that rather than having a role within the follicle, ERα action is necessary to the re-modelling of extra-follicular tissue. While elevated LH levels have been associated with the cystic phenotype of the knock-out animals, increased permeability of the basement membrane may also contribute to the formation of the haemorrahgic cysts.

The final aim of this study was to examine whether oocytes obtained from ERKO females were capable of fertilisation and further development. Previous reports have suggested a direct role for oestrogen receptors in oocyte function (Wu *et al.,* 1992;1993) and during pre-implantation development (Hiroi *et al.,* 1999). The results presented here show that fertilisation rate and development to blastocyst stage was similar for both ERKO and WT derived oocytes. Couse *et al,* (1999) also reported very similar rates of fertilization. In order to further examine whether development was compromised, total cell numbers and the incidence of cell death within the blastocyts was compared. Mouse blastocysts undergo cell death in the inner cell mass as a normal feature of development perhaps as a mechanism to eliminate defective cells and regulate total cell number which may be critical for later

development (Brison and Shultz, 1997). We found no differences either in the total cell number or in the numbers of apoptotic cells between the groups, suggesting that the loss of ERα mediated action during oocyte growth and maturation is not essential at least until the implantation stage of development. Although pre-implantation development is unaffected by the lack of ERα, as measured by the parameters used in this study, an effect could become obvious at a later stage of development. It is becoming increasingly clear that many aspects of oocyte development, such as genomic imprinting (which occur during the growth and maturation phase of the follicle) may not become apparent until post-implantation of the embryo. In order to absolutely examine whether ERα defficiency during oocyte growth and maturation produces no long-term effects, blastocyst transfers need to be performed in order to obtain viable offspring.

In conclusion, the results of this study, and published work from others, is helping determine whether oestrogen is necessary to follicular development and oocyte viability. The results discussed here have demonstrated that follicles are capable of growth and development and that oocytes can fertilise and undergo pre-implantation development in the absence of ERα mediated actions. Further studies are needed to investigate the role of ERα within the thecal interstitial layers of the follicle, especially in relation to tissue re-modelling. To rule out an oocyte effect, embryo transplantation studies need to be carried out.

Although the actions of ERα do not seem to play an obligatory role in ovarian function it cannot be discounted altogether. Oestrogen receptors preferentially form hetrodimers (α and β) on ligand binding but in the absence of one homodimers form. It is possible therefore that ERα may play a role in normal follicular development but

that in its absence ERβ can substitute and normal events occur. Also the presence of other oestrogen receptors cannot be discounted.

In order to study the effects of oestrogen, mice have been generated carrying deletions in both the α and β oestrogen receptors and also in the aromatase gene (Fisher *et al.,* 1998). These latter animals are incapable of producing ovarian oestrogen. Although examination of the ovaries of these animals has suggested that oestrogen is likely to facilitate some aspects of ovarian function, the data obtained needs to be interpreted with caution. All of these knock-out genotypes have altered steroidal and/or hormonal profiles which arise as secondary effects of the deletion making it difficult to attribute any abnormal phenotype to the gene deletion. The involvement of oestrogen within the ovary needs to examined perhaps in more sophisticated ways either by generating tissue targeted gene deletion or through the use selective agonists and antagonists. Finally it is worth noting that while the laboratory mouse can reveal much, the data obtained may not be relevant to all species.

Chapter Seven

General Discussion

7.1 The aim of this thesis

The growth and development of the ovarian follicle is a long and complex process that takes many weeks in lower mammals and months in primates. Throughout this process the oocyte maintains close contact with the surrounding somatic cells and through bi-directional communication acquires the developmental programs necessary for fertilisation and embryonic development. After the onset of puberty, the cyclic rise and fall of the circulating gonadotrophins is the primary control of follicular development and of the species-specific number of oocytes released within in each ovulatory cycle. The exogenous administration of gonadotrophin preparations can over-ride this control system, allowing a larger than normal number of follicles to mature to the ovulatory stage. This technique has been widely used in both agricultural and clinical settings as a means of obtaining large numbers of oocytes for use in assisted reproductive technologies such as IVF. Until recently most preparations of gonadotrophins used for ovulation induction contained both FSH and LH and little was known about the individual roles that these play in the development of the follicle and maturation of the oocyte. With the availability of recombinant forms of both FSH and LH it is now possible to determine more precisely what effects each of these have on both the follicle and oocyte. To that end, the main aim of this thesis was to address the basic question what effects gonadotrophins have on oocyte development within the follicle prior to ovulation and how does this effect the quality of the embryo after fertilisation?

7.2 Summary of results.

The work presented in this thesis relied heavily on the use of an *in vitro* system that supports the development of murine follicles from the late pre-antral stage through to the pre-ovulatory stage of development. Since its development, this culture system would undergo periods in our laboratory during which it would not be possible to sustain the growth of intact follicles. After two days in culture the three-dimensional structure of the follicle would be lost due to rupture of the basement membrane. Apart from making it difficult to assess follicular growth rates, the contribution of the extra-cellular matrices (including the antral fluid) may be lessened which could have had an impact on the maturation of the oocyte. A primary objective therefore, was to optimise the *in vitro* system thereby increasing the numbers of follicles reaching the antral stage by the end of the culture period. It was found that inclusion of ascorbic acid in the culture media greatly increased the percentages of follicles that could maintain an intact basement membrane *in vitro*, in part at least, by modulating matrix metalloproteinase activity. Apart from promoting collagen synthesis, ascorbic acid acts as an anti-oxidant. It was found that under conditions that induced oxidative stress within follicles, the addition of ascorbic acid reduced the degree of apoptotic cell death. Granulosa cells of growing follicles sequester ascorbic acid and the ability to uptake this vitamin may confer a developmental advantage to follicles at the time of selection into the antral phase. Little is known about the nutritional requirements of growing follicles and the results of these studies contribute to that knowledge

Having established a culture system that yielded high numbers of intact follicles made it possible to investigate the individual roles that FSH and LH have on

follicular development and what effects that varying gonadotrophin exposure had on the viability of the enclosed oocytes. These experiments were successful in establishing that the supplementation of FSH with LH has no effect on follicular growth and morphology *in vitro*. The second aim, investigating the viability of the oocytes obtained from follicles, proved more problematic to determine. The results obtained varied enormously from culture to culture, under the conditions used, making it difficult to statistically determine the effect of the different treatments. Nonetheless there was an indication that the addition of low concentrations of LH did not augment oocyte quality whereas high concentrations resulted in fewer oocytes capable of completing pre-implantation development.

It had been hoped to use an *in vivo* model, the hypogonadal mouse, to investigate any indirect effects of gonadotrophins that may have arisen as a result of inter-follicular and/or extra-ovarian effects. Using a similar experimental regime to that used in the *in vitro* experiments, different gonadotrophin regimes were administered to hypogonadal mutant mice prior to inducing ovulation. Histological examination of the ovaries, including the saline controls, indicated that large-pre-ovulatory follicles were present in all the groups and there were no notable effects of any gonadotrophin treatment on follicular development. This indicated that the doses of gonadotrophins used for superovulation were sufficient to induce an ovarian response but the ovulatory mechanism was deficient in some animals. It was interesting to note however, that only ovaries exposed to FSH and the higher concentration of LH responded and released oocytes. Furthermore there was an indication that only with this treatment regime was sufficient oestrogen produced to increase uterine weight.

In addition to gaining information on the function of the ovary, it had been hoped that any effects on the oocytes obtained from the different treatments could have been examined using IVF. This proved difficult to accomplish for a number of reasons. It was found that high doses of superovulatory gonadotrophins were required to induce a response in these animals. Even then these regimes did not consistently induce ovulation in all of the animals with some mice responding while others did not. As a result few oocytes were available for IVF. Oocytes that were obtained either by superovulation alone or after prior stimulation with gonadotrophins fertilised but arrested before reaching the blastocyst stage. This indicated that the high doses of superovulatory gonadotrophins that were required to induce ovulation were detrimental to oocytes. No conclusions could be drawn as to how FSH and LH individually or in combination affected oocyte quality.

Although the results of these *in vivo* experiments were disappointing, they did raise some interesting questions. It is unknown what effects FSH and LH have on follicular development prior to the so-called 'gonadotrophin responsive' stage. Firstly, do follicles need to be exposed to gonadotrophins prior to this stage in order to become sensitive to gonadotrophin action later in development? Secondly, what effects do gonadotrophins or their mediators have on oocyte maturation during the earlier growth stages? Finally, did the increased production of oestrogen induced by the higher concentration of LH administration facilitate the ovulatory process? Further studies using this animal model may answer some of these questions.

A consequence of follicular gonadotrophin stimulation is the production of the sex steroids and the inhibin family of glycoproteins, all of which have the potential to act as mediators of gonadotrophin action. As part of the investigation

into the effects of FSH and LH, it was found that alteration to the gonadotrophin stimulation regime resulted in alterations in the profile of steroids produced by the follicles. Where LH was included in the treatment, follicles began to synthesise appreciably higher levels of both androstenedione and oestradiol at an earlier stage of development. Therefore, it seemed a logical next step to begin to investigate the effects of directly altering the follicular steroid environment. These experiments clearly showed that elevation of oestrogens during follicular development adversely affected the fertilisation rate of the oocytes while elevating androgens enhanced fertilisation rate.

Both oestrogen and androgen receptors are found in the granulosa cells of follicles and their expression patterns appear to be developmentally regulated. To date, there is no evidence that the oocyte has an androgen receptor whereas oestrogen receptors have been described and implicated in directly promoting some aspects of oocyte maturation. It has yet to be determined if androgens can directly act on the oocyte but they can indirectly affect the oocyte as a result of paracrine signalling via the granulosa cells. Previous studies from our laboratory have shown that androgens can directly promote growth and antral formation of murine follicles *in vitro* and the finding that elevation of androgens promotes fertilisation, confirms that this steroid, independently of oestrogen, is a necessary component of the signalling system in follicular and oocyte development.

There are a number of reports in both rodents and primates that suggest that oestrogen does not play an obligatory role in follicular development. Determining a role for this steroid has become more complex as it is now known that more than one oestrogen receptor (ERα and ERβ) exist in the mammalian ovary. Transgenic mice

carrying deletions for each of these receptors have been generated and each has a different phenotype. The βERKO mouse is fertile (although litter sizes are reduced) while the ERKO mouse is not. The cause of infertility in the ERKO mouse may be as a direct result of the lack of an active ovarian receptor or as consequence of the perturbations to the endocrine system that occurs in these animals. Using the ERKO mouse model, studies presented in this thesis have clarified this situation and shown that lack of the ERα receptor does not prevent follicular development or adversely affect the ability of the oocyte to fertilise and undergo further development at least until the pre-implantation stage. The embryos generated in these studies were heterozygous for ERα as the sperm used to fertilise the oocytes was obtained from F1 mice: ERKO males are infertile. Therefore, it is unknown whether any effect of this receptor is necessary in the development of the embryo. Given that the absence of either of the oestrogen receptors is not detrimental to the fertilisation and development of the oocyte questions whether exposure to oestrogen is necessary in promoting oocyte maturation. Similar experiments to those described here using the double knock-out mouse would resolve this question.

Two of the studies presented here have indicated that oestrogen may facilitate the ovulatory process. Using the hypogonadal mouse as model, it was found that only those animals that produced higher concentrations of oestrogen in response to LH stimulation ovulated, and mice lacking the ERα receptor produced fewer oocytes when administered with superovulatory doses of gonadotrophins. Further evidence supporting this theory comes from the BERKO mouse, which although fertile, has reduced litter sizes.

7.3 Conclusions

The gonadotrophins are vital to the latter stages of follicular growth and development. It is generally acknowledged that FSH is the primary gonadotrophin that drives follicular development whereas both FSH and LH contribute to follicular steroidogenesis. Whether LH contributes factors that affect the maturation of the oocyte has been the subject of some debate. The relationship between gonadotrophin stimulation, follicular development and steroid production is likely to be tightly controlled *in vivo* resulting in the ovulation of optimally viable oocytes. Little is known about how intervention resulting in changes to the follicular environment affects the developmental competence of the oocyte. The studies presented within this thesis have gone part way towards redressing this.

The main findings from these investigations are that FSH alone is capable of inducing follicular growth and producing viable oocytes and that LH has little effect on the rate of follicular growth and development. However, inclusion of LH in the stimulatory protocols induced changes in the production of the sex steroids. Under the influence of LH, follicles began to produce androgens and oestrogens at an earlier time–point in their development. Although follicular development and oestrogen production can proceed in the absence of LH stimulation *in vitro* it is likely that *in vivo* some LH is necessary to promote sufficient oestrogen to ensure that the reproductive tract is primed for an ensuing pregnancy.

Steroids have been implicated in determining follicular growth, development and fate. The conclusions drawn from the research presented here do not support this view, as alteration in the steroidal environment had little effect on follicles. However,

the steroidal environment of the follicle does impact upon the oocyte contained within it. In a previous study we have shown that androgens directly influence the growth and development of murine follicles. The work presented here has extended this knowledge and shown that the activities of androgens are also important for the development of the oocyte. The ratios of androgen and oestrogen production shifts towards increased oestrogen production as the follicle approaches the ovulatory stage. Inappropriate stimulation by oestrogen before this stage can affect oocyte viability. Whether the actions of oestrogen, either directly or indirectly, play any role in oocyte maturation remains to be elucidated.

In conclusion, manipulating the ovary (such as through the administration of exogenous gonadotrophins), while inducing multiple follicles to reach the ovulatory stage, can alter the steroidal environment within those follicles and subsequently affects oocyte maturation. While the idea that steroids influence oocyte competence is not new, the information gained from these present studies indicates that the oocyte needs to be exposed to the correct **pattern** of steroid exposure in order to mature correctly.

7.4 Experimental models

Many aspects of reproduction have been examined in animals (commonly mice) that have natural occurring mutations, such as the hypogonadal mouse, or have been transgenically altered to either over-express or carry a deletion for certain genes. These include 'knockouts' for the FSH receptor, oocyte specific growth factors such as GDF9, steroidogenic enzymes and the steroid receptors. Mice have also been engineered to over-express the LHβ sub-unit and follistatin. Most of these models have perturbed ovarian function for example, the LHβ over-expressing mouse develops ovarian tumours and the ERKO mouse develops an ovarian pathology that leads to infertility. The resulting ovarian pathologies could arise as either as a direct result of gene deletion/over-expression or as a result of the altered endocrine environment. While these models have been useful in determining what the effects of deletion (or over-expression) have on the reproductive process, whether or not these manipulations directly impact upon intra-ovarian function has been more difficult to determine.

The technologies that support follicular and oocyte development in vitro have developed tremendously over recent years. Culture systems have been developed in many species including rodents, primates and domestic animals. However, the vast majority of these *in vitro* systems have been devised using the mouse as a model. The ability to isolate ovarian components (tissue fragments, intact follicles or oocyte-granulosa complexes) and manipulate the *in vitro* environment, has led to a better understanding of what effects factors, such as the gonadotrophins and steroids, have on follicular growth and development directly.

The value of utilising both *in vitro* and *in vivo* models to address specific questions in reproduction has, I believe, been demonstrated by the work contained within this thesis. Using both models made it possible to evaluate more precisely what effects ERα deletion had on reproductive function. Similarly while *in vitro* experiments indicated that follicular development did not require LH stimulation, the in vivo model indicated that, in the case of hypogonadism, ovarian stimulation with this gonadotrophin might be a requirement for adequate steroidogenesis.

7.5 Future Work

Bi-directional communication between the oocyte and somatic cells ensures certain aspects of follicular and oocyte growth proceeds in a co-ordinated manner. There has been much research on oocyte control of somatic cell function but far less is known about the signalling that the oocyte receives from the somatic cells. The findings of this current work point to a role for the steroids in this signalling pathway and that the oocyte needs to be exposed to the correct pattern of androgens and oestrogens in order to mature optimally.

7.5.1 What is the correct steroid pattern?

In the experiments described in Chapter Five, follicles were exposed to altered steroidal environments. The results of those experiments led to the conclusion that these alterations had an impact on oocyte viability and that, in order to mature correctly, the oocyte needs to be exposed to the correct pattern of steroids. However, in those experiments steroid levels were raised throughout the culture period that encompassed follicular growth from the pre-antral to antral stage of development. From the current results it cannot be established at what point in follicular development androgens and oestrogens exert their effects. Future studies are needed to address this in order to establish this.

7.5.2 Where do steroids act?

Androgen and oestrogen receptors have been described in the somatic compartments of the follicle in a number of species and the expression of these receptors appears to be developmentally regulated *in vivo*. One possible consequence

of altering the follicular steroidal environment could be that the receptor pattern is also altered. This could result in the inappropriate differentiation and/or function of the somatic cells. Apart from acting through the somatic cells, steroids could also act directly on the oocyte. mRNA for oestrogen receptors has been reported in the oocytes of mice and of humans but no androgen receptor in the oocyte has yet been described. It is possible that the expression of oocyte receptors is tightly controlled and is transient limiting the actions of steroids to particular developmental stages. Further studies are needed to investigate the expression patterns of steroid receptors in both the somatic cells and the oocyte. Experiments such as those described in Chapter Five could be used to elucidate how alteration to the steroid environment alters these expression patterns.

Two of the studies described within this thesis suggested that oestrogen might have a role in ovarian function at the time of ovulation. Only *hypogonadal* mutant mice that had produced higher levels of oestrogen ovulated. I also found that the ERKO mouse did not ovulate at the same rate as their wild type counterparts. This would implicate a role for oestrogen, acting through the Erα receptor, in facilitating either the number of follicles reaching the ovulatory stage or in the ovulatory process itself. This aspect of oestrogen action would be worth further investigation.

7.5.3 What initiates the mechanism of genomic imprinting?

One of the most important aspects of oocyte maturation is the epigenetic modification of the genome. Genomic imprinting is an epigentic form of gene regulation that results in the differential expression of the two parental alleles in a gene pair. Whether an allele is silenced or active is dependent upon parental origin.

Imprinted genes participate in a number of developmental processes including regulation of embryonic development, placental function and foetal growth. They have also been implicated in tumour suppression, genetic and behavioural disorders.

In the oocyte, maternal imprinting is established during the growth phase (Kono, 1998). Analysis of eight known imprinted genes in growing oocytes has shown, that the imprinting signals for each of these genes were not imposed together, but occurred at specific times (Hata *et al.,* 2002). The exact nature of the marks that imprint genes has yet to be discovered but the mechanism that enforces it is likely to be DNA methylation. The great majority of imprinted genes studied so far show differences in methylation patterns between the parental alleles. Methylation is carried out by DNA methyltransferases (Dnmts) which introduce methyl groups in to unmethylated DNA. Three members of this family, Dnmt1, 3a and 3b have been implicated in methylating germ cell DNA. Knocking –out the methylase genes in mice results in embryo lethality or developmental defects. It is thought that Dnmt1is responsible for the maintenance of methylation whereas Dnmt 3a and 3b may be responsible for *de novo* methylation (Reik *et al.*, 2001). An oocyte specific form of Dnmt1 (Dnmt1o) has been described whose expression increases commensurate with oocyte growth, when imprints are established but little is known whether this can initiate new methylation patterns. Little is known about the expression of Dnmt 3a and 3b in oocytes however, Dnmt $3a^{-/-}$ and Dnmt $3b^{+/-}$ mice fail to establish maternal methylation patterns (Hata *et al.,* 2002*).*

Given that the imprints are imposed at different time-points during oocyte growth it would seem logical to assume that the expression patterns of the Dnmts are also regulated in a timed manner. While there is some information on the expression

of Dnmt1o very little is known about the expression patterns of Dnmt 3a and 3b. It is not yet clear which of these enzymes are responsible for *de novo* methylation in the oocyte. Potentially the steroids could play a role in this process and aberrations in imprinting could arise as a result of inappropriate steroid stimulation either directly or as a result of gonadotrophin manipulation. I believe that this is an important aspect of oocyte maturation and my current work is focussing on establishing the expression patterns of Dnmt expression throughout oocyte growth and what effect alteration in steroid environment has upon these.

7.5.4 Beyond blastocysts

The end point for many of the investigations contained within this thesis was to examine oocyte developmental competence up to the point of pre-implantation development. Based on that criteria conclusions were drawn, for example, regarding the role of the ERα receptor in oocyte viability. It is now known that the effects of some aspects of oocyte maturation, such as genomic imprinting, do not become apparent until later in development. It may well be that any aberrant effects in oocyte maturation do not become apparent until the implantation stage or beyond. Ultimately, in order to truly test whether oocytes were affected or not by different follicular environments, embryo transfers will need to be done.

7.6 Implications for ART

ART is widely used in both clinical and agricultural situations and the number of techniques being developed is rapidly expanding. As well as being used to treat infertility, preserve endangered and valuable species, these techniques now include the cloning of domestic species and the production of embryonic stem cells. Although some of these techniques, such as IVF, have been used for many years, none have particularly high success rates. The majority of these techniques rely upon a supply of large numbers of optimally mature oocytes. In order to gain these, it is common practice to stimulate the ovary with high concentrations of gonadotrophins and yet we still know remarkably little about how FSH and LH influence the developmental competence of the oocyte. I believe that the studies contained within this thesis have contributed to this knowledge and that they suggest areas worthy of future investigations.

References

Austin CR (1995) Gametes – The Oocyte. *eds* J Grusinkas and J Yorvich. Cambridge University Press.

Adashi EY (1994) Endocrinology of the ovary *Hum Reprod* **9**, 815-827.

Adashi EY (1998) The IGF family and folliculogenesis *J Reprod Immunol* **39**,13-19.

Albrecht KH, Eicher EM (2001) Evidence that Sry is expressed in pre-Sertoli cells and Sertoli and granulosa cells have a common precursor *Dev Biol* **240**, 92-107.

Amsterdam, Koch Y, Lieberman ME, Linder HR (1975) Distribution of binding sites for human chorionic gonadotropin in preovulatory follicles of the rat *J Cell Biol* **67,** 894-900

Anderiesz C, Trounson AO (1995) The effect of testosterone on the maturation and developmental capacity of murine oocytes in vitro *Hum Reprod* **10**, 2377-2381.

Armstrong DT, Dorrington JH (1977) Estrogen biosynthesis in the ovaries and testes. *Adv Sex Horm Res* **3**, 217-258.

Baker SJ, Spears N (1999) The role of intra-ovarian interactions in the regulation of follicle dominance. *Hum Reprod* **5**, 153-165.

Baker SJ, Sršeň V, Lapping R, Spears N (2001) Combined Effect of Follicle-Follicle Interactions and Declining Follicle-Stimulating Hormone on Murine Follicle Health In Vitro. *Biol Reprod* **65**, 1304-1310.

Baker TG, Neal P (1973) Initiation and control of meiosis and follicular growth in ovaries of the mouse *Ann Biol Anim Biochem Biophys* **13,** 137-144

Baker TG (1972) Gametogenesis. *Acta Endocrinol Suppl (Copenh)* **166,** 18-41.

Baker TG (1963) A quantitative and cytological study of germ cells in human ovaries. *Proc R Soc London Ser* **158** 417-433

Balasch J, Miro F, Burzaco I, Casamitjana R, Civico S, Ballesca JL, Peurto B, Vanrell JA (1995) The role of LH in human follicle development and oocyte fertility;evidence from *in-vitro* fertilisation in a woman with long-standing hypogonadotrophic hypogonadism *Hum Reprod* **10**, 1678-1683.

Balen AH, Tan SL, Jacobs HS (1993) Hypersecretion of LH - a significant cause of subfertility and miscarriage *British Journal of Obs and Gyn* **100**, 1082-1089.

Bartolomei MS, Tilghman SM (1997) Genomic imprinting in mammals. *Ann Rev Genet* **31**, 493-525.

Beato M (1989) Gene regulation by steroid hormones *Cell* **56,** 335-344

Beekman JM, Allan GF, Tsai WF, Tsai MJ, O'Malley BE (1993) Transcriptional activation by the estrogen receptor requires a conformational change in the ligand-binding domain. *Mol Endocrinol* **7,** 1266-1274

Behrman HR, Preston SL, Aten RF, Rinaudo D, Zreik TG (1996) Hormone induction of ascorbic acid transport in immature granulosa cells *Endo* **137**, 4316-4321.

Bergh C, Selleskog U, Hillensjo T (1997) Recombinant human gonadotropins stimulate steroid and inhibin production in human granulosa cells *Eur J Endocrinol* **136**, 617-623.

Besmer P, Manova K, Duttlinger R, Huang E, Parker A, Gyssler C, Bacharova R (1993) The kit –ligand (steel factor) and its receptor c-kit/W: pleiotropic roles in gametogenesis and melanogenesis *Development Suppl.* 125-137

Billiar RB, Loukides JA, Miller MM (1992) Evidence for the presence of the estrogen receptor in the ovary of the baboon (Papio anubis) J *Clin Endocrinol Metab* **75,**1159-65

Billig H, Furuta I, Hsueh AJW (1993) Estrogens inhibit and androgens enhance ovarian granulosa cell apoptosis *Endo* **133**, 2204-2212.

Bley MA, Saragueta PE, Baranao JL (1997) Concerted stimulation of rat granulosa cell deoxyribonucleic acid synthesis by sex steroids and follicle-stimulating hormone *J Steroid Biochem Mol Biol* **62**, 11-19.

Bloom and Fawcett. A Textbook of Histology (1962) 8th edition published by WB Saunders Company, London

Boatman DE (1997) Responses of gametes to the oviductal environment *Hum Reprod* **12**, 133-149.

Borsuk E (1991) A nucleate fragments of parthenogenetic eggs and of maturing oocytes contain complementary factors required for the development of a male pronucleus *Mol Reprod Dev* **29,** 150-156

Brison DR, Schultz RM (1997) Apoptosis during mouse blastocyst formation: evidence for a role for survival factors including TGF beta. *Biol Reprod* **56**, 1088-1096.

Brison DR, Leese HJ (1994) The role of exogenous energy substrates in blastocoele fluid accumulation in the rat *Zygote* **2**, 69-77

Bronson RA, McLaren A (1970) Transfer to the mouse oviduct of eggs with and without the zona pelucida *J Reprod Fertil* **22,** 129-137

Brown JB (1987) Pituitary control of ovarian function-Concepts derived from gonadotrophin therapy *Aust NZJ Obstet Gynaencol*

Buccione R, Vanderhyden BC, Caron PJ, Eppig JJ (1990) FSH-induced expansion of the mouse cumulus oophorus *in vitro* is dependent upon a specific factor(s) secreted by the oocyte *Dev Biol* **138**, 16-25

Byers M, Kuiper GGJM, Gustafsson J, Park-Sarge O (1997) Estrogen receptor-Beta mRNA expression in rat ovary: down regulation by gonadotropins *Mol Endo* **11**, 172-182

Byrd JA, Pardue SL, Hargis BM (1993) Effect of ascorbate on luteinizing hormone stimulated progesterone biosynthesis in chicken granulosa cells in vitro. *Comp Biochem Physiol Comp Physiol* **104**, 279-281.

