The Influence of Germ Cell-specific *Dazl* on Follicle Growth and Development in Mice

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Declaration

Except where acknowledgement is made by reference, the experiments detailed in this thesis were the unaided work of the author. No part of this work has previously been accepted for any other degree, nor is any part of it being concurrently submitted in candidature for another degree.

Elaine Anne Watson
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Abbreviations

Ab	Antibody
ABC	Avidin Biotin Complex
ALK	Activin Receptor-Like Kinase
AMH	Anti-Mullerian Hormone (same as MIS)
ANOVA	Analysis of Variance
AR	Androgen Receptor
ArKO	Aromatase Knockout
ATP	Adenosine Triphosphate
AZF	Azoospermia Factor Region
Bcl-2	B-Cell Lymphoma-2
BDNF	Brain-Derived Neurotrophic Factor
BMP	Bone Morphogenetic Protein
BMPR	Bone Morphogenetic Protein Receptor
BSA	Bovine Serum Albumin
$CaCl_2$	Calcium Chloride
cAMP	cyclic Adenosine Monophosphate
CARD	Caspase Recruitment Domain
cDNA	Complementary DNA
CL	Corpus Luteum
CMC	Culture Media Control
CREB	CRE Binding Protein
DAB	Diaminobenzidine
DAGS	Donkey Anti-Goat Serum
DARS	Donkey Anti-Rabbit Serum
dH_20	Distilled Water
ddH_20	Double Distilled Water
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
Daz	Deleted in Azoospermia
Dazl	Deleted in Azoospermia-like Genes
DAZAP	Deleted in Azoospermia Associated Protein
DZIP	Deleted in Azoospermia Interacting Protein
DED	Death Effector Domain
DiI	Octadecyl Indocarbocyanines
DiO	Oxacarbocyanines
d.p.c	Days Post Coitum

E	Glutamic Acid
ECM	Extracellular Matrix
EDTA	Ethylene Diaminetetra-Acetic Acid
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Oestrogen Receptor
ERα	Oestrogen Receptor-α
ERβ	Oestrogen Receptor-β
Fig-α	Factor in the Germ Line-α
Fox	Forkhead Transcription Factor
FP	Follicles Pooled
FSH	Follicle Stimulating Hormone
FSHR	Follicle Stimulating Hormone Receptor
g	Gram
G	Glycine
GCIF	Granulosa Cell-Inhibitory Factor
GDF	Growth Differentiation Factor
GDP	Guanosine Diphosphate
GFP	Green Fluorescent Protein
GnRH	Gonadotrophin-Releasing Hormone
GPCR	G-Protein Coupled Receptor
GTP	Guanosine Triphosphate
H	Histidine
haf	Human Amniotic Fluid
hCG	Human Chorionic Gonadotrophin
HCl	Hydrochloric Acid
HDL	High Density Lipoprotein
H&E	Haematoxylin and Eosin
Het	Heterozygous
H_2O	Water
H_2O_2	Hydrogen Peroxide
HRP	Horse-Radish Peroxidase
3β-HSD	3β-hydroxysteroid dehydrogenase
IGF	Insulin-like Growth Factor
IGFBP	Insulin-like Growth Factor Binding Protein
IMS	Industrial Methylated Spirit
INSL3	Insulin-like 3 (Leydig Cells)
IU	International Unit
kDa	Kilodalton

KO	Knockout
L	Litre
LDL	Low Density Lipoprotein
LH	Luteinising Hormone
LHR	Luteinising Hormone Receptor
LOX	Lysyl Oxidase
M	Molar
MAPK	Mitogen-Activated Protein Kinase
α-MEM	α-Minimal Essential Medium
$MgCl_2$	Magnesium Chloride
MIS	Mullerian Inhibiting Substance
ml	Millilitre
mm	Millimetre
MMP	Matrix Metalloproteinase
MPF	Maturation-Promoting Factor
mRNA	Messenger Ribonucleic Acid
MS	Microsoft
MT	Membrane-type
mu	Mouse Uterus
mrv	Mouse Right Ventrical
Mvh	Mouse Vasa Homologue
MW	Molecular Weight Marker
n	Number
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
$NaH_2PO_42H_2O$	Sodium Dihydrogen Orthophosphate
$NaHPO_4$	Sodium Hydrogen Orthophosphate (Anhydrous)
NGF	Nerve Growth Factor
NOBOX	Newborn ovary homeobox-encoding gene
NSB	Non-Specific Binding
NT-4	Neurotrophin-4/5
OD	Optical Density
oFF	Ovine Follicular Fluid
p	Probability
PA	Plasminogen Activator
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PCOS	Polycystic Ovary Syndrome
PGC	Primordial Germ Cell

PKA	Protein Kinase A
PKB	Protein Kinase B
PMSG	Pregnant Mare Serum Gonadotrophin
PR	Progesterone Receptor
PUM2	Pumilio-2
QC	Quality Control
rpm	Revolutions per Minute
RNA	Ribonucleic Acid
RRM	RNA Recognition Motif
scc	Side-chain Cleavage
Scp3	Synaptonemal Complex Protein-3
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error of the Mean
SMAD	SMA and Mothers Against Decapentaplegic
Sry	Sex-determining Region Y
StAR	Steroidogenic Acute Regulatory Protein
TE	Tris-EDTA
TBE	Tris Borate EDTA
TBS	Tris Buffered Saline
TGF	Transforming Growth Factor
TIMP	Tissue Inhibitor of Matrix Metalloproteinases
TNF	Tumour Necrosis Factor
TrkA	Tyrosine Kinase-A
TrkB	Tyrosine Kinase-B
TSH	Thyroid-Stimulating Hormone
UT	Untreated
UV	Ultra Violet
v	Volts
v/v	Volume to Volume Ratio
VEGF	Vascular Endothelial Growth Factor
wt	Wild-Type
WT-1	Wilm's Tumour
w/v	Weight to Volume Ratio
$ZnCl_2$	Zinc Chloride
ZP	Zona Pellucida
µg	Microgram
µl	Microlitre
µm	Micrometre

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Abstract

The *DAZ (Deleted in Azoospermia)* gene is located on the human Y chromosome and is associated with male infertility in humans. An autosomal form of this gene (*DAZ-like (Dazl)*) is present in all mammals and is expressed in germ cells, the Dazl itself being an RNA binding protein. Transgenic homozygous *Dazl* knockout (KO) male and female mice are infertile due to loss of almost all germ cells in early neonatal life. However, in heterozygous (het) females there is a significant increase in ovulation rate and litter size in comparison to their homozygous wild type (wt) littermates, in spite of lower levels of plasma Follicle Stimulating Hormone (FSH) (McNeilly *et al.*, 2000), suggesting an alteration in follicle sensitivity to FSH.

The aims of this study were to assess follicle numbers in ovaries and follicle FSH-sensitivity in het mice compared to wt mice. The follicle reserve of untreated, 10IU FSH-treated and ovine Follicular Fluid (oFF)-treated day 21 pre-pubertal mice was assessed to determine whether variations in follicle reserve existed which resulted in increased litter sizes. The untreated het mice had a significantly greater percentage of antral follicles ($p<0.05$) compared to wt mice, and this difference was eliminated when endogenous FSH levels were suppressed by oFF treatment or FSH levels were increased by exogenous FSH treatment, suggesting greater FSH-sensitivity. Furthermore, antral follicles from untreated het mice had a greater granulosa cell area and follicle area. To determine FSH-sensitivity, follicles derived from day 21 *Dazl* wt and het mouse ovaries were cultured in the presence of different concentrations of recombinant human FSH and their growth and development compared. Individual pre-antral follicles (180-210μm) were micro-dissected and cultured for 6 days in 1, 0.1 or 0.01IU FSH. At all doses of FSH, follicles from the het mice were significantly larger than the follicles from the wt mice (1IU FSH $p<0.01$; 0.1IU FSH $p< 0.05$ and 0.01IU FSH $p<0.01$), whilst their endocrinology was not significantly different at 1IU and 0.1IU FSH. However, at 0.01IU FSH the follicles from the het mice secreted significantly greater levels of oestradiol ($p<0.001$) and inhibin A ($p<0.05$) suggesting greater maturity.

Co-cultures demonstrated that significant dominance occurred in follicles from wt ($p<0.01$) and het ($p<0.05$) mice when co-cultured in physical contact. However, when co-cultured physically separated, although one follicle became larger, this failed to reach significance. Follicles from wt and het mice co-cultured in physical contact resulted in one follicle becoming larger but this also failed to reach significance, although the larger follicle tended to be from a het mouse.

However, in contrast to the results when wt/wt or het/het combination of follicles were co-cultured, when het and wt follicles were cultured together, but physically separated, one follicle did become significantly larger ($p<0.05$), and follicles from het mice were significantly more likely to become the dominant follicle ($p<0.01$).

Immunohistochemistry demonstrated that there was no difference in levels of staining for Anti-mullerian hormone (AMH) and cleaved caspase-3 in primary, secondary and antral follicles from untreated, FSH-treated and oFF-treated wt and het mice. However, there were significantly more secondary follicles from het FSH-treated mice which stained positively for AMH but were negative for cleaved caspase-3 ($p<0.01$) (i.e. healthy), and less that stained negative for AMH and were positive for cleaved caspase-3 ($p<0.01$) (i.e. potentially becoming atretic), than wt. Therefore, while follicle activation from the reserve pool and atresia rates did not differ overall between follicles from wt and het mice in untreated and oFF-treated mice, there was evidence of a greater activation or maintenance of healthy follicles after FSH treatment in het mice.

In conclusion, the present studies suggest that, although *Dazl* is an oocyte-specific gene, the putative protein(s) that *Dazl* affects through RNA binding in the oocyte targets granulosa cells. The influence of *Dazl* on follicle development occurs through the single copy of *Dazl* enhancing FSH sensitivity, but the exact mechanism of *Dazl* on the granulosa cells is unknown. The studies presented in this thesis suggest that follicles from the *Dazl* het mice have accelerated growth because of a reduced threshold for FSH that allows them to remain for a longer duration within the critical FSH threshold for follicle growth. In addition, this study suggests that the percentage of healthy and atretic follicles in the *Dazl* wt and het mice is similar in untreated and oFF-treated mice, but that after FSH treatment there are more healthy follicles in het mice. It could be concluded that, in the adult after follicle activation and recruitment into the pool of growing follicles, a single copy of *Dazl* results in maintained follicle growth, because of increased FSH-sensitivity. Thus, more larger follicles achieve dominance and these subsequently ovulate, leading to an increased litter size.

Chapter 1

General Introduction

1.1 Introduction

During gestation in humans there are approximately seven million germ cells in the fetal ovary. However, by birth this number will have reduced to approximately two million (Baker, 1963). This finite number of oocytes gradually depletes until there are no oocytes left and the menopause is experienced (Gosden and Faddy, 1994; Hillier, 1994). From the two million oocytes at birth only approximately 400 will ovulate, with the remaining 99.98% of follicles becoming atretic (Hillier, 1994). In rodents a similar reduction in germ cells occurs. However, the primordial follicle pool is not fully complete until a few days after birth (Borum, 1961; Rugh, 1990). This drastic reduction in oocytes is postulated to be the mechanism ensuring that the oocytes reaching ovulation are the healthiest, and additionally this process, coupled with recruitment, selection and follicle dominance, is thought to prevent ovulation rates exceeding the average for the species (Tilly, 2001; Tajima *et al.,* 2002; Manabe *et al.,* 2004).

The studies in this thesis arose from previous work carried out on the *Deleted in Azoospermia-like* (*Dazl*) mouse model. This model demonstrated both male and female mice were infertile shortly after birth (Ruggiu *et al.*, 1997; McNeilly *et al.,* 2000) and in female adults the *Dazl* heterozygous (het) mice had increased litter sizes compared to wild-type (wt) mice (McNeilly *et al.,* 2000; McNeilly *et al.,* Unpublished (Appendix A)). The work outlined in this thesis concentrates on the effect of one copy of the *Dazl* gene compared to two copies of the gene on ovarian follicle growth and development.

The follicle reserve provides the life-time supply of oocytes and therefore this reserve and the rate of depletion is critical for the reproductive lifespan. Although recent studies have proposed that oocyte renewal occurs throughout life in the mammal (Johnson *et al.,* 2004; Johnson *et al.,* 2005a). The follicle reserve in untreated *Dazl* wt and het mice were analysed to determine if one copy of the *Dazl* gene affected the size of the reserve or the rate of follicle activation compared to two copies of the gene. Additionally, treatment of mice *in vivo* with follicle stimulating hormone (FSH) or ovine follicular fluid (oFF) demonstrated the effects of increased or decreased circulating FSH and its effects on follicle growth and development. The rate of follicle activation and atresia are important in the rate of depletion of the follicle reserve. Follicle activation and atresia in the *Dazl* wt and het mice was studied by examining anti-mullerian hormone (Amh) expression and Cleaved Caspase-3 activity in untreated, FSH-treated and oFF-treated mice.

This thesis also examined the levels of matrix metalloproteinase-2 and -9 (MMP) as the extracellular matrix (ECM) is important in follicle atresia. Additionally, follicle selection and dominance is important in regulating ovulation rates and this thesis reported on this through co-culturing follicles from *Dazl* wt and het mice.

Although the effect of *DAZL* in humans is of importance the mouse model provides an ideal system to fully determine the effects of this gene on follicle growth and endocrinology under controlled conditions. FSH is vital for follicle growth beyond the early antral stage of development and stimulates oestradiol, inhibin and activin levels which, in turn, regulate FSH secretion (Figure 1.1; Zeleznik *et al.,* 1985; Hillier, 1994; Cortvrindt *et al.,* 1997). Luteinising Hormone (LH) is also essential for oestradiol production and for the latter stages of follicle maturation, progesterone production and ovulation (Hillier, 1994; Xu *et al.,* 1995). In addition, the endocrine system is fundamental for regulating follicle development and many aspects were analysed in this thesis.

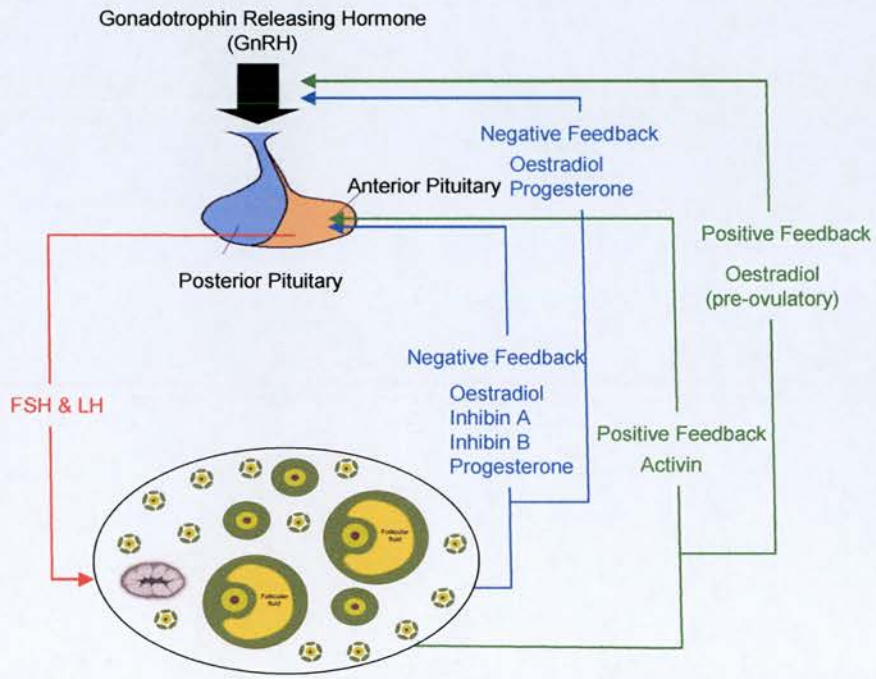


Figure 1.1 The ovarian feedback system. (Pituitary image from Wilkipedia and corpus luteum from www.nelsonginecologia.med.br).

1.2 Follicle Development: an Overview

1.2.1 Cellular and Molecular Mechanisms Regulating Initiation and Maintenance of Primordial Oocyte and Follicle Growth and Meiotic Progression

The regulation of the initiation and maintenance of the ovarian follicles requires fine control and co-ordination between germ and somatic cells (McLaren and Buehr, 1990; Eppig *et al.,* 2002). Without such regulation of follicle formation and activation, the ovarian reserve which provides a female with her lifetime supply of follicles would be incompletely formed and potentially diminished at an accelerated loss. There are various candidate genes which play an important role in germ and somatic cell development and these will be discussed below.

Oogenesis is the formation of gametes within the ovaries. During early fetal development germ cells move from the yolk sac epithelium into the developing ovaries (Picton, 2001). Primordial germ cells (PGCs), called oogonia, develop into oocytes in the early stages of fetal ovary development (Ginsburg *et al.,* 1990; Rugh, 1990). The oogonia grow and progress through meiosis prophase 1 (leptotene – pachytene) when they are 'primary oocytes'. Primary oocytes arrest in meiotic prophase 1 (diplotene stage) and can remain in this stage for up to fifty years in the human (Borum, 1961; Picton, 2001). The maximum number of germ cells in the mouse is at embryonic day 11.5. Thereafter, this number rapidly declines (Nakatsuji and Chuma, 2001). Oocyte formation and development is dependent upon several oocyte factors such as *Factor in the Germ Line-α* (*Fig-α or FIGLA*) (Liang *et al.,* 1997; Soyal *et al.,* 2000).

1.2.1.1 Primordial Germ Cell (PGC) Formation and Migration

Mouse gestation takes approximately three weeks. During this time the PGCs form from embryonic cells which originate from the epiblast (McLaren, 1992). Murine germ cells develop at the epiblast where the extra-embryonic ectoderm, primitive streak, allantois and epiblast meet (Tam and Zhou, 1996; Gilbert, 2003). Embryonic day 7 marks the formation of PGC in the mouse (Ginsburg *et al.,* 1990). The PGCs then migrate to the endoderm from the primitive streak, where after, the PGCs migrate towards the genital ridge (embryonic day 10.5; McLaren and Buehr, 1990). This migration occurs through 'amoeboid-like movement' and 'passive transfer' (McLaren, 2000; Hutt *et al.*, 2006b). Once within the genital ridge the PGC present multiply (McLaren, 2000). PGC multiplication takes place over a 2-3 day period from approximately 40 cells to 24,000 cells. The duration of each PGC multiplication takes approximately 16 hours (Tam and Snow, 1981; McLaren, 2000).

At the genital ridge sex cords develop which consist of germline cysts (Bristol-Gould *et al.,* 2006a). These cysts consist of groups of germ cells (oogonia) which soon begin to enter meiosis (and subsequently remain at the diplotene stage just prior to birth) and between 20.5 and 22.5 days post coitum (d.p.c) these cysts breakdown (Pepling and Spradling, 1998; Pepling and Spradling, 2001). This breakdown occurs as somatic cells aid the breakdown of the cysts forming primordial follicles (Pepling and Spradling, 2001).

The somatic cells (granulosa and theca cells) are also essential for normal follicle development (as discussed in Section 1.10) (Eppig *et al.,* 2002). *Kit* Ligand and its receptor c-*Kit* are expressed in the granulosa cell and oocyte respectively (Motro and Bernstein, 1993). Both of these are expressed during PGC migration, and animals with natural mutations (for example, the *white spotting* loci mutation which encodes c-*Kit* and *Steel* loci mutation which encodes *Kit* Ligand) experience abnormal PGC migration (Mahakali Zama *et al.,* 2005; Hutt *et al.,* 2006b).

1.2.2 Meiotic Progression

During follicle development oocytes remain arrested in the diplotene stage of meiosis until 9-10 hours before ovulation (Rugh, 1990). The correct balance of cyclic adenosine monophosphate (cAMP) levels is essential for maintaining meiosis with high levels of cAMP having an inhibitory effect on recommencement of meiosis (Lamb *et al.,* 1991; Richard, 2006). When oocytes from *Xenopus* were treated with progesterone cAMP levels declined (Maller *et al.,* 1979) and therefore treatment with such hormones could stimulate meiotic progression. In the mouse progesterone treatment does not effect oocyte maturation (Batten *et al.,* 1989).

Other growth factors involved in regulating meiotic progression are*: Msh4/5, Dmc1, Atm, Spo11,* epidermal growth factor (EGF), *Leydig and Insulin-like 3* (*INSL3*) (Epifano and Dean, 2002; Richard, 2006). *Msh4/5, Dmc1, Atm* and *Spo11* are all genes that are important in meiosis and any mutations of these genes causes infertility in animals due to depletion of the oocytes supply in the ovary (Epifano and Dean, 2002). These genes are involved in various stages of meiosis from the double-strand breaks until during chromosome crossover and mutations in any of these proteins results in infertility (Villeneuve and Hillers, 2001; Barnett *et al.,* 2006).

Insl3 is a member of the insulin family and is expressed within the theca cells within the ovarian follicle and is promoted by LH (Kawamura *et al.,* 2004; Richard, 2006). Treatment of rat oocytes with *Insl3* stimulates meiotic progression by decreasing cAMP production (Kawamura *et al.,* 2004). Furthermore, EGF also stimulates meiotic progression even in follicles that are in the early stages of antral development and this effect was inhibited by treatment with cAMP (Dekel and Sherizly, 1985). These studies demonstrate that cAMP plays an important role in the maintenance of meiosis progression through regulation from factors such as progesterone, LH and Insl3 (Dekel and Sherizly, 1985; Kawamura *et al.,* 2004; Richard, 2006). Gap junctions provide the communication channel for which cAMP produced by granulosa cells transports to the oocyte (Heikinheimo and Gibbons, 1998). Low levels of cAMP increases the activity of the oocyte Maturation-Promoting Factor (MPF) and stimulates meiosis (Kovo *et al.,* 2006).

1.2.3 Initiation of Primordial Oocyte and Follicles

Primordial follicles form before or shortly after birth in the mouse when the somatic cells surround the oocyte (Borum, 1961; Rugh, 1990). In humans these follicles can remain in this resting state for up to fifty years (Picton *et al.*, 1998). However, it is not known what factor(s) activates these follicles from the ovarian reserve, although many factors have been identified that can activate primordial follicles. Oocyte growth is one marker of primordial follicle activation as the oocyte grows in the early stages of development, whereas granulosa cell proliferation and antral fluid are responsible for follicle size in the latter stages of development (Hirshfield, 1991).

1.2.4 Candidate Genes and Growth Factors from Germ Cells and Somatic Cells

Newborn Ovary Homeobox-Encoding gene (Nobox) is expressed within germ cell cysts and primordial and growing oocytes. When this gene was disrupted, heterozygous mice had normal morphology whereas *Nobox* null mice were infertile with no oocytes present at week 6 (Suzumori *et al.,* 2002; Rajkovic *et al.,* 2004). These *Nobox* knockout (KO) mice demonstrated increased oocyte loss after birth and also primordial follicle activation was prevented signifying its importance in oocyte and follicle initiation (Rajkovic *et al.,* 2004). Furthermore, animals lacking *Nobox* also experienced reduced levels of other genes which are fundamental for oocyte and follicle development such as *Growth Differentiation Factor-9* (*Gdf-9*) (Rajkovic *et al.,* 2004). More recent studies have demonstrated that *Nobox* could potentially regulate the transcription of *Gdf-9* in mice as *Gdf-9* contains a region to which Nobox can bind (Choi and Rajkovic, 2006). Similarly in the *Dazl* KO mice the females are infertile by day 5 whereas heterozygous females have increased litters compared to wt females (Ruggiu *et al.,* 1997; McNeilly *et al.,* 2000; McNeilly, Unpublished (Appendix A and B)). This demonstrates the importance of *Dazl* in PGC formation.

PGC formation is reliant upon genes such as *Bone Morphogenetic Protein-4* (*Bmp-4*) and *Bmp-8b* working concurrently (Ying *et* al., 2000; Ying *et al.,* 2001). Studies of *Bmp-4* and *Bmp-8b* KO mice have demonstrated that these animals have no or decreased numbers of PGCs and animals heterozygous for *Bmp-4* have lower numbers of PGCs compared to controls (Lawson *et al.,* 1999; Ying *et al.,* 2000; Ying *et al.,* 2001), indicating that *Bmp-4* and *Bmp-8b* are fundamental for PGC formation. However, although such members of the Transforming Growth Factor-β (TGF-β) family are important in ensuring the correct environment for follicle formation and development (Ying *et al.,* 2001) the signalling pathway has also been found to be essential (Kaivo-oja *et al.*, 2006). SMA and Mothers Against Decapentaplegic Smad-2 and Smad-3 are involved the signalling pathways used by both GDF-9 and activin (Kaivo-oja *et al.*, 2006). *Smad-3*-mutated mice have impaired fertility, with a normal ovarian reserve compared to controls but with fewer activated follicles, suggesting that *Smad-3* is fundamental for follicle activation (Tomic *et al.,* 2002; Kaivo-oja *et al.*, 2006). In addition, as discussed in Section 1.8.3 Amh is another member of the TGF-β family which is important in follicle growth initiation (Durlinger *et al.,* 1999; McGee *et al.,* 2001).

Another germ cell-specific transcription, *Fig-α*, has been found to be essential for follicle formation in both the human and the mouse (Liang *et al.,* 1997; Soyal *et al.*, 2000; Huntriss *et* al., 2002; Bayne *et al.*, 2004) with messenger mRNA (mRNA) expression increasing at the time primordial follicle formation occurs (Bayne *et al.*, 2004). Mice lacking *Fig-α* had no primordial follicles and it is suggested infertility occurs as a result of failure of primordial follicle formation (Liang *et al.*, 1997; Soyal *et al.,* 2000). Furthermore, *Fig-α* (possibly together with other factors) has also been shown to play a role in synchronizing the expression of zona pellucida (*ZP*) genes *Zp1*, *Zp2*, and *Zp3* which are essential for fertilisation (Liang *et al.*, 1997; Soyal *et al.*, 2000). *In vitro Fig-α* can bind to the E box of *Zp1*, *Zp2*, and *Zp3* promoters and in turn affect their expression (Liang *et al.*, 1997; Bayne *et al.*, 2004). The importance of mutations of the *Zp* genes can be demonstrated by the *Zp3* null mice, which are infertile although oocyte maturation is normal (Picton *et al.,* 1998). Similarly, *Zp2* null mice are also infertile. However, *Zp1* mice have impaired fertility (Rankin *et al.,* 2001; Barnett *et al.,* 2006).

Oct-4 is another transcription factor and expression is localised in the mouse embryo to the germ cells it is postulated to play a role in regulating early follicle formation and development (Ovitt and Scholer, 1998; Pesce *et al.,* 1999; Pesce and Scholer, 2000; Epifano and Dean, 2002). As *Oct-4* levels decline somatic cells begin to differentiate from totipotent cells during fetal development (Pesce and Scholer, 2000; Pesce and Scholer, 2001). Additionally, murine Oct-4 mRNA expression decreases as the oocyte matures, especially after fertilisation (Ovitt and Scholer, 1998).

Nerve growth factor (*NGF*) is expressed within the oocyte and granulosa cells of primordial follicles in the human fetus and their receptor tyrosine kinase-A (TrkA) is expressed mainly in the oocyte with some expression in the granulosa cells (Abir *et al.,* 2005). This growth factor has also been found to be required for the breakdown of the germline cysts and mice deficient in this factor have reduced numbers of activated follicles (Dissen *et al.,* 2001; Barnett *et al.*, 2006). Therefore, this factor is essential in follicle initiation and is involved in PGC formation.

The role of the oocyte-somatic cell interaction is also essential for primordial follicle development and initiation. Studies that blocked post-natal murine *Kit* Ligand and c-*Kit* communication observed inhibited primordial follicle development and follicle activation form the ovarian reserve, but did not affect follicle formation (Yoshida *et al.*, 1997). Furthermore, *in vitro* studies of cultured rat ovaries demonstrated that when treated with *Kit* Ligand the number of activated follicles (primary to pre-antral) was greater compared to controlled ovaries (Parrott and Skinner, 1999). However, a more recent study by Hutt *et al.*, (2006a) repeated a similar *in vitro* study where rabbit and mouse ovarian strips were cultured with and without recombinant mouse *Kit* Ligand. The results concluded that *Kit* Ligand is fundamental in follicle activation in the mouse where it stimulated this process whereas it did not in the rabbit, suggesting species differences (Hutt *et al.,* 2006a). *Forkhead Transcription Factor-2* (*Foxl2*) *and Foxl3* are also important factors in follicle activation with follicles unable to develop beyond the primordial stage of development in *Foxl2* KO mice whereas in *Foxl3* KO mice follicle activation is stimulated (Barnett *et al.,* 2006). In addition, *Fox03* KO mice have increased rates of follicle activation (Castrillon *et al.,* 2003).

Other factors that regulate primordial oocyte and follicle formation include; Neurotrophin-4/5 (NT-4) and brain-derived neurotrophic factor (BDNF) which signal through the tyrosine kinase-B (TrkB) receptors are also pivotal for follicle activation (Paredes *et al.,* 2004; Barnett *et al.,* 2006). Previous studies in fetal human ovaries observed the majority of NT-4 expression is localised in the oogonia (Anderson *et al.,* 2002). Further studies demonstrated approximately half of *TrkB* KO mice had abnormal ovary morphology with reduced numbers of oocytes and *in vitro* studies also observed that survival of oocytes in primordial follicles was inhibited when treated with antibodies that blocked NT-4 and BDNF (Spears *et al.,* 2003). These studies all suggest that both NT-4 and BDNF and the TrkB receptor are important for oocyte and follicle formation, survival and development (Anderson *et al.,* 2002; Spears *et al.,* 2003).

Somatic cells are also essential for ovarian development as it is such cells that are involved in regulating sexual differentiation (Vainio *et al.*, 1999; McLaren, 2000). Wilm's tumour (WT-1) is a repressor protein which is central for ovarian development (Kreidberg *et al.,* 1993; Logan *et al.*, 2003). When the *Wt-1* gene is disrupted the genital ridge deteriorates (Kreidberg *et al.,* 1993; Epifano and Dean, 2002) and therefore will prevent gonad development. Similarly, *Wnt-4* gene a member of the *Wnt* family has also been shown to be important in oocyte formation as in mutant *Wnt-4* mice, the ovaries have at least a 90% reduction in oocyte numbers compared to controls (Vainio *et al.*, 1999). This gene is expressed in the mesenchyme at 11d.p.c. However, at 11.5d.p.c when sexual differentiation occurs, *Wnt-4* levels declines in males and continues to be expressed in the developing ovary (Vainio *et al.*, 1999). Furthermore, lack of expression of the *Sex-determining Region Y* (*Sry*) gene between 10.5-11.5d.p.c in the somatic cells enables the embryo to develop into a female (Koopman *et al.,* 1990; Hacker *et al.,* 1995).

1.2.5 Follicle Growth and Initiation: Pre-antral Development

Shortly after birth in the mouse, oocytes have entered the diplotene stage of prophase 1 of meiosis and are arrested at this stage (Borum, 1961). Once in this quiescent state, each oocyte is surrounded by flattened granulosa cells and is then referred to as a primordial follicle (Borum, 1961; Gougeon, 1996). The arrest of meiosis is maintained by cAMP, possibly through the protein kinase A (PKA) pathway (Downs and Hunzicker-Dunn, 1995). A reduction of cAMP is associated with the onset of meiosis (Conti *et al.,* 2002). Oocytes remain arrested at this stage of meiosis until the PKA pathway is no longer stimulated (Kovo *et al.,* 2006) after the gonadotrophin surge.

A follicle is referred to as "transitionary or intermediary" when some granulosa cells are cuboidal whilst others remain flattened. Such follicles are still regarded as being in the resting follicle pool (Gougeon and Chainy, 1987; Gougeon, 1996; Smitz and Cortvrindt, 2002). Primordial and transitionary follicles are located in small clusters in the outer region of the ovarian cortex, whereas growing follicles (primary-antral) are located in the cortico-medullary region which is highly vascularised (Peters, 1969; Picton, 2001). Each day a number of follicles are activated from the follicle reserve. More follicles are activated than will ultimately ovulate and a number of these will be lost during development with the number of oocytes ovulating, depending upon the species and strain (Gougeon, 1996; Baker and Spears, 1999). Which factors trigger the initial development of primordial follicles to primary follicles is unknown, although factors such as AMH, progesterone and oestradiol inhibit this recruitment process (Durlinger *et al.,* 1999; Kezele *et al.,* 2002; Kezele and Skinner, 2003; Tremellen *et al.*, 2005). Oestradiol and progesterone treatment *in vitro* halved the number of primordial rat follicles activated compared to controls (Kezele and Skinner, 2003).

Follicle initiation occurs throughout life, although during the pre-pubertal years, when plasma FSH and LH are at a basal level, follicles only reach the early antral stage, beyond which increased gonadotrophin levels are required for stimulation. This was demonstrated in FSHβ-KO and Follicle Stimulating Hormone Receptor (FSHR) KO mice where follicle growth was inhibited and no pre-ovulatory follicles were present (Hillier, 1994; Abel *et al.,* 2003). However, once puberty is reached, follicles are able to fully develop due to stimulation from FSH and LH released from the anterior pituitary (Hillier, 1994).

Pre-antral development is a slow process which takes approximately 10 days in mice. Graafian follicles are observed by day 21 (Picton, 2001). Oocyte growth is the earliest recognisable event in follicle growth and, once begun, the granulosa cells become cuboidal in shape. When an oocyte is encapsulated by a single layer of these 'cuboidal' granulosa cells, it is referred to as a primary follicle (Mandl and Zuckerman, 1952; Gougeon, 1996). At this stage, granulosa cell proliferation accelerates and a basement membrane forms around the outer layer of the granulosa cells. Stromal cells are recruited to the basement membrane where they form the theca layer (Peters, 1969; Senbon *et al.*, 2003). However, at present what initiates the development of the theca layer is unknown. The granulosa cells of a primary follicle continue to proliferate, increasing the follicle size until there are several layers of granulosa cells (Pedersen and Peters, 1968).

The oocyte secretes proteins forming the ZP, which separates the oocyte from the granulosa cells (Bleil and Wassarman, 1980). The formation of the ZP is observed in primary follicles (Odor and Blandau, 1969). The ZP also provides an Extracellular Matrix (ECM) which anchors the cumulus granulosa cells (Richards, 2001a; Fortune *et al.,* 2001; Picton, 2001). This stage of follicle development can occur independently of gonadotrophins (Kumar *et al.,* 1997) but follicle development can be stimulated by gonadotrophins since they are capable of responding to FSH as FSHRs are present at this stage (O'Shaughnessy *et al.,* 1996; Durlinger *et al.*, 2001).

Early follicular growth is characterised by granulosa cell proliferation. However, oocyte growth accelerates and contributes significantly to overall follicle size at this stage. Oocyte growth in rodents initiates when the oocyte is surrounded by more than 9 granulosa cells (Lintern-Moore and Moore, 1979; Picton, 2001). Oocyte-granulosa cell interactions regulate primordial follicle development and growth and involve TGF-β family members, such as *GDF-9, BMP-15, AMH* and inhibin A and B (Durlinger *et al.*, 1999; Knight and Glister, 2003; Webb *et al.,* 2003). However, although granulosa cell proliferation requires factors secreted from the oocyte (such as Gdf-9), oocyte growth and development is in turn dependent on the granulosa cells surrounding it (Buccione *et al.,* 1990; Eppig *et al.*, 2002).

Once an oocyte has acquired more than one layer of granulosa cells surrounding it, the follicle is referred to as a secondary follicle (Gougeon, 1996; Eppig, 2001). Follicles in mice begin to reach this stage at 10-12 days after birth (Matzuk *et al.*, 2002). During this growth phase, the follicles move to the ovarian medulla, and the theca develops into two distinct layers, the theca *interna* and theca *externa*. The theca interna is required for vascularisation to enable further follicle development (Rugh, 1990; Gougeon, 1996; Smitz and Cortvrindt, 2002). At this stage of development of 'full length' gonadotrophin receptors are expressed in the follicle (O'Shaughnessy *et al.*, 1997).

1.2.6 Follicular Antral Development

As the granulosa cells proliferate, they secrete a viscous fluid containing varying levels of hormones and proteins which begins to accumulate between granulosa cells and eventually forms a single follicular antrum (McNatty *et al.*, 1975; De Jong and Sharpe, 1976). Mouse follicles begin to reach this stage of development 14-24 days after birth (Matzuk *et al.*, 2002). Early antral follicles have small patches of follicular fluid between granulosa cells and once these patches of follicular fluid have amalgamated the follicles are referred to as antral follicles (Hirshfield, 1991; Smitz and Cortvrindt, 2002). Antral fluid accumulation within the follicle contributes significantly to follicle growth (Hirshfield, 1989; Hirshfield, 1991).

In mice the oocyte reaches its maximum diameter and volume when the follicle contains an antral cavity (Hirshfield, 1989; Hirshfield, 1991). At this point, the oocyte is nearly fully developed, although it is still secreting proteins and regulating the expression of receptors (such as Luteinising Hormone Receptor (LHR)) that are vital for future follicle development (Eppig *et al.*, 1997b; Yoshida *et al.*, 1997; Eppig, 2001). It is at this stage that oocytes acquire the capacity to resume meiosis from dictiate phase (prophase) (Sorensen and Wassarman, 1976; Rugh, 1990). Antral cavity formation requires FSH, marking the beginning of the gonadotrophin-dependent phase. In this phase FSH is essential for further follicle growth and development, this is demonstrated by FSHβ-KO and FSHR-KO female mice ovaries which only contain pre-antral follicles (Abel *et al.*, 2003).

FSH stimulates mitosis of granulosa cells and this action is modulated by local factors, which themselves are stimulated or inhibited by FSH (Hillier, 1994; Cortvrindt *et al.*, 1997). For example, FSH stimulates GC proliferation and oestradiol synthesis which in turn inhibits FSH secretion at the pituitary (Zeleznik *et al.*, 1985). The granulosa cells continue proliferating and differentiating rapidly, forming two distinct populations of cells. The oocyte is surrounded by cumulus cells which are themselves surrounded by follicular fluid. The mural granulosa cells are located nearest the basement membrane (Peters, 1969; Canipari, 2000).

The mural granulosa cells located nearest the antral cavity are referred to as peri-antral granulosa cells (Eppig, 2001). The formation of follicular fluid separates the mural and cumulus granulosa cells both physically and functionally (Matzuk *et al.*, 2002). In the final stages of follicular development, the mural granulosa cells cease proliferating, whereas the cumulus granulosa cells continue proliferating until ovulation occurs (Hirshfield, 1986; Vanderhyden *et al.*, 1992).

Follicle growth and development require both endogenous hormones and intra-ovarian factors that are produced locally, and act locally on their receptors (Adashi, 1994). Cumulus granulosa cells stimulated by the gonadotrophin surge secrete hyaluronic acid which is important for cumulus expansion. Mural granulosa cells differentiate after ovulation to become luteal cells (Eppig, 1979; Vanderhyden *et al.*, 1992). Rat granulosa cell P450 side chain cleavage (scc) expression was greatest in the granulosa cells at the basement membrane and expression decreased the closer the cells were to the antrum (Zlotkin *et al.*, 1986). LHR, P450scc and P450 aromatase (CYP19) are all expressed at higher levels in mural granulosa cells than in cumulus cells (Amsterdam *et al.*, 1975; Zlotkin *et al.*, 1986; Eppig *et al.*, 1997b). At this stage of development the mouse follicle reaches an average diameter of 600µm, with an oocyte averaging 80µm in diameter, surrounded by approximately 50-60,000 granulosa cells (Picton, 2001; Wassarman, 2002).

1.3 Ovarian Follicle Reserve – Are the Oocytes Replenished During Life?

Prior to the 1950s there were a number of studies suggesting that the ovarian follicle pool was replenished, and that oocyte renewal occurred every menstrual cycle (Allen, 1923). Other studies in the 1950s provided substantial evidence that the follicle pool was finite (Zuckerman, 1951). The subject of ovarian follicle reserve has been a 'central dogma' since the 1950s. This states that the number of oocytes present at, or shortly after birth, is finite and there is no further formation of oocytes at any stage during later life (Baker, 1963). In humans at 5 months post fertilisation, there are approximately $7x10^6$ germ cells, but by birth this is already reduced to $2x10^6$ cells (Baker, 1963). This finite resource of oocytes is rapidly reduced by follicle atresia, with 99.9% of follicles undergoing atresia throughout the reproductive lifespan (Hillier, 1994; Richards, 1994). Reduced follicle numbers are thus signs of an aging ovary (Jones and Krohn, 1961; Peters, 1969).

However, in 2004 the dogma of a fixed ovarian reserve was challenged by a study suggesting that some germ cells within the ovary replenished the declining number of ovarian follicles throughout life (Johnson *et al.,* 2004), and that, as in the testis, postnatal renewal occurs in the ovary. The initial study examined oocyte 'atresia and clearance' rates and suggested that germ cell renewal occurred accounting for 77 new follicles per day (Johnson *et al.,* 2004). The estimate of follicle loss per day due to atresia was based on a mathematical model devised by Faddy *et al.* (1987) that examined follicle development and atresia rates in mice and demonstrated that follicle depletion was high in the first 20 days in CBA/Ca mice (Faddy *et al.,* 1987; Johnson *et al.*, 2004). However, the strain of mouse used in Johnson's study was not the same as in Faddy's study (Byskov *et al.*, 2005) and previous studies have shown that there are great variations between mouse strains and follicle reserve (Canning *et al.*, 2003). Furthermore the study on the atresia rates of follicles carried out by Johnson *et al.* (2004) has also been under scrutiny (Telfer *et al.,* 2005). Classifying follicles as 'atretic' is complex and involves analysis of both granulosa and oocyte morphology. Johnson *et al.* (2004) only examined oocyte morphology and defined an atretic follicle as one where the oocyte was 'convoluted, condensed or fragmented' (Ratts *et al.,* 1995; Matikainen *et al.,* 2001; Byskov *et al.*, 2005). In addition, although a follicle is classified as atretic, it does not necessarily mean it is not functional. Previous studies have demonstrated that although follicles are showing signs of atresia they are still capable of functioning normally (Hirshfield, 1989).

The authors proposed that these 'germ stem cells' are present on the surface epithelium of the ovary (Johnson *et al.,* 2004), although later it was proposed the bone marrow was the source of these germ cells (Johnson *et al.,* 2005a). Similar histological findings to Johnson *et al.* (2004) were observed as far back as 1969 in a study that observed small atretic oocytes were present in the ovarian surface epithelium. It was suggested that small oocytes might penetrate through the surface epithelium of the ovary into the 'peri-ovarian space' during infancy (Peters, 1969). What causes the epithelium to separate allowing the oocytes through remains unknown (Peters, 1969) or whether this actually happens is a matter of conjecture.

In order to determine whether germline stem cells were present in the mouse ovary the expression of mouse *Synaptonemal Complex Protein-3* (*Scp3*) was studied as this gene was stated as being only expressed in the initial stages of meiosis (Johnson *et al.*, 2004). However, previous studies demonstrated that *Scp3* was still expressed to some extent in female mice at postnatal day 7 and therefore suggests that this is not solely expressed in the early stages of meiosis (Hodges *et al,* 2001; Byskov *et al.*, 2005). If germline stem cells do exist in adult mice why does the ovarian reserve decline and become exhausted as stem cells do not normally lose their function (Byskov *et al.*, 2005)?

Further studies suggested that the bone marrow functions as a source of germ cells and these are delivered to the ovary in the circulation. Peripheral blood and bone marrow transplants were capable of generating oocytes in mice where the ovarian reserve had been diminished through induced oocyte death (Johnson *et al.*, 2005a). This study met with much criticism as it did not establish whether oocytes could recover after treatment with doxorubicin. In addition, the time required for oocyte regeneration was far faster (within a 24 hour period) than the time required for normal oogenesis in the mouse, implying that oocytes can recover following treatment with doxorubicin (Johnson *et al.,* 2005a; Johnson *et al.,* 2005b; Telfer *et al.*, 2005).

To further examine the theory of bone marrow as a source of new germ cells which are delivered to the ovary by the peripheral blood studies were carried out by other laboratories. One study examined parabiosis of animals, a process whereby two mice are surgically joined and develop a 'common' blood system. This study examined mice labelled with the green fluorescent marker GFP joined to mice which were not labelled (Eggan *et al.,* 2006) to determine whether blood can play a part in ovarian function in the same strain of mice (C57BL/6) used by the Johnson *et al.* (2004 and 2005a) studies (Eggan *et al.*, 2006). If the bone marrow was the source of germ cells, it would be expected that mice which express GFP paired with non-GFP mice would contain oocytes with the genetic background of the other animal (Eggan *et al.*, 2006; Powell, 2006). However, this was not the case. Furthermore, after oocyte retrieval the phenotype of these oocytes was consistent with that of the animals from which they were derived (Eggan *et al.*, 2006). These studies are not directly comparable, since one study examined immature oocytes (Johnson *et al.*, 2005a) whereas the other examined ovulated oocytes (Eggan *et al.*, 2006). Many would argue that this is not important, as small oocytes will develop to become ovulated oocytes (Powell, 2006). However, Johnson *et al.* (2004 and 2005a) have stated that they are unsure whether observed regenerated oocytes in this particular mouse strain (C57BL/6) are capable of ovulating, and have postulated that the oocytes may simply regulate the hormone balance of the ovarian cycle (Powell, 2006).

Furthermore, Eggan *et al.* (2006) also demonstrated that, after intensive treatment with both cytoxan and busulfan, the ovarian reserve was not entirely exhausted. Johnson *et al.* (2004) also demonstrated that busulfan treatment destroyed 95% of the ovarian follicle reserve but did not fully exhaust the ovary, but rather decreased numbers. Therefore, suggestions that factors within the blood can rescue ovulation in animals after sterilising with these chemicals seem unlikely. It is more likely, that only ovulation is impaired by this treatment but these animals are still have viable growing follicles that given time are capable of ovulating (Johnson *et al.,* 2005a; Eggan *et al.*, 2006).

A previous study also found that ovarian reserve was not fully exhausted after gamma-radiation treatment (Guigon *et al.,* 2003). Animals were treated on post-natal day 5 with gamma-radiation and hormone levels and follicle growth were assessed 24 hours later. Although 99% of primordial follicles disappeared within this time the follicles that were already growing were not affected (Guigon *et al.,* 2003). Indeed follicles that were growing demonstrated normal development when examined by *in situ* hybridization for LHR, inhibin subunits and P450 aromatase when compared to control mice. However, as a result of the follicle reserve being largely depleted, these animals experienced sterility very early at 4 months (Guigon *et al.*, 2003), again supporting the general consensus that the ovarian reserve is not replenished, and that oocytes may be more resistant to chemical treatment than previously thought. Interestingly, busulfan treatment has been proven to destroy germline stem cells due to its toxicity. Hence, it is unlikely that germline stem cells are capable of regenerating new oocytes in busulfan-treated mice (Byskov *et al.,* 2005; Hutt and Albertini, 2006).

A recent study by Kerr *et al.* (2006) supported the claims by Johnson *et al.* (2004; 2005a) that, rather than declining, primordial follicles within the ovary are maintained and supports the theory of follicle renewal. However, this study did not find any evidence of the presence of germline stem cells in these ovaries in the same mouse strain as that used by Johnson *et al.* (2004; 2005a). A further study in human ovaries demonstrated that the follicle reserve is not fixed but rather is continually adapting, with follicles regressing but also differentiating (Bukovsky *et al.*, 2004). This study proposed that new follicles differentiate from cells within the ovarian *tunica albuginea* and possibly explains why ovulation continues for decades despite a high rate of atresia. This may also explain the selection process of the healthiest oocytes (Bukovsky *et al.*, 2004). These recent studies postulated that oocyte renewal in mice is similar to that in lower vertebrates such as frogs, where oocyte renewal occurs throughout life (Johnson *et* al., 2004; Jamnongjit and Hammes, 2005).

The debate about germ cell renewal remains open and ongoing. The general consensus is still that female mammals are born with a finite resource of follicles, and that the size of this reserve and its rate of depletion determine reproductive lifespan. At present few studies (Bukovsky *et al.*, 2004; Kerr *et al.,* 2006) support Johnson's study and the majority of the available data suggests that the ovarian follicle pool (at birth or shortly after birth) is finite and no renewal occurs. The mouse is an ideal model for determining the factors that impinge upon follicle reserve and development and the average lifespan is such that the ovary is not fully exhausted of follicles by the time of natural death (Peters, 1969). Studies undertaken in mice have concluded that the total number of oocytes and the rate of depletion of the follicle reserve are strain-dependent (Jones and Krohn, 1961; Peters, 1969; Canning *et al.,* 2003).

1.4 Assessing the Ovarian Follicle Reserve

There are several means of assessing the relative size of the ovarian follicle reserve. The following section evaluates the various methods available to determine follicle numbers.

1.4.1 Ovarian Follicle Counts

Numerous methods have been reported for the quantification of ovarian follicle numbers in various species, yet several decades later, there is still no universal method of follicle counting. Firstly, the number of sections counted in every ovary differs and the correction factor used to calculate total follicle numbers also varies. Secondly, the classifications used to categorise follicles also differs between laboratory groups, resulting in large variations between studies.

Allen (1923) illustrated that oogenesis changes throughout the oestrous cycle. However, the method used by Allen has been criticised as this led to counting some follicles twice due to the method of counting every section (Zuckerman, 1951). The standard method of counting ovarian follicles is to classify them into easily distinguishable and suitable groups, and count every follicle with the nucleus in the middle of the oocyte in every section (Zuckerman, 1951). However, this is not ideal for two reasons: firstly, it is very labour intensive, and secondly over-lap between sections can result in the same follicle being counted twice (Zuckerman, 1951). Further studies by Zuckerman (1951) found that counting all the follicles on every fifth or tenth section of the ovary solved both of these problems. In order to calculate the total number of follicles for the entire ovary, follicle numbers were multiplied by five or ten respectively.

This multiplication method of follicle counting has proved popular. The starting section for the counts is usually randomly selected. For example, if counting every tenth section the first section counted can be any one of the first ten sections. A study tested the accuracy of the multiplication method and demonstrated that the variation between follicle counts using the multiplication method or counting every section was so great that the multiplication method was described as 'highly inaccurate' (Tilly, 2003). However, this method was inaccurate by only 3-11% and was found to be the most accurate method available and has remained popular. This therefore became the standard method of follicle counting (Tilly, 2003).

One of the factors found to alter the reliability of this method was the use of the correction factor. As described previously when counting every tenth section a correction factor of ten was used to account for the other sections not counted (Zuckerman, 1951; Flaws *et al.*, 2001a). However, Flaws *et al.* (2001a) used a multiplication factor of eighty. This multiplication factor was justified since every tenth section was counted, and therefore numbers were multiplied by ten. However, as every section was 8µm, thick a further multiplication of eight was included to allow for the depth of every section. Abercrombie (1946) examined the numbers of nuclei from microtome sections. This study concluded that the thickness of a section and the length of the nuclei are essential factors when carrying out ovarian follicle counts. Therefore, a correction factor was applied to counts taking these factors into consideration (Abercrombie, 1946). This method has been used in various studies. However, this multiplication factor results in extremely large follicle numbers, and the relevance of the section thickness is questioned (Flaws *et al.*, 2001a; Tilly, 2003).

Although the multiplication enables an approximation of follicle numbers from analysing one tenth of an ovary, ovaries are not cylindrical and this method does not account for the changing shape of the ovary. Although section thickness is important, as long as the same multiplication factor is used throughout the study it does not matter for comparative purposes in the same study whether the raw counts are multiplied by ten or eighty as all the values will have been calculated in the same manner (Tilly, 2003). However, it is difficult, if not impossible, to compare results and conclusions from different studies on the same or different strains of mice using different counting methods (Canning *et al.*, 2003).

In addition to differences in the correction factor used there are also variations in the criteria used to classify ovarian follicles into groups (Table 1.1). Follicles are often classified according to their granulosa cell morphology. However, even by this method there can be great variation, as demonstrated by laboratories that count six stages of follicle development (Mandl and Zuckerman, 1952) compared to other studies that classify follicles into only two groups (small/medium or pre-Graafian; Hirshfield and Midgley, 1978a). Other studies classify the follicles according to oocyte diameter (Zuckerman, 1951) or to a compilation of criteria, including follicle diameter, antrum formation and granulosa cell morphology (Bucci *et al.*, 1997). Yet another method that is commonly used is the Fractionator/Optical or Fractionator/Physical Disector method (Britt *et al.*, 2000; Myers *et al.*, 2004). However, this also encounters follicle classification problems as mentioned above. Every method of analysing follicle counts has its merits and drawbacks (Table 1.2). However, the diversity of methods makes drawing comparisons and conclusions from different studies difficult.

Study	Follicle Classification	Number of Classification Groups	Follicle Counting Method
Arai (Cited in Zuckerman, 1951)	Oocyte diameter	3	Every section of the ovary.
Zuckerman (1951)	Granulosa cell morphology	6	Count every 5th or 10th section and multiply by 5 or 10 respectively as the correction factor.
Hirshfield and Midgley (1978a)	Follicle diameter	3	50 random sections per ovary analysed.
Flaws *et al.* (2001a)	Granulosa cell morphology	3	Count every 10th section and multiply by fraction i.e. 10 and thickness of section as the correction factor.
Canning *et al.* (2003)	Not stated in report	3	Count every 5th section and multiply by 5 as the correction factor.
Britt *et al.* (2000)	Granulosa cell morphology	3	Fraction of thickness of section.
Bucci *et al.* (1997)	Granulosa cell morphology, Diameter of follicles and 'Nature of Antrum'	3	Counted every 10th section.
Myers *et al.* (2004)	Granulosa cell morphology	5	Fractionator/Physical Disector method.

Table 1.1 Summary of the main follicle counting methods (adapted from Myers et al.*, 2004).*

Method	Advantages	Disadvantages
Canning *et al.* 2003 1.Both ovaries from every animal were fixed in Bouins and serial sectioned at 8µm. 2.Sections stained with haematoxylin and picric methyl blue. 3.Only follicles with a nucleus in the oocyte were counted. 4.Count follicle from both ovaries in every 5th section and multiply final numbers by 5 (the correction factor).	1. Not all tissue is used. Can therefore analyse the rest of the ovary for other factor e.g. apoptosis. 2. It provides a realistic estimate of total follicle numbers throughout the ovary/animal.	1. Previous studies demonstrated large variation in follicle numbers using this method compared to counting the entire ovary. 2. Is only representative of follicle numbers if follicles are equally distributed throughout the ovary. 3. Ideally counts should be carried out 3 times by 3 different people. 4. Time consuming.
Flaws *et al.* 2001a 1.The same method as above but instead of multiplying by only the correction factor: multiply the final numbers by the correction factor and then multiplied by the section thickness.	Same Advantages as above.	Same disadvantages as above. 5. Multiplying with correction factor alone is a more accurate estimate of follicle numbers.
Myers *et al.* 2004 1.Ovaries were fixed in Bouins and one from each animal was randomly selected to be embedded in paraffin wax and the other ovary in glycolmethacrylate resin. Ovaries were then serial sectioned at 20µm (glycolmethacrylate resin embedded ovaries) and at 3µm (paraffin wax embedded ovaries). 2.Primordial and primary follicle numbers were determined by the following two methods: **Fractionator/Optical Disector (glycolmethacrylate sections)** - Sections were randomly started within the first 3 sections and thereafter every 3rd section was counted under a microscope. Sections were stained with periodic acid-Schiff and counterstained with haematoxylin. Three levels of sampling were used; Firstly a counting frame was 'superimposed' over the ovary and moved along the X and Y axes and the follicles counted. Secondly, the first 3µm of tissue is ignored as it is likely to be affected with cutting artefacts. Thirdly, the next 10µm of the 20µm section was optically sectioned and oocytes that were not on the exclusion border line counted. The oocyte nucleus number was associated to follicle numbers. **Fractionator/Physical Disector Design (paraffin sections)** - Every 10th and 11th section was used. Sections were stained with a modified Masson trichrome stain and every follicle was counted if it appeared in one section but not in another. A counting frame was superimposed over the ovary and oocyte nucleus number was equated to follicular number. **Quantification of Secondary, Antral and Atretic Follicles** Follicles at secondary stage and beyond were recorded by exact counts from digital images of consecutive 20µm sections. As before oocyte nuclear number was associated to follicle numbers.	1. Prevents any problems associated with ovary volume changes due to swelling or shrinking during fixation. 2. Ignores top and bottom of the section as it is likely to be affected with cutting artefacts. 3. The whole ovary is analysed.	1. Uses the whole ovarian sample. 2. Only one ovary was analysed per animal. 2. Secondary follicles, antral follicles and atretic follicles were only counted in one ovary from each animal. 3. Time consuming. 4. Ideally counts should be carried out 3 times by three different people.

Table 1.2 Advantages and disadvantages of main methods used for follicle counts.

1.4.2 Production Line Hypothesis

The production line theory proposes that there are 'gradients' which result in reductions of chiasma frequencies between the first and last formed oocytes (Henderson and Edwards, 1968). The oocytes formed first with high chiasmatic occurrence are believed to be the first to ovulate compared to those oocytes formed later in fetal development where chiasmatic occurrence is lower and which ovulate later in life (Henderson and Edwards, 1968; Speed and Chandley, 1983). Those oocytes that ovulate later in life with low chiasmatic occurrence may show some chromosomal irregularities (Speed and Chandley, 1983). Later radio-labelling studies in two strains of mice demonstrated that the time of oocyte maturation after puberty was dependent upon the time at which meiosis was entered into the fetus, thereby supporting a 'production line' system of activation (Polani and Crolla, 1991). However, further studies in foetal mice ovaries failed to demonstrate the existence of a production line. Any abnormalities that were observed occurred over several days, and occurred as frequently in the early stages of oocyte maturation as in the latter stages of oocyte maturation (Speed and Chandley, 1983).