Byskov AG (1978) The anatomy and ultrastructure of the rete system in the fetal mouse ovary *Biol Reprod* **19**, 720-735.

Calogero AE, Burrello N, Barone N, Palermo I, Grasso U, D'Agata R (2000) Effects of progesterone on sperm function: mechanisms of action *Hum Reprod* **15 (1)**, 28-45.

Camp TA, Rahal JO, Mayo KE (1991) Cellular localization and hormonal regulation of follicle-stimulating hormone and luteinizing hormone messenger RNAs in the rat ovary *Mol Endocrinol* **5,** 1405-1417

Canipari R (1994) Cell-cell interactions and oocyte growth *Zygote* **2**, 343-345.

Catt KJ (1996) Intracelluar Signalling. In Hillier SG, Kitchener HC, Neilson JP (eds) Scientific Essentials of Reproductive Medicine. WB Saunders Company Ltd, London. 1996

Carroll J, Jones KT, Whittingham DG (1996) Ca2+ release and the development of Ca2+ release mechanisms during oocyte maturation: a prelude to fertilization. *Rev.Reprod* **1**, 137-143.

Casan EM, Raga F, Bonilla-Musoles F, Polan ML (2000) Human oviductal gonadotropin-releasing hormone: possible implications in fertilization, early embryonic development, and implantation *J Clin Endocrinol Metab* **85**, 1377-1381.

Chappel SC, Howles C (1991) Re-evaluation of the roles of LH and FSH in the ovulatory process *Hum Reprod* **6 (9)**, 1206-1212.

Clark BJ, Soo SC, Caron KM, Ikeda Y, Parker KL, Stocco DM (1995) Hormonal and developmental regulation of the steroidogenic acute regulatory protein *Mol Endocrinol.* **9**, 1346-1355

Clarke IJ (1996) The Hypopthalmo-Pituitary Axis. In Hillier SG, Kitchener HC, Neilson JP (eds) Scientific Essentials of Reproductive Medicine. WB Saunders Company Ltd, London.

Cheng G, Weihua Z, Makinen S, Mekela S, Saji S, Warner M, Gustafsson JA and Hovatta O (2002) A role for the androgen receptor in follicular atresia of estrogen receptor beta knockout mouse ovary. *Biol Reprod* **66:** 77-84

Coney P, Yoshimura Y, Hosoi Y, Bongiovanni A, Wallach E (1987) Effect of aromatase inhibitors on the histology of the cycling rat ovary *Gynecol Obstet Invest* **23**, 177-183.

Cortvrindt RG, Hu Y, Liu J, Smitz JE (1998) Timed analysis of the nuclear maturation of oocytes in early pre-antral mouse follicle culture supplemented with recombinant gonadotropin. *Fert Steril* **70**, 1114-1125.

Couse JF, Bunch DO, Lindzey J, Schomberg DW, Korach KS (1999) Prevention of the polycystic ovarian phenotype and characterization of ovulatory capacity in the estrogen receptor- alpha knockout mouse *Endo* **140**, 5855-5865.

Couse JF, Curtis HS, Korach KS (2000) Receptor null mice reveal contrasting roles for estrogen receptor alpha and beta in reproductive tissues. *J Steroid Biochem Mol Biol* **74**, 287-296.

Couse JF, Korach KS (1999) Reproductive phenotypes in the estrogen receptor-alpha knockout mouse. *Ann.Endocrinol (Paris)* **60**, 143-148.

Couse J F, Lindzey J, Grandien K, Gustaffson J, and Korach K S. (1997) Tissue distribution and quantitative analysis of estrogen receptor alpha and beta mRNA in wild type and ER alpha- knockout mouse. *Endo* **138,** 4613-4621.

Danforth DR (1995) Endocrine and paracrine control of oocyte development. *American Journal of Obstet.Gynecol.* **172**, 747-752.

Danniellian PS, White R, Lees JA, Parker MG (1992) Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors *EMBO* **11,** 1025-1033

Deane HW (1952) Histochemical observation on the ovary and oviduct of the albino rat during the estrus cycle. *American Journal of Anatomy* **91**, 363-413

Devreker F, Englert Y (2000) In vitro development and metabolism of the human embryo up to the blastocyst stage. *Eur.J Obstet.Gynecol.Reprod Biol* **92**, 51-56.

Devreker, F. and Hardy, K (1997) Effects of glutamine and taurine on preimplantation development and cleavage of mouse embryos in vitro. *Biol Reprod* **57**, 921-928..

Dierich A, Sairam MR, Monaco L, Fimia GM, Gansmuller A, LeMeur M, Sassone-Corsi P (1998) Impairing follicle-stimulating hormone (FSH) signaling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance *Proc Natl Acad Sci U S A* **95**,13612-7

Dierschke DJ, Chaffin CL, Hute RJ (1994) Role and site of estrogen action in follicular atresia. *Trends in Endocrine Metabolism* **5**, 215-219.

Dong J, Albertini DF, Nishimori K, Kumar TR, Lu N, Matzuk MM (1996) Growth differentiation factor-9 is required during early ovarian folliculogenesis *Nature* **10,** 531-535.

Downs S, Eppig JJ (1987) Induction of mouse oocyte maturation *in vivo* by perturbations of purine metabolism. *Biol Reprod* **36,** 431-437

Driancourt MA, Thuel B (1998) Control of oocyte growth and maturation by follicular cells and molecules present in follicular fluid. *Reprod Nutr Dev* **38**, 345-362.

Driancourt MA, Reynaud K, Smitz J (2001) Differences in follicular function of 3-month old and mature cows. *Reprod* **121,** 463-724

Drummond AE, Baillie AJ, Findlay JK (1999) Ovarian estrogen receptor alpha and beta mRNA expression: impact of development and estrogen *Mol Cell Endocrinol* **149**, 153-161.

Drummond AE, Findlay JK (1999) The role of estrogen in folliculogenesis. *Mol Cell Endocrinol* **151**, 57-64.

Ducibella T, Huneau D, Angelichio E, Xu Z, Shultz RM, Kopf GF, Fisson R, Madoux S, Ozil JP (2002) Egg –to –embryo transition is driven by differential responses to Ca2+ oscillation number. *Dev Biol.* **250,** 280-291

Dupont S, Krust A, Gansmuller A, Dierich A, Chambon P, Mark M (2000) Effect of single and compound knockouts of estrogen receptors (ERα, ERβ and ERαβ) on mouse reproductive phenotypes. *Develop* **127**, 4277-4291.

Edwards RG (2000) The role of embryonic polarities in pre-implantation growth and implantation of mammalian embryos. *Hum Reprod* **15 (6)**, 1-8.

Eisenbach M, Tur-Kaspa I (1999) Do human eggs attract spermatozoa? *BioEssays* **21**, 203-210.

Elvin JA, Yan C, Matzuk MM (2000) Oocyte-expressed TGF-beta superfamily members in female fertility. *Mol Cell Endocrinol* **159**, 1-5.

Endo T, Aten RF, Wang F, Behrman HR (1993) Co-ordinate induction and activation of metalloproteinase and ascorbate depletion in structural luteolysis. *Endo* **133**, 690-698.

Enmark E, Petito-Huikko M, Grandien K, Fried G, Lagerkrantz S, Nordenkjold M, Gustaffson J (1997) Human estrogen receptor β-gene structure, chromosomal location and expression pattern. *J Clin Endocrinol Metab* **82,** 4258-4265

Eppig JJ (1997) Coordination of nuclear and cytoplasmic oocyte maturation in eutharian mammals *Reprod Fertil* **8**, 485-489.

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

Eppig JJ, Downs SM (1988) Gonadotropin-induced murine oocyte maturation in vivo is not associated with decreased cyclic adenosine monophosphate in the oocyte-cumulus cell complex. *Gamete Res* **20**, 125-131.

Eppig JJ, O'Brien MJ (1996) Development in vitro of mouse oocytes from primordial follicles. *Biol Reprod* **54**, 197-207.

Eppig JJ, Schroeder AC (1989) Capacity of mouse oocytes from preantral follicles to undergo embryogenesis and development to live young after growth, maturation and fertilization in vitro. *Biol Reprod* **41**, 268-276.

Eppig JJ, Wigglesworth K, Pendola F, Hiroa Y (1997) Murine oocytes suppress expression of LH receptor mRNA by granulosa cells. *Biol Reprod* **56**, 976-984.

Eppig JJ, Scultz R, O'Brien M, Chesnel F (1994) Relationship between the developmental programs controlling nuclear and cytoplasmic maturation of the mouse oocyte. *Develop Biol* **50,** 531-536

Erickson GF (1983) Primary cultures of ovarian cells in serum-free medium as models of hormone-dependent differentiation. *Mol Cell Endocrinol* **29**, 21-49.

Esch FS, Shimasaki S, Mercado M, Cooksey K, Ling N, Ying S, Ueno N,Guillemin R (1987) Structural characterization of follistatin: a novel follicle-stimulating hormoen release-inhibiting polypeptide from the gonad. *Mol Endocrinol* **1,** 849-855

Espey LL (1994) Current status of the hypothesis that mammalian ovulation is comparable to an inflammatory reaction. *Biol of Reprod* **50**, 233-238.

Espey LL (1998) Ovulation, Evolving Scientific and Clinical Concepts. *ed* E Adashi. Proceedings of Serono Synposia.

Espey LL, Coons PJ (1976) Factors which influence ovulatory degradation of rabbit ovarian follicles. *Biol Reprod* **14**, 233-245.

Evans RM (1988) The steroid and thyroid hormone super family. *Science* **240**, 889-895

Faddy MJ, Gosden RG (1995) A mathematical model of follicle dynamics in the human ovary. *Hum Reprod* **10**, 770-775.

Farookhi R (1985) Effects of aromatizable and nonaromatizable androgen treatments on LH receptors and ovulation induction in immature rats. *Biol of Reprod* **33**, 363-369.

Fauser BC (1997) Follicular development and oocyte maturation in hypogonadotrophic women employing recombinant follicle-stimulating hormone: the role of oestradiol. *Hum Reprod Update* **3**, 101-108.

Findlay JK, Drummond AE (1999) Regulation of the FSH Receptor in the Ovary. *Trends Endocrinol Metab* **10**, 183-188.

Findlay JK, Drummond AE, Britt KL, Dyson M, Wreford NG, Robertson DM, Groome NP, Jones ME, Simpson ER (2000) The roles of activins, inhibins and estrogen in early committed follicles. *Mol Cell Endocrinol* **163**, 81-87.

Fisher C, Graves K, Parlow AF, Simpson ER (1998) Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the *cyp19*gene. *Proc Natl Acad Sci USA* **95,** 6965-6970

Fleming R, Retika P, Deshpanda N, Jamieson ME, Yates RWS, Lyall H (2000) Supression of LH during ovarian stimulation; effects differ with purified urinary FSh and recombinant FSH. *Hum Reprod* **15,** 1440-1445

el-Fouly MA, Cook B, Nekola M, Nalbandov AV (1970) Role of the ovum follicular luteinisation. *Endo* **87,** 286-293.

Fowler E, Edwards RG (1957) Induction of superovulation and pregnancy in mature mice treated by gonadotrophins. *J Endocrinol* **15,** 374-384

Fraser LR (1999) New insights into possible causes of male fertility. *Hum Reprod* **14,** 38-46

Galway AB, Lapolt PS, Tsafriri A, Dargan CM, Boime I, Hsueh AJ (1990) Recombinant follicle-stimulating hormone induces ovulation and tissue plasminogen activator expression in hypophysectomized rats. *Endocrinology* **127,** 3023-8

Gandolfi TA, Gandolfi F (2001) The maternal legacy to the embryo: cytoplasmic components and their effects on early development. *Theriogenology* **55**, 1255-76.

Gelety TJ, Magoffin DA (1997) Ontogeny of steroidogenic enzyme gene expression in ovarian thecal-interstitial cells in the rat: regulation by a paracrine theca differentiating factor prior to achieving luteinizing hormone responsiveness. *Biol Reprod* **56,** 938-945

Giannelli G, Pozzi A, Stetler-Stevenson WG, Gardner HA, Quaranta V (1999) Expression of matrix metalloprotease-2-cleaved laminin-5 in breast remodeling stimulated by sex steroids. *Am J Pathol* **154**, 1193-1201.

Gibson MJ, Kasowski H, Dobrjansky A (1994) Continuos gonadotropin-releasing hormone infusion stimulates dramatic gonadal development in hypogonadal female mice. *Biol Reprod* **50**, 680-685.

Gibson MJ, Wu TJ, Miller GM, Silverman AJ (1997) What nature's knockout teaches us about GnRH activity: hypogonadal mice and neuronal grafts. *Horm Behav* **31**, 212-220.

Gilbert SF (2000) Developmental Biology 6th Edition, Sinauer Associates, USA

Gondas B (1970) In Buth WR, Crooke AC, Ryle M (eds). Gondaotrophins and Ovarian Development. Edinburgh

Gosden RG (2002) Oogenesis as a foundation for embryogenesis. *Mol Cell Endocrinol* **186**, 149-153.

Gosden RG, Krapez J, Briggs D (1997) Growth and development of the mammalianm oocyte. *Bioessays* **19,** 875-882

Gosden RG (1995) Gametes – The Oocyte. *eds.* In Grusinkas JG andYorvich JL (eds.). Cambridge University Press.

Gosden RG, Bownes M (1995) Cellular and molecular aspects of oocyte development. In Grudzinskos JG, Yovich JL (eds.). Gametes-The Oocyte, Cambridge University Press

Gosden RG, Boland NI, Spears N, Murray AA, Chapman M, Wade JG, Zohdy N, Brown N (1993) The biology and technology of follicular oocyte development in vitro. *Reprod Med Rev* **2**, 129-152.

Gosden RG, Hunter RHF, Telfer E, Torrance C, Brown N (1988) Physiological factors underlying the formation of ovarian follicular fluid. *J. Reprod. Fert* **82,** 813-825.

Gougeon A (1996) Regulation of ovarian follicular development in primates: Facts and hypotheses. *Endocrine Reviews* 121-155.

Green S, Walter P, Greene G, Krust A, Goffin C, Jensen E, Scrace G, Waterfield M, Chambon P (1986) Cloning of the human oestrogen receptor cDNA. *J Steroid Biochem* **24**, 77-83.

Greep RO, Van Dyke H, Chow B F (1942). Gonadotropins of the swine pituitary: various biological effects of purified thylakentrin (FSH) and pure metakentrin (ICSH). *Endocrinology* **30**, 635-649

Hagglund AC, Ny A, Leonardsson G, Ny T (1999) Regulation and localization of matrix metalloproteinases and tissue inhibitors of metalloproteinases in the mouse ovary during gonadotropin- induced ovulation. *Endo* **140**, 4351-4358.

Halpin DMG, Charlton HM (1988) Effects of short-term injection of gonadotropin on ovarian follicle development in hypogonadal (hpg) mice. *J Reprod Fert* **82**, 393-400.

Halpin DMG, Jones A, Fink G, Charlton HM (1986) Postnatal ovarian follicle development in hypogonadal (hpg) and normal mice and associated changes in the hypothalamic-pituitary ovarian axis. *J Reprod Fert* **77**, 287-296.

Hardy K (1999) Apoptosis in the human embryo. *Rev.Reprod* **4**, 125-134.

Hardy K, Handyside A, Winston RML, becker DL (1996) Expression of intracellular junction during preimpnatation development of the human embryo. *Mol Hum Reprod* **2,** 621-632

Hashizume K, Tsujii H, Rokutanda M (1995) Effects of gonadotropin administration on follicular growth and in vitro fertilization in female hypogonadal mice. *Exp.Anim* **44**, 241-244.

Hata K, Okano M, Lei H, Li E (2002) Dnmt3L cooperates with the Dnmt3 family of de novo DNA methyltransferases to establish maternal imprints in mice. *Development* **129**, 1983-1993.

Hedin L, Rodgers RJ, Simpson ER, Richards JS (1987) Changes in content of cytochrome P450(17)alpha, cytochrome P450scc, and 3-hydroxy-3-methylglutaryl CoA reductase in developing rat ovarian follicles and corpora lutea: correlation with theca cell steroidogenesis. *Biol Reprod* **37,** 211-23

Henttu PM, KalkhovenE, Parker MG (1997) Af-2 activity and recruitment of steroid receptor coactivator 1 to the estrogen recptor depend on a lysine residue conserved in nuclear receptors *Mol Cell Biol* **17,** 198-204

Hibbs MS, Hasty KA, Seyer JM, Kang AH and Mainardi CL (1985) Biochemical and immunological characterization of the secreted forms of human neutrophil gelatinase. *J Biol Chem* **260**, 2493-2500.

Hill LJ, Hammar K, Smith PJ, Gross DJ (1999) Stage-dependent effects of epidermal growth factor on Ca^{2+} efflux in mouse oocytes. *Mol Reprod Dev* **53,** 244-253.

Hillier S, Whitelaw P, Smyth C (1994) Follicular oestrogen synthesis: the 'two-cell, two-gonadotrophin' model revisited. *Mol Cell Endocrinol* **100**, 51-4

Hillier SG (1994) Current concepts of the role of FSH and LH in folliculogenesis. *Hum Reprod* **9**, 188-191.

Hillier SG (2001) Gonadotropic control of ovarian follicular growth and development. *Mol Cell Endocrinol* **179**, 39-46.

Hillier SG, Tetsuka M (1998) An anti-inflammatory role for glucocorticoids in the ovaries? *J Reprod Immunol.* **39**, 21-27.

Hillier SG, Yong EL, Illingworth PJ, Baird DT, Schwall RH, Mason AJ (1991) Effect of recombinant activin on androgen synthesis in cultured human thecal cells. *J Clin Endo Metab* **72**, 1206-1211.

Hiroi H, Momoeda M, Inoue S, Tsuchiya F, Matsumi H, Tsutsumi O, Muramatsu M, Taketani Y (1999) Stage-specific expression of estrogen receptor subtypes and estrogen responsive finger protein in preimplantational mouse embryos. *Endocr.J.* **46**, 153-158.

Hirshfield AN (1991) Development of follicles in the mammalian ovary. *International Review of Cytology* **124**, 43-99.

Hirshfield AN (1991) Thecal cells may be present at the outset of follicular growth. *Biol Reprod* **44**, 1157-1162.

Ho Y, Wigglesworth K, Eppig JJ, Schultz RM (1995) Preimplantation development of mouse embryos in KSOM: augmentation by amino acids and analysis of gene expression. *Mol Reprod Dev* **41**, 232-238.

Hogan B, Beddington R, Constantini F, Lacy E (1994) Manipulating the Mouse Embryo. 2nd Edition. Cold Spring Harbor Press. USA

Homburg R (1998) Adverse effects of luteinizing hormone on fertility: fact or fantasy. *Baillieres.Clin Obstet.Gynaecol.* **12**, 555-563.

Howles CM (2000) Role of LH and FSH in ovarian function. *Mol Cell Endocrinol* **161**, 25-30.

Hsueh AJ, McGee EA, Hayashi M, Hsu SY (2000) Hormonal regulation of early follicle development in the rat ovary. *Mol Cell Endocrinol* **163**, 95-100.

Hsueh AJW (1986) Paracrine mechanisms involved in granulosa cell differentiation. *Clinical Endocrinology and Metabolism* **15,** 117-134.

Hu Y, Cortvrindt R, Smitz J (2002) Effects of aromatase inhibition on in vitro follicle and oocyte development analyzed by early preantral mouse follicle culture. *Mol Reprod Dev* **61**, 549-559.

Huang M, Li J, Teoh H, Man RY (1999) Low concentrations of 17 beta-oestradiol reduce oxidation modification of low-density lipoproteins in the presence of vitamin C and vitamin E. *Free Radical Biology and Medicine* **27**, 438-441.

Huet C, Pisselet C, Mandon-Pepin B, Monget P, Monniaux D (2001) Extracellular matrix regulates ovine granulosa cell survival, proliferation and steroidogenesis: relationships between cell shape and function. *J Endocrinol* **169**, 347-360.

Huhtaniemi IT, Aittomaki K (1998) Mutations of follicle-stimulating hormone and its receptor: effects on gonadal function. *Eur.J Endocrinol* **138**, 473-481.

Huot RI, Shain SA (1988) Differential metabolism of dehydroepiandrosterone sulfate and estrogen conjugates by normal or malignant AXC/SSh rat prostate cells and effects of these steroid conjugates on cancer cell proliferation in vitro. *J Steroid Biochem* **29**, 617-621.

Hutchison LA, Findlay JK, deVoss FL, Robertson DM (1987) Effects of bovine inhibin, transforming growth factor β and bovine activin –A on granulosa cell differentiation *Biochem Biophys Res Commun* **146,** 1405-1412

Hutz RJ, Wagner N, Krause P, Fisher, Syed N, Dierschke DJ, Monniaux D, Tomanek M (1993) Localization of estrogen receptors in rhesus monkey ovary. *Am J Primatol* **31,** 299-309

Hutz RJ (1989) Disparate effects of estrogens on in vitro steroidogenesis by mammalian and avian granulosa cells. *Biol Reprod* **40**, 709-713.

Igarashi M (1977) Augmentative effect of ascorbic acid upon induction of human ovulation in clomiphene-ineffective anovulatory women. *Int.J Fertil* **22**, 168-173.

Ignar-Trowbridge DM, Nelson KG, Bidwell MC, Curtis SW, Washburn TF, McLachlan JA, Korach KS (1992) Coupling of dual signaling pathways: epidermal growth factor action involves the estrogen receptor. *Proc Natl Acad Sci U.S.A* **89**, 4658-4662.

Jia C, Kessel B, Welsh TH, Hsueh AJW (1985) Androgen inhibition of FSH stimulated LH receptor formation in cultured rat granulosa cells. *Endocrinology* **117**, 13-22.

Katzenellenbogen BS, Sun J, Harrington WR, Kraichley DM, Ganessunker D, Katzenellenbogen J (2001) Structure-function relationships in estrogen receptors and the

characterization of novel selective estrogen receptor modulators with unique pharmacological profiles. *Ann NY Acad Sci* **949,** 6-15

Kaye PL (1997) Preimplantation growth factor physiology. *Rev.Reprod* **2**, 121-127.

Kezele PR, Nilsson EE, Skinner MK (2002) Insulin but not insulin-like growth factor-1 promotes the primordial to primary follicle transition. *Mol Cell Endocrinol* **192**, 37-43.

Knight PG (1996) Roles of inhibins, activins, and follistatin in the female reproductive system. *Front Neuroendocrinol* **17**, 476-509.

Knight PG, Glister C (2001) Potential local regulatory functions of inhibins, activins and follistatin in the ovary. *Reproduction* **121**, 503-12.

Kono T (1998) Influence of epigenetic changes during oocyte growth on nuclear reprogramming after nuclear transfer. *Reprod Fertl Dev* **10**, 593-598.

Korach KS (2000) Estrogen receptor knock-out mice: molecular and endocrine phenotypes. *J Soc Gyneco lInvestig* **7**, S16-S17.

Kovacs GT, Baker HWG, Burger HG, Lee J, Summerbell D (1990) Prognosis of pregnancies concieved by donor insemination with respect to the lat follicular phase LH level. *British Jl Obs Gyn* **97**, 654-656.

Kramer MM, Harman MT, Brill AK (1933) Disturbances of reproduction and ovarian changes in the guinea pig in relation to vitamin C deficiency .*American J Physiol* **106**, 611-622

Krege JH, Hodgin JB, Couse JF, Enmarks E, Warner M, Mahler JF, Sar M, Korach KS, Gustafsson J, Smithies O (1998) Generation and reproductive phenotypes of mice lacking estrogen receptor β. *Proc. Natl. Acad. Sci. U. S. A.*, 15677-15682.

Kumar TR, Wang Y, Lu N, Matzuk MM (1997) Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. *Nat Genet* **15,** 201-4

Kuo SM, Lin CP (1998) 17beta-estradiol inhibition of ascorbic acid accumulation in human intestinal Caco-2 cells. *Eur.J Pharmacol.* **361**, 253-259.

Kuiper GGJM, Enmark E, Pelto- Huikko M, Nilsson S and Gustaffson J (1996) Cloning of a novel estrogen receptor expressed in rat prostate and ovary *Proceedings of the National Academy of Science U.S A.* **93:** 5925-5930

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the bacteriophage T4. *Nature* **220**, 680-685

Lambeth J.D and Stevens VL (1985) Cytochrome P-450scc: Enzymology, and the regulation of intramitochondrial delivery to the enzyme. *Endocrine Res.* **10,** 283-309.

Langan T (1968) Histone phosphorylation stimulated by cAMP. *Science* **162,** 579-581

Latham KE, Sapienza C (1998) Localization of genes encoding egg modifiers of paternal genome function to mouse chromosomes one and two. *Develop* **125**, 929-935.

Latham KE, Schultz RM (2001) Embryonic genome activation. *Front Biosci.* **6**, 748-759.

Layman LC, McDonough PG (2000) Mutations of follicle stimulating hormone-beta and its receptor in human and mouse: genotype/phenotype. *Mol Cell Endocrinol* **161**, 9-17.

Leese HJ, Tay JI, Reischl J and Downing SJ (2001) Formation of fallopian tubal fluid: role of a neglected epithelium *Reproduction* **121:** 339-346

Levin ER (2001) Cell localization, physiology, and nongenomic actions of estrogen receptors. *J Appl Physiol* **91**, 1860-1867.

Levy DP, Navarro JM, Schattman GL, Davis OK, Rosenwaks Z (2000) The role of LH in ovarian stimulation: Exogenous LH: let's design the future. *Hum Reprod* **15**, 2258-2265.

Lewis KA, Gray PA, Blount AL, MacConell LA, Wiater E, Bilezikjian LM, Vale W (2000) Betaglycan binds inhibin and can mediate functional antagonism of activin signalling. *Nature* **404,** 411-414

Li E, Bestor TH, Jaenisch R (1992) Targeted mutation of the DNA methyltransferase gene esults in embryonic lethality. *Cell* **69**, 915-926.

Ling N, Ying SY, Ueno N, Shimasaski S, Esch F, Hotta M, Guillemin R (1986) Pituitary FSH is released by a heterodimer of the β subunits of two forms of inhibin. *Nature* **321,**779-782

Lintern-Moore S, Moor GMP (1979) The initiation of follicle and oocyte growth in the mouse ovary. *Biol Reprod* **20,** 773-778

Liu HC, He Z, Rosenwaks Z (2002) In vitro culture and in vitro maturation of mouse preantral follicles with recombinant gonadotropins. *Fert Steril* **77 (2)**, 383.

Liu X, Andoh K, Abe Y, Kobayashi J, Yamada K, Mizunuma H, Ibuki Y (1999) A comparative study on transforming growth factor-beta and activin A for preantral follicles from adult, immature, and diethylstilbestrol-primed immature mice. *Endo* **140**, 2480-2485.

Liu YX, Hsueh AJ (1987) Plasminogen activator activity in cumulus-oocyte complexes of gonadotropin-treated rats during the periovulatory period *Biol Reprod* **36**,1055-62

Lonning PE, Geisler J, Dowsett M (1998) Pharmacological and clinical profile of anastrozole. *Breast Cancer Res Treat* **49 Suppl 1,** S53-S57.

Lopata A, Kohlman D, Johnson I (1983) The fine structure off normal and abnormal human embryos developed in culture. In, Beier HM, Lindner HR (eds). Fertilization of the human egg in vitro. Springer, Heidelberg.

Loumaye E, Engrand P, Shoham Z, Hillier SG, Baird DT. On behalf of the recombinant LH study group (2003) Clinical evidence for an LH 'ceiling' effect induced by administration of recombinant human LH during the late follicular phase of stimulated cycles in WHO type 1 and type 11 anovulation. *Hum Reprod* **18,** 314-322

Lubahn DB, Moyer JS, Goldug TS, Couse JF, Korach KS, Smithies O (1993) Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proc Nat Acad Sci U.S.A.* **90**, 11162-11166.

Luck MR, Jeyaseelen I, Scholes RA (1995) Ascorbic acid and fertility. *Biol Reprod* **52**, 262-266.

Luck MR, Junglas B (1987) Catecholamines and ascorbic acid as simulators of bovine ovarian oxytocin secretion *J. of Endo* **114**, 423-430

Lutz LB, Cole LM, Gupta MK, Kwist KW, Auchus RJ, Hammes SR (2001) Evidence that androgens are the primary steroids produced by Xenopus laevis ovaries and may signal through the classical androgen receptor to promote oocyte maturation. *Proc Natl Acad Sci U.S.A* **98,** 13728-13733.

Magoffin DA, Jakimiuk AJ (1998) Inhibin A, inhibin B and activin A concentrations in follicular fluid from women with polycystic ovary syndrome. *Hum Reprod* **13,** 2693-2698.

Manandhar G, Sutovsky P, Joshi HC, Stearns T, Schatten G (1998) Centrosome reduction during mouse spermiogenesis. *Dev Biol* **15,** 424-34

Mann RJ, Keri RA, Nilson JH (1999) Transgenic mice with chronically elevated luteinizing hormone are infertile due to anovulation, defects in uterine receptivity, and midgestation pregnancy failure. *Endo* **140**, 2592-2601.

Mannaerts B, Uilenbroek J, Schot P, de Leeuw R (1994) Folliculogenesis in hypophysectomised rats after treatment with rhFSH. *Biol of Reprod* **51**, 72-81.

Mason H (2000) Function of the polycystic ovary. *Hum Fertil (camb)* **3**, 80-85.

Mason AJ, Niall HD, Seeburg PH (1986) Structure of two human ovarian inhibins. *Biochem Biophys Res Commun* **135,** 957-964

Matzuk M, Ginegold M, Su J, Hsueh A, Bradley A (1992) a-inhibin is a tumour-supressor gene with gonadal specificity in mice. *Nature* **360,** 313-319

McGee E, Spears N, Minami S, Hsu SY, Chun SY, Billig H, Hsueh AJ (1997) Preantral ovarian follicles in serum-free culture: suppression of apoptosis after activation of the cyclic guanosine 3',5'- monophosphate pathway and stimulation of growth and differentiation by follicle-stimulating hormone. *Endo* **138**, 2417-24.

McIntush EW, Smith MF (1998) MMP and TIMPs in ovarian function. *Rev Reprod* **3**, 23-30.

McLaren A the embryo (1992) In Short RV, Austin CR (eds) Reproduction in Mammals: Embtyonic and Fetal Development. Cambridge University Press, Canbridge.

McNatty KP, Smith DM, Makris A, Osathanondh R, Ryan KJ (1979) The microenvironment of the human antral follicle: interrelationships among the steroid levels in antral fluid, the population of granulosa cells, and the status of the oocyte in vivo and in vitro. *J Clin Endocrinol Metab* **49**, 851-860.

Merck FB, Boticelli CR, Albright JT (1972). An intrafollicular response to oestrogen by granulose cells in the rat ovary: an electron microscope study. *Endocrinology*. **90:** 992-1007.

Mermillod P, Oussaid B, Cognie Y (1999) Aspects of follicular and oocyte maturation that affect the developmental potential of embryos. *J Reprod Fertil Suppl* **54**, 449-460.

Meur SK, Sanwal PC, Yadav MC (1999) Ascorbic acid in buffalo ovary in relation to oestrous cycle. *Indian J Biochem Biophys.* **36**, 134-135.

Milewich L, Chen GT, MacDonald PC, Peterson JA (1981) Ascorbic acid inhibition of aromatase activity in human placenta tissue. *J Steroid Biochem* **14**, 185-193.

Miro F, Hillier SG (1996) Modulation of granulosa cell deoxyribonucleic acid synthesis and differentiation by activin. *Endocrinol* **137,** 464-468

Miro F, Hillier SG (1992) relative effects of activin and inhibin on steroid hormone synthesis in primate granulosa cells. *Endocrinol* **129,** 3388-3394

Miyazaki S, Hashimoto N, Yoshimoto Y, Kishimoto T, Igusa Y, Hiramot Y (1986) Temporal and spatial dynamics of the periodic increase in intracellular free calcium at fertilization of golden hamster eggs. *Dev Biol* **118,** 259-267

Monk M (1990) Changes in DNA methylation during mouse embryonic development in relation to X-chromosome activity and imprinting. *Philos.Trans.R.Soc.Lond B Biol Sci* **326**, 299-312.

Moor RM, Dai Y, Lee C, Fulka J, Jr. (1998) Oocyte maturation and embryonic failure. *Hum.Reprod.Update.* **4**, 223-236.

Morodomi T, Ogata Y, Sasaguri Y, Morimatsu M, Nagase H (1992) Purification and characterization of matrix metalloproteinase 9 from U937 monocytic leukaemia and HT1080 fibrosarcoma cells. *Biochem J* **285(Pt 2),** 603-611.

Murphy G, Reynolds J, Werb Z (1985) Biosynthesis of tissue inhibitor of metalloproteinases by human fibroblasts in culture. Stimulation by 120O tetradecanoylphorbol 13-acetate and interleukin 1 in parallel with collagenase. *J Biol Chem* **260**, 3079-3083.

Murray AA, Gosden RG, Allison V, Spears N (1998) Effect of androgens on the development of mouse follicles growing in vitro. *J Reprod Fert* **113**, 27-33.

Muttukrishna S, Groome N, Ledger W (1997) Gonadotropic control of secretion of dimeric inhibins and activin A by human granulosa-luteal cells in vitro. *J AssistReprod Genet* **14**, 566-574.

Muttukrishna S, Knight PG (1991) Inverse effects of activin and inhibin on the synthesis and secretion of FSH and LH by ovine pituitary cells. *J Mol Endocrinol* **6,** 171-178

Nicholson SM, Bruzzone R (1997) Gap junctions: getting the message through. *Curr. Biol* **7**, 340-344

Nilsson N, Makela S, Eckardt T, Michel T, Thomsen J, ,Andersonn G, Enmark E, Pettersson K, Warner M, Gustafsson J (2001). Mechanisms of estrogen action. *Phys Rev.* **4,** 1535-1565

Nilsson E, Parrott JA, Skinner MK (2001) Basic fibroblast growth factor induces primordial follicle development and initiates folliculogenesis. *Mol Cell Endocrinol* **175**, 123-30.

Nilsson E, Skinner MK (2001) Cellular interactions that control primordial follicle development and folliculogenesis. *J.Soc.Gynecol.Investig.* **8**, S17-20.

Nokatani A, Shimaski S, dePaola LV, Erickson GF, Ling N (1991) Cyclic changes in follistatin messenger ribonucleic acid and its proteins in the rat ovary during the oestrus cycle. *Endocrinol* **129,** 603-611

Obata Y, Kono T (2001) Maternal primary imprinting is established at a specific time for each gene throughout oocyte growth. *J Biol Chem* **277**, 5285-5289.

Oelsner G, Serr Dm, Maschiach S, Blankstein S, Snyder M Lunefield B (1978) The study of induction of ovulation with menotropins; analysis of 1897 treatment cycles. *Fertil Steril* **30,** 538-544

Onate SA, Boonyaratanakornkit V, Spencer TE, Tsai SY, Tsai MJ, Edwards DP, O'Malley BW (1998) The steroid-receptor co-activator-1 contains multiple receptor interacting and activation domains that cooperatively enhance the activation function 1 (AF1) and AF2 of steroid receptors. *J Biol Chem* **273,** 1201-12108

Osborn JC, Moor RM (1983) The role of steroid signals in the maturation of mammalian oocytes. *J.Steroid Biochem.* **19**, 133-137.

O'Shaughnessy PJ, Dudley K, Rajapaksha WR (1996) Expression of FSH- receptor mRNA during gonadal development. *Mol Cell Endo* **125**, 169-175.

O'Shaughnessy PJ, Gray SA (1997) Gonadotropin-dependent and gonadotropin-independent development of inhibin subunit mRNA levels in the mouse ovary. *Endo* **136**, 2060-2065.

O'Shaughnessy PJ, McLelland D, McBride MW (1997) Regulation of LH -receptor and FSH -receptor mRNA levels during development in the neonatal mouse ovary. *Biol Reprod* **57**, 602-608.

Oswald J, Engemann S, Lane N, Mayer W, Olek A, Fundele R, Dean W, Reik W, Walter J (2000) Active demethylation of the paternal genome in the mouse zygote. *Curr Biol* **10**, 475-478.

Padh H (1991) Vitamin C: newer insights into its biochemical functions. *Nutr.Rev.* **49**, 65-70.

Padh H, Aleo JJ. (1985). Stability of ascorbic acid in solution. *IRCS Med Sci* **13**:1028

Paesi FJA (1952) The effect of small doses of oestrogen on the ovary of the immature rat. *Acta Endocrinol (Copenh)* **11,**251-268

Parlow AF (1958) A rapid bioassay method for LH and factors stimulating LH secretion. *Federation Proceedings* **85**, 501-520

Parrott JA, Skinner MK (1998) Thecal cell-granulosa cell interactions involve a positive feedback loop among keratinocyte growth factor, hepatocyte growth factor and Kit ligand during follicular development. *Endocrinol.* **139,** 2240-2245

Parrott JA, Skinner MK (1999) Kit ligand actions on ovarian stromal cells: effects on theca cell recruitment and steroid production. *Mol Reprod Develop* **55**, 55-64.

Parrott JA, Skinner MK (1999) Kit-Ligand/Stem Cell factor induces primordial follicle development and initiates folliculogenesis. *Endocrinol* **140,** 4262-4271

Paszkowski T, Clarke RN (1999) The Graafian follicle is a site of L-ascorbate accumulation. *J Assist Reprod Genet* **16**, 41-45.

Patsoula E, Loutradis D, Drakakis P, Kallianidis K, Bletsa R, Michalas S (2001) Expression of mRNA for the LH and FSH receptors in mouse oocytes and preimplantation embryos. *Reprod* **121**, 455-61.

Pelletier G, Labrie C, Labrie F (2000) Localization of oestrogen receptor alpha, oestrogen receptor beta and androgen receptors in the rat reproductive organs. *J Endocrinol* **165**, 359-370.

Peters H, Byskov AG, Grinsfed J (1981) The development of the ovary during childhood in health and disease. In Coults JRT (ed). Functional morphology of the human ovary. MTP press, Lancaster.

Pfeffer F, Casanueva E, Kamar J, Guerra A, Perichart O, Vadillo-Ortega F (1998) Modulation of 72-kilodalton type IV collagenase (Matrix metalloproteinase-2) by ascorbic acid in cultured human amnion-derived cells. *Biol Reprod* **59**, 326-329.

Phillips PH, Lardy HA, Boyer PD, Werner GM (1941) The relationship to reproduction of ascorbic acid in the cow. *Journal of Dairy Science* **24**, 153-158.

Picazo RA, Illera JC, Illera M (2000) Development of a long-term serum-free culture system for immature granulosa cells from diethylstilboestrol-treated prepubertal rabbits: influence of androstenedione and fibronectin on FSH-induced cytodifferentiation. *J Reprod Fertil* **119**, 279-285.

Pico L, Clegg KB (1982) Quantitative changes in total RNA, total poly(A) and ribosomes in early mouse embryos *Dev Biol* **89,** 362-378

Pinnell SR (1985) Regulation of collagen biosynthesis by ascorbic acid: a review. *Yale J Biol Med* **58**, 553-559.

Pintauro SJ, Bergan JG (1982) Effects of ascorbic acid on in vitro steroidogenesis in guinea pigs. *J Nutr* **112**, 584-591.

Quinn P, Barros C, Whittingham DG (1982) Preservation of hamster oocytes to assay fertilizing capacity of human spermatozoa. *J. Reprod. Fertil* **66**, 161-168

Rabinovici J, Spencer S, Jaffe R (1990) Recombinant human activin-A promotes proliferation of human luteinized preovulatory granulosa cells. *J Clin Endocrinol* **71,** 1396-1398

Rao IM, Mills TM, Anderson E, Mahesh VB (1991) Heterogeneity in granulosa cells of developing rat follicles *Anat Rec* **229,** 177-185

RankinTL, O'Brien M, Lee E, Wigglesworth K, Eppig J, Dean J (2001) defective zonae pellucidae in Zp2-null mutant mice disrupt folliculogenesis,fertility and development *Develop* **128,** 1119-1126

Rankin T, Soyal S, Dean J (2000) The mouse zona pellucida: folliculogenesis, fertility and preimplantation development *Mol Cell Endocrinol* **163,** 21-25

Rankin T, Familari M, Lee E, Ginsberg A, Dwyer N, Blanchette-Mackie J, Drago J, Westphal H, Dean J (1996) Mice homozygous for an insertional mutation in the ZP3 gene lack a zona pellucida and are infertile *Develop* **122,** 3847-3855

Recombinant Human FSH Product Development Group (1998). Recombinant follicle stimulating hormone: development of the first biotechnology product for the treatment of infertility. *Hum Reprod Update.* **4**, 862-881.

Regan L, Owen EJ, Jacobs HS (1990) Hypersecretion of LH, infertility and miscarriage. *Lancet* **336**, 1141-1144.

Reik W, Dean W, Walter J (2001) Epigenetic reprogramming in mammalian development. *Science* **293**, 1089-93.

Reynaud K, Driancourt MA (2000) Oocyte attrition. *Mol Cell Endocrinol* **163**, 101-108.

Reynolds H, Nathan P, Srivastava LS, Hess EV (1982) Release of estradiol from fetal bovine serum by rat thymus, spleen, kidney, lung and lung macrophage cultures. *Endo* **110**, 2213-2215.

Richards JS (1980) Maturation of ovarian follicles: actions and interactions of pituitary and ovarian hormones on follicular cell differentiation. *Phys Rev* **60**, 51-89.

Richards JS (1994) Hormonal control of gene expression in the ovary. *Endocr Rev* **15**, 725-751.

Richards JS (2001) The ovarian follicle - a perspective in 2001. *Endo* **142**, 2184-2193.

Risma KA, Hirshfield AN, Nilson JH. Elevated luteinizing hormone in prepubertal transgenic mice cause hyperandrogenemia, precocious puberty and substantial ovarian pathology. *Endocrinol* **138,** 3540-3547

Risma KA, Clay CM, Nett TM, Wagner T, Yun J, and Nilson JH (1995) Targeted over-expression of LH in transgenic mice leads to infertility, polycystic ovaries and ovarian tumours. *Proc Nat Acad Sci U.S.A.* **92,** 1332-1326.

Rivier C, Vale W (1991) Effect of recombinant activin-A on gonadotropin secretion in the female rat *Endocrinol* **129,** 2463-2465

Roberts AJ, Skinner MK (1990) Estrogen regulation of thecal cell steroidogenesis and differentiation: thecal cell-granulosa cell interactions. *Endo* **127**, 2918.

Robertson DM, Hertan R, Farnworth PG (2000) Is the action of inhibin mediated via a unique receptor? *Rev Reprod* **5**, 131-135

Robertson DMP, Klein FI, deVos RI, McLachlan RE, Wettenhall MTW, Hearn H, Burger DM, de Kreste D (1987) The isolation of polypeptides with FSH supressing activity from bovine follicular fluid which are structurally different to inhibin. *Biochem Biophys Res Commun* **149,** 744-749

Robker RL, Richards JS (1998) Hormonal control of the cell cycle in ovarian cells: proliferation versus differentiation. *Biol Reprod* **59**, 476-482.

Robker RL, Russell DL, Yoshioka S, Sharma SC, Lydon JP, O'Malley BW, Espey LL, Richards JS (2000) Ovulation: a multi-gene, multi-step process. *Steroids* **65**, 559-570.

Rodgers HF, Irvine CM, Van Wezel IL, Lavranos TC, Luck MR, Sado Y, Ninomiya Y, Rodgers RJ (1998) Distribution of the alpha1 to alpha6 chains of type IV collagen in bovine follicles. *Biol Reprod* **59**, 1334-1341.

Rodgers HF, Lavranos TC, Vella CA, Rodgers RJ (1995) Basal lamina and other extracellular matrix produced by bovine granulosa cells in anchorage-independent culture. *Tissue Res.* **282**, 463-471.

Rodgers RJ, Lavranos TC, Van Wezel IL, Irving-Rodgers HF (1999) Development of the ovarian follicular epithelium. *Mol Cell Endocrinol* **151**, 171-179.

Rose UM, Hanssen RG, Kloosterboer HJ (1999) Development and characterization of an in vitro ovulation model using mouse ovarian follicles. *Biol Reprod* **61**, 503-511.

Rosenfeld CS, Roberts RM, Lubahn DB (2001) Estrogen receptor- and aromatase-deficient mice provide insight into the roles of estrogen within the ovary and uterus. *Mol Rep Develop* **59**, 336-46.

Rosenfeld CS, Wagner JS, Roberts RM, Lubahn DB (2001) Intraovarian actions of oestrogen. *Reprod* **122**, 215-26.

Rosselli M, Reinhart K, Imthurn B, Keller PJ, Dubey RK (2000) Cellular and biochemical mechanisms by which environmental oestrogens influence reproductive function. *Hum.Reprod.Update.* **6**, 332-350.

Rothchild I, In Bardin CW, Milgrom E, Mauvais-Jarvis P (eds) Progesterone and Progestins. New York, Raven Press, 1983

Russell JB, Knezevich KM, Fabian KF, Dickson JA (1997) Unstimulated immature oocyte retrieval: early versus midfollicular endometrial priming. *Fert Steril* **67**, 616-620.

Salustri A, Yanagishita M, Hascall VC (1990) Mouse oocytes regulate hyaluronic acid synthesis and mucification by FSH-stimulated cumulus cells. *Dev Biol* **138**, 26-32.

Samborskaia EP, Ferdman TD (1966) Mechanism of abortion induced by ascorbic acid. *Biulletin Eksperimetalnoi Biologii I Mediitsny* **62**, 96-98.

Sanyal S, Datta S (1979) Ascorbic acid depleting action of PGE1 in rat adrenal and ovary and the effect of cycloheximide and actinomycin D treatment. *Indian J Med.Res* **70**, 674-677.

Sar M, Welsch F (1999) Differential expression of estrogen receptor-beta and estrogen receptor-alpha in the rat ovary. *Endo* **140**, 963-971.

Saunders PT (1998) Oestrogen receptor beta (ER beta). *Rev.Reprod* **3**, 164-171.

Saunders PT, Maguire SM, Gaughan J, Millar MR (1997) Expression of oestrogen receptor beta (ER beta) in multiple rat tissues visualised by immunohistochemistry. *J Endocrinol* **154**, 13-16.

Saunders PT, Millar MR, Williams K, Macpherson S, Harkiss D, Anderson RA, Orr B, Groome NP, Scobie G, Fraser HM (2000) Differential expression of estrogen receptor-alpha and -beta and androgen receptor in the ovaries of marmosets and humans. *Biol Reprod* **63**, 1098-1105.

Schams D and Berisha B (2002) Steroids as local regulators of ovarian activity in domestic animals. *Domestic Animal Endocrinology* **23:** 53-65

Sharma SC, Clemens JW, Pisarska MD and Richards JS (1999) Expression and function of estrogen receptor subtypes in granulosa cells: regulation by estradiol and forskolin. *Endocrinology* **140:** 4320-4334

Schatten H, Schatten G, Mazia D, Balczon R, Simerly C (1986) Behavior of centrosomes during fertilization and cell division in mouse oocytes and in sea urchin eggs. *Proc Natl Acad Sci USA,* **83, 105-109**

Schatten G, Simerly C, Schatten H (1985) Microtubule configurations during fertilization, mitosis, and early development in the mouse and the requirement for egg microtubule-mediated motility during mammalian fertilization. Proc Natl Acad Sci USA **82,**4152-6

Schomberg DW, Couse JF, Mukherjee A, Lubahn DB, Sar M, Mayo KE, Korach KS (1999) Targeted disruption of the estrogen receptor-alpha gene in female mice: characterization of ovarian responses and phenotype in the adult. *Endo* **140**, 2733-2744.

Sharma SC, Clemens JW, Pisarska MD, Richards JS (1999) Expression and function of estrogen receptor subtypes in granulosa cells: regulation by estradiol and forskolin. *Endoy* **140**, 4320-4334.

Shoham Z, Howles CM, Zalel Y, Weissman A, Insler V (1994) Induction of follicular growth and production of a normal hormonal milieu in spite of using a constant low dose of luteinizing hormone in women with hypogonadotrophic hypogonadism. *Hum Reprod* **9**, 431-436.

Shoham Z, Jacobs HS, Insler V (1993) LH increase leads to infertility and increased miscarriages. *Fert Steril* **58**, 1153-1161.

Schoot BCJM, Harlin J, Shoham Z, Mannaerts BMJL, Lahlou N, Bouchard P, Coelingh HJTB, Fauser BCJM (1994) Recombinant human follicle-stimulating hormone and ovarian response in gonadotrophin deficient women. *Hum Reprod* **9,**1237-1242

Schultz RM Preimplantaion development (1999). In, Fauser BCJM, Rutherford AJ, Strauss JF, Van Streirteghem A (eds). Molecular biology in reproductive medicine. Parthenon Publishing, London

Schultz RM Regulation of zygotic gene activation in the mouse (1993) *Bioessays* **15,** 531-538

Sicinski P, Donaher JL, Geng Y, Parker SB, Gardner H, Park MY, Robker RL, Richards JS, McGinnis LK, Biggers JD, Eppig JJ, Bronson RT, Elledge SJ, Weinberg RA (1996) Cyclin D2 is an FSH-responsive gene involved in gonadal cell proliferation and oncogenesis. *Nature* **5**,470-474

Solovyeva EV, Hayashi M, Margi K, Barkrats C, Klein C, Amsterdam A, Hsueh AJ, Tsafiri A (2000) Growth differentiation factor-9 stimulates rat thecal-interstitial biosynthesis. *Biol Reprod.* **63,** 1214-1218

Sills ES, Levy DP, Moomjy M, McGee M, Rosenwaks Z (1999) A prospective, randomized comparison of ovulation induction using highly purified follicle-stimulating hormone alone and with recombinant human luteinizing hormone in in-vitro fertilization. *Hum Reprod* **14**, 2230-2235.

Simon AM, Goodenough PA, Li E, Paul DL, Goodenough DA (1997) Female infertility in mice lacking connexin 37. *Nature* **385**, 525-529.

Simoni M, Gromoll J, Nieschlag E (1997) The follicle-stimulating hormone receptor: biochemistry, molecular biology, physiology and pathophysiology. *Endocr Rev* **18**, 739-773.

Smith MF, McIntush EW, Ricke WA, Kojima FN, Smith GW (1999) Regulation of ovarian extracellular matrix remodelling by metalloproteinases and their tissue inhibitors: effects on follicular development, ovulation and luteal function. *J Reprod Fertil Suppl* **54**, 367-381.

Smyth CD, Miro F, Whitelaw PF, Howles CM, Hillier SG (1993) Ovarian thecal/intestitial androgen synthesis is enhanced by a *FSH*-stimulated paracrine mechanism. *Endo* **133**, 1532-1538.

Snow MHL, Monk M (1983) Emergence and migration of moise primordial germ cells. In McLaren A, Wylie Rc (eds) Current problems in germ cell differentiation. University Press, Cambridge.

Sorensen RA, Wassarmen DM (1976) Relationship between growth and meiotic maturation of the mouse oocyte. *Develop Biol* **50**, 531-536.

Spears N, Boland NI, Murray AA, Gosden RG (1994) Mouse oocytes derived from in vitro grown primary ovarian follicles are fertile. *Hum Reprod* **9**, 527-532.

Spears N, Murray AA, Allison V, Boland NI, Gosden RG (1998) Role of gonadotrophins and ovarian steroids in the development of mouse follicles in vitro. *J Reprod Fert* **113**, 19-26.

Spears N, Baker S, Srsen V, Lapping R, Mullan J, Nelson R, Allison V (2002) Mouse ovarian follicles secrete factors affecting the growth and development of like-sized follicles *in vitro*. *Biol Reprod* **67,** 1726-1733

Stansfield DA, Flint AP (1967) The entry of ascorbic acid into the corpus luteum in vivo and in vitro and the effect of luteinizing hormone. *J Endocrinol* **39**, 27-35.

Surani MA, Barton SC, Norris ML (1984) Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. *Nature* **308**, 548-550.

Swann K (1996) Soluble sperm factors and Ca2+ release in eggs at fertilization. *Rev.Reprod* **1**, 33-39.

Sugino H, Sugino K, Nakamura T (1994) the activin binding protein. In Burger H, Findlay J, Robertsin D, deKrester D, Petraglia F (eds) *Frontiers in Endocrinology Vol.3* Inhibin and Inhibin Related Proteins. Ares Serono Symposia

Tam PP, Gad JM, Kinder SJ, Tsang TE, Behringer RR (2001) Morphogenetic tissue movement and the establishment of body plan during development from blastocyst to gastrula in the mouse. *BioEssays* **23**, 508-17.

Tesarik J, Mendoza C (1995) Nongenomic effects of 17 beta-estradiol on maturing human oocytes: relationship to oocyte developmental potential. *J Clin Endocrinol Metab* **80**, 1438-1443.

Tesarik J, Mendoza C (1997) Direct non-genomic effects of follicular steroids on maturing human oocytes: Oestrogen versus androgen antagonism. *Hum Reprod Update.* **3**, 95-100.

Tetsuka M, Hillier SG (1996) Androgen receptor gene expression in rat granulosa cells: The role of FSH and steroid hormones. *Endo* **137**, 4392-4397.

Tetsuka M, Hillier SG (1997) Differential regulation of aromatase and androgen receptor in granulosa cells. *J Steroid Biochem Mol Biol* **61**, 233-239.

Thomas A, Okamoto S, O'Shea F, MacLachlan V, Besonku M, Healy D (1989) Do raised serum LH levels during stimulation for IVF predict outcome? *Brit J Obs Gyna* **97**, 654-656.