1.4.3 Follicle Reserve – Mathematical Modelling

Another means of calculating follicle reserve is through mathematical models. One model for determining human ovarian follicle reserve depends on calculation of exponential follicle decay (Faddy *et al.,* 1992; Faddy, 2000). This simplistic model examines follicle decline throughout the reproductive lifespan with follicle numbers gradually declining with age (Faddy *et al.,* 1992). However, further analysis demonstrated that human follicle numbers do not decline exponentially, but that the rate of decline in the human ovary doubles at the age of 37.5 years, leading to a bi-exponential model of decline (Faddy *et al.,* 1992). The rate of depletion of ovarian follicles continues until the menopause occurs when there are still approximately 1000 follicles remaining in the ovary, suggesting that this is the 'threshold' for human menopause (Faddy *et al.,* 1992). If a bi-exponential decline in the ovarian reserve did not occur the menopause was calculated to occur around 71 years of age (Faddy *et al.*, 1992).

Another model for analysing follicle growth and numbers is the Compartmental Model (Faddy *et al.,* 1987). This model categorises follicles into stages of development and considers the number of follicles that progress from one stage of development to another in addition to the number of follicles that become atretic (Faddy *et al.,* 1987; Faddy, 2000). Using this model when examining human ovaries it was predicted that there was an activation rate of one follicle every two days by the late forties, compared to 37 follicles per day 25 years earlier in life. However, this model was based solely on healthy adult humans, as insufficient data could be collected for children (Faddy and Gosden, 1995).

A further study in sheep developed a different mathematical model to determine ovarian reserve which incorporated the rate of follicle proliferation, differentiation, and apoptosis (Clement *et al.*, 1997; Clement *et al.*, 2002). This model was based on the number of oocytes that were capable of ovulating and therefore incorporated the selection process during follicle development. Therefore, although this model does not calculate the full ovarian reserve, it calculates the ovulation rate from the ovarian reserve (Clement *et al.*, 1997).

A more recent model examined follicle activation and progression from primordial through primary to secondary follicles in CD1 mice to determine whether the ovarian follicle reserve was capable of supplying enough follicles through to the end of the reproductive lifespan (Bristol-Gould *et al.*, 2006b). This study failed to find any evidence of germline stem cells but supported the central dogma of a fixed ovarian reserve and by assuming a 'fixed pool' of follicles determined the maximum number of primary follicles occurs between 4-6 months of age (Bristol-Gould *et al.*, 2006b).

Although mathematical models can estimate follicle numbers, they do not necessarily allow for differences between species and strains of animals, the rate of decline which may vary depending on the age of the individual, follicle activation rates or the number of healthy follicles present. For example, in humans a bi-exponential decline is observed, whereas an exponential decay is observed in rodents (Faddy *et al.,* 1992; Faddy, 2000). These mathematical models provide an insight into human ovaries where analysis of follicle numbers is difficult, and although not entirely accurate they do give an indication of reproductive lifespan (Faddy and Gosden, 1996). Together these factors comprise of the essential components of a mathematical model for estimating follicle numbers. However, such models remain only an estimate and are species dependent (Faddy *et al.,* 1992; Faddy, 2000).

1.5 Ovarian Reserve and Exogenous Steroids and Gonadotrophins

Gonadotrophins and steroids regulate follicle growth and development. Therefore, any variation in levels of either gonadotrophins or steroids could alter the follicle reserve. The following section discusses how gonadotrophins and steroids affect the follicle reserve. Removal of the pituitary gland has been found to reduce the loss of primordial follicles (Meredith *et al.*, 1986) suggesting that high levels of gonadotrophins increase the rate of depletion of the ovarian reserve. However, in women with low plasma FSH, the ovarian reserve was depleted at an accelerated rate compared to those with normal FSH levels (Mais *et al.,* 1995).

Studies examining hypogonadal mice which expressed transgenic human FSH demonstrated that these mice had increased ovarian weight (four-fold compared to controls). In addition, primordial follicle numbers also increased two-fold but primary follicle numbers were unchanged compared to controls. However, numbers of secondary and antral follicle were greater in these mice compared to hypogonadal deficient controls although the ovulation rates remained similar (Allan *et al.*, 2006). This study demonstrates that FSH stimulates the ovarian follicle numbers compared to control mice. The mechanisms by which it does so remain unknown although it is postulated that FSH delays atresia and so increases the size of the ovarian reserve (Hirshfield, 1989; Hirshfield, 1991; Allan *et al.*, 2006).

Raised LH/FSH ratios can stimulate androgen production which has been associated with increased atresia (Hillier and Ross, 1979; Mann *et al.*, 1999). Studies in women, with basal FSH levels, receiving fertility treatment demonstrated that high LH levels increased oocyte yield compared to patients with low LH levels (Weghofer and Feichtinger, 2006). Interestingly, transgenic mice with chronically elevated LH levels demonstrated normal ovarian reserves compared to controls at 21 days. However, by 35 days mice with elevated LH had ovaries which contained 45% fewer primordial follicles compared to controls. In addition, ovaries of mice with high levels of LH also contained cysts and abnormally-shaped granulosa cells (Flaws *et al*, 1997). The reduced ovarian follicle reserve was further depleted by 3 months, when the transgenic mice had 68% less primordial follicles and 53% less primary follicles (Flaws *et al.*, 1997). There are two possible explanations for this increased depletion of the ovarian reserve. Firstly, elevated LH could stimulate primordial follicles into the growing follicle pool. However, Flaws *et al.* (1997) did not see a correlated increase in primary and antral follicle numbers but found a decline in primary follicle numbers compared to the controls. Alternatively, it was proposed that elevated LH levels could result in a toxic effect and induce atresia (Flaws *et al.*, 1997). These studies conclude that elevated levels of LH accelerate the depletion of the ovarian reserve.

The effect of oestrogen on the ovarian reserve is demonstrated in *Aromatase* KO (*ArKO*) mice, which are deficient in oestrogen. These had fewer primordial and primary follicles compared to control mice suggesting that endogenous oestrogen is fundamental for the size of the ovarian follicle reserve (Britt *et al.*, 2004). Whereas, the ovarian follicle reserve was greater in size compared to controls when rats were treated with progesterone, co-treatment with oestrogen and progesterone did not affect the size of the follicle reserve (LaPolt *et al.*, 1998).

However, progesterone-treatment of neonatal rats demonstrated that follicle formation was not completed *in vivo,* with 10% of follicles still to be assembled in these animals between postnatal day 0 and day 7 (Kezele and Skinner, 2003) Furthermore, animals with elevated progesterone had reduced follicle development with no corpus luteum (CL) formation compared to controls (LaPolt *et al.*, 1998). Other studies have shown that progesterone treatment in adult mice also resulted in the cessation of ovulation (Telfer *et al.,* 1991) demonstrating that it had an inhibitory effect of follicle development. Similar to progesterone, oestrogen-treated rats also demonstrated a reduction in follicle activation (Kezele and Skinner, 2003).

Androgen treatment of oestrogen-primed hypophysectomised rats *in vivo* increased follicle atresia (Hillier and Ross, 1979) whereas androgen treatment of follicles *in vitro* stimulated follicle growth and development of murine follicles (Murray *et al.,* 1998). Treatment of ewes with testosterone during fetal development reduced ovarian reserve, but increased follicle activation. Therefore, testosterone increased the decline in ovarian reserve and led to the onset of the menarche at an earlier age (Steckler *et al.,* 2005). Although increased activation of the follicles contributes to increased loss of follicles, the ovarian reserve is also diminished by testosterone however the mechanisms by which it does so remain unknown (Steckler *et al.,* 2005).

1.6 Follicle Recruitment and Selection

Although the endocrine system plays a fundamental role in the regulation of follicle selection, inter-follicular factors are also essential in regulating follicle dominance (Baker and Spears, 1999). There are three possible mechanisms by which such intra-ovarian interactions may occur; paracrine, autocrine or juxtacrine regulation. Paracrine regulation involves signals from one cell type that act locally on a different cell type, whereas autocrine regulation involves a signal that acts upon the same cell. Juxtacrine regulation involves cell-cell or cell-matrix contact from cells that are in close proximity to each other (Armstrong and Webb, 1997; Baker and Spears, 1999).

There are two stages of follicle selection during the growing phase; recruitment and selection. Recruitment is the process whereby follicles are activated from the resting pool (Armstrong and Webb, 1997; Fortune *et al.*, 2001) and growth is initiated (Gruijters *et al.*, 2003). The number of follicles that are recruited is dependent upon the size of the follicle reserve and the number of follicles activated reduces with age but the reserve is diminished at a faster rate (Gosden *et al.,* 1983; Faddy, 1992; Themmen, 2005). Selection is the process whereby a number of the growing cohort of follicles are selected for further growth and development and is species-specific (Armstrong and Webb, 1997; Fortune *et al.*, 2001).

Follicle recruitment and selection are controlled by both endocrine hormones and intra-ovarian factors (Zhao *et al.*, 2000). In mice there is a brief increase in FSH that occurs when follicles initiate growth although recruitment and growth can still occur to the early antral stage of development in the absence of FSH (Scaramuzzi *et al.,* 1993; Dierich *et al.,* 1998; Themmen and Huhtaniemi, 2000; Fortune *et al.,* 2001; Webb *et al.,* 2003). Studies of bovine ovaries demonstrated that primordial follicles were located on the outer most aspect of the ovary. Staining for Von Willebrand factor (factor VIII) demonstrated that vascularisation of the ovary was not present in this area suggesting that these follicles are avascular (van Wezel and Rodgers, 1996). As primordial follicles are avascular local factors such as Amh are thought to determine the recruitment rate of follicles (Baker and Spears, 1999; Themmen, 2005). The larger the follicle becomes, the more responsive it becomes to growth factors and hormones such as FSH and LH, possibly due to increased expression of receptors in larger follicles (Zhao *et al.,* 2000). The more responsive an antral follicle is to FSH the more likely the follicle is to survive selection and become dominant.

Follicle selection is the process whereby follicles are selected for further development and rescued from atresia by FSH. However, this process operates in the post-pubertal years (Themmen, 2005). Follicle selection is regulated by endocrine factors such as FSH, oestradiol, inhibin and activin. However even at this stage, the number of follicles in the growing cohort exceeds the number that will proceed to ovulation, and further selection takes place that reduces this cohort. Studies of bovine follicles demonstrated that the decline in FSH secretion at follicle selection is due to an increase in oestradiol and inhibin A secretion from the largest follicle (Bleach *et al.*, 2001). Negative feedback by oestradiol and inhibin lowers FSH levels to a point where smaller subordinate follicles are unable to respond to the circulating FSH levels (Webb *et al.*, 2003).

The follicle(s) that demonstrates greatest FSH-sensitivity is selected and becomes dominant. The dominant follicle secretes increasing levels of oestradiol and inhibin which feed back to the pituitary (Hillier, 1994; Baker and Spears, 1999; Valdez *et al.*, 2005). After dominant selection the oestradiol levels in the follicular fluid drastically reduces, however, this reduction in synthesis is not a result of reduced androstenedione but correlates with reduced aromatase expression (Valdez *et al.,* 2005). The dominant follicle is usually the largest in the growing cohort and is able to respond to lower FSH levels and continue developing by beginning to respond to FSH-induced LHRs, whereas smaller subordinate follicles are unable to withstand this reduced FSH (Baker and Spears, 1999; Webb *et al.*, 2003; Zeleznik, 2004).

Follicle growth and development is driven by the gonadotrophins which regulate intra-ovarian factors that provide the correct local environment to achieve maximal growth and development. Any slight change in this balance results in atresia (Webb *et al.,* 2003). In addition, granulosa cell LHR expression post follicle selection enables the dominant follicle to respond to both FSH and LH (Fortune *et al.,* 2001). Dominant follicles in multi-ovular species may arise in equal numbers from each ovary, from systematic ovulation from alternative ovaries, or may be completely random (Baker and Spears, 1999).

1.7 Steroidogenesis

1.7.1 Oestrogen Production

All sex steroids are produced from cholesterol (Knobil and Neill, 1994). Steroidogenesis occurs in pre-pubertal and fetal ovaries, and cholesterol side-chain cleavage (P450scc) and 17α-hydroxylase enzyme are expressed (Adashi, 1994). However, once a follicle reaches the antral stage of development FSH and LH are produced and secreted in increasing quantities. The steroid hormone oestradiol is produced in the granulosa cells, whereas androgens are produced in the theca cells (Zeleznik *et al.,* 1985; Nayudu and Osborn, 1992; Adashi, 1994). The main androgen produced in mice is androstenedione and the main oestrogen is oestradiol (Knobil and Neill, 1994; Britt and Findlay, 2002). Androgen production is an essential requirement for oestradiol production. However, granulosa cells lack the ability to produce androgen *de novo* and, therefore, production is dependent upon co-operation between the theca and granulosa cells (Adashi, 1994). The production of oestrogen is also reliant upon LH and FSH acting on the theca and granulosa cells respectively (Figure 1.2).

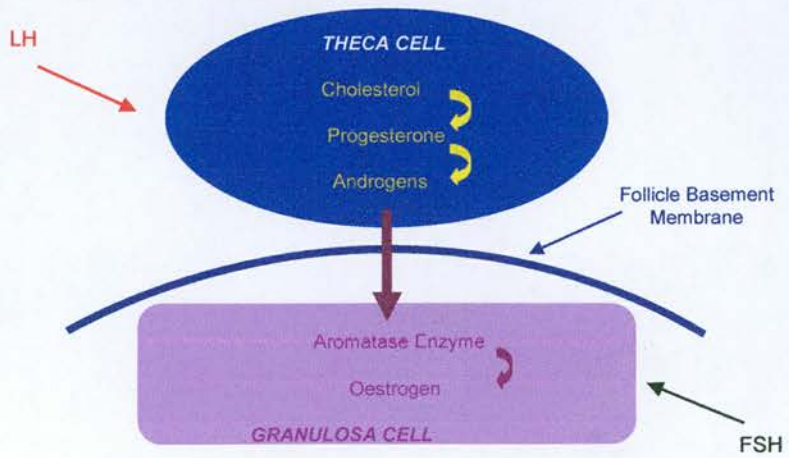


Figure 1.2 An illustration of the two-cell two gonadotrophin model.
Cholesterol is converted to androgens in the theca cells. The androgens diffuse across the basement membrane to the granulosa cells where they are aromatised to oestrogen.

Oestrogen is required for follicle growth, particularly in the latter stages of development, and stimulates granulosa cell proliferation and FSHR and LHR expression (Byers *et al.*, 1997; Britt *et al.*, 2004). Interestingly, although LH is required for driving the conversion of cholesterol to androgen, the pre-ovulatory LH surge actually results in a decline of oestrogen receptor-β (ERβ) expression (Byers *et al.*, 1997).

Theca cells receive a rich blood supply and can therefore make use of circulating cholesterol (Knobil and Neill, 1994). Low-density lipoproteins (LDL) are associated with steroidogenesis in humans although high-density lipoproteins (HDL) are coupled with steroidogenesis in rodents (Knobil and Neill, 1994). LH stimulates androgen synthesis in the theca cells (Knobil and Neill, 1994; Themmen and Huhtaniemi, 2000).

Initially, cholesterol is cleaved to form pregnenolone by the P450scc enzyme (CYP11A), which is located in the mitochondria. This process necessitates transport of cholesterol to the inner mitochondria membrane – a process regulated by steroidogenesis acute regulatory protein (StAR; Knobil and Neill, 1994; Elvin *et al.*, 1999a). Pregnenolone is then converted to progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD) and then to androgen by 17α-hydroxylase (Adashi, 1994; Knobil and Neill, 1994). Androgens then diffuse across the basement membrane where they are aromatised into oestrogen. The cytochrome (P450) aromatase enzyme (CYP19), is responsible for aromatising the exogenous androgens to oestrogen, and is expressed only in granulosa cells in the ovary (Yong *et al.*, 1994; Britt *et al.*, 2000). Studies have concluded that maximal FSH activation results in lower levels of cAMP compared to maximal LH activation (Yong *et al.*, 1994). For example, P450scc genes were stimulated by LH/high cAMP levels and cytochrome P450arom genes were stimulated by FSH/low cAMP levels in isolated human granulosa cells (Yong *et al.*, 1994).

Oestrogen levels are crucial for ovarian follicle selection since increased oestradiol production by the mature follicle(s) inhibits FSH secretion from the pituitary. This suppression of plasma FSH results in the inhibition of the growth of less advanced follicles which have a lower FSH sensitivity (Zeleznik *et al.*, 1985; Zeleznik and Kubik, 1986). Together with FSH, oestradiol treatment *in vivo* stimulates granulosa cell LHR expression, therefore oestradiol is fundamental for pre-ovulatory follicle development *in vivo* (Quirk *et al.*, 2004). Once the menopause is reached oestrogen synthesis terminates due to the lack of follicles, and FSH levels increase due to the lack of negative feedback from oestradiol (Themmen and Huhtaniemi, 2000). Granulosa cells are the source of oestradiol and progesterone, the two main steroids involved in ovarian steroidogenesis. Higher levels of oestradiol and progesterone compared to androgens are found in large healthy antral follicles, but this ratio is reversed in smaller follicles and in atretic follicles (Adashi, 1994).

Oestrone is the initial oestrogen synthesised in humans but it is quickly converted into oestradiol by 17α-hydroxysteroid dehydrogenase in the granulosa cells (Adashi, 1994). FSH stimulates increased granulosa cell proliferation, aromatase expression and, in turn, increased oestradiol production (Hillier *et al.*, 1994; Yong *et al.,* 1994; Rosenfeld *et al.*, 2001). *In vitro,* oestradiol synthesis is increased when follicles are cultured with increasing doses of FSH (Nayudu and Osborn, 1992). There are two oestrogen receptors (ER: ERα and ERβ) expressed in the ovaries (Rosenfeld *et al.,* 2001). Granulosa cells express more ERβ compared to ERα receptors (Britt and Findlay, 2002). The presence of ERs within the granulosa cells suggests that oestrogen has an autocrine action on granulosa cells (Hillier, 2001).

The role of oestrogen has been studied in ERα and ERβ KO mice. ERα KO mice are infertile whereas, ERβ KO mice are fertile but experience impaired fertility, resulting in reduced litter sizes (Rosenfeld *et al.,* 2001; Britt and Findlay, 2002). In the ERα and ERβ KO mice models follicle development is normal until the antral stage of development but these follicles fail to ovulate in ERα KO mice (Dupont *et al.,* 2000). Such studies report that the intra-ovarian action of oestrogen is essential for follicle growth, although an earlier study concluded that 'physiological' levels of oestrogen has only a slight effect *in vitro* (Spears *et al.,* 1998). Follicle growth was not impaired by treatment of follicles with anti-oestrogens, suggesting that intra-follicular actions of oestradiol are not required for follicle growth and development *in vitro* (Spears *et al.*, 1998). Follicle cultures have shown that oestradiol secretion increases with the duration of culture leading to increased oestradiol levels in the culture media although oestradiol synthesis differed in follicles of a similar size (Fehrenbach *et al.*, 1998). Oestradiol synthesis was dependent on the presence of FSH and its concentration in the culture media which regulated P450arom expression in the granulosa cells (Yong *et al.*, 1994; Fehrenbach *et al.*, 1998). However, maximum biosynthesis of progesterone and P450 expression only occurred when stimulated by LH, therefore, maximum oestradiol synthesis is dependent upon LH in pre-ovulatory follicles (Yong *et al.,* 1994).

Oestradiol synthesis increases throughout follicle development and reaches a maximum when the follicle is at its greatest diameter. Thereafter, if ovulation does not occur, oestradiol levels decline, as a result of reduced P450 activity in the granulosa cells (Valdez *et al.,* 2005). Oestrogens amplify the actions of FSH in pre-ovulatory follicles by stimulating granulosa cell proliferation which, in turn, increases FSHR expression (Findlay and Drummond, 1999). Oestradiol also stimulates Insulin-like Growth Factor-1 (Igf-1) signalling, which stimulates ERβ expression, suggesting autocrine regulation of granulosa cell proliferation (Richards *et al.,* 2002).

1.7.2 Progesterone Production

Progesterone is secreted by the ovarian follicle only at the later stages of follicle development, and its synthesis is stimulated by LH (Yong *et al.*, 1994; Gilbert, 2003). Progesterone secretion is at basal levels until just prior to the LH surge when levels increase sharply and continue to increase post-ovulation (Yong *et al.*, 1994; Tortora and Grabowski, 2000). At this stage in primates, oestradiol secretion, together with progesterone, prepare the endometrium for implantation (Tortora and Grabowski, 2000). The luteal stage of the ovarian cycle in mammals is characterised by increasing levels of serum progesterone (Gilbert, 2003).

Progesterone is synthesised by granulosa cells. However, progesterone receptor (PR) expression in the granulosa cells does not occur until late in follicle development when oestradiol levels exceed a critical threshold, and the surge of FSH and LH is triggered that results in ovulation. Oestrogen levels then fall and progesterone levels increase in response to LH stimulation (Figure 1.3; Natraj and Richards, 1993; Hillier, 2001; Gilbert, 2003). Progesterone levels rise and are maintained unless fertilisation does not occur. The corpus luteum then regresses (Gilbert, 2003). The LH surge stimulates transient PR expression in the mural granulosa cells (Park and Mayo, 1991; Richards *et al.*, 2002). PR expression is fundamental for ovulation to occur since mice deficient in PR expression are unable to ovulate even when treated with exogenous hormones (Richards *et al.*, 2002).

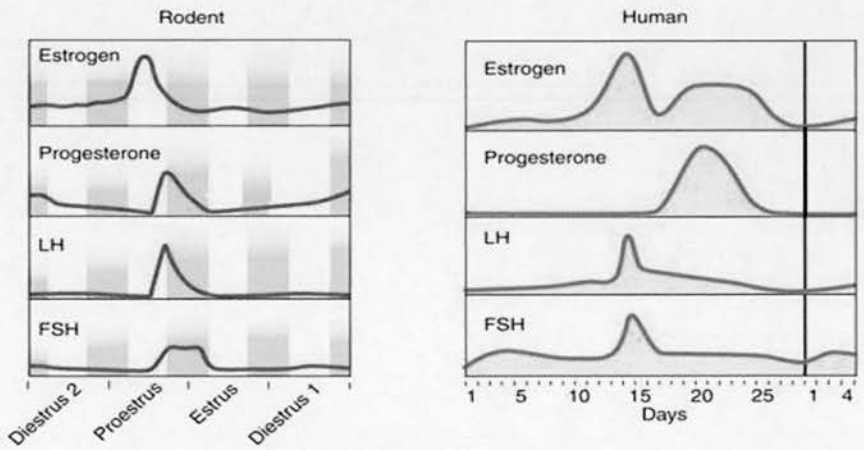


Figure 1.3 Summary of LH, FSH, oestrogen and progesterone secretion during rodent and human follicle development (Staley and Scharfman, 2005).

1.7.3 Androgens

Androgens are synthesised in the theca cells and diffuse across the basement membrane to the granulosa cells, where the androgen receptors (AR) are expressed (Knobil and Neill. 1994; Hillier, 2001). AR expression increases in follicles near antral formation compared to pre-antral follicles in rhesus monkeys treated with testosterone (Fortune, 2003). Androgen mRNA expression increases as FSHR mRNA expression increases in primate follicles (Weil *et al.*, 1999). In addition, atresia has been found to be negatively correlated with AR expression in granulosa cells suggesting that androgens do not play a role in atresia and implies that AR is a marker of a healthy follicle (Fortune, 2003). Additionally, murine follicles cultured in the presence of recombinant human FSH increased AR expression, whilst maturation of follicle growth in cultured follicles was inhibited when treated with anti-androgen anti-serum (Murray *et al.,* 1998). Furthermore, androstenedione treatment *in vitro* stimulates ovine follicle growth and differentiation, but inhibits oocyte 'survival' in smaller follicles (Thomas *et al.*, 2003).

Other growth factors involved in regulating follicle growth and development include EGF and transforming growth factor-α (TGF-α) which are 'closely related' proteins (Conti *et al.*, 2005). EGF regulates folliculogenesis by stimulating granulosa cell proliferation (Conti *et al.*, 2005). Studies on human granulosa cells demonstrated that proliferation rates were stimulated when treated with EGF, whereas rat granulosa cells required fibroblast growth factor and EGF treatment combined to stimulate proliferation (Gospodarowicz and Bialecki, 1979).

1.8 Transforming Growth Factor-β (TGF-β)

TGF-β family members are dimeric glycoproteins with a cleavage site which is required for activation (van Rooij *et al.,* 2002; Rey *et al.*, 2003; Salmon *et al.*, 2004; La Marca *et al.*, 2005). The TGF-β superfamily is a family of growth and differentiation factors that includes activin, inhibin, AMH, GDF-9 and the BMPs (Lin *et al.,* 2003). These growth factors bind to specific membrane proteins (receptor types I and II) and act in both paracrine and autocrine manners (Knight and Glister, 2003). Factors such as Gdf-9 play a fundamental role in the control and regulation of the differentiation and proliferation of granulosa cells (Elvin *et al.*, 1999a; Elvin *et al.*, 1999b). The members of the TGF-β family are expressed as prepropeptides, with a pre-domain, pro-domain and mature-domain. Removal of the pre-domain (signal domain) results in activation of the pro-domain leading to the formation of the active mature-factor (Figure 1.4; McNatty *et al.*, 2004).

Figure 1.4 Structure of the TGF-β family members (Adapted from McNatty et al., 2004).

1.8.1 TGF-β Signalling

The TGF-β family signal by forming 'heteromeric complexes' of type I and II receptors on the theca and granulosa cell membranes (Figure 1.5; Knight and Glister, 2003). When the ligand binds to the type II receptor it recruits the type I receptor which is then trans-phosphorylated and in turn activates the Smad pathway (Figure 1.5; Knight and Glister, 2003). Depending on the ligand bound to the receptor different Smads are activated. For example, Bmp-15 activates Smad-1 and Smad-5, whereas activin activates Smad-2 and Smad-3 (Elvin *et al.*, 1999a). These Smads then combine with Smad-4, and this complex translocates to the cell nucleus where gene expression is altered (Figure 1.5; Knight and Glister, 2003). However, with 7 type I receptors and 5 type II receptors identified so far, different members of the TGF-β superfamily are able to activate different receptor combinations, and the specific receptors that mediate the actions of many of the TGF-β family are still to be identified (McGee *et al.*, 2001; Durlinger *et al.*, 2002; Knight and Glister, 2003).

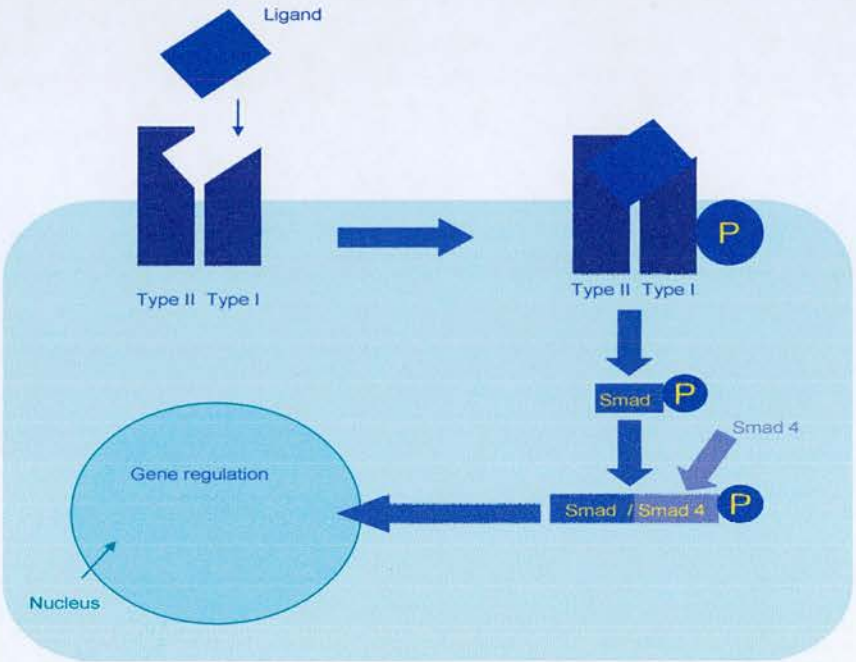


Figure 1.5 TGF-β signalling summary (Adapted from Knight and Glister, 2003).

1.8.2 Inhibin, Activin and Follistatin

The inhibins are heterodimeric glycoproteins that consist of an α-subunit and either one of the β-subunits (βA and βB). The α-subunit is linked to one of two β-subunits by 4 disulphide bridges. Inhibin A is formed when the α-subunit is linked to a βA-subunit, and inhibin B is formed when the α-subunit is linked to a βB-subunit (Figure 1.6; Welt and Schneyer, 2001; Chada *et al.,* 2003). Activin consists of homodimers or heterodimers of the β-subunits i.e. activin A = βA-βA, activin B = βB-βB and activin AB = βA-βA (Figure 1.6; Knight and Glister, 2001). Although structurally unrelated to activin, follistatin plays a fundamental role in activin regulation through binding to activin and preventing binding to its receptor (Knight and Glister, 2001). Inhibin, activin and follistatin all have profound effects upon the ovary due to the effect they have on FSH levels (Glister *et al.,* 2001). For example, activin expression is increased in granulosa cell culture media when treated with FSH (Glister *et al.*, 2001).

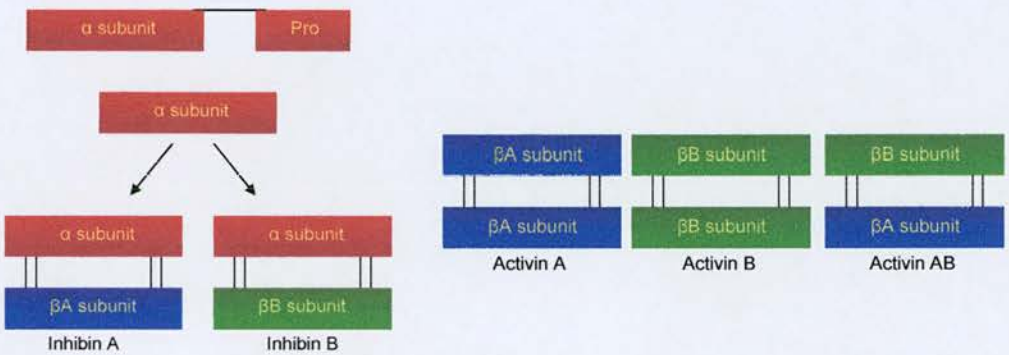


Figure 1.6 Inhibin and activin structure (Adapted from Inhibin, http://www.inhibin.com/Home/Static.aspx?PageID=14).

As their names imply, inhibins suppress FSH secretion, whilst activins stimulate its production from the pituitary gland (Halvorson and DeCherney, 1996). These peptides play an essential role in the reproductive system (Smyth *et al.*, 1994). Inhibin and activins are secreted by the granulosa cells and follicular fluid is a source of inhibin, activins and follistatin (Halvorson and DeCherney, 1996). Inhibin is expressed in the granulosa cells throughout follicle development (Campbell and Baird, 2001) and seems to be a key regulator within the ovary (Halvorson and DeCherney, 1996). Studies by Groome *et al.* (1996) demonstrated the presence of inhibin B in the plasma of humans during the menstrual cycle, suggesting they regulate gonadotrophin levels through pituitary feedback (Halvorson and DeCherney, 1996). Inhibin A secretion in sheep is positively correlated to oestrogen secretion and increases in the latter stages of follicle development (Campbell and Baird, 2001). Treatment with follicular fluid suppresses plasma FSH and inhibits follicle growth (De Jong and Sharpe, 1976; Wallace and McNeilly, 1985).

The production and secretion of these factors varies depending on the stage of folliculogenesis. The granulosa cells produce inhibins upon stimulation by FSH (Smyth *et al.*, 1994). Inhibin B secretion is greater in the early stages of follicle development whereas inhibin A is secreted in large amounts by pre-ovulatory follicles and the corpus luteum in the primate but not other species. Additionally, high levels of inhibin α-subunit are secreted from small antral follicles but this has no inhibitory action upon FSH. Inhibin α subunit is produced in excess and this is thought to enable binding to the β-subunits as they are synthesised, resulting in the formation of inhibins rather than activins (Braw-Tal, 1994; Drummond *et al.*, 2000).

Inhibin B is principally expressed by pre-antral and small antral follicles whereas inhibin A is the chief inhibin produced by pre-ovulatory follicles (Groome *et al.*, 1996; Welt and Schneyer, 2001; Hohmann *et al.*, 2005). Inhibin B secretion is greatest during the early stages of follicle development and its suppressive actions on FSH are responsible for the decline in plasma FSH levels, thus closing the "FSH window" (the period of time that FSH levels exceed the threshold for full follicle maturation) (Hohmann *et al.*, 2005). Groome *et al.* (1996) demonstrated that inhibin B is the main inhibin in follicular fluid and inhibin B is thought to play a fundamental role in follicle selection (Chada *et al.*, 2003; Hohmann *et al.*, 2005). As inhibin B secretion declines, inhibin A secretion increases. Inhibin A is responsible for suppressing FSH levels as ovulation approaches (Hohmann *et al.*, 2005). Inhibin A levels continue to increase after the dominant follicle(s) have been selected and inhibin A is secreted in large amounts by the corpus luteum in primates (Ying, 1987; Campbell and Baird, 2001; Hohmann *et al.*, 2005).

FSH stimulation increases both inhibin A and B although at different stages of follicle development (Hohmann *et al.*, 2005). Inhibin increases LH-stimulated thecal androgen production, in a paracrine manner and hence follicular oestradiol production (Glister *et al.*, 2001). In the FSH KO mouse, inhibin secretion is at basal levels due to absence of FSH stimulation of the follicle, demonstrating that FSH stimulation is required for the activation of inhibin secretion. However, secretion of the α-subunit of inhibin is less affected than secretion of the β-subunit in these mice (Hirst *et al.*, 2004). Recently inhibin has also been suggested to play a role in the inhibition of oocyte maturation (Knight and Glister, 2003). Fertilised bovine oocytes treated with inhibin A demonstrated inhibited development compared to controls (Silva *et* al., 1999). While a specific receptor for inhibin was proposed to be the inhibin binding protein (InhBP) (Bernard *et al.*, 2001) it now appears that betaglycan, the TGFβ RIII, acts to increase the affinity of inhibin to bind activin type II receptors through the β chain of inhibin, thus blocking activin binding and activation of the activin receptor (Lewis *et al.*, 2000; Vale *et al.*, 2004). It is generally thought that inhibins act to suppress FSH at the pituitary through binding to the activin type II receptor and thus prevent activin activation (Knight and Glister, 2001; Newton *et al.*, 2002).

Activin stimulates FSHβ-subunit synthesis and secretion (Ulloa-Aguirre and Timossi, 1998) and has various roles within the follicle including stimulating granulosa cell proliferation, promoting FSHR expression, oestradiol synthesis and inhibin production. In addition, activin inhibits progesterone production (Braw-Tal, 1994; Glister *et al.*, 2001; Knight and Glister, 2003). Furthermore, FSH and activin stimulate LHR expression and to a lesser extent FSHR expression (Kishi *et al.*, 1998). In the presence of moderate to high levels of FSH, activin induces FSHR expression, whereas in the presence of low levels of FSH activin inhibits FSHR expression. However, the mechanisms involved are not understood (Xiao *et al.,* 1992; Simoni *et al.,* 1997; Findlay and Drummond, 1999). Furthermore, when granulosa cells were pre-treated with activin the granulosa cells became more sensitive to FSH and LH, with increased steroidogenesis and secretion of growth factors (Xiao *et al.,* 1992).

Activin receptors are expressed on theca cells (Knight and Glister, 2003). In addition, activin receptors (types I and II) are located within the oocyte, and activin is therefore thought to be involved in oocyte maturation (Newton *et al.*, 2002; Knight and Glister, 2003). Activin receptor expression was greatest in the rat oocyte (Cameron *et al.,* 1994). Activin together with FSH stimulated follicle growth and oestradiol synthesis in pre-pubertal mice, whereas activin alone blocked the stimulatory effects of FSH in adult mice (Yokota *et al.,* 1997; Fortune, 2003). Recent studies on activin A have demonstrated that its effects are dependent on the age of the animal. Activin A stimulates follicle growth of small pre-antral follicles in pre-pubertal mice, but inhibits FSH-stimulated growth of antral follicles in adult mice (Mizunuma *et al.*, 1999).

Mice deficient in activin type II receptors demonstrated impaired follicle growth, with development arresting in the early antral stage, suggesting that activin is essential for the final stages of granulosa cell proliferation and differentiation (Knight and Glister, 2003). However, these mice had low FSH levels, which could explain the arrest in follicle development (Knight and Glister, 2003). Inhibin levels exceed activin levels as the follicle develops. Activin inhibits and inhibin stimulates thecal androgen production, and inhibin levels increase beyond activin levels in the latter stages of follicle development, when increased secretion of androgen is required for oestrogen synthesis (Hillier, 2001).

Follistatin binds the β-subunit of activin and neutralises its actions by preventing binding to the type II receptor (Lin *et al.*, 2003), thereby suppressing FSH secretion by preventing activin action. Follistatin also binds to inhibin through the β-subunit, although this does not seem to have the same inhibitory effect as it does on activin (Knight and Glister, 2001; Lin *et al.*, 2003). Follistatin mRNA and protein are expressed by granulosa cells of most species, and expression increases as follicles develop to the pre-ovulatory stage but declines in follicles undergoing apoptosis (Lin *et al.*, 2003).

However, excessive expression of follistatin prevents development beyond the primary follicle stage and ultimately these mice are infertile; suggesting that follistatin regulates the action of TGF-β family members (in particular activin) that are required for follicle growth beyond this stage (Guo *et al.,* 1998; Picton, 2001).

1.8.3 Anti-Mullerian Hormone (AMH)

AMH is a member of the TGF-β family and was initially identified in the fetal testis where it promotes the regression of the Mullerian ducts. However, it is also expressed in the ovary after birth when follicles are activated from the follicle reserve (Themmen, 2005). Amh signals, as do other members of the TGF-β family, through 'heterodimeric serine-threonine kinase receptors', resulting in activation of the Smad pathway, as detailed in Section 1.8.1. The Amh type II receptor is expressed on the cell membrane of granulosa cells of pre-antral and early antral follicles, and theca cells of pre-antral and antral follicles (McGee *et al.*, 2001; Durlinger *et al.*, 2002; Rey *et al.*, 2003). However, Amh is not expressed by theca cells, though the Amh type II receptor is expressed by theca cells of atretic follicles (McGee *et al.*, 2001; Rey *et al.*, 2003). The type II receptor recruits a type I receptor and there are three type I receptors thought to be involved in AMH signalling: Activin Receptor-Like Kinase-6 (ALK6) (also named Bone Morphogenetic Protein Receptor 1B (BMPRI-B)), ALK2 (also named ActRI) and ALK3 (also named BMPRI-A) (Rey *et al.*, 2003). The type I receptor ALK2 is expressed within the ovary, but its cellular localisation remains unknown. ALK6 is not expressed in the foetal ovary, but is expressed by the oocytes of small follicles, and by the oocyte and granulosa cells of larger follicles (Durlinger *et al.*, 2002). Studies examining the role of ALK receptors in mouse Amh signalling demonstrated that Amh is essential for ALK2 (but not ALK6) activation (Visser *et al.,* 2001). Basal AMH levels are gonadotrophin-independent in pre-ovulatory follicles (Rey *et al.*, 2003). Amh has been found to inhibit follicle activation (Durlinger *et al.,* 2001). However Amh increases pre-antral follicle growth when stimulated by FSH (McGee *et al.*, 2001). FSH stimulates *Amh* transcription via activation of the adenylate cyclase, cAMP and PKA pathway (Lukas-Croisier *et al.*, 2003).

1.8.3.1 *Amh* KO Mouse Model

Amh KO female mice are fertile, and it is postulated that *Amh* plays a role in inhibiting follicle development and, in turn, influences reproductive lifespan (Durlinger *et al.*, 2002). Follicle reserve activation is accelerated in *Amh* KO mice compared to wt litter mates, and *Amh* KO mice have a larger cohort of growing follicles (Durlinger *et al.,* 1999). Heterozygous *Amh*-null mice have a follicle reserve between that of *Amh* KO and wild-type mice, suggesting that *Amh* effects are dose-dependent (Durlinger *et al.,* 1999; Gruijters *et al.*, 2003).

Amh KO mice with lower FSH serum levels have increased numbers of growing follicles compared to wt controls, and follicles treated with Amh *in vitro have* a smaller diameter compared to controls suggesting that Amh reduces FSH-sensitivity by inhibiting follicle growth and, in turn, inhibits aromatase expression (Durlinger *et al.*, 2001; Durlinger *et al.*, 2002; Pigny *et al.*, 2003). However, studies with cultured human granulosa cells found no direct effect of FSH on *Amh* mRNA levels though cAMP increased *Amh* mRNA levels (Voutilainen and Miller, 1987; van Rooij *et al.,* 2002). Conversely, other studies in mice found that FSH-stimulated pre-antral follicle growth was inhibited by Amh (Durlinger *et al.*, 2001; Durlinger *et al.*, 2002). Further studies in rodents have concluded that FSH inhibits Amh and Amh type II receptor expression in the ovary (La Marca *et al.*, 2005). Interestingly, those follicles with greatest Amh expression are pre-antral which are not FSH-dependent (La Marca *et al.,* 2005).

Amh inhibits the number of follicles recruited from the primordial follicle reserve and is important for the recruitment and selection of follicles (Salmon *et al.*, 2004). *Amh* deficiency results in accelerated activation of the primordial follicle pool and increased numbers of pre-antral and early antral follicles. This, in turn, results in exhaustion of the follicle reserve at an earlier age (Durlinger *et al.,* 1999; Durlinger *et al.*, 2002). Over-expression of *Amh* during fetal life increases follicle death, and accelerates follicle reserve exhaustion after birth (Behringer *et al.*, 1990; Knight and Glister, 2003). In addition, when treated with human mullerian inhibiting substance (MIS) day 16 female mice ovaries contained seminiferous tubules implying that *MIS* influences the ovary to become more like the testis (Behringer *et al.*, 1990). Serum AMH levels are positively correlated to antral follicle numbers and, therefore, are often used as a marker for ovarian follicle numbers in humans (Gruijters *et al.*, 2003; McIlveen *et al.,* 2006).

Amh levels are greatest in cumulus granulosa cells, but *Amh* inhibits oocyte maturation (Kuroda *et al.*, 1991; Salmon *et al.,* 2004). The inhibition of follicle recruitment by Amh is postulated to occur by inter-follicle communication. Growing follicles secrete Amh and inhibit primordial follicle activation (Salmon *et al.,* 2004). However, the regulation of primordial follicle activation is not due solely to *Amh* and other factors such as *Kit* Ligand and *Gdf-9* are also thought to play a role (Parrott and Skinner, 1999; Durlinger *et al.,* 2002; Shimizu, 2006).

1.8.4 Growth Differentiation Factor-9 (GDF-9)

Gdf-9 is a protein which belongs to the TGF-β family and is secreted by the oocyte (Vitt *et al.*, 2000). Gdf-9 expression is observed in the mouse oocyte throughout all stages of development except in primordial follicles (McGrath *et al.,* 1995; Dong *et al.,* 1996) and it is essential for follicular growth beyond primary follicle development (Dong *et al.,* 1996; Elvin *et al.,* 2000a; Knight and Glister, 2003).

GDF-9 mRNA and protein are expressed in the cumulus granulosa cells and in mural granulosa cells which neighbour the cumulus stalk (Duffy *et al.,* 2003; McNatty *et al.,* 2004). Gdf-9 expression in rat oocytes of antral follicles was not consistent, possibly explaining why follicle development differs from one follicle to another (Vitt *et al.*, 2000). The fundamental role of *Gdf-9* in follicle development was demonstrated in *Gdf-9* KO mice where follicle development arrested at the primary follicle stage, the oocytes deteriorated and, as a result, mice were infertile (Dong *et al.,* 1996; Erickson and Shimasaki, 2000; Vitt *et al.*, 2000; Knight and Glister, 2003). Furthermore, in *Gdf-9* KO mice the somatic cells of the follicle undergo morphological changes after the oocyte has deteriorated, and secrete oestrogen and progesterone, like corpora lutea (Elvin *et al.,* 1999b; Erickson and Shimasaki, 2000). Furthermore, *Gdf-9* KO mice have elevated levels of FSH and LH (Erickson and Shimasaki, 2000).

The *Gdf-9* KO mouse has characteristic follicles with a maximum of one layer of granulosa cells. However, in contrast to inhibited granulosa cell growth, the follicles in these KO mice had enlarged oocytes compared to controls (Erickson and Shimasaki, 2000; Fortune, 2003). The oocytes in *Gdf-9* null mice also grow at an accelerated rate (Carabatsos *et al.,* 1998). In addition, mice deficient in *Gdf-9* had follicles with abnormal granulosa cells and no theca layer was present, suggesting that *Gdf-9* was also required for theca recruitment (Knight and Glister, 2003). However, follicles in the *Gdf-9* KO ovary reach only the primary stage and theca recruitment has not usually occurred by this stage of development (Erickson and Shimasaki, 2000). Therefore, although *Gdf-9* is an oocyte-specific factor it determines granulosa and theca development (Knight and Glister, 2003).

Treatment with Gdf-9 has several effects on follicle development in mice including stimulating follicle growth, enhancing inhibin-α production, promoting cumulus cell expansion, and inhibiting LHR expression (Vitt *et al.*, 2000). The receptors for Gdf-9 have not been identified, although it is thought to bind to TGFβ type receptor ALK5 and BMPR-II (Vitt *et al.*, 2000; McNatty, *et al.,* 2004). Studies so far suggest that Gdf-9 does not stimulate, but rather inhibits FSH-stimulated cAMP production (Vitt *et al.*, 2000). Treatment of cultured granulosa cells with Gdf-9 induces proliferation but reduces FSH-induced differentiation, and inhibits steroidogenesis and LH receptor development. In addition *Gdf-9* suppressed FSH stimulation of cAMP production (Vitt *et al.*, 2000). However, *Gdf-9* is not the only gene that is expressed within the oocyte that regulates follicle growth (for example, *Bmp-15* is co-expressed with *Gdf-9* throughout follicle development; Vitt *et al.*, 2000).

1.8.5 Bone Morphogenetic Proteins (BMPs)

There are several *Bmps* and their receptors are expressed in oocyte, granulosa cells and the theca cells of the follicle (Figure 1.7; Table 1.3; Onagbesan *et al.*, 2003). A list of the *Bmps* present in the ovary is detailed below in Table 1.4. *Bmp-4* and *Bmp-7* are expressed in the theca cells and *Bmp-6* and *Bmp-15* are expressed in the oocyte, with all the Bmp receptors expressed in the granulosa and theca cells or the oocyte (Figure 1.7; Shimasaki *et al.*, 1999; Onagbesan *et al.*, 2003; Brankin *et al.*, 2005). BMP-15 is involved in follicle regulation and development, and regulates granulosa cell proliferation (Otsuka *et al.*, 2000). In a similar manner to *Gdf-9*, *Bmp-15* plays a pivotal role in folliculogenesis (Eppig *et al.*, 2002). The fundamental importance of *Bmp-15* in ovarian development has been highlighted in extensive studies over the last few years (Otsuka *et al.*, 2000). BMP-15 binds to the type I receptor ALK6 and BMPRII in granulosa cells of rats and humans (Moore *et al.*, 2003b; McNatty *et al.*, 2004). The different BMPs bind to different type I and type II receptors and a list of those identified in the ovary to date are detailed below in Table 1.5.

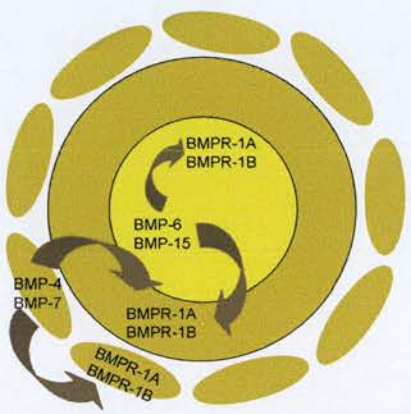


Figure 1.7 BMP and BMPR expression in the rat follicle.
This figure demonstrates the communication between the oocyte, granulosa and theca cells in BMP signalling.

Receptor	Expression
BMPR-IA	Oocytes, Granulosa Cells, Theca Cells
BMPR-IB	Oocytes and Granulosa Cells
BMPR-II	Granulosa Cells
ActR-IA	Oocyte and Theca Cells
ActR-II	Oocyte and Theca Cells

Table 1.3 Summary of BMP receptor expression (Erickson and Shimasaki, 2003; Knight and Glister, 2003).

BMP	Receptor Type I	Receptor Type II
BMP-2	BMPR-II	BMPR-IA
BMP-4		BMPR-IB
BMP-6	BMPR-II	ActR-IA
BMP-7	ActR-II ActR-11B	BMPR-IB
BMP-15	BMPR-II	BMPR-IB

Table 1.4 Summary of BMP ligand and receptor signalling adapted from Shimasaki et al. (2004).

BMP-15 is an oocyte-specific factor similar in structure to *Gdf-9* (Dube *et al.,* 1998) that acts on granulosa cells (Otsuka *et al.*, 2000; Otsuka *et al.*, 2001b). Unlike *Gdf-9* KO mice, *Bmp-15* (*Gdf-9B*) KO mice are fertile but their fertility is impaired, with a decline in ovulation rate and reduced fertility rates, suggesting that (unlike sheep) *Bmp-15* is not required for folliculogenesis, but enhances fertility in mice (Yan *et al.,* 2001; Fortune, 2003). However, homozygous Inverdale and Hanna sheep, which a have natural mutation of the BMP-15 gene, are infertile but heterozygotes have an increased ovulation rate (Galloway *et al.,* 2000). Bmp-15 mRNA and protein are expressed in rodent oocytes at all stages of follicle development, including primordial follicles (Otsuka *et al.*, 2000). BMP-15 stimulates granulosa cell proliferation, and inhibits FSH-stimulated progesterone synthesis, but does not affect oestradiol synthesis (Otsuka *et al.*, 2000; Otsuka *et al.*, 2001a; Otsuka *et al.*, 2001b).

Further studies demonstrated that BMP-15 inhibits the expression of several factors that are regulated by FSH including: StAR, P450scc, 3β-HSD, LHR, inhibin, activin and FSHR (Otsuka *et al.*, 2001b). A list of natural agonists and antagonists of the *BMP* family are summarised below in Table 1.6. The stimulation of granulosa cell mitosis by BMP-15 is probably FSH-independent, as Bmp-15 mRNA and protein are expressed in pre-antral follicles (Otsuka *et al.*, 2000; Brankin *et al.,* 2003). BMP-15 seems to have an inhibitory effect on FSH action through inhibiting FSHR expression (Otsuka *et al.*, 2001b). In addition, BMP-15 was found to reduce expression of genes activated by FSH as well as inhibiting FSHR expression (Otsuka *et al.*, 2001b). This inhibitory action of genes regulated by FSH is postulated to be up-stream of cAMP signalling and occurs as a result of inhibiting FSHR numbers (Otsuka *et al.*, 2001a; Otsuka *et al.*, 2001b). BMP-15 also inhibits progesterone synthesis suggesting that *BMP-15* could be an oocyte factor that inhibits luteinisation (Otsuka *et al.*, 2000).

BMP	Localisation	Animal	Ovarian Effect of BMP
BMP-2	Granulosa Cells	Sheep Rat	1. Stimulated GC differentiation. 2. Inhibits GC progesterone production. 3. Stimulates FSH-induced oestradiol and inhibin production.
BMP-3b	Theca Cells	Rat	1. Possible autocrine action on theca externa.
BMP-4	Theca Cells	Sheep Rat Murine	1. Required for PGC formation. 2. Inhibits GC progesterone production. 3. Stimulates oestrogen production
BMP-5	Granulosa Cell	Rat	1. Inhibits GC progesterone production. 2. Increases cyclin D2 expression. 3. Stimulates GC proliferation.
BMP-6	Oocyte Granulosa Cells	Sheep Rat Porcine	1. Inhibits GC progesterone production. 2. Stimulates oestrogen production.
BMP-7	Theca Cells	Rat	1. Increases GC proliferation in early antral follicles. 2. Reduces ovulation rate. 3. Inhibits GC serum progesterone levels. 4. Enhanced P450 expression in GC. 5. Stimulates oestrogen production.
BMP-8a BMP-8b	Decidual Cells Placenta	Murine	1. Required for PGC formation.
BMP-15	Oocyte	Murine	1. In KO's fertility is mildly impaired. 2. Stimulates GC proliferation (FSH – independent). 3. Inhibits FSH stimulated GC progesterone synthesis.

Table 1.5 Summary of BMPs from various species, their expression within the ovary, the effect they have within the follicle and on fertility (Otsuka et al., 2000; Lee et al., 2001; Souza et al., 2001; Erickson and Shimasaki, 2003; Shimasaki et al., 2004; Hashimoto et al., 2005; Hunter et al., 2005; Pierre et al., 2005; Juengel et al., 2006).

Natural Agonists of BMPs	Natural Antagonist of BMPs
Twisted gastrulation	Follistatin (BMP-4, -7 and -15)
Prostaglandin E2 Receptor (BMP-2)	DAN/Dante
Low levels of follistatin (BMP-7)	Inhibin (BMP-2 and -7)
	Smad6
	noggin (BMP-2, -4, -7, -15)
	Chordin
	Connective-tissue Growth Factor (BMP-4)
	BMP and Activin Membrane Bound Inhibitor (BAMBI)
	FSH (BMP-6)
	Gremlin

Table 1.6 Summary of Natural agonists and antagonists of BMPs (Oelgeschlager et al., 2000; Paria et al., 2001; Wiater and Vale, 2003; Arikawa et al., 2004, Pangas et al., 2004; Shimasaki et al., 2004; Tanaka et al., 2004).

Mutations in either of these TGFβ family members alters the ovulation rate (McNatty *et al.*, 2004). Animals with mutations for either *BMP-15* or *GDF-9* levels have an increased ovulation rate and those with mutations for *BMPRIB* gene (as observed in the Booroola sheep) also have increased ovulation rates (Souza *et al.,* 2001; McNatty *et al.*, 2004). However, low or null *BMP-15* or *GDF-9* inhibits follicle growth and hence ovulation rates (McNatty *et al.*, 2004). *BMP-15* and *GDF-9* double heterozygous sheep mutants have a greater ovulation rate than single mutants for either of these growth factors (Hanrahan *et al.,* 2004; McNatty, *et al.*, 2004). After ovulation the cumulus oophorus was extremely fragile with a reduced number of fertilised oocytes in mice deficient in both *Bmp-15* and *Gdf-9* (Su *et al.*, 2004), implying that both *Bmp-15* and *Gdf-9* are required for a stable cumulus oophorus complex in mice.

1.9 The role of the oocyte in follicle growth

The oocyte is fundamental in regulating granulosa cell development (Buccione *et al.,* 1990). Two studies in 1990 demonstrated that the presence of the oocyte is essential for the production of hyaluronic acid and in turn cumulus cell expansion (Buccione *et al.,* 1990; Salustri *et al.,* 1990). Further studies demonstrated when oocytes from secondary follicles were cultured with primordial follicles. Follicle growth was accelerated in the primordial follicles when a more developmentally-advanced oocyte was cultured with the follicle (Eppig *et al.*, 2002). These findings suggest that overall co-ordination of follicle development is reliant on communication between the oocyte and granulosa cells (Buccione *et al.,* 1990). However, more recent studies have concluded oocyte-granulosa cell communication is 'bi-directional' as oocyte growth requires support from granulosa cells, and granulosa cells require growth factors from the oocyte to achieve full maturation (Eppig *et al.*, 2001; Picton *et al.,* 2003; Su *et al.*, 2004). When mouse oocytes were cultured on follicle cells, the oocyte diameter increased on average by 4.7μm compared to oocytes cultured in medium (Herlands and Schultz, 1984). This demonstrates that there are many complex feedback systems between the oocyte and somatic cells which are fundamental in providing the correct environment for normal growth and maturation. Bi-directional communication between the oocyte and granulosa cells is essential for normal follicle growth and maturation. However, even though oocytes are absent in *Dazl* KO mice the cells present in the ovary are still steroidogenically active (McNeilly *et al.*, 2000).

On resumption of meiosis the germinal vesicle breaks down (Manabe *et al.,* 2004). Removal of oocytes from antral follicles causes them to resume meiosis, suggesting that the granulosa cells prevent the oocyte resuming premature meiosis (Eppig, 2001). Oocytes *in vivo* do not resume meiosis until the LH surge unless they succumb to atresia. However, *in vitro* oocytes resume meiosis unless cultured with mural granulosa cells, when this does not occur. This implies that mural granulosa cells secrete an inhibiting substance that prevents the resumption of oocyte meiosis (Peters, 1969; Tsafriri and Channing, 1975). This inhibitory substance was demonstrated to be present in the follicular fluid as oocytes cultured *in vitro* with follicular fluid demonstrated inhibited maturation (Tsafriri and Channing, 1975; Tsafriri and Pomerantz, 1984).

1.10 Cell-Cell Communication

1.10.1 Oocyte-Somatic Communication

Communication between the oocyte and the granulosa cells is maintained through the formation of gap junctions and follicles with disrupted gap junctions experience impaired follicle growth and oocyte development (Anderson and Albertini, 1976; Buccione *et al.,* 1990; Eppig *et al.*, 1997a). Gap junctions provide essential channels for small molecular weight compounds (<1kDa) from the granulosa cells to reach the oocyte (Buccione *et al.,* 1987; Kidder and Mhawi, 2002). Gap junctions are present in primordial to pre-ovulatory follicles and enable the transfer of low molecular weight nutrients and growth factors between the oocyte and the granulosa cells and between the granulosa cells (Anderson and Albertini, 1976; Mitchell and Burghardt, 1986; Sommersberg *et al.,* 2000; Wassarman, 2002; Senbon *et al.*, 2003). These junctions have been identified in the murine fetal ovary as early as embryonic day 13 and as the embryo matures the number of gap junctions increases (Mitchell and Burghardt, 1986). In addition, transzonal projections (cell channels that cross between the granulosa cells and the oocyte) provide a direct channel for communication between the granulosa cell and the oocyte and their formation is inhibited by FSH (Albertini *et al.*, 2001). Reduced transzonal projections has been associated with poor oocyte viability (Carabatsos *et al.,* 1998) demonstrating its importance in follicle development.

Gap junction channels are formed by the end-end attachment of connexins from adjoining cells. Cellular communication through those channels is regulated by calcium and by the characteristics of the connexins themselves (Bruzzone *et al.,* 1996; Sommersberg *et al.,* 2000; Gittens *et al.,* 2005). Connexins expressed in the murine antral follicle include Connexin-32, -37, -43, and -45 (Wright *et al.,* 2001). The connexins involved in cellular communication are dependent upon the cell type within the follicle. Oocyte growth is impaired in mice deficient in Connexin-43 and follicles are unable to develop multiple granulosa cell layers (Juneja *et al.,* 1999).

Murine oocytes from mice lacking Connexin-43 had inhibited oocyte growth and granulosa cell development was also impaired (Ackert *et al.*, 2001). Mice deficient in Connexin-37 (gap junctions between oocyte and cumulus granulosa cells) or Connexin-43 (gap junctions between granulosa cells) lack follicles with multi-granulosa cell layers and subsequently are infertile (Simon *et al.*, 1997; Ackert *et al.*, 2001). Connexin-43 null mice had impaired oocyte growth with abnormal ZP formation (Ackert *et al.*, 2001). Up-regulation of Connexin-43 reduced apoptosis of granulosa cells (Sasson *et al.*, 2003b).

However, not all molecules can move in this manner and larger molecules transfer by endocytosis (Gilchrist *et al.*, 2004), a process whereby the cell membrane pinches off into the cell so transporting larger molecules into the cell. Oocyte-somatic cell communication is also through paracrine signalling (Otsuka *et al.*, 2000; Brankin *et al.*, 2003). There are many factors secreted by the oocyte that act on the granulosa cells, for example the TGF-β family (Dong *et al.*, 1996; Otsuka *et al.*, 2000) and it is through paracrine signalling that Dazl is thought to act within the follicle. However, as mentioned previously bi-directional communication is required to enable follicle maturation.

1.10.1.1 Granulosa Cell Proliferation, Differentiation and Steroidogenesis

The role of the oocyte in granulosa cell proliferation is shown as cell division increases in cumulus granulosa cells compared to mural granulosa cells (Vanderhyden *et al.*, 1992). Thymidine labelling of rat ovaries demonstrated that no thymidine was localised to mural granulosa cells in antral follicles, implying the cells had differentiated (Hirshfield, 1986). The effect of the oocyte on granulosa cell proliferation was illustrated when rat oocytes were co-cultured with rat granulosa cell and DNA synthesis was stimulated in the granulosa cells. This effect was also observed, although to a lesser extent, when granulosa cells were cultured with oocyte-conditioned media, demonstrating that the oocyte secretes a soluble factor which can stimulate granulosa cell proliferation (Lanuza *et al.*, 1998).

When oocyte-granulosa cell contact was prevented, the development of cumulus cells was inhibited demonstrating that cell contact is essential for successful follicle growth and development (Vanderhyden *et al.*, 1993; Vanderhyden and Macdonald, 1998). The different phenotypes of the cumulus granulosa cells and mural granulosa cells are determined by the influence of the oocyte, the granulosa cells nearest the oocyte becoming the cumulus cells, whilst those further away where the oocyte secreted factors are less likely to influence are the mural granulosa cells (Vanderhyden *et al.*, 1990; Vanderhyden *et al.*, 1992; Gilchrist *et al.*, 2004).

Oocytes also regulate granulosa cell steroidogenesis, and have been demonstrated to inhibit progesterone production and LHR expression as well as sustaining oestradiol synthesis by granulosa cells (Vanderhyden and Macdonald, 1998; Eppig *et al.,* 1997b). Mural granulosa cells differ from cumulus granulosa cells. They are more steroidogenic, have a higher LHR expression and have a lower proliferation rate (Eppig *et al.,* 1997b). The effect of the oocyte on LHR expression is dependent upon the developmental stage of the oocyte, with mature oocytes from pre-ovulatory follicles capable of suppressing LHR expression more than pre-antral follicles in pre-ovulatory mural granulosa cells where LHR is abundant (Eppig *et al.*, 1997b).

1.10.1.2 Oocyte Specific Factors Involved in Follicle Development

1.10.1.2.1 GDF-9 & BMPs

In mammals *Bmps* are expressed in the oocyte, granulosa and theca cells. *Bmp-6* and *Bmp-15* are oocyte-specific *Bmps* similar to *Gdf-9* (McGrath *et al.,* 1995; Otsuka *et al.,* 2000; Vitt *et al.,* 2000; Erickson and Shimasaki, 2003; Onagbesan *et al.,* 2003). The receptors for these Bmps are expressed both within the oocyte and the granulosa cells (Erickson and Shimasaki, 2003; Knight Glister, 2003). BMP-6 and BMP-15 have been shown to inhibit progesterone production in the granulosa cells, demonstrating paracrine communication between the oocyte and the granulosa cells. Previous studies have demonstrated that the oocyte secretes a factor that stimulates cumulus cell proliferation (Eppig *et al.,* 1993). BMP-15 has a stimulatory effect on granulosa cell proliferation, an effect which was shown to be FSH-independent (Otsuka *et al.,* 2000; Yang *et al.,* 2003; Brankin *et al.,* 2005). Initial studies of BMP-6 and its actions in the rat ovary demonstrated that although *Bmp-6* is an oocyte-secreted factor, it does not have the same actions as BMP-15. Instead BMP-6 regulates FSH signalling by reducing cAMP production and unlike BMP-15 does not affect granulosa cell proliferation (Otsuka *et al.,* 2001a). Oocyte specific *Bmp-15* is important in follicle development. Although its absence in the mouse does not result in infertility, it can impair fertility (Otsuka *et al.,* 2000; Yan *et al.,* 2001). This effect is species dependent (Shimasaki *et al.*, 2004), again demonstrating the complexity of oocyte-somatic cell communication. For example, murine *Bmp-15* null mice have impaired fertility, whereas *BMP-15* mutated sheep are sterile (Galloway *et al.,* 2000; Yan *et al.,* 2001).

Gdf-9 again demonstrates that the importance of the oocyte in follicle development. *Gdf-9* is expressed in the murine oocyte at all stages of follicle development (McGrath *et al.,* 1995; Matzuk *et al.,* 2000). In the absence of this oocyte factor animals are infertile with follicles not capable of surviving past the primary follicle stage of development (Dong *et al.,* 1996). Further studies demonstrated that *Gdf-9* regulates cumulus cell expansion and also stimulates progesterone production in the granulosa cells (Elvin *et al.,* 1999a; Elvin *et al.,* 2000b).

1.10.1.2.2 *Kit* Ligand – c-*Kit*

Kit Ligand is expressed in the granulosa cells and its receptor c-*Kit* in the oocyte (Manova *et al.,* 1990; Joyce *et al.,* 1999). KO mice for either *Kit* Ligand or c-*Kit* are infertile, and the follicles are unable to develop normally (Driancourt *et al.,* 2000). Murine oocytes grown *in vitro* were stimulated 67% by *Kit* Ligand compared to controls (Packer *et al.*, 1994). Previous studies have also demonstrated the importance of paracrine signalling by *Kit* Ligand (expressed in granulosa cells) and *Kit* (expressed in the theca). *Kit* Ligand stimulated androstenedione but not progesterone production in cultured bovine theca cells (Parrott and Skinner, 1997). In the mouse *Kit* Ligand increased follicle testosterone levels (Reynaud *et al.,* 2000). These oocyte factors not only work independently but also interact with each other. For example, oocyte factor BMP-15 increases *Kit* Ligand expression in rat granulosa cells, and Bmp-15 expression in the oocyte was inhibited by *Kit* Ligand (Otsuka and Shimasaki, 2002; Thomas and Vanderhyden, 2006). Gdf-9 inhibits *Kit* Ligand expression in small pre-antral follicles and mural granulosa cells (Joyce *et al.*, 2000; Thomas and Vanderhyden, 2006).

1.10.1.2.3 Other Factors

Oocyte communication is fundamental in follicle development and several studies suggest that oocyte-secreted factors regulate FSH action by inhibiting the expression of P450scc, progesterone production and LHR, and by stimulating hyaluronic acid and oestradiol synthesis (Vanderhyden *et al.,* 1990; Vanderhyden and Tonary, 1995; Eppig *et al.,* 1997b; Otsuka *et al.*, 2001b). Hyaluronic acid is produced in the cumulus granulosa cells after the gonadotrophin surge, and stimulates oocyte-cumulus expansion, whereby the spaces between the granulosa cells expand (Eppig, 1979; Eppig *et al.,* 1997b; Eppig, 2001; Richards *et al.*, 2002). *In vitro* studies of mouse oocyte-cumulus complexes have demonstrated that FSH stimulates hyaluronic acid synthesis but LH does not (Eppig, 1979). Oocyte factors are thought to regulate hyaluronic acid synthesis, as mural granulosa cells (which do not normally synthesise hyaluronic acid) synthesised hyaluronic acid when treated with oocyte-conditioned media (Salustri *et al.,* 1990; Elvin *et al.,* 1999a). The mural granulosa cells remain in the ovary after ovulation and luteinise and together with the theca cells, they establish the corpus luteum (Eppig, 2001). The oocyte also regulates the production of progesterone by inhibiting its synthesis and, therefore, may suppress precocious luteinisation (Vanderhyden and Tonary, 1995; Eppig, 2001).

Furthermore, factors such as FSH are also influential in the regulation of oocyte-somatic cell interaction (Thomas and Vanderhyden, 2006). Oocyte growth and development in FSHR-KO mice is impaired (Yang *et al.*, 2003). Although the FSHR is expressed in the granulosa cells, FSH signalling is fundamental for normal oocyte maturation again demonstrating the importance of oocyte-somatic cell interactions (O'Shaughnessy *et al.,* 1996; Yang *et al.*, 2003). In addition, murine granulosa cells are required to provide energy sources (which is required for fertilisation) such as glucose to the oocyte. This occurs through gap junctions, and without cumulus cells, minimal levels of glucose is absorbed by the oocyte (Saito *et al.*, 1994; Sutton *et al.*, 2003). At present little is known about the germ cell factor *Dazl* and its influence upon granulosa cells. A single copy of this oocyte-specific gene has a dramatic effect on follicle development, maturity and litter sizes compared to when two copies are present (McNeilly *et al.*, Unpublished; Appendix A). All of the above studies have demonstrated the importance of bi-directional oocyte-somatic cell communication and its effect on the follicle growth and development.

1.10.2 Theca and Granulosa Cell Communication

Normal follicle development requires granulosa-theca cell interaction. The following section will discuss the factors that are secreted by the granulosa and theca cells and how they interact with one another. Most of the research on morphology and growth has been carried out in cattle. However, it does demonstrate the importance of the interactions in other species and highlights potential areas of importance in the mouse.

1.10.2.1 Effect on Morphology, Growth and Development

A recent study by Tajima *et al.* (2006) demonstrated the importance of granulosa cell and theca cell communication with regards to cell structure and morphology. When granulosa cells were co-cultured with theca cells, the granulosa cells became more convex and developed multi-layers of cells compared to the mono-layer of concave cells when granulosa cells were cultured alone (Tajima *et al.*, 2006). Similarly, when theca cells were cultured alone, the cells were sparse and concave shaped compared to those co-cultured with granulosa cells (Tajima *et al.*, 2006). Co-cultures of bovine granulosa and theca cells stimulated proliferation of both cell types, in the early stages of folliculogenesis. However, in follicles in the latter stages of development, only theca cell proliferation was stimulated when cultured with granulosa cells, and granulosa cell growth was independent of theca cells (Tajima *et al.*, 2006).

1.10.2.2 Hormone, Steroid and Growth Factor Production

Androstenedione levels significantly increased when granulosa-theca cells were co-cultured compared to granulosa cells cultured on their own (Tajima *et al.*, 2006). This supports previous studies in the bovine (Fortune, 1986) and suggests that theca and granulosa cell communication is important in steroidogenesis.

Granulosa-theca cell interactions and their outcome on follicle hormone production are dependent upon the stage of culture and the type of theca cell cultured. Theca *interna* cells inhibited progesterone production in the granulosa cells whereas theca *externa* cells had no effect (Bosc and Nicolle, 1997). Since the cells in the theca *externa* are physically further from the granulosa cells, the effects of granulosa cell factors would be expected to be less. However, both theca *interna* and *externa* inhibited oestradiol production by the granulosa cells (Bosc and Nicolle, 1997). This result was unexpected as, detailed in Section 1.7.1, oestrogen production requires interaction between the theca and the granulosa cells (Hillier *et al.,* 1994). Briefly, the theca cells convert cholesterol to androgens when stimulated by LH and the androgen diffuses across the basement membrane to the granulosa cells where it is aromatised to oestrogen (Hillier *et al.,* 1994). Without this interaction between the granulosa and theca cells synthesis of oestrogen is not possible.

Activin is produced in the granulosa cells (Halvorson and DeCherney, 1996), and activin receptors are expressed in the granulosa cells and the oocyte (Cameron *et al.,* 1994; Knight and Glister, 2003). Thecal activin inhibits LH-stimulated androgen production and in turn, oestradiol production (Hsueh *et al.,* 1987; Knight and Glister, 2003). Inhibin and activin are both secreted by the granulosa cells, but have opposing effects with regards androgen production, with activin inhibiting and inhibin stimulating androgen production (Hsueh *et al.*, 1987). These granulosa cell factors influence thecal cell activity (androgen synthesis) which in turn affects oestrogen synthesis by the granulosa cells.

As mentioned in Section 1.8.5, *Bmps* are expressed in specific cell types of the follicle, and their receptors are expressed within the oocyte, granulosa cells and theca cells to varying degrees (Table 1.3) (Erickson and Shimasaki, 2003). *Bmp-4* and *Bmp-7* are expressed in the theca cells and Bmp-4 and Bmp-7 receptors are expressed by granulosa cells and are responsible for stimulating oestrogen synthesis and inhibiting progesterone production (Lee *et al.,* 2001; Knight and Glister, 2003) – again demonstrating the importance of cell-cell interaction in follicle development BMP-7 stimulates P450 aromatase expression in the granulosa cells and therefore, oestrogen production (Lee *et al.,* 2001).

These studies emphasise the importance of communication between different components of the follicle with regards cell morphology, growth and hormone and steroid production. Once activated from the resting pool the ovarian follicle is continually developing. This requires stringent control of growth factors, hormones and steroids. If any factor is secreted in abnormal amounts, the development of the follicle (and in turn fertility) may be affected drastically. Normal follicle development is reliant on oocyte and somatic cell communication to ensure that the correct environment is established to allow follicle maturation and fertility.

1.11 FSH and LH

The ovarian cycle is regulated by gonadotrophin-releasing hormone (GnRH) secreted from the hypothalamus that acts upon the gonadotrophs in the anterior pituitary gland to stimulate the secretion of FSH and LH. These hormones in turn act upon specific receptors expressed in the ovary (Themmen and Huhtaniemi, 2000; Burns *et al.*, 2001; Vassart *et al.,* 2004). Both FSH and LH are produced by the gonadotrophs in the pituitary (Ulloa-Aguirre and Timossi, 1998). FSH is a member of the glycoprotein family that includes LH, human chorionic gonadotrophin (hCG) and thyroid-stimulating hormone (TSH) (Mercer and Chin, 1995). All share a common α-subunit, with a specific β-subunit that determines the biological effect of the hormone (Kumar *et al.,* 1997; Simoni *et al.*, 1997; Hillier, 2001; Vassart *et al.,* 2004). The FSHβ-subunits are highly conserved between species as demonstrated by Maurer and Beck (1986) who analysed the nucleotide sequence of bovine FSHβ and compared this sequence with the FSHβ sequence from other mammals (Maurer and Beck, 1986; Ulloa-Aguirre and Timossi, 1998). FSH and LH are large proteins and although the β-subunits are specific to each hormone there is a similarity in amino acid sequence between hormones. However, the FSHβ-subunit also differs from LHβ in that it has a longer N-terminal, although the importance of this feature remains unknown (Ulloa-Aguirre and Timossi, 1998; Themmen and Huhtaniemi, 2000).

1.11.1 Follicle Development and FSH

FSH is necessary for normal follicle growth and development and is required for granulosa cell differentiation and proliferation (Sasson *et al.,* 2003a). FSH is also responsible for follicle dominance. The follicle(s) that first express the FSHR can continue to respond to falling levels of FSH and will become dominant. All other FSH-dependent follicles that do not express sufficient FSHR will become atretic. FSH stimulates granulosa cell proliferation, increases follicular fluid production and stimulates oestradiol secretion and the expression of the LHR (Zeleznik and Kubik, 1986; Burns *et al.*, 2001; Senbon *et al.,* 2003).

Even when LHR expression is induced on the granulosa cells, low levels of FSH continue to be required for their maintenance (Adashi, 1994). Rat granulosa cell cultures demonstrated that LHR expression is reliant upon FSH, and after exposure to FSH LHR expression initiates within 24-48 hours. However, the effect of FSH on LHR expression is also dependent on oestradiol and progesterone (Rani *et al.,* 1981). Spears *et al.* (1998) demonstrated the importance of FSH (with this particular culture method) for pre-antral follicles to develop to Graafian follicles *in vitro*. FSH is also essential for granulosa cell proliferation and differentiation, and reduces atresia rates of hamster follicles *in vitro* (Roy and Greenwald, 1989). Lack or low secretion of FSH results in underdeveloped secondary sex characteristics as a result of low oestrogen secretion (Themmen and Huhtaniemi, 2000). Layman *et al.* (1997) reported a human case in which there were mutations of the FSHβ gene and this postponed puberty. The importance of FSH action in the ovary was demonstrated by FSHβ KO and FSHR KO models. Females were infertile, with follicles unable to develop beyond the pre-antral stage. Expression of P450arom and P450scc were down-regulated in mice null for FSHβ, demonstrating the essential role of FSH in follicular steroid production (Dierich *et al.,* 1998; Burns *et al.*, 2001).

Although FSH alone was capable of maintaining follicle growth, a delicate balance of other growth factors and hormones was also essential for follicle development (Canipari, 2000). FSH was not essential for FSHR expression, as FSHβ-KO mice had increased FSHR expression (Burns *et al.*, 2001). Granulosa cell proliferation drives follicle growth to the stage where they become responsive to FSH. FSH also induces increased proliferation of cumulus cells relative to mural cells (Hirshfield, 1986; Erickson and Shimasaki, 2000; Eppig, 2001). This suggests that the oocyte is important in granulosa cell responsiveness to FSH, and that the oocyte determines the fate of granulosa cells and their function (Eppig *et al.,* 1997a; Eppig *et al.,* 1997b; Erickson and Shimasaki, 2000). However, FSH *in vitro* increases mural granulosa cell proliferation and suppresses cumulus granulosa cell proliferation (Vanderhyden *et al.*, 1992).

Follicle growth in the latter stages of pre-antral follicle development can be advanced by FSH *in vivo* and *in vitro* (Hirshfield and Midgley, 1978b; Webb *et al.*, 2003). FSH is essential for antral growth, and stimulates follicle growth at this stage of development *in vivo* and *in vitro* (Picton and McNeilly, 1991). FSH also plays a critical role in the regulation of follicular development by increasing responsiveness to both itself and to other hormones and growth factors in the granulosa cell. Inadequate levels of FSH result in follicle atresia (Hirshfield, 1989; Hillier, 1994).

Follicles become FSH dependent at the early antral stages of development. However, as the follicle matures, sensitivity to FSH increases and less FSH is required for continuing development (Zeleznik *et al.,* 1985; Hillier, 1994). Initially sufficient levels of FSH are present to promote the growth and development of several follicles. However, as FSH levels fall and follicles become gonadotrophin-dependent, subordinate follicles become atretic (Zeleznik *et al.,* 1985; Hillier, 1994). Although there is a threshold requirement for FSH to stimulate follicle growth there is also a maximum threshold beyond which FSH can no longer stimulate development (Brown, 1978).

1.11.2 FSHR Structure

The FSHR and LHR belong to the G-protein coupled receptor (GPCR) family that is distinguished by seven hydrophobic transmembrane helices. Gonadotrophin receptors are located on the granulosa and theca cell membrane (Simoni *et al.,* 1997), although FSHR expression has also been observed in the oocyte (Roy and Greenwald, 1986). G proteins consist of α, β and γ subunits (Figure 1.8). The β and γ subunits are normally 'tightly bound' to one another (Ulloa-Aguirre and Timossi, 1998).

The N-terminal domain of the FSHR is extracellular, and the C-terminal domain is intracellular (Jiang *et al.,* 1995; Bogerd *et al.*, 2005). Once FSH is bound to the receptor, the G-protein is activated by the exchange of guanosine diphosphate (GDP) to guanosine triphosphate (GTP), resulting in the α-subunit dissociating from the $\beta\gamma$-subunits. This stimulates the enzyme adenylate cyclase, activating the conversion of adenosine triphosphate (ATP) to cAMP (Hillier, 2001; Conti, 2002). This increase in cAMP activates the PKA pathway. Upon activation, the catalytic and regulatory PKA units dissociate, activating protein phosphorylation (PKA) and leading in turn to the activation of cAMP response element binding protein (CREB) (Figure 1.8; Simoni *et al.,* 1997; Conti, 2002). However, other studies dispute whether the PKA pathway alone is sufficient for FSH-induced gene activation. Studies on female rats demonstrate that protein kinase B (PKB) is also necessary for activation of granulosa cell differentiation by FSH (Gonzalez-Robayna *et al.*, 2000; Richards, 2001b; Zeleznik *et al.,* 2003). Although the PKA pathway is the main pathway involved in FSH signalling, other pathways are also involved, including the protein kinase C (PKC) pathway, and increased intracellular calcium levels. Furthermore, the PKA pathway also activates the mitogen-activated protein kinases (MAPKs) pathway (Flores *et al.,* 1992; Richards, 2001b). Studies in porcine granulosa cells demonstrated that cAMP stimulates calcium accumulation in granulosa cells. However, this was not considered to involve the PKA pathway (Flores *et al.,* 1992). The contribution of these different pathways in follicular regulation remains unclear (Flores *et al.,* 1992; Simoni *et al.,* 1997; Themmen and Huhtaniemi, 2000; Zeleznik *et al.,* 2003).

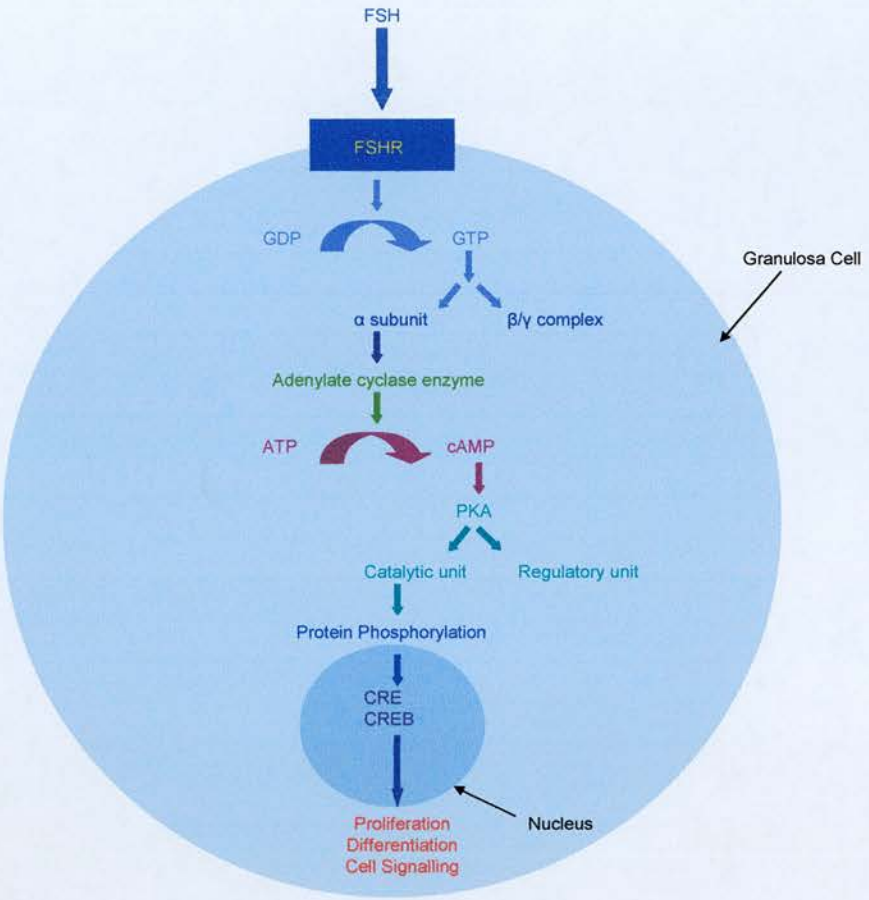


Figure 1.8 FSH signalling pathway (adapted from Richards, 2001b).
FSH activates cAMP and PKA pathway resulting in cell proliferation and differentiation.

The FSHR undergoes 'alternative splicing' resulting in four different mRNA transcripts. These splice variants are both age- and species-dependent and could explain why FSH affects follicle growth at specific stages and development (O'Shaughnessy *et al.,* 1996; Findlay and Drummond, 1999). Additionally, activation of these various splice variants of FSHR at different time points and physiological states may alter the biological effects of FSH (e.g. cellular growth versus steroidogenesis; Padmanabhan *et al.*, 1999).

1.11.3 FSHR Expression

FSHRs are localised solely on granulosa cells (Camp *et al.,* 1991; Simoni *et al.,* 1997; Eppig, 2001; Hillier, 2001). FSHR mRNA in the mouse was expressed by the granulosa cells in the ovary of primordial follicles (at birth), and FSHR mRNA levels rose until day 10, when follicles in the initial growing cohort reached the stage of antral development (O'Shaughnessy *et al.,* 1996; Findlay and Drummond, 1999), although some studies have demonstrated that FSHR mRNA is not present in primordial follicles but is initiated in primary follicles (Tisdall *et al.,* 1995).

Further studies have demonstrated the presence of FSHRs and FSHR mRNA in human, pig and hamster oocytes of primary through to pre-ovulatory follicles. FSHR expression in oocytes declines when follicles became atretic (Oxberry and Greenwald, 1982; Roy and Greenwald, 1986; Meduri *et al.*, 2002). These findings suggest that FSH may act directly upon the oocyte and suggest that FSH action in the oocyte is required for follicle growth (Meduri *et al.*, 2002). Patsoula *et al.* (2001) confirmed the presence of both FSH and LH receptors in the mouse oocyte and in the early stages of development of the embryo. However, studies on oocyte-granulosa cell complexes demonstrated that FSH did not promote or enhance the ability of the oocyte to undergo maturation, yet in the presence of FSH with other growth factors, oocyte maturation was more effective (Eppig *et al.,* 1998).

Expression of the FSHRs is first detected in primary follicles (Simoni *et al.,* 1997), although their function at this stage is unknown, as the follicle is thought to be gonadotrophin-independent (Findlay and Drummond, 1999). Insufficient levels of plasma FSH, reduced sensitivity to FSH or reduced FSHR levels are associated with apoptosis (Burns *et al.*, 2001). Although FSHR expression is reliant upon circulating FSH, previous studies in hypogonadal mice that are deficient in circulating FSH have demonstrated expression of FSHR mRNA transcripts (O'Shaughnessy *et al.*, 1994). Although FSHR expression has been demonstrated in primary follicles, studies suggest that these receptors are not 'coupled' to cAMP, as treatment of bovine cortical strips with dibutyryl cAMP did not increasing the ratio of primary follicles to secondary follicles (Derrar *et al.,* 2000; Fortune, 2003). In rats the ovary begins to respond to FSH from day 4 with increased cAMP levels which correspond to FSHR mRNA expression (Simoni *et al.,* 1997). cAMP levels increased in response to FSH in day 4 rats. However, cAMP levels did not respond to LH until day 7 (Sokka and Huhtaniemi, 1990).

Treatment with pregnant mare serum gonadotrophin (PMSG) increased rat granulosa cell FSHR expression (LaPolt *et al.,* 1992; Simoni *et al.,* 1997). However, induction of ovulation with hCG or with a high dose of recombinant FSH reduced FSHR expression, suggesting a 'biphasic' pattern of FSHR expression (LaPolt *et al.,* 1992; Simoni *et al.,* 1997). FSH is important in regulating FSHR expression. FSHR expression in rat granulosa cells *in vivo* increased after treatment with moderate levels of FSH, and FSH stimulated granulosa cell proliferation in granulosa cells that contain FSHRs. However, the hypogonadal mouse still expresses FSHRs suggesting that other factors are also involved in this process (O'Shaughnessy *et al.,* 1994; Simoni *et al.,* 1997). Therefore, FSHR expression is not solely dependent upon the presence of gonadotrophins. Indeed, some members of the TGF-β family (such as activin) enhance FSHR expression, whereas BMP-15 suppresses FSHR expression (Otsuka *et al.*, 2001b; Inoue *et al.*, 2003).

1.11.4 FSH Desensitisation

FSH desensitisation is the process by which FSHR numbers either decrease ('down-regulation') or receptor uncoupling occurs, usually as a result of over exposure to the hormone (Simoni *et al.,* 1997). Both LH and FSH share the adenylyl cyclase signalling pathway. Granulosa cells express both FSH and LH receptors and can become desensitised by high levels of either hormone (Jonassen and Richards, 1979; LaPolt *et al.*, 1992). The effect of FSH on FSHR expression is dependent upon the stage of follicle development (LaPolt *et al.,* 1992). Continuous FSHR stimulation which can occur naturally when there are deficiencies in inhibin, and FSH levels are elevated as a result of a lack of negative feedback results in cAMP down-regulation, and in turn reduced steroidogenesis (Themmen and Huhtaniemi, 2000; Amsterdam *et al.*, 2002; Amsterdam *et al.*, 2003).

1.11.5 Regulation of Ovarian Genes by FSH

FSH is involved in follicle growth and development and follicle selection by modifying specific genes and growth factors involved in these processes (Ulloa-Aguirre and Timossi, 1998). Steroids secreted from the ovary both stimulate and inhibit GnRH secretion, thereby altering FSH synthesis (Themmen and Huhtaniemi, 2000). Follicular steroidogenesis is dependent upon the action of FSH and LH on the granulosa and theca cells (Knobil and Neill, 1994; Simoni *et al.*, 1997). FSH regulates several genes in the ovary which, in turn, regulate follicle growth and development, and also regulates several genes involved in steroidogenesis including P450 aromatase, P450scc, StAR and 3β-HSD (Otsuka *et al.*, 2001). Other important factors include *IGF-1*, *Vascular Endothelial Growth Factor* (*VEGF*), *Connexin 43*, *Kit* Ligand and *Fox-01* (Richards, 1994; Shi and LaPolt, 2003; Zimmermann *et al.,* 2003; Otsuka *et al.*, 2005). The FSH-dependent factors discussed here include; inhibin, activin, oestradiol, *IGF*, *Connexin-43* and *Kit* Ligand.

As described in Section 1.8.2, FSH stimulation increases granulosa cell proliferation and the secretion of inhibins and activins (Hirshfield and Midgley, 1978b; Ying, 1987; Simoni *et al.,* 1997; Findlay and Drummond, 1999). Additionally, as described in Section 1.7.1, elevated levels of FSH increase granulosa cell proliferation, increasing the expression of cytochrome P450 aromatase and therefore oestradiol synthesis (Vanderhyden *et al.*, 1992; Hillier, 2001). In addition, oestradiol augments FSH action on granulosa cells (Otsuka *et al.*, 2005), and promotes LHR expression by the granulosa cells in the latter stages of development (Eppig *et al.,* 1997; Hillier, 2001). At this stage the follicle is able to respond to both FSH and LH (Vitt *et al.*, 2000), and is increasingly sensitive to gonadotrophins allowing the follicle(s) to achieve dominance and to undergo the final pre-ovulatory changes necessary for eventual ovulation.

Igf-1 is required for follicle development. *Igf-1* KO mice are not responsive to gonadotrophins and follicles growth is arrested at the pre-antral stage of development (Fortune, 2003). *Igf-1* is produced by granulosa cells and theca cells, and Igf-1 in turn stimulates FSH action within the ovary. The mechanism by which this occurs remains unknown (Wang and Chard, 1999; Hillier, 2001; Richards *et al.*, 2002). Receptors for Igf are expressed solely in theca cells, and androgen production is stimulated by Igf-1 in the rat theca cells *in vitro* (Hernandez *et al.,* 1988; Hillier, 2001). PKB is a 'downstream target' of Igf-1, a pathway that is also activated by FSH. Hence, FSH can imitate some of the effects of Igf-1 through activation of this pathway (Richards, 2001b).

In FSHβ subunit-deficient mice, *Igf-1* expression is not affected compared to controls but Igf-1 receptor numbers in granulosa cells are reduced (Richards *et al.*, 2002). Zhou *et al.* (1997) demonstrated that *Igf-1* levels in FSH KO mice remain normal, suggesting that FSH may not be required for *Igf-1* regulation. However, *Igf-1* KO mice have reduced expression of FSHR (Zhou *et al.*, 1997), suggesting that, although FSH may not regulate *Igf-1* transcription, *Igf-1* may stimulate FSHR. *Vegf* is produced by granulosa and theca cells and plays an important role in angiogenesis. It is also required for pre-ovulatory follicle development (Zimmermann *et al.*, 2003). *Vegf* is also regulated by FSH, and mRNA levels increase *in vitro* after FSH stimulation (Sasson *et al.*, 2003a).

The gap junction protein Connexin-43 is expressed by granulosa cells and is implicated in cell signalling within the follicle (Sommersberg *et al.,* 2000). *Connexin-43* gene expression increases in larger follicles (Sommersberg *et al.,* 2000). FSH induces the expansion of cumulus cells, which are connected to each other through gap junctions within the follicle. However studies showed that the production of equine *Connexin-43* did not vary during follicle maturation whereas rat *Connexin-43* levels were up-regulated by FSH treatment (Sommersberg *et al.,* 2000; Dell'Aquila *et al.*, 2004). Connexin-43 expression is also related to the health of the follicle, and granulosa cells from quails had reduced Connexin-43 expression in follicles showing signs of apoptosis (Krysko *et al.,* 2004).

Kit Ligand plays an important role in follicle development. It is expressed only by mural granulosa cells and expression is greatest in antral follicles (Driancourt *et al.*, 2000; Fortune, 2003). Follicle growth is blocked in the primary stage of development in *Kit* Ligand KO mice (Huang *et al.,* 1993; Gittens *et al.*, 2005). Murine *Kit* Ligand mRNA expression is present in small pre-antral follicles but expression is greatest in large antral follicles (Manova *et al.,* 1993; Fortune, 2003). Treatment of neo-natal rat ovaries with *Kit* Ligand led to an increased number of growing follicles, suggesting that *Kit* Ligand may be important in primordial follicle activation (Parrott and Skinner, 1999; Fortune, 2003).

Kit Ligand and c-*Kit* interaction also promotes follicle recruitment and growth, stimulating oocyte and theca growth, and protects the follicle from atresia (Eppig, 2001; Senbon *et al.*, 2003). Treatment of mouse follicles with *Kit* Ligand enhanced oocyte maturation compared to follicles with no treatment (Reynaud *et al.,* 2000). FSH up-regulates the expression of *Kit* Ligand dose dependently and this may explain why *Kit* Ligand expression is more abundant in more advanced follicles (Driancourt *et al.*, 2000). The *Kit* Ligand receptor, c-*Kit*, is expressed in the oocyte. Oocyte growth has been reported to have 3 stages: stage 1, which is not reliant upon gap junctions with granulosa cells and is stimulated by *Kit* Ligand; stage 2, which requires both gap junctions and *Kit* Ligand, and stage 3, which is dependent entirely upon granulosa cell communication (Klinger and DeFelici, 2002; Wassarman, 2002). Whilst *Kit* Ligand can initiate oocyte growth, it does not promote full maturation (Klinger and DeFelici, 2002).

1.11.6 FSHR-KO and FSHβ-KO Mice

Follicles fail to develop to the antral stage in the absence of adequate levels of FSH. However, follicle development also requires LH. Without the development of LH receptors on theca cells, follicle development arrests at the gonadotrophin-dependent stage (Dierich *et al.,* 1998; Filicori and Cognigni, 2001). The initial role of FSH in follicle development is demonstrated by FSHR-KO and FSHβ-KO models (Kumar *et al.,* 1999; Themmen and Huhtaniemi, 2000; Abel *et al.,* 2003). Female mice are infertile and follicle development is blocked prior to the late pre-antral stage of development (Dierich *et al.,* 1998; Burns *et al.*, 2001). Ovaries of these mice contain follicles at all stages of pre-antral development but no Graafian follicles or corpora lutea (Dierich *et al.,* 1998; Themmen and Huhtaniemi, 2000; Burns *et al.*, 2001).

FSHβ-KO mice have a well developed theca layer which stains positive for 17α-hydroxylase mRNA. Therefore, recruitment and formation of the theca layer is not dependent upon FSH (Burns *et al.*, 2001). The phenotypes of FSHR-KO and FSHβ-KO mice are similar for the first 2 months. These mice have ovaries which are significantly smaller than littermates and thin uteri, as a result of lack of FSH stimulation of follicle growth, leading to reduced ovarian mass, reduced oestradiol levels and poor uterine development. The majority developed ovarian tumours (in follicle cells) at one year (Themmen and Huhtaniemi, 2000; Danilovich *et al.,* 2001; Abel *et al.*, 2003; Kumar, 2005). The tumours observed in these mice may result from the elevated LH levels or from reduced inhibin levels which are postulated to suppress tumour formation (Abel *et al.*, 2003). However, after two months, the phenotypes of FSHR-KO and FSHβ-KO mice begin to diverge with age, with FSHβ-KO mice demonstrating increased ovarian and uterine mass. However, this may be due to other factors activating the FSH receptor in these mice (Abel *et al.*, 2003).

In addition, mice deficient in FSH demonstrate increased expression of FSHR mRNA but decreased LHR, P450scc, cytochrome P450 aromatase, inhibin and activin α-subunit mRNA expression (Burns *et al.,* 2001) suggesting FSH is fundamental to their regulation. In the FSHR-KO and FSHβ-KO mice ovaries, inhibin B was detectable but not inhibin A, suggesting that FSH stimulation of the granulosa cells is required for inhibin βA transcription (Hirst *et al.*, 2004). Inhibin B is produced by smaller follicles whilst inhibin A is produced by larger follicles, explaining why ovaries of FSHR-KO and FSHβ-KO mice which lack antral follicles have undetectable inhibin A levels. However, serum inhibin B levels were significantly lower than controls suggesting that only low levels of inhibin B were expressed within the ovaries of these mice and released into the bloodstream (Hirst *et al.*, 2004). An inactivating mutation of the FSH receptor has been identified in the extracellular domain of the receptor, in FSH-resistance and infertility (Perez Mayorga *et al.*, 2000).

FSH binds to its receptor through the β-subunit. However, the α-subunit is also essential for binding and the binding activity of FSHβ is reduced by 90% relative to the αβ dimer (Dias, 2005). Heterozygous FSHR mice are fertile although their fertility is impaired compared to wt mice (Burns *et al.*, 2001), whereas heterozygous FSHβ mice are fertile and do not display any phenotype compared to controls (Kumar, 2005). FSHβ-KO female mice had normal levels of serum oestradiol but reduced progesterone levels and increased levels of LH compared to control animals (Kumar, 2005). In contrast, adult FSHR-KO mice had high levels of LH and FSH and low oestrogen levels (Kumar, 2005). FSHβ-KO mice were capable of ovulating when treated with PMSG/hCG, and when mated these mice ovulated and had a normal size litter (Themmen and Huhtaniemi, 2000; Kumar, 2005). However, animals over-expressing FSH were infertile, with high serum oestradiol and progesterone levels, and ovarian cysts (Kumar *et al.,* 1999; Themmen and Huhtaniemi, 2000).

1.11.7 LH and Follicle Development

The LHR is expressed in theca cells during pre-antral development and LH is required for androgen production for subsequent conversion to oestradiol for ovulation (Knobil and Neill, 1994; Robker and Richards, 1998; Gonzalez-Robayna *et al.*, 2000; Webb *et al.,* 2003). LHR-KO and LHβ-KO are infertile, with follicles capable of reaching antral development that then degenerate. However, LH is not essential for folliculogenesis *in vitro,* though addition of LH *in vitro* does stimulate follicle maturation (Demeestere *et al., 2005*). *In vitro* mouse follicles treated with both FSH and LH increased the percentage of oocytes that advanced through the early stages of meiosis from 46% to 76% (Cortvrindt and Smitz, 1998). Once follicles are stimulated by FSH, expression of LHR occurs in the granulosa cells (Robker and Richards, 1998; Webb *et al.,* 2003), and is greatest in the mural granulosa cells of mice and rats nearest the basement membrane but absent in cumulus granulosa cells (Vitt *et al.*, 2000; Eppig, 2001; Eppig *et al.*, 1997b).

The exact role of the LHR in granulosa cell function is unclear. Some have postulated that LH expression increases aromatase expression and, hence, increases oestradiol synthesis. However, other studies have found that oestradiol secretion is unaltered by LH, through progesterone secretion increases (Berndtson *et al.,* 1995; Fortune *et al.*, 2001). Bovine granulosa cell cultures showed that oestradiol synthesis was inhibited by high LH but progesterone synthesis was stimulated (Berndtson *et al.,* 1995). LH activates the cAMP pathway and inhibits aromatase expression and oestradiol synthesis at high levels (Conti, 2002). The LH surge stops granulosa cell proliferation and promotes luteinisation and corpus luteum development (Knobil and Neill, 1994; Gonzalez-Robayna *et al.*, 2000). LH is released in low pulses which increase during the follicular phase (McNeilly *et al.,* 2003). The LH surge is activated by increased follicular oestradiol secretion and is necessary for ovulation, otherwise antral follicles will die (Hillier, 1994; Fortune *et al.,* 2001). Additionally, LH stimulates androgen and aromatase synthesis which directly influences oestrogen secretion (Hillier, 1994). The LH surge coincides with maximal LHR expression on the granulosa cells. Elevated levels of LH result in apoptosis or premature luteinisation of pre-ovulatory follicles (Hillier, 1994; Xu *et al.,* 1995).

1.11.8 LHR Expression

Follicle formation is not completed until a few days after birth and LHRs are not expressed in such follicles until shortly after birth (Dufau, 1998). LHRs belong to the GPCR family (Hillier, 2001). Both LH and hCG bind to the LH receptor with high affinity and activate the adenylate cyclase, cAMP and PKA pathway as described in Section 1.11.2 (Dufau, 1998). LHRs are expressed in the theca cells of small pre-antral follicles. However, at the pre-ovulatory stage of development the mural granulosa cells also begin to express LHRs (Richards, 1994; Eppig *et al.,* 1997b; Hillier, 2001; Robert *et al.,* 2003). LHR expression within the theca cells is localised mainly to the theca *interna* (Xu *et al.,* 1995; Misrahi *et al.,* 1996). LHR expression is greater furthest from the oocyte suggesting that the oocyte inhibits LHR (Robert *et al.,* 2003).

Oestradiol enhances granulosa cell cAMP production by FSH which is required for FSH stimulation of LHR expression (Richards and Kersey, 1979), and treatment with FSH stimulates granulosa cell LHR expression *in vivo* and *in vitro* (Eppig *et al.,* 1997a; Hillier, 2001). Initiation of LHR expression in the granulosa cells is fundamental for follicle dominance (Xu *et al.,* 1995). LHR expression in the theca cells is enhanced by rising FSH which stimulates inhibin secretion which, in turn, enhances thecal androgen production leading to greater levels of oestradiol and further stimulation of LHR expression (Hillier *et al.*, 1994). LHRs that are 'inactivated' results in infertility in women (Huhtaniemi, 2000).

1.12 Follicular Atresia

As previously mentioned, 99.98% of ovarian follicles will undergo the process of atresia (Hillier, 1994; Manabe *et al.*, 2004), though this process is not fully understood (Hughes and Gorospe, 1991). In addition, the concept of follicles being 'rescued' from atresia has been controversial and extensive studies have shown that once apoptosis is initiated it can only be delayed (Hirshfield, 1989). Although previous studies have claimed that follicular atresia only occurs at the antral stage of development, Himelstein-Braw *et al.* (1976) found that atresia occurs at all stages of follicle development, and is a gradual process (Hirshfield, 1989).

Two types of follicle atresia have been identified; antral and basal atresia (Irving-Rodgers *et al.*, 2001). Antral atresia is identified by the appearance of follicle atresia initially in the membrana granulosa cells nearest the antral cavity, and by the presence of pyknotic nuclei in the granulosa cells. In contrast, basal atresia appears to initiate around the basement membrane, and instead of pyknotic nuclei, apoptotic bodies are observed in the granulosa cells (Irving-Rodgers *et al.*, 2001; Amsterdam *et al.*, 2003). During follicular atresia, vascularisation of the follicles declines and is eventually lost entirely, resulting in disruption of oxygen reaching the follicle (Webb *et al.*, 2003), perhaps triggering granulosa cell apoptosis (Berardinelli *et al.,* 2004). Many studies have investigated when follicle atresia occurs, and whether such follicles can be rescued from their atretic fate. Treating atretic antral follicles with PMSG *in vivo* results in increased follicle growth but this growth is a result of an enlarged antral cavity and not increased granulosa cell numbers, suggesting that follicle atresia can be delayed but not reversed by FSH (Hirshfield, 1989).

Cell death occurs either from necrosis or apoptosis (Hughes and Gorospe, 1991). Necrosis occurs when ruptured dying cells release toxic factors into the body triggering an inflammatory response. Apoptosis is characterised by reabsorption of the cells by phagocytosis and does not trigger an inflammatory response (Hughes and Gorospe, 1991; Hsu and Hsueh, 1997). The reabsorbance of the follicle can take several days (Gosden and Spears, 1997). Apoptosis is derived from the Greek meaning 'dropping off' and is the cellular process involved in ovarian follicle atresia and is sometimes referred to as programmed cell death (Glamoclija *et al.,* 2005; Hussein, 2005). Various studies have established that granulosa apoptotic cell death is fundamental to the process of ovarian follicle atresia in vertebrates (Krysko *et al.,* 2004).

Apoptosis causes changes in the morphology of the cells characterised by: cell shrinkage, apoptotic bodies, pyknotic nuclei in granulosa cells and granulosa cells detach from the basement membrane (Tilly *et al.,* 1991; Uma *et al.*, 2003; Manabe *et al.*, 2004). Biochemically apoptosis is identified by rapid DNA cleavage (Hughes and Gorospe, 1991; Hussein, 2005).

Apoptosis can be identified morphologically *in vitro* when the granulosa cells and oocyte, have darkened, although the initial stages of apoptosis cannot be recognised microscopically (Hirshfield, 1989; Hartshorne, 1997). Several factors such as ascorbic acid and FSH can inhibit apoptosis of granulosa cells *in vitro* (Tilly and Tilly, 1995). There are two main mechanisms that have been implicated in ovarian apoptosis: the mitochondria pathway and the surface receptor pathway (Hsu and Hsueh, 1998; Tilly, 2001; Hussein, 2005).

Ovarian expression of mitochondria *B-Cell Lymphoma-2* (*Bcl-2*) suppresses apoptosis by preventing the release of cytochrome c from the mitochondria, therefore inhibiting the activation of caspases (Hsu and Hsueh, 1997; Hsu and Hsueh, 1998; Matikainen *et al.*, 2001; Pru and Tilly, 2001; Amsterdam *et al.*, 2003). In *Bcl-2* KO mice the ovary has few primordial follicles and oocytes after birth compared to wt or het mice, but granulosa apoptosis is not affected in these animals, implying that cell death is very cell specific (Tilly, 1996; Ratts *et al.*, 1995). Pro-apoptotic proteins such as *Bax* allow cytochrome c to be released from the mitochondria, which then bind Apaf-1, activating caspase-9 and a caspase cascade (Figure 1.9; Johnson, 2003; Hussein, 2005). *Bax* KO mice have depleted ovarian reserves and suppressed Bcl-2 (Gosden and Spears, 1997; Tilly, 2001). Apoptosis can also be triggered by binding of molecules such as Tumour Necrosis Factor-α (TNF-α) to membrane receptors and activation of a caspase cascade.

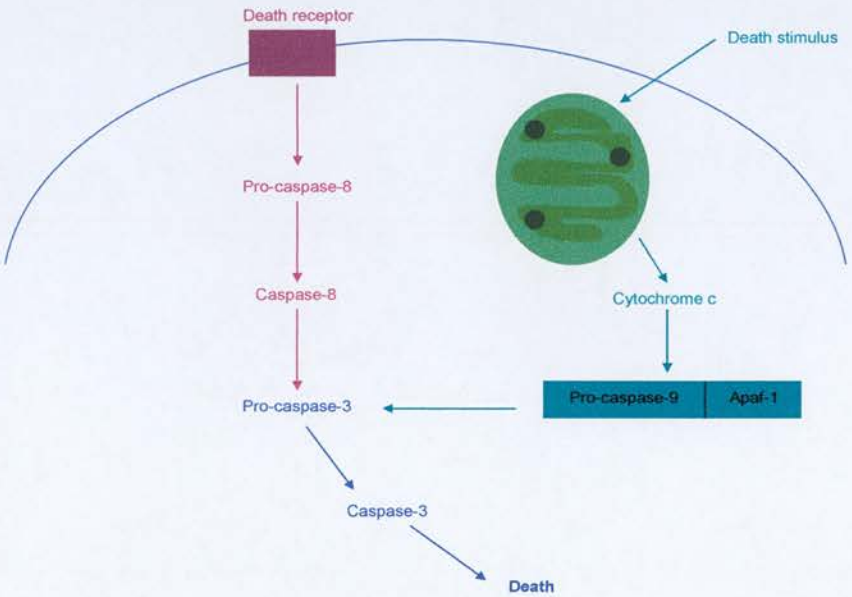


Figure 1.9 Apoptosis pathways in granulosa cells (adapted from Tilly, 2001).
Both the death receptor pathway and the death stimuli pathway activate the caspase family. Apaf-1= apoptotic peptidase activating factor-1.

The caspases remain in their inactive form until activation occurs (Matikainen *et al.,* 2001; Quirk *et al.,* 2004). *Caspase-3* KO mice have a similar ovarian reserve with equivalent numbers of oocytes compared to wt littermates at birth (Matikainen *et al.*, 2001) suggesting that *Caspase-3* only affects atresia postnatally. However, oocyte atresia induced by anti-cancer drugs does not require activation of Caspase-3, whereas Caspase-2 is associated with oocyte death (Takai *et al.,* 2003).

At present there are 14 members of the caspase family in mammals, and a number of these have been identified as important in ovarian apoptosis (Johnson and Bridgham, 2002). The caspase family can be subdivided into initiator and effector caspases (Fulton *et al.,* 2005). All are synthesised as "inactive zymogens" that require cleavage at the C-terminus to be activated (Figure 1.10; Johnson and Bridgham, 2002; Lavrik *et al.*, 2005). Every caspase consists of a pro-domain, with one or multiple heterodimerisation domains. This variation accounts in part for the different actions of the caspase family (Johnson and Bridgham, 2002). Some initiator Caspases (e.g. Caspase-8 and -9) contain a death effector domain (DED), whereas others (e.g. Caspase-4 and -5) contain a caspase recruitment domain (CARD). When bound these domains activate the caspases and apoptosis (Figure 1.10; Johnson and Bridgham, 2002).

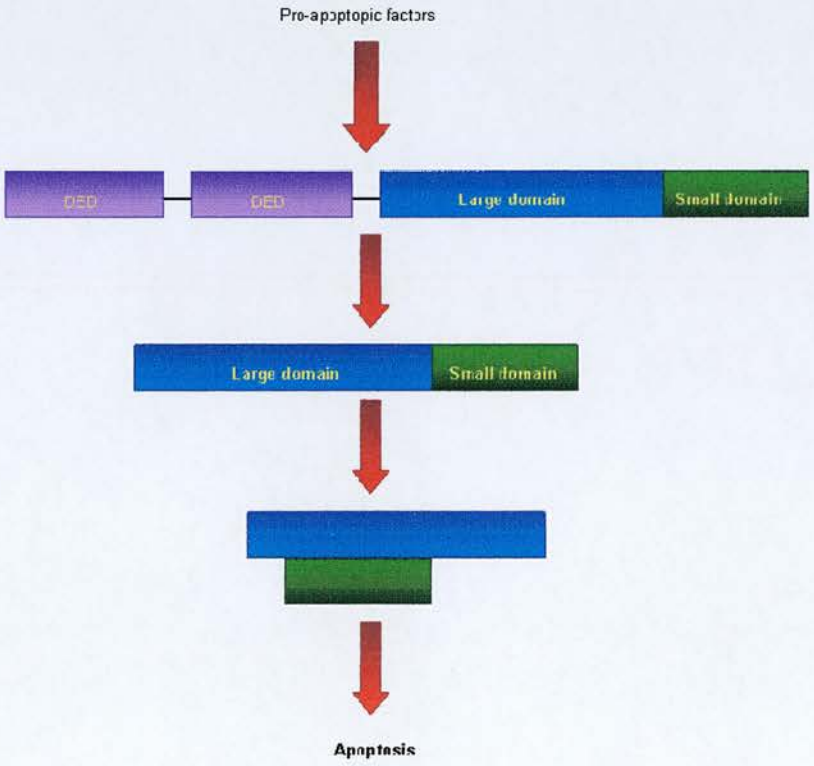


Figure 1.10 Caspase-3 activation in the ovary (adapted from Johnson and Bridgham, 2002). DED is the death effector domain. Activation occurs when the large and small domains are cleaved (Lavrik et al., 2005).

Initiation of follicular atresia is dependent upon the stage of the follicle. In primordial to early pre-antral follicles, initiation of atresia occurs in the oocyte, followed by cell death in the granulosa cells (Glamoclija *et al.,* 2005). However, atresia in late pre-antral and pre-ovulatory follicles is characterised by the appearance of apoptotic granulosa cells located at the basement membrane (Manabe *et al.,* 2004; Glamoclija *et al.,* 2005).

Atresia can occur at any point during follicle development (Manabe *et al.,* 2004). It has not been observed in theca layers during the initial stages of atresia, though atretic cells were occasionally identified in the theca cells of antral follicles (Palumbo and Yeh, 1994; Manabe *et al.,* 2004). The majority of follicles undergoing atresia are in the late pre-antral to early antral stages of development, when follicle growth is largely dependent upon stimulation by gonadotrophins (Tilly, 1996). Atresia is associated with alterations in the oestradiol to progesterone ratio, and oestradiol levels in follicular fluid from atretic follicles are significantly lower than in healthy follicles, whereas progesterone levels are higher (Yu *et al.*, 2004). Oocyte atresia is associated with poor c-*Kit*-*Kit* Ligand binding and reduced EGF levels in follicles (Packer *et al.,* 1994; Markstrom *et al.,* 2002).

The cell cycle is regulated by the cyclin-dependent kinase family and the D-type cyclins are essential for cell cycle entry (Quirk *et al.,* 2004). Granulosa cell apoptosis primarily occurs at the G1 to S phase of the cell cycle and factors such as oestradiol shorten this transition phase, therefore protecting against apoptosis (Quirk *et al.,* 2004). In addition, hypophysectomised rodent follicles demonstrated increased cyclin D_2 mRNA and protein levels after treatment with oestrogen or FSH, implying that these essential regulators of the cell cycle can reduce the level of apoptosis (Robker and Richards, 1998). The gonadotrophins are the main regulators of apoptosis, either inhibiting the process directly or indirectly through regulating the expression of other factors that prevent apoptosis (Hirshfield, 1989; Markstrom *et al.,* 2002). For example, *Igf-1* expression, which is stimulated by FSH, occurs only in healthy follicles and is thought to inhibit apoptosis (Yu *et al.*, 2004). Apoptosis is also inhibited by the activation of the cAMP pathway (Johnson, 2003).

After the LH surge follicles are less susceptible to atresia. Although the exact mechanisms are not understood, PR expression may play a role (Markstrom *et al.,* 2002). The theca layer undergoes apoptosis only when the follicle is in the more advanced stages of atresia (Johnson, 2003), although the mechanisms by which the theca undergoes apoptosis may differ from apoptosis of the granulosa cells (Berardinelli *et al.,* 2004). Since the vascularisation of the theca *interna* provides the granulosa cells with nutrients and gases, apoptosis is initiated in the mural granulosa cells as the follicular vascular network is disrupted (Berardinelli *et al.,* 2004).

1.13 The Extracellular Matrix (ECM)

The ECM is a highly complex scaffold composed of collagen, proteoglycans and various glycoproteins (e.g. fibronectin and laminin) and undertakes an important role in maintaining tissue structure, cell-cell interactions, cell migration and epithelium formation (Gilbert, 2003). Unlike most organs in the body where tissue remodelling is kept to a minimum after formation and development, ovarian function is dependent upon continual remodelling throughout the reproductive cycle (Smith *et al.*, 1999). The ECM is required to create the correct environmental conditions for ovarian follicle growth and development (Visse and Nagase, 2003). It achieves this through regulating gene expression, cell proliferation and apoptosis (McIntush and Smith, 1998). Collagen is the most abundant component of the ECM with collagen IV found in the follicle basement membrane (Zhao and Luck, 1995). The ECM also determines which materials pass through the ECM to reach the cells within the follicle (Hess *et al.,* 1998; Rodgers *et al.*, 2000).