Tilly JL, Tilly KI (1995) Inhibitors of oxidative stress mimic the ability of FSH to suppress apoptosis in cultured rat ovarian follicles. *Endo* **136**, 242-252.

Toledo SP, Brunner HG, Kraaij R, Post M, Dahia PL, Hayashida CY, Kremer H Themmen AP(1996) An inactivating mutation of the luteinizing hormone receptor causes amenorrhea in a 46,XX female *J Clin Endocrinol Metab* **81**,3850-3854

Tora L, White J, Brou C, Tasset D, Webster N, Scheer E, Chambon P (1989) The human oestrogen receptor has two independent nonacidic activation functions. *Cell* **59,** 477-487

Tsuji M, Ito Y, Terada N, Mori H (1989) Ovarian aromatase activity in scorbutic mutant rats unable to synthesize ascorbic acid. *Acta Endocrinol (Copenh)* **121**, 595-602.

Ueneo N, Ling N, Ying S-Y, Esch F, Shimaske S, Guillemin R (1987) Isolation and partial characterisation of follistatin, a novel Mr 35000 monomeric protein which inhibits the release of follicle-stimulating hormone. *Proc Natl Acad Sci USA* **84,** 8282-8286

van Cappellen WA, Meijs-Roelofs HMA, Kremer P, van Leeuwen ECM, de Leeuw R, de Jong FH (1995) Recombinant FSH (Org 32489) induces follicle growth and ovulation in the adult cyclic rat. *J Endo* **144**, 39-47.

Vanderhyden BC, MacDonald EA (1998) Mouse oocytes regulate granulosa cell steroidogenesis throughout follicular development. *Biol Reprod* **59**, 1296-1301.

Vale N, Rivier J, Vaughan J, McLintock R, Corrigan A, Woo W, Karr D, Spress J (1986) Purification and characterization of an FSH releaising protein from porcine ovarian follicular fluid. *Nature* **321,** 776-779

Vassali A, Matzuk MM, Gardner HAR, Lee K-F, Jaenisch R (1994) Activin/inhibin βB subunit gene disruption leads to defects in eyelid development and female reproduction. *Genes and Development* **8,** 414-427.

Vendola K, Zhou J, Wang J, A Bondy C (1999) Androgens promote insulin-like growth factor-I and insulin-like growth factor-I receptor gene expression in the primate ovary. *Hum Reprod* **14**, 2328-2332.

Vendola K, Zhou J, Wang J, Famuyiwa OA, Bievre M, Bondy CA (1999) Androgens promote oocyte insulin-like growth factor I expression and initiation of follicle development in the primate ovary. *Biol Reprod* **61**, 353-357.

Wandji SA, Srsen V, Voss AK, Eppig JJ, Fortune JE (1996) Initiation *in vitro* of growth of bovine primodial follicles. *Biol Reprod* **55**, 942-948.

Wang XN, Greenwald GS (1993) Human chorionic gonadotrophin or human recombinant follicle-stimulatin hormone (FSH)-induced ovulation and subsequent fertilization and early eambryo development in hypophysectomised FSH-primed mice *Endocrinol* **13,** 2009-2016

Warne D, Hughes J-N, Cedrin Durnerin L, Decosterd G, Loumaye E (2000) The efficacy and safety of recombinant versus-urinary-human chorionic gonadotrophins in ovulation induction; results of a double blind clinical study. *Program of the 15th Annual meeting of the European Society of Human Reproduction and Embryology*. p46

Wassarman PM (1988) The Mammalian Ovum. In 'The Physiology of Reproduction'. (Eds E Knobil and J Neill) pp 69-102. Raven Press, Ltd.: New York.

Wassarman PM (1999) Zona pellucida glucoprotein MZP3: a versatile player during mammalian fertilization. *J Reprod Fert* **116**, 211-216.

Wassarman PM, Jovine L, Litscher ES (2001) A profile of fertilization in mammals. *Nat Cell Biol* **3**, 59-64.

Watson AJ, Barcroft LC (2001) Regulation of blastocyst formation. *Front Biosci.* **6**, 708-730.

Weil S, Vendola K, Zhou J, Bondy CA (1999) Androgen and follicle-stimulating hormone interactions in primate ovarian follicle development. *J.Clin Endocrinol.Metab.* **84**, 2951-2956.

Weston AM, Zelinski-Wooten MB, Hutchison JS, Stouffer RL, Wolf DP (1996) Developmental potential of embryos produced by IVF from GnRH antagonist- treated macaques stimulated with rhFSH alone or in combination with LH. *Hum Reprod* **11**, 608-613.

Williams PC (1940) Effect of stieboestrol on the ovaries of hypophysectomized rats. *Nature* **145**, 388-389.

Whitelaw PF, Smyth CD, Howles CM, Hillier SG (1992) Cell-specific expression of aromatase and LH receptor mRNAs in rat ovary. *J Mol Endocrinol* **9,** 309-312

Wu J, Nayudu PL, Kiesel PS, Michelmann HW (2000) Luteinizing hormone has a stage-limited effect on preantral follicle development in vitro. *Biol of Reprod* **63**, 320-327.

Wu TC, Jih MH, Wang L, Wan YJ (1994) Expression of activin receptor11 and 11B mRNA isoforms in mouse reproductive organs and oocytes *Mold Reprod Dev* **38,** 9-15

Wu TC, Wang L, Wan YJ (1992) Expression of estrogen receptor gene in mouse oocyte and during embryogenesis. *Mol. Reprod. Dev.*, **33:** 407-412.

Wu T-CJ, Wang L, Won Y-JY (1993) Detection of estrogen receptor mRNA in human oocytes and cumulus oocyte complexes using RT-PCR. *Fert Steril* **59**, 54-59.

Xia P, Younglai EV (2000) Relationship between steroid concentrations in ovarian follicular fluid and oocyte morphology in patients undergoing intracytoplasmic sperm injection (ICSI) treatment. *J Reprod Fertil* **118**, 229-233.

Xiao S, Robertson DM, Findlay JK (1992) Effects of activin on follicle-stimulating hormone (FSH) –suppressing protein /follisatatin on FSH receptors and differentiation of cultured rat granulosa cells. *Endocrinol* **131,** 1009-1016

Yanagimachi R (1978) Calcium requirment for sperm-egg fusion in mammals. *Biol Reprod* **19**, 949-95

Yang P, Kriatchko A and Roy SK (2002) Expression of ER-alpha and ER-beta in the hamster ovary: differential regulation by gonadotropins and ovarian steroid hormones. *Endocrinology* **143:** 2385-23988.

Yong EL, Hillier SG, Turner M, Baird DT, Ng SC, Bongso A, Ratnam SS (1994) Differential regulation of cholesterol side-chain cleavage (P450scc) and aromatase (P450arom) enzyme mRNA expression by gonadotrophins and cyclic AMP in human granulosa cells. *J Mol Endocrinol* **12**, 239-249.

Yong EL, Turner M, Baird DT, Hillier SG (1992) Molecular basis of gonadotrophin action on human granulosa cell function. *Ann Acad Med Singapore* **21,**510-6

Zafeirous S, Loutradis D, Michalas S (2000) The role for gonadotrophins in follicular development and their use in ovulation induction protocols for assisted contraception. *Euro J Contracept Reprod Health Care* **5,** 157-167

Zelinski-Wooten MB, Hess DL, Wolf DP, Stouffer RL (1994) Steroid reduction during ovarian stimulation impairs oocyte fertilization but not folliculogenesis, in rhesus monkeys. *Fert Steril* **61**, 1147-1155.

Zeleznik AJ, Hillier SG (1984) The role of gonadotropins in the selection of the preovulatory follicle *Clin. Obst. Gynecol.* **27,** 927-940

Zeleznik AJ, Hillier SG (1996) The Ovary: Endocrine function. In Hillier SG, Kitchener HC, Neilson JP (eds) Scientific Essentials of Reproductive Medicine. WB Saunders Company Ltd, London. 1996

Zernicka-Goetz M, Ciemerych MA, Kubiak JZ, Tarkowski AK, Maro B (1995) Cytostatic factor inactivation is induced by a calcium-dependent mechanism present until the second cell cycle in fertilized but not in parthenogenetically activated mouse eggs. *J Cell Sci* **108**,469-74

Zhang FP, Poutanen M, Wilbertz J, Huhtaniemi I (2001) Normal prenatal but arrested postnatal sexual development of luteinizing hormone receptor knockout (LuRKO) mice *Mol Endocrinol* **15,**172-83

Zhao Y, Luck MR (1995) Gene expression and protein distribution of collagen, fibronectin and laminin in bovine follicles and corpora lutea. *J Reprod Fert* **104**, 115-123

Ziomek CA, Johnson MH (1980) Cell surface interaction induces polarization of mouse eight cell blastomeres at compaction *Cell* **21,** 935-942

Appendix A. Suppliers Addresses

All chemicals used during this thesis investigation from **Sigma-Aldrich, Merck Ltd** or **Boehringer Mannheim Lewes, U.K**.

Accumedic Ltd London, UK.

Astecair Weston- Super-Mare, UK.

Alpha Innotech Corp. San Leando, CA. U.S.A

Becton Dickinson and Co. New Jersey USA.

BDH Supplies see Merck Ltd.

Bibby Sterilin Ltd Aberbargoed, UK

Bio-Rad Hemel Hempstead, UK

Boehringer Mannheim see Roche

Fisher Scientific UK Loughborough, UK

Flowgen Stafford, UK

Forma Scientific Marietta, OH, USA

Intervet Milton Keynes, UK

Invitrogen Renfrew, UK

Iwaki see Bibby Sterilin Ltd

Leica UK Ltd. Milton Keynes, UK

Linkam Tadworth, UK

Merck Lutterworth, UK

MJ Research Mass, USA

Molecular Probes Ltd. Eugene, OR, USA

Nikon Tokyo, Japan

Oswel Southampton, UK

Pierce Rockford, IL. U.S.A

Phoenix Pharmaceuticals Ltd Gloucester, UK

Promega Southampton, UK

Quiagen Crawley, UK

Reichart-Jung see Leica UK Ltd.

Roche Lewes, UK

Roebling Berlin, Germany

Sigma-Aldrich Company Ltd. Poole, UK

Sigma St. Louis. MO. U.S.A

Sherwood-Davis Gosport, UK

TAAB Laboratories Ltd. Aldermaston, UK

University Technologies International Inc Calgary, Alberta, Canada

Vector Laboratories Burlingame, CA, USA

Zeiss (Carl Zeiss Ltd) Hertfordshire, UK

Appendix B. IVF media

T6 Stock Solutions

Stock A (100ml)

Sodium Chloride	5.719g
Pottasium Chloride	0.106g
Magnesium Chloride hexahydrate	0.096g
diSodium Hydrogen Phosphate	0.129g
Sodium Lactate (60% syrup)	4.652g
Glucose	1.0g

Stock B (100ml)

Sodium Hydrogen Carbonate	2.101g
Phenol Red	0.01g

Stock C (10ml)

Sodium Pyruvate	0.055g

Stock D (10ml)

Calcium Chloride dihydrate	0.262g

KSOM Stock solutions

Stock A (100ml)

Sodium Chloride	5.55g
Potassium Chloride	0.186g
Potassium diHydrogen Phosphate	0.048g
Magnesium Sulphate heptahydrate	0.049g
Sodium Lactate (60% syrup)	1.869g
Glucose	0.036g
EDTA	0.004g

Stock B (100ml)

Sodium Hydrogen Carbonate	2.101g
Phenol red	0.01g

Stock C (10ml)

Sodium Pyruvate	0.022g

Stock D (10ml)

Calcium Chloride dihydrate	0.146g

Appendix C. PCR Protocols

Tail Tip Lysis Buffer

Tris pH 8	100mM
Sodium Chloride	20mM
EDTA pH 8	5mM
SDS	0.2%

TBE Buffer pH 8.3

Tris	10.9g
Boric Acid	5.5g
EDTA	0.93g
ddH2O	1000ml

PCR Screening for ERα gene

PCR Reaction mixture

ddH_2O (MilliQ-autoclaved)	10.875 µl
10x PCR buffer (Invitrogen)	2.5 µl
25mM $MgCl_2$ (Sigma)	1.5 µl
DMSO (Sigma)	2.5 µl
5M Betaine (Sigma)	5.0 µl
dNTPs (Promega, 1:1:1:1)	0.25 µl
100mM Spermidine (Sigma)	0.25 µl
Platinum Taq (Invitrogen)	0.125 µl
Primer Mix (Oswel)	1 µl
DNA sample	1 µl

Primer Sequences

5' CTACGGCCAGTCGGGCAT	(WT)
5' AGACCTGTAGAAGGCGGGAG	(WT)
5' TGAATGAACTGCAGGACGAG	(ERKO)
5' AATATCACGGGTAGCCAACG	(ERKO)

The individual primers (100µm) were mixed at a ratio of 1:1:1:1 and stored frozen at

–20°C.

PCR Parameters

1.Preheat @ 94°C for 1 minute,
2. Melt @ 94°C for 40 sec
3. Anneal @ 60°C for 40sec
4. Extend @ 72°C for 1.5 min.
5. Steps 2-4 34 more times
6. Extend 72°C for 5 min.

PCR screening for *hpg* mutation

PCR reaction mixture

ddH_2O (MilliQ-autoclaved)	12.2 µl
10x PCR buffer (Invitrogen)	2.5 µl
50mM $MgCl_2$ (Sigma)	1.5 µl
dNTPs (Promega, 1:1:1:1)	0.2 µl
Platinum Taq (Invitrogen)	0.1 µl
Primer Mix (Oswel)	1.0 µl
DNA sample	7.5 µl

Primer Sequences

5' CACATCTGTAGCCACAGTCC	(WT)
5' AGCTGGGAGGCTGCTGTCACTGG	(*hpg* deletion)
5' GCTTGGAGAGCTGTAAGGTC	(WT)

The individual primers (100µm) were mixed at a ratio of 2:1:2 and stored frozen at

–20°C.

PCR parameters

1.Preheat @ 92°C for 4min 45sec
2. Melt @ 94°C for 15 sec
3. Anneal @ 60°C for 15 sec
4. Extend @ 72°C for 1 min.
5. Steps 2-4 19 more times

Gel Loading Buffer

Glycerol (Sigma)	5ml
0.5M EDTA (Sigma)	40µl
Orange G Dye (BDH)	few grains
ddH_2O	10ml

Ethidium Bromide (Gel Visualisation)

Ethidium Bromide (Sigma) 5mg/ml

Appendix D. Published work

The effect of elevated androgens and oestrogens on follicular development and oocyte competence *in vitro*.

A. A. Murray, R. Smith, V. Srsen, S.G. Hillier[1] and N. Spears. Dept. of Biomed. Sc., [1] Dept. Repro. and Dev., University of Edinburgh, George Square, Edinburgh. EH8 9XD

During late stages of follicular development the co-ordinated actions of FSH and LH produce androgen and oestrogen. The precise effects of these steroids on follicular development and subsequent oocyte maturity have still to be established. Exogenous administration of gonadotrophins e.g. in assisted reproductive technologies, may lead to intra-follicular steroid concentrations being perturbed resulting in poor oocyte quality. We have been investigating the effects of androgen and oestrogen on follicular growth and oocyte competence using the mouse as a model. Individual pre-antral follicles were dissected from the ovaries of pre-pubertal mice and placed in 96 U-well plates. Media was supplemented with serum (from hypogonadal mice) and recombinant FSH. In further treatments the level of intrafollicular androgen, oestrogen or both was elevated by supplementing media with an aromatase inhibitor (Arimidex: to increase androgen), diethyly stilbestrol (DES: to increase oestrogen), or in combination (increasing androgen and oestrogen). At the end of the culture period, oocytes underwent IVF. Follicular growth was unaffected in any group and TUNEL staining indicated there was no increase in follicular apoptosis across treatments. Fertilisation rates were similar in oocytes obtained from follicles grown in control media (69%), or when oestrogen or androgen was elevated (65% and 67% respectively). However fertilisation rate was reduced in oocytes obtained from follicles exposed to both elevated androgen and oestrogen (44%). Furthermore within the latter group no fertilised oocytes completed development to the blastocyst stage, whereas blastocyts were observed in all other groups. We conclude that oocytes require exposure to a specific pattern of androgens and oestrogens to undergo optimal maturation.

The Effect of Alpha Oestrogen Receptor Deletion on Ovarian Function

A. A. Murray, D. Edwards, R. E. Smith, C.S. Rosenfeld, D.B. Lubahn, S.G. Hillier and N. Spears

An intraovarian role for oestrogen remains controversial. Some studies have demonstrated that oestrogen positively effects follicular development and prevents atresia. Others have concluded that any autocrine/paracrine effects are not obligatory and that the ovary can function when the actions of this steroid are blocked.

Until recently it was thought that oestrogen exerted its effects via a single receptor (now termed ERα). However, a second receptor (ERβ) has now been discovered. Both ER isoforms are expressed within mammalian ovaries although their distribution patterns appear to be different. Transgenic mice have now been generated which carry deletions in either one or both of these receptor types (ERKO, BERKO and αβERKO respectively), providing a unique opportunity to distinguish their intraovarian functions.

The ERKO mouse has a distinct ovarian phenotype. Adult ovaries develop haemorrhagic cysts resulting in anovulation and infertility. While this may suggest a direct role for this receptor in ovarian function, one consequence of ER□deletion is perturbed steroidal and gonadotrophin secretory profiles and it is possible that these alterations give rise to the ovarian phenotype indirectly.

The aim of this study was to examine more directly the effects of ERKO on ovarian function in pre-pupertal mice. We specifically set out to investigate follicular development, ovulatory capability and determine whether resultant oocytes could complete pre-implantation development.

Using an *in vitro* culture system that permits the development of follicles from the pre antral to antral stages, we found that, when compared to their wild type counterparts, follicles isolated from ERKO mice grew to similar sizes and reached similar stages of development. Treatment with gonadotrophins *in vivo* induced ERKO prepubertal animals to ovulate but with less success than wild type mice. When superovulated oocytes were fertilised *in vitro* and cultured to the blastocyst stage, there were no significant differences between wild type and ERKO oocytes with respect to rates of either fertilisation or blastocyct development. Scrutiny of the blastocysts for apoptosis using TUNEL labelling and confocal microscopy revealed no differences in either the total cell numbers or the proportion of apoptotic cells.

From these results we conclude that follicles can grow in the absence of ERα and that the actions of this receptor are not essential to the ovulatory process. However as fewer oocytes were obtained from ERKO mice, it may have an auxiliary secondary role in promoting ovulation or the number of follicles available to ovulate. Finally absence of ERα mediated action during oocyte growth and appears to be inconsequential at least until the implantation stage of development.

ASCORBIC ACID PROMOTES FOLLICLE INTEGRITY AND SURVIVAL IN INTACT MURINE OVARIAN FOLLICLES *IN VITRO*

Alison A Murray, MD Molinek, SJ Baker, SG Hillier & N Spears

Ascorbic acid (Vitamin C) is a dietary requirement for primates, guinea pigs and a few other species. A range of body tissues accumulate ascorbic acid with high concentrations being observed in the gonads. Within the ovary, ascorbic acid has been localised to the theca interna, granulosa cells and the corpus luteum with changes of concentration observed throughout the oestrus cycle. There appear to be three main roles for this vitamin within the ovary: prevention of free radical damage regulating biosynthesis of steroid hormones, and stimulating biosynthesis of collagen. While there is much information on the role of ascorbic acid during corpus luteum formation, little is known about its role during follicular growth and development. We have been investigating this using an isolated follicle culture system. The culture system allows the growth of pre-antral follicles through to Graafian stages and the oocytes obtained can be fertilised and live young produced (Spears et al,1994). It closely mimics growth in vivo and is therefore a highly physiological model. Individual pre-antral follicles were isolated from 3-week old mice and cultured in 96 well plates. a -MEM media was supplemented with 5% serum and 1 IU/ml rh FSH. A further two groups of follicles were cultured in the same media but with the addition of 5 or 50 μg/ml ascorbic acid. Follicles were examined for integrity, measured and moved to fresh media daily, for 5 days. Spent culture media were analysed for oestradiol. In a second experiment, follicles were isolated and cultured as before, but in the absence of serum, with or without ascorbic acid. At the end of a 24-hr culture period, genomic DNA was extracted from the follicles, DIG labelled and analysed for laddering using agarose electrophoresis and Southern blotting. Our results show that addition of ascorbic acid to culture media increased the percentage of follicles maintaining their integrity throughout the culture period in a dose dependent manner. However the growth rate of intact follicles was unaffected by the presence or absence of ascorbic acid. Oestradiol production was decreased by the addition of ascorbic acid to the media. Analysis of DNA laddering (as an indication of apoptosis) showed that ascorbic acid markedly decreased fragmentation and therefore promotes the survival of cultured ovarian follicles.

PRODUCTION OF INHIBIN A, INHIBIN B AND ACTIVIN A BY MOUSE FOLLICLES IN CULTURE

Shanthi Muttukrishna, AA Murray, J Asselin, M Molinek, WL Ledger, NP Groome & Norah Spears

Inhibin and activin are ovarian glycoprotein hormones that modulate pituitary FSH production, whilst pituitary gonadotrophins control the production of these hormones by the ovary. We have used whole mouse follicle cultures as a model to study factors controlling follicular inhibin and activin production, as these cultures mimic follicle development *in vivo*. This study investigated the effects of FSH and LH on the production of inhibins and activin A by cultured mouse follicles. Preantral follicles were dissected from the ovaries of 3 week old mice and cultured over a 6-day period. The follicles were cultured in 96 well plates in alpha MEM medium and 5% serum. Serum was obtained from mutant hypogonadal (mhpg) mice, which have no measurable circulating gonadotrophins. Individual follicles were cultured in gonadotrophin free media or in the presence of 1 IU rFSH/ml. This latter group was further subdivided and LH was added to some of the cultures at 0.01IU/ml or 0.05IU/ml. On day 2 of culture the follicles were placed in fresh media and 50% of the media was exchanged on days 4 and 6. Spent media was pooled and stored frozen until assayed. This data shows that inhibin A, inhibin B and activin A are detectable in growing mouse follicle culture media and levels of hormones rise as follicles mature *in vitro*. Levels of inhibins and activin A were undetectable in control media. Concentrations of inhibins and activin A increased with time in the gonadotrophin free culture medium although levels remained low. Addition of FSH stimulated secretion of both inhibin A and B. The addition of LH to the media with FSH did not elevate production of either inhibin A or B further. Activin A was detectable in all experimental groups. However, the addition of gonadotrophins had little effect on the concentrations of activin A. Our preliminary results suggests that while inhibins are under the control of FSH action, activin secretion is gonadotrophin independent.

EFFECT OF GONADOTROPHINS DURING FOLLICULAR GROWTH ON OOCYTE VIABILITY AND SUBSEQUENT DEVELOPMENT

Alison A. Murray, Michael Molinek, S.G. Hillier and Norah Spears.

Many assisted reproductive technologies (such as *in vitro* fertilisation, or IVF) require the administration of exogenous gonadotrophins (FSH and LH) as a means of collecting multiple oocytes. Clinical reports have suggested that inappropriate LH stimulation may be detrimental to pregnancy rates, although how this effect is mediated is unclear. We have been investigating the effects of gonadotrophin stimulation on follicular growth and oocyte viability, using the mouse as a model.

Individual pre-antral follicles were dissected from ovaries of pre-pubertal mice. Follicles were placed into individual wells of 96 U-well plates. Media were supplemented with recombinant FSH and mouse serum: serum was obtained from hypogonadal animals which have no measurable circulating gonadotrophins. In two further treatments, media were also supplemented with either low or high concentrations LH. Follicles were measured and moved daily. At the end of the culture period, oocytes were matured and IVF was carried out. Oocytes from superovulated animals were used as IVF controls. Follicles can grow to pre-ovulatory sizes in the presence of FSH alone (Spears *et al.* 1998). We show here that follicular development was unaffected by either low or high LH concentration. Oocytes obtained from follicles grown in FSH alone or with a low concentration of LH fertilised and developed to blastocyst at similar rates. However fewer oocytes obtained from follicles grown in the presence of high LH fertilised and subsequent development to the blastocyst stage was also retarded.

)GY OF REPRODUCTION 62, 599–605 (2000)

adotropin Induction of Ovulation and Corpus Luteum Formation in Young ɔgen Receptor-α Knockout Mice[1]

l S. Rosenfeld,[3] Alison A. Murray,[5] Gretchen Simmer,[4] Martha G. Hufford,[4] Michael F. Smith,[3] ı Spears,[5] and Dennis B. Lubahn[2,3,4]

tments of Animal Sciences[3] and Biochemistry and Child Health,[4] University of Missouri at Columbia, ıbia, Missouri 65211

tment of Biomedical Sciences,[5] University Medical School, Edinburgh EH8 9AG, United Kingdom

RACT

ogen receptor-α (ERα) knockout (ERαKO) female mice ertile. Initially, they exhibit normal follicular develop- ıut by 4–5 wk of age, they begin to develop hemorrhagic ı cysts. Follicles in adult ERαKO female mice progress to afian stage, but there are no corpora lutea (CL). To test ır ERα is required for ovarian folliculogenesis, ovulation, formation, eCG and hCG were used to ovulate 3- to 5- ERαKO and wild-type (WT) sibling mice. Gonadotropin stration resulted in ovulation in both ERαKO and WT Gonadotropin-treated ERαKO females that ovulated pro- 7.09 ± 0.77 oocytes per mouse, whereas gonadotropin- WT female mice had 16.17 ± 0.84 oocytes. Surprisingly, ed ERαKO ovarian follicles developed into CL that had morphology. Gonadotropin-treated ERαKO mice had 3- gher concentrations of serum progesterone than did con- αKO mice that had been administered saline rather than ıtropins. Thus, the CL in gonadotropin-treated ERαKO ppeared to be steroidogenically functional. On the basis e findings, ovarian folliculogenesis, ovulation, and CL for- can occur in the absence of ERα, although to a lesser than in WT mice.

ODUCTION

nulosa cells synthesize estrogen, which regulates hy- amic and pituitary function, but whether estrogen act- ı one or both of its known receptors has any direct within the ovary remains unsettled. Some reports ndicated that estrogen is locally required for normal n folliculogenesis [1–4], while others suggested no ı, 6].

dence supporting a direct intraovarian effect of estro- rough its cognate receptors has been based on hy- sectomy studies [7], ovarian follicle cultures [1, 2, 4], dministration of either estrogen receptor (ER) antag- [8, 9] or antiaromatase compounds [10, 11]. Estrogen en reported to modulate granulosa cell gap junction ion [7], steroidogenesis [12–15], FSH and LH recep- ɔression [16, 17], and ovarian follicular development t also inhibits granulosa cell apoptosis [19]. In rats ıd rabbits [21, 22], estrogen seems required for main- tenance and function of corpora lutea (CL), even in the absence of gonadotropins.

In contrast, other groups have shown that estrogen does not locally affect ovarian folliculogenesis [5, 6]. For example, addition of the antiestrogenic compound ICI 182,780 and/or antiestrogen antibodies to ovarian follicular cultures of late primary mouse ovarian follicles did not affect the growth and development of the follicles to the preovulatory stage [6].