During follicular growth the ECM undergoes marked changes in composition. Bovine granulosa cells exhibit a 21-doubling during growth from a primordial to pre-ovulatory follicle (van Wezel and Rodgers, 1996; Rodgers *et al.*, 1998). During bovine folliculogenesis (primordial to pre-ovulatory) follicular surface area will have increased 36,000 fold (McIntush and Smith, 1998). Therefore, to support this growth the ECM must undergo profound remodelling (Rodgers *et al.*, 2000; Rodgers *et al.*, 2003). FSH stimulation of follicle growth results in an increase in follicular volume and this requires the ECM of the follicles to undergo continual remodelling (Hirshfield and Midgley, 1978b; Curry and Osteen, 2001).

Various factors affect both the breakdown and re-synthesis of the ECM. An important family in tissue decomposition are the MMPs (Brew *et al.,* 2000; Stamenkovic, 2003; Imai *et al.,* 2003). The MMPs consists of four sub-families: seralysins, adamlysins, astracins and matrix metalloproteinases which all contain a common 'Met-turn motif' (Stamenkovic, 2003). This family has an essential role in ECM degradation and remodelling in the ovary during folliculogenesis, ovulation and corpus luteum establishment (Imai *et al.,* 2003). Another enzyme family involved in ECM remodelling is the plasminogen activator (PA) system which 'converts plasminogen to plasmin'. Plasmin activation triggers MMP activation and leads to ECM remodelling (Vassalli *et al.,* 1984; Ny *et al.,* 2002). However, the enzymes involved in ECM synthesis are also important for follicle remodelling. For instance lysyl oxidase (LOX) cells stimulates the final 'cross-linking of collagen' a fundamental component of the ECM and is expressed in granulosa cells (Harlow *et al.,* 2003; Rodgers *et al.,* 2003). LOX levels are reduced in rat granulosa cell cultures treated with FSH (Harlow *et al.,* 2003). Ascorbic acid

which is fundamental for collagen synthesis is another factor required to maintain the integrity of the follicle basal lamina (Murray *et al.*, 2001).

The following section will deal with the components of the follicular ECM, MMP activation and regulation by their tissue-inhibitors (TIMPs), and with the role of the MMPs and TIMPs in follicle growth and development. Degradation of the follicular ECM in this thesis will focus on the gelatinase MMPs (MMP-2 and MMP-9) and their degradation of the substrates gelatin and collagen IV.

1.13.1 The Follicular Basal Lamina

The follicle basement membrane or basal lamina separates granulosa from theca cells (Rodgers and Irving-Rodgers, 2002) and is composed of collagen IV and laminin which are cross-linked (Rodgers *et al.*, 2000; Gilbert, 2003). The surface area of the basal lamina of bovine ovarian follicle doubles 19 times during follicular growth in line with increases in follicle volume and cell number (Rodgers *et al.,* 1998). Changes not only occur in the surface area but also in ECM composition (Table 1.7). The ratio of collagen IV to laminin in the basal lamina varies throughout follicle growth. Furthermore, different types of laminin and collagen (Table 1.7) can be sub-classified, and the ratios of these components can vary providing a very complex structure (Rodgers *et al.*, 2000). The collagen IV family comprises six types of collagen (α chains) and these can be arranged into at least three different groupings (Sado *et al.,* 1998). MMP-2 and MMP-9 degrade collagen IV, a major component of the basal lamina (Rodgers *et al.,* 1998; Curry and Osteen, 2001). Granulosa cells secrete many of the components that constitute the basal lamina (Zhao and Luck, 1995; Rodgers *et al.,* 1998; Rodgers and Irving-Rodgers, 2002), but basal lamina synthesis appears to require both theca and granulosa cells (Rodgers *et al.,* 1998). For example, TIMP-2 is localised mainly in the theca of ovine follicles but can activate pro-MMP-2 which is secreted by the granulosa cells (Smith *et al.,* 1995; Curry *et al.,* 2001), suggesting that ECM synthesis and degradation requires both theca and granulosa cells.

Matrix Components of the Follicular Basal Lamina

Developmental Stage	Laminin	Collagen	Other
Primordial	α1, β2, γ1	Type IV α1, α2, α3, α4, α5, α6	
Pre-antral	α1, β2, γ1	Type IV α1, α2, α3, α4, α5, α6	Nidogen & perlecan
Antral	α1, β2, γ1	Type IV α1, α2	Nidogen & perlecan
Atretic Antral	α1, β2, γ1	Type IV α1, α2, +/- α2	Nidogen & perlecan

Table 1.7 The constitution of the basal laminae in bovine ovarian follicles (adapted from Rodgers et al., 2003).

A function of the basal lamina of the ovarian follicle is to prevent molecules and plasma proteins with a high molecular weight penetrating into the follicle (Rodgers and Irving-Rodgers, 2002). Previous studies have determined that small proteins constitute a large percentage of bovine follicular fluid follicles (Andersen *et al.,* 1976). However, the molecular weight of a protein is not the only criterion for traversing the basal lamina, and the electrical charge of the molecule also influences whether or not it may cross the basal lamina (Rodgers *et al.,* 2003). Thus, proteins of a similar molecular weight but opposite electrical charge have differing abilities to cross the blood-follicle barrier (Hess *et al.*, 1998). In addition to providing a matrix to which various growth factors and or binding proteins such as follistatin and activin can bind the basal lamina prevents large molecules and proteins synthesised by the oocyte and granulosa cells from leaving the follicle (Rodgers and Irving-Rodgers, 2002). Therefore, the basal lamina plays a pivotal role in controlling the movement of molecules and proteins into and out of the follicle, by restricting growth factors from leaving the follicle and by sequestering growth factors, so controlling the micro-environment of the follicle (Rodgers and Irving-Rodgers, 2002; Rosso, *et al.*, 2004).

1.13.2 Basal Lamina and Follicle Growth, Atresia and Ovulation

Specific components of the basal lamina (such as collagen type I in the granulosa cells and fibronectin in the theca externa) have been associated with increased follicle growth, whereas follicular atresia was associated with increased levels of fibronectin, laminin and collagen type IV in the granulosa cell layer (Huet *et al.*, 1997). The basal lamina stimulates murine mural granulosa cell differentiation, and LHR expression and progesterone secretion by granulosa cells increased when cells were cultured on bovine ECM (Eppig *et al.*, 1997b). Compared to controls, there was a two-fold increase in LH/hCG receptor expression by granulosa cells grown *in vitro* in the presence of bovine ECM components (Furman *et al.,* 1986).

The basal lamina of a healthy follicle is thought to be continuous, but in atretic and luteinising follicles it becomes 'fragmented'. Furthermore, atretic follicles were found to have theca cells penetrating into the follicle (Bagavandoss *et al.*, 1983; Markstrom *et al.*, 2002). However, more recent studies have demonstrated that the basal lamina in bovine atretic follicles does not degenerate or become thickened as previously thought. Rather, the basal lamina begins to fold, with the folding becoming more extensive as atresia progresses thereby possibly preventing growth factors diffusing across the basement membrane (Irving-Rodgers *et al.*, 2002; Rodgers *et al.*, 2003).

This may explain why atresia occurs initially in the granulosa cells nearest the basement membrane, and progresses to the granulosa cells adjacent to the antral cavity (Irving-Rodgers *et al.,* 2002; Rodgers and Irving-Rodgers, 2002). As the integrity of the basement membrane is degraded (basal atresia only) macrophages can cross the basal lamina into the follicle (Rodgers and Irving-Rodgers, 2002). The basal lamina is degraded extensively during ovulation (Rodgers and Irving-Rodgers, 2002) and this is thought to involve increased MMP secretion by the granulosa cells (Smith *et al.*, 1999).

1.13.3 Matrix Metalloproteinases (MMPs)

The MMPs are a large family of enzymes which require zinc for catalytic activity (McIntush and Smith, 1998; Kim *et al.,* 2001). MMPs play a vital role in the degradation of the ECM and are expressed throughout the ovary. They and their inhibitors are fundamental for normal ovarian physiology and follicular atresia results in activation of the MMPs (Huet *et al.,* 1998; Smith *et al.,* 2002; Curry and Osteen, 2003). Once activated MMPs participate in the degradation of many components of the ECM, including collagen, gelatin and laminin (Allan *et al.,* 1995; Visse and Nagase, 2003). However, although MMP-2 and MMP-9 bind to collagen 1, they do not degrade it (Allan *et al.,* 1995). MMPs were named and numbered in order of their discovery (Curry and Osteen, 2001; Curry and Osteen, 2003). MMPs and their inhibitors (TIMPs) have a key role in controlling the extent of tissue remodelling throughout the organism, and in the ovarian cycle MMPs play a major role in follicle growth and ECM remodelling (Curry and Osteen, 2001). Follicle rupture requires increased activity of MMPs, which 'cleave denatured' collagen (gelatin) and type IV collagen (Smith *et al.*, 1999). Ewes with no circulating gonadotrophins showed an increase in MMP-2 and MMP-9 activity regulation by the gonadotrophs (Huet *et al.,* 1998).

1.13.4 MMP Structure

The members of the MMP family are subdivided into 6 classes; collagenases, gelatinases, stromelysins, matrilysins, Membrane-Type (MT) and other MMPs (Table 1.8) (Nagase, 1997; Visse and Nagase, 2003). Structurally, members of the MMP family have many features in common. The MMPs contain a signal peptide, a pro-peptide domain (which must be cleaved for activation), a catalytic domain (containing the zinc binding site) and a hemopexin-like domain (Figure 1.11; Sternlicht and Werb, 2001; Curry and Osteen, 2001).

MMPs exhibit several similarities in both structure and function. The MMPs have a common HEBXHXBGBXH motif that is required for zinc binding at the catalytic site where H (histidine), E (glutamic acid) and G (glycine) are not variable, B is a 'hydrophobic residue' and X is a variable residue (Sternlicht and Werb, 2001; Stamenkovic, 2003). However, the MMPs can be distinguished and sub-divided according to their structure and the components of the ECM they degrade. Differences in MMP structure enable specific components of the ECM to be targeted and degraded (Table 1.8; Curry and Osteen, 2001; Curry and Osteen, 2003).

MMP Sub-Family	MMPs
Collagenases	MMP-1, -8, -13, -18
Gelatinases	MMP-2, -9
Stromelysins	MMP-3, -10
Matrilysins	MMP-7, -26
Membrane-Type (MT)	MMP-14, -15, -16, -17, -24, -25
Other MMPs	MMP-12, -19, -20, -22, -23, -28

Table 1.8 Summary of MMP sub-family members (adapted for Visse and Nagase, 2003).

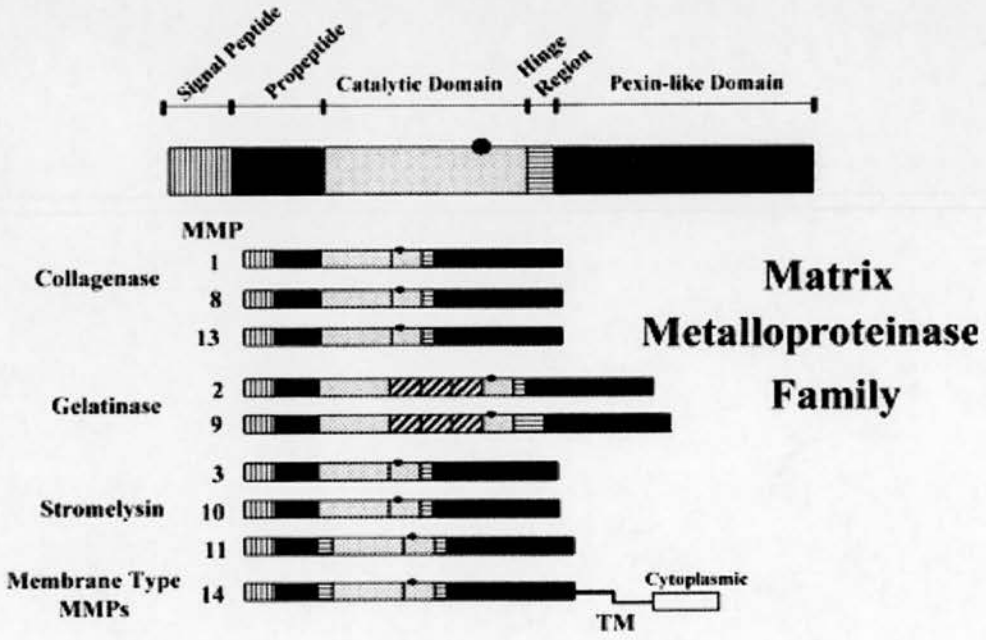


Figure 1.11 Schematic representation of the MMP family (from Curry and Osteen, 2001).
All MMPs contain a signal peptide, pro-peptide, catalytic domain and a hemopexin-like domain. For activation the pro-peptide domain has to be cleaved to reveal the active site.

1.13.5 MMP Activation

MMPs act upon the ECM by a common mechanism involving cleavage by the catalytic domain (Curry and Osteen, 2003). The MMPs are secreted in an inactive form (pro-MMP) and the active site is revealed when cleavage occurs (either extracellularly or on the cell membrane for MT MMPs). The exception to extracellular activation is MMP-11 which can be activated intracellularly by protein-processing enzymes which cleave the pro-peptide domain (Santavicca *et al.,* 1996; Curry and Osteen, 2001). The two domains common to all MMPs (present in all except MMP-23) are the pro-domain and the catalytic domain. The pro-peptide of all MMPs (except MMP-23) contains a 'cysteine switch' which once opened through the removal of the pro-peptide domain exposes the active zinc site (Van Wart and Birkedal-Hansen, 1990; Visse and Nagase, 2003).

Extracellular activation of pro-MMPs is affected by other members of the MMP family, by the plasminogen-activator plasmin system or by chemical induction *in vitro* e.g. sodium dodecyl sulphate (SDS) (Curry and Osteen, 2001; Curry and Osteen, 2003; Visse and Nagase, 2003; Riley, Personal Communication). The addition of SDS results in a conformational change of the protein which reveals the active site but does not cleave the pro-domain (Riley, Personal Communication). Some MMPs can activate other members of the MMP family. MT2-MMP has been shown to activate MMP-2 through interacting with pro-MMP-2 (Morrison *et al.,* 2001). MMP activity is controlled by other proteinases and limited by, 'serum-borne and tissue-derived' metalloproteinase inhibitors (TIMPs) (Curry and Osteen, 2003).

1.13.6 Tissue Inhibitors of Matrix Metalloproteinases (TIMPs)

The MMPs are regulated by serum-borne inhibitors (such as the less selective protein inhibitor α_2-macroglobulin) and by TIMPs which are specific inhibitors of the MMPs (Brew *et al.,* 2000). To date there are 4 members of the TIMP family (TIMPs 1-4; detailed in Table 1.9; Curry *et al.,* 2001). TIMPs are produced locally to inhibit MMPs and high levels are found in the ovary (Curry and Osteen, 2001). However, their localisation within the follicle and their affinity for the various MMPs varies (Stamenkovic, 2003). In general, when MMP activity exceeds TIMP capacity, degradation of the ECM occurs, and when TIMP capacity is greater than MMP activity, accumulation of ECM occurs (Smith *et al.*, 1999). However, other factors such as substrate availability and MMP and TIMP interactions also affect the degradation and deposition of the ECM.

TIMP	FUNCTION
TIMP-1	Inhibits all known MMPs. Binds to MMPs in their active form and to the latent form of MMP-9.
TIMP-2	Inhibits all known MMPs but has high affinity for the latent and active forms of MMP-2. Also has an important role in the activation of MMP-2.
TIMP-3	Inhibits all known MMPs. This inhibitor is initially secreted and then binds to the ECM.
TIMP-4	Inhibits all known MMPs.

Table 1.9 Function and role of members of the TIMP family (Curry et al., 2001)

1.13.7 TIMPs Structure and Action

TIMPs bind to MMPs with a 1:1 stoichiometry (Curry and Osteen, 2003). The N- terminal domain interacts with the MMP catalytic site so preventing degradation of the ECM (Visse and Nagase, 2003). However, MMP inhibition by TIMPs is not their sole role. In addition, TIMP-2 assists in the activation of MMP-2 (Brew *et al.,* 2000; Curry and Osteen, 2001; Jo *et al.,* 2004). The N-terminus of TIMP-2 binds to the extracellular domain of the active site of MT1-MMP. Pro-MMP-2 then binds to the C-terminal domain of TIMP-2 forming a pro-MMP-2/TIMP-2/MT1-MMP complex resulting in the cleavage of the MMP-2 pro-domain and activation of MMP-2 (Butler *et al.,* 1998; Brew *et al.,* 2000; Curry and Osteen, 2001).

1.13.8 MMPs and the Ovarian Follicle

As mentioned previously the follicular ECM undergoes continual remodelling throughout the reproductive cycle. The MMPs and their inhibitors play a role in ECM remodelling during follicle growth and development (Curry and Osteen, 2001). The MMPs also activate certain growth factors. MMP-2 and MMP-9 cleave TGF-β1 and TGF-β2 activating these factors (Yu and Stamenkovic, 2000; Stamenkovic, 2003). Ovarian MMP expression fluctuates according to the stage of follicle development. In rat follicles, MMP-2 and MMP-9 protein increased in the later stages of folliculogenesis, with MMP-2 confined to granulosa, theca and interstitial cells whereas MMP-9 was confined in theca and interstitial cells (Bagavandoss, 1998). In contrast, studies in rats (day 23) have found MMP-2 expression solely in the theca cells of pre-antral to pre-ovulatory follicles (Liu *et al.,* 1998; Richards *et al.*, 2002). Furthermore, MMP expression can be regulated through hormones. A marked increase of cellular MMP-2 and MMP-9 expression was observed in rat ovaries after injection with PMSG (Bagavandoss, 1998; Curry and Osteen, 2003).

1.13.8.1 MMP-2 and MMP-9 Expression and Follicle Development

The MMP gelatinases, like many members of the MMP family, are secreted as pro-MMPs and are activated by opening of the cysteine switch, exposing the active site (Curry and Osteen, 2003; Visse and Nagase, 2003). The composition of the basal lamina and the levels of MMPs and TIMPs play a crucial role in follicle development by regulating the environment for follicle growth, atresia and ovulation. MMP-2 and MMP-9 (gelatinases) are expressed in pre-ovulatory follicles of several species including bovine and equine ovaries (McIntush and Smith, 1998; Imai *et al.,* 2003; Riley *et al.,* 2004). Although MMP-2 is expressed during follicle development, MMP-2 KO mice are fertile suggesting MMP-2 is not pivotal to follicle growth and development (Itoh *et al.,* 1997; Smith *et al.,* 1999). The latent and active forms of MMP-2 have a molecular weight by PAGE of 72kDa and 66kDa respectively, whilst latent and active forms of MMP-9 have a molecular size of 92kDa and 86kDa respectively (Fridman *et al.,* 1995; Smith *et al.,* 2005). However, another larger isoform of MMP-2 (110kDa) has been identified in bovine follicular fluid and serum. This newly identified isoform may be produced by the dimer of active and latent MMP-2 (Kim *et al.,* 2001).

1.13.9 TIMPs and the Ovarian Follicle

MMP activity is tightly controlled and studies have demonstrated that as follicle MMP levels increase there is an increase in TIMP activity (Curry and Osteen, 2001; Curry and Osteen, 2003). TIMP-1 is expressed in the ovine granulosa cell layer, whereas TIMP-2 is expressed in the theca cell layer (Smith *et al.*, 1995). TIMP-1 null mice have modified levels of both oestradiol and progesterone as TIMP-1 stimulates granulosa cell production of oestradiol proposing that TIMPs also play a role in controlling follicle growth through regulating steroid production (Curry *et al.*, 2001).

1.14 *Deleted in AZoospermia (DAZ)*

The *Dazl* gene belongs to the *DAZ* family of genes which also consists of *DAZ* and *BOULE*. The *DAZ (Deleted in AZoospermia)* gene is located on the Y chromosome in 'old world primates' (Cooke *et al.,* 1996; Ruggiu *et al.,* 1997; Venables *et al.*, 2001; Reynolds and Cooke, 2005). An autosomal form of this gene (*DAZ-like* gene – *DAZL*) is expressed in germ cells, which when deleted is associated with infertility. In mice, the only homologous form of this gene is found on chromosome 17 and has been named *Dazl* (Cooke *et al.*, 1996; Ruggiu *et al.,* 1997). *DAZL and DAZ* are thought to have evolved from their ancestor *Boule* (Saunders *et al.*, 2003).

DAZ is thought to have arisen by translocation of the *DAZL* gene to the Y chromosome followed by a process of 'amplification and pruning' during primate evolution (Reijo *et al.,* 2000; Tsui *et al.,* 2000; Cauffman *et al.,* 2005). DAZ and DAZL demonstrate a high level of homology, whereas Boule is less specifically related (Collier *et al.,* 2005). *Boule* is not necessary for female gametogenesis whereas *Dazl* is essential (Ruggiu *et al.,* 1997). The DAZ proteins are RNA binding proteins, and have an RNA recognition motif (RRM), which controls gene expression in germ cells through regulating mRNA expression (Reijo *et al.,* 2000; Tschanter *et al.*, 2004; Cauffman *et al.,* 2005). Although the *DAZ* family are expressed in germ cells, its sex-specific expression differs between species (Karashima *et al.*, 2000). Deletions in any of the *DAZ* family result in fertility problems (Reynolds and Cooke, 2005).

1.14.1 Gene Structure

Dazl is essential for oogenesis and spermatogenesis, and a drastic reduction in germ cells is observed in *Dazl* null mice (Saunders *et al.,* 2003). The *DAZ* genes encode proteins that contain an RRM and 8-18 copies of the DAZ repeat (Reynolds and Cooke, 2005). *Dazl* is required for germ cells to proceed through from 'meiotic prophase' (Saunders *et al.,* 2003). In humans *DAZL* is 90% identical to human *DAZ* and is also expressed in germ cells (Slee *et al.,* 1999). The high homology between BOULE, DAZ and DAZL is demonstrated by the restoration of meiotic activity when Xenopus *Dazl* cDNA was introduced to *boule* null flies and the *Xdazl* (Houston *et al.*, 1998).

Human DAZ and DAZL and murine Dazl are RNA-binding proteins and both bind to poly U and G (Tsui *et al.,* 2000). The exact mechanisms and role of the DAZ/Daz family is complicated, since DAZ/Daz proteins act at numerous points during male germ cell development (Reijo *et al.,* 2000). *BOULE* is essential for meiosis and, therefore, loss of this gene prevents meiosis resulting in sterility in flies (Houston *et al.,* 1998; Houston and King, 2000; Reynolds and Cooke, 2005). In the mouse and human, Dazl and DAZL respectively are expressed in the embryonic ovary and in maturing follicles (but not in the menopausal ovary) suggesting the role and function of *Dazl* are far more diverse than that of *BOULE* (Ruggiu *et al.,* 1997; Nishi *et al.,* 1999; Pan *et al.,* 2002).

The *DAZ* gene family of RNA-binding proteins all contain at least one copy of the 24 amino acid DAZ repeat (Tsui *et al.,* 2000; Reynolds and Cooke, 2005). Whereas *BOULE* and *DAZL* contain only a single DAZ repeat, humans have four *DAZ* genes on the Y chromosome (Reynolds and Cooke, 2005), *DAZ1, DAZ2, DAZ3* and *DAZ4*. These are all longer than the *DAZL* gene and have a greater number of tandem repeats (Szmulewicz *et al.,* 2002). The *DAZ* genes are arranged in two clusters which each contain either *DAZ1* and *DAZ2* or *DAZ3* and *DAZ4* (Fernandes *et al.,* 2002; Reynolds and Cooke, 2005).

Structurally DAZ and DAZL have a high homology and there is also a high degree of homology between human *DAZ* and murine *Dazl* as demonstrated by the fact that human *DAZ* is capable of rescuing the effects of *Dazl* KO in male mice to varying degrees (Tsui *et al.*, 2000; Vogel *et al.*, 2002). Although in humans *DAZ* is a Y-linked gene it has also been detected in human females by Southern blot hybridisation although its source and function remains unknown (Cooke *et al.*, 1996). Although a murine *Dazla* probe detected *DAZ* in humans, it could not be identified in the mouse suggesting that *Daz* is present only in primates and not in the mouse (Cooke *et al.*, 1996). Meiosis is regulated by *DAZ* genes and loss or mutations of these genes results in infertility (Reynolds and Cooke, 2005).

1.14.2 Dazl Expression in the Testis

Both male and female transgenic *Dazl* KO mice are infertile (Ruggiu *et al.*, 1997). In human males deletions of gene(s) located on the azoospermia factor region (AZF) on the Y chromosome have been associated with up to ten percent of male infertility (Slee *et al.*, 1999; Schrans-Stassen *et al.*, 2001). The group of *DAZ* genes is located on the AFZc region (de Vries *et al.*, 2002; Reynolds and Cooke, 2005).

Deletion of the *Dazl* gene, which has high homology with *DAZ* in humans, also causes infertility in males due to the germ cells being unable to mature. This is characteristic of patients suffering from deletions in the AZF region (Slee *et al.*, 1999; Tsui *et al.*, 2000; Schrans-Stassen *et al.*, 2001), and male infertility is frequently associated with deletions in the Azoospermia Factor (AZF) region of the Y chromosome. This region can be further subdivided into three sections (AZFa, AZFb and AZFc) and 10% and 15% of males that experience oligozoospermia or azoospermia respectively have mutations on the AZFc region of the Y chromosome (Fernandes *et al.*, 2002; Dada *et al.*, 2004; Reynolds and Cooke, 2005).

Human DAZ and DAZL (and murine Dazl) are not located exclusively to the cytoplasm but are also expressed in the nuclei at various stages of testicular development (Reijo *et al.*, 2000). *Dazl* KO males have a three-fold reduced testicular mass with germ cells absent in these animals (Ruggiu *et al.*, 1997), and het males have a lower sperm count than wt males. Interestingly, het males have a far higher percentage of 'abnormal sperm' than wt males (Ruggiu *et al.*, 1997). A reduction in normal germ cells in the testis of het males is also observed to an equivalent level in human males with no fertility problems therefore the effect of *Dazl* het mice may not be fully identifiable in males (Ruggiu *et al.*, 1997).

The extent to which male fertility is compromised is dependent upon the *DAZ* genes that are deleted (Reynolds and Cooke, 2005). Deletion of *DAZ1* and *DAZ2* resulted in extreme oligozoospermia whereas deletion of *DAZ3* and *DAZ4* had no disastrous effect on fertility (Fernandes *et al.,* 2002; Reynolds and Cooke, 2005). Further studies established that deletion of one of the human *DAZ* gene clusters resulted in an intermediary phenotype, suggesting that the effect of *DAZ* on spermatogenesis was dose dependent (de Vries *et al.,* 2002).

Treatment of rats with GnRH-antagonist to inhibit gonadotrophin secretion demonstrated similar Dazl expression in both GnRH-antagonist treated animals and control animals suggesting that Dazl expression is not hormone dependent (Rocchietti-March *et al.,* 2000). Injection of wt germ cells into *Dazl* KO testis function showed that these cells were capable of supporting the germ cells and the testis of these mice functioned normally, suggesting that *Dazl* affected germ cell development (Rilianawati *et al.,* 2003). Human *DAZL* is expressed in both male and female embryonic gonads prior to and during meiosis (Brekhman *et al.,* 2000; Reynolds and Cooke, 2005). Murine *Dazl* is expressed throughout spermatogenesis until maturation. However its actions and target mRNAs are thought to differ throughout this development (Reynolds *et al.,* 2005). *Dazl* mutations initially result in reduced numbers of germ-cells, and then cause meiotic failure at puberty (Slee *et al.,* 1999).

1.14.3 Dazl Expression in the Ovary

During embryonic development and in the pre-pubertal ovary, Dazl is located in the cytoplasm of the oocyte but in adults, Dazl expression is located at the ZP (Ruggiu *et al.,* 1997). *Dazl* has been detected as early as 12.5d.p.c in both male and female mouse embryos, when the cells present in the gonads were solely PGCs but were not present in mice with null germ cells indicating that *Dazl* was expressed solely in the germ cells (Seligman and Page, 1998). At day 15 post-coitum the *Dazl* KO mouse ovary seems normal, with many cells at the pachytene stage of development. However by 19d.p.c the ovary appears abnormal, with low numbers of oocytes present, with many of those that are present undergoing atresia (Ruggiu *et al.,* 1997).

In the murine female ovary Dazl is expressed at all stages of follicle development but its expression is dependent upon the stage of follicle development (Ruggiu *et al.,* 1997), and a study of *DAZL* expression in human follicles demonstrated expression in the granulosa cells of primordial follicles and in the theca interna of developing follicles (Cauffman *et al.,* 2005). *Dazl* KO female mice have smaller ovaries than either wt or het ovaries (Ruggiu *et al.,* 1997). In the neonate *Dazl* KO ovary, there are no oocytes present at postnatal day 4 (Ruggiu *et al.*, 1997; McNeilly *et al.*, 2000; Saunders *et al.,* 2003). Interestingly, het ovaries were larger than wt ovaries (McNeilly *et al.*, 2000) and *Dazl* heterozygotes had larger litters, with an average of 12 pups per litter, compared to wt mice with an average of 7 pups per litter (McNeilly *et al.*, Unpublished; Appendix A).

Ovarian follicle counts in heterozygous adult *Dazl* mice have demonstrated that these mice have a significantly greater percentage (43%) of follicles at the antral stage of development compared to wt mice (27%) (Appendix B), but have a smaller follicle pool in adulthood than wt animals (McNeilly *et al.*, Unpublished; Appendix B). Het mice had lower levels of plasma FSH and higher inhibin B levels than wt mice. If het follicles produce more inhibin B per follicle, this would imply the presence of greater numbers of small follicles in these mice, and could potentially explain the greater litter sizes (McNeilly *et al.,* 2000). However, despite the increase in the number of small follicles, similar levels of inhibin A and oestradiol were secreted by het and wt follicles, suggesting that not all follicles are capable of ovulating (McNeilly *et al.,* 2000).

DAZL is expressed in the human fetus, and DAZL is important for oogenesis in human (Tsai *et al.,* 2000). Studies in the human ovary have found DAZL expression in the granulosa cells of primordial follicles at week 23 gestation, in contrast with previous findings of its localisation solely in the oocyte. However, gap junctions between the oocyte and granulosa cells enable proteins to pass between the oocyte and the granulosa cells, and DAZL is also capable of this movement (Brekhman *et al.,* 2000). In the sheep, DAZL expression is similar with that of other genes associated with meiosis such as DMC1 (disrupted meiotic cDNA1) suggesting that DAZL plays a role in meiosis (Mandon-Pepin *et al.,* 2003).

Human DAZL is expressed in the cytoplasm of female germ cells (Nishi *et al.,* 1999). DAZL mRNA and protein was also found to be expressed in the human corpus luteum (Pan *et al.,* 2002), with protein expression greatest in the early luteal phase and was localised to the cytoplasm of the granulosa-luteal cells (Pan *et al.,* 2002). The expression of DAZL in the CL suggests that it has roles other than cell proliferation as during the early stages of CL formation only a small percentage of cells (5% of granulosa-luteal cells and 15% of theca-luteal cells) proliferate (Pan *et al.,* 2002). DAZL protein has also been reported to be localised to the theca *interna* of developing follicles. However, it is not present in menopausal women, suggesting that DAZL is pivotal in oogenesis and follicle development (Nishi *et al.,* 1999; Pan *et al.,* 2002).

1.14.4 Mechanisms of Action

Dazl is an RNA binding protein that has a profound effect on germ cell differentiation in all animals (Venables *et al.,* 2001). Although the effect of the DAZ/ DAZL genes is unknown, several proteins have been identified that are postulated to interact with DAZ and DAZL (Moore *et al.*, 2003a). The effect of Dazl proteins is also species dependent, and knocking out *Dazl* across various species has demonstrated an important role in different aspects of germ cell development. For example, in *Xenopus* it prevents PGCs from surviving and migrating, whereas in *Dazl* KO mice, both male and females demonstrate meiotic failure (Venables *et al.,* 2001).

There are 34 possible targets of Dazl protein though these targets have yet to be identified in germ cells (Venables *et al.,* 2001). In the *Dazl* KO mouse mitotic division terminates in both male and female germ cells (Ruggiu *et al.,* 1997). Normal development of germ cells in both male and female *Dazl* KO at 15d.p.c is followed by a reduction of germ cells at 19d.p.c suggesting that germ cell proliferation is normal. However there is a dramatic increase in germ cell loss in both males and females (Ruggiu *et al.,* 1997). In female *Dazl* null mice, the germ cells are lost by day 17.5d.p.c and do not complete meiotic prophase (Saunders *et al.,* 2003).

It has been postulated that Dazl proteins play a role in translation of mRNAs in the germ cells, in particular Twine, a Cdc25 phosphate which is pivotal in cell cycle regulation through stimulating cyclin-dependent kinases. Twine protein levels were extremely low in mutant Boule *Drosophila* (the Dazl homologue) (Maines and Wasserman, 1999; Tsui *et al.,* 2000). Cdc25 is thought to be one of the targets of *Drosophila* Boule, and influences G2/m phase transition indicating the importance of Boule in meiosis (Maines and Wasserman, 1999). Similar to *Drosophila* Cdc25A homologue, murine *Dazl* can bind to Cdc25A mRNA suggesting an effect of *Dazl* on regulation of the cell cycle (Jiao *et al.,* 2002). Recent studies on the male mouse have demonstrated that Dazl stimulates mouse vasa homologue (Mvh), a protein which is essential for gametogenesis, and *Dazl* KO mice have lower expression of Mvh protein (Reynolds *et al.,* 2005). Pumilio-2 (PUM2) is a protein involved in the preservation of germ line stem cells and is also able to bind to DAZL through the RRM. Its expression has been found to overlap with DAZ/DAZL expression (Moore *et al.,* 2003a; Reynolds and Cooke, 2005).

There are several proteins identified by two-hybrid screening that might act together with *DAZ/DAZL*. These proteins include: DAZAP1 (DAZ Associated Protein), DAZAP2, DZIP1 (DAZ –interacting protein) and DZIP2. However, the exact roles that these have when these proteins interact with *Dazl* are unknown (Tsui *et al.,* 2000; Dai *et al.,* 2001; Moore *et al.,* 2004; Reynolds and Cooke, 2005). DAZAP1 is expressed in both human and rat ovaries luteal cells, and expression levels decrease as the luteal stage progresses (Pan *et al.,* 2005). DAZAP1 was not associated with polyribosomes, suggesting that together these proteins do not play a role in translation. However, DAZ and DAZAP1 may act together to regulate expression of germ cell genes (Dai *et al.*, 2001). Localisation of Dazl within the polyribosomes of the mouse testis suggest that it is also important in translation (Dai *et al.*, 2001) and again suggests that Dazl has multiple functions. These studies confirm that *Dazl* gene expression is vital in germ cell development and survival. However, the influence and effect of *Dazl* upon follicle growth and development in *Dazl* wt and het mice remains to be discovered.

1.15 Hypothesis and Aims of this Thesis

To date little is known about the role of *Dazl* on ovarian follicle development, and the majority of research on *Dazl* has been undertaken in the male mouse. However, both male and female *Dazl* KO mice are infertile (Ruggiu *et al.,* 1997; Slee *et al.,* 1999; McNeilly *et al.*, 2000), and there are significant differences in litter sizes, with *Dazl* het animals having larger litters and lower plasma FSH levels compared to *Dazl* wt mice (McNeilly *et al.*, 2000; McNeilly *et al.*, Unpublished; Appendix A).

The hypothesis of this thesis was that a single copy of the *Dazl* gene increased the FSH-sensitivity of follicles compared to follicles with two copies of the *Dazl* gene. To address this hypothesis the effects of *Dazl* on ovarian follicle growth and development were investigated by:

a. Comparing ovarian follicle reserve and growing follicle numbers in untreated *Dazl* wild-type and heterozygous KO mice. In addition, follicle numbers were compared in *Dazl* wt and het mice treated *in vivo* with recombinant human FSH (to increase plasma FSH levels) or treatment with oFF containing inhibin (to suppress plasma FSH levels).

b. Determining FSH-sensitivity of follicles from day 21 *Dazl* wt and het mice and comparing growth rates of follicles cultured *in vitro* with different concentrations of FSH.

c. Determining the percentage of healthy activated follicles and atretic follicles in untreated *Dazl* wt and het mice, and in *Dazl* wt and het mice treated *in vivo* with FSH or oFF.

Chapter 2

General Material and Methods

All reagents and equipment used in the studies carried out in this thesis are recorded below and supplier information is detailed in full in Appendix C.

2.1 Animal Experimentation

Animal experimentation was carried out according to the United Kingdom Home Office Act 1967 under project licence PPL/603232, and those involved with the animal work held Home Office personal licences. The *Dazl* mice were produced by 'targeted mutation' of exons 6 and 7 and the majority of exon five in embryonic stem cells and generated as detailed in Ruggiu *et al.* (1997) and McNeilly *et al.* (2000). All animals used were kept under normal conditions with a period of 14 hours light and 10 hours of darkness and fed *ad libitum*. Tail tips and ear tags were taken under light halothane anaesthesia at day 14 *post partum* for genotyping (Section 2.3). Animals were weaned at day 21 *post partum* when they were used for the experiments.

2.2 Tissue Collection and Fixation

Animals were killed by halothane asphyxiation followed by spinal dislocation prior to dissection. The ovaries were removed and either fixed for immunohistochemistry (Section 2.4) or dissected for follicle cultures (Section 2.6). Ovary fixation was carried out for 3 hours using Bouin's solution (Bios) prior to transfer into 70% (v/v) ethanol and processing into paraffin blocks (Section 2.4.1). Bouin's fixation results in cross-linking of proteins giving a stable structure to the tissue whilst maintaining antigenic sites.

2.3 Genotyping

All animal tail tips and ear punches were taken by Dr. J. McNeilly and were genotyped by Miss M. Laird, MRC Human Reproductive Sciences Unit, Edinburgh, by a standard in-house procedure as described.

2.3.1 Digestion

Either 1-2mm of tail or the entire ear punch was placed in a sterile Eppendorf tube and 100µl 25mM NaOH (BDH, Poole, UK), 0.2mM Ethylene Diaminetetra-Acetic Acid (EDTA; Sigma, Gillingham, UK) was added to the tails and 50µl to the ear punches. Samples were digested in the above solution for 20 minutes at 95°C (Omnigene PCR Machine, Thermo Hybaid, Ashford, UK) using the manual programme, ensuring that 'Tube Control' was selected. After the samples were centrifuged for 5 minutes at 13,000 revolutions per minute (rpm) and 100µl (tail tips) or 50µl (ear punches) 40mM Trizma HCl (Sigma) added. The samples were then vortexed and stored at -20°C.

2.3.2 Polymerase Chain Reaction (PCR)

The primers for *Dazl* were as follows: J889 (CAGTGGCTTTTGGAAATTATCA) with either K19 (TCAGCATGCATTCTTTCACTGCTTTC) for wt or K20 (GCTTCCTCTTGCAAAACCAC) for KO (MWG, London, UK; Ruggiu *et al.*, 1997; Figure 2.1). The PCR solution reagents (see below) were added to 5µl samples of digested ear punch or tails and the PCR ran (machine PTC 200, MJ Research, Waltham, Massachusetts, USA) using the programme below.

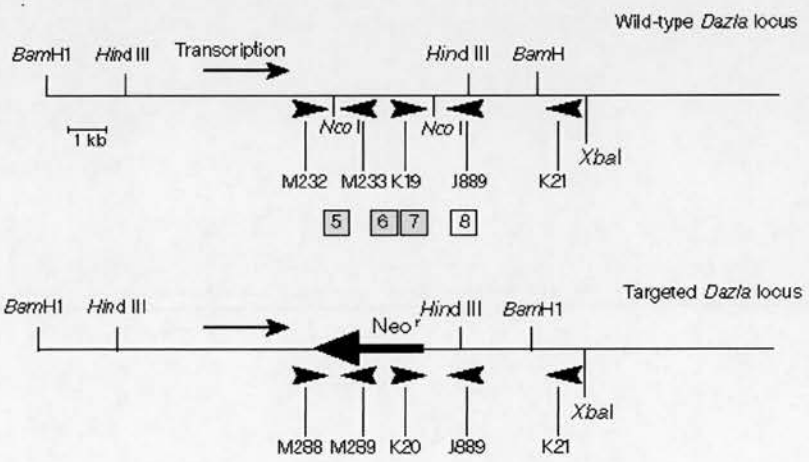


Figure 2.1 Location of Dazl primers (from Ruggiu et al., 1997)

Stage	Temperature	Duration	
Initial Denaturing	95°C	5 minutes	
Denaturation	94°C	1 minute	Repeated a further 34 Cycles.
Specific Primer Annealing Temp	60°C	45 seconds	
Extension	72°C	1 minute	
Final Extension	72°C	10 minutes	

PCR Solution

10x Buffer	5µl (ABgene, Epsom, UK)
25µM $MgCl_2$	4µl (ABgene)
ddH_2O	39.1µl
10µM U660.U661	0.6µl (MWG)
10µM U662.U663	0.6µl (MWG)
10nM DNTPs	0.5µl (Larova, Teltow, Germany)
Hotstart Taq	0.25µl (ABgene)

2.3.3 Agarose Gel Electrophoresis

On completion of the PCR, a 1:5 dilution of Orange G loading dye (see below) was added to each sample. A 2% agarose gel was prepared and 10µl of a 100bp ladder (Fermentas, Generuler, York, UK) was included in every gel as a reference marker and 25µl per sample was loaded per well. A water blank was added to every gel to ensure no contamination. The addition of ethidium bromide (Sigma) allowed the visualisation of DNA bands under UV illumination. The gels were run at 100v in 1x running buffer (10x running buffer = 108g Tris (Roche Diagnostic, Burgess Hill, UK), 55g Boric Acid (BDH), 40ml 0.5M EDTA (BDH) made to final volume of 1L) for approximately 1 hour (Bio-Rad Power Pack, Hemel Hempstead, UK). Gels were visualised on a UV transilluminator and photographed (Figure 2.2) using an integrated camera (Gene Flash, Syngene, Bio Imaging, Cambridge, UK).

Orange G

Orange G	0.025g (Sigma)
Ficoll	3g (Sigma)
EDTA	1.86g (BDH)
Tris-EDTA (TE)*	10ml (Sigma)
5M NaOH	Adjust to pH 7 (BDH)

*1ml Tris pH 7.5 and 0.2ml EDTA, made to a final volume of 100ml with dH_20 and autoclaved.

2% Agarose Gel (100ml)

Agarose	2g (Roche Diagnostic)
1x Tris-Borate EDTA (TBE)	100ml
Ethidium Bromide (10mg/ml)	5µl (Sigma)

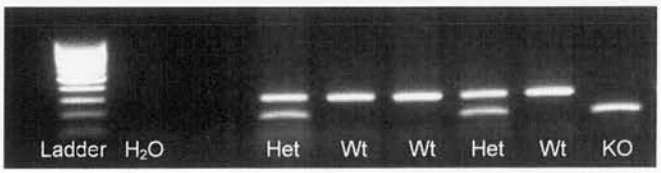


Figure 2.2 Gel of Dazl genotype.

2.4 Immunohistochemistry

2.4.1 Tissue Processing and Sectioning

The ovaries were embedded into paraffin blocks by the MRC Human Reproductive Sciences Unit, Histology Department, Edinburgh. Paraffin processing was carried out using an automatic processor (TP 1050, Leica, Milton Keynes, UK) using a standard method as detailed by Bancroft and Stevens (1996).

Once embedded, paraffin wax blocks (Bayer Diagnostics, Newbury, UK) were cut into 5μm sections on a hand-operated microtome RM 2125 (Leica). The sections were floated on ice-cold water followed by warm water (42°C) then placed onto charged slides (Superfrost Plus, VWR, Lutterworth, UK) and dried overnight at 50°C. Following this, slides were dewaxed in xylene for 10 minutes and re-hydrated through a series of decreasing percentages of alcohols (100%, 100%, 95% and 70%; 20 seconds in each) and finally into tap water.

2.4.2 Haematoxylin and Eosin (H&E) Staining

After following the procedures in Section 2.4.1, the slides were placed in 0.4% (w/v) haematoxylin (BDH) for 5 minutes, after which they were rinsed in tap water. The slides were then washed in 1% (v/v) HCl (BDH) for 10 seconds followed by a further rinse in tap water, and then placed in Scott's Water (containing: 10g potassium hydrogen carbonate, 100g magnesium sulphate and 5L H_2O) for 30 seconds, rinsed in tap water, immersed in eosin (BDH) for 30 seconds and finally rinsed in tap water. Slides were then de-hydrated in increasing concentrations of alcohols (v/v) (70%, 95%, 100%, 100%; 20 seconds each) before being cleared in histoclear (VWR) and xylene (VWR) for 5 minutes each. Finally, a coverslip was placed over the sample using Pertex fixative (CellPath, Powys, UK). During the process of staining the haematoxylin is oxidised, staining the cell nuclei blue. Eosin is a red acidic dye that stains epithelial and squamous cells.

2.4.3 Antigen Retrieval

Antigen retrieval was carried out to recover epitopes that undergo changes during the fixation process. Cross-linking of various unrelated proteins occurs and results in a loss of immunoreactivity by the antigen (Boenisch *et al.*, 2001).

Antigen retrieval was carried out by pressure cooking the slides in 2L 0.01M citrate buffer (pH 6) in a Tefal Clipso pressure cooker and boiled at full pressure for 5 minutes after which the pressure was released and the slides left to cool in the buffer for a further 20 minutes. The slides were then cooled with tap water prior to immersion in 1x Tris-buffered saline (TBS) (60.55g Tris, (Roche Diagnostic); 82.66g NaCl, (BDH) pH 7.4 with HCl and made to final volume of 1 litre with H_2O).

2.4.4 Methanol/Peroxide Block

Slides were incubated in a methanol solution containing 3% hydrogen peroxide (H_2O_2) (BDH) for 30 minutes at room temperature to block endogenous peroxidase activity. Following incubation, slides were washed (2x 5 minutes) in 1x TBS.

2.4.5 Avidin-Biotin Block and Serum Block

The slides were incubated at room temperature using an Avidin-Biotin blocking kit (Vector SP2001, Peterborough, UK) following the manufacturer's protocol to reduce endogenous non-specific binding. Briefly, 0.1% avidin was added neat to the slides for 30 minutes in a humidified staining box before being washed twice in TBS for 5 minutes each. The slides were then incubated in 0.01% Biotin for 20 minutes at room temperature in a humidified staining box prior to washing twice in 1x TBS. Blocking using appropriate serum (1 part blocking serum (Autogen Bioclear, Calne, UK)/4 parts TBS/5% Bovine Serum Albumin (BSA; Roche Diagnostic); Table 2.1) was then carried out for 30 minutes at room temperature in a humidified staining box.

2.4.6 Primary and Secondary Antibodies

The appropriate primary antibody (Ab) (Table 2.1) was diluted in 1 part blocking serum (Autogen Bioclear)/4 parts TBS/5% BSA (Roche Diagnostic) and sufficient was added to cover the sections. Positive and negative controls were included in every immunohistochemistry experiment carried out. Positive controls were sections from mouse ovaries which had previously stained positive for AMH and Cleaved Caspase-3. The negative control involved every stage of the experiment except that serum was added in place of the primary Ab. The slides were incubated overnight at 4°C in a humidified staining box.

After overnight incubation the slides were washed in 1x TBS three times for 5 minutes each. A specific biotin-coupled secondary Ab was diluted in the appropriate serum (Table 2.1) and incubated for 1 hour at room temperature in a humidified staining box. Following this sections were washed twice in 1x TBS.

Antigen	Blocking Serum	Primary Ab	Secondary Ab
Mullerian Inhibiting Substance	Rabbit Serum (Autogen Bioclear)	Mullerian Inhibiting Substance (1:500) Santa Cruz (Wiltshire, UK)	Rabbit Anti-Goat Biotin (1:500) DAKO (Cambridgeshire, UK) Raised in rabbit.
Cleaved Caspase-3	Goat Serum (Autogen Bioclear)	Cleaved Caspase-3 (1:500) Cell Signalling (Hertfordshire, UK)	Biotinylated Anti-Goat (1:500) Vector

Table 2.1 Summary of blocking sera, primary and secondary antibodies.

2.4.7 Horse-radish Peroxidase (HRP)

The avidin biotin complex (ABC)-HRP detection system was used to amplify the signal from the secondary Ab. The ABC-HRP (DAKO) complex was prepared as per manufacturer's instructions. Briefly, 1 drop of solution A and 1 drop of solution B was added to 5ml of 0.05M Tris HCl. This solution was prepared 20 minutes in advance at room temperature allowing the complex to form. The ABC-HRP was added to the slide and incubated for 30 minutes at room temperature in a humidified staining box and then washed three times in 1x TBS.

2.4.8 Developing / Counterstaining / Mounting

Staining was developed using diaminobenzidine (DAB; DAKO) which produces a brown stain. The DAB solution was prepared by adding 1 drop of neat DAB solution in 1ml DAB buffer. The DAB buffer is an Imidazole-HCl buffer which contains hydrogen peroxide and an anti-microbial agent (pH 7.5) (DakoCytomation Product Information). Once stained, the slides were placed in tap water to stop the reaction. The slides were then counterstained in haematoxylin (BDH) for 5 minutes, washed in 1% (v/v) HCl (BDH) alcohol (1% (v/v) HCl in 70% Industrial Methylated Spirit (IMS: 70ml IMS; 30ml H_2O), Fischer Scientific, Loughborough, UK) for 10 seconds, and washed in Scott's Water for 30 seconds. The slides were then dehydrated and mounted as described in Section 2.4.2.

2.5 Stereology

2.5.1 Follicle Counts

Follicle counts were carried out to determine total number of follicles in the ovaries of the *Dazl* wt and het mice, as described by Benedict *et al.* (2000). Every 10^{th} section of ovary was analysed for follicles that contained a nucleus within the oocyte. The benefits of this system and other counting methods are outlined in Chapter 1 (Table 1.2).

Ovarian sections were stained with H&E as described in Section 2.4.2. Follicles were classified using the parameters in Table 2.2 and visualised at x20 magnification on a live image on Image-Pro with Stereology plug in (Media Cybernetics, Berkshire, UK). Examples of follicle classification are shown in Figure 2.3.

Follicle Classification	Characteristic
Primordial	Oocyte surrounded by a single layer of flattened granulosa cells.
Transitionary	Some granulosa cells are cuboidal but some still remain flattened.
Primary	Full layer of granulosa cells surrounds the oocyte enclosed by a basement membrane.
Secondary	The start of a 2nd layer of cuboidal granulosa cells and the presence of a distinct theca layer.
Antral	Eight or more layers of granulosa cells and the presence of fluid-filled patches.
Unclassifiable	Large oocyte surrounded by a single layer of flattened granulosa cells.

Table 2.2 Follicle classification for follicle counts (Gougeon, 1996; Gougeon and Busso, 2000; Smitz and Cortvrindt, 2002).

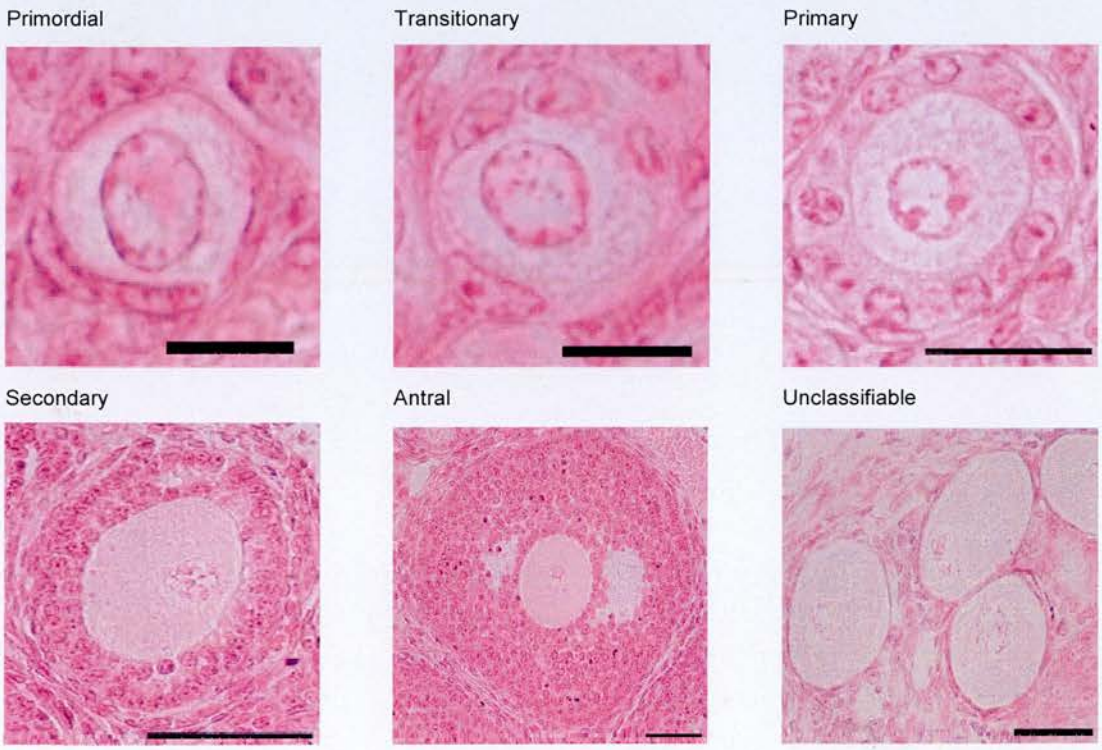


Figure 2.3 Examples of follicles in each classification. Scale bars = 10μm for Primordial (x100) and Transitionary (x100), 20μm for Primary (x100) and 50μm for Secondary (x40), Antral (x40) and Unclassifiable (x40).

Follicle counts and follicle areas were measured using a BH2 Olympus Microscope (Olympus, UK) with attached digital camera (Hitachi HV-C20A, Tokyo, Japan) using Image Pro Plus™ software with a Stereology 5.1 plug-in (Media Cybernetics).

Follicle Counts

To calibrate the settings of the programme:

1. Click on the stereologer taskbar
2. File – Open Settings – select Stereovalues
3. Select Experimental Design – no overlay

Manual tag was selected on the Image-Pro programme and the classes of follicles added (Figure 2.4). Data was analysed in Microsoft (MS) Excel.

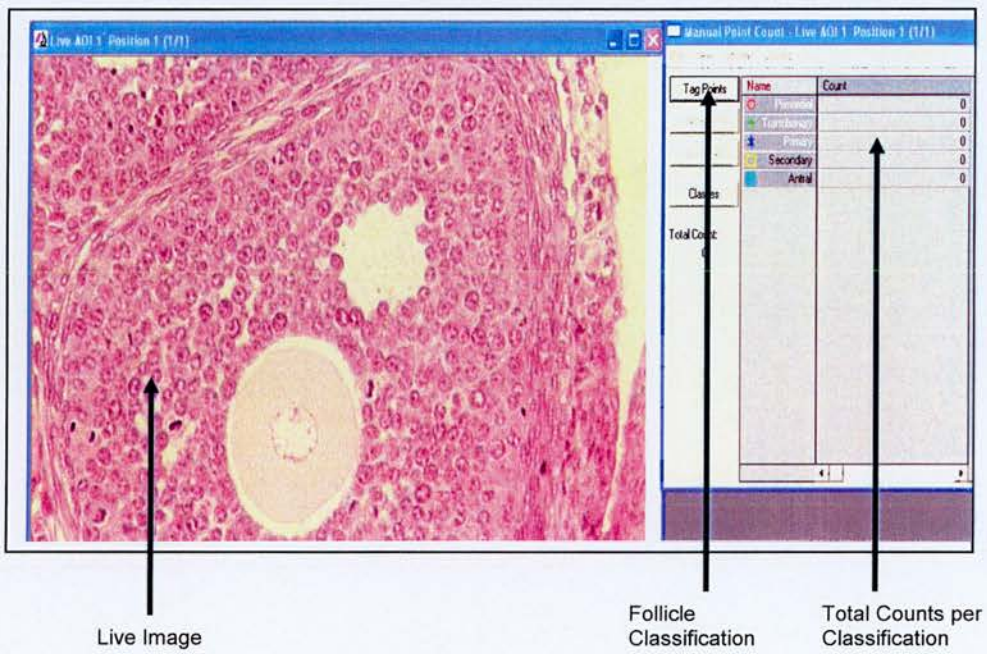


Figure 2.4 Methods of Follicle Count - Manual Tag.

2.5.2 Measurement of Ovary/Follicle/Oocyte/Granulosa Cell Areas and Diameters

The areas of ovary, follicle, granulosa cells and oocytes for primary, secondary and antral follicles were analysed. In addition, the follicular fluid area of antral follicles was also measured. The methods used for measuring the areas using the Image Pro Plus™ software with a Stereology 5.1 plug-in system are described in detail below. The magnification used was x20 for follicle, oocyte and granulosa cell area and diameters and x4 magnification for ovary areas.

Image Pro Taskbar:

1. Click on the stereologer taskbar.
2. File – Open Settings – Stereovalues.
3. Experimental Design – No overlay.
4. Measurement – Calibration – select spatial – select relevant magnification.

Stereologer taskbar:

1. Experimental Design – Analysis – select relevant magnification.

Areas were measured by two means:

1. Using the Bean-shaped button on the Image Pro programme taskbar. After determining the area of interest ensure the 'Live Off' button on the Stereologer taskbar is clicked. The next steps are (Figures 2.5 and 2.6):

- Go to File and select Fill.
- Go to Measure and select Count – count the objects.
- On the Count taskbar go to File and select DDE Options and DDE to Excel.

2. Select Measure on the Image Pro programme taskbar, click on measurements and select the relevant form of measurement and export to MS Excel (Figure 2.7).

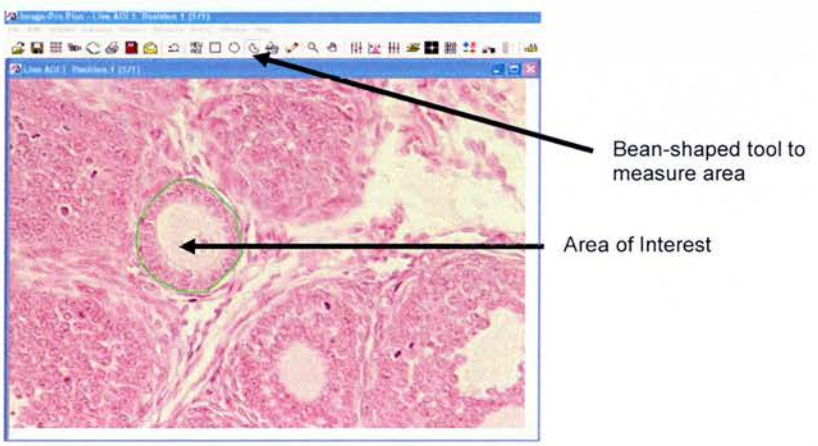


Figure 2.5 Selecting the Area of Interest Using Bean-shaped Tool.

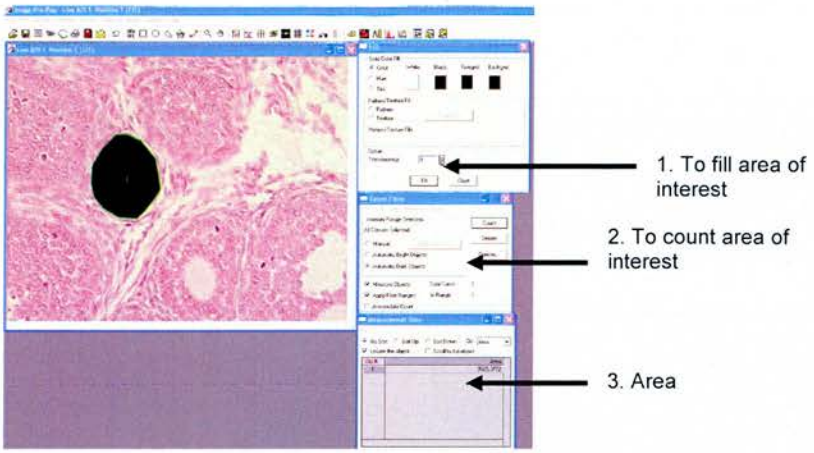


Figure 2.6 Filling the Area of Interest.

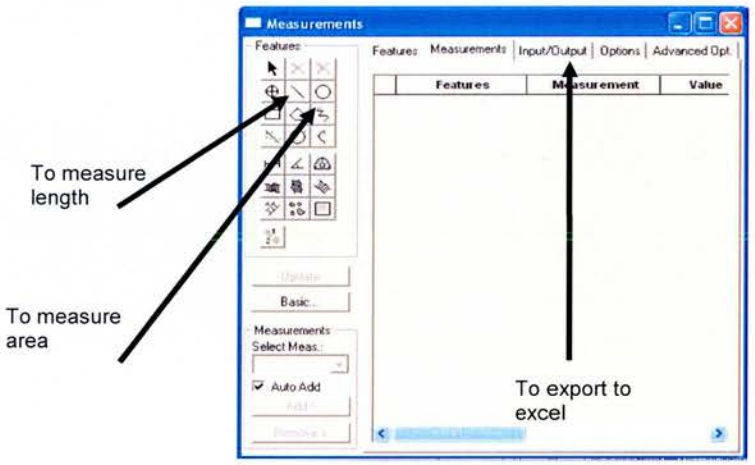


Figure 2.7 Example of Method 2 used to Measure Areas and Diameters.

2.6 Follicle Cultures

Follicle culture was used to analyse the growth and development of follicles dissected from *Dazl* wt and het mice under controlled conditions *in vitro*. Culturing the follicles using the following method enabled analysis of factors which affect the growth and development of ovarian follicles.

2.6.1 Culture System

Leibovitz L-15 medium (Invitrogen) was used for collecting ovaries and the follicular dissection. Each 50ml bottle was adjusted to 285-293mOsm/kg H_2O with sterile water (Sigma) using an osmometer (Roebling, Berlin, Germany). Leibovitz medium was kept for a maximum of 5 days at 4°C before being discarded. Leibovitz was supplemented with 0.3% w/v BSA (BSA, Fraction V, Tissue Culture Grade, Sigma) and sterile filtered (0.22µm large filter, Iwaki, Stone, UK). Medium was prepared one day in advance and kept at 4°C. Prior to dissecting the ovaries, 1ml was aliquoted into watch glass dishes (BDH) and warmed to 37°C. All dissection procedures were undertaken in aseptic conditions using the culture system outlined in Figure 2.8. Ovaries were dissected, placed into a watch glass containing 1ml Leibovitz L-15 medium (Invitrogen, Paisley, UK) and any excess tissue was removed.

The ovaries were cut in half and each placed into a fresh watch glass also containing Leibovitz. One half of an ovary was worked on at any time for a maximum of 15 minutes whilst the other parts of the ovary were maintained at 37°C.

Follicles were dissected using an inverted dissecting microscope (Zeiss, Welwyn Garden City, UK) fitted with a heated stage (Linkam, Tadworth, UK) and an ocular micrometer. Pre-antral follicles were manually dissected from mice using insulin needles (Sherwood, Gosport, UK) and acupuncture needles (Acumedic, London, UK), which were mounted in steel holders. The follicles were visualised under a dissecting microscope and were manually isolated using finely pulled sterile glass pipettes coated with a 10% BSA solution in H_2O. Follicles from each animal were placed in fresh Leibovitz and kept on a heated plate at 37°C until follicles from both ovaries from every mouse had been pooled.

The morphological appearance and size of the follicles were assessed post dissection. Follicles selected for culture fitted the following criteria: (1) 180-220µm in diameter, (2) a central spherical oocyte, (3) a small theca layer and (4) the follicle was spherical. Follicles that fitted these criteria were individually transferred from the collection dish into row B of 96-well plates (Bibby-Sterlin Ltd., Stone, UK).

2.6.2 Follicle Culture Set-up

Two rows of wells containing culture media were prepared in advance and allowed to equilibrate overnight. Follicles were cultured individually in 96-well plastic plates, each well containing 30µl of α-minimum essential medium (α-MEM; Invitrogen), and covered with 75µl filtered silicon oil (0.45µm filters, Iwaki; silicon fluid, Merck, Beeston, UK) to prevent evaporation.

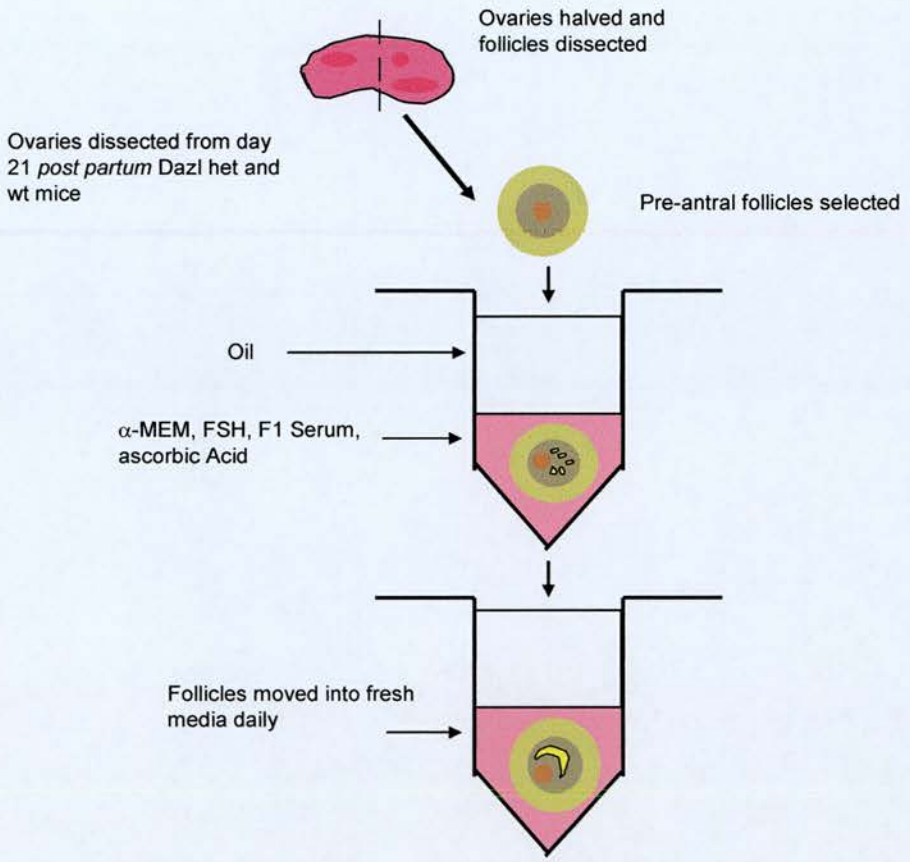


Figure 2.8 Follicle culture method (adapted by permission of Dr. A. Murray).

A reverse pipetting technique was used in order to prevent bubbles in the culture medium. This involved pressing the pipette plunger past the first stop to the second stop stage prior to putting the tip into the media. The tip was then placed in the media and the plunger released. The plunger was then pressed to the first stop stage to expel the media.

2.6.3 Culture Medium

Fresh medium was prepared every two days as detailed by Murray *et al.* (2001) and left to equilibrate in the incubator for at least one hour before transferring the follicles into wells containing fresh medium. The osmolarity was maintained between 285-293mOsm/kg H_2O with sterile water. The α-MEM was supplemented with 5% (v/v) mature F1 mouse serum, 0.5% ascorbic acid (Sigma) and various concentrations of recombinant FSH (rFSH; Puregon 3001IU/vial; batch number 311209, Cambridge, UK). All culture media were filtered using a 0.2µm filter top (Iwaki).

All mouse serum was collected from 6-week-old F1 female mice by Dr. Alison Murray or Dr. Judy McNeilly. The mice were anaesthetised and blood extracted by cardiac puncture. Blood was aliquoted into Eppendorf tubes and left for 15 minutes to clot. The Eppendorfs were centrifuged at 13,000rpm for 10 minutes. Serum was pooled, centrifuged for a further 10 minutes at 13,000rpm and stored in 200µl aliquots at -70°C. Once defrosted the serum was only frozen and thawed once more before being discarded. Ascorbic acid (Sigma) solution (5mg per ml of α-MEM) was prepared and stored in 20µl aliquots for a maximum of one month at -70°C, and used only once after defrosting.

2.6.4 Incubation

Follicles were incubated at 37°C and 5% CO_2 (Forma Scientific, Ohio, USA) in high humidity (detailed by Smitz and Cortvrindt, 1998). The duration of each culture period is detailed in the appropriate chapter. Every day, follicles were transferred into the next row of 96-well plates using glass pipettes (coated with 10% BSA solution) and the follicle diameter measured in fresh α-MEM. Follicles were measured using a calibrated eyepiece graticule fitted to the dissecting microscope as described by Murray *et al.* (2001). Follicles were moved and measured quickly and precisely thus ensuring that they were out of the incubator for the shortest possible time. The diameters of all follicles were recorded, and any antral formation noted.

2.7 Enzyme-Linked Immunosorbent Assay (ELISA)

The concentration of oestradiol, progesterone, inhibin A and inhibin B released into the culture medium was measured by specific ELISAs (in-house). Media from individual follicle cultures was stored at -20°C. ELISAs detect the levels of a particular substance present in the culture media and comparison with a standard curve allows the calculation of the concentration of that component in the sample. NUNC 96-well immuno-plates (Invitrogen Life Technologies, Paisley, UK) were used for all oestradiol, progesterone and inhibin A plate assays. For inhibin B assays 96-well ELISA plates (Greiner Bio-one, Stonehouse, UK) were employed. The oestradiol and progesterone ELISAs are competition ELISAs and work on the principle that the greater the level of oestradiol or progesterone in the media, the greater the displacement of the biotin-labelled protein. This displacement reduces the streptavidin binding and, therefore, reduces the colour product in the wells (Figure 2.9).

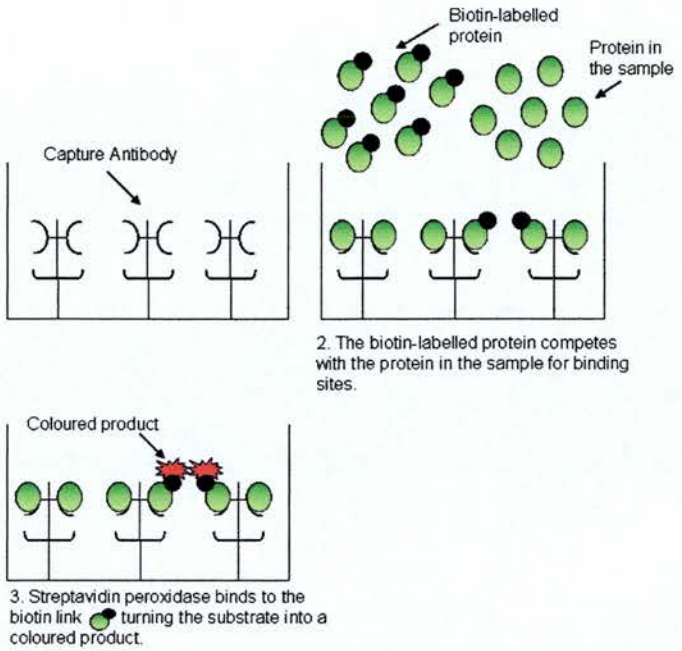


Figure 2.9 Competition ELISA method for oestradiol and progesterone assays (adapted from Dr. C. Dunn, MRC, Edinburgh).

2.7.1 Oestradiol ELISA

Follicular oestradiol secretion was analysed using an ELISA plate assay based on the assay detailed by Glasier *et al.* (1989). The cross reactivity of this assay with other steroids is <0.1% and for oestrone is 10.6%. The inter-assay coefficients of variation for the oestradiol ELISA were 35% (low) and 33% (high). Due to the minimal volume of samples the intra-assay variation was not determined. A representative standard curve is shown below with high and low Quality Controls (QCs) of 164.5pg/50µl and 6.14pg/50µl respectively (Figure 2.10).

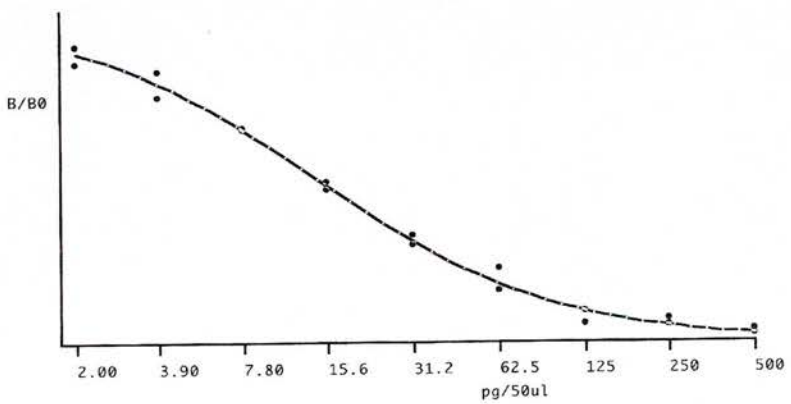


Figure 2.10 Representative image of oestradiol ELISA standard curve.

Plates were coated in a buffer containing a 1:1000 dilution of coating Ab (see below) donkey anti-goat serum (DAGS; The Binding Site, Birmingham, UK). A total volume of 100µl was added per well. Plates were then sealed and incubated overnight at room temperature.

Coating Buffer

Sodium Carbonate	4.24g (BDH)
Sodium Bicarbonate	4.04g (BDH)

Dissolved in 1L of dH_2O and adjusted to pH 9.6 with HCl (BDH).

The incubate was removed the following day, and the plate washed 10 times with wash solution (see below) and dried. In all ELISA methods, all plates were washed with 1x wash solution. A standard curve (Figure 2.11) was added to every plate with the top standard reading 500pg/50µl. Stock oestradiol (provided by Prof. S.G Hillier) was diluted in α-MEM culture medium to 500pg/50µl. This was diluted 1:2 to generate a range of standards down to 2pg/50µl. Low and high QCs were prepared in a similar manner.

Wash Solution (25x Concentrate)

Tris	302g (Roche Diagnostic)
NaCl	450g (Sigma)
Tween 20	25ml (Sigma)

Made to a final volume of 2L with dH_2O (pH 7.5).

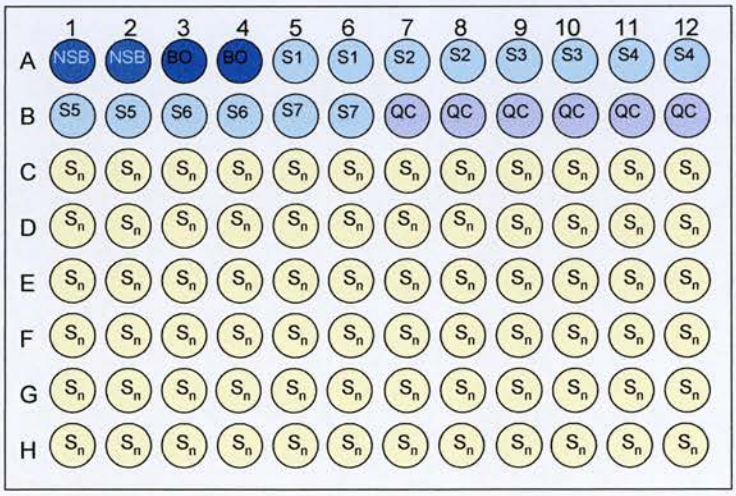


Figure 2.11 Format of oestradiol ELISA plate,
where: NSB = non-specific binding, Bo = total binding, QC = quality control, S1 etc = standard and S_n = sample.

150µl of α-MEM was added to wells 1 and 2 (of each plate) to measure non-specific binding (NSB). 100µl of assay buffer (see over page) was added to wells 3 and 4 to measure total binding (Bo). The remaining wells had a total volume of 50µl of standard, QC or sample (s_n) (samples were diluted 1:25 in α-MEM) added in duplicate (see Figure 2.11).

0.1M Phosphate Gelatin Buffered Saline (P.G.B.S) Assay Buffer

$NaHPO_4$	11.46g (BDH)
NaH_2PO_4 $2H_2O$	2.61g (BDH)
NaCl	9.0g (Sigma)
Gelatin	1.0g (Sigma)
Thiomersalate	0.1g (BDH)

Made to a final volume of 1L with dH_2O and dissolved on a mixer with heat and adjusted to pH 7.4.

Assay buffer (50µl) was added to every well containing a standard, QC or sample. The primary Ab (sheep anti-oestradiol; Lot: BW 26/9/80; Gift to Prof. A. S. McNeilly from Prof. R. Webb, University of Nottingham, UK) was diluted in assay buffer to give a 1:750,000 working solution and 50µl was added to every well containing Bo, a standard, QC or sample. Finally, 50µl of neat oestradiol-biotin diluted to a working concentration of 1:80,000 in assay buffer was added to all wells. The plates were sealed in a plastic container to maintain temperature and humidity and then incubated overnight at room temperature.

The following day, incubates were removed and the plate washed ten times. 100µl of streptavidin-horse-radish peroxidase (Streptavidin-HRP; BD Bioscience, Oxford, UK) diluted 1:2000 in 0.1M P.G.B.S containing 1% casein (BDH)) was added to each well and the plate sealed, then placed in a plastic container and incubated for one hour at room temperature. The plate was then washed a further ten times and dried completely. 200µl of substrate solution (see below) was added to each well using a multi-channel pipette (Biohit Proline, Torquay, UK). The plate was covered with foil and incubated for 15 minutes at room temperature. On reaching an appropriate level of development, 50µl of stopping solution (1:10 dilution of concentrated sulphuric acid (BDH)) was added to each well. The optical density (OD) was then read at 490nm for 0.1 seconds on the Victor Plate Reader (Wallac, Milano, Italy; see Section 2.7.4).

Substrate Solution (light sensitive – cover with foil)

O-Phenylenediamine dihydrochloride	30mg (Sigma P8412)
Hydrogen Peroxide	30µl (Sigma)
Substrate Buffer	75ml

Substrate Buffer

Citric Acid	10.3g (Sigma)
Na_2HPO_4 Dihydrate	17.79g (BDH)

Deionised H_2O to 1L.

2.7.2 Progesterone Assay

Progesterone secretion was analysed using an ELISA plate assay based on the assay detailed by Wulff *et al.* (2001). The cross reactivity of this assay was <20% for 5-pregnan 3,20 dione, <10% 11-deoxycorticosterone, <5% 17-hydroxy progesterone, <0.25% hydrocortisone and <0.15% all other steroids. The inter-assay coefficient of variations for the progesterone ELISA could not be calculated as there were only sufficient samples for one ELISA plate. Due to the minimal volume of samples only one assay was carried out and, therefore, the inter-assay the intra-assay variations were not determined. A representative of the standard curve is detailed below with high, mid and low QCs of 0.78ng/ml, 2.10ng/ml and 8.77ng/ml respectively (Figure 2.12).

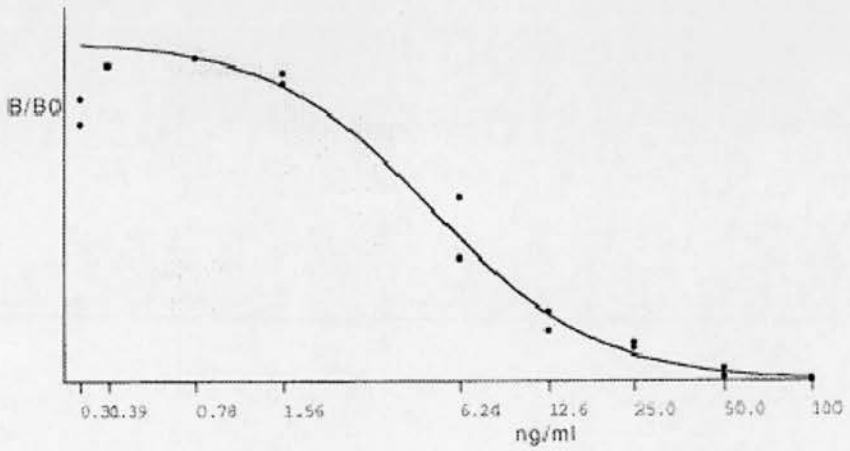


Figure 2.12 Representative image of progesterone ELISA standard curve.

Plates were coated with 100µl coating donkey anti-rabbit serum (DARS; MP Biomedicals, Stretton, UK) per well, diluted 1:1000 in coating buffer (see below) and incubated overnight at room temperature.

Coating Buffer

Sodium Carbonate	4.24g (BDH)
Sodium Bicarbonate	4.04g (BDH)

Dissolved in 1L of dH_2O and adjusted to pH 9.6 with HCl (BDH).

The incubate was removed the following day and the plate washed 10 times (see below). A 9-point standard curve was added to every plate by doubling dilutions of 1000pg/50µl to 3pg/50µl. Standards and quality controls were prepared by the MRC Human Reproductive Sciences Unit, Assay Department, Edinburgh.

150µl phosphate citrate assay buffer (see below) was added to wells 1 and 2 of each plate to measure NSB, and 100µl of assay buffer to wells 3 and 4 to measure Bo. A total volume of 50µl of standard, QC or sample (diluted 1:10 in α-MEM) was added to the plate in duplicate (Figure 2.11). The primary Ab, SAPU anti-P4 serum (obtained from rabbits immunised with 11-alpha-progesterone-bovine albumin conjugate; Diagnostic Scotland), was diluted in phosphate/citrate assay buffer to a working concentration of 1:50,000. 50µl of this was added to every well except NSBs. The plate was sealed and incubated at room temperature overnight.

Wash Solution (25x Concentrate)

Tris	302g (Roche Diagnostic)
NaCl	450g (Sigma)
Tween 20	25ml (Sigma)

Made to a final volume of 2L with dH_2O (pH 7.5).

Phosphate/Citrate Assay Buffer

NaH_2PO_4	17.85g (BDH)
Citric acid	7.75g (Sigma)
Gelatin	1.0g (Sigma)
Thiomersalate	0.1g (BDH)

Made to a final volume of 1L with dH_2O, adjusted to pH 6 and stored at 4°C.

P_4-11-glucuronide biotin complex (prepared in-house by the MRC Human Reproductive Sciences Unit, Assay Department) was diluted to a working concentration of 1:7000 in phosphate/citrate assay buffer and 50µl added per well. The plate was sealed and incubated for 3 hours at room temperature, after which the incubate was removed and the plate washed 5 times and dried. A total volume of 100µl per well of streptavidin-HRP (Streptavidin-HRP (BD Bioscience) diluted 1:2000 in 0.1M P.G.B.S containing 1% casein (BDH) was added and incubated for 1 hour at room temperature. The plate was washed as before, then 200µl of substrate solution (see below) added per well. The plate was covered with foil and left at room temperature for 15-30 minutes. 50µl of stopping solution (1:10 dilution of concentrated sulphuric acid (BDH)) was added to each well and the OD read on a Victor Plate Reader (Wallac, Section 2.7.4).

Substrate Solution (light sensitive – cover with foil)

O-Phenylenediamine dihydrochloride	30mg (Sigma P8412)
Hydrogen Peroxide	30µl (Sigma)
Substrate Buffer	75ml

Substrate Buffer

Citric Acid	10.3g (Sigma)
Na_2HPO_4 Dihydrate	17.79g (BDH)

Deionised H_2O to 1L.

Two-site Sandwich ELISA

2.7.3 Inhibin Assays

Inhibin A and inhibin B were measured using a two-site Sandwich ELISA method (Figure 2.13) as described previously (Groome *et al.*, 1994; Groome *et al.*, 1996) and validated for measurement in the mouse (McNeilly *et al.*, 2000). The concentration of the QCs for inhibin A were 30pg/ml, 95pg/ml and 500pg/ml for high, medium and low QCs respectively and were 50pg/ml, 125pg/ml and 250pg/ml for inhibin B for high, medium and low QCs respectively. The inter-assay variation was 24% (low), 10% (mid) and 22% (high) for inhibin A and 20% (low), 20% (mid) and 30% (high) for inhibin B. An example of the standard curve for inhibin A and inhibin B is demonstrated below (Figures 2.14 and 2.15 respectively).

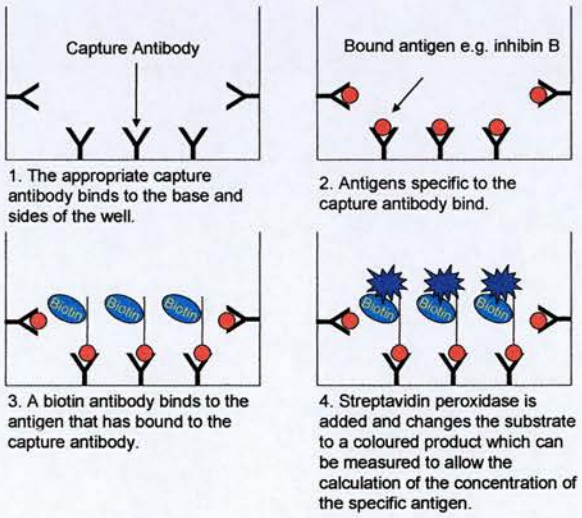


Figure 2.13 Two-site sandwich ELISA method for inhibin A and inhibin B assays (adapted from Dr. C. Dunn, MRC, Edinburgh).

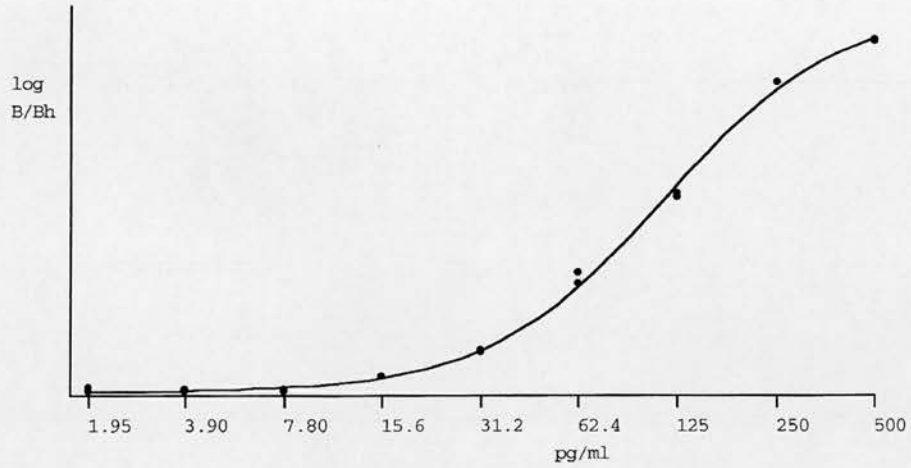


Figure 2.14 Representative image of inhibin A ELISA standard curve.

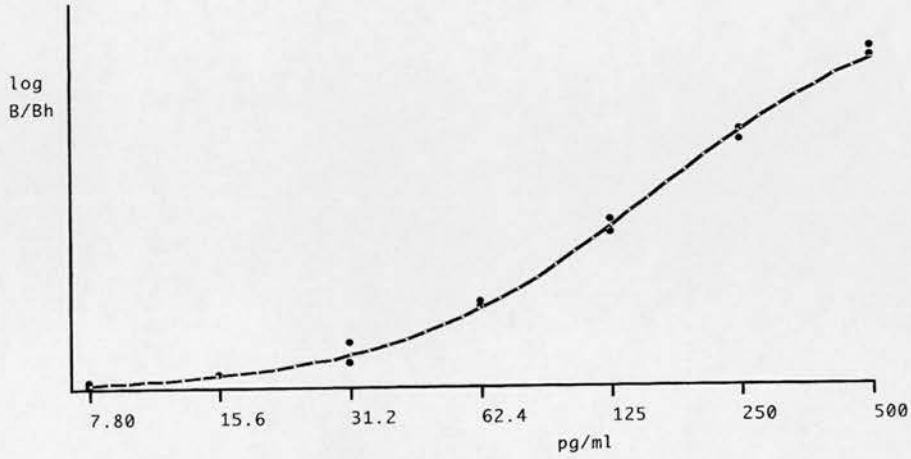


Figure 2.15 Representative image of inhibin B ELISA standard curve.

Capture antibodies for inhibin A (Ab E_4) and inhibin B (Ab C_5) were diluted in coating buffer (see below) to 10µg/ml and 20µg/ml respectively. 50µl was added to each well and incubated overnight at 4°C.

Coating Buffer

Sodium Carbonate	4.24g (BDH)
Sodium Bicarbonate	4.04g (BDH)

Dissolved in 1L of dH_2O and adjusted to pH 9.6 with HCl (BDH).

The incubate was removed and the plate washed 10 times (see below) and dried. On the 3rd wash for inhibin B, an extra 5-minute wash in phosphate-buffered saline (PBS) (Sigma) on a plate shaker was required. The plate was then washed a further 7 times.

Wash Solution (25x Concentrate)

Tris	302g (Roche Diagnostic)
NaCl	450g (Sigma)
Tween 20	25ml (Sigma)

Made to a final volume of 2L with dH_2O (pH 7.5).

A standard curve was added to every plate with doubling dilutions ranging from 500pg/ml to 7.8pg/ml for inhibin B. Standards were prepared by diluting inhibin B stock (Prof N. Groome) in α-MEM culture media. A standard curve for inhibin A ranging from 500pg/ml to 1.95pg/ml was added to every second plate. Standards and QCs were prepared (by the MRC Human Reproductive Sciences Unit, Assay Department, Edinburgh) by diluting dimeric inhibin A (Prof N. Groome) in α-MEM culture media.

Culture media samples were diluted 1:100 and 1:50 in α-MEM for inhibin A and inhibin B respectively. 100µl of either standard, QC or sample were added to 1.5ml polypropylene microtubes. To every microtube 50µl of 6% w/v SDS (Roche Diagnostic) solution was added and the tubes vortexed prior to boiling the samples for three minutes. The samples were removed from the water bath and allowed to cool for 10 minutes (at room temperature). When cooled, 50µl of 6% (w/v) H_2O_2 solution was added to every standard, QC or sample and incubated for 30 minutes at room temperature. Finally, 100µl of assay buffer (see below) was added to each tube and mixed. A 96-well plate was then set up as in Figure 2.11 with NSBs, Bos, standards, QCs and samples in duplicate. The plate was then shaken for 5 minutes and incubated overnight at room temperature for inhibin A or at 4°C for inhibin B.

Assay Buffer

Tris	1.21g (Roche Diagnostic)
NaCl	0.9g (Sigma)
BSA	10.0g (Sigma A-3294)
Triton X-100	5.0ml (Sigma)
dH_2O	100ml

Adjust pH to 7.5 with HCl (BDH) and filter through 0.8µm filter. Add 0.1g Na azide (Sigma) and 1ml of 0.06% amido black solution (60mg Amido Black (Sigma), 100ml dH_2O, freeze at -20°C).

The incubate was removed and the plate washed 10 times (Section 2.7.1). R_1alkaline phosphatase (Prof N. Groome) was diluted 1:100 in assay buffer and 50µl was added to each well and incubated for 2.5 hours. The incubate was removed and the plate washed a further 10 times. After the 5th wash, the plate was filled with wash solution and placed on a plate shaker for 5 minutes (for inhibin B only). 50µl of substrate solution (Invitrogen Life Technologies) was then added to each well. The plate was then shaken and allowed to incubate for 2.5 hours (for inhibin A) or 3 hours (for inhibin B) at room temperature. Finally, 50µl of amplifier solution (Invitrogen Life Technologies) was added to each well and incubated until the colour of the top standard had an OD reading of between 2.0 and 2.5 as determined by development of a claret colour. 50µl of 0.4M HCl (BDH; stop solution) was then added to each well and the plate read on a Victor Plate Reader (Wallac, Section 2.7.4).

2.7.4 Assay Data Collection

Plates for all assays were read at 490nm on a Victor Plate Reader (Wallac) linked to an Apple MacIntosh (Mac) Computer. Data was analysed using AssayZap (a universal assay programme which enables results to be calculated in pg/µl). Each assay had its own specific method file which specified the number of Bo, NSBs and standards run on every plate. The concentrations in unknown samples were calculated from the standard curve.

2.8 Protein Assay

Protein was assayed using the Bio-Rad DC Protein Assay Kit (Hemel Hempstead, UK). Nine standards ranging from 0-500µg/protein were diluted in 0.01% SDS. Media and follicle extracts were diluted 1:2 in 0.01% SDS. Due to small quantities of samples, the assay was not performed in duplicate. A total volume of 20µl of standard (in duplicate) or sample was added to a 96-well plate, and 20µl of working solution (1:100 dilution of Solution S in Solution A) and 80µl of Solution B were added to each well. The plate was incubated on a plate shaker at room temperature for 15 minutes and OD read on Labsystems Multiskan EX plate reader at 690nm (VWR).

2.9 Zymography

Gelatin zymography was used to detect active and latent forms of gelatinases, in particular MMP-2 and MMP-9. Gelatin is the substrate required for these gelatinases, was included in the resolving gel. Activity was identified by the presence of a white band on the coomassie-blue-stained gel. Latent forms of gelatinases could also be identified, as SDS in the gel allows exposure of the active site.

2.9.1 Resolving Gel and Stacking Gel

Gelatin gels were prepared by making a 7.5% resolving gel (0.75mm thick) and a 4% stacking gel (0.75mm thick). The resolving gel (see below) was prepared first. Gelatin dissolved in dH_2O (Sigma; bovine skin type III Bloom 225) was heated and dissolved in a microwave (Sharp Compact 700, John Lewis, Edinburgh, UK; 70watts for 50 seconds, mixed and repeated) prior to use. The solution was then pipetted into the gel former of a Bio-Rad Mini Protean II Mini-gel System, covered with water-saturated butanol (equal volumes of H_2O and butanol (Sigma) added together and mixed vigorously) and left to set at room temperature for 45 minutes.

The water-saturated butanol was rinsed off and the gel washed with water and 1x Tris buffer (6.06g Tris (BDH), made to final volume of 100ml with dH_2O and adjusted to pH 6.8) 5 times. The gel was covered with Tris buffer (diluted 1:4 in dH_20) and then with cling film and left overnight at 4°C to allow polymerisation to occur.

Resolving Gel (volume required for 2 gels)

ddH_2O	3.85ml (MilliPore)
Tris 1.5M (pH 8.8)	2.5ml (BDH)
Gelatin 10mg/ml	1.0ml (Sigma)
10% SDS	100µl (Bio-Rad)
10% ammonium persulphate	50µl (Sigma)
Acrylamide/bisacrylamide	2.5ml (Bio-Rad)
TEMED	5µl (Sigma)

The stacking gel was prepared the next day as described below. Tris buffer (1x) was poured off and the resolving gel rinsed with water and dried with blotting paper. Combs (0.75mm thick, 15 lanes) were placed securely between the plates. The stacking gel was pipetted between the plates onto the resolving gel and was topped up continually until set, to accommodate any shrinkage.

Stacking Gel (volume required for 2 gels)

dH_2O	3.05ml (MilliPore)
Tris 0.5M (pH 6.8)	1.25ml (BDH)
10% SDS	50µl (Bio-Rad)
10% ammonium persulphate	50µl (Sigma)
Acrylamide/bisacrylamide	0.65ml (Bio-Rad)
TEMED	5µl (Sigma)

2.9.2 Electrophoresis

Combs were carefully removed from the gels and the plates were then attached to the cooling core of the electrophoresis apparatus. 7µl of sample was mixed with 7µl of loading buffer (see below). The central chamber was filled with 1x running tank buffer (as detailed below) until the wells were covered. Then 14µl of sample was loaded into each well. Every gel had one well loaded with 15µl molecular weight standard markers (Bio-Rad prestained broad range ladder) and at least one well with a human amniotic fluid (haf) positive control. The gels were run at 100v for 2 hours (Bio-Rad Power Pack 3000) and the resolving gel then washed in 50ml TritonX-100 wash buffer (5ml Triton X-100 (BDH), 200ml 1x TBS) for 15 minutes, followed by a second wash on a plate shaker (Stuart Scientific, Stone, UK) for 45 minutes at room temperature.

Loading Buffer

Glycerol	10ml (BDH)
SDS	1g (Bio-Rad)
Bromophenol Blue	20mg (Bio-Rad)

Dissolved in 50ml of dH_2O (MilliPore).

Running Tank Buffer x10

Tris 0.25M	30.3g (BDH)
Glycine 1.9M	144g (Sigma)
SDS	10g (Bio-Rad)

Dissolved in 1 litre of H_2O and pH adjusted to 8.8.

2.9.3 Digestion and Staining

The gels were washed twice for 2 minutes at room temperature in 50ml each of 1x wash buffer (as below) and then incubated in 50ml digestion buffer (as detailed below) for 20 hours at 37°C in a sealed container. Digestion buffer was removed and the gels washed twice (1x wash buffer) at room temperature for 5 minutes. The wash solution was drained and the gels then incubated (on plate shaker) in 50ml of 0.5% (w/v) Coomassie Brilliant Blue R250 (Bio-Rad) staining solution at room temperature for 3 hours.

Wash Buffer 10x (TBS)

Tris 0.5M	6.06g (BDH)
NaCl 1.5M	90g (BDH)

Dissolve in 750ml H_2O and adjust to pH 8.0 with 5N HCl. Make up to a final volume of 1 litre.

Digestion Buffer

Tris 50mM	6.07g (BDH)
NaCl 0.2M	11.69g (BDH)
$CaCl_2$ 5mM	735mg (BDH)
$ZnCl_2$ 1mM	1ml (BDH)
Brij-35	660µl of 30% solution (Sigma)

Dissolve in 750ml H_2O and adjust to pH 7.6 with 5N HCl. Make up to a final volume of 1 litre. Store at 4°C.

2.9.4 Destaining and Gel Quantification

The staining solution was removed and each gel incubated on a plate shaker (Stuart Scientific) in 50ml of destaining solution (see below) for 30 minutes at room temperature. The solution was replaced with fresh destain solution for a further 60 minutes. Once destaining was satisfactory, the gels were transferred into 50ml H_2O. Gels were scanned using a Bio-Rad GS700 Imaging Densitometer and semi-quantified by densitometry using Bio-Rad Quantity One software.

Destaining Solution

Methanol	300ml (BDH)
Glacial acetic acid	100ml (BDH)
dH_20	600ml

2.10 Statistical Analysis

Prior to statistical analysis, data was assessed for normality distribution and log transformed if required. Normal distribution was determined by plotting the data against a normal distribution curve using Statistical Programm for Social Sciences (SPSS; SPSS Inc., Chicago, USA) software. Results were expressed as mean $\pm$ standard error of the mean (SEM) and a p value <0.05 was taken as significant. Data was analysed using MS (Phoenix Software, York, UK) Excel, GraphPad Prism (GraphPad Software, San Diego, USA) or SPSS. The specific statistical analysis tests used are described in each chapter.

2.10.1 Correlation Analysis

All data for correlation analysis was Log10 transformed in MS Excel to ensure a Gaussian distribution and then analysed using GraphPad Prism. Following the Log10 transformation Pearson's Correlation was carried out and a two-tail p value was computed. The r^2 (correlation coefficient) is a measure of the relationship between the two variables being analysed.

2.10.2 One-Way Analysis of Variance (ANOVA)

Data was analysed by a parametric one-way ANOVA followed by Tukey's post-hoc test to determine significance. Tukey's test was chosen as it is more stringent than other options available and the best means of analysis for data with both small and large numbers of groups. This option in GraphPad Prism also allows for unequal sample sizes. Another method for one-way analysis of variance the Chi-square (two-tail) test was used to analyse data with one of two possible outcomes i.e. burst or survived.

2.10.3 Student's t-test

This analysis was used to compare the means of the two groups and this was used for data with only two data sets e.g. latent MMP-2 data for follicles from wt versus het mice. Student's t-tests carried out were:

1. Unpaired and one-tail – FSH dose response follicle growth.
2. Unpaired and two-tail – any other data sets.

Chapter 3

Ovarian Follicle Morphology of Wild-type Mice and Mice Heterozygous for the *Dazl* Gene

3.1 Introduction

Initial studies concluded that *Dazl* het mice have higher ovulation rates, demonstrated by significantly larger litter sizes compared to *Dazl* wt mice (McNeilly *et al.,* Unpublished; Appendix A). These studies also concluded that *Dazl* wt and het mice had a similar percentage of primordial follicles, but the *Dazl* wt mice had a significantly greater proportion of pre-antral follicles compared to *Dazl* het mice. Furthermore, the *Dazl* het mice had a significantly greater proportion of antral follicles (McNeilly *et al.,* Unpublished; Appendix B). This suggests that either the same number of follicles are activated from adult *Dazl* wt and het mice but follicles from *Dazl* het mice are less likely to succumb to atresia, or that the *Dazl* het mice have an increased number of activated follicles possibly due to increased FSH sensitivity, resulting in recruitment of a larger cohort of follicles and subsequently larger litter sizes. The experiments in this chapter were to carryout a detailed analysis of ovarian counts and follicle morphology in day 21 *Dazl* wt and het mice.

3.1.1 Ovarian Follicle Reserve

As discussed in Chapter 1 the ovarian follicle reserve dogma has been challenged recently. One of the major obstacles in determining oocyte regeneration and follicle reserve is the lack of a universal method for quantifying follicle numbers. Many methods of follicle quantification have been developed, as detailed in Chapter 1 (Section 1.4). Assessing the follicle reserve includes measuring: FSH levels, AMH levels, inhibin B levels, ovarian follicle counts and using mathematical models to determine rates of follicle depletion (Faddy *et al.,* 1992; Mais *et al.,* 1995; Ficicioglu *et al.,* 2003; Yong *et al.,* 2003; Tremellen *et al.,* 2005*)*. However, none of these methods is entirely accurate (as discussed in Chapter 1) though each method gives an approximate indication of the ovarian reserve. From the methods detailed above, follicle counts appear to give the most accurate indication of actual follicle reserve. In this study the method of follicle counting described by Zuckerman (1951) was used, using the follicle classification of Gougeon, 1996, Gougeon and Busso (2000) and Smitz and Cortvrindt (2002).

The method used in this study classifies follicles into six classes (primordial, transitionary, primary, secondary, antral and unclassifiable). However these are not the same classifications used previously in the *Dazl* follicle counts undertaken by McNeilly *et al.,* (Unpublished; Appendix B). The expansion of the classes of follicle should ensure a better detailed analysis of follicle progression, and should enable any differences in activation from the resting pool and growth rates of follicles to be determined.

There are various methods of determining follicle classification, including oocyte diameter, follicle diameter, granulosa morphology and antral development (Zuckerman, 1951; Bucci *et al.,* 1997; Myers *et al.,* 2004; Table 1.1). The majority of studies today use granulosa cell morphology to determine follicle classification and this method was chosen for this study as it enabled the results to be compared with those from other studies. Furthermore, the diameters of oocyte and follicles (primary-antral follicles) were also recorded. This was carried out in primary, secondary and antral groups of follicles only as it was not completely accurate in the smaller follicles as it was difficult to determine follicle structure with no ZP present. Every 10^{th} section of ovary was analysed in this study, as previous reports have concluded that this counting method ensures that no follicle is counted twice on consecutive sections (Zuckerman, 1951). This method enabled us to count follicle numbers in a representative part of the ovary and it gave an approximation of the total follicle numbers within the ovary. However, as stated previously, the ovary is not cylindrical and therefore multiplying (by 10) will not give precise follicle numbers, though this method has been found to be accurate to within 3-11% (Tilly, 2003).

3.1.2 FSH and Follicle Growth

FSH plays a pivotal role in the growth and development of the ovarian follicle. It activates many factors that are associated with the latter stages of follicle development, such as LHR expression and proteoglycan synthesis (Hirshfield, 1991). Treatment with FSH accelerates follicle growth (animals treated with PMSG have higher follicle mitotic levels compared with controls), stimulates granulosa cell proliferation (Hirshfield and Midgley, 1978b; Hirshfield, 1986; Hirshfield, 1991), stimulates follicle maturation and also reduces atresia rates (Gosden *et al.*, 1983; Hirshfield, 1989). FSH is required for antral development, and FSH treatment has become routine in both laboratory animals and human IVF treatment to induce follicle maturation (LaPolt *et al.*, 1992). Pre-pubertal mice can ovulate when treated with recombinant FSH alone (Rugh, 1990).

Follicle counts of ovaries from animals treated with FSH (10IU) allow us to observe patterns of follicle growth and development in the *Dazl* wt and het mice. This should help us to determine whether follicles from *Dazl* het mice were more or less FSH sensitive than follicles from *Dazl* wt mice. FSH treatment is believed to 'rescue' follicles from atresia, as ovulation was blocked in ewes treated with follicular fluid to inhibit FSH secretion (Hirshfield and Midgley, 1978b; McLeod and McNeilly, 1987). In addition, studies in humans have established that follicles numbers are reduced at an accelerated rate when FSH levels are extremely low whereas FSH reduced atresia rates (Mais *et al.*, 1995).

FSH (10IU) was used to treat mice as this dosage stimulated follicle growth, and previous studies by the laboratory group had used this dose of FSH (McNeilly, Personal Communication). Other studies have demonstrated that ovulation can be stimulated in adult rats when given as little as 7.8IU recombinant FSH (Galway *et al.,* 1990). Other studies by LaPolt *et al.* (1992) reported stimulation of follicle growth in rats treated with 10IU PMSG. Treatment with PMSG not only stimulates granulosa cell proliferation but also enhances follicle maturation, with follicles showing increased LHR expression (Hirshfield, 1989; LaPolt *et al.,* 1990).

3.1.3 Ovine Follicular Fluid and Follicle Growth

The main site of inhibin production in ovarian follicles is the granulosa cells. Once synthesised, inhibin is secreted into the follicular fluid (Henderson and Franchimont, 1983), and has a negative feedback action on FSH secretion by inhibiting its secretion by the pituitary gland. When treated with follicular fluid (charcoal stripped to remove the steroids), circulating levels of FSH in animals are suppressed within hours (without affecting LH levels) (Chappel and Selker, 1979; Wallace and McNeilly, 1985; Campbell *et al.*, 1991; Knox *et al.*, 1991). Furthermore, treatment with follicular fluid also causes an increase in follicular atresia due to the suppressed FSH levels (McLeod and McNeilly, 1987).

The dose of oFF for the following study had been previously used by the laboratory group and was sufficient to suppress FSH levels (McNeilly, Personal Communication; Crawford *et al.,* 2002). Treatment with bovine follicular fluid inhibits FSH secretion to basal levels, and significantly increased the number of follicles with apoptotic granulosa cells in follicular fluid treated animals compared to control animals (Jolly *et al.,* 1997). Similarly, treatment of ewes with oFF has been shown to suppress FSH, oestradiol, inhibin and androstenedione levels and LHR expression in the theca cells (Baird *et al.,* 1990; Campbell *et al.,* 1999). Follicle counts of animals treated with oFF should enable the effects of suppression of FSH to basal levels on follicle growth and development to be observed in *Dazl* wt and het mice.

3.1.4 Aims of Study

Studies have shown that a depleted ovarian reserve is an important factor in infertility (Tremellen *et al.*, 2005). For this reason, the number of ovarian follicles in day 21 *Dazl* wt and het animals was analysed to determine whether increased litter sizes in the het animals were due to increased follicle numbers. Although previous studies were carried out in adults, the study in this and subsequent chapters were carried out on day 21 mice. However, follicle dissection is more successful in younger mice (around day 21) which lack corpora lutea (Spears, Personal Communication). Therefore, in order to make all the results comparable the follicle counts were also carried out in day 21 mice. FSH sensitivity of the follicle was assessed both *in vivo* (this chapter) and *in vitro* (Chapter 4) to give a more comprehensive understanding of how the *Dazl* gene affects follicle development at two time points.

The studies described in this chapter were designed to determine the number of follicles and the effects of either increasing (FSH-treated) or decreasing (oFF-treated) plasma FSH concentrations in wt and het *Dazl* mice. In addition, the areas of the oocyte, granulosa cell mass, follicle and whole ovary were measured to determine whether there was any variation in ovarian morphology between *Dazl* wt and het mice. These measurements were made in day 21 mice and were the source of follicles used for *in vitro* culture in other experiments (Chapter 4 and Chapter 5).

The aims of the follicle morphology analysis were to determine:

1. Whether follicle diameter or area is the better measurement of follicle size.
2. Whether oocyte diameter or area is the better measurement of oocyte size.
3. The relationship between follicle area and granulosa cell number.
4. Whether oocyte size (area) is related to follicle size (area).

3.2 Materials and Methods

3.2.1 Treatment Groups, Tissue Collection and Fixation

There were three treatment groups of animals:

1. Untreated
2. 10IU FSH (recombinant human FSH; Puregon)
3. 100µl oFF (charcoal stripped)

Animals were injected (subcutaneous) by Dr. Judy McNeilly twice daily for three days and culled three hours after the last injection. Day 21 mice were used since follicle dissecting is easier when there are no corpora lutea present (Spears, Personal Communication). Ovaries from day 21 *Dazl* het or wt mice (numbers detailed below in Table 3.1) were removed and fixed as detailed in Section 2.2.

Treatment	Number of Ovaries	Number of Animals
Untreated	10 wt / 10 het	5 wt / 5 het
10IU FSH	6 wt / 6 het	3 wt / 3 het
oFF	6 wt / 6 het	3 wt / 3 het

Table 3.1 Treatment groups and numbers of ovaries analysed for follicle counts and oocyte, follicle and ovary areas.

FSH and oFF preparation

The dose of 10IU FSH was used previously within the laboratory group and the animals responded to this dose of FSH. FSH was diluted in sterile physiological saline (Baxter, Glasgow, UK) and injected subcutaneously (by Dr. Judy McNeilly) twice daily for three days and mice were culled three hours after the last injection.

Ovine follicular fluid was collected as described (McNeilly, 1985; Campbell *et al.*, 1991) by Dr Judy McNeilly. Briefly, follicular fluid was collected from all follicles where there was any fluid present, charcoal stripped (Knox *et al.*, 1991) and tubes rotated overnight in the cold room. The follicular fluid was stored at -20°C until required. Animals were injected with 100µl oFF (subcutaneous) by Dr. Judy McNeilly twice daily for three days and culled three hours after the last injection.

3.2.2 Genotyping

Genotyping as described in Section 2.3 was carried out by Miss Mhairi Laird on tail tips taken from the animals post-cull. The genotype of the ovaries used for ovarian follicle counts remained unknown until full morphological analysis was completed.

3.2.3 Haematoxylin and Eosin (H&E) Staining

H&E staining was carried out on every tenth section of every ovary using the standard method described in Section 2.4.2 as detailed in Table 3.1.

3.2.4 Follicle Counts, Follicle Areas and Diameters

The advantages and disadvantages of this system and other counting methods are discussed in Chapter 1 (Section 1.4). Every tenth section of ovary was analysed for follicles containing a nucleus within the oocyte, and the final number multiplied by ten. Follicles were classified according to the parameters described in Chapter 2 (Section 2.5.1). Follicle, oocyte and granulosa cell areas and diameters were measured at a magnification of x20, and ovary areas at x4 magnification as described in Section 2.5.2.

Every ovary was counted, the total number of follicles in each category calculated for each animal and the means for the animals in each group were analysed (Appendix D). The proportion of follicles in the various follicle classes were then calculated (Appendix D).

3.2.5 Statistical Analysis

Follicle Counts: One-way ANOVA was conducted on Log10 transformed raw follicle counts and percentages of follicles and the significance calculated by two tail, unpaired t-test.

Ovary Areas: Ovary areas were analysed by one-way ANOVA (Log10 transformed data) followed by Tukey's multiple comparison test.

Correlation of Diameters & Areas, Granulosa Cell and Oocyte Areas and Granulosa Cell Areas and Granulosa Cell Counts: Correlations between follicle and oocyte diameter and areas, granulosa cell and oocyte areas, and granulosa cell areas and granulosa cell counts were assessed on Log10 transformed data and parametric correlation was used to obtain Pearson's r^2 value.

Area Distributions for Follicle, Granulosa Cell & Oocyte Areas: All follicle, granulosa cell and oocyte area distributions were analysed by one-way ANOVA (non-parametric). Data was unable to be Log transformed since not all ovaries contained follicles with an oocyte containing a nucleus in each classification area. To estimate differences in the distributions of antral follicles and granulosa cell areas between wt and het animals, a Chi-squared test was carried out on those follicles above and below 80,000μm^2. The 80,000μm^2 area was chosen as the oFF treated animals had no antral follicles above this size and, therefore, suggests that follicles greater than 80,000μm^2 were gonadotrophin dependent in this breed of mice.

3.3 Results

3.3.1 Follicle Counts

3.3.1.1 Raw Follicle Counts

No variation between genotypes was observed in any of these treatment groups at any stage of follicle development for the total number of follicles counted (Figure 3.1). Further analysis of the total number of pre-antral and antral follicles (Figure 3.2) demonstrated no differences between wt and het counts in all treatment groups at both the pre-antral and antral stages of development.

3.3.1.2 Percentage of Follicle Counts

For untreated animals the proportions of primordial, transitionary, primary, secondary and unclassifiable follicles was not statistically significant for het and wt mice. However, het animals had significantly more antral follicles ($p<0.05$) compared to wt animals (Figure 3.3 (A)). However, following treatment with 10IU FSH (Figure 3.3 (B)) or oFF (Figure 3.3 (C)), there was no difference in the percentage of follicles at any stage of follicle development between wt and het mice.

The percentage of follicles from het mice in each treatment group (Figure 3.4 (A)), was similar to the percentage of follicles at primordial, transitionary, secondary and unclassifiable stages of development of wt mice (Figure 3.3). However, there was a significantly greater percentage of primary follicles from wt untreated animals compared to both wt FSH-treated animals ($p<0.05$) and wt oFF-treated animals ($p<0.05$) (Figure 3.4 (A)). Similarly, there was a significantly greater percentage of antral follicles in the wt (10IU) FSH-treated animals compared to wt untreated ($p<0.05$) and wt oFF-treated ($p<0.05$) animals. At this stage of follicle development the percentage of antral follicles was significantly greater ($p<0.05$) for the untreated wt animals than for oFF-treated wt animals. The percentage of follicles between all treatment groups in het animals was not significantly different for the three treatment groups at all stages of follicle development (Figure 3.4. (B)).

3.3.2 Correlations between Follicle or Oocyte Diameter and Area

The correlation between diameter and area was calculated for both follicles and oocytes to determine the most accurate method of analysing follicle and oocyte size. The correlation between mean follicle diameter and area in both wt and het mice was high for primary follicles (r^2=0.91 (wt) and r^2= 0.95 (het), Figures 3.5 (A) and (B)), secondary follicles (r^2=0.95 (wt) and r^2=0.68 (het), Figures 3.6 (A) and (B)) and antral follicles from wt mice (r^2=0.84 Figure 3.7 (A)) but not antral follicles from het mice (r^2=0.1, Figure 3.7 (B)). Similarly, there was a high correlation between mean oocyte diameter and area in both the wt and het mice in primary follicle (r^2=0.79 (wt) and r^2=0.86 (het), Figures 3.5 (C) and (D)), secondary follicles from wt mice (r^2= 0.83, Figure 3.6 (C)) but not for secondary follicles from het mice (r^2=0.4, Figure 3.6 (D)). However, the correlation between oocyte diameter and area of antral follicles was high for both wt and het mice (r^2=0.72 and 0.84 respectively) (Figures 3.7 (C) and (D)).