Estrogen needs to bind to its cognate receptor to exert its effects. Currently, two estrogen receptors, ERα [23] and ERβ [24, 25], have been characterized. Estrogen receptor-α knockout (ERαKO) female mice are infertile, as a result of pubertal hemorrhagic ovarian cyst formation [3]. Women who have mutations of the aromatase gene [26] and mice that have targeted disruption of the aromatase gene, cyp19, are infertile, and no CL are present in cyp19-deleted mice [27, 28]. On the basis of naturally occurring human aromatase deficiency cases [26] and targeted gene-disrupted mice [3, 27, 28], it may be postulated that estrogen/ER is required for normal ovarian function. However, disruption of these genes may cause other systemic effects such as elevated serum concentrations of LH [27, 29] that hinder interpretation of the direct effects of estrogen within the ovary. To examine the ovarian function of ERαKO female mice, gonadotropins were used to ovulate prepubescent ERαKO and WT female mice.

MATERIALS AND METHODS

Animals and Genotyping

ERαKO and wild-type (WT) female mice of a mixed C57BL/6J/129 background were used according to institutional animal care protocols. They were housed at the University of Missouri Animal Sciences Research Center laboratory animal facility and maintained ad libitum on mouse chow formulation 5001 (Purina, St. Louis, MO) and water. They were on a 12D:12L cycle. The genotypes of the mice were determined on the basis of ERα polymerase chain reaction (PCR) analysis [3].

Examination of Ovaries from Adult ERαKO and WT Female Mice

Ovaries from nontreated 6- to 8-wk-old ERαKO (n = 25) and WT (n = 25) mice were fixed in Bouin's fixative (Sigma Chemical Co., St. Louis, MO) and histologically examined, as described below.

Gonadotropin Treatment of Young ERαKO and WT Female Mice

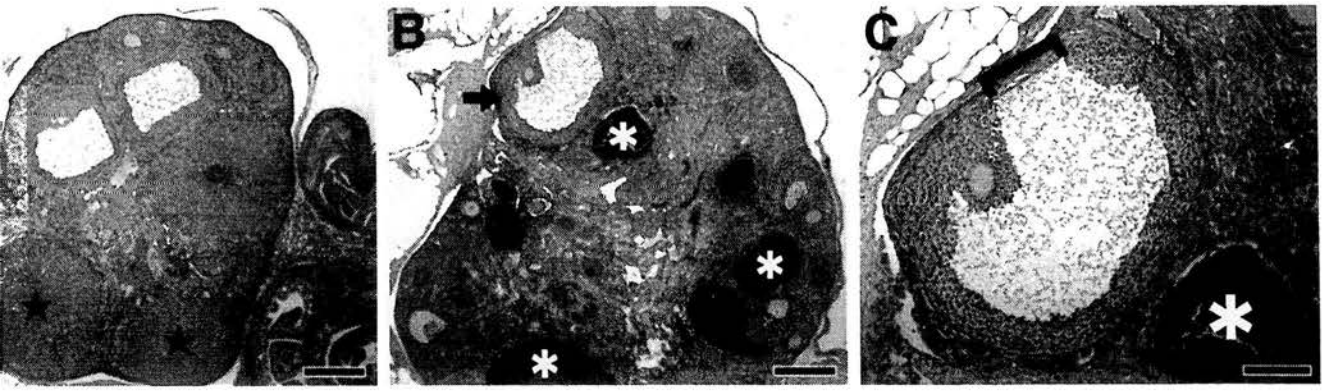
Three- to 5-wk-old ERαKO and WT female mice were given either 5 (Intervet, Cambridge, UK) or 10 IU of eCG

ɔrk was supported by a USDA National Needs Fellowship to C.S.R. his work was presented at the Serono Ovarian Workshop, Hous- 1998.

ɔondence: Dennis B. Lubahn, University of Missouri, 163 ASRC, t Campus Drive, Columbia, MO 65211. FAX: 573 882 6827; e- bahnd@missouri.edu

d: 17 May 1999.
ision: 28 June 1999.
d: 14 October 1999.

)06-3363. http://www.biolreprod.org



Histological examination of adult WT and ERαKO ovaries. **A**) Histological examination of normal adult WT ovaries revealed many CL (stars). ation bar = 500 μm. **B**) Ovaries from ERαKO female mice contained many hemorrhagic ovarian cysts (asterisks). However, occasional graafian ollicles (arrow) were present. Magnification bar = 500 μm. **C**) Higher magnification of graafian ovarian follicle from adult ERαKO ovary in **B** reveals that there was differentiation of granulosa cells with cumulus cells surrounding the ovary and follicular antral fluid in the central of the ovarian follicle. However, there was abnormal stratification of granulosa cells, with a portion of the follicle lined by a single layer of icketed area). Magnification bar = 200 μm.

Chemical Co.) i.p., followed 48–54 h later by 5 IU ; (Sigma Chemical Co.). Age-matched control WT RαKO female mice received 0.9% saline (Sigma :al Co.). Mice were anesthetized with CO_2 and killed vical dislocation. In order to recover ovulated oo- ovaries and oviducts from gonadotropin-treated WT .6) and ERαKO (n = 24) female mice 12 h post- ere placed in potassium simplex optimized medium M; Specialty Media, Phillipsburg, NJ) or M2 medium Chemical Co.) in the presence of 300 μg/ml of nidase (Sigma Chemical Co.). The ampullary region oviduct was examined under a Nikon SMZ stereo- cope (Nikon, Melville, NY), and the ovulated oo- vere counted. To further examine CL formation and progesterone concentrations in gonadotropin-treated O and WT mice, sera and ovaries from gonadotropin- line-treated WT and ERαKO female mice were col- 48 h post-hCG.

gy

ries and oviducts from gonadotropin- and saline- WT and ERαKO female mice were fixed in either s fixative (Sigma Chemical Co.) or 4% paraformal- : (w:v; Electron Microscopic Sciences, Fort Wash- , PA) and embedded in paraffin or glycomethacrylate ciences, Inc., Warrington, PA), respectively. Two- to thick sections were cut and stained with Gill's he- ylin (Fisher Scientific, St. Louis, MO) and eosin · Scientific). Ovaries and oviducts were photographed a Spot 2 digital camera (Diagnostic Instruments, Inc., g Heights, MI), and images were printed with a Fuji raphy 3000 printer (Fuji, Tokyo, Japan).

terone RIA

ncentrations of progesterone in serum were deter- with a Coat-a-Count progesterone kit (Diagnostics cts Corp., Los Angeles, CA), as previously described To validate the progesterone RIA for mice, serum ovariectomized WT and ERαKO mice were used as ve controls. Since progesterone has previously been to peak in mice at about Day 6 of pregnancy [31], from WT 6-day post-coitus mice were used as pos- ontrols. Serum from 3- to 5-wk-old WT and ERαKO mice three days after either hCG or 0.9% saline were assayed for serum progesterone concentrations. Undiluted and serial dilutions of 1:1, 1:2, and 1:4 of serum were used to determine parallelism. Radioactivity was measured by an LKB Wallac beta counter (Wallac Inc., Gaithersburg, MD).

Statistical Analysis

The number of ERαKO and WT female mice that ovulated in response to gonadotropins was analyzed by chi-square analysis. The numbers of oocytes ovulated in WT and ERαKO mice and serum progesterone concentrations were calculated as the mean ± SEM. The following comparisons were analyzed by Student's *t*-test: gonadotropin-treated WT (n = 4) versus gonadotropin-treated ERαKO (n = 9) female mice, gonadotropin-treated ERαKO (n = 9) versus saline-treated ERαKO female mice (n = 9) and gonadotropin-treated WT (n = 4) versus saline-treated WT female mice (n = 10).

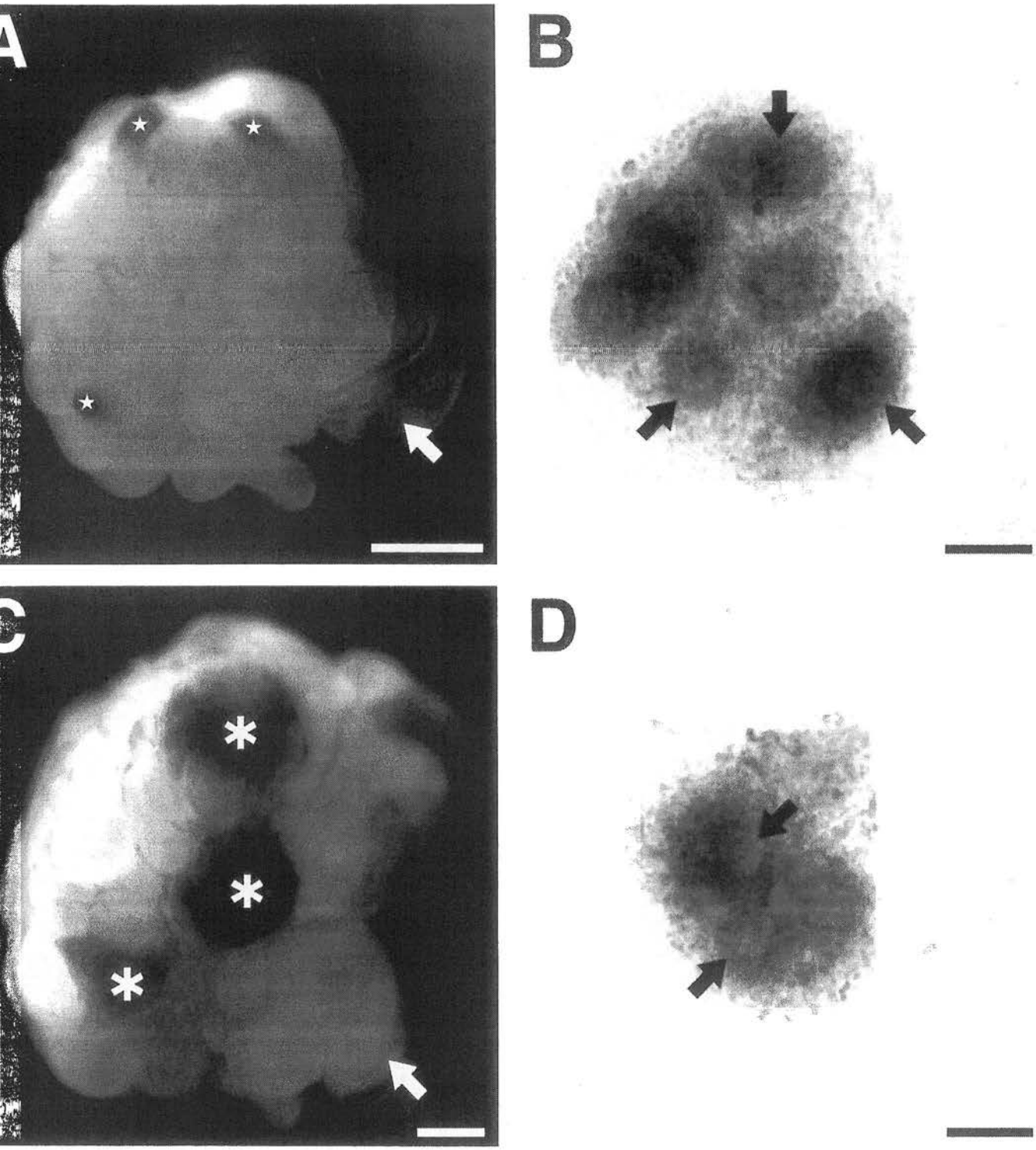
RESULTS

Adult ERαKO and WT Female Mice

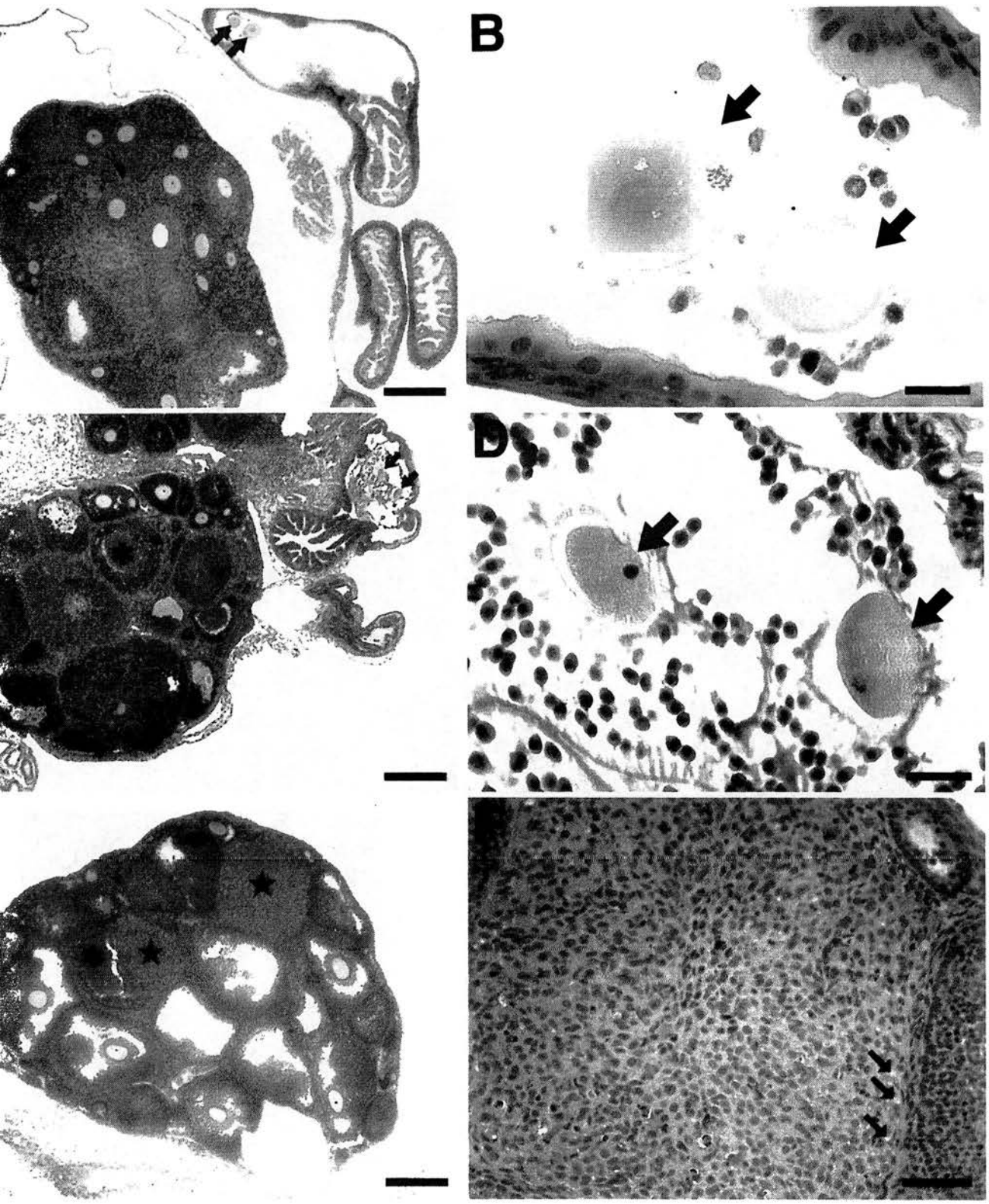
Histological analysis confirmed previous results [3] that the ovaries of ERαKO mice were dominated by hemorrhagic cysts (Fig. 1, B and C). These structures were absent in normal adult WT female ovaries (Fig. 1A). However, in contrast to previous results [32], ERαKO female mice developed graafian ovarian follicles (Fig. 1, B and C), although they were abnormal. There was abnormal stratification of granulosa cells, with some areas of the follicle surrounded by multiple layers of cells but other regions having a single layer of squamous-appearing cells (Fig. 1, B and C). As noted earlier [3,32], CL did not form in the ovaries of adult ERαKO female mice (compare Fig. 1, B and C, with Fig. 1A).

Gonadotropin Treatment of Young ERαKO and WT Female Mice

Gonadotropin treatment of young 3 to 5-wk-old WT (Fig. 2, A and B; Fig. 3, A and B) and ERαKO (Fig. 2, C and D; Fig. 3, C and D) mice resulted in ovulation of oocytes with expanded cumulus cells into the ampulla of the oviduct. Fewer gonadotropin-treated ERαKO female mice ovulated than gonadotropin-treated WT mice (11 of 24 ver-



2. Subgross examination of ovaries from gonadotropin-treated young WT and ERαKO mice. **A**) Subgross examination of ovary and oviduct from nadotropin-treated WT female mouse revealed that the oviduct was dilated (white arrow) with ovulated oocytes in the lumen of the oviduct. Corpora orrhagica were present in the ovary (stars). Magnification bar = 500 μm. **B**) Ovulated oocytes with expanded cumulus cells (arrows) from the same adotropin-treated WT female mouse as depicted in **A.** Total oocytes ovulated from this gonadotropin-treated WT female mouse was nine. Magnifi-on bar = 250 μm. **C**) Ovary and oviduct from a gonadotropin-treated ERαKO female mouse had mild dilatation of the oviduct with ovulated oocytes he lumen (white arrow). Within the ovary, there were multiple hemorrhagic ovarian follicular cysts (asterisks). The hemorrhagic ovarian follicular s were larger than the corpora hemorrhagica observed in the ovary of the gonadotropin-treated WT mouse depicted in **A.** Magnification bar = 500 **D**) Expulsion of ovulated oocytes from the oviduct of this gonadotropin-treated ERαKO female mouse represented in **C** revealed that the oocytes e surrounded by expanded cumulus cells. Four oocytes were ovulated in this gonadotropin-treated ERαKO female mouse. Magnification bar = 250



Histology of ovaries and oviduct from young gonadotropin-treated WT and ERαKO female mice. **A**) Ovary and oviduct from a 5-wk-old pin-treated WT female mouse 12 h post-hCG revealed that ovulated oocytes (arrows) were present in the ampullary region of the oviduct. ion bar = 500 μm. **B**) WT ovulated oocytes (arrows) in the oviduct as depicted in **A** were surrounded by expanded cumulus cells. Magni- ır = 50 μm. **C**) Ovary and oviduct from a 5-wk-old gonadotropin-treated ERαKO female mouse 12 h post-hCG illustrated that gonadotropin- αKO female mice were able to ovulate oocytes (arrows) into the oviduct, even though hemorrhagic ovarian cysts were beginning to develop Magnification bar = 500 μm. **D**) Higher magnification of ERαKO ovulated oocytes (arrows) in the oviduct demonstrated that they were d by many expanded cumulus cells. Magnification bar = 50 μm. **E**) Gonadotropin-treated 5-wk-old ERαKO female ovary 12 h post-hCG has CL formation (stars). Similar to **C,** hemorrhagic ovarian cysts are beginning to form (asterisk). Magnification bar = 500 μm. **F**) Higher ion of upper CL from ovary of gonadotropin-treated ERαKO mouse depicted in **E.** The CL was composed of various cell types, including containing lipid droplets (small arrows). Magnification bar = 200 μm.

LE 1. Serum progesterone concentrations of gonadotropin- and sa-treated ERαKO and WT female mice. Serum from ovariectomized six-day-post-coitus pregnant mice were used as negative and positive trols, respectively.

	Serum progesterone[a] (ng/ml)	
ıale mice	WT	ERαKO
ılt ovariectomized	0.41 ± 0.09 (n = 5)	1.56 ± 0.60 (n = 5)
ne-treated	1.28 ± 0.26[b] (n = 10)	1.98 ± 0.59[c] (n = 9)
ıadotropin-treated	17.68 ± 2.51[b] (n = 4)	6.41 ± 1.50[c] (n = 9)
gnant	24.13 ± 1.19 (n = 4)	—

ean ± SEM.
Both gonadotropin-treated WT[b] and ERαKO[c] female mice had a statis-lly significant elevation in serum progesterone concentrations com-ed to those of their respective genotype controls that received only ne ($P < 0.01$); however, gonadotropin-treated ERαKO females had er serum progesterone concentrations than did gonadotropin-treated female mice ($P < 0.01$).

s 23 of 26; $P < 0.01$). In addition, ERαKO mice ovulated ver oocytes than gonadotropin-treated WT mice (7.09 ± 7 versus 16.17 ± 0.84; $P < 0.01$). As shown in Figures and 3C, the ERαKO female mice were only just begin-ıg to develop hemorrhagic ovarian follicular cysts at this e. The hemorrhagic ovarian cysts could be distinguished m corpora hemorrhagica (Fig. 2A) by their larger size d the presence of nonluteinized granulosa cells surround-g them. CL were present in gonadotropin-treated ERαKO ce (Fig. 3, E and F). Multiple cell types were evident thin the CL and some cells contained lipid droplets (Fig.) suggesting that they were steroidogenically functional.

ogesterone Results

Serum concentrations of progesterone were low in both T (0.41 ± 0.09 ng/ml) (n = 4) and ERαKO (1.56 ± 60) (n = 4) female mice that had been previously ovari-omized (Table 1). Serum concentrations of progesterone 6-day post-coitus WT mice were, as expected, elevated 4.13 ± 1.19 ng/ml; n = 4), and corresponded to the peak culating concentrations of progesterone noted previously pregnant mice [31]. Together, these negative and posi-e controls support the validity of the assay. Three days er gonadotropin treatments, ERαKO females had statis-ally significant lower serum progesterone concentrations < 0.01) than WT mice (6.41 ± 1.5 ng/ml versus 17.68 2.51 ng/ml). However, gonadotropin-treated ERαKO fe-ales had 3-fold higher concentrations ($P < 0.01$) of serum ogesterone than ERαKO age-matched female mice that d received only saline (6.41 ± 1.5 ng/ml versus 1.98 ± 59 ng/ml). This rise in progesterone correlates with the pearance of CL in gonadotropin-treated ERαKO females. expected, gonadotropin-treated WT mice also had high-progesterone concentrations ($P < 0.01$) than control WT ce (17.68 ± 2.51 ng/ml versus 1.28 ± 0.26 ng/ml). The vated progesterone concentrations in gonadotropin-treat-WT versus ERαKO female mice is consistent with the reased number of CL present in WT compared to ERαKO nale mice.

SCUSSION

On the basis of these studies, ovarian folliculogenesis, ulation, and CL formation can occur in the absence of α, although to a lesser extent than in WT mice.

As there was a decrease in the percentage of gonadotro-n-treated ERαKO female mice that ovulated as well as in number of oocytes ovulated per mouse, ERα could have an auxiliary ovarian role. Ovarian ERα might, for example, facilitate ovarian follicular development and maturation, so that there are fewer antral follicles induced to undergo ovulation in ERαKO mice. Estrogen is known to promote FSH-induced ovarian follicular growth in other species [1, 4], although it is unclear which ER(s) mediates the effect. Possibly, ERβ rather than ERα is involved.

ERβ mRNA and its protein have been detected in granulosa cells at various stages of ovarian follicular development in the rat [24, 33–39], human [40], and cow [41]. Multiple alternative spliced variants of ERβ have been identified within the ovary [42]. Therefore, to determine whether estrogen has a direct role within the ovary, all of the currently described ER and splice variants need to be considered. Furthermore, other novel estrogen receptors may exist in the ovary [43–45] and the uterus [46]. Presently, ERβ appears to be the predominant ER in the ovary.

Unlike the ERαKO mouse, the ERβKO female mouse is fertile, although there are fewer ovarian follicles and subsequently fewer ovulations [47]. Consequently, there are fewer CL and smaller litter sizes than in WT mice [47]. Gonadotropin-treated ERβKO [47], like ERαKO female mice, also respond subnormally to gonadotropins. In both instances, fewer oocytes are released than in WT counterparts. Therefore, neither known ER is essential, but each may be needed to provide full ovarian function.

One difference between the two mutant mouse strains is that adult ERαKO mice cannot be induced to ovulate [32]. Possibly, the young ERαKO mice can be induced to ovulate because they have not been exposed to prolonged and elevated levels of LH and subsequently have not developed hemorrhagic ovarian cysts. If the LH induction of these cysts could be blocked, adult ERαKO females might be capable of undergoing normal ovulation and CL formation. There seems little doubt that such cysts arise in response to elevated levels of LH [48, 49], but the basis of the pathology is unclear. There may be weakening of the follicular basement membrane, which ruptures before ovulation, allowing entry of blood into the follicle.

Mice that are unable to synthesize estrogen because of targeted disruption of the P450 aromatase gene are able to progress up through the antral stage of ovarian follicular development, but they do not form CL [27, 28]. This phenotype is consistent with the hypothesis that estrogen is not necessary for ovarian folliculogenesis but that it might be required for ovulation and CL formation. However, deletion of the P450 aromatase gene results in other systemic effects. Testosterone, FSH, and LH are all elevated in these mutant female mice [27] and could thus underlie the failure of these mice to ovulate.

In conclusion, we have shown that contrary to previous data [32] and implied expectations [20–22], a proportion of ERαKO mice can be induced to ovulate and develop what appear to be functionally normal CL. To further understand the differences in response to gonadotropin treatment within ERαKO female mice, studies are underway to quantitate endogenous gonadotropins in these mice. Additionally, future studies include quantitating the number of ovarian follicles and CL in gonadotropin-treated ERαKO mice. It remains to be determined whether ovulated ERαKO oocytes can be fertilized and undergo normal development if transferred to recipient WT female mice. The data presented in this paper combined with the ERβKO studies [47] suggest both ERα and ERβ are needed for full ovarian function or that alternative mechanisms exist, such as a novel estrogen receptor.

WLEDGMENTS

hors wish to thank Dr. Matt Lucy, Dr. H. Allen Garverick, Dr. hu Ganjam, Dr. Mohan Manikkam, Paul D. Fell, Elisabeth L. ie Kardis, Melissa Larson, Dr. Alan Ealy, and Ali Bagegni for and technical assistance. We are grateful to Dr. R. Michael his critical review of the manuscript.