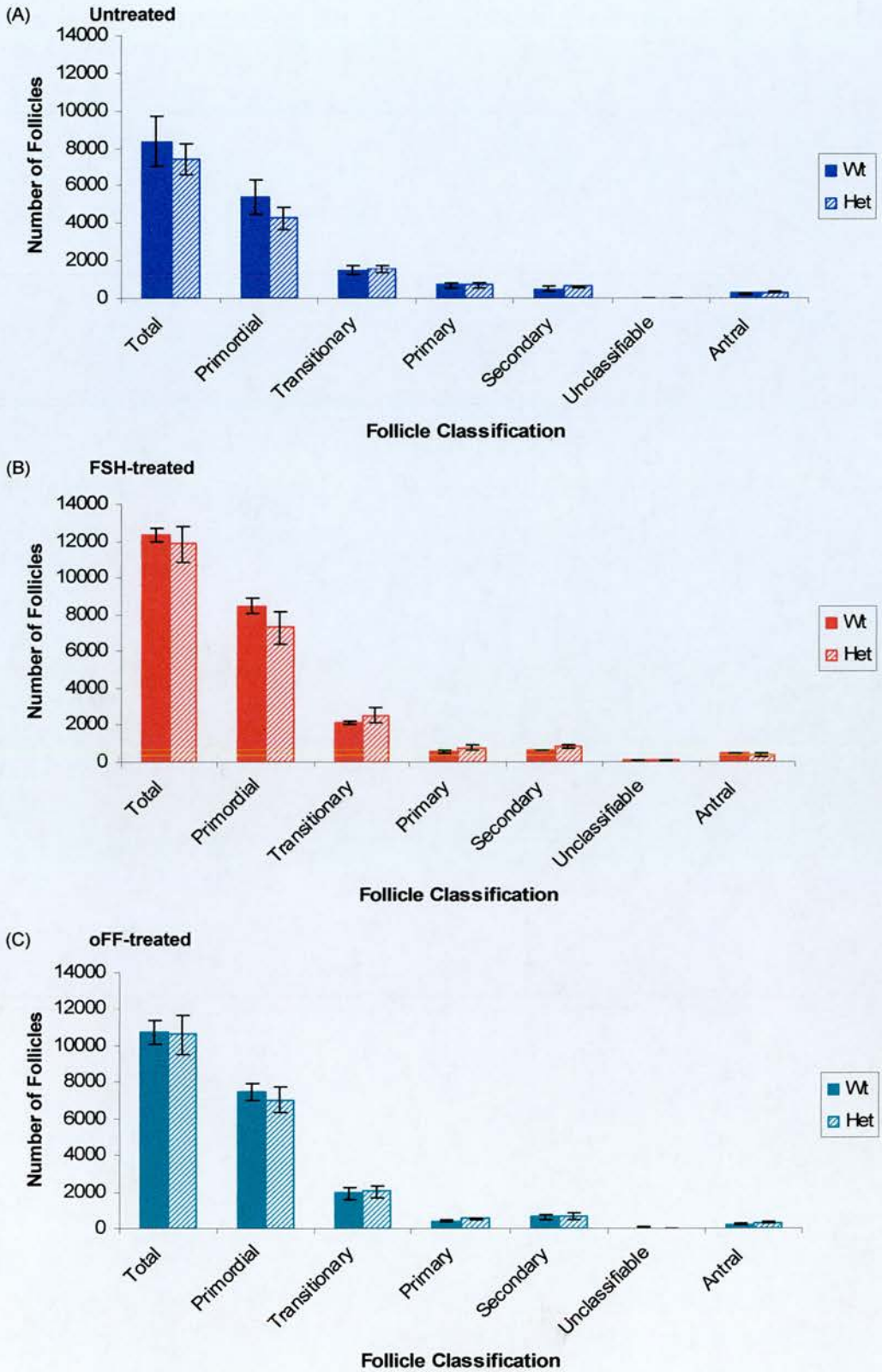


Figure 3.1 Total raw ovarian follicle counts for Dazl wt and het (A) untreated animals (B) 10IU FSH-treated animals and (C) oFF-treated animals. The values presented are the sum of follicles per animal and the mean ± S.E.M. of all ovaries from wt and het animals in each classification (n=5 untreated wt and het mice, n= 3 wt and het FSH-treated mice and n=3 oFF-treated wt and het mice).

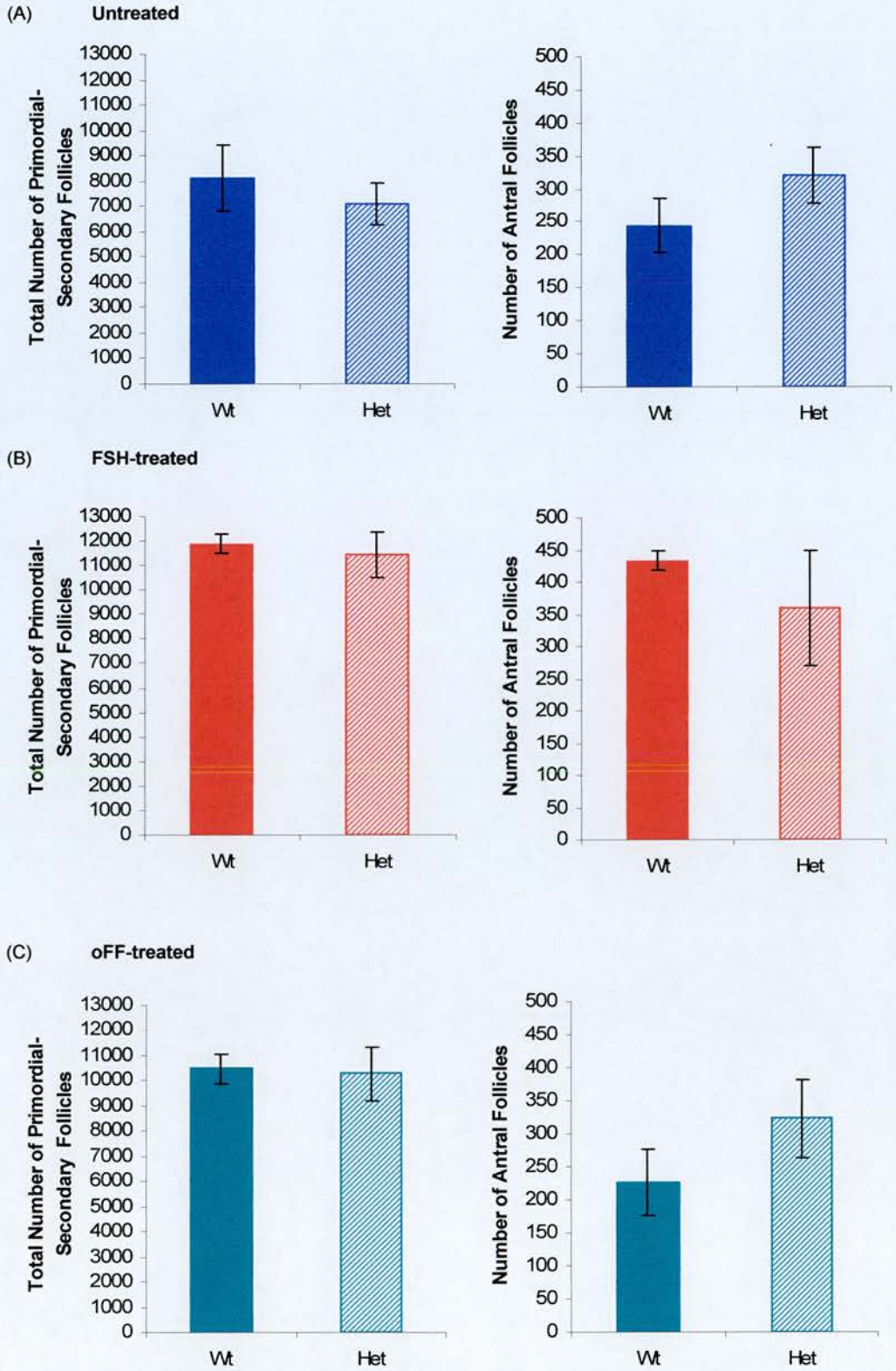


Figure 3.2 Total number of pre-antral and antral follicle counts for (A) untreated wt and het mice, (B) 10IU FSH-treated animals and (C) oFF-treated animals. Primordial, Transitionary, Primary, Unclassifiable and Secondary follicles were grouped together to get the pre-antral follicles value. Values are means ± S.E.M. The values presented here are the sum of follicles per mouse and then the mean of wt and het follicles in every treatment group (n=5 untreated wt and het mice, n= 3 wt and het FSH-treated mice and n=3 oFF-treated wt and het mice).

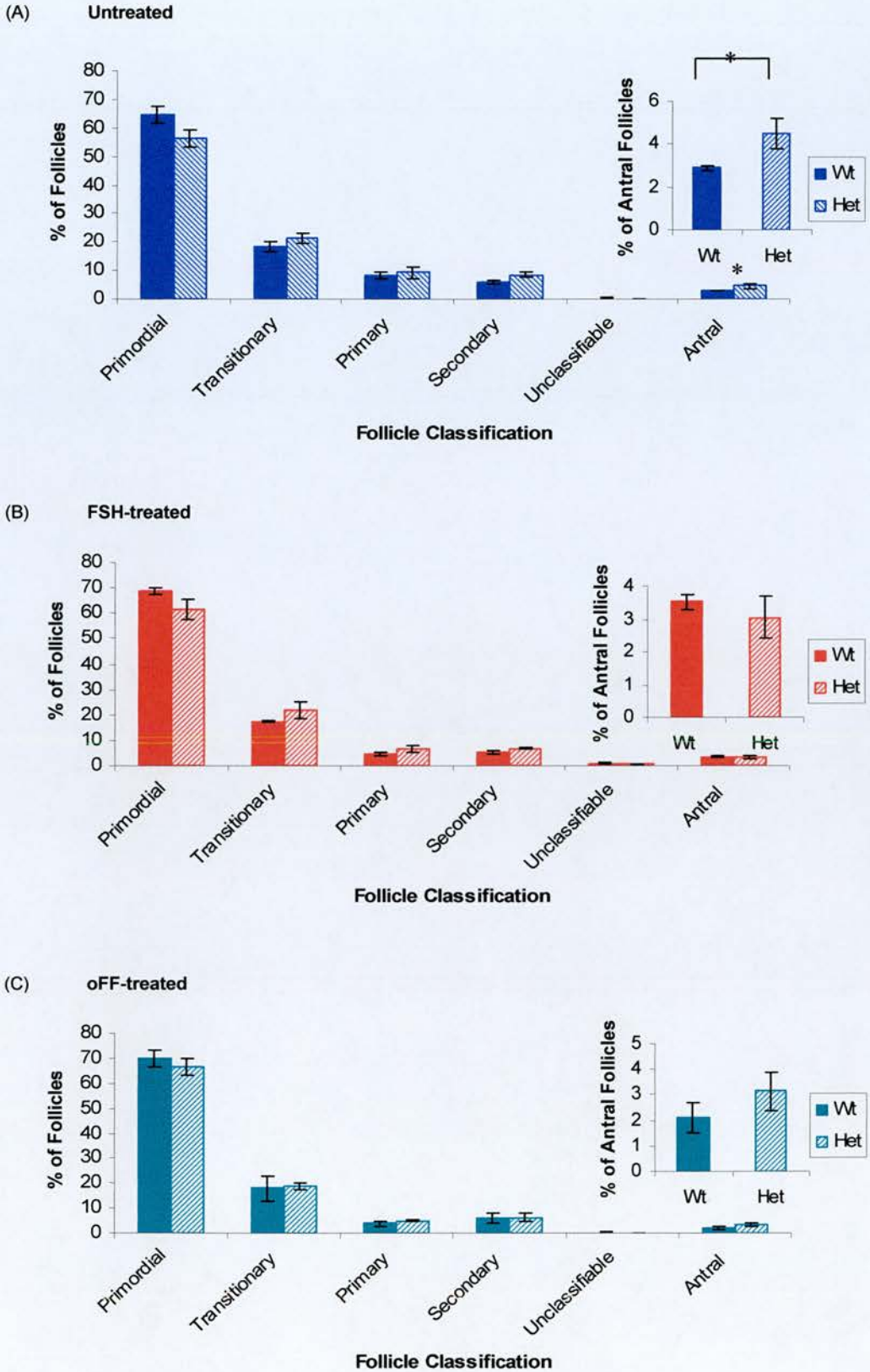


*Figure 3.3 The percentage of follicles in each category of follicle development for Dazl wt and het (A) untreated animals, (B) 10IU FSH-treated animals and (C) oFF-treated animals. * $p<0.05$. The percentage of follicles from each animal was calculated and the mean ± S.E.M reported (n=5 untreated wt and het mice, n= 3 wt and het FSH-treated mice and n=3 oFF-treated wt and het mice).*

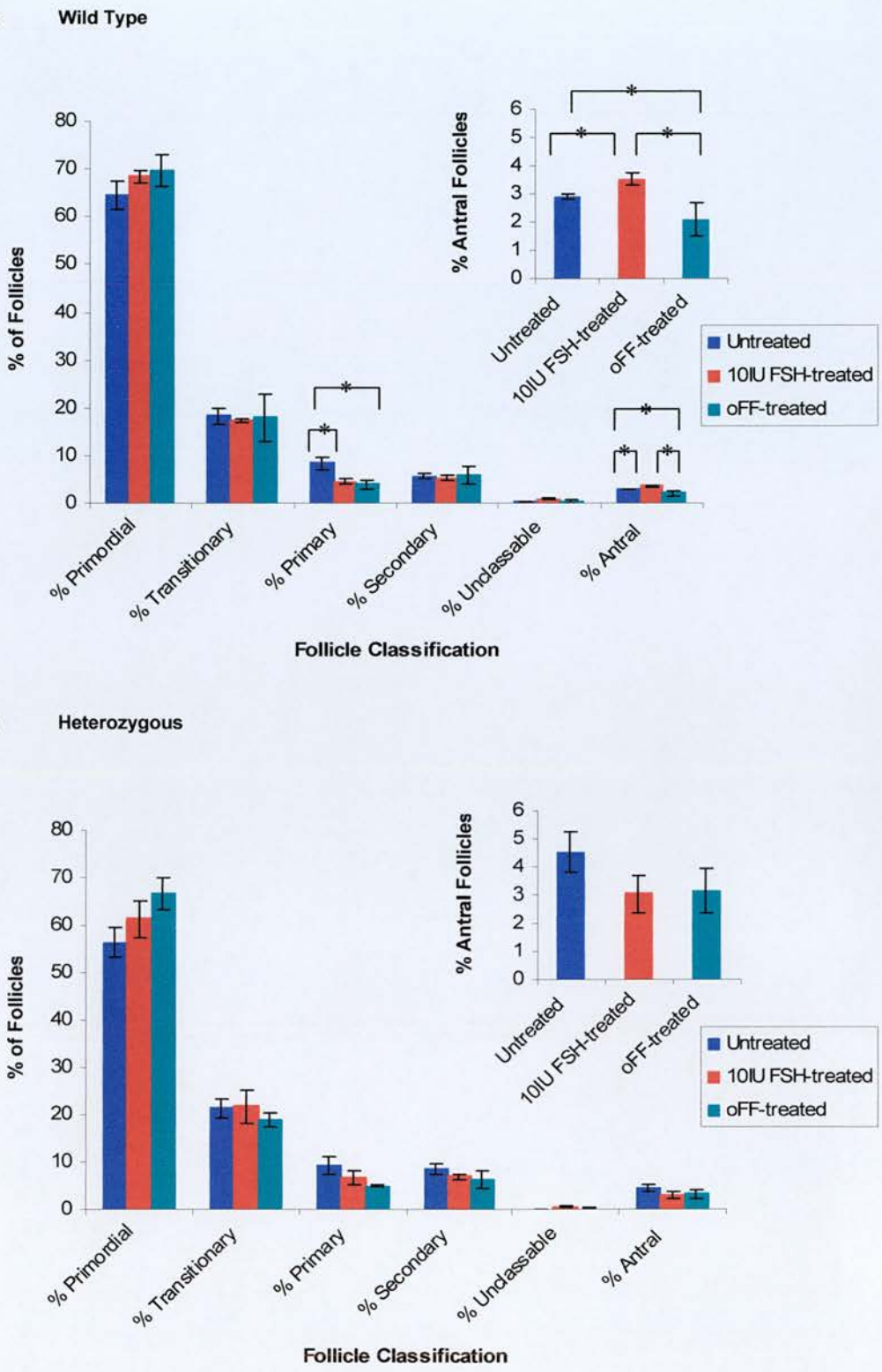


*Figure 3.4 The percentage of follicles from (A) wt animals and (B) het animals at every stage of follicle development between treatment groups. * $p<0.05$. The total percentage of follicles from each animal was calculated and the mean ± S.E.M values reported (n=5 untreated wt and het mice, n= 3 wt and het FSH-treated mice and n=3 oFF-treated wt and het mice).*

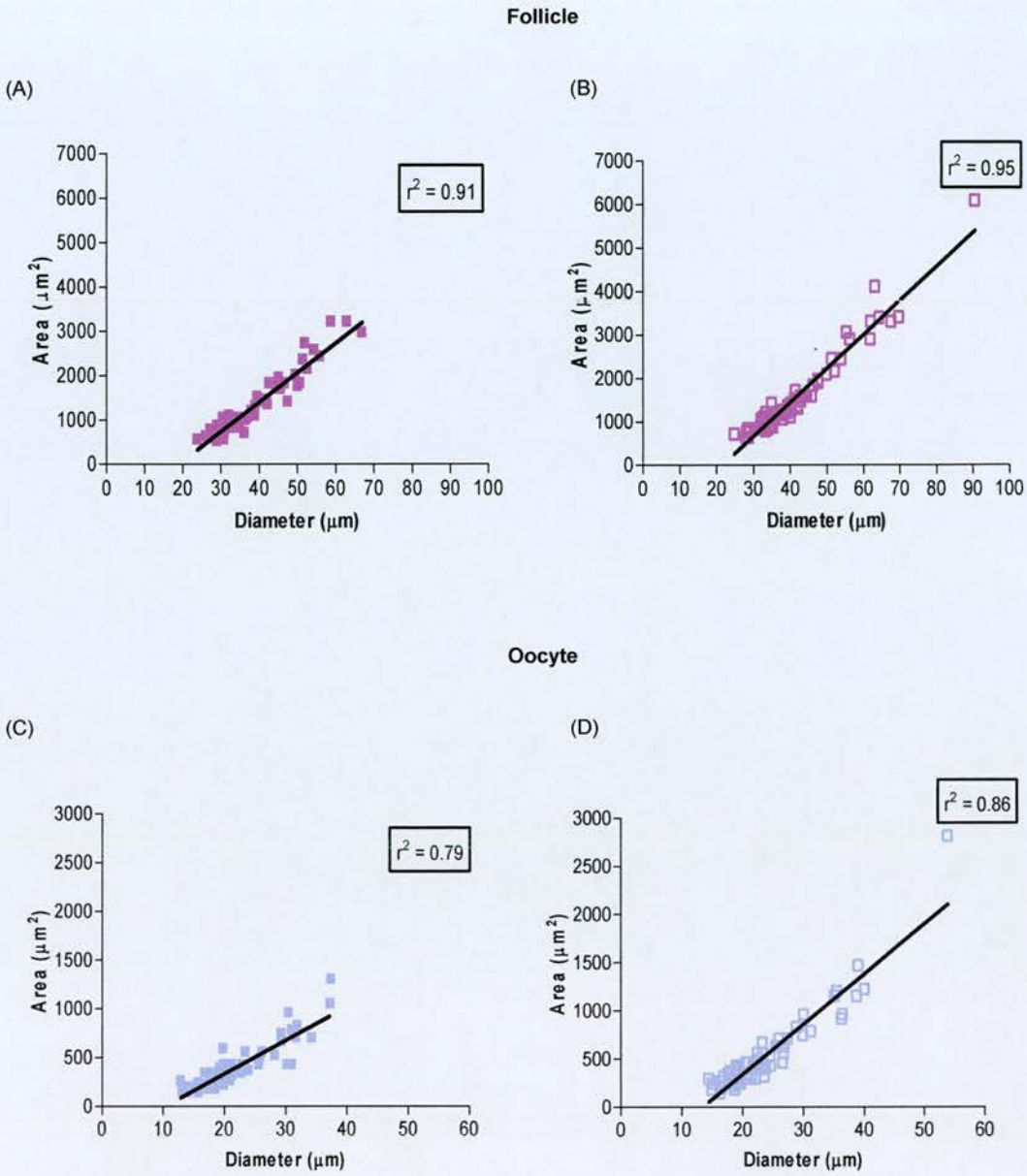


Figure 3.5 Correlations between primary ((A) and (B)) follicle and ((C) and (D)) oocyte diameter and area for untreated ((A) and (C) n=70) wt and ((B) and (D) n=63) het mice.

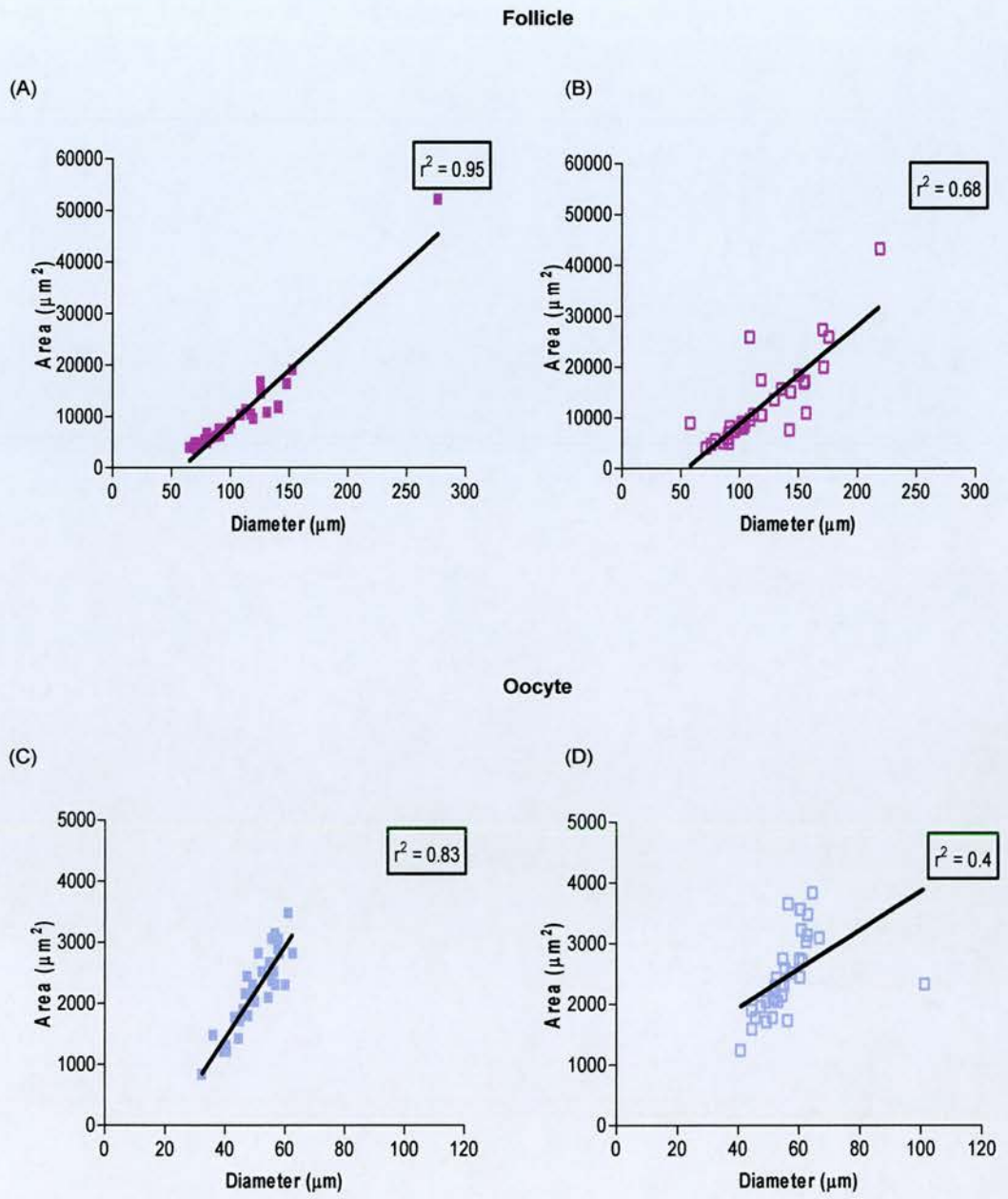


Figure 3.6 Correlations between secondary ((A) and (B)) follicle and ((C) and (D)) oocyte diameter and area for untreated ((A) and (C) n=32) wt and ((B) and (D) n=34) het mice.

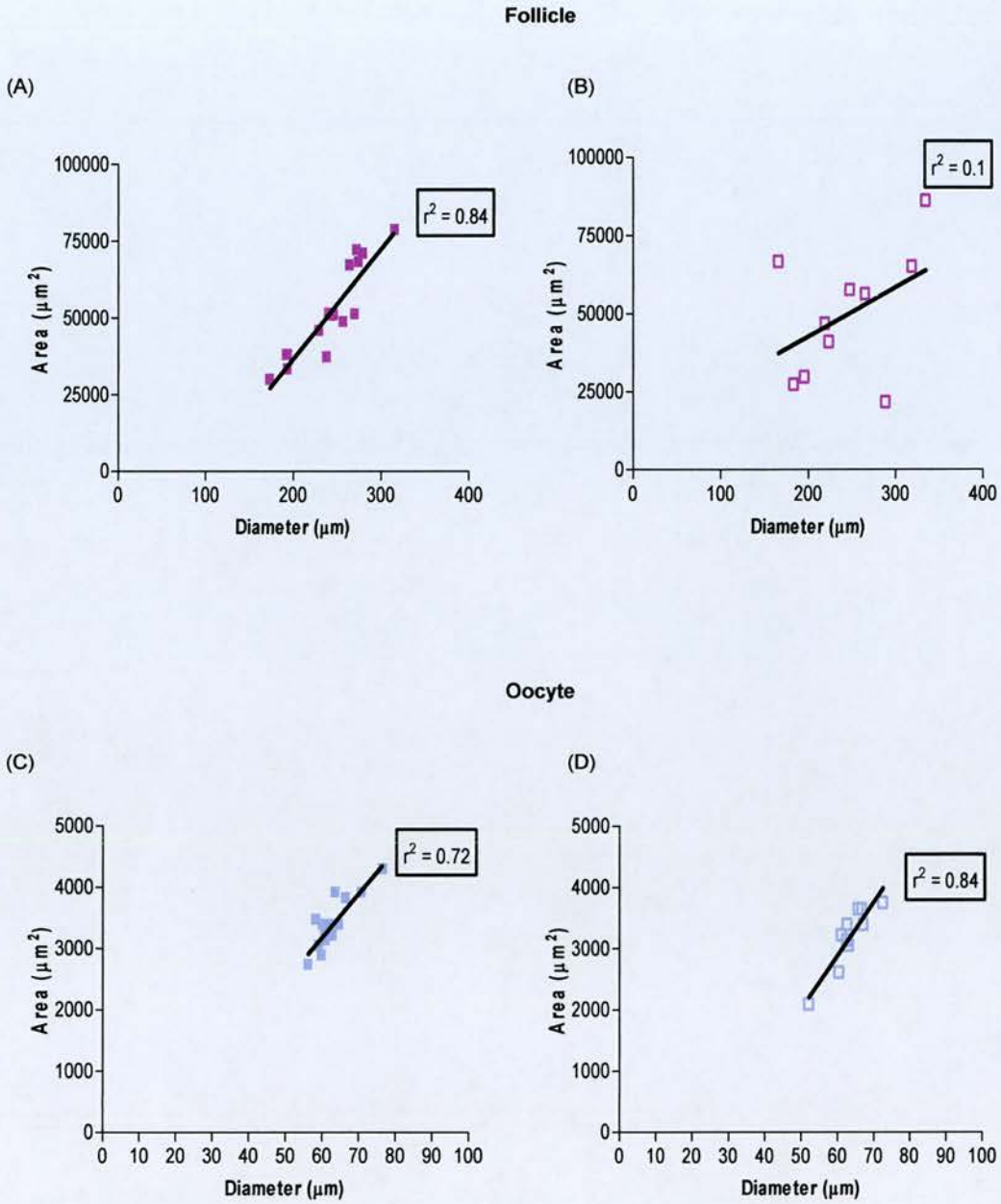


Figure 3.7 Correlations between antral ((A) and (B)) follicle and ((C) and (D)) oocyte diameter and area for untreated ((A) and (C) n=16) wt and ((B) and (D) n= 10) het mice.

3.3.3 Ovary Area

The mean ovary area per treatment group was analysed to determine any variation in the overall morphology between the *Dazl* wt and het mice. Analysis was carried out both between genotypes and between treatment groups (Figure 3.8). There was no significant difference between the ovary areas of wt mice compared to het mice in all treatment groups. The mean ovary area for both wt and het animals increased significantly ($p<0.001$ (wt); $p<0.05$ (het)) compared to the untreated animals when the mice were treated with 10IU FSH, whilst there was no difference between the ovary areas for untreated and oFF-treated wt and het mice. However, there was a significant difference ($p<0.001$) between ovary area of FSH-treated and oFF-treated wt mice, although, there was no significant difference between ovary area of FSH-treated and oFF-treated het mice ($p>0.05$).

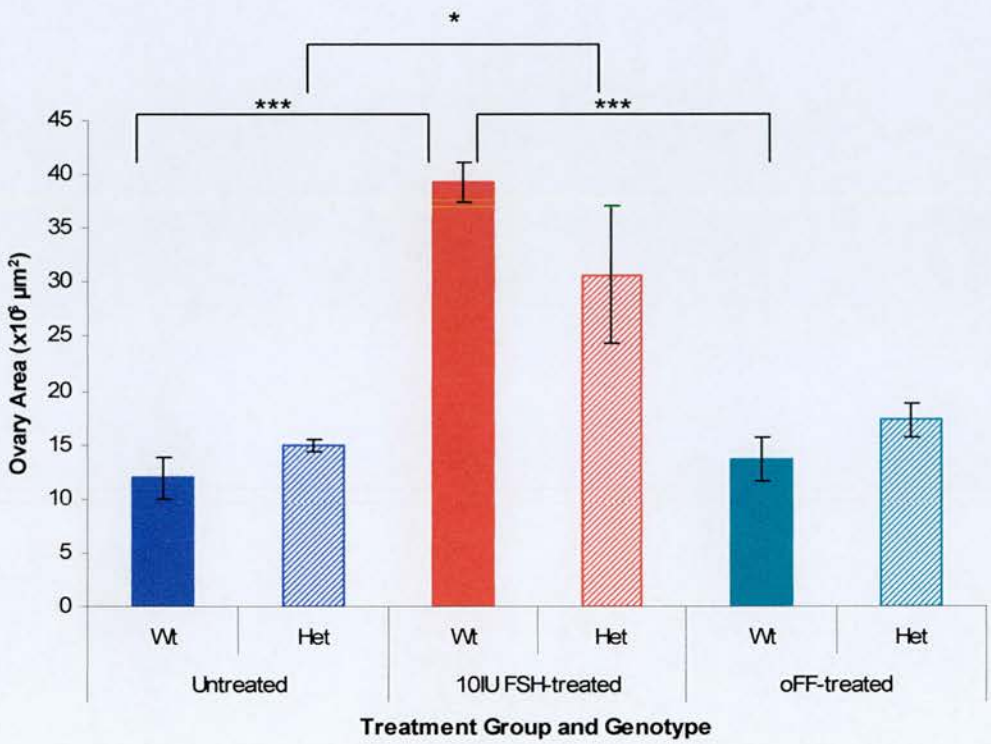


*Figure 3.8 Ovary area of wt and het animals from untreated, 10IU FSH-treated and oFF-treated groups. * $p<0.05$ and *** $p<0.001$. The total ovarian volume was calculated and the mean ovarian volume of every animals was then determined. The mean ± S.E.M of wt and het ovarian volume was then calculated (n=5 untreated wt and het mice, n= 3 wt and het FSH-treated mice and n=3 oFF-treated wt and het mice).*

3.3.4 Correlation between Granulosa Cell Area and Granulosa Cell Counts

To determine whether granulosa cell area was related to granulosa cell numbers, the areas of follicles from wt and het mice at primary (n=3 follicles), secondary (n=4 follicles) and antral (n=4 follicles) stages were selected at random from one untreated wt and het animal, measured and the number of granulosa cells in each area counted. The correlation between the granulosa cell area and granulosa cell counts was extremely high for follicles from both wt and het mice (Figure 3.9). Therefore, measuring granulosa cell area is representative of granulosa cell counts and any difference in areas is due to variation in granulosa cell numbers and not granulosa cell size.

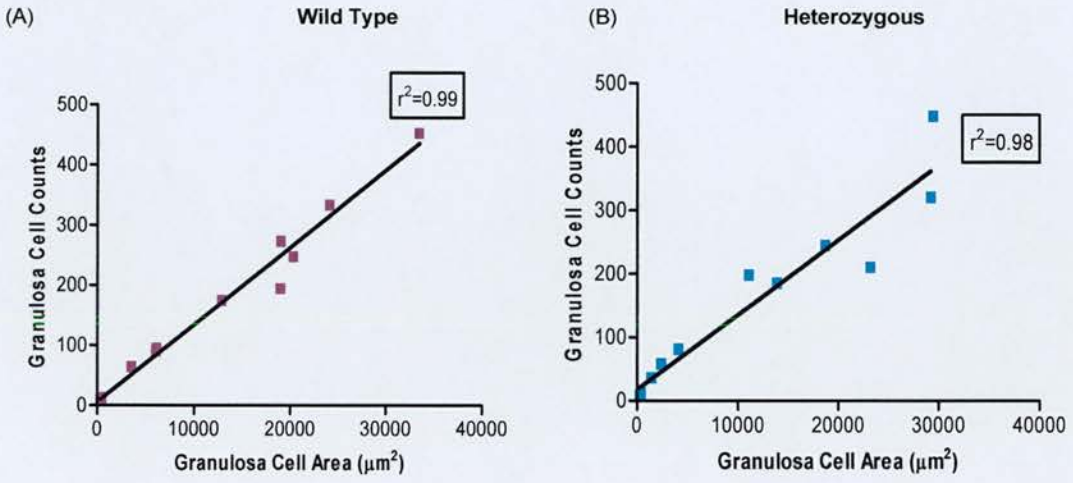


Figure 3.9 The correlation between granulosa cell area and granulosa counts in follicles from (A) wt and (B) het mice. n=3 primary follicles, n=4 secondary follicles and n= 4 antral sized follicles for both wt and het mice.

3.3.5 Area Distributions

Primary, secondary and antral follicles were measured in three ways: firstly the area of the entire follicle was measured, secondly, oocyte area was measured and thirdly any follicular fluid was measured. Finally, the granulosa cell volume was calculated by subtracting the oocyte area and follicular fluid area from the follicle area. As shown in 3.3.4 this area measurement directly reflects the number of granulosa cells in that area of the follicle. In order to determine any significant trends in this data set, the data was analysed in terms of percentages of follicles at different size groupings as illustrated in Figure 3.9.

3.3.5.1 Follicle Area Distribution

Primary Follicles: There was no difference in the overall distribution of primary follicle area between wt and het animals in untreated, 10IU FSH-treated and oFF-treated animals (Figure 3.10) with most follicle areas falling within the 501-2000μm^2 range in all treatment groups. However, in the untreated het animals primary follicles were significantly more likely ($p<0.01$) to be in the 1001-1500μm^2 range compared to follicles from wt animals.

Secondary Follicles: The distribution of the areas of secondary follicles did not vary between the genotypes in all the treatment groups (Figure 3.11). The majority of the follicles in this category had an area between 2501-12500μm^2 in all of the treatment groups.

Antral Follicles: The area distributions for antral follicles in untreated animals were considerably different (Figure 3.12 (A)). The het mice had significantly more antral follicles greater than 80,000μm^2 ($p<0.001$), compared to antral follicles in wt mice. In the 10IU FSH-treated and oFF-treated wt and het animals there was no significant difference in area distributions (Figures 3.12 (B) and (C)).

Antral follicle area distributions were significantly different in wt mice under the various treatments (Figure 3.13 (A)). 10IU FSH-treated wt animals had significantly more larger ($p<0.001$) antral follicles compared to the untreated and oFF-treated animals. However, there was no significant difference in follicle areas for oFF-treated antral follicles compared to the untreated antral follicles. Similarly, for het animals, antral follicle area was significantly greater ($p<0.001$; Figure 3.13 (B)) in the 10IU FSH-treated mice compared to the untreated and oFF-treated groups. There was no significant difference in the size of the antral follicles between untreated and oFF-treated het animals ($p>0.05$).

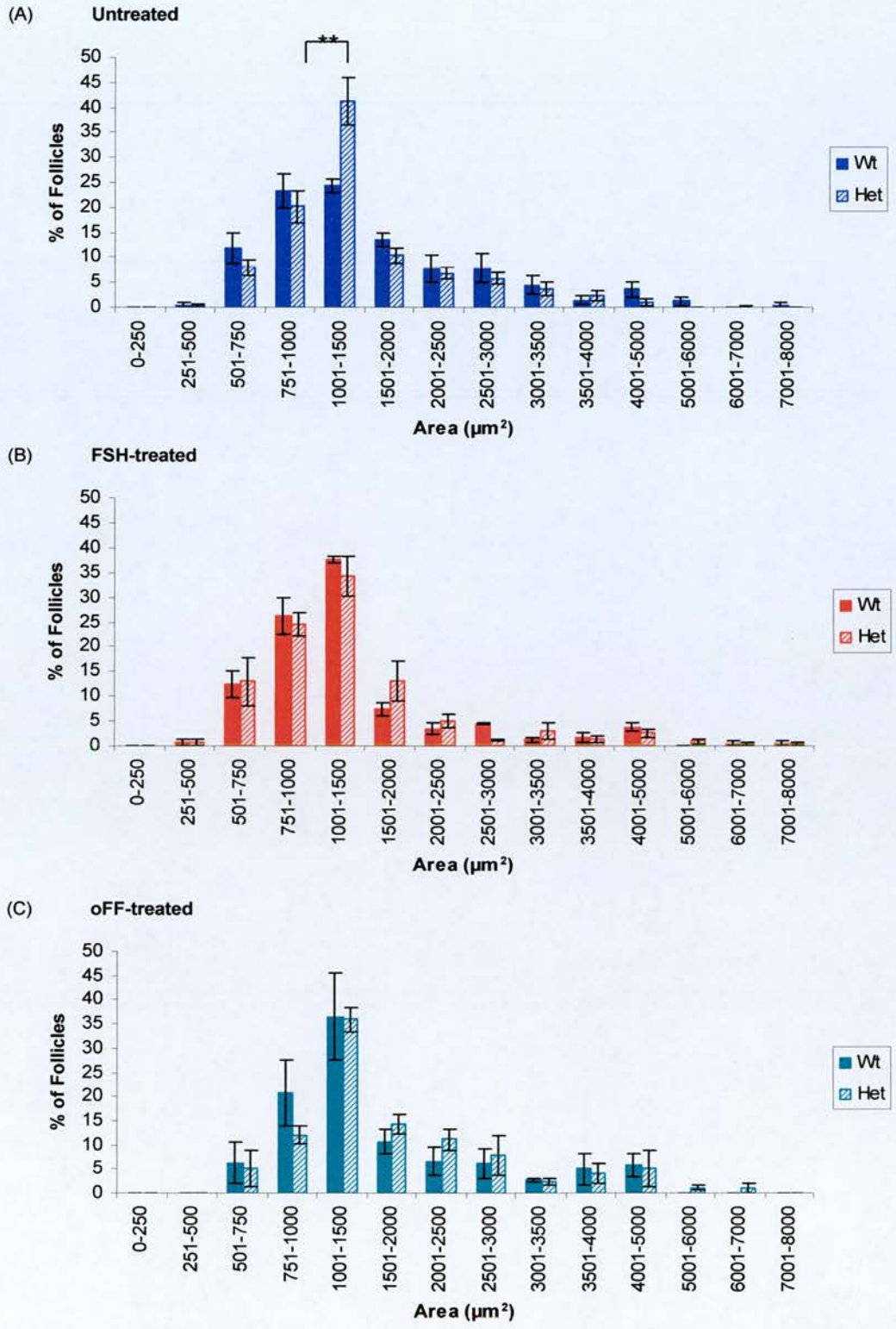


*Figure 3.10 Area distributions of primary follicles from (A) untreated animals, (B) 10IU FSH-treated animals and (C) oFF-treated wt and het animals. ** p<0.01. The total number and percentage of follicles in every size range per ovary was calculated and the mean per animal was determined followed by the mean ± S.E.M for wt and het mice in every treatment group were calculated (n=5 untreated wt and het mice, n= 3 wt and het FSH-treated mice and n=3 oFF-treated wt and het mice).*

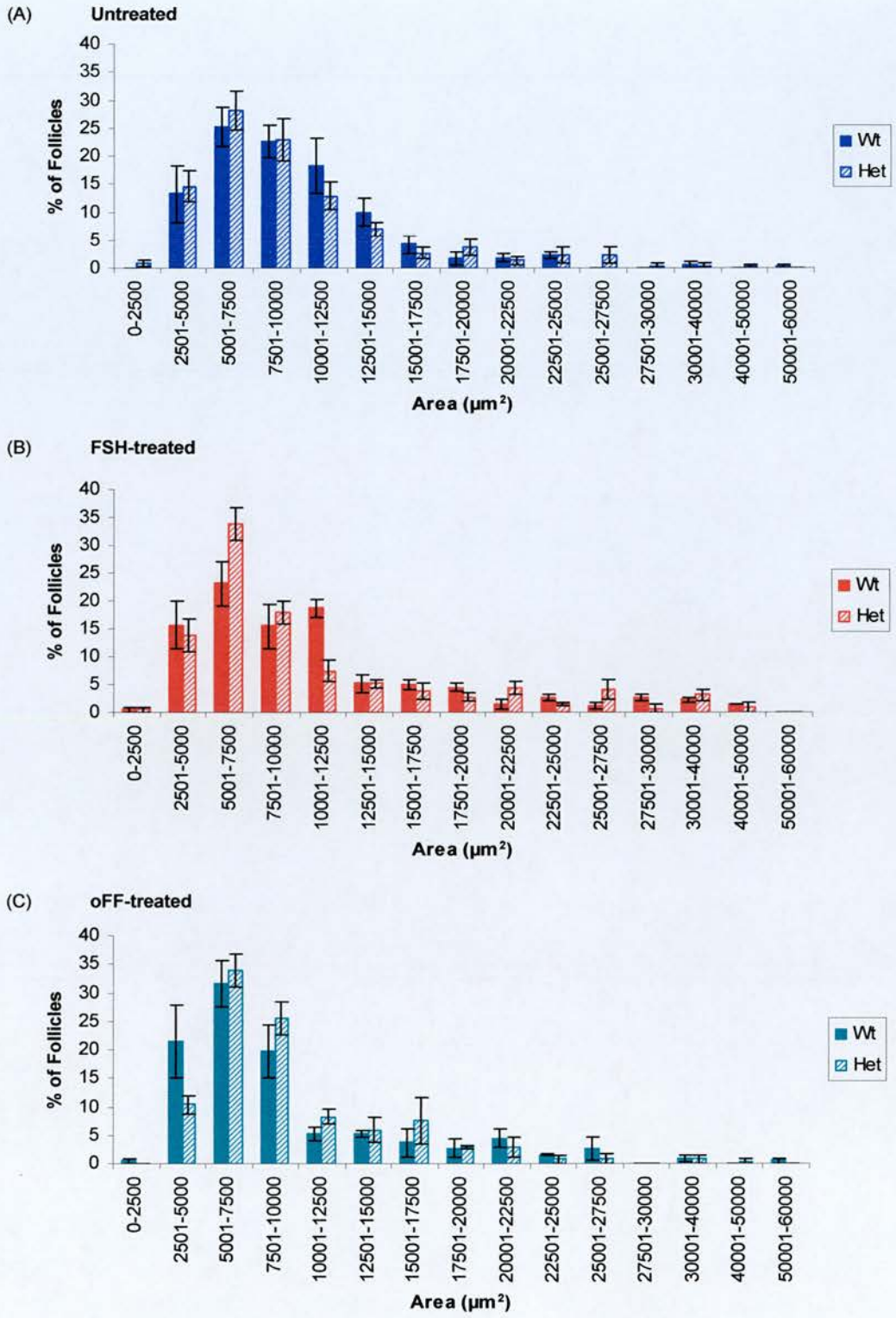


Figure 3.11 Area distributions of secondary follicles from (A) untreated animals, (B) 10IU FSH-treated animals and (C) oFF-treated wt and het animals. The total number and percentage of follicles in every size range per ovary was calculated and the mean per animal was determined followed by the mean ± S.E.M for wt and het mice in every treatment group were calculated (n=5 untreated wt and het mice, n= 3 wt and het FSH-treated mice and n=3 oFF-treated wt and het mice)

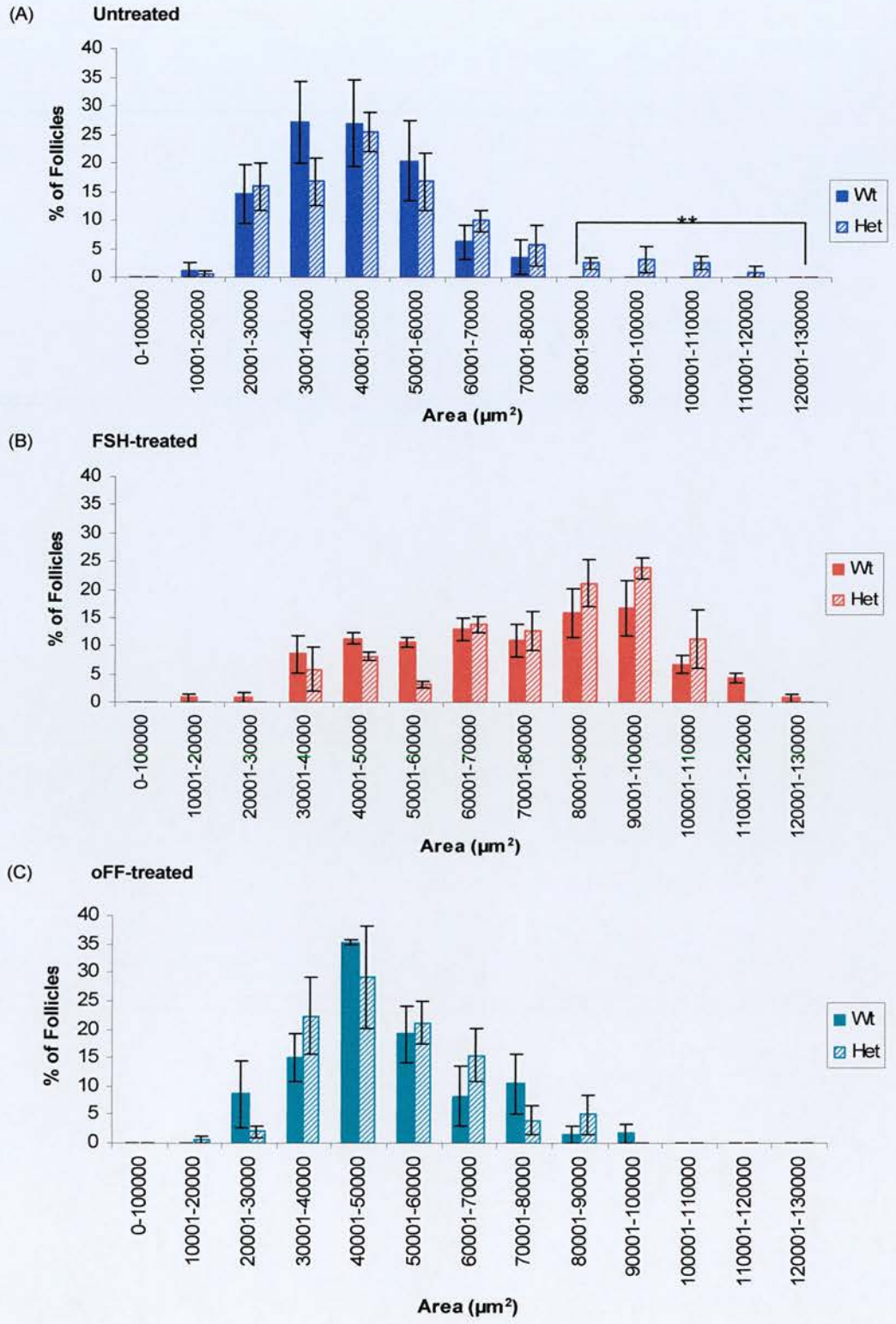
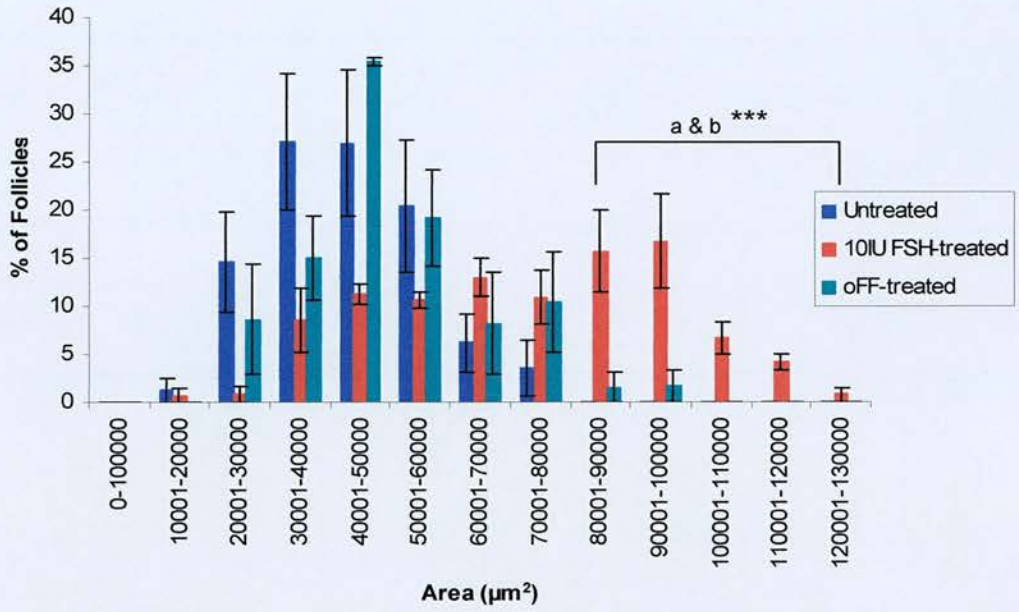


*Figure 3.12 Area distributions of antral follicles from (A) untreated animals, (B) 10IU FSH-treated animals and (C) oFF-treated wt and het animals. ** p<0.01. The total number and percentage of follicles in every size range per ovary was calculated and the mean per animal was determined followed by the mean ± S.E.M for wt and het mice in every treatment group were calculated (n=5 untreated wt and het mice, n= 3 wt and het FSH-treated mice and n=3 oFF-treated wt and het mice).*

(A) **Wild Type**



(B) **Heterozygous**

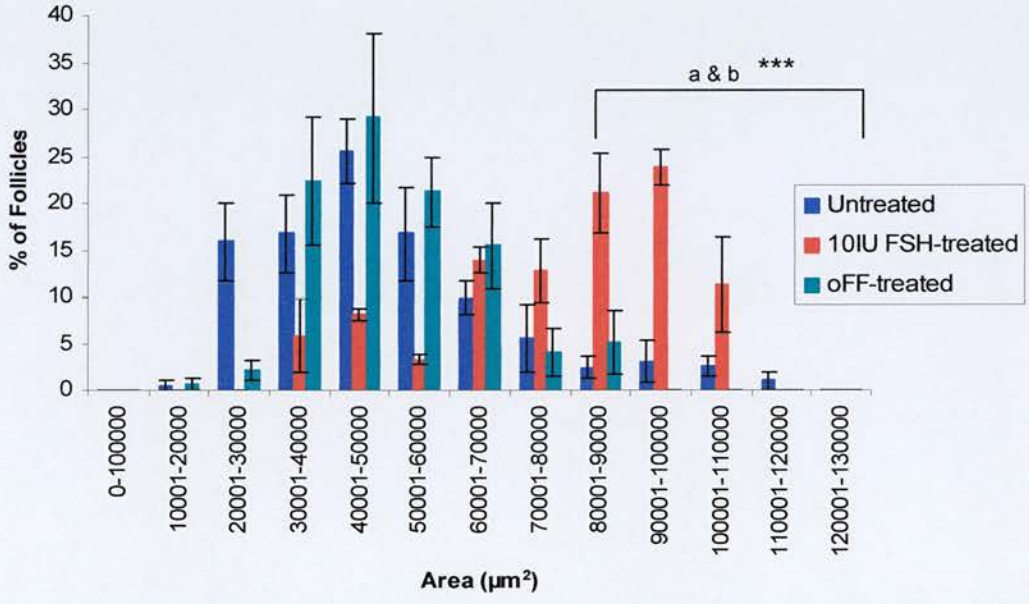


*Figure 3.13 Area distributions of antral follicles from (A) wt and (B) het animals. *** p<0.001. The total number and percentage of follicles in every size range per ovary was calculated and the mean per animal was determined followed by the mean ± S.E.M for wt and het mice in every treatment group were calculated (n=5 untreated wt and het mice, n= 3 wt and het FSH-treated mice and n=3 oFF-treated wt and het mice). a= untreated versus 10IU FSH-treated, b=10IU FSH-treated versus oFF-treated*

3.3.5.2 Granulosa Cell Area Distributions

Primary follicles. There was no significant difference for primary follicle granulosa cell area distributions of wt and het mice in each treatment group (Figure 3.14). Granulosa cell areas fell largely within the 1-2000μm^2 range in the untreated, 10IU FSH-treated and oFF-treated animals.

Secondary follicles. The granulosa cell areas of secondary follicles did not differ significantly for wt and het animals in all treatment groups (Figure 3.15). Most secondary granulosa cell areas fell within the 1-10,000μm^2 range in the untreated, 10IU FSH-treated and oFF-treated animals.

Antral follicles. In untreated animals, the granulosa cell areas of antral follicles were greater for het compared to wt mice (Figure 3.16 (A)). A significantly higher proportion of antral follicles from het mice ($p<0.05$) had granulosa cell areas greater than 80,000μm^2 compared to follicles of wt mice. The distribution of antral follicle granulosa cell areas did not differ significantly between 10IU FSH-treated and oFF-treated wt and het mice (Figures 13.16 (B) and (C)).

The distribution of granulosa cell area for antral follicles differed significantly between the treatment groups (Figure 3.17). The percentage of follicles from 10IU FSH-treated animals was more likely to be greater than 80,000μm^2 ($p<0.001$), compared to untreated and oFF-treated wt and het animals. There was no difference in the distribution of granulosa cell area between untreated and oFF-treated animals where no antral follicles were greater than 80,000μm^2 in either untreated and oFF-treated wt mice and oFF-treated het mice. A small number of antral follicles from het mice were larger than 80,000μm^2, however this was not significant.

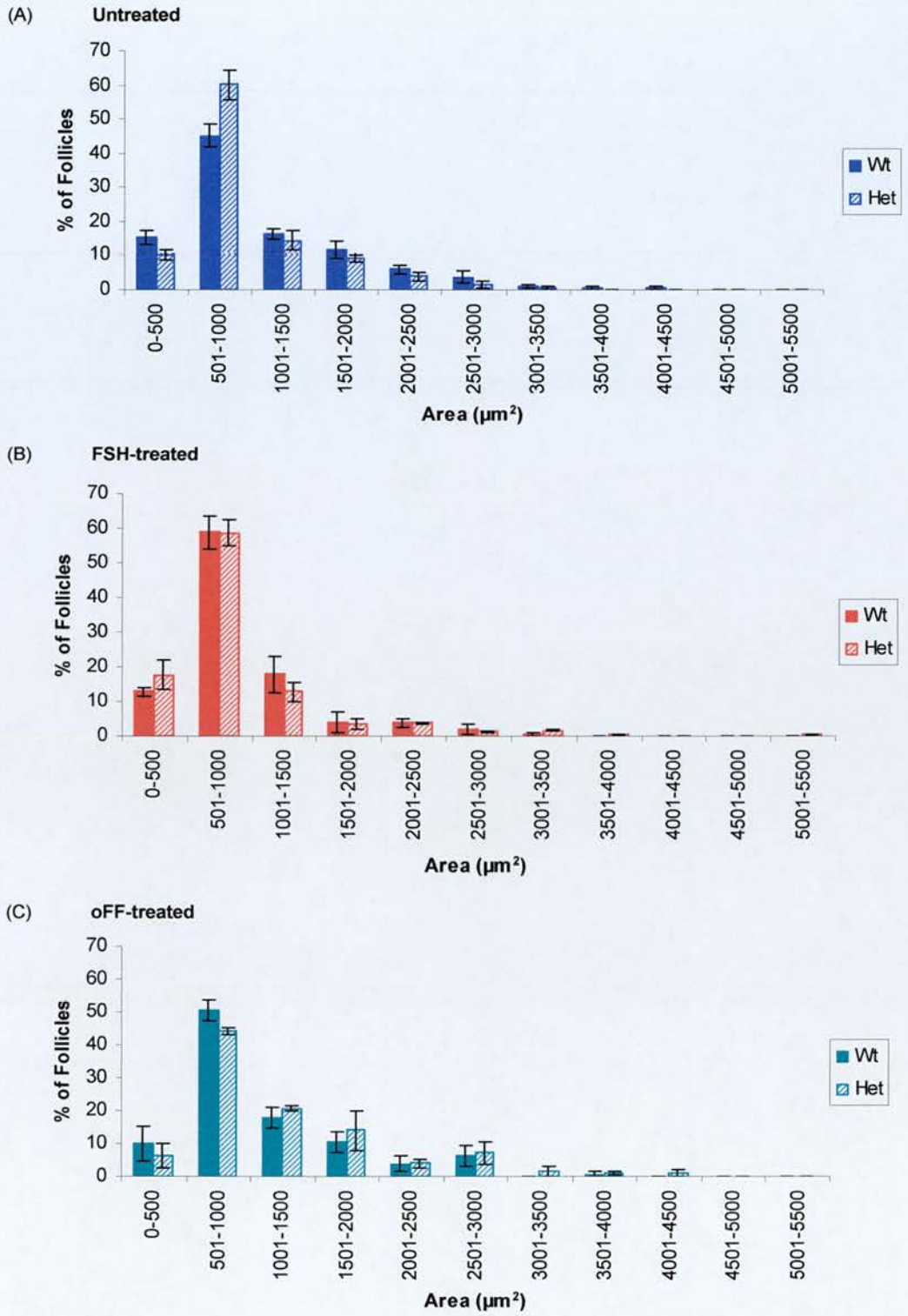


Figure 3.14 Granulosa cells area distributions for primary follicles from wt and het (A) untreated animals, (B) 10IU FSH-treated animals and (C) oFF-treated animals. The total number and percentage of follicles with a granulosa cell area in every size range was calculated per ovary and the mean per animal was determined followed by the mean ± S.E.M for wt and het mice in every treatment group were calculated (n=5 untreated wt and het mice, n= 3 wt and het FSH-treated mice and n=3 oFF-treated wt and het mice).