ICES

Langton RE, Daniel SAJ. Follicle-stimulating hormone and es- regulate antrum-like reorganization of granulosa cells in rat al follicle cultures. Biol Reprod 1990; 43:65–72.
u PL, Osborn SM. Factors influencing the rate of preantral and growth of mouse ovarian follicles *in vitro*. J Reprod Fertil 1992; –362.
n DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies eration of reproductive function but not prenatal sexual devel- after insertional disruption of the mouse estrogen receptor Proc Natl Acad Sci USA 1993; 90:11162–11166.
f SCJ, Figueriredo JR, Beckers JF, Bevers MM, van der Donk n den Hurk R. Effects of fetal bovine serum, FSH, and 17β- ol on the culture of bovine preantral follicles. Theriogenology 44:217–226.
P, Yoshimura Y, Hosoi Y, Bongiovanni A, Wallach E. Effect natase inhibitors on the histology of the cycling rat ovary. Gy- Obstet Invest 1987; 23:177–183.
N, Murray AA, Allison V, Boland NI, Gosden RG. Role of otrophins and ovarian steroids in the development of mouse *in vitro*. J Reprod Fertil 1998; 113:19–26.
FB, Botticelli CR, Albright JT. An intercellular response to es- by granulosa cells in the rat ovary; an electron microscopy Endocrinology 1972; 90:992–1007.
ama T, Nakano R, Iwao M. Evidence for a role of endogenous n on follicular growth in immature female rats. Endocrinol Jpn 28:715–723.
o R, Nakayama T, Iwao M. Inhibition of ovarian follicle growth nemical antiestrogen. Horm Res 1982; 16:230–236.
aj N, Shetty G, Vijayalakshmi K, Bhatnagar AS, Mougdal NR. of blocking oestrogen synthesis with a new generation aroma- nhibitor CGS 16949A on follicular maturation induced by preg- nare serum gonadotrophin in the immature rat. J Endocrinol 142:563–570.
G, Bhatnagar AS, Mougdal NR. Blockade of estrogen synthesis n aromatase inhibitor affects luteal function of the pseudopreg- t. J Steroid Biochem Mol Biol 1995; 55:347–353.
PCK, Armstrong DT. Further evidence in support of a short- edback action of estrogen on ovarian androgen production. Life 80; 27:415–420.
TH Jr, Zhuang L-Z, Hsueh AJW. Estrogen augmentation of otropin-stimulated progestin biosynthesis in cultured rat granu- lls. Endocrinology 1983; 112:1916–1924.
e JE, Hansel W. The effects of 17β-estradiol on progesterone on by bovine theca and granulosa cells. Endocrinology 1979; 34–1838.
s AJ, Skinner MK. Estrogen regulation of thecal cell steroido- s and differentiation: thecal cell-granulosa cell interactions. En- ology 1990; 127:2918–2929.
ds JS, Ireland JJ, Rao MC, Bernath GA, Midgley AR Jr, Reich- Jr. Ovarian follicular development in the rat: hormone receptor ion by estradiol, follicle stimulating hormone, and luteinizing ne. Endocrinology 1976; 99:1562–1570.
ds JS, Jonassen JR, Rolfes AI, Kersey KA, Reichert LE Jr. sine 3′,5′ monophosphate, LH receptor, and progesterone pro- n during granulosa cell differentiation: effects of estradiol and Endocrinology 1979; 104:765–773.
berg RL, Vaitukaitis JL, Ross GT. Estrogen and follicle stim- hormone interactions on follicle growth in rats. Endocrinology 90:1492–1498.
H, Furuta I, Hsueh AJW. Estrogens inhibit and androgens en- ovarian granulosa cell apoptosis. Endocrinology 1993; 133: 2212.
nove EM. Preservation of functional corpora lutea in the rat by n treatment. Endocrinology 1966; 79:1011–1015.
n JM. Maintenance by oestrin of the luteal function in hypoph- nized rabbits. J Physiol 1937, 90:435–439.
22. Keyes PL, Nalbandov AV. Maintenance and function of corpora lutea in rabbits depend on estrogen. Endocrinology 1967; 80:938–946.
23. Green S, Walter P, Kumar V, Krust A, Bornert J-M, Argos P, Chambon P. Human oestrogen receptor cDNA: sequence, expression, and homology to v-*erb*-A. Nature 1986; 320:134–139.
24. Kuiper GGJM, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson J-A. Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 1996; 93:5925–5930.
25. Mosselman S, Polman J, Dijkema R. ERβ: identification and characterization of a novel human estrogen receptor. FEBS Lett 1996; 392: 49–53.
26. Conte FA, Grumbach MM, Ito Y, Fisher CR, Simpson ER. A syndrome of female pseudohermaphrodism, hypergonadotropic hypogonadism, and multicystic ovaries associated with missense mutations in the gene encoding aromatase (P450arom). J Clin Endocrinol Metab 1994; 78:1287–1292.
27. Fisher CR, Graves KH, Parlow AF, Simpson ER. Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the *cyp19* gene. Proc Natl Acad Sci USA 1998; 95:6965–6970.
28. Honda S, Harada N, Ito S, Takagi Y, Maeda S. Disruption of sexual behavior in male aromatase-deficient mice lacking exons 1 and 2 of the cyp19 gene. Biochem Biophys Res Commun 1998; 252:445–449.
29. Rissman EF, Wersinger SR, Taylor JA, Lubahn DB. Estrogen receptor function as revealed by knockout studies: neuroendocrine and behavioral aspects. Horm Behav 1997; 31:232–243.
30. Niswender KD, Li J, Powell MR, Loos KR, Roberts RM, Keisler DH, Smith MF. Effect of variants of interferon-τ with mutations near the carboxyl terminus on luteal lifespan in sheep. Biol Reprod 1997; 56: 214–220.
31. McCormack JT, Greenwald GS. Progesterone and oestradiol-17β concentrations in the peripheral plasma during pregnancy in the mouse. J Endocrinol 1974; 62:101–107.
32. Korach KS. Insights from the study of animals lacking functional estrogen receptor. Science 1994; 266:1524–1527.
33. Byers M, Kuiper GGJM, Gustafsson J-A, Park-Sarge O-K. Estrogen receptor-β mRNA expression in rat ovary: down-regulation by gonadotropins. Mol Endocrinol 1997; 11:172–182.
34. Saunders PTK, Maguire SM, Gaughan J, Millar MR. Expression of oestrogen receptor beta (ERβ) in multiple rat tissues visualised by immunohistochemistry. J Endocrinol 1997; 154:R13–R16.
35. Tesuka M, Milne M, Hillier SG. Expression of oestrogen receptor isoforms in relation to enzymes of oestrogen synthesis in rat ovary. Mol Cell Endocrinol 1998; 141:29–35.
36. Sar M, Welsch F. Differential expression of estrogen receptor-β and estrogen receptor-α in the rat ovary. Endocrinology 1999; 140:963–971.
37. Hiroi H, Inoue S, Watanabe T, Goto W, Orimo A, Momoeda M, Tsutsumi O, Taketani Y, Muramatsu M. Differential immunolocalization of estrogen receptor α and β in rat ovary and uterus. J Mol Endocrinol 1999; 22:37–44.
38. Sharma SC, Clemens JW, Pisarska MD, Richards JS. Expression and function of estrogen receptor subtypes in granulosa cells: regulation by estradiol and forskolin. Endocrinology 1999; 140:4320–4334.
39. O'Brien ML, Park K, In Y, Park-Sarge O-K. Characterization of estrogen receptor-β (ERβ) messenger ribonucleic acid and protein expression in rat granulosa cells. Endocrinology 1999; 140:4530–4541.
40. Enmark E, Pelto-Huikko M, Grandien KAJ, Lagercrantz S, Lagercrantz J, Fried G, Nordenskjold M, Gustafsson J-A. Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. J Clin Endocrinol Metab 1997; 82:4258–4265.
41. Rosenfeld CS, Yuan X, Manikkam M, Calder MD, Garverick HA, Lubahn DB. Cloning, sequencing, and localization of bovine estrogen receptor-β within the ovarian follicle. Biol Reprod 1999; 60:691–697.
42. Petersen DN, Tkalcevic GT, Koza-Taylor PH, Turi TG, Brown TA. Identification of estrogen receptor β_2, a functional variant of estrogen receptor β expressed in normal rat tissues. Endocrinology 1998; 139: 1082–1092.
43. Kudolo GB, Elder MG, Myatt L. A novel oestrogen-binding species in rat granulosa cells. J Endocrinol 1984; 102:83–91.
44. Kudolo GB, Elder MG, Myatt L. Further characterization of the second oestrogen-binding species of the rat granulosa cell. J Endocrinol 1984; 102:93–102.
45. Hillier SG, Saunders PTK, White R, Parker MG. Oestrogen receptor mRNA and a related RNA transcript in mouse ovaries. J Mol Endocrinol 1989; 2:39–45.
46. Das SK, Taylor JA, Korach KS, Paria BC, Dey SK, Lubahn DB. Estrogenic responses in estrogen receptor-alpha deficient mice reveal

a distinct estrogen signaling pathway. Proc Natl Acad Sci USA 1997; 94:12786–12791.
Krege JH, Hodgin JB, Couse JF, Enmark E, Warner M, Mahler JF, Sar M, Korach KS, Gustafsson J-A, Smithies O. Generation and reproductive phenotypes of mice lacking estrogen receptor β. Proc Natl Acad Sci USA 1998; 95:15677–15682.
Risma KA, Clay CM, Nett TM, Wagner T, Yun J, Nilson JH. Targeted overexpression of luteinizing hormone in transgenic mice leads to infertility, polycystic ovaries, and ovarian tumors. Proc Natl Acad Sci USA 1995; 92:1322–1326.
49. Risma KA, Hirshfield AN, Nilson JH. Elevated luteinizing hormone in prepubertal transgenic mice causes hyperandrogenemia, precocious puberty, and substantial ovarian pathology. Endocrinology 1997; 138: 3540–3547.

Reproduction (2001) **121**, 89–96

Research

Role of ascorbic acid in promoting follicle integrity and survival in intact mouse ovarian follicles *in vitro*

A. A. Murray[1], M. D. Molinek[1], S. J. Baker[1], F. N. Kojima[2], M. F. Smith[2], S. G. Hillier[3] and N. Spears[1]*

[1]*Department of Biomedical Sciences, Hugh Robson Building, University of Edinburgh, George Square, Edinburgh EH8 9XD, UK;* [2]*Animal Sciences Unit, University of Missouri-Columbia, 160 Animal Sciences Center, Columbia, MO 65211, USA; and* [3]*Department of Reproductive and Developmental Sciences, University of Edinburgh, Chalmers Street, Edinburgh EH3 9EW, UK*

ic acid has three known functions: it is necessary lagen synthesis, promotes steroidogenesis and acts antioxidant. Within the ovary, most studies have ıtrated on the role of ascorbic acid in luteal forma- d regression and little is known about the function vitamin in follicular growth and development. lar growth and development were investigated in ıdy using an individual follicle culture system that the growth of follicles from the late preantral stage afian morphology. Follicles were isolated from ertal mice and cultured for 6 days. Control media ıed serum and human recombinant FSH. Further of follicles were cultured in the same media but ıe addition of ascorbic acid at concentrations of 28 or 280 μmol l^{-1}. Addition of ascorbic acid at the concentration significantly increased the percentage of follicles that maintained basement membrane integrity throughout culture ($P < 0.001$). Ascorbic acid had no effect on the growth of the follicles or on oestradiol production. Metalloproteinase 2 activity tended to increase at the higher concentration of ascorbic acid and there was a significant concomitant increase in the activity of tissue inhibitor of metalloproteinase 1 ($P < 0.01$). Follicles cultured without the addition of serum but with FSH and selenium in the culture media underwent apoptosis. Addition of ascorbic acid to follicles cultured under serum-free conditions significantly reduced apoptosis ($P < 0.05$). From these data it is concluded that ascorbic acid is necessary for remodelling the basement membrane during follicular growth and that the ability of follicles to uptake ascorbic acid confers an advantage in terms of granulosa cell survival.

Introduction

:tions of ascorbic acid (vitamin C) are among t understood of all the vitamins. Ascorbic acid is a requirement for primates, and a few other mammals, ack the necessary hepatic enzymes for its synthesis. amin has been associated with conditions such as the common cold and cancer, and the recom- l daily requirement has been the subject of many .

ovaries, and other endocrine tissues, accumulate nounts of ascorbic acid. Within the ovaries, ascor- l accumulates in the granulosa, thecal and luteal eane, 1952) and it has long been associated with (Luck *et al.*, 1995). Ascorbic acid has been used to ertility in cows (Phillips *et al.*, 1941) and enhances ct of clomiphene on induction of ovulation in (Igarashi, 1977). Conversely, high dosages have been implicated in spontaneous abortion in both women and rats (Samborskaia and Ferdman, 1966). The ovarian content of ascorbic acid changes throughout the oestrous cycle. In response to the LH preovulatory surge, ascorbic acid uptake by the ovaries is blocked and tissue content is depleted: an action that formed the basis of an early bioassay (Parlow, 1958). Ovaries produce increasing concentrations of progesterone in response to LH. Studies on luteinizing granulosa cells have shown that ascorbic acid stimulates production of progesterone (Byrd *et al.*, 1993) and that increasing progesterone concentrations block the uptake of ascorbic acid (Stansfield and Flint, 1967). Therefore, the action of LH may indirectly control the fluctuations in ascorbic acid concentration observed throughout the ovarian cycle. In addition, ascorbic acid acts as a co-factor in the amidation of some proteins and has been implicated in the regulation of oxytocin secretion by ovaries (Luck and Junglas, 1987).

The role of ascorbic acid in promoting collagen biosynthesis has been studied extensively (Pinnell, 1985). During follicular growth, ovulation and formation of corpora lutea, basement membranes and the extracellular matrix

ondence
orah.Spears@ed.ac.uk

1470-1626/2001

are undergoing constant remodelling and, therefore, have high requirements for collagen. Early studies implicated ascorbic acid in the regulation of the Graafian follicular basement membrane; lack of ascorbic acid causes degeneration of follicle membranes and high doses inhibit collagenolytic activity in mature follicles (Kramer *et al.*, 1933; Espey and Coons, 1976).

The matrix metalloproteinases (MMPs) and their inhibitors, tissue inhibitors of metalloproteinases (TIMPS), are members of an enzyme family associated with the remodelling of extracellular matrix within ovaries (Smith *et al.*, 1999). Many studies of the actions of MMPs and TIMPs within ovaries have concentrated on their functions during the periovulatory period (Hagglund *et al.*, 1999), but little is known about their expression and control throughout follicular development. Vitamin C deficiency has been associated with premature rupture of placental membranes. Addition of ascorbic acid to cultured human amnion cells resulted in a decrease in MMP activity (Pfeffer *et al.*, 1998), which indicates that ascorbic acid may play a role in the control of these enzymes.

Although there is much information on the role of ascorbic acid during formation of corpora lutea, little is known about its role during follicular growth and development. In the present study the role of ascorbic acid during follicular growth and development was studied using an isolated follicle culture system. The culture system used allows the growth of preantral follicles through to Graafian stages and the oocytes obtained can be fertilized and live young produced (Spears *et al.*, 1994). The culture system closely mimics growth *in vivo* and is therefore a highly physiological model.

Materials and Methods

Animals

C57Bl/6 × CBA/Ca F1 female mice aged 21–25 days were housed in an environmentally controlled room on a 14 h light:10 h dark photoperoid. Food and water were available *ad libitum*.

Follicle isolation and culture

The mice were killed by cervical dislocation and their ovaries were removed and placed on watch glasses containing Leibovitz L-15 medium (Gibco-BRL, Renfrew) supplemented with 3 mg BSA ml^{-1} (Fraction V; Sigma Chemical Co, Poole). Individual preantral follicles (approximately 200 ± 20 μm in diameter) were dissected manually using fine needles and allocated randomly to the 'U' shaped wells of microtitre plates (Iwaki, Tokyo) containing 30 μl α-minimal essential medium (α-MEM; Gibco-BRL) overlaid with 75 μl silicone fluid (Gibco-BRL). The culture media was supplemented with 1 iu recombinant human FSH (hrFSH; Serono-Ares, Geneva) ml^{-1}, 5% mouse serum and ascorbic acid as described below. Media were prepared freshly at 2 day intervals. The α-MEM was supplied as 1 × liquid. The formulation includes ascorbic acid at a concentration of 50 mg l^{-1}; however, liquid media is prepared in batches and supplied with a minimum 3 months shelf life. Therefore, it is likely that the ascorbic acid component would have decayed in the media used.

Experiment 1: effects of ascorbic acid on basement membrane integrity, follicular growth and morphology

Follicles were cultured in control media (as above) or with the addition of either 5 or 50 μg L-ascorbic acid ml^{-1} sodium salt (Sigma) to the culture media (28 and 280 μmol l^{-1}, respectively). Ascorbic acid stock solution (5 mg ml^{-1}) was prepared in α-MEM and aliquots were stored at –70°C for 1 month only. Follicles were examined once a day; the basement membrane was defined as intact where follicles maintained their three-dimensional morphology. Follicular diameter was measured using a precalibrated ocular micrometer. Data presented on follicular growth were obtained from follicles that remained intact on the final day of culture. During the first 2 days of culture, all follicles were moved to fresh wells of media. Follicles that had lost basement membrane integrity within the first 2 days of culture were discarded from the experiment (as this may have been caused by damage during dissection). Intact follicles were moved into fresh media once a day between days 3 and 6 of culture. Ruptured follicles were not moved, but instead 15 μl fresh media was exchanged for 15 μl spent media each day. Thirty-six follicles were allocated to each treatment group in each run of the experiment. The experiment was repeated twice, giving a total of 72 follicles per treatment.

At the end of culture, follicles from all treatments were fixed in 4% (w/v) paraformaldehyde (Sigma, Poole) and embedded in LR White resin (Taab Laboratories, Alderton). Sections (3 μm thickness) were cut and stained with haematoxylin–eosin.

Experiment 2: effects of ascorbic acid on production of oestradiol and progesterone

Spent culture media from days 4 and 5 of culture (see Expt 1) were analysed for oestradiol and progesterone using ELISA (Biostat, Cambridge). Oestradiol was analysed in the media separately each day, whereas progesterone was analysed from the combined media of days 4 and 5. For oestradiol, the inter- and intra-assay coefficients of variation were both ≤ 5% and the assay sensitivity was ≤ 18 pmol l^{-1}. For progesterone, the inter- and intra-assay coefficients of variation were both ≤ 5% and the assay sensitivity was ≤ 0.48 nmol l^{-1}. The data presented are from ≥ 25 individual measurements from each treatment group.

Experiment 3: detection of metalloproteinase and tissue inhibitor of metalloproteinases

Gelatin zymography and reverse zymographic analyses were conducted for detection of MMP and TIMP activities,

pectively, in cultured mouse follicles. Freshly isolated licles, corresponding to the diameters of follicles on ch day of culture, were also analysed. These results t shown) indicated that MMP activity was detectable in licles of a similar size to those cultured for 2 days. On basis of these initial experiments, follicles were tured for 2 days in the same concentrations of ascorbic d as in Expt 1, after which they were analysed for MMP d TIMP activity.

latin zymographic and reverse zymographic analyses

Gelatin zymography was conducted with 7.5% (w/v) S–PAGE gels containing 1 mg gelatin ml^{-1} (Sigma, St uis, MO), under non-reducing conditions as described by obs *et al.* (1985) and Morodomi *et al.* (1992). Follicles (ten icles per sample) were dissolved directly in 20 µl S–PAGE loading buffer (Laemmlli, 1970) without a reduc- agent to avoid the possible loss of MMP and TIMP activ- associated with tissue extraction from small samples. The nples were subjected to electrophoresis at room tempera- e (10 µl per lane). After electrophoresis, SDS was eluted m gels in four changes of buffer containing 50 mmol s–HCl l^{-1} (pH 7.5), 5 mmol $CaCl_2$ l^{-1}, 5 µmol $ZnCl_2$ l^{-1}, 2% (w/v) NaN_3 and 2.5% (v/v) Triton X-100 (Sigma) for –90 min at room temperature. The gels were then incu- ed in the same buffer without Triton X-100 at 37°C for h, stained with Coomassie brilliant blue R250 and tained in 30% (v/v) methanol and 1% (v/v) formic acid. eas of MMP activity were identified as clear bands of di- ted gelatin. Molecular weight markers (Gibco-BRL) and rified human pro-matrix metalloproteinase 2 (MMP-2; a from H. Nagase, University of Kansas) were used to de- mine the molecular sizes of pro- and active forms of atinases. The intensity of bands was determined by use of ChemiImager 4000 Low Light Imaging System (Alpha otech Corp, San Leando, CA). All gelatinase activity was ibited by including 10 mmol EDTA l^{-1} or 10 mmol enanthroline l^{-1} in the incubation buffer (data not shown).

Reverse gelatin zymography was conducted with 12% v) SDS–PAGE gels containing gelatin (1 mg ml^{-1}) under n-reducing conditions (Reverse Zymography Kit; iversity Technologies International Inc, Calgary). The ne samples as described above (10 µl per lane) were ojected to electrophoresis at room temperature. After ctrophoresis, SDS was eluted from the gels and the gels re incubated at 37°C for 30 h as described above. Gels re stained with GelCode® Blue Stain Reagent (Pierce, ckford, IL) and washed in water. TIMP activity was ntified as dark bands of undigested gelatin. Molecular ight markers and purified recombinant ovine tissue ibitor of metalloproteinase 1 (E. W. McIntush and M. F. ith, unpublished) were used to determine the molecular es of TIMPs. Intensity of bands was determined as cribed above.

n each run of the experiment, ten follicles were analysed treatment group. The experiment was repeated three times, giving a total of 30 follicles per treatment.

Experiment 4: effects of ascorbic acid on apoptosis

Follicles were dissected as described above and assigned randomly to three groups to determine whether ascorbic acid can prevent apoptosis. Each group was cultured in 5% CO_2 at 37°C for 24 h in one of the following media: (i) α-MEM supplemented with 1 iu hrFSH ml^{-1} (control group); (ii) α-MEM supplemented with 1 iu hrFSH ml^{-1} and 280 µmol ascorbic acid l^{-1} (experimental group); and (iii) α-MEM supplemented with 5% serum, 280 µmol ascorbic acid ml^{-1}, 0.5 ng sodium selenite ml^{-1} (Sigma) and 1 iu hrFSH ml^{-1} (positive control group, as inclusion of serum in culture media inhibits follicular degeneration through apoptosis).

DNA extraction and labelling

Genomic DNA was extracted from an average of eight follicles per culture tray using a commercial kit, according to the manufacturers' instructions (QIAmp Tissue Kit; Qiagen Ltd, Crawley). Extracted DNA was eluted in a final volume of 150 µl (to maximize yield) and ethanol precipitated (45 µl potassium acetate (8 mol l^{-1}), 400 µl ethanol) and resuspended in 20 µl ddH_2O. Extracted DNA fragments were 3′ end-labelled with digoxigenin using a 3′ end labelling oligonucleotide kit (Boehringer Mannheim, Lewes) before separation by electrophoresis on a 2% (w/v) agarose gel. The gels were Southern blotted overnight onto positively charged nylon membranes (Boehringer Mannheim), baked at 120°C for 30 min and the labelled fragments were detected using the DIG detection kit (Boehringer Mannheim) according to the manufacturers' instructions. Developed membranes were scanned using an Imaging Densitometer (Model GS-670; Bio-Rad, Hemel Hempstead) and analysed with the Molecular Analyst program (Bio-Rad). Density readings were recorded for bands corresponding to approximately 185, 370 and 555 base pairs on each lane run and mean densities were calculated. Density was calculated relative to the positive control group (iii), supplemented with serum, which was assigned an arbitrary value of 1.

In each run of the experiment, eight follicles were analysed in each treatment group. The experiment was repeated three times, giving a total of 24 follicles per treatment.

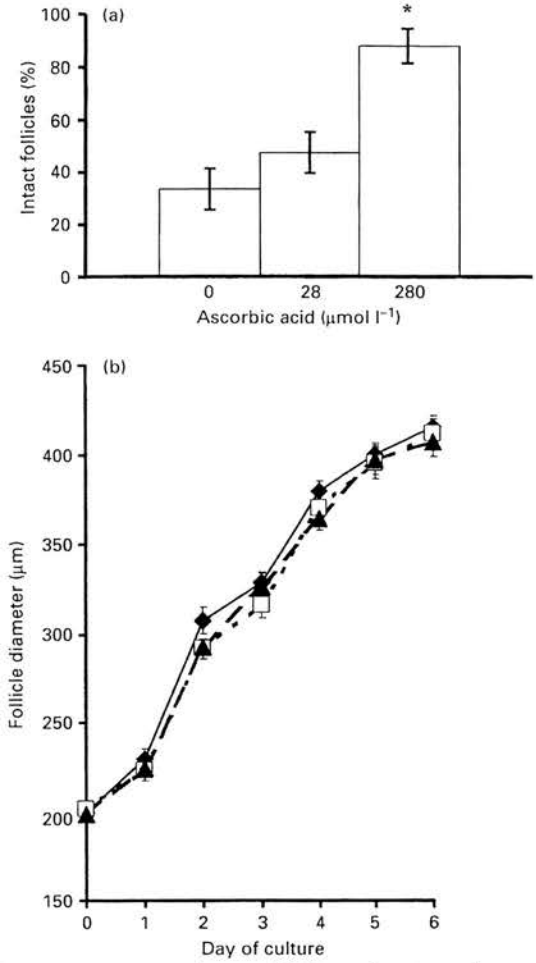
Statistical analysis

Probability values were determined by ANOVA. Paired comparisons were made using Student's *t* test where appropriate.

Results

Experiment 1: effects of ascorbic acid on basement membrane integrity, follicular growth and morphology

In the absence of ascorbic acid, 33% of follicles maintained integrity of the basement membrane throughout the



ig. 1. (a) Percentage of mouse follicles with an intact basement membrane at the end of 6 day culture. Follicles were cultured in ontrol media or in media supplemented with either 28 or 80 μmol ascorbic acid l^{-1}. Values are mean ± SEM ($n = 72$). Significantly different from control value ($P < 0.001$). (b) Growth ates of mouse follicles cultured in control media (◆) and in nedia supplemented with either 28 (□) or 280 (▲) μmol ascorbic cid l^{-1}. Values are mean ± SEM ($n = 72$).

days of culture. A non-significant increase in the per-entage of follicles remaining intact (47%) was observed n follicles cultured in 28 μmol ascorbic acid l^{-1}. However, vhen follicles were cultured in 280 μmol ascorbic acid l^{-1} here was a significant increase in the percentage of ollicles remaining intact (88%; $P < 0.001$) compared with ontrols (Fig. 1a).

Follicular growth rate was unaffected by ascorbic acid oncentration. Follicles that remained intact in the control nd ascorbic acid-treated groups had reached similar sizes y day 6 of culture (Fig. 1b). Histological examination of follicles at the end of culture showed that intact follicles were of similar size and had large antral cavities regardless of treatment (data not shown).

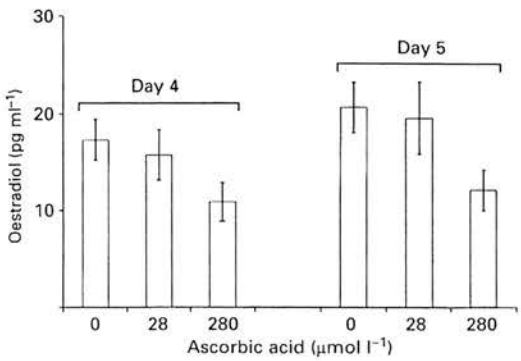


Fig. 2. Production of oestradiol by mouse follicles on days 4 and 5 of culture. Mouse follicles were cultured in media containing 0, 28 or 280 μmol ascorbic acid l^{-1}. Data are mean ± SEM ($n \geq 25$).