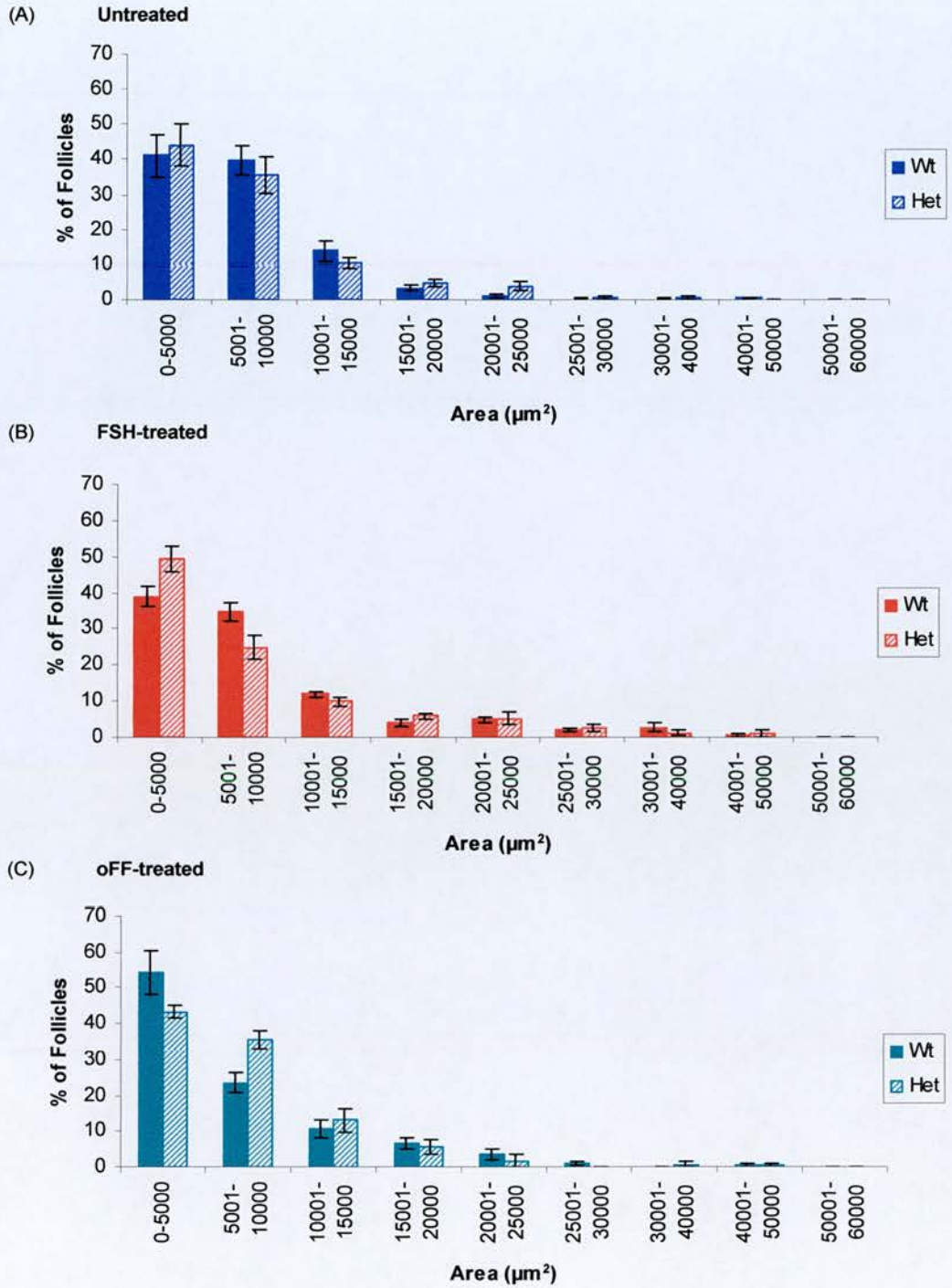


Figure 3.15 Granulosa cells area distributions for secondary follicles from Dazl wt and het (A) untreated animals, (B) 10IU FSH-treated animals and (C) oFF-treated animals. The total number and percentage of follicles with a granulosa cell area in every size range was calculated per ovary and the mean per animal was determined followed by the mean ± S.E.M for wt and het mice in every treatment group were calculated (n=5 untreated wt and het mice, n= 3 wt and het FSH-treated mice and n=3 oFF-treated wt and het mice).

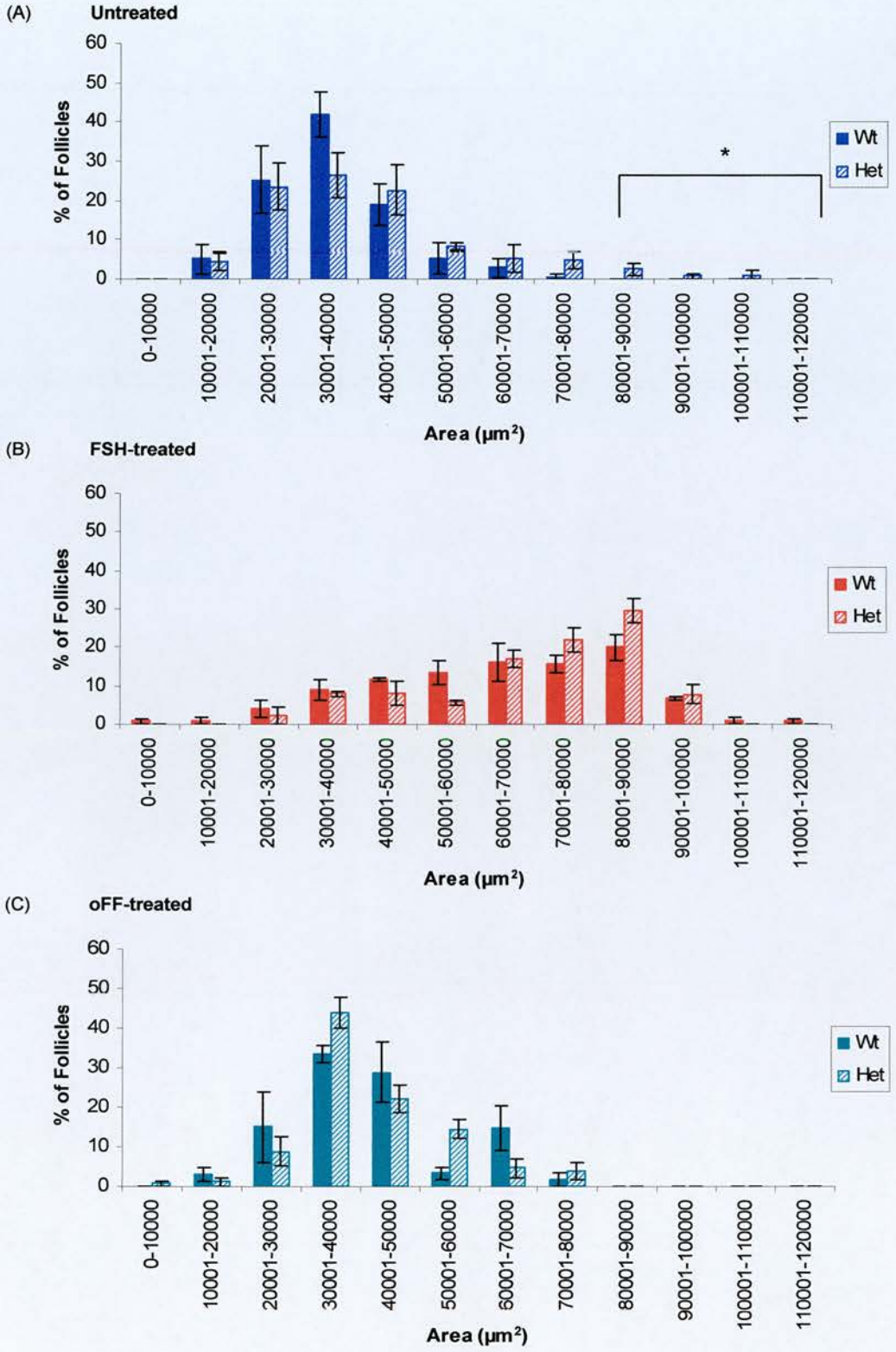
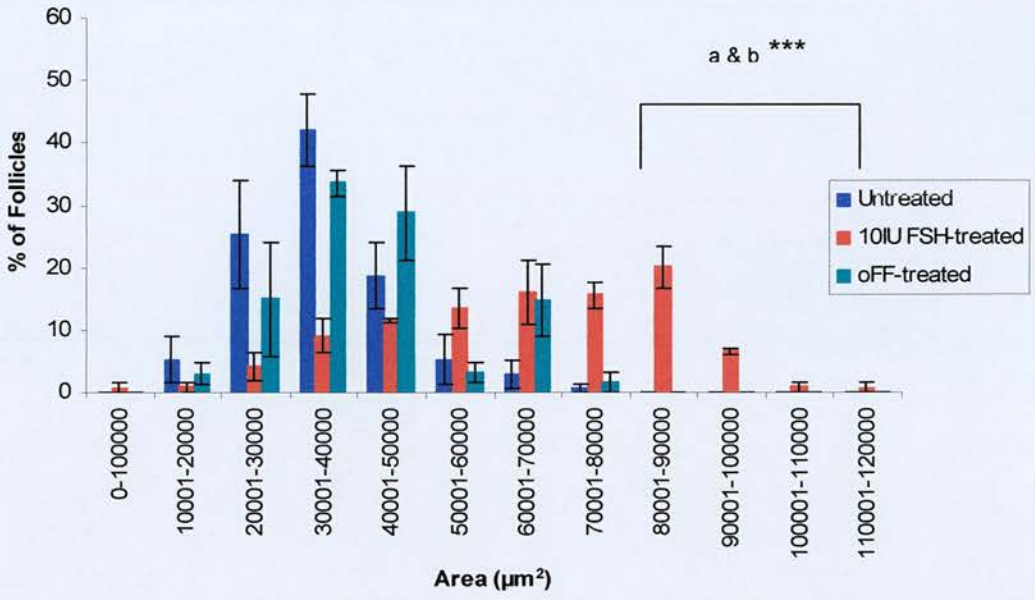


*Figure 3.16 Granulosa cells area distributions for antral follicles from Dazl wt and het (A) untreated animals, (B) 10IU FSH-treated animals and (C) oFF-treated animals. * $p<0.05$. The total number and percentage of follicles with a granulosa cell area in every size range was calculated per ovary and the mean per animal was determined followed by the mean ± S.E.M for wt and het mice in every treatment group were calculated (n=5 untreated wt and het mice, n= 3 wt and het FSH-treated mice and n=3 oFF-treated wt and het mice).*

(A) **Wild Type**



(B) **Heterozygous**

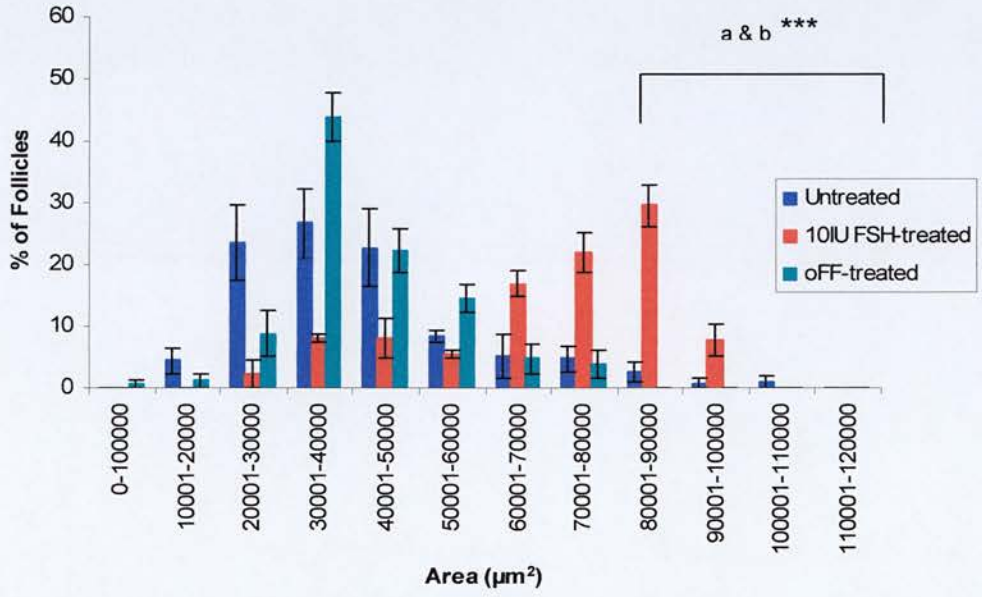


*Figure 3.17 Granulosa cells area distributions for antral follicles from (A) wt and (B) het animals. *** p<0.001. The total number and percentage of follicles with a granulosa cell area in every size range was calculated per ovary and the mean per animal was determined followed by the mean ± S.E.M for wt and het mice in every treatment group were calculated (n=5 untreated wt and het mice, n= 3 wt and het FSH-treated mice and n=3 oFF-treated wt and het mice). a= untreated versus 10IU FSH-treated, b=10IU FSH-treated versus oFF-treated.*

3.3.5.3 Oocyte Area Distribution

The area distributions of oocytes from primary (Figure 3.18), secondary (Figure 3.19) and antral (Figure 3.20) follicles were not affected by genotype for untreated, 10IU FSH-treated and oFF-treated animals.

3.3.6 Correlation between Granulosa Cell and Oocyte Areas

3.3.6.1 Correlation between Granulosa Cell and Oocyte Areas of Primary Follicles

There was a high positive correlation between the granulosa cell area and oocyte area for primary follicles in every treatment group (Figure 3.21). In the untreated animals the r^2 value was 0.74 in the wt animals, and 0.72 in the het animals (Figures 3.21 (A) and (B)). This correlation was slightly lower in the primary follicles of 10IU FSH-treated animals (r^2=0.63 for the wt and 0.64 for the het animals; Figures 3.21 (C) and (D)). In primary follicles of oFF-treated animals, the correlation remained high (r^2=0.76 for the wt and 0.77 for het animals; Figures 3.21 (E) and (F)). The correlation between granulosa cell area and oocyte area of primary follicles was similar in wt and het mice in all treatment groups.

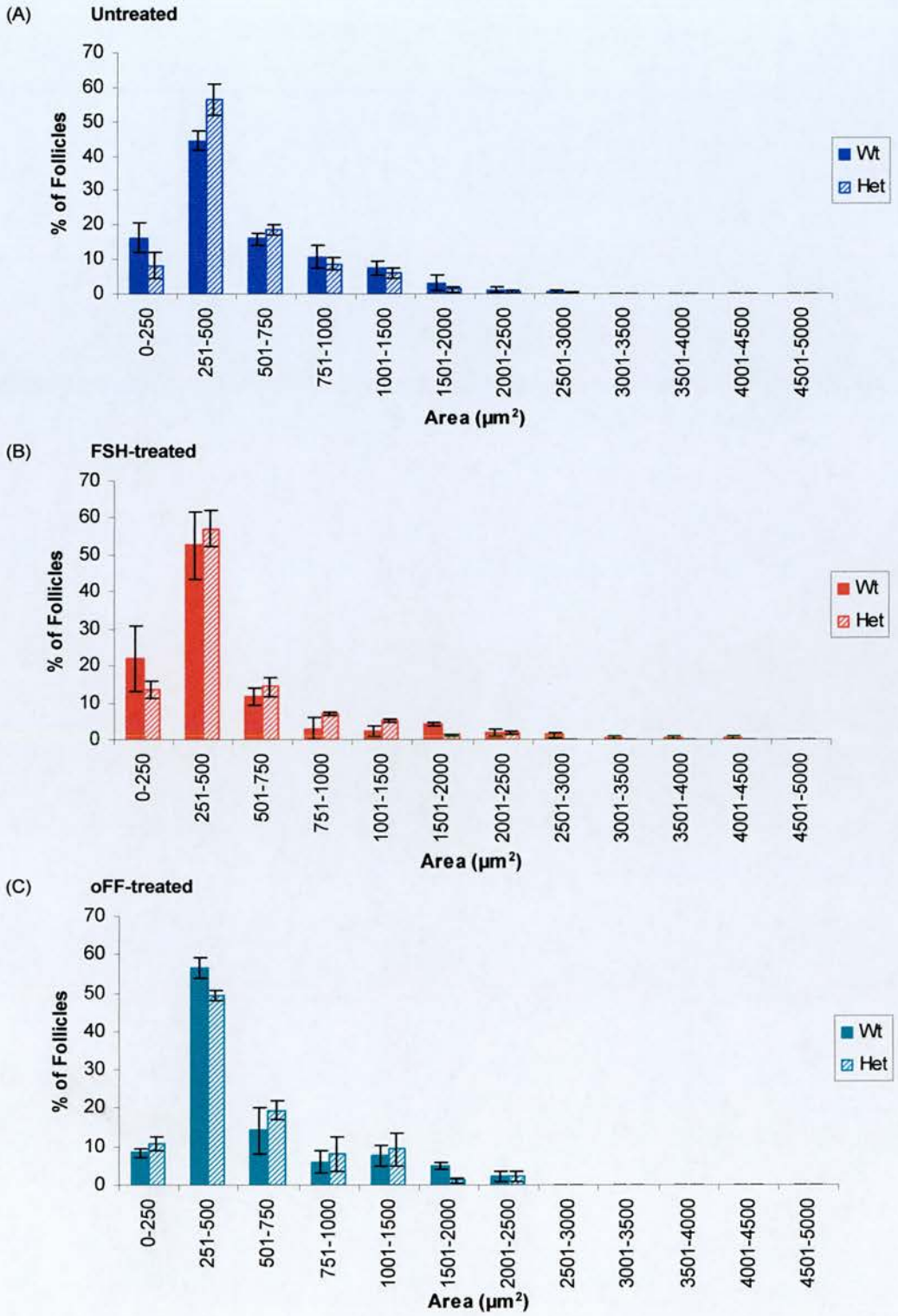


Figure 3.18 Oocyte area distributions for primary follicles from Dazl wt and het (A) untreated animals, (B) 10IU FSH-treated animals and (C) oFF-treated animals. The total number and percentage of follicles with a oocyte area in every size range was calculated per ovary and the mean per animal was determined followed by the mean ± S.E.M for wt and het mice in every treatment group were calculated (n=5 untreated wt and het mice, n= 3 wt and het FSH-treated mice and n=3 oFF-treated wt and het mice).

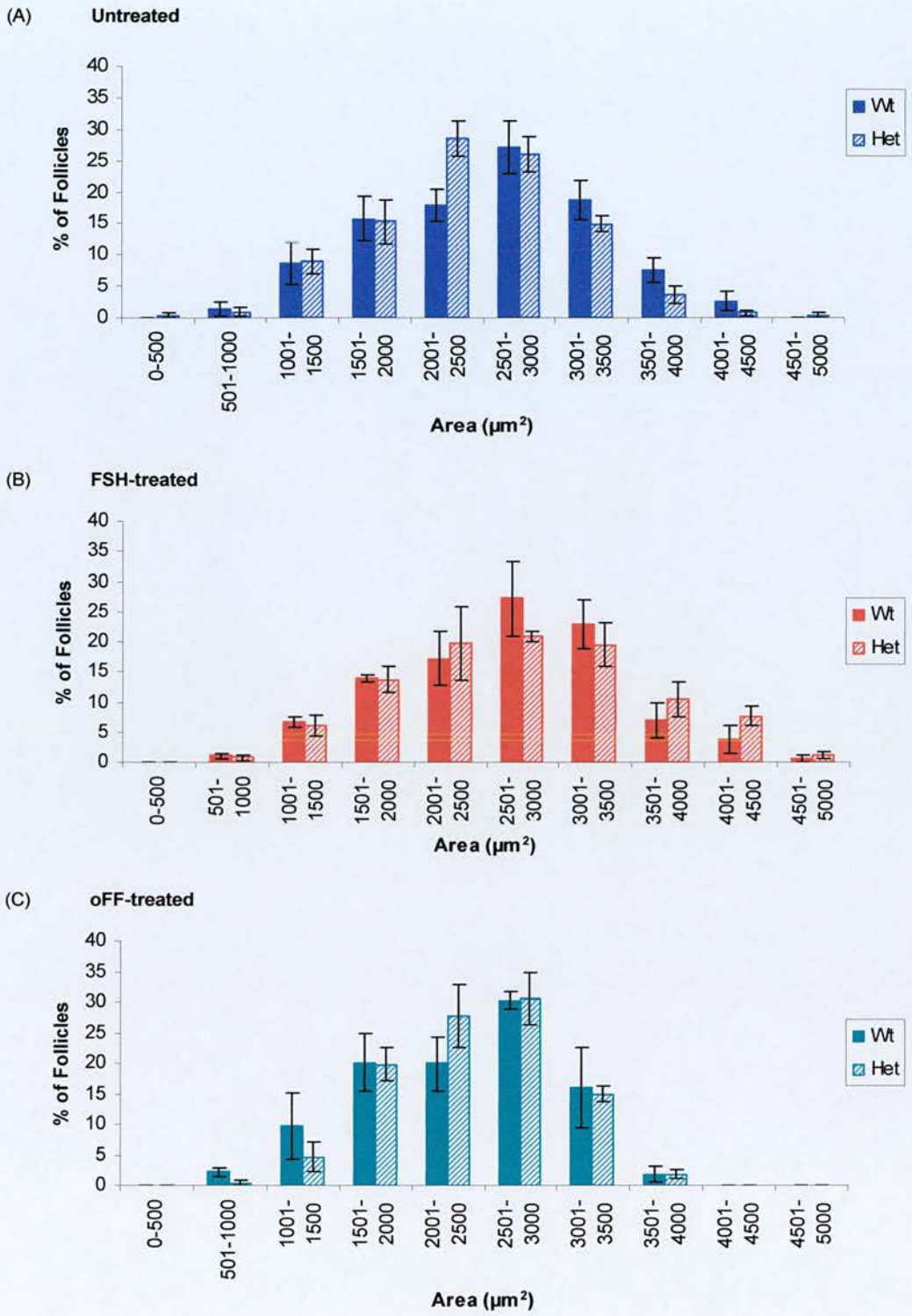


Figure 3.19 Oocyte area distributions for secondary follicles from Dazl wt and het (A) untreated animals, (B) 10IU FSH-treated animals and (C) oFF-treated animals. The total number and percentage of follicles with a oocyte area in every size range was calculated per ovary and the mean per animal was determined followed by the mean ± S.E.M for wt and het mice in every treatment group were calculated (n=5 untreated wt and het mice, n= 3 wt and het FSH-treated mice and n=3 oFF-treated wt and het mice).

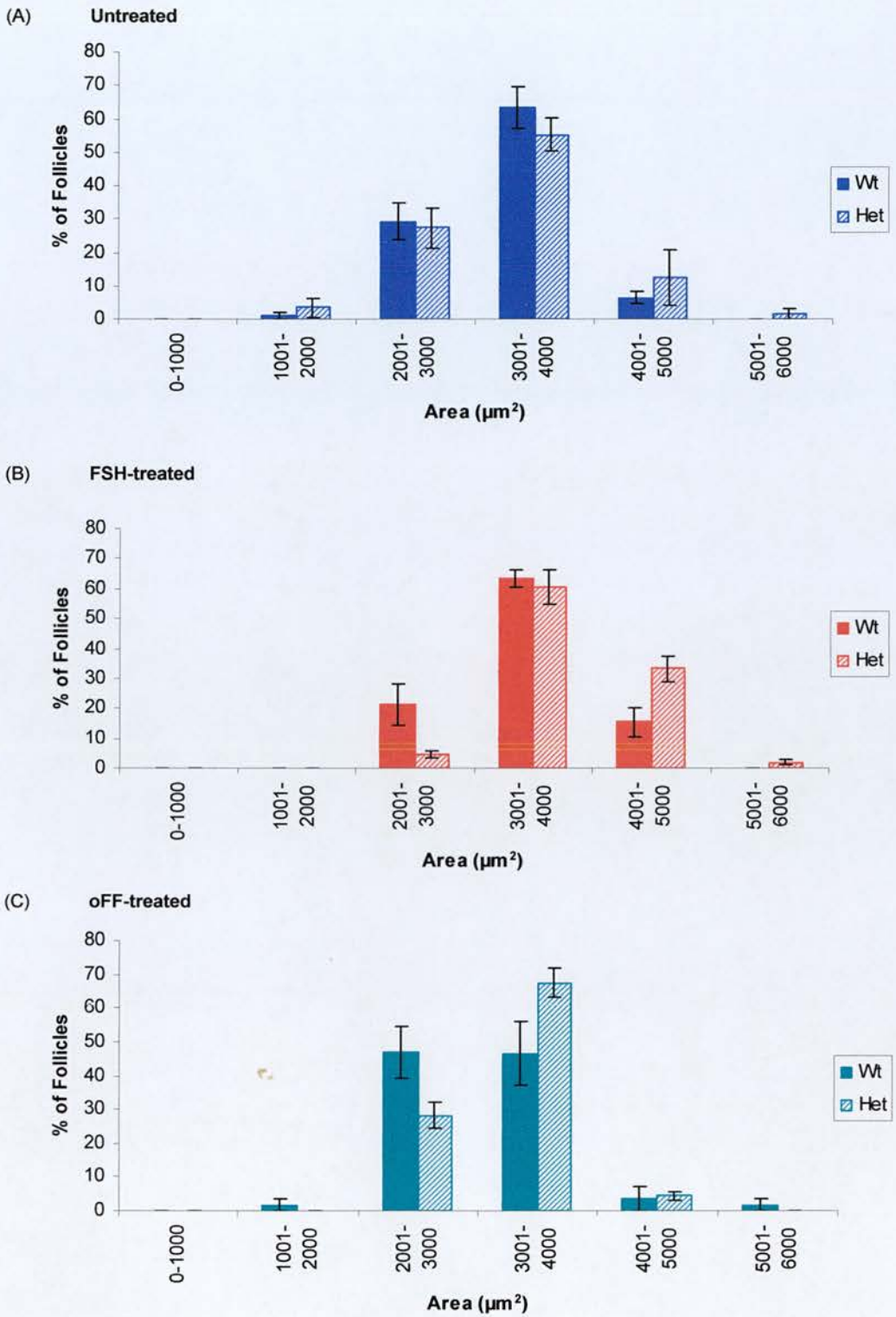


Figure 3.20 Oocyte area distributions for antral follicles from Dazl wt and het (A) untreated animals, (B) 10IU FSH-treated animals and (C) oFF-treated animals. The total number and percentage of follicles with a oocyte area in every size range was calculated per ovary and the mean per animal was determined followed by the mean ± S.E.M for wt and het mice in every treatment group were calculated (n=5 untreated wt and het mice, n= 3 wt and het FSH-treated mice and n=3 oFF-treated wt and het mice).

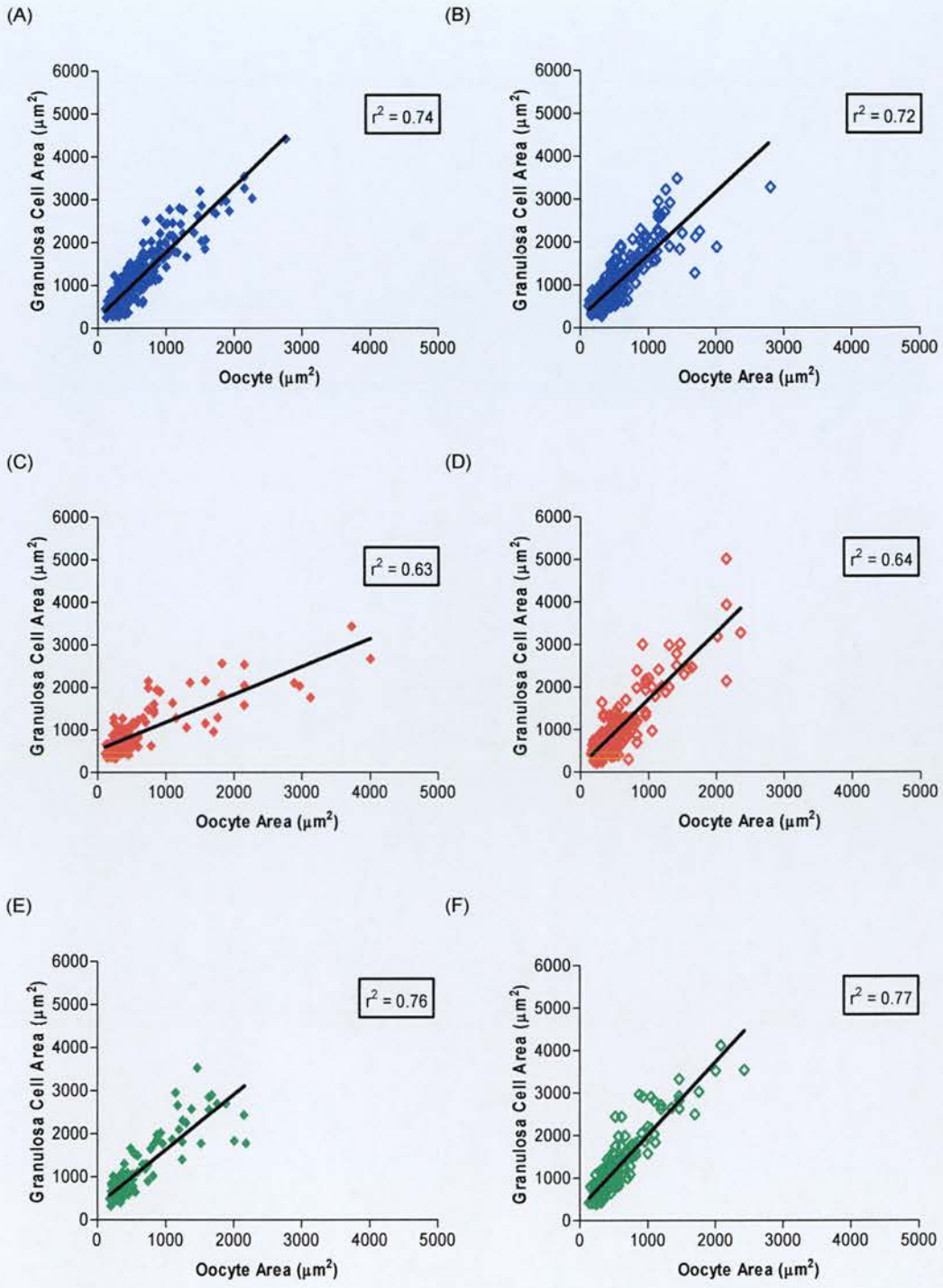


Figure 3.21 Correlation between primary follicle oocyte and granulosa cell areas for ((A) and (B)) untreated, ((C) and (D)) 10IU FSH-treated and ((E) and (F)) oFF-treated follicles from wt ((A) n=333, (C) n= 153 and (E) n=122) and het ((B) n=332, (D) n=226 and (F) n= 152) mice. wt = ◆ *and het =* ◇

3.3.6.2 Correlation between Granulosa Cell and Oocyte Areas of Secondary Follicles

The correlation between granulosa cell area and oocyte area for secondary follicles of untreated animals was relatively low (r^2=0.42 for wt and 0.38 for het mice) (Figures 3.22 (A) and (B)). In the 10IU FSH-treated animals, the correlation was also low (r^2=0.46 for wt and 0.45 for het mice) (Figures 3.22 (C) and (D)). Similarly, oFF-treated animals also had a low granulosa cell area and oocyte area correlation (r^2= 0.44 for wt and 0.47 for het mice; Figures 3.22 (E) and (F)). In all treatment groups the correlation between granulosa cell area and oocyte area of secondary follicles was similar between follicles from *Dazl* wt and het mice.

3.3.6.3 Correlation between Granulosa Cell and Oocyte Areas of Antral Follicles

The correlation between granulosa cell area and oocyte area was very low for antral follicles of untreated mice (r^2=0.1 for wt and 0.19 for het mice; Figures 3.23 (A) and (B)). A similar r^2 value was found for antral follicles of animals injected with 10IU FSH (r^2=0.00006 for wt and 0.02 for het mice; Figures 3.23 (C) and (D)). Furthermore, another extremely low correlation coefficient was observed between granulosa cell area and oocyte area for oFF-treated animals (r^2=0.01 for the wt and 0.04 for the het mice) (Figures 3.23 (E) and (F)). Therefore, there was no correlation between granulosa cell area and oocyte area of antral follicles of untreated, 10IU FSH-treated and oFF-treated mice. The lack of correlation observed in follicles from wt and het animals was similar between genotypes in all treatment groups.

3.3.7 Oocyte Area in vivo at time of Culture

This analysis compared the stage of oocyte maturation (by measuring the oocyte area from follicles within the size range selected for culture) in follicles from wt and het mice. The oocyte area between follicles (diameter of 180-220μm) from wt and het mice was not different between the two genotypes (Figure 3.24).

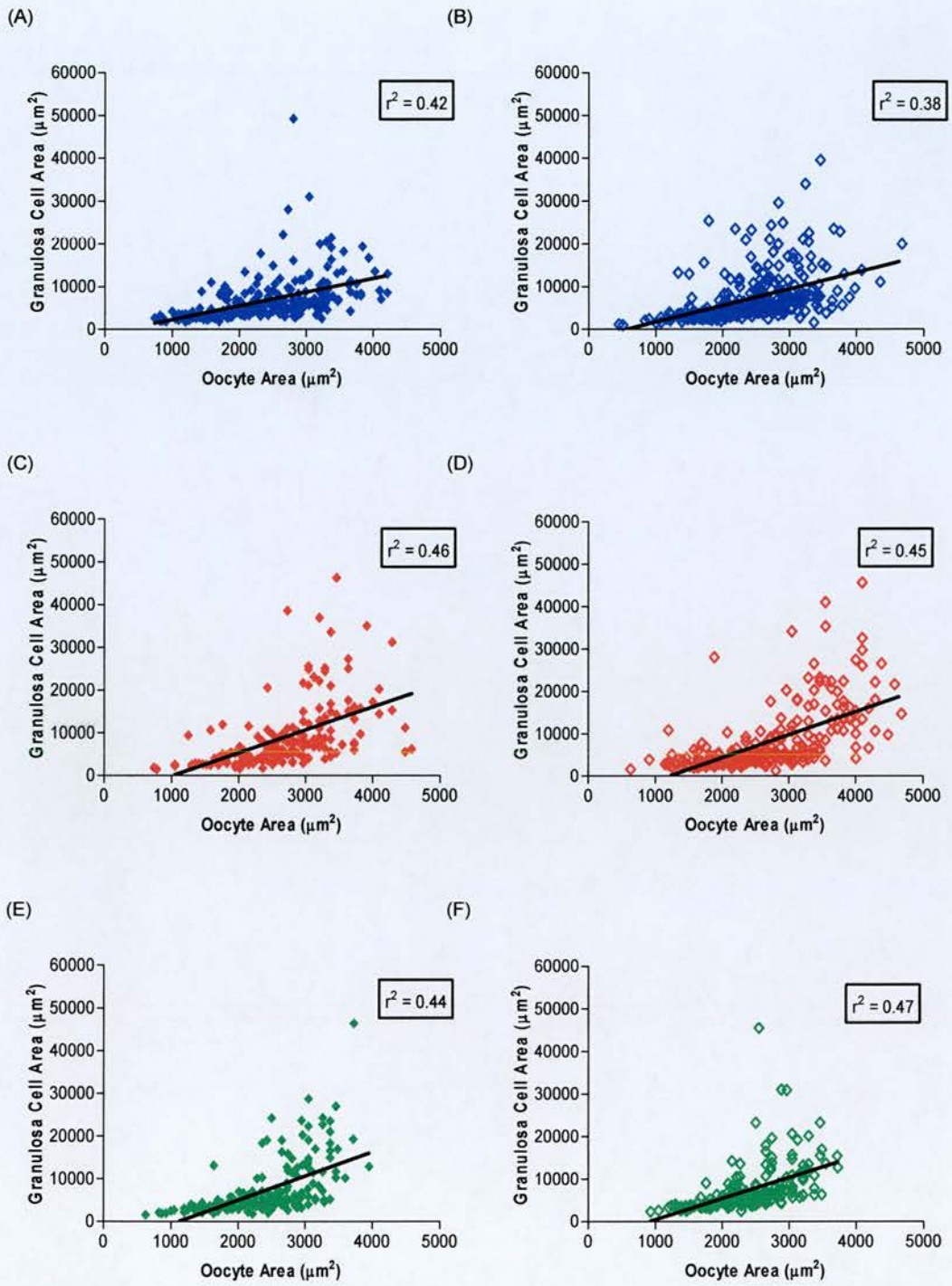


Figure 3.22 Correlation between secondary follicle oocyte and granulosa cell areas for ((A) and (B)) untreated, ((C) and (D)) 10IU FSH-treated and ((E) and (F)) oFF-treated follicles from wt ((A) n=242, (C) n=186 and (E) n=188) and het ((B) n=297, (D) n=241 and (F) n=200) mice. wt = ◆ and het = ◇

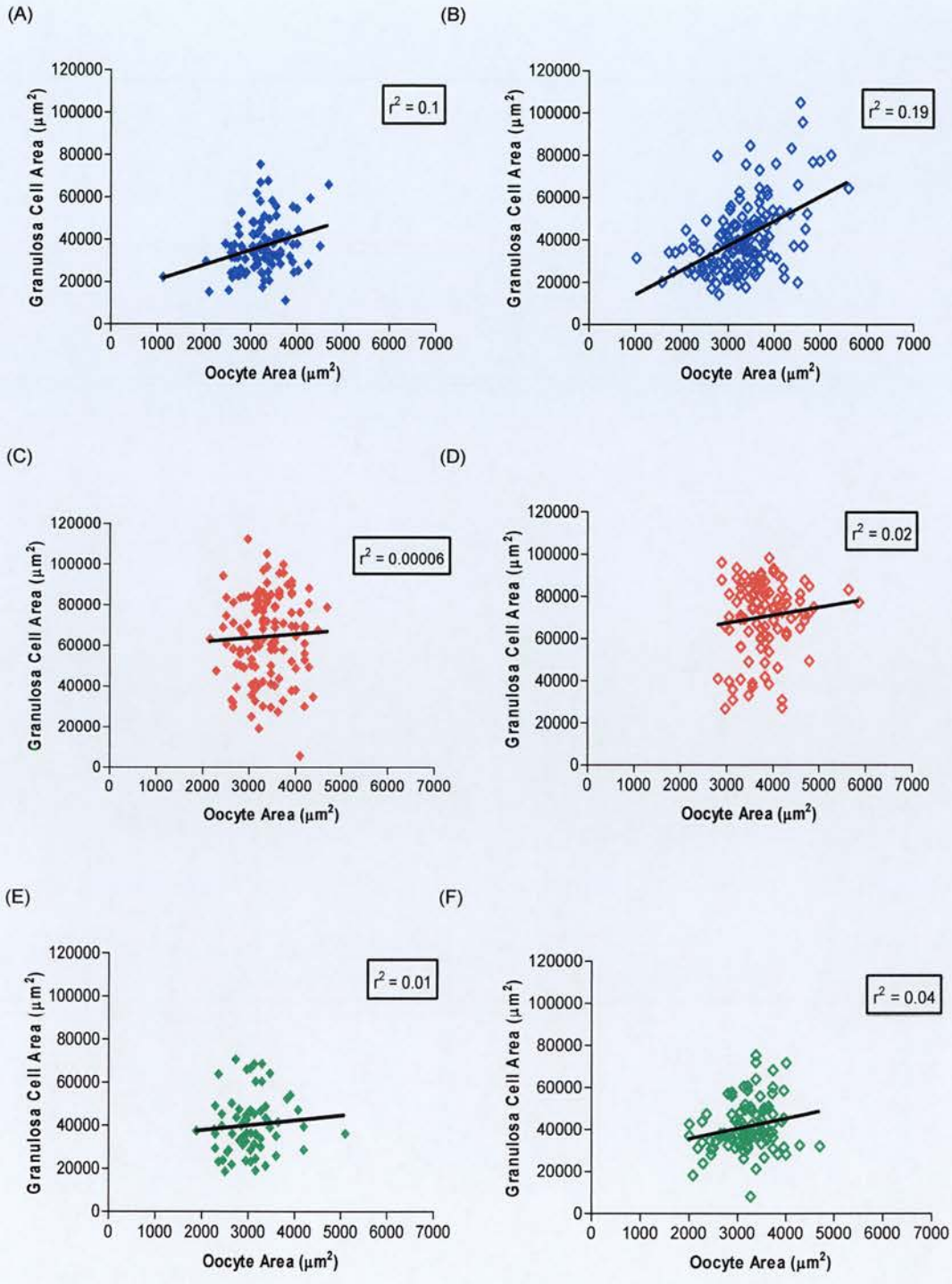


Figure 3.23 Correlation between antral follicle oocyte and granulosa cell areas for ((A) and (B)) untreated, ((C) and (D)) 10IU FSH-treated and ((E) and (F)) oFF-treated follicles from wt ((A) n=108 , (C) n=122 and (E) n=67) and het ((B) n=140, (D) n=105 and (F) n=93) mice. wt = ◆ and het = ◇

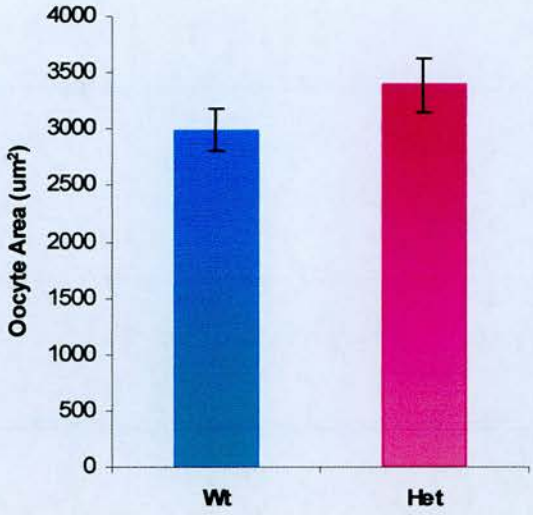


Figure 3.24 Untreated oocyte area of follicles 180-220μm in diameter Values are means ± S.E.M. wt n= 4 follicles and het n=6 follicles.

3.4 Discussion

3.4.1 Ovarian Counts

The data presented in this chapter demonstrated that untreated day 21 *Dazl* het animals have a significantly greater percentage of antral follicles compared to untreated *Dazl* wt animals (Figure 3.3). This supports previous studies in the *Dazl* adult mice which also observed that the het animals had a larger percentage of antral follicles (McNeilly *et al.,* Unpublished; Appendix B). However, there is no published data on ovarian counts in the *Dazl* mouse strain to our knowledge and due to high variation between strains of mice (Canning *et al.,* 2003) these data are not comparable with other strains. Coupled with previous data on the *Dazl* mice which illustrated that the adult *Dazl* het mice have lower plasma FSH levels but a larger percentage of antral follicles and greater ovulation rates this data suggest that the follicles from the het animals exhibit greater sensitivity to FSH (McNeilly *et al.,* 2000). However, the exact mechanism of this sensitivity is unknown and could be a result of increased FSHR numbers on the granulosa cells, increased binding of FSH to the receptor or greater stimulation of the FSH pathway (Simoni *et al.*, 1997; Themmen and Huhtaniemi, 2000).

Furthermore, the untreated day 21 *Dazl* wt and het mice showed similar percentages of primordial, transitionary, primary and secondary follicles (Figure 3.3). Previous studies in the *Dazl* KO mice illustrated that shortly after birth there were no oocytes present in the ovary (Ruggiu *et al.,* 1997; McNeilly *et al.,* 2000) and this current study demonstrated that the total numbers of follicles observed at day 21 did not differ between the genotypes suggesting there is no intermediate phenotype. However by adulthood the ovaries from the het animals have a lower number of total follicles compared to ovaries from wt animals (McNeilly *et al.,* Unpublished; Appendix B). This suggests that the het animals demonstrate greater activation of follicles from the reserve or increased atresia compared to wt follicles. This supports various studies which conclude that the oocyte regulates follicle growth (Vanderhyden *et al.,* 1990; Eppig, 2001).

Although follicle activation has not been studied in the *Dazl* mouse model the *Amh* KO mouse also demonstrates a similar number of follicles shortly after birth compared to the wt animals. However, the *Amh* KO mice which have lower FSH levels have a greater activation rate from the follicle reserve which results in faster exhaustion of the reserve in these mice (McGee *et al.*, 2001; Durlinger *et al.*, 2002; Pigny *et al.*, 2003). A similar observation in the *Dazl* mice suggests that *Dazl* could be a factor involved in follicle initiation and activation from the follicle reserve, although this is likely only to be important during adulthood.

However, since the follicle reserve is depleted in the *Dazl* adult mice (McNeilly *et al.*, Unpublished; Appendix B) but not in the pre-pubertal mice, this suggests that this difference could be due solely to greater FSH-sensitivity in the *Dazl* mice. Since plasma FSH levels are greater in the post-pubertal animal (Hillier, 1994) this difference in follicle numbers would be expected to be more marked in the adult mice.

There was no difference observed in the follicle counts between ovaries from wt and het animals after treatment with either FSH or oFF at any point of folliculogenesis (Figures 3.1 and 3.3). Treatment with PMSG or recombinant FSH stimulates follicle growth and development *in vivo* (Galway *et al.*, 1990; LaPolt, *et al.*, 1992). However, the window between the FSH threshold required for follicle development and insufficient FSH is very narrow (Brown, 1978). This suggests that after treatment with FSH the follicles from the wt and het animals are able to respond similarly due to adequate FSH and therefore diminishes the differences observed in the untreated wt and het antral follicle numbers (Figure 3.25). Treatment with oFF suppresses FSH levels to basal levels and increases follicle atresia rates (Wallace and McNeilly, 1985; McLeod and McNeilly, 1987; De Jong and Sharpe, 1976). This study observed no difference in antral follicle numbers of oFF-treated and FSH-treated *Dazl* het and wt mice. However, in animals treated with follicular fluid antral development was not observed, as FSH levels were too low to maintain and stimulate full follicle development (Baird *et al.*, 1990; Campbell *et al.*, 1999). Therefore, no differences between wt and het follicle development would be expected as the only differences observed in the untreated animals were seen at the antral stage of development. However, the mice in this current study were pre-pubertal and therefore FSH was already at basal levels. Therefore, a similar response would be expected for oFF and untreated animals (Wallace and McNeilly, 1985; McLeod and McNeilly, 1987; Hillier, 1994).

Figure 3.25 FSH sensitivity in Dazl follicles.

This figure demonstrates follicles from untreated het animals are capable of exceeding the FSH threshold compared to follicles from wt untreated animals. The FSH and oFF-treated wt and het animals have a similar response with follicles from the FSH-treated animals able to exceed the FSH threshold due to exogenous FSH whereas, the oFF-treated follicles are not capable of exceeding the FSH threshold.

When follicle numbers were analysed across the treatment groups for the wt and het animals it was concluded that the untreated wt animals had a greater percentage of primary follicles compared to FSH-treated and oFF-treated wt animals (Figure 3.4). The untreated wt animals also had a significantly greater percentage of antral follicles compared to the oFF-treated animals and the FSH-treated animals had a larger percentage of antral follicles compared to the untreated and oFF animals. Previous studies have demonstrated that primary follicles express the FSHR (Tisdall *et al.,* 1995; O'Shaughnessy *et al.,* 1996; Simoni *et al.,* 1997). Although FSH is not required at this stage of development follicle growth *in vitro* was stimulated by FSH (Eppig *et al.,* 1998). Therefore, this suggests that the FSH-treated mice had a reduced number of primary follicles and increased number of antral follicles compared to untreated animals due to increased activation and growth in these animals. Treatment with oFF, as previously mentioned, suppresses FSH levels, which in turn increases atresia rates (Hirshfield, 1989; Baird *et al.,* 1990). This possibly explains the reduced number of primary and antral follicles observed in the oFF-treated wt animals compared to untreated wt animals. Furthermore, the effect of oFF suppressing the FSH which is required for antral development explains the significant reduction in the number of antral follicles observed in the wt oFF-treated mice compared to untreated wt animals (McLeod and McNeilly, 1987).

The data presented in this chapter demonstrated no difference in the percentage of follicles at any stage of development in the het animals across all treatment groups (Figure 3.4). Previous studies in the *Dazl* mouse strain have demonstrated that the het animals had reduced plasma FSH levels but increased ovulation rates compared to wt animals (McNeilly *et al.,* 2000). The similar number of follicles across the treatment groups supports Brown's theory which suggests that the FSH threshold between which follicles cannot develop and those that can develop is very small (Brown, 1978). This suggests that the follicles from the het animals are more responsive to FSH and can respond even to basal levels, enabling antral development in the presence of low FSH. There were differences in the number of antral follicles observed in oFF and untreated het animals but these were not significant. However, the health of the follicles in the oFF-treated animals would be expected to be poorer than untreated animals due to lower FSH (Hirshfield, 1989).

Previous studies in rats have concluded that excessive levels of FSH (i.e. 20IU recombinant human FSH) result in down-regulation of the FSHRs and therefore the stimulation of follicle growth is inhibited (LaPolt *et al.,* 1992). This current study treated *Dazl* mice with 10IU FSH which has been previously demonstrated to stimulate follicle growth in rodents (Galway *et al.,* 1990; LaPolt *et al.,* 1992). Future work in the *Dazl* mouse model should assess different doses of FSH to determine the FSH threshold below which the het animals have increased antral development and beyond which the differences are diminished.

Follicles in this study were recorded as unclassifiable if they did not fit into any of the primordial-antral categories and contained a large oocyte with a single layer of flattened granulosa cells (Figure 2.3). The number of unclassifiable follicles was similar both between wt and het animals as well as between treatment groups. No other studies have demonstrated such follicles in the *Dazl* strain but similar structures have been observed in other mouse models (Britt *et al.,* 2004). The *Gdf-9* KO and the *Aromatase* KO mouse model have also demonstrated such structures within the ovary (Carabatsos *et al.,* 1998; Britt *et al.,* 2004). However, this could simply be a characteristic of animals in which oocyte secreted factors have been genetically manipulated.

Throughout the analysis of the ovaries in this current study there was no observation of oocyte regeneration. Johnson *et al.* (2004) observed oocytes in the outer surface of the ovary which they concluded to be signs of oocyte regeneration. The method used in the experiments presented here examined only a tenth of the actual ovary and therefore such structures could be present in the sections that were not counted. Furthermore, this method used H&E staining and therefore did not assess stem cell factors in the ovaries to determine if regeneration occurs (Johnson *et al.*, 2005a). However, no signs of regeneration were observed in this study suggesting that no regeneration occurred.

Studies during the 1920-1950s on ovarian follicle counts debated whether oocyte renewal occurs, with studies presenting conflicting evidence for and against this (Allen, 1923; Zuckerman, 1951). From this time the central dogma stated that the ovary does not replenish oocytes throughout life, and the number of oocytes formed at birth or shortly after birth is finite (Zuckerman, 1951; Baker, 1963; Gosden, 2004). However, the publication of the paper by Johnson *et al.* (2004) brought this issue back to the centre of debate. Since this publication, numerous studies have been carried and with mixed results with some supporting the Johnson paper and concluding that germ cell replenishment occurs throughout life in mice ovaries, whereas others oppose this belief and are critical of the methods and interpretation of the results (Byskov *et al.,* 2005; Telfer *et al.,* 2005). The methods which are universally accepted to determine ovarian reserve are diverse and include: FSH levels, ovarian volume, follicle counts, inhibin B levels, AMH levels, oestradiol levels and age (Flaws *et al.*, 2001b; Ficicioglu *et al.*, 2003; Themmen, 2005). In mice the most common method of assessing the ovarian reserve is through careful follicle counts (Zuckerman, 1951, Canning *et al.,* 2003; Myers *et al.,* 2004).

The method used for the analysis of the follicle counts in this study is not 100% accurate. However, no method used to assess the ovarian reserve is completely accurate (Faddy, 2000; Bukulmez and Arici, 2004). The method used in this study is accurate to within 3-11% of the total counts, however (Tilly, 2003). The more modern method of the Fractionator/Optical Disector method of counting follicles that was not available during this study should be assessed for accuracy in future studies of ovarian counts (Myers *et al.,* 2004). This method analyses the entire ovary but is not subjective to the problem of follicles being counted twice (Myers *et al.,* 2004). Furthermore, the ovarian follicle counts recorded in this study did not assess whether the follicles were healthy or atretic but counted all follicles. Therefore, assessing the morphology of the follicle as described by Irving-Rodgers *et al.* (2001) and determining the variation between healthy and atretic follicles could provide more information into folliculogenesis in the *Dazl* mouse model. With an increased ovulation rate in adult *Dazl* mice (McNeilly *et al.*, 2000; Appendix A), it would be expected that these mice would have a greater number of healthy follicles compared to wt mice.

3.4.2 Granulosa, Follicle and Oocyte Size

The correlation between follicle/oocyte diameter and area was assessed to decide which parameters were the most accurate in measuring follicles. Previous studies have used either oocyte/follicle diameter or granulosa cell morphology (Hirshfield and Midgley, 1978a; Hirshfield and Midgley, 1978b; Bucci *et al.,* 1997; Myers *et al.;* 2004). The results presented in this chapter demonstrate that there is a high correlation between diameter and area of untreated primary and secondary follicles from wt and het mice and antral follicles from wt animals (Figures 3.5, 3.6 and 3.7). The correlation between these parameters was lower in untreated antral follicles from het mice. In addition, the correlation between the oocyte diameter and area was also high in the follicles from untreated, primary follicles from wt and het mice, secondary follicles from wt mice and antral follicles from both wt and het mice.

Smaller follicles would be expected to have a higher correlation between area and diameter as in these follicles there is little variation with less granulosa cell layers and proliferation (Gougeon and Busso, 2000). As follicles mature granulosa cell proliferation is responsible for the increase in follicle size and follicles grow at different rates (Hirshfield and Midgley, 1978a; Hirshfield and Midgley, 1978b). Therefore, the correlation would be expected to be lower in antral follicles. The difference between the high correlation found in the antral follicles from wt mice and the low correlation found in the antral follicles of het mice suggests that the het mice have increased proliferation rates compared to wt mice. Furthermore, previous studies have shown that antral follicles in the ovary are not spherical and together with the findings in this chapter, suggests that follicle area is a more accurate measurement of measuring follicles than diameter (Zuckerman, 1951).

Oocyte growth is greatest in the early stages of follicle development with growth completed by the time of antral development (Mandl and Zuckerman, 1952; Hirshfield, 1991). This suggests that the oocytes from the secondary follicles from het mice are maturing at a different rate to oocytes from secondary follicles of wt animals. It also proposes that the oocyte growth of the het animals is not as stringently regulated to that of wt animals.

The correlation between granulosa cell area and granulosa cell counts should determine whether granulosa cell area was representative of number of granulosa cells. The data presented in this thesis concludes that granulosa cell area is representative of granulosa cell numbers (Figure 3.9). Previous studies assessing granulosa cell proliferation have examined proliferation rates through analysing thymidine expression (Hirshfield, 1986). However, such a method was not possible in this study. Other studies included actual counts of granulosa cells (Wassarman, 2002). However, the finding that granulosa cell area is representative of granulosa cell numbers allows the use of granulosa cell area as a measure of granulosa cell numbers in this study.

The correlation between granulosa cell area and oocyte area was analysed to determine whether a single copy of the *Dazl* gene altered the rate of oocyte growth and granulosa cell proliferation compared to two copies of the *Dazl* gene. The data recorded in this chapter shows the correlations between follicle and oocyte areas from wt and het animals in all treatment groups were similar (Figures 3.21, 3.22 and 3.23). As the follicles mature from primary to secondary to antral stages of development the correlation decreases. As a follicle matures, oocyte growth slows and granulosa cell proliferation increases. Hence, the correlation would be expected to decrease in these more advanced follicles (Figure 3.26; Hirshfield, 1991).

The studies carried out in this chapter demonstrate that antral follicles from untreated het mice had increased granulosa cell areas and follicle areas in antral follicles but similar oocyte areas compared to antral follicles from untreated wt animals (Figures 3.12, 3.16 and 3.20). In addition both primary and secondary follicles were similar in size in both untreated genotypes (Figures 3.10-3.19). Granulosa cell proliferation is greatest during the latter stages of development, particularly antral development (Hirshfield, 1991). *Dazl* could possibly play a role in controlling the expression of factors that regulate granulosa cell proliferation and in turn increase FSH-sensitivity. The study presented in this chapter examined granulosa cell proliferation through recording granulosa cell areas and not granulosa cell differentiation. Therefore, future studies should involve assessing granulosa cell differentiation in this strain of mice to determine whether follicles from *Dazl* het mice are indeed more developmentally advanced than those from wt mice.