Experiment 2: effects of ascorbic acid on oestradiol and progesterone production

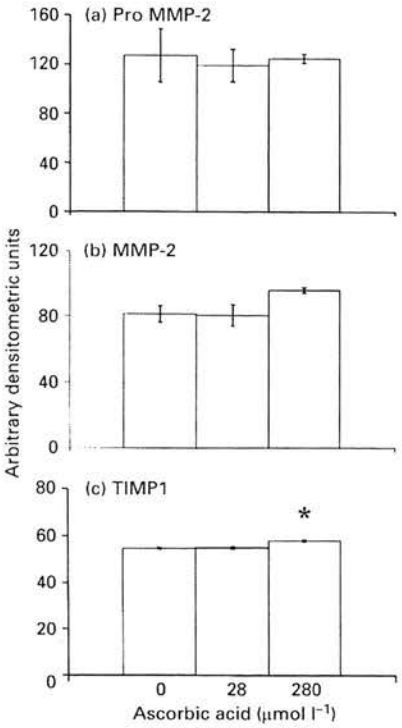
Oestradiol analysis was performed on media collected on days 4 and 5 of culture. Media were collected from both intact follicles and those that had lost their basement membrane integrity. The results indicate that oestradiol production increased between day 4 and day 5 in all treatments. Addition of ascorbic acid to the media reduced the production of oestradiol whether or not follicles had maintained basement membrane integrity, although this decrease was not significant (Fig. 2). Progesterone production was not detected in any group.

Experiment 3: detection of metalloproteinase and tissue inhibitor of metalloproteinase

The primary gelatinolytic bands (M_r 62 000 and 72 000) corresponded to the pro- and active forms of MMP-2 (gelatinase A) and comigrated with the pro- and active forms of recombinant human MMP-2. The larger gelatinolytic band (pro-MMP-2) was the predominant form present within mouse follicles. Addition of ascorbic acid at 280 μmol l^{-1} resulted in a small but significant increase ($P < 0.01$) in TIMP-1 activity, whereas 28 μmol ascorbic acid l^{-1} did not. MMP-2 activity increased at the higher concentration but the increase was not significant (Fig. 3).

Experiment 4: effects of ascorbic acid on apoptosis

Control follicles grown in the absence of serum showed high levels of apoptosis (measured as the degree of nuclear DNA laddering). Addition of ascorbic acid to the serum-free media (experimental group) significantly reduced the incidence of DNA fragmentation ($P < 0.05$) to nearer the values of the positive control group, which also contained serum and selenium (Fig. 4).



3. Effect of ascorbic acid concentration on (a) gelatinolytic -matrix metalloproteinase 2 (MMP-2), (b) activated MMP-2 (c) tissue inhibitor of metalloproteinase 1 activities in mouse cles. Values were assigned arbitrary densitrometric units. ues are mean ± SEM ($n = 30$). *Value is significantly different n control ($P < 0.01$).

Discussion

common with other endocrine tissues, the ovaries sites of ascorbic acid accumulation, which fluctuates response to stimuli such as LH, cyclic AMP and staglandins. Most studies have concentrated on the ll-known effects of ascorbic acid on steroidogenesis in ponse to these factors (Sanyal and Datta, 1979). wever, it has been suggested that the high ascorbic acid ntent measured within ovarian tissue is in excess of that uired solely for steroidogenesis. As the ovaries are sites ntensive tissue remodelling, ascorbic acid is probably uired as a co-factor in collagen production (Luck *et al.*, 95). Much attention has been paid to the ability of orbic acid to act as an antioxidant (Padh, 1991); as h tissue remodelling and steroidogenesis are processes t produce reactive oxygen species, it is likely that ascor- acid serves this function within the ovaries. The results he present study indicate that the multi-functional activ- s of ascorbic acid contribute to follicular development.

The hormonal influences that affect follicular growth development have been well documented but little is known about the physical processes that occur throughout the growth phase. During follicular growth and expansion there is rapid production of the basal lamina that separates the thecal and granulosa compartments. This has been estimated as a 3×10^4 times increase in mice (Gosden *et al.*, 1993). In the *in vitro* system described in the present study, murine follicles grew from approximately 200 μm to approximately 415 μm in diameter during culture for 6 days, resulting in a 4.3 times increase in follicular surface area. Although ascorbic acid had no effect on the growth rate or morphology of the follicles, there was a marked increase in the percentage of follicles able to maintain basement membrane integrity. Without ascorbic acid, only 33% of the follicles had retained an intact membrane after 6 days in culture compared with 88% when the medium was supplemented with 280 μmol ascorbic acid l^{-1}. Although this is a higher concentration than that found commonly in human serum, it correlates reasonably well with the high concentrations of ascorbic acid found in human follicular fluid (Luck *et al.*, 1995). These results are in good agreement with a recent report by Rose *et al.* (1999), in which addition of selenium and ascorbic acid also increased the percentage of follicles able to maintain their spherical morphology when cultured *in vitro*. In the culture system used in the present study, no selenium was added to the media and therefore the effect seen can be attributed solely to the addition of ascorbic acid. A very early study with scorbutic guinea pigs noted that these animals were anovulatory and a marked degeneration of the follicular wall was observed (Kramer *et al.*, 1933). A major component of the follicular basal lamina is collagen IV, which both granulosa and theca cells can produce *in vitro* (Rodgers *et al.*, 1995; Zhao and Luck, 1995). Ascorbic acid is known to promote collagen synthesis, both at the level of the gene, and as a co-factor in the secretion and stabilization of the protein (Pinnell, 1985). Therefore, it could be assumed that the growing follicle would have a high requirement for ascorbic acid if it is to produce sufficient basal lamina components to maintain expansion of this membrane during development. The results presented here support this idea. There are also unpublished data (V. Srsen and N. Spears, unpublished) which show that follicles grown in the presence of ascorbic acid require a more stringent permeabilization treatment before fixation, providing further evidence for a role of ascorbic acid in basal lamina formation and stabilization. Rodgers *et al.* (1998) reported that in bovine follicles the distribution of the collagen IV chains within the basal lamina changes composition during follicular development. As ascorbic acid was present in the media throughout the culture period, it was not possible to determine whether it was required at all stages of development or, for example, only during pre- or post-antrum formation.

Extracellular matrix components are constantly remodelled by the action of extracellular proteases, mainly MMPs and TIMPs. As such, MMPs and TIMPs are obvious candidates for modulating the ovarian follicle basement

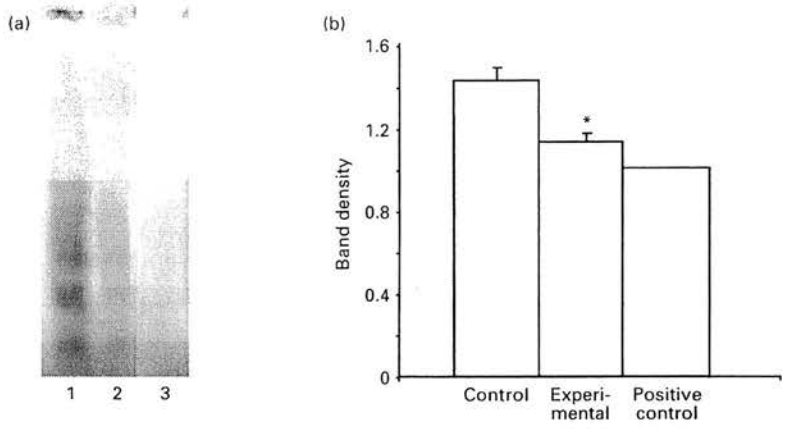


Fig. 4. (a) Representative gel of mouse follicles showing laddering of DNA into approximately 185 bp multiples. Lane 1: control group (cultured in the absence of serum). Lane 2: experimental group (cultured in the absence of serum but with the addition of 280 µmol ascorbic acid l^{-1}). Lane 3: positive control group (cultured in the presence of serum, 280 µmol ascorbic acid l^{-1} and 5 µg selenium ml^{-1}). (b) Densities of apoptotic bands in control and experimental groups, calculated relative to the positive control group, which was assigned an arbitrary value of 1. Values are mean ± SEM ($n = 24$). *Value is significantly different from control ($P < 0.05$).

membrane. The collagenous component of the ovarian stroma includes interstitial collagen types I and III, whereas collagen type IV is the collagenous component of the basement membrane. Collagens can be degraded by MMP-2, which is activated during structural luteolysis, concurrent with a marked depletion in ascorbic acid (Endo *et al.*, 1993). In humans, vitamin C deficiency has been associated with increased rupture of placental membranes and addition of ascorbic acid to cultured human amnion cells decreased MMP-2 expression and activity (Pfeffer *et al.*, 1998). These reports indicate that ascorbic acid influences the actions of MMPs. In the present study, the pro- and active forms of MMP-2 (gelatinase A) were detected within cultured mouse follicles collected on day 2, just before the period during which most cultured follicles began to rupture. Although similar MMP-2 and TIMP-1 activities were present in control follicles and in those cultured with the lower concentration of ascorbic acid, a significant increase in TIMP-1 was observed at the higher concentration of ascorbic acid used; this is the same treatment in which a significantly higher percentage of follicles had the ability to maintain basement membrane integrity. TIMP and MMP activities frequently increase simultaneously (Murphy *et al.*, 1985): in this instance there was a concomitant increase in the active form of MMP-2, although this increase was not significant. Although the results of the present study add further evidence for a role of ascorbic acid in mediating MMP activity within growing follicles, it seems most likely to be acting through a mechanism that allows both formation and expansion of the basement membrane, and hence requires both MMPs and TIMPs.

Absence of serum from the culture media induced apoptosis in the preantral follicles within a short period (24 h of culture), as detected by DNA laddering. Addition of ascorbic acid to the serum-free media reduced the degree of apoptosis within these follicles. Tilly and Tilly (1995) described a similar effect of antioxidants (including ascorbic acid) in short term cultures of rat antral follicles. These authors concluded that oxidative stress could play a role in follicular atresia by inducing an apoptotic mechanism. Ascorbic acid accumulation has been described within follicles at all stages of development; in the small follicles of buffalo (Meur *et al.*, 1999), Graafian follicles of humans (Paszkowski and Clarke, 1999) and, in the present study, in late preantral follicles of mice. Granulosa cells, under the influence of FSH, take up ascorbic acid actively (Berhman *et al.*, 1996) and although this is consistent with the idea of ascorbic acid being required as a co-factor in collagen synthesis and hence basal lamina expansion, it is likely that ascorbic acid also functions as an antioxidant preventing cell death. At the late preantral stage of development, follicles acquire responsiveness to gonadotrophins and develop rapidly; therefore it is feasible that an ability to accumulate ascorbic acid confers an advantage to a follicle and promotes survival.

In the present study ascorbic acid did not affect production of oestradiol. This finding is in contrast to other systems, in which addition of ascorbic acid *in vitro* adversely affected aromatase activity through alteration of pH (Milewich *et al.*, 1981). High concentrations of ascorbic acid are inhibitory to cholesterol side chain cleavage (Pintauro and Bergan, 1982), which subsequently affects androgen production and directly decreases aromatase activity (Sanyal and Datta, 1979; Tsuji *et al.*, 1989). The

ct of ascorbic acid on progesterone could not be deter-
ned, as progesterone concentrations were not measur-
e in any media in the present study. However, the lack
progesterone production shows that the granulosa cells
follicles unable to maintain their basement membrane
l not undergone premature luteinization. The relation-
p between ascorbic acid and oestradiol appears com-
x. Circulating ascorbic acid concentrations are low in
man using oral contraceptives, perhaps through an
ibitory effect of oestradiol on ascorbic acid uptake by
intestine (Kuo and Lin, 1998). When high doses of
orbic acid were administered exogenously to pregnant
men, oestradiol production increased, resulting in
rtion (Samborskaia and Ferdman, 1966). However,
afian follicles, which have high concentrations of
tradiol in the follicular fluid, are the sites of ascorbic
d accumulation (Paszkowski and Clarke, 1999). High
centrations of oestradiol are required to inhibit the
dation of low-density lipoproteins (LDL) *in vitro*.
wever, in the presence of ascorbic acid, concentrations
oestradiol close to physiological concentrations can
protect LDLs from oxidation (Huang *et al.*, 1999).
hough the effects of ascorbic acid on oestradiol may be
ue specific, further investigations are needed to deter-
ne the effects of ascorbic acid on oestradiol production
hin follicles.

n conclusion, ascorbic acid greatly increased the
centage of follicles able to maintain their basement
mbrane *in vitro* but did not affect growth rate. Ascorbic
d also increased the production of MMP-2 and TIMP-1
the cultured follicles, which implicates this vitamin in
dulating MMP remodelling of the basement membrane.
corbic acid reduced the degree of apoptosis within
icles subjected to oxidative stress (serum-free cultures)
icating that accumulation of ascorbic acid would be
vantageous for survival. The effects of ascorbic acid on
tradiol production require further investigation.

The authors would like to thank V. Allison and R. Smith for
ert technical assistance. They are grateful to V. Srsen for trans-
on of references and U. Rose for advice on the concentrations
scorbic acid used to supplement culture media. The recombi-
t FSH was a gift from Serono-Ares, Geneva, Switzerland.
Spears is a Royal Society University Research Fellow and the
llcome Trust supported this study.

References

rman HR, Preston SL, Aten RF, Rinaudo P and Zreik TG (1996) Hormone induction of ascorbic acid transport in immature granulosa cells *Endocrinology* **10** 4316–4321

d JA, Pardue SL and Hargis BM (1993) Effect of ascorbate on luteinizing hormone stimulated progesterone biosynthesis in chicken granulosa cells *in vitro*. *Comparative Biochemistry and Physiology C* **104** 279–281

ne HW (1952) Histochemical observation on the ovary and oviduct of he albino rat during the estrus cycle *American Journal of Anatomy* **91** 363–413

o T, Aten RF, Wang F and Behrman HR (1993) Co-ordinate induction and activation of metalloproteinase and ascorbate depletion in structural luteolysis *Endocrinology* **133** 690–698

Espey LL and Coons PJ (1976) Factors which influence ovulatory degradation of rabbit ovarian follicles *Biology of Reproduction* **14** 233–245

Gosden RG, Boland NI, Spears N, Murray AA, Chapman M, Wade JC, Zhody NI and Brown N (1993) The biology and technology of follicular oocyte development *in vitro*. *Reproductive Medicine Review* **2** 129–152

Hagglund AC, Ny A, Leonardsson G and Ny T (1999) Regulation and localization of matrix metalloproteinases and tissue inhibitors of metalloproteinases in the mouse ovary during gonadotrophin-induced ovulation *Endocrinology* **140** 4351–4358

Hibbs MS, Hasty KA, Seyer JM, Kang AH and Mainardi CL (1985) Biochemical and immunological characterization of the secreted forms of human neutrophil gelatinase *Journal of Biological Chemistry* **260** 2493–2500

Huang M, Li J, Teoh H and Man RY (1999) Low concentrations of 17 beta-estradiol reduce oxidative modification of low-density lipoproteins in the presence of vitamin C and vitamin E *Free Radical Biology and Medicine* **27** 438–441

Igarashi M (1977) Augmentative effect of ascorbic acid upon induction of human ovulation in clomiphene-ineffective anovulatory women *International Journal of Fertility* **22** 168–173

Kramer MM, Harman MT and Brill AK (1933) Disturbances of reproduction and ovarian changes in the guinea-pig in relation to vitamin C deficiency *American Journal of Physiology* **106** 611–622

Kuo SM and Lin CP (1998) 17beta-estradiol inhibition of ascorbic acid accumulation in human intestinal Caco-2 cells *European Journal of Pharmacology* **361** 253–259

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the bacteriophage T4 *Nature* **220** 680–685

Luck MR and Junglas B (1987) Catecholamines and ascorbic acid as stimulators of bovine ovarian oxytocin secretion *Journal of Endocrinology* **114** 423–430

Luck MR, Jeyaseelan I and Scholes RA (1995) Ascorbic acid and fertility *Biology of Reproduction* **52** 262–266

Meur SK, Sanwal PC and Yadav MC (1999) Ascorbic acid in buffalo ovary in relation to oestrus cyle *Indian Journal of Biochemistry and Biophysics* **36** 134–135

Milewich L, Chen GT, MacDonald PC and Peterson JA (1981) Ascorbic acid inhibition of aromatase activity in human placenta tissue *Journal of Steroid Biochemistry* **14** 185–193

Morodomi T, Ogata Y, Sasaguri Y, Morimatsu M and Nagase H (1992) Purification and characterization of matrix metalloproteinase 9 from U937 monocytic leukaemia and HT1080 fibrosarcoma cells *Biochemical Journal* **285** 603–611

Murphy G, Reynolds J and Werb Z (1985) Biosynthesis of tissue inhibitor of metalloproteinases by human fibroblasts in culture. Stimulation by 12-O tetradecanoylphorbol 13-acetate and interleukin 1 in parallel with collagenase *Journal of Biological Chemistry* **260** 3079–3083

Padh H (1991) Vitamin C: newer insights into its biochemical functions *Nutrition Reviews* **49** 65–70

Parlow AF (1958) A rapid bioassay method for LH and factors stimulating LH secretion *Federation Proceedings* **85** 501–520

Paszkowski T and Clarke RN (1999) The Graafian follicle is the site of L-ascorbate accumulation *Journal of Assisted Reproduction and Genetics* **16** 41–45

Pfeffer F, Casenueva E, Kamar J, Guerra A, Perichart O and Vadillo-Ortega F (1998) Modulation of 72-kilodalton type IV collagenase (matrix metalloproteinase-2) by ascorbic acid in cultured human amnion-derived cells *Biology of Reproduction* **59** 326–329

Phillips PH, Lardy HA, Boyer PD and Werner GM (1941) The relationship of ascorbic acid to reproduction in the cow *Journal of Dairy Science* **24** 153–158

Pinnell SR (1985) Regulation of collagen synthesis by ascorbic acid: a review *Yale Journal of Biology and Medicine* **58** 553–559

Pintauro SJ and Bergan JG (1982) Effects of ascorbic acid on *in-vitro* steroidogenesis in guinea pigs *Journal of Nutrition* **112** 584–591

Rodgers HF, Lavranos TC, Vella CA and Rodgers RJ (1995) Basal lamina

and other extracellular matrix produced by bovine granulosa cells in anchorage-independent culture *Tissue Research* **282** 463–471

Rodgers HF, Irvine CM, van Wezel IL, Lavranos TC, Luck MR, Sado Y, Ninomiya Y and Rodgers RJ (1998) Distribution of the alpha6 chains of type IV collagen in bovine follicles *Biology of Reproduction* **59** 1334–1341

Rose UM, Hanssen RG and Koosterboer HJ (1999) Development and characterization of an *in vitro* ovulation model using mouse ovarian follicles *Biology of Reproduction* **61** 503–511

Samborskaia EP and Ferdman TD (1966) Mechanism of abortion induced by ascorbic acid *Biulletin Eksperimentalnoi Biologii I Meditsiny* **62** 96–98

Sanyal S and Datta S (1979) Effect of ascorbic acid in *in vitro* rat adrenal and ovarian steroidogenesis *Indian Journal of Experimental Biology* **17** 86–88

Smith MF, McIntush EW, Ricke WA, Kojima FN and Smith GW (1999) Regulation of ovarian extracellular matrix remodelling by metalloproteinase and their tissue inhibitors: effects on follicular development, ovulation and luteal function *Journal of Reproduction and Fertility Supplement* **54** 367–381

Spears N, Boland NI, Murray AA and Gosden RG (1994) Mouse oocytes derived from *in vitro* grown primary follicles are fertile *Human Reproduction* **9** 527–532

Stansfield DA and Flint AP (1967) The entry of ascorbic acid into the corpus luteum *in-vivo* and *in-vitro* and the effect of luteinising hormone *Journal of Endocrinology* **39** 27–35

Tilly JL and Tilly KI (1995) Inhibitors of oxidative stress mimic the ability of follicle-stimulating hormone to suppress apoptosis in cultured rat ovarian follicles *Endocrinology* **136** 242–252

Tsuji M, Ito Y, Terada N and Mori H (1989) Ovarian aromatase activity in scorbutic mutant rats unable to synthesize ascorbic acid *Acta Endocrinologica (Copenhagen)* **121** 595–602

Zhao Y and Luck MR (1995) Gene expression and protein distribution of collagen, fibronectin and laminin in bovine follicles and corpora lutea *Journal of Reproduction and Fertility* **104** 115–123

Received 21 March 2000.
Accepted 5 July 2000.

Follicular Development In Vitro

Alison Murray, M.I.Biol. and Norah Spears, D.Phil.

ABSTRACT—*There has been tremendous interest in recent years in the culture of oocytes and follicles. Although much of the research using follicle culture aims to increase understanding of the regulation of follicle development, an important goal has been to develop a method that will eventually allow maturation of human oocytes from the primordial follicle to the mature Graafian stage. We are still some way from this at present, although it has now been achieved in the mouse. In this article, we consider various methods of follicle culture for primordial, preantral, and antral follicles. In vitro development of primordial follicles has used primarily whole ovaries or ovarian fragments as a source of follicles. Culture of later stages of follicle development uses mainly isolated follicular units, either whole (with an intact basement membrane and, in some cases, attached thecal cells) or nonintact (oocyte–somatic cell complexes, which may or may not have remnants of basement membranes and/or thecal cells attached).*

KEYWORDS: Follicle, oocyte, culture

A human female fetus produces around 6 to 7 million germ cells during ovarian development: by birth, the vast majority of these have been lost, with the ovary typically containing around 1 million oocytes surrounded by granulosa cells in primordial follicles.[1] Because only around 400 of these oocytes are ever likely to reach maturity and be ovulated, there is clearly a large pool of primordial follicles potentially available for in vitro maturation. Although much of the research using follicle culture aims to increase understanding of the regulation of follicle development, an important technical goal throughout has been to develop a method that will allow maturation of human oocytes from the primordial follicle to the mature Graafian stage, although we are far from achieving this at present.

In this article, we outline follicle culture techniques currently available, concentrating on the mouse (the only species to date in which mature, viable oocytes have been obtained from primordial follicles) and humans and other primates (with clinical end points in mind). We consider only techniques culturing oocytes taken at a fairly early stage of maturation; techniques involving the final maturation of oocytes will be addressed in a later article. We have not managed to cover all aspects of follicle culture in this short review (there has been extensive work in this area of late). Neither have we always been able to cite all references, but we have given appropriate examples.

OVERVIEW OF FOLLICLE DEVELOPMENT IN VIVO

The process of folliculogenesis within the ovary begins during prenatal life with the formation of primordial follicles, each primordial follicle consisting of an oocyte (held in meiotic arrest) sur-

Department of Biomedical Sciences, University of Edinburgh, Edinburgh, Scotland, UK.

Reprint requests: Dr. Spears, Department of Biomedical Sciences, University of Edinburgh, Hugh Robson Building, George Square, Edinburgh EH8 9XD, Scotland, UK.

1526-8004,p;2000,18,02,109,122,ftx,en;sre00053x.

rounded by a few flattened pregranulosa cells and enclosed by a basement membrane. Throughout follicle growth, the oocyte and somatic cells are interdependent: the development of each regulates and is regulated by the other.[2]

Primordial follicles are quiescent but they leave the resting pool at a steady rate to begin their growth phase, some beginning development shortly after formation whereas others may lie dormant until near the end of reproductive life. However, it is not until after the onset of puberty that these growing follicles, in response to rising levels of gonadotropins, can mature fully and ovulate. Although large numbers of follicles can leave the primordial pool and begin to grow, very few will be selected to release their oocytes for fertilization. The "default" pathway for the vast majority of follicles is to undergo atresia and die unless they can respond to the correct stimuli at the appropriate developmental stage.

After the initial loss of oocytes prior to puberty, the main subsequent loss of primordial follicles from the resting pool is due to entry into the growth phase. The mechanisms that regulate initiation of follicular growth from the primordial pool are poorly understood and it has yet to be determined whether follicles are released from growth inhibition or directly stimulated to develop, although there is some evidence that oocyte-derived factors may be involved.[3,4] Once follicles have started to grow, follicular development can be considered as a two-phase process: a slow growth phase that is not directly influenced by the gonadotropins and a faster growth phase in which responsiveness to follicle-stimulating hormone (FSH) and luteinizing hormone (LH) is obligatory if the follicle is to proceed to the preovulatory stage. The transition between these two stages can be characterized by the formation of a fluid-filled antral cavity and the production of steroid hormones. The stages and sizes of human and mouse follicles are shown diagrammatically in Figure 1.

On leaving the primordial pool, the first signs of growth are an increase in oocyte size and proliferation of the surrounding granulosa cells. Until the early antral stage, follicular growth proceeds slowly, taking many weeks in rodents and months in larger animals. As this process traverses many cyclic changes in circulating levels of gonadotropins and as it can proceed in animal models that are naturally or surgically rendered incapable of producing pituitary gonadotropins, development through to this early antral stage has been deemed "gonadotropin independent." However, gonadotropins may indirectly influence initiation of growth as a consequence of gonadotropin-stimulated factors produced by larger follicles.[4]

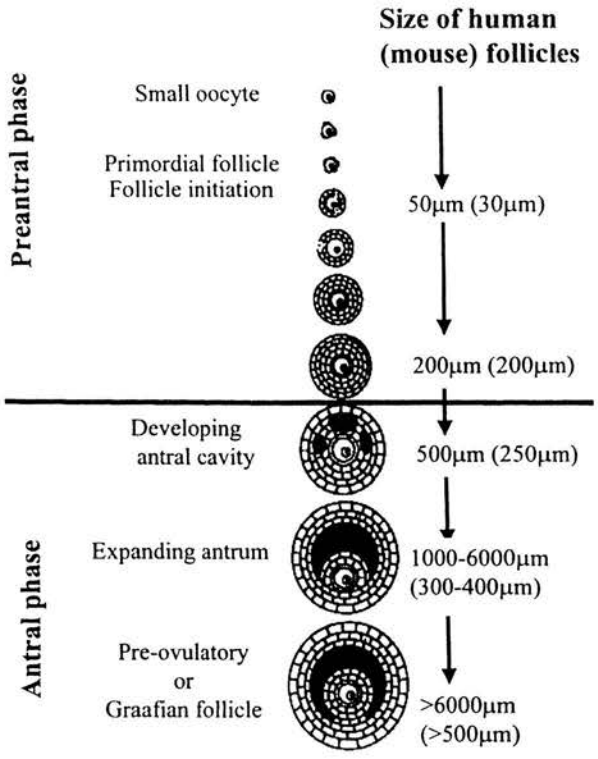


Figure 1. Diagrammatic illustration of stages and sizes of developing follicles in the human and mouse. (Adapted from Gosden et al.[8])

By the early antral stage, the follicle has a multilaminar granulosa cell layer and has also acquired vascularized and distinct layers of thecal cells that are separated from the granulosa cells by the basement membrane. Thecal cells are derived from the surrounding interstitial cells, although the mechanisms that initiate their differentiation and organization are as yet unknown and it has been suggested that their precursors may be present at the outset of follicular growth.[5] By this stage of follicular development the oocyte has grown to, or near to, its full size (80 μm in rodents and 120 μm in humans) and has increased in volume ~300-fold. During the late preantral to early antral period, oocytes synthesize molecules necessary for the resumption of meiosis and have become competent to resume meiosis and undergo fertilization, although subsequent embryonic development is arrested.[6] In all species studied so far, most follicles that simultaneously begin growth reach the late preantral stage of development, with few lost to atresia.[7] However, from this cohort only those follicles that have acquired the ability to respond to the cyclic rise in circulating gonadotropins are capable of proceeding on through the antral stages.

Antrum formation occurs when 2000 to 3000 granulosa cells are present, regardless of species and of the final size of the follicle.[8] Fluid begins to

accumulate within the small spaces between granulosa cell layers. These eventually coalesce to form a large antral cavity. Follicular fluid is filtered from the blood supply that penetrates the thecal layers of the follicle. Proteins of high molecular weight are excluded, and the concentrations of glucose, lipids, and amino acids differ from those found in plasma. During later stages of development fluid is accumulated rapidly, accounting in large part for the marked expansion in follicular size,[9] and contains a variety of molecules including steroids, growth hormone binding proteins, and proteoglycans. It may also act as a "sink," diluting or concentrating metabolites from different cell types of the follicle: the actions of oocyte-derived factors may, for example, be allowed to exert an influence only over their immediate surrounding cells before being "diluted out" within the follicular fluid, hence limiting their action on the granulosa cells positioned farthest from the oocyte. From the growing cohort of antral follicles, only a proportion of follicles are able to respond to the rising levels of FSH and consequently a large number of them die at the early antral stage of development. In humans, approximately 20 follicles are selected and continue through to the preovulatory stages of development.[10] The growth rate of the selected follicles must now accelerate, as from this population some (usually one in humans) will attain dominance and must be ready to release their oocytes for fertilization 4 days later in the rodent and about 28 days in the human. In that later growth phase, granulosa cells rapidly proliferate and differentiate into two populations, the mural granulosa cells, which are adjacent to the basement membrane, and the cumulus cells, which surround the oocyte. The oocycte, surrounded by the cumulus cells, becomes embedded within the follicular fluid connected by a stalk of cells to the mural cells. The follicles also acquire the ability to synthesize steroid hormones. Androgen substrate (mainly androstenedione) is synthesized by the thecal cells under the influence of LH and then converted to estrogen (principally estradiol) in response to FSH within the granulosa cells.[11]

Although the gonadotropins are necessary for follicle development, there are many other growth factors and cytokines that modulate their actions (for references see refs. 12–14), but these are not covered in this article.

CULTURE OF WHOLE OVARIES AND TISSUE FRAGMENTS

Techniques for the in vitro culture of whole ovaries have been described for almost 70 years[15] and have been used to investigate gonadotropin influence and ovulatory processes.[16–20] Although this type of culture preserves normal tissue interactions, it is severely limited in that long-term maintenance of organ explants is difficult. It is possible to maintain the cortical regions of the ovary to some extent, but the inner medulla region is subject to anoxia, depletion of nutrients, and accumulation of metabolites leading to necrosis. Because of these limitations and the time required for full follicular development (especially in larger species), the culture of adult ovaries is of limited use and perhaps best confined to the study of particular ovarian events such as the effects of blood flow and ovulation.[21] Ovarian fragments or tissue slices can also be used to overcome some of the problems associated with mass (see Fig. 3). In addition, a number of methods such as suspending or floating organ or tissue slices in culture have been developed, thus maximizing gas and nutrient diffusion and reducing necrosis. Although follicles can grow under these culture conditions, at least until the antral stage,[18] it is difficult to follow their individual progress.

Another point to consider in the culture of whole ovaries or ovarian fragments is the interaction between follicles. Tissue culture may be one way to study these interactions; conversely, interactions between follicles add another layer of complexity when considering the development of any one follicle from within the tissue.

Initiation and Growth of Primordial Follicles

The reserves of primordial follicles within the ovary represent a vast store of gametes that could be available for in vitro development. It has been estimated that each square millimeter of ovarian cortex in a young woman contains hundreds of follicles at the primordial and preantral stages. Removal of small pieces of the cortex is unlikely to alter reproductive capability and should it be possible to mature the oocytes within these pieces, it might be feasible to greatly increase the number of gametes available for infertility treatment.

Whereas recent cultures representing later stages of follicle development have tended to use isolated follicles, whole organ and tissue fragments have been used to maximal advantage in primordial follicle cultures (isolated follicles tend to do poorly at this stage[22,23]).

One of the most intriguing questions in the development of ovarian follicles is what governs the release or initiation of primordial follicles into the growth phase. To begin to answer this question and to investigate the viability of using primordial folli-

cles as a source of gametes, a number of investigators have turned their attention to the growth of primordial follicles in vitro. Much of this work has been carried out in domestic species (see ref. 24 for review)—here we cover culture of rodent and primate primordial follicles only.

Location of Growing Follicles

The ovaries from pre- and neonatal rodents contain mostly primordial follicles, and whole ovaries from mice, rats, and hamsters have been placed directly in culture[25–27] or used as fragments.[28] Human fetal tissue has been utilized, but this is not a common source of material.[29] In primates, the majority of primordial follicles are found in the cortex of the ovary and tissue pieces from this region have been used,[30–32] although there is great variation in the number of primordial follicles obtained.[33]

In all species studied so far, high percentages of primordial follicles spontaneously begin to grow when placed in culture, but there appear to be differences in the location of the growing follicles when comparisons are made between whole ovaries and tissue pieces. Wandji et al,[31] examining baboon cortical pieces, observed that 76% of primordial follicles had begun to grow throughout the fragments after 4 days in serum-free cultures. By contrast, Eppig and O'Brien[25] noted that in whole fetal mouse ovaries, only some primordial follicles had begun their growth phase and these growing follicles were confined to the medullary region of the ovary, with those in the cortical region remaining quiescent. These results could reflect species differences or be due to variations in culture techniques, fragments having more access to nutrients and air than the innermost portion of the whole ovary. Growth of primordial follicles is characterized by an enlargement of the oocyte surrounded by a layer of rounded granulosa cells as shown in Figure 2.

Factors Regulating Growth Initiation

Potential regulatory factors that may affect primordial follicle entry into the growth phase have been investigated in vitro. A study using juvenile rat ovaries has implicated kit ligand (KL) in primordial follicle development: spontaneous primordial follicle development was blocked by using an antibody to c-kit (the KL receptor), and after treatment with KL a higher proportion of the primordial follicles had undergone development.[27] Inclusion of FSH in the culture media may increase the percentage of follicles able to grow to the preantral

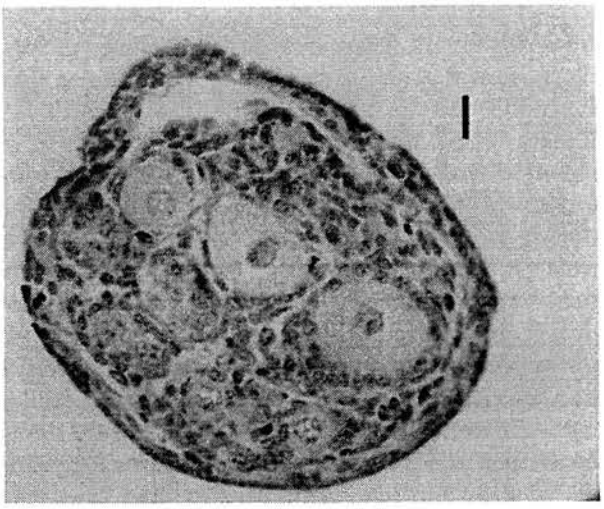


Figure 2. Tissue fragment of a newborn mouse ovary after 7 days in culture. Growing follicles are seen in the tissue, which contained only primordial follicles at the start of culture. Bar represents 20 μm. (From Molinek M, Spears N, and Telfer E, unpublished data.)

stages, perhaps by acting as a survival factor and preventing atresia.[32] In the absence of serum, insulin has also been implicated in the transition of primordial to growing follicles.[26]

Development of Oocytes from Primordial Follicles

To date, the only species for which an oocyte obtained from a primordial follicle has been matured, fertilized and resulted in the birth of live young has been the mouse, and a combination of first organ and later isolated oocyte–somatic cell cultures was used.[25] Although the success rate using this system was low, it has clearly demonstrated that complete maturation of oocytes is possible in vitro and offers the hope that this may soon be possible in other species. In a more recent study, Liu et al[34] used a different approach to generate mature oocytes from primordial follicles. Newborn ovaries were grafted into adult animals and removed after 14 days. It was found that preantral follicles had formed in the grafts and could subsequently be isolated and placed into culture. At the end of the culture period, oocytes were able to resume meiosis, but it remains to be seen whether live young can be produced.

Hovatta et al[35] initially cultured human cortical slices for a period of 7 to 9 days, after which the growing follicles were isolated either mechanically or enzymatically. The isolated follicles were then cultured for a further 2 weeks. During that time follicular structures started to degenerate; this degra-

dation occurred sooner when follicles were isolated enzymatically.

Some attention has been paid to the effects of cryopreservation prior to culture as this may have particular relevance in the clinical situation where storage of material may be desirable, for example, in restoration of fertility to patients undergoing cancer treatments. The evidence suggests that freezing and storage are detrimental neither to the numbers nor to the potential of follicles that are activated subsequent to cryopreservation.[30,33,36]

CULTURE OF ISOLATED FOLLICULAR UNITS

Short-term cultures of antral follicles and their oocytes have been successfully used to investigate the final stages of follicular growth and oocyte maturation.[37,38] However, over the past decade a number of in vitro systems have evolved that support the growth and development of follicular units from preantral through to preovulatory stages. Some of these culture systems have produced mature oocytes capable of fertilization and live young have been born, albeit with limited success rates.[39,40] The vast majority of these in vitro culture systems have been devised using rodents as models. The ovaries of rodents are readily available and lack the fibrous tissue associated with the ovaries of larger species. Many studies have utilized follicles obtained from juvenile mice and rats; these ovaries have few antral follicles, lack corpora lutea, and contain large numbers of follicles at similar stages of preantral development. In addition, the time course of complete follicular development is relatively short in these species, making it possible to grow follicles in a manner that closely resembles the in vivo situation. Although rodents have been used extensively in developing culture systems, some progress has been made in domestic species (not covered in this review; see ref. 41) and humans.[42] Human material is difficult to obtain, and few studies of the in vitro growth of follicles have been reported. In tissue samples obtained from women undergoing surgery, the number of follicles at the preantral stage of development is negatively correlated with age and hence few follicles may be available for study.[42] The growth rate and antral development of follicles have been shown to be dependent on the initial starting size of the follicles and the age of the animals from which they are obtained. Attempts to culture isolated follicles from mice of less than 6–8 days of age have not been successful; when placed in culture, the oocytes become detached from their surrounding granulosa cells and fail to mature.[25,43]

Methods of Isolation

Follicular units can be isolated either mechanically or by enzymatic digestion. Enyzmatic digestion using collagenase and deoxyribonuclease (DNase) has been successfully used to isolate follicles from mice,[39,44] rats,[45,46] hamsters,[47] and humans.[42] Small pieces of tissue are incubated with the enzymatic mixture, and by mechanically agitating the pieces through progressively smaller pipette tips, individual follicles are released. The degree of damage to the follicular units is dependent on the stringency of the treatment and on the type of tissue being used. Digestion of ovary samples from hamsters and humans results in follicles with an intact basement membrane but with no attached thecal cells, compared with mice and rats that lose both thecal cells and basement membrane integrity. Effectively, in the latter species, the "follicles" collected are oocyte–somatic cell complexes. Although these complexes appear devoid of a basement membrane and attached thecal cells, it is likely that some of these components are transferred into the culture to a greater or lesser degree depending on the methods used.

Mechanical isolation of follicles is a more labor-intensive method of isolating follicles but ensures that the basement membrane of the follicle remains intact and preserves follicular architecture. Small pieces of tissue are teased apart and follicles dissected from these using fine needles. The main criteria used in selection of follicles by this method are size, a centrally placed oocyte surrounded by tightly packed granulosa cell layers, and some attached thecal or stromal cells. The methods of isolation and examples of the different culture techniques used are illustrated in Figure 3.

Culture Conditions

To a certain extent, the method of isolation determines the conditions of the culture into which follicles are placed. Follicle units that have been isolated by enzymatic means and lack a basement membrane need to be cultured under conditions that prevent the dissociation of the granulosa cell from the oocytes, as contact between the two cell types is necessary if the oocyte is to grow and mature. This has been achieved by placing these isolated complexes on collagen-impregnated membranes, by embedding them in collagen gels,[25,44] or by allowing them to plate down onto substrate adhesive surfaces.[45,46] Follicles with the basement membrane intact (isolated either mechanically or enzymatically) have been cultured by embedding

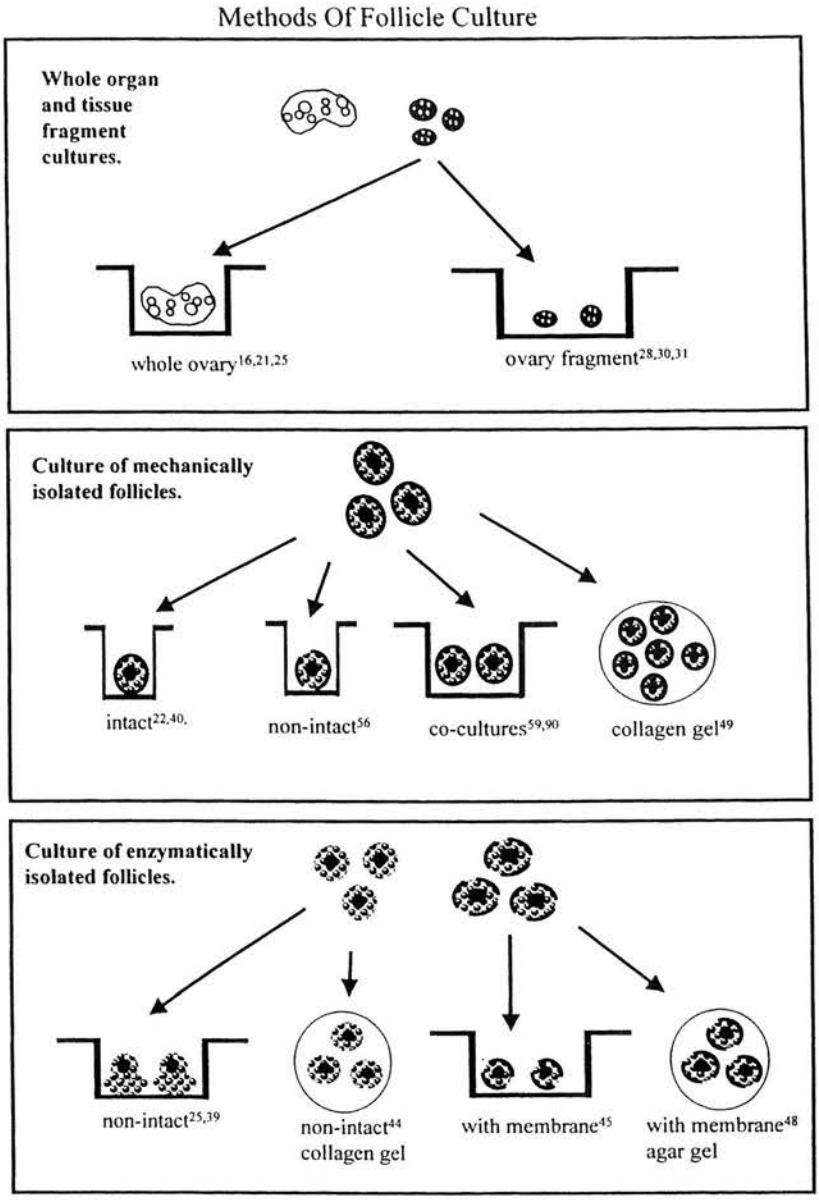


Figure 3. Diagram illustrating the methods of isolation and types of culture systems used. Example references are given for each method.

them in agar or collagen gels[42,48,49] or by placing follicles on various surfaces bathed in culture medium. With the latter culture techniques, follicles can be maintained as intact units if cultured under conditions that prevent adhesion to culture dishes.[40,50–55] Where follicles are placed on surfaces allowing adhesion, follicles tend to rupture during culture, but all cell types remain present in the culture.[45,56]

Each type of culture system has advantages and disadvantages, and selection of one over the other is very dependent both on the experimental end point and on the type of information desired. The culture of intact follicles ensures that each cell type (theca, granulosa, and oocyte) is present and allows the investigation of how each of these contributes to both follicular development and growth and maturation of the oocyte. However, this type of culture may not support development of antral follicles from larger species, where diffusion of nutrients and oxygen diffusion may be problematic These problems could be overcome, and larger numbers of oocytes maintained in vitro, by using a culture system such as that developed foryoocyte–somatic cells that are "open" to the culture media although the contribution of the extracellular matrices (including antral fluid) is then excluded. Culture systems in which all elements of the follicular unit are included but the three-dimensional structure is lost may address some of these questions, although here growth may be impeded and basement membrane or thecal cell components may no differentiate in a manner that resembles the in vivo situation.[49] Imbedding follicles in a matrix may be preferable for gaining information on growth kinetics but is not so suitable a method for examining

Follicle cultures have already revealed much about how gonadotropins regulate follicle development and how the oocyte controls granulosa cell function. Use of ovarian material from transgenic mice has, in particular, proved a powerful tool.[89,95] Similarly, cultures should prove useful in examination of other aspects of follicle development that are currently little understood, such as the initiation of primordial follicle growth or signals the oocyte receives from somatic cells.

Other processes, too, have yet to be elucidated. During oocyte growth, specific epigenetic modifications occur resulting in genetic imprinting, a process that ensures the expression of only one parental allele. Incorrect imprinting can affect embryo and fetal development and even cause health problems in later life. The mouse born from development from the primordial stage in vitro suffered from some late-onset health problems perhaps because imprinting was perturbed.[62] Again, culture techniques can help us to address this issue and animal models of this are a necessary tool before clinical application can proceed.

It is envisaged that future use of in vitro systems will begin to unravel some of these mechanisms. Knowledge of how oocytes become fully competent will be of particular use if this technology is to be practical in clinical settings. The process of oocyte maturation in the human is very long and the technical difficulties that need to be overcome are many. The hope is that, with the knowledge gained from other models, it may be possible to accelerate this process in humans.

REFERENCES

1. Baker TG. Oogenesis and ovulation. In: Austin CR, Short RV, eds. Reproduction in Mammals. 2nd ed. Cambridge: Cambridge University Press; 1982:17–45
2. Eppig JJ. Intercommunication between mammalian oocytes and companion somatic cells. Bioessays 1991;13:569–574
3. Gougeon A. Regulation of ovarian follicular development in primates: facts and hypotheses. Endocr Rev 1996;17: 121–155
4. Vendola K, Zhou J, Wang J, Famuyiw OA, Bievre M, Bondy CA. Androgens promote oocyte insulin-like growth factor I expression and initiation of follicle development in the primate ovary. Biol Reprod 1999;61:353–357
5. Hirshfield AN. Thecal cells may be present at the outset of follicular growth. Biol Reprod 1991;44:1157–1162
6. Eppig JJ, O'Brien M, Wigglesworth K. Mammalian oocyte growth and development in vitro. Mol Reprod Dev 1996; 44:260–273
7. Fortune JE. Ovarian follicular growth and development in mammals. Biol Reprod 1994;50:225–232
8. Gosden RG, Boland NI, Spears N, et al. The biology and technology of follicular oocyte development in vitro. Reprod Med Rev 1994;2:129–152
9. Hirshfield AN. Development of follicles in the mammalian ovary. Int Rev Cytol 1991;24:43–99
10. Hillier SG. Current concepts of the role of FSH and LH in folliculogenesis. Hum Reprod 1994;9:188–191
11. Armstrong DT, Dorrington JH. Estrogen biosynthesis in the ovary and testes. In: Thomas JA, Singhal RL, eds. Regulatory Mechanisms Affecting Gonadal Hormone Action. Vol 2. Baltimore: University Park Press; 1979:217–258
12. Udoff LC, Adashi EY. Autocrine/paracrine regulation of the ovarian follicle. Endocrinologist 1999;9:99–106
13. Armstrong DG, Webb R. Ovarian follicle dominance: the role of intraovarian growth factors and novel proteins. Rev Reprod 1997;2:139–146
14. Norman RJ, Brannstrom M. Cytokines in the ovary: pathophysiology and potential for pharmacological intervention. Pharmacol Ther 1996;69:219–236
15. Martinovitch PN. The development in-vitro of the mammalian gonad-ovary and ovogenesis. Proc R Soc Lond (Biol) 1938;125:232–249
16. Gregoraszccuk EL,Stoklosowa S, Wojtusiak A. Organ culture as a model of studying follicular development and function of postnatal mouse ovaries. Acta Biol Hung 1997; 48:431–438
17. Ryle M. Morphological responses to pituitary gonadotrophins by mouse ovaries in-vitro. J Reprod Fertil 1969;20: 307–312
18. Baker TG, Neal P. Gonadotrophin-induced maturation of mouse Graafian follicles in organ culture. In: Biggars JD, Schuetz AW, eds. Oogenesis. Baltimore: University Park Press; 1972:377–396
19. Lambertson CJ, Greenbaum DF, Wright KH, Wallach EE. In vitro studies of ovulation in the perfused rabbit ovary. Fertil Steril 1976;27:178–187
20. Channing CP, Tsafriri A. Mechanism of action of luteinizing hormone and follicle-stimulating hormone on the ovary in vitro. Metabolism 1977;26:413–468
21. Bonello N. Inhibition of nitric oxide: effects on IL-1 beta–enhanced ovulation rate, steroid hormones and ovarian leukocyte distribution at ovulation in the rat. Biol Reprod 1996;54:436–445
22. Abir R, Franks S, Mobberley MA, Moore PA, Margara RA, Winston RM. Mechanical isolation and in vitro growth of preantral and small antral human follicles. Fertil Steril 1997;68:682–688
23. Abir R, Roizman P, Fisch B, et al. Pilot study of isolated early human follicles cultured in collagen gels for 24 hours. Hum Reprod 1999;14:1299–1301
24. Fortune JE, Kito S, Byrd DD. Activation of primordial follicles in vitro. J Reprod Fertil Suppl 1999;54:439–448
25. Eppig JJ, O'Brien MJ. Development in vitro of mouse oocytes from primordial follicles. Biol Reprod 1996;54: 197–207
26. Yu N, Roy SK. Development of primordial and prenatal follicles from undifferentiated somatic cells and oocytes in the hamster prenatal ovary in vitro: effect of insulin. Biol Reprod 1999;61:1558–1567
27. Parrott JA, Skinner MK. Kit-ligand/stem cell factor induces primordial follicle development and initiates folliculogenesis. Endocrinology 1999;140:4262–4271
28. Blandeau R, Warrick E, Runery RE. In vitro cultivation of fetal mouse ovaries. Fertil Steril 1965;16:705–715
29. Hartshorne GM. Fetal ovarian tissue in vitro. Assist Reprod Rev 1996;6:72–82
30. Hovatta O, Silye R, Abir R, Krausz T, Winston RM. Extracellular matrix improves survival of both stored and fresh human primordial and primary ovarian follicles in long-term culture. Hum Reprod 1997;12:1032–1036
31. Wandji SA, Srsen V, Nathanielsz PW, Eppig JJ, Fortune JE. Initiation of growth of baboon primordial follicles in vitro. Hum Reprod 1997;12:1993–2001
32. Wright CS, Hovatta O, Margara R, et al. Effects of follicle-stimulating hormone and serum substitution on the in vitro growth of human ovarian follicles. Hum Reprod 1999; 14:1555–1562
33. Qu J, Godin PA, Nissolle M, Donnez J. Distribution and epidermal growth factor receptor expression of primordial follicles in human ovarian tissue before and after cryopreservation. Hum Reprod 2000;15:302–310
34. Liu J, Van Der Elst J, Van Den Broecke R, Dumortier F, Dhont M. Maturation of mouse primordial follicles by com-

80. Findlay JK. An update on the roles of inhibin, activin and follistatin as local regulators of folliculogenesis. Biol Reprod 1993;48:15–23
81. Smitz J, Cortvrindt R. Inhibin A and B secretion in mouse preantral follicle culture. Hum Reprod 1998;13:927–935
82. McGee EA, Chun SY, Lai S, He Y, Hsueh AJ. Keratinocyte growth factor promotes the survival, growth, and differentiation of preantral ovarian follicles. Fertil Steril 1999;71: 732–738
83. Li R, Phillips DM, Mather JP. Activin promotes ovarian follicle development in vitro. Endocrinology 1995;136:849–856
84. Smitz J, Cortvrindt R, Hu Y, Vanderstichele H. Effects of recombinant activin A on an in vitro culture of mouse preantral follicles. Mol Reprod Dev 1998;50:294–304
85. McGee EA, Smith R, Spears N, Nachtigal M, Ingraham H, Hsueh AJW. Mullerian inhibitory substance induces growth of preantral ovarian follicles. Biol Reprod 2001;64: 293–298
86. Yokota H, Yamada K, Liu X, et al. Paradoxical action of activin A on folliculogenesis in immature and adult mice. Endocrinology 1997;138:4572–4576
87. Parrott JA, Skinner MK. Developmental and hormonal regulation of KGF expression and action in the ovarian follicle. Endocrinology 1998;139:228–235
88. Mizunuma H, Liu X, Andoh K, et al. Activin from secondary follicles causes small preantral follicles to remain dormant at the resting stage. Endocrinology 1999;140:37–42
89. Hayashi M, McGee EA, Min G, et al. Recombinant growth differentiation factor-9 (GDF-9) enhances growth and differentiation of cultured early ovarian follicles. Endocrinology 1999;140:1236–1244
90. Spears N, de Bruin JP, Gosden RG. The establishment of follicular dominance in co-cultured mouse ovarian follicles. J Reprod Fertil 1996;106:1–6
91. Johnson LD, Albertini DF, McGinnis LK, Biggers JD. Chromatin organization, meiotic status and meiotic competence acquisition in mouse oocytes from cultured ovarian follicles. J Reprod Fertil 1995;104:277–284
92. Hartshorne GM, Sargent IL, Barlow DH. Meiotic progression of mouse oocytes throughout follicle growth and ovulation in vitro. Hum Reprod 1994;9:352–359
93. Merriman JA, Whittingham DG, Carroll J. The effect of follicle stimulating hormone and epidermal growth factor on the developmental capacity of in vitro matured mouse oocytes. Hum Reprod 1998;13:690–695
94. Eppig JJ, Wigglesworth K, O'Brien M. Comparison of embryonic developmental competence of mouse oocytes grown with and without serum. Mol Reprod Dev 1992;32: 33–40
95. Sicinski P, Donaher JL, Geig Y, et al. Cyclin D2 is an FSH-responsive gene in gonadal cell proliferation and oncogenesis. Nature 1996;384:470–474

A.M. was supported by MRC grant R33713.