Previous studies on the *Dazl* mice concluded that het mice had larger litters than wt mice and therefore this suggests that more follicles from the het mice were capable of maturing further than follicles from wt mice (McNeilly *et al.*, 2000; McNeilly *et al.*, Unpublished; Appendix A). Examination of factors such as cumulus and mural granulosa cell morphology, LHR and PR expression in the granulosa cells would determine whether these developmental markers of follicle development are expressed to similar levels in wt and het mice when granulosa cell proliferation has stopped and differentiation has began (Hirshfield, 1991; Eppig *et al.*, 1997b).

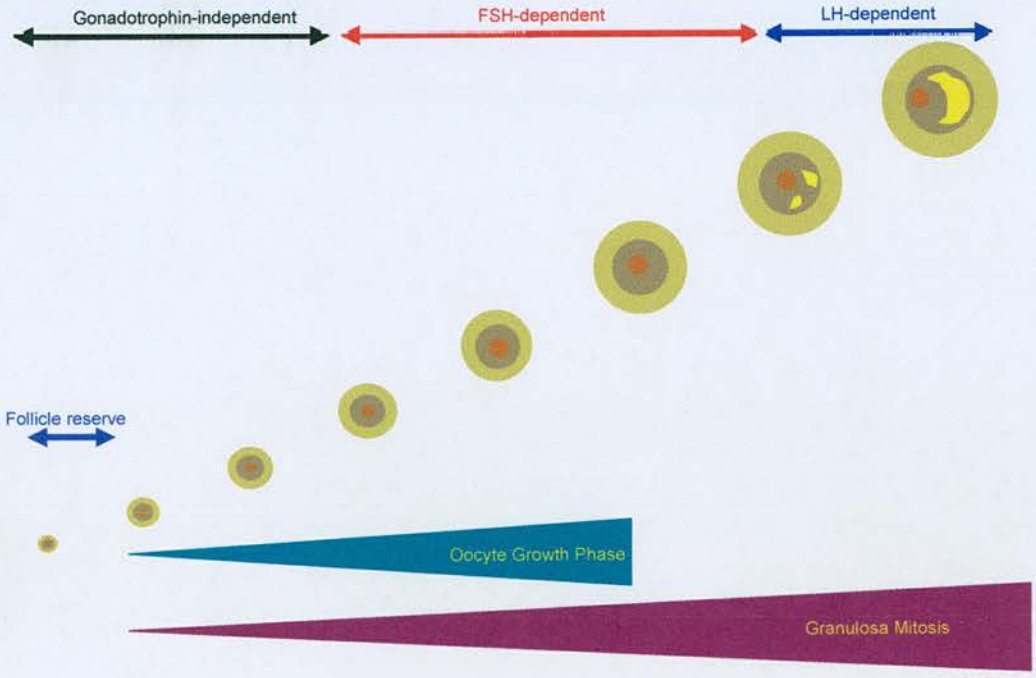


Figure 3.26 Stages of oocyte and follicle growth.

Oocyte growth is greater in the early stages of follicle development whereas the latter stages of development are characterised by greater granulosa cell proliferation.

Other strains of animals also demonstrate increased ovulation rates viz. the Booroola sheep, where homozygous animals have greater ovulation rates compared to hets (McNatty *et al.*, 2001; Souza *et al.*, 2001; Shimasaki *et al.*, 2004). This strain has increased ovulations with follicles that are smaller (but in greater quantity) as a result of increased FSH-sensitivity and an enhanced cAMP response (McNatty *et al.*, 1985; Shimasaki *et al.*, 2004). However, the data presented here conclude that *Dazl* het mice have larger follicles with larger litters suggesting that *Dazl* may not work through stimulating cAMP (McNeilly *et al.*, 2000). Although McNeilly *et al.* (Unpublished; Appendix A) demonstrated that there was increased litter size the ovulation rate was directly not measured. Therefore, het animals could demonstrate increased survival of embryos compared to wt mice. Future studies would involve assessing ovulation rate in these animals. However, with the data in this study coupled with the litter sizes, suggest that *Dazl* het mice have increased ovulation rates (McNeilly *et al.*, Unpublished; Appendix A).

Animals heterozygous for oocyte *Gdf*-9 and *Bmp-15* also have increased ovulation rates with infertile KO animals (Dong *et al.,* 1996; McNeilly *et al.,* 2000; Shimasaki *et al.,* 2004). However, there are some species-specific differences. Mutations in *BMP-15* cause infertility in sheep, but *Bmp-15* KO mice have only impaired fertility (Yan *et al.,* 2001; McNatty *et al.,* 2004; Shimasaki *et al.,* 2004). Although *DAZ* and *Dazl* demonstrate a high homology, with the human gene able to partially rescue the murine gene, this does not necessarily mean these genes function in a similar manner across species (Tsui *et al.,* 2000). Both *Gdf-9* and BMP-15 stimulate granulosa cell mitosis (Erickson and Shimasaki, 2001; Otsuka *et al.,* 2001b; Shimasaki *et al.,* 2004). From the studies reported here it is suggested that a single copy of the *Dazl* gene stimulates granulosa cell proliferation compared to two copies of the gene. However, how one copy of the *Dazl* gene stimulates granulosa cell proliferation to a greater extent than two copies remains unknown. Future studies are required to assess levels of factors in follicles from wt and het mice (such as *Nobox, Kit* Ligand and *Gdf-9*) which are postulated to be involved in follicle activation (Dong *et al.*, 1996; Yoshida *et al.*, 1997; Rajkovic *et al.,* 2004).

The results outlined here suggest that *Dazl* het mice are more FSH-sensitive than *Dazl* wt mice. Other oocyte-secreted factors also affect FSH sensitivity of the follicle (Vitt *et al.,* 2000; Jin *et al.,* 2005). For example, *Gdf-9* KO mice have increased granulosa cell mitosis but have inhibited granulosa cell differentiation (Vitt *et al.,* 2000). The presence of Gdf-9 down-regulated FSH cAMP signalling and reduced FSH sensitivity (Vitt *et al.,* 2000). Similarly both BMP-15 and *Kit* Ligand (via its interaction with oocyte receptor c-*Kit*) were also postulated to stimulate granulosa cell proliferation as suggested by increasing steroidogenesis which is often associated with increased granulosa cell proliferation. However, these factors are also associated with down-regulation of the FSHR and in turn inhibit differentiation (Otsuka *et al.,* 2001b; Jin *et al.,* 2005). Future work on the *Dazl* strain is required to determine whether granulosa differentiation is altered in the het animals compared to wt animals.

As mentioned previously there was no difference in follicle, granulosa cell and oocyte size in FSH-treated and oFF-treated wt and het mice (Figures 3.10-3.20). There is very little difference between inadequate and sufficient FSH for follicle maturation (Brown, 1978). This study supports Brown's data and concludes that 10IU FSH is sufficient to stimulate growth and development (LaPolt *et al.,* 1992). Therefore, this level of endogenous FSH is adequate for follicle development in both genotypes and any variation observed in untreated animals would be lost. Similarly, as discussed above, the detrimental effect of oFF on follicle development reduces FSH levels (Baird *et al.,* 1990) to beyond a point at which follicles from wt and het animals respond in a similar manner.

There was no difference in follicle, granulosa cell and oocyte areas from antral follicles of wt animals when treated with oFF compared to untreated animals (Figures 3.13 (A), 3.17 (A) and 3.20). A similar trend was observed in antral follicles from untreated and oFF-treated animals. This is not surprising as these mice were studied on day 21 and were therefore pre-pubertal, when FSH levels would be at a basal level (similar to animals treated with oFF) (McLeod and McNeilly, 1987; Baird *et al.,* 1990). Furthermore, antral follicles from untreated and oFF-treated wt and oFF-treated het mice were all below 80,000μm^2, whereas antral follicles from het mice had some follicle above this size (Figure 3.12). However, this number was not significant, although a greater number of activated and advanced follicles would result in a greater cohort of follicles and the possibility of an increased ovulation rate. FSH-treated animals had larger follicles and granulosa cell areas but similar oocyte areas compared to both untreated and oFF-treated animals with the same genotype. FSH stimulates granulosa cell proliferation and the production of steroids and growth factors that enhance follicle development (Hirshfield, 1986; Hirshfield, 1991). This may explain the observed differences between the treatment groups. Moreover, the increase in granulosa cell and follicle areas in the FSH-treated animals (both wt and het) suggests that FSHR down regulation did not occur (LaPolt *et al.,* 1992; Simoni *et al.*, 1997; Themmen and Huhtaniemi, 2000).

The oocytes in primary, secondary and antral follicles from wt and het animals in all treatment groups were similar in size, which is surprising as *Dazl* is an oocyte-derived factor (Figure 3.18, 3.19 and 3.20). Other mouse models with oocyte-specific factors such as *Gdf-9* KO mice have follicles with enlarged oocytes (Carabatsos *et al.,* 1998). The mechanism by which the *Dazl* gene works is still unknown (Venables *et al.,* 2001; Reynolds and Cooke, 2005). However, the data presented in this study suggest that this gene effects granulosa cell proliferation and therefore FSH-sensitivity. Recent studies have demonstrated that there is bi-directional communication between oocytes and granulosa cells and follicle growth is reliant on this communication (Eppig, 2001; Senbon *et al.,* 2003).

Factors such as gap junctions play a vital role in follicle development. *Connexin-37* KO mice (which lack gap junctions between the oocyte and the granulosa cell) are infertile with arrested follicle development in the pre-antral stages of development and oocyte growth is also inhibited (Carabatsos *et al.,* 2000). The effect of the oocyte on granulosa cell proliferation can be observed since granulosa cells nearest the oocyte have a greater proliferation rate, and oocytectomy reduces the rate of granulosa cell proliferation (Hirshfield, 1986; Hirshfield, 1991; Vanderhyden *et al.,* 1992). Although the oocyte requires factors from the granulosa cells to mature, the oocyte seems to regulate the overall growth and development of the follicle. When more mature oocytes are cultured with primordial follicles, these follicles develop faster compared to controls (Eppig *et al.,* 2002).

Furthermore, FSH stimulated Connexin-43 expression (gap junctions between granulosa cells which are required for development of follicles beyond the secondary stage of development) although increased expression of Connexin-43 inhibited proliferation and improved differentiation of the granulosa cells (Sommersberg *et al.,* 2000; Ackert *et al.,* 2001).

As mentioned previously the exact mechanism of *Dazl* on follicle growth and development remains unknown at present. The exact target(s) of *Dazl* are still unclear, although there are at least 34 possibilities with which *Dazl* could interact (Venables *et al.,* 2001). A single copy of the *Dazl* gene could stimulate gap junctions in the oocyte and enhance bi-directional communication and development to a greater extent than animals with two copies of the gene. Alternatively, the ability of *Dazl* to bind to Cdc24A (which is critical for cell cycle regulation) suggests that this interaction could stimulate the proliferation of granulosa cells, as observed in this study (Jiao *et al.,* 2002). However, further analysis is required to determine which of the factors known to interact with *Dazl* are present in the ovary, and how such factors differ between *Dazl* wt and het animals.

3.4.3 Ovarian Volume

The present data demonstrated no significant difference in ovarian volume between wt and het animals (in all treatment groups) although untreated het animals had a larger percentage of antral follicles. (Figure 3.8) However, previous studies on *Dazl* adult mice found that het animals had larger ovaries compared to wt mice (Ruggiu *et al.,* 1997; McNeilly *et al.,* 2000). This variation in ovarian morphology between studies could be due to the different methods in measuring ovary morphology (McNeilly *et al.,* 2000) or the use of adult mice compared to the pre-pubertal mice used in this study. Therefore, a direct comparison cannot be made. Furthermore, the exact mechanisms of *Dazl* remain unknown (Moore *et al.*, 2003a; Reynolds and Cooke, 2005) and *Dazl* could have a greater influence in adulthood explaining this variation in results. If the follicles from *Dazl* het mice are more FSH-sensitive then the differences between wt and het mice would be more noticeable in adulthood when FSH levels would be greater. Previous studies have demonstrated that ovarian volume can be used as a measurement of the ovarian follicle reserve with larger ovaries indicating larger reserves of antral follicles (Flaws *et al.,* 2001b). This was not observed in this study, however. Although there was a significant increase in the percentage of antral follicles in untreated het mice, this only accounted for a small number of antral follicles and, therefore, would not necessarily influence the total ovary area. However, this may not be noticeable until adulthood when antral follicle development is stimulated with increased levels of FSH (Hillier, 1994). In addition, the method used to analyse the ovary areas differs with those used by Flaws *et al.* (2001b) using ultrasound scans compared to the analysis of ovary area (every 10^{th} section) reported here.

Using recombinant FSH we have shown that FSH treatment increased ovarian volume in FSH-treated wt and het animals (Figure 3.8). As mentioned previously FSH treatment stimulates follicle granulosa cell proliferation and follicle development (Hirshfield and Midgley, 1978b, Schipper *et al.,* 1998). Therefore ovarian follicles would be expected to be larger when treated with FSH and have an increased ovarian volume. Furthermore, FSH treatment also protects follicles from atresia (Hirshfield, 1989; Kumar, 2005). Therefore, ovaries treated with FSH would be expected to be larger due to a greater total number of follicles because of a lower atresia rate compared to untreated and oFF-treated mice (Hirshfield and Midgley, 1978b; McLeod and McNeilly, 1987). Data on untreated and oFF-treated animals presented in this study demonstrated no difference between ovary volumes between treatment groups. However, since these animals were day 21 pre-pubertal mice, FSH levels would be at a basal level, and granulosa cell mitosis would also be low, and similar to levels in oFF-treated mice (Hillier, 1994). These treatment groups in wt and het animals have a similar number of total follicles and hence ovarian volume would be expected to be similar (Flaws *et al.,* 2001b). Although het animals had more larger antral follicles than het oFF-treated animals this was not significant.

3.4.4 Oocytes in Culture

Oocyte size is often used as a measurement of oocyte maturation and oocyte growth is accelerated in the initial stages of follicle development (Mandl and Zuckerman, 1952). The data presented in this study demonstrated no difference in oocyte size *in vivo* from follicles that were selected for culture in Chapters 4 and 5 (Figure 3.24). Previous studies have shown that oocyte maturation is influenced by oocyte-secreted factors and oocytes from *Gdf-9* null mice have larger oocytes than wt mice (Carabatsos *et al.,* 1998; Erickson and Shimasaki, 2000; Fortune, 2003).

However, the follicles cultured in the following chapters had similar sized oocytes and therefore can be assumed to be at the same developmental stage irrespective of genotype. This suggests that although *Dazl* is expressed solely in the oocyte of the murine ovary it does not necessarily determine oocyte development and implies that this gene is involved in regulation of somatic cell development. Studies carried out by Pan *et al.* (2005) have shown that human *DAZL* and *DAZAP1* (*Dazap1* expressed mainly in the oocyte and theca cells in rats) interact with each other and have been postulated to be involved in controlling mitosis. This is further supported by previous studies carried out in this chapter where granulosa cell proliferation was stimulated by one copy of the *Dazl* gene compared to two copies. Future studies examining the expression of *Dazap1* would be beneficial to determine its role in granulosa cell mitosis in follicles from *Dazl* wt and het mice.

3.4.5 Conclusion

The data presented in this study concludes that untreated *Dazl* het mice have a significantly greater percentage of antral follicles compared to wt mice. This difference was diminished in oFF-treated and FSH-treated mice, where both het and wt mice ovaries contained similar percentages of antral follicles. Furthermore, untreated *Dazl* het mice had larger antral follicles with larger granulosa cell areas compared to antral follicles from wt mice, but had oocytes of a similar size. This suggests that a single copy of the *Dazl* gene stimulates granulosa cell proliferation, possibly through increased FSH-sensitivity compared to two copies of the gene. Treatment with either oFF or FSH diminished these differences. Treatment with 10IU FSH was sufficient to raise plasma FSH levels above the required threshold to a level where follicles from wt and het mice respond in a similar manner, with larger follicles, more antral follicles and larger ovaries compared to untreated animals. In addition, treatment with oFF possibly suppressed FSH below the threshold to a level where follicles from both genotypes acted and developed in a similar manner. The similar ovary volume in untreated wt and het mice was unexpected as previous studies in adult mice showed that *Dazl* het mice had larger ovaries. However, the effect of different numbers of copies of this gene may be more marked in adults than at day 21. The exact mechanism of action of Dazl is unknown but this data suggests that the follicles from *Dazl* het mice have greater FSH-sensitivity compared to follicles from *Dazl* wt mice. These observations led to the next study in which this concept was tested by determining the effect of different doses of FSH on follicle growth *in vitro* in a completely controlled environment.

Chapter 4

FSH-sensitivity of Follicles from *Dazl* Wt and Het Mice

4.1 Introduction

Follicle growth is reliant upon various growth factors, with development involving synchronisation of both locally- and pituitary gland-produced growth factors. Gonadotrophins play an essential role in follicle growth and maturation, as demonstrated by the infertility problems encountered in animals that lack crucial genes or receptors that affect gonadotrophin stimulation or secretion. The impact of such factors can be observed in mice which lack the FSHR or growth factor *Gdf-9* and which are both infertile and lack any antral follicles (Dong *et al.,* 1996; Kumar *et al.,* 1997). More recently the importance of bi-directional signalling between the oocyte and granulosa cells and its importance in follicle maturation has been highlighted (Albertini *et al.,* 2001). Recent experiments have demonstrated that the oocyte controls follicle development but granulosa cells are also fundamental for oocyte maturation by stimulating factors such as oestradiol which are essential for normal follicle development (Yong *et al.,* 1994). Oocytes are essential for regulating granulosa cell differentiation and the development of the cumulus cell phenotype (Eppig *et al.,* 1997a; Eppig *et al.,* 1997b). Removal of antral oocytes from follicles and culturing them with granulosa cells resulted in increased LHR expression in cumulus cells when LHR expression is associated with the mural granulosa cell phenotype thereby demonstrating the importance of the oocyte in regulating granulosa cell differentiation (Eppig *et al.,* 1997b). Growth factors Gdf-9 and BMP-15 are expressed in the oocyte and regulate FSH activity and stimulate granulosa cell mitosis (Otsuka *et al.,* 2005).

4.1.1 FSH Sensitivity

FSH stimulates follicle development and granulosa cell proliferation (Vanderhyden *et al.,* 1992). During early follicle development the gonadotrophins induce mitotic activity in the follicle and in the latter stages they stimulate gene expression required for full maturation (Richards, 1994). Ovarian FSHR expression increases in the presence of FSH (Findlay and Drummond, 1999), although recurrent exposure to high FSH leads to a decline in FSH response, apparently due to a decline of cAMP response that is followed by reduced oestradiol and progesterone synthesis (Amsterdam *et al.*, 2002).

The exact mechanisms involved in desensitisation to FSH are unknown (Amsterdam *et al.*, 2002) although the receptor uncouples from the G-protein and FSHR expression is reduced (Simoni *et al.*, 1997; Themmen and Huhtaniemi, 2000; Amsterdam *et al.*, 2002). FSH binding to the FSHR *in vitro* depends upon both temperature and salt concentration. Above 30°C the binding of FSH to the FSHR becomes 'non-reversible' (Andersen *et al.*, 1983; Simoni *et al.*, 1997).

Increased FSH-sensitivity can result from; increased FSHR expression, increased binding of FSH to the FSH receptor or increased stimulation of the signalling pathway (Zeleznik and Kubik, 1986; Simoni *et al.*, 1997; Themmen and Huhtaniemi, 2000). The experiments in this chapter analyse follicle response to lowering doses of FSH (and thereby FSH-sensitivity). The causes of any changes in FSH-sensitivity in this study may involve effects on granulosa cell proliferation, inhibin or oestradiol secretion, *Kit* Ligand, Gdf-9 or Bmp-15 expression as well as FSHR expression or FSH signalling (Zeleznik and Kubik, 1986; Nayudu and Osborn, 1992; Halvorson and DeCherney, 1996; Vitt *et al.*, 2000; Otsuka *et al.*, 2001b; Jin *et al.*, 2005).

4.1.2 Follicle Endocrinology

As detailed in Chapter 1, oestrogen and progesterone are important for folliculogenesis. Oestradiol 17β is the most abundant oestrogen in the ovary (Knobil and Neill, 1994) and low oestradiol production is associated with abnormal growth and atresia (Adashi, 1994; Fehrenbach *et al.*, 1998). Secretion of both oestradiol and progesterone is regulated by FSH and LH respectively, through the cAMP pathway (Yong *et al.*, 1994). The oocyte also regulates steroidogenesis, as oocyte removal from Graafian follicles results in 'spontaneous luteinisation' and elevated progesterone biosynthesis (Vanderhyden and Macdonald, 1998).

The endocrinology of the follicle is a marker of its development and maturation. Oestradiol and progesterone are markers of the latter stages of follicle development (Zeleznik *et al.*, 1985; Nayudu and Osborn, 1992; Yong *et al.*, 1994). Measuring oestradiol and progesterone levels in the culture media enables the development of individual follicle to be assessed, allowing any differences between follicles from *Dazl* wt and het animals to be determined. Oestradiol secretion is greatest in the follicle that becomes dominant and is stimulated by FSH (Nayudu and Osborn, 1992; Valdez *et al.*, 2005). Progesterone secretion increases as oestradiol levels drop and its secretion is maximal at the time of the LH surge and continues to increase after ovulation (Yong *et al.*, 1994; Hillier, 2001).

Other growth factors also have an essential role in follicle growth and development by suppressing plasma FSH (Section 1.8.2). Inhibin A and B are produced in the granulosa cells (though at different stages of follicle development). Inhibin B is secreted in the early stages of follicle development and is an indicator of the number of small follicles and, therefore, a possible marker of ovarian reserve (Groome *et al.*, 1996; Hohmann *et al.,* 2005). Although inhibins suppress FSH, FSH in turn stimulates granulosa cell inhibin synthesis (Smyth *et al.*, 1994; Hohmann *et al.*, 2005).

4.1.3 MMP-2 and MMP-9 Expression and Follicle Development

Both MMP-2 and MMP-9 have the ability to bind to and degrade gelatin as a result of the common 'fibronectin-like sequence' located in their catalytic domain (Curry and Osteen, 2003). The gelatinases play a fundamental role in the degradation of the basal lamina that separates granulosa and theca cells. The basal lamina is composed of type-IV collagen, laminin and fibronectin (Smith *et al.,* 2002; Curry and Osteen, 2003; Visse and Nagase, 2003). Alterations in MMP activity in the *Dazl* follicles could explain the observed differences between genotypes in the rate of bursting of follicles during culture.

In rat antral follicles, protein and mRNA expression of MMP-2 is localised to the granulosa, theca and stroma cells, whereas MMP-9 expression is localised in the theca and stroma cells (Bagavandoss, 1998; Curry *et al.,* 2001; Curry and Osteen, 2003; Smith *et al.,* 2005). However, Liu *et al.* (1998) found MMP-2 only in the theca cells of rat follicles at all stages of development. Both gelatinases have been detected by zymography in equine follicular fluid, with greater expression of MMP-2 compared to MMP-9 in both pre-ovulatory and ovulatory follicles (Riley *et al.,* 2004). In rats, gelatinase expression increases considerably in the latter stages of follicle development, and MMP-2 expression always exceeds MMP-9 expression (Bagavandoss, 1998; Hess *et al.,* 1998; Smith *et al.,* 2002). However, disruption of the murine MMP-2 gene did not affect fertility (Smith *et al.,* 1999). Granulosa cell gelatinase expression increased in response to treatment with FSH (Liu *et al.,* 1998; Ke *et al.,* 2004) suggesting the gelatinases are regulated by gonadotrophins.

The zymography method detailed by Riley *et al.* (2004) allows both latent and active MMP-2 and MMP-9 levels to be assessed. This method is well established for both bovine and equine follicles and previous studies within this laboratory have used this method on pooled murine follicles (Riley *et al.,* 2004; Thomas *et al.,* 2001; Spears and Riley, Personal Communication). MMP secretion by individual murine follicles bordered on the detection limits of this method (Riley, Personal Communication). However, MMP-2 and MMP-9 measurements in pooled follicles or in whole ovaries, may mask subtle differences in individual follicles from the *Dazl* wt and het animals.

MMP-2 and MMP-9 expression in rodents has also been assessed by immunohistochemistry (Bagavandoss, 1998; Curry and Osteen, 2003). Immunohistochemistry only examines expression and therefore this method was not used in this study as this would not necessarily reflect MMP activity and therefore the extent of degradation. The zymography method allows quantification of MMP activity by providing adequate substrate (Riley, Personal Communication). However, this method does not allow measurement of the TIMP inhibitors of MMPs (Smith *et al.*, 1999; Brew *et al.,* 2000).

4.1.4 Aims of Study

The size of a follicle and its endocrinology are markers of follicle development (Nayudu and Osborn, 1992; Spears *et al.,* 1998; Zhao *et al.,* 2000). The aims of the experiments described in this chapter were to assess the FSH-sensitivity of follicles from *Dazl* het mice compared to follicles from *Dazl* wt mice by culturing follicles with: 1IU, 0.1IU or 0.01IU FSH and monitoring their growth rate. Previous studies have shown that 1IU of FSH stimulates follicle growth to the pre-ovulatory stage of development (Spears *et al.,* 1998; Murray *et al.,* 2001). 0.1IU FSH and 0.01IU FSH were chosen to study the effects of low FSH (Spears, Personal Communication). The endocrinology of these follicles was determined by ELISAs measuring oestradiol, inhibin A and inhibin B secretion. During these cultures, some follicles burst on days 3 and 4. Hence, MMP-2 and MMP-9 production by follicles on day 2 of culture was examined.

4.2 Methods

4.2.1 Follicle Culture

To study paracrine and autocrine actions and the effects of specific growth factors on the development of the follicle, individual follicles were dissected and cultured in α-MEM media as detailed in Section 2.6 (Fehrenbach *et al.,* 1998; Murray *et al.,* 1998). This culture system enabled comparison of the growth and development of pre-antral follicles from *Dazl* wt and het mice when treated with various concentrations of FSH.

The isolated follicles were cultured for the standard six-day period in three treatment groups with: 1IU, 0.1IU or 0.01IU FSH (Section 2.6). The follicles across all treatments groups and plates were randomised whenever possible so that wt and het follicles were treated with the FSH doses being examined in the culture. The total number of follicles cultured in these treatment groups is detailed in Table 4.1. Follicle growth and antral development was recorded daily. However, follicles from the *Dazl* strain of mice were dark and therefore accurate determination of antral development in these follicles was more difficult. Therefore, this data was not used for analysis. Following culture, the follicles were frozen at -70°C and the culture media at -20°C.

Genotype	1IU FSH	0.1IU FSH	0.01IU FSH
Dazl Wt	78 follicles 19 animals	58 follicles 11 animals	32 follicles 7 animals
Dazl Het	82 follicles 14 animals	45 follicles 9 animals	39 follicles 6 animals

Table 4.1 Individual cultured follicle numbers and animals.

4.2.1.1 FSH Batch Comparison

Previous studies have found variations in the response of follicles with different batches of FSH (Spears, Personal Communication). The response of follicles from F1 mouse strain, *Dazl* wt and *Dazl* het mice to both the old and new batches of FSH was tested at a concentration of 1IU FSH and follicle growth was recorded.

4.2.2 ELISAs

ELISAs for oestradiol, inhibin A, inhibin B and progesterone were carried out according to the standard protocols described in Section 2.7. Table 4.2 details the day of culture at which samples were assayed. Samples of culture media stored at -20°C were defrosted and aliquots were diluted in α-MEM culture media.

The oestradiol and progesterone antibodies for the ELISA assays have previously been validated (Glasier *et al.*, 1989; Wulff *et al.*, 2001). The ELISAs used to measure inhibin A and inhibin B are well established and have been used to determine inhibin concentrations in both plasma and culture media in several species, from humans to mice (Groome *et al.*, 1996; McNeilly *et al.*, 2000). Inter-assay coefficients of variation are detailed in Table 4.3. The inter-assay coefficient of variation for progesterone could not be calculated as this assay was only run once on one plate.

Genotype	Oestradiol Day 6	Inhibin A and B Day 6	Inhibin B Day 2 and 6	Progesterone Day 6
Wt 1IU FSH	n=59	n=53	n=48	No Sample
Het 1IU FSH	n=57	n=70	n=53	No Sample
Wt 0.1IU FSH	n=43	n=25	n=23	No Sample
Het 0.1IU FSH	n=25	n=26	n=17	No Sample
Wt 0.01IU FSH	n=27	n=13	n=17	No Sample
Het 0.01IU FSH	n=29	n=26	n=4	No Sample
Wt Follicles (368μm D6)	n=13	n=17 (A) & 10 (B)	Day 2 only, n=17	n=6
Het Follicles (368μm D6)	n=18	n=21 (A) & 19 (B)	Day 2 only, n=16	n=9

Table 4.2 Sample numbers and day of culture on which samples were taken for assay.

ELISA	LQC	MQC	HQC
Oestradiol	35%	No MQC	33%
Inhibin A	24%	10%	22%
Inhibin B	20%	20%	30%

Table 4.3 Oestradiol, inhibin A and inhibin B ELISA: Inter-assay coefficients of variation.

4.2.3. Zymography

Follicle cultures for zymography were carried out by Dr. Norah Spears and Dr. Alison Murray (Department of Biomedical Sciences, The University of Edinburgh). Follicles were isolated and cultured (Section 2.6) for a two-day period in α-MEM supplemented with 1IU FSH, 0.5% ascorbic acid and 3% BSA (fatty acid free). Following culture, the follicles were washed in PBS and fixed in Bouin's solution (Bios) (n=2 wt and het follicles) or snap frozen at -70°C (n=10 wt and het follicles). Culture media were stored at -20°C. To concentrate culture media for assay, 20μl aliquots were freeze-dried overnight (6-8mbar, Vacuum Pump: Savant Instruments Inc, New York; Freeze Drying Unit; E.C. Apparatus, New York) and reconstituted in 7μl dH_20, then 7μl of loading buffer was added. This process enabled 20μl of culture media to be dissolved in only 7μl of dH_20 by freezing the solution and then under vacuum removing the ice and leaving a powder.

A blank culture medium sample was run in every culture media gel as a negative control. Individual follicles were homogenised in 80µl of 0.01% SDS for one minute, centrifuged for 20 minutes at 13,000rpm, and the supernatant of each sample collected in an individual fresh Eppendorf tube and stored at -20°C. A protein assay (Section 2.8) was carried out on the supernatant from these follicle samples. A total of 1ng of protein was added in duplicate to each well of a PAGE gel. However, to achieve this, follicle samples were also freeze-dried overnight (6-8mbar) and reconstituted in 7µl dH_20. A total volume of 14µl (7µl sample and 7µl loading buffer) was loaded per well and zymography was carried out as described in Section 2.9. The gels were incubated in digestion buffer for 21 hours. Media samples were destained for 1.5 hours, whereas follicle samples were destained for a further 4 hours to allow detection of lower levels of MMP production.

All gels were semi-quantified as detailed in Section 2.9.4 with all gels being analysed by myself to improve accuracy from subjectivity. This was carried out by drawing a box the area of the bands on the gel – which then gave a reading. A similar reading was taken below every band to get the background per band. This ensured that the background reading was accurate if the destaining was not consistent over the entire gel. The background reading was subtracted from the actual band reading.

4.2.4 Statistical Analysis

Data for follicle growth were analysed using one-way ANOVA, followed by one-tailed, unpaired Student's t-test for data with two variables, and with Tukey's Post-Hoc multiple comparison test for data with three or more variables. Chi-squared analysis was used to analyse numbers of burst and non-burst follicles. One-way ANOVA of Log10 transformed data was performed for oestradiol, inhibin A and inhibin B secretion. This enabled analysis of the data between genotypes and between FSH doses. Unpaired, two-tailed Student's t-tests were carried out after log transformation of the data on 360µm sized follicles from wt and het mice. MMP activity in culture media was analysed by one-way ANOVA (Non-Parametric) with Dunn's multiple test (due to there being 4 variables). Zymography data for MMP production from follicle samples (both media and follicle data) was analysed by the Mann-Whitney test. This non-parametric analysis was used since some data consisted of zero values and therefore could not be log transformed to assume normality. A normal distribution of data had to be assumed for analysis of the correlations between MMP production and follicle diameter. Non-parametric testing using the Spearman test would not have given an r^2 value and, therefore, the correlations were analysed using the Pearson Correlation test (two-tailed).

4.3 Results

4.3.1 Follicle Growth

The FSH batch used at the start of this study was insufficient to enable the study to be completed. It was therefore necessary to compare the effects of the old and new FSH preparations on follicle growth in F1, *Dazl* wt and *Dazl* het mice (Figures 4.1 (A), (B) and (C)). There was no difference in the effects of the two batches of FSH on the growth of follicles from F1 and *Dazl* het mice (Figures 4.1 (A) and (C)). However, growth of follicles from *Dazl* wt mice treated with the new batch of FSH was greater ($p<0.05$) than with the older FSH batch on day 3 (Figure 4.1 (B)), although this difference in growth was not significant by day four and beyond. Hence, all experiments presented in this thesis were obtained from the new FSH batch.

When compared to follicles from F1 mice the follicles from *Dazl* wt and het mice were markedly smaller (Figure 4.1 (D)). On day four of culture, follicles from het mice were significantly greater in size ($p<0.01$) than follicles from wt mice. However, there was no difference at this stage between follicles from *Dazl* (wt and het) and F1 mice on day four. By day five of the culture, differences between follicles from wt and het mice remained ($p<0.05$), and follicles from F1 mice were substantially larger ($p<0.01$) compared to follicles from wt mice. At the end of the culture, follicles from het mice were still significantly larger than follicles from wt mice ($p<0.05$). Furthermore, follicles from F1 mice were also larger than follicles of either *Dazl* wt or het mice ($p<0.001$ and $p<0.01$, respectively).

Follicle growth was assessed for all genotypes at each FSH dose tested. Follicles from wt animals cultured in the presence of 1IU FSH varied greatly ($p<0.01$) on day three between experiments. However, by day four and beyond this variation had disappeared (Appendix E (A)). There was no difference in growth of follicles from wt mice cultured in the presence of 0.1IU and 0.01IU FSH between experiments (Appendix E (C) and (E)). Similarly, follicle growth from het mice cultured in the presence of 1IU, 0.1IU and 0.01IU FSH was not significantly different between experiments (Appendix E (B), (D) and (F)).

Follicles from wt and het animals showed a dose response when cultured with increasing FSH levels (Figure 4.2). Follicles from het mice were significantly greater in diameter on day 6 ($p<0.01$, $p<0.05$ and $p<0.01$) than follicles from wt mice when cultured with 1IU, 0.1IU and 0.01IU FSH (Figure 4.3).

4.3.2 Incidence of Burst Follicles

On days three and four of culture many follicles burst. However, fewer follicles from het mice burst ($p<0.05$) during culture when treated with 1IU and 0.1IU FSH compared to follicles from wt mice (Figure 4.4). There was no variation in the incidence of bursting between follicles from wt and het mice cultured with 0.01IU FSH (Figure 4.4).

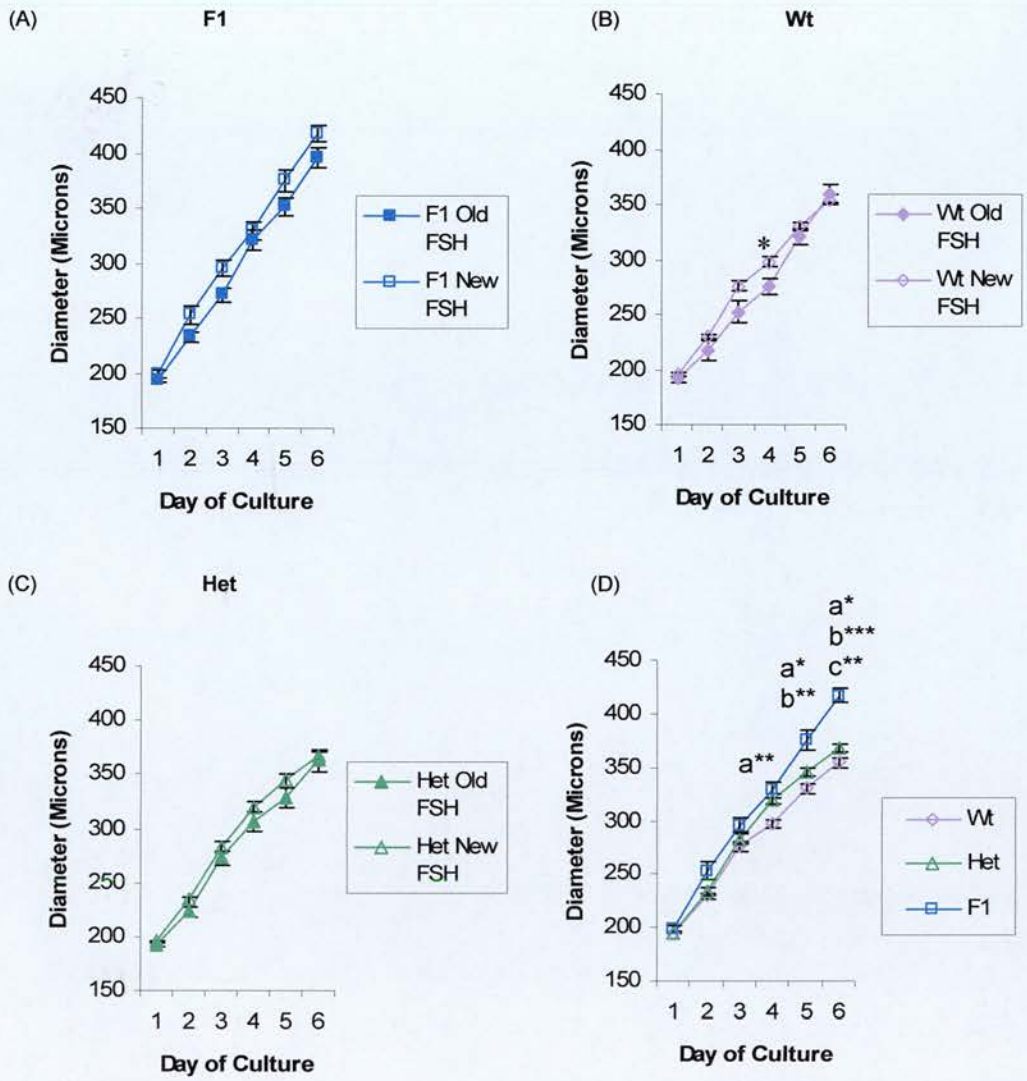
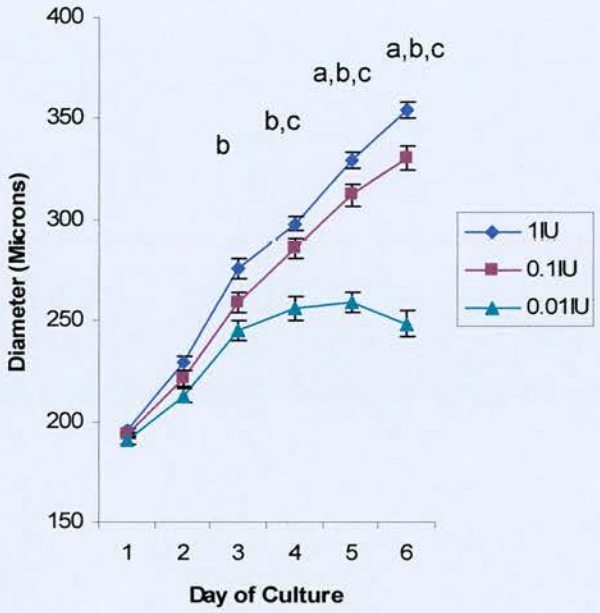


*Figure 4.1 The effect of two different FSH batches on follicle growth in vitro. (A) F1 (n=29 old FSH and n=8 new FSH), (B) Dazl wt (n= 15 old FSH and n=78 new FSH) and (C) Dazl het (n=27 old FSH and n=82 new FSH) mice. (D) Growth rates of F1 (n=8) and Dazl wt (n=78) and Dazl het (n=82) mice follicles cultured in the presence of 1IU FSH. a= follicles from wt mice compared to het mice, b= follicles from F1 mice compared to wt mice and c= follicles from F1 mice compared to het mice. * p<0.05, ** p<0.01 and *** p<0.001. Values are means ± S.E.M.*

(A) **Wild Type**



(B) **Heterozygous**

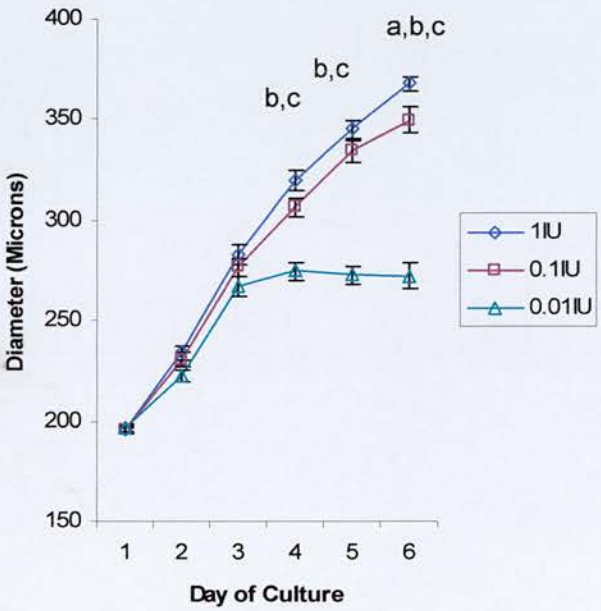


Figure 4.2 Follicle growth of Dazl (A) wt follicles cultured in the presence of 1IU FSH (n=78), 0.1IU FSH (n=58) and 0.01IU FSH (n=32)and (B) het follicles cultured in the presence of 1IU FSH (n=82), 0.1IU FSH (n=45) and 0.01IU FSH (n=39). a= follicles treated with 1IU FSH compared to 0.1IU FSH, b= follicles treated with 1IU FSH compared to 0.01IU FSH and c= follicles treated with 0.1IU FSH compared to 0.01IU FSH. For clarity all p values are taken as <0.05, see text for all details. Values are means ± S.E.M.

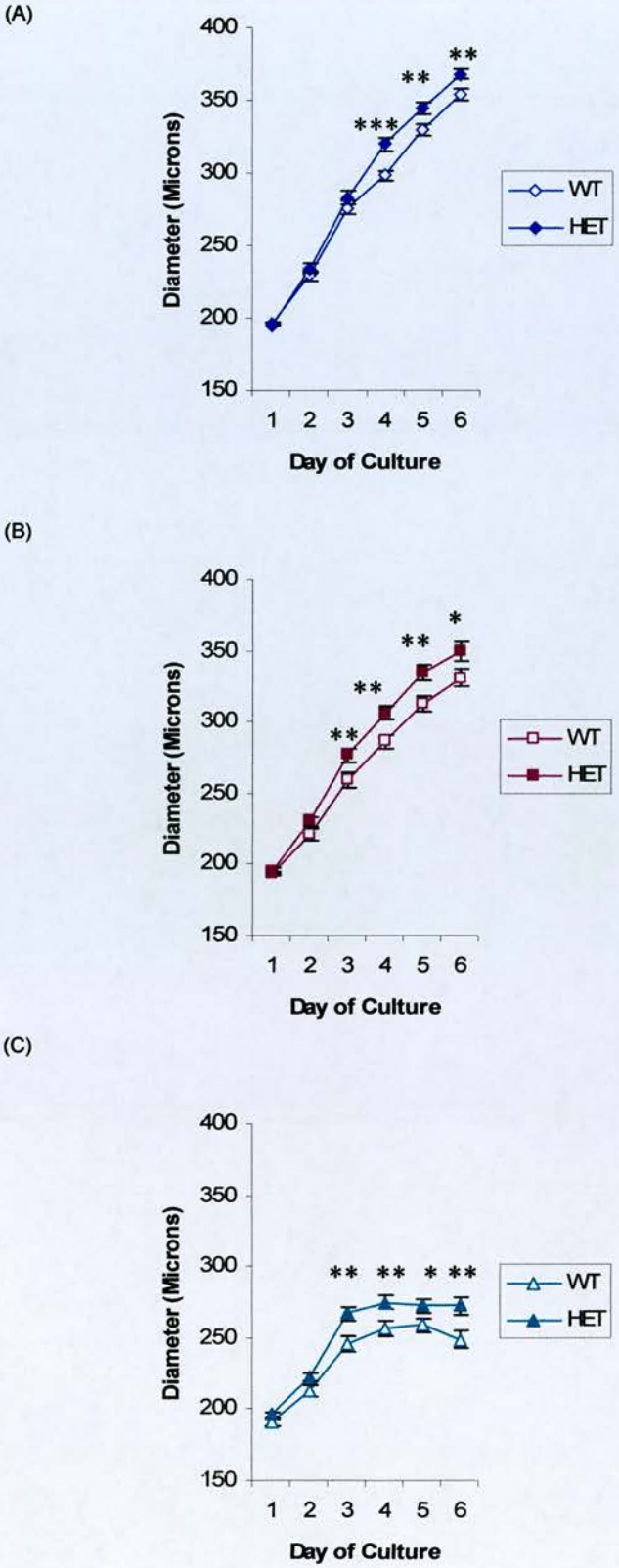


*Figure 4.3 Growth rates for Dazl wt and het follicles cultured with (A) 1IU FSH (wt n=78 and het n=82) (B) 0.1IU FSH (wt n=58 and het n=45) and (C) 0.01 IU FSH (wt n=32 and het n=39). * p<0.05, ** p<0.01 and *** p<0.001. Values are means ± S.E.M.*

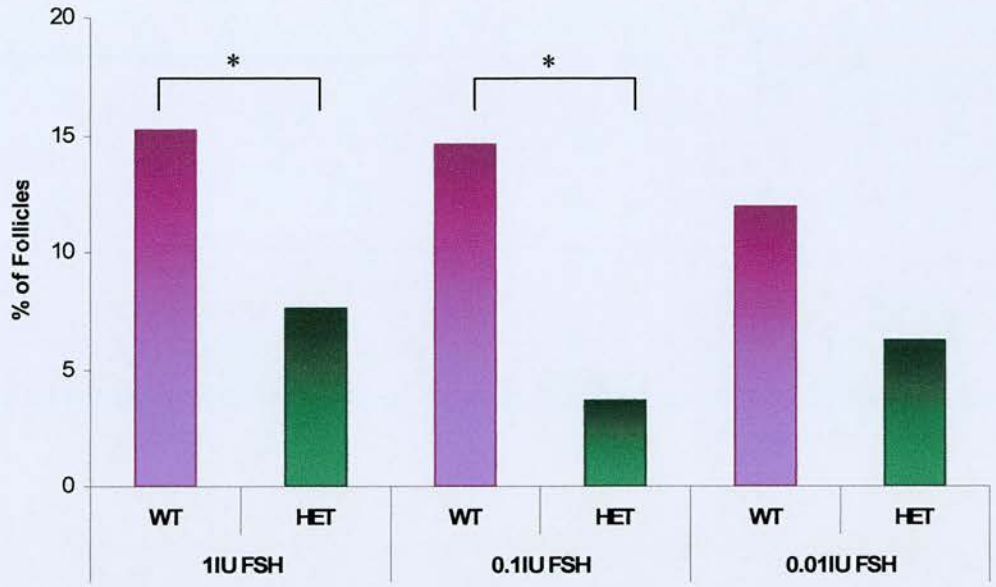


*Figure 4.4 The percentage of burst follicles on days 3 and 4 of culture for wt and het follicles in 1IU FSH (n= 17 and 7 respectively), 0.1IU FSH (n= 12 and 2 respectively) and 0.01IU FSH (n= 6 and 3 respectively). * p<0.05. Values are percentage of observed burst follicles during culture.*

4.3.3 Oestradiol, Inhibin A and Inhibin B Secretion

Oestradiol secretion by follicles cultured with 1IU and 0.1IU FSH (Figure 4.5) did not differ between genotypes. However, follicles from *Dazl* het mice cultured with 0.01IU FSH secreted considerably less oestradiol ($p<0.001$) compared to follicles from *Dazl* wt mice (Figure 4.5). Due to sample volume constraints assays were carried out on as many samples as possible although not all samples could be assayed. Due to there being significant variation between oestradiol secretion by *Dazl* wt and het mice when treated with 0.01IU FSH the growth rate of the follicles in this FSH dose group assayed was examined. This ensured that the growth rate of the follicles assayed for oestradiol secretion was the same as the growth of all follicles treated with 0.01IU FSH (Figure 4.3 (C)). This analysis demonstrated no difference in the size, on day six, of the follicles from either genotype and cultured with 0.01IU FSH and the follicles that were later assayed (Figure 4.6), therefore the assayed follicles were representative of the follicle treatment group.

There was no difference in inhibin B secretion on day six of culture by follicles from wt and het animals cultured with all doses of FSH (Figure 4.7). Inhibin A secretion did not differ on day six for follicles from wt and het mice cultured with either 1IU or 0.1IU FSH. However, follicles from het mice cultured with 0.01IU FSH secreted higher levels of inhibin A ($p<0.05$) than follicles from wt mice (Figure 4.7). Inhibin A secretion was considerably greater than inhibin B secretion for follicles from both wt and het animals cultured with 1IU ($p<0.001$) or 0.01IU FSH ($p<0.01$ and $p<0.001$ respectively; Figure 4.7). Although there was no significant difference in inhibin A and inhibin B secretion for follicles from wt and het mice cultured with 0.1IU FSH, both wt and het follicles secreted higher inhibin A levels (Figure 4.7). Inhibin B levels secreted by follicles of either wt or het mice cultured with 1IU FSH were not significantly different to levels secreted by follicles cultured with 0.1IU FSH. However, inhibin B levels secreted for both wt and het mice were significantly lower with 0.01IU FSH compared to 1IU FSH ($p<0.001$ for both wt and het mice) or 0.1IU FSH ($p<0.01$ for wt mice and $p<0.001$ for het mice; Figure 4.7).

Follicles from wt and het mice cultured with 1IU or 0.1IU FSH secreted greater levels of inhibin B on day six compared to day two (Figure 4.8). Follicles cultured with 0.01IU FSH secreted similar levels of inhibin B on day two and day six of culture (Figure 4.8). Follicles from het mice cultured with 1IU or 0.1IU FSH secreted similar levels of inhibin B on day two but significantly higher ($p<0.05$) levels on day six compared to follicles from wt mice (Figure 4.8). However, follicles from het mice cultured with 0.01IUFSH secreted similar levels of inhibin B on days two and six of culture compared to follicles from wt mice (Figure 4.8).

To determine whether inhibin B levels *in vitro* during the early stages of culture could predict follicle survival, levels of inhibin B secreted by follicles of wt and het mice on day two of culture were measured and analysed according to their outcome during culture (i.e. survived culture or did not survive culture). Inhibin B levels on day two did not differ in the follicles that survived the culture period compared to those that did not survive at all doses of FSH (Figure 4.9).

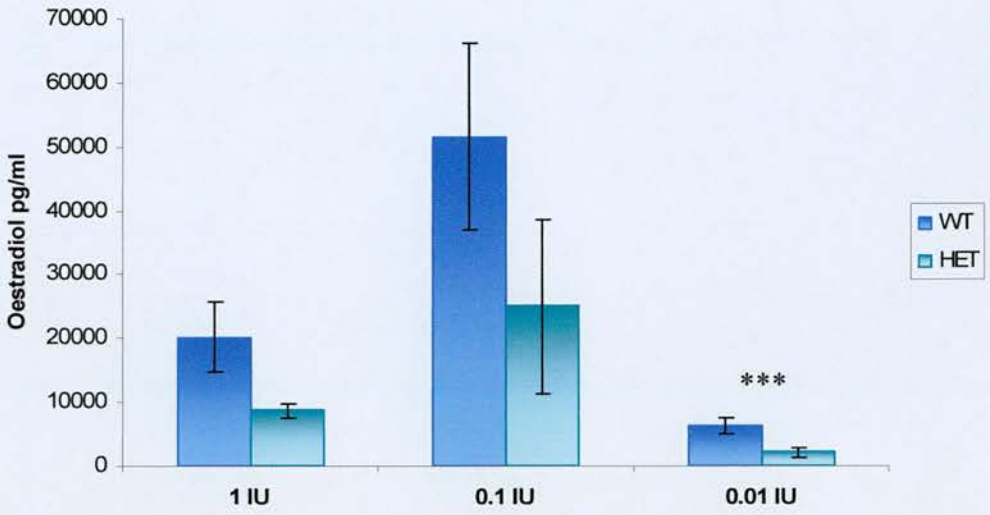


*Figure 4.5 Oestradiol secretion by follicles from Dazl wt and het mice cultured with 1IU FSH, 0.1IU FSH and 0.01IU FSH on day 6. Wt and het oestradiol secretion at 0.01IU FSH = *** p<0.001. Values are means ± S.E.M (n= 59 follicles wt 1IU FSH, n=57 follicles het 1IU FSH, n=43 follicles wt 0.1 IU FSH, n=25 follicles het 0.1IU FSH, n=27 follicles wt 0.01IU FSH and n=29 follicles het 0.01IU FSH).*

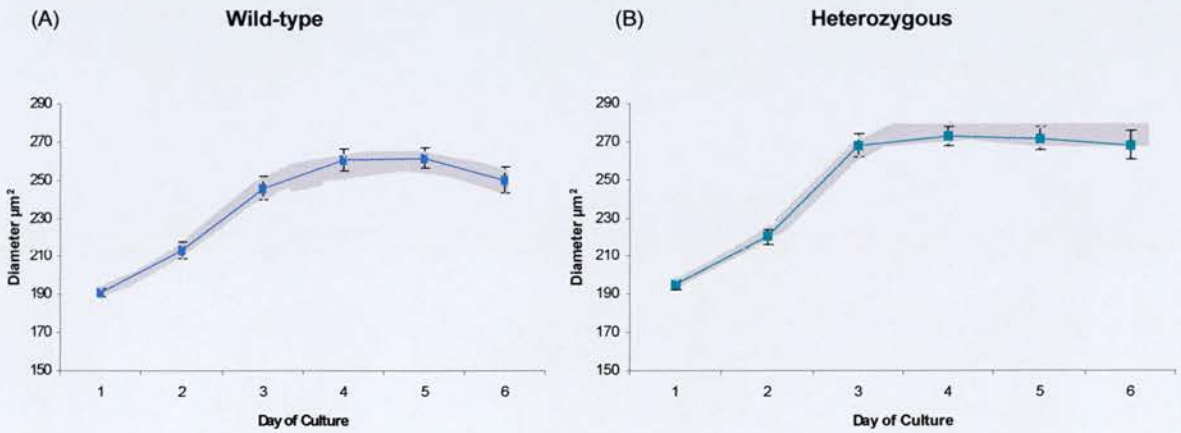


Figure 4.6 Growth rates of follicles from wt (A) and het (B) mice analysed for oestradiol secretion cultured with 0.01IU FSH. Values are means ± S.E.M (n=27 follicles wt 0.01IU FSH and n=29 follicles het 0.01IU FSH). The grey shading demonstrates the growth range for all follicles cultured with 0.01IU FSH in comparison to those assayed for oestradiol and cultured with 0.01IU FSH.

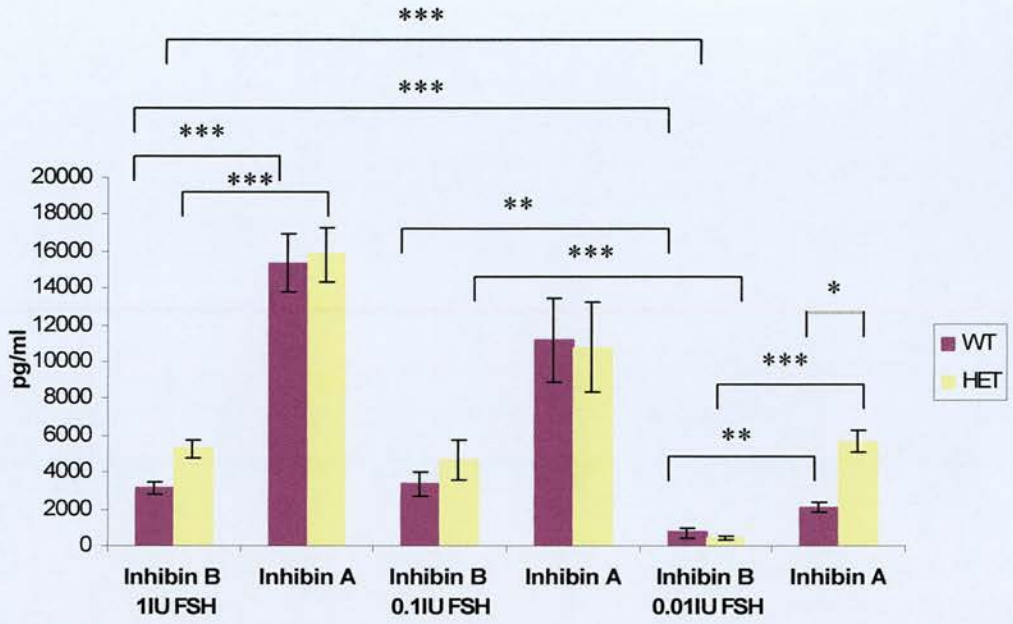


*Figure 4.7 Inhibin B and inhibin A secreted by follicles from Dazl, wt and het mice cultured with 1IU FSH, 0.1IU FSH and 0.01IU FSH on day 6. * p<0.05, ** p<0.01 and *** p<0.001. Values are means ± S.E.M (n=53 wt 1IU FSH, n=70 follicles het 1IU FSH, n=25 follicles wt 0.1IU FSH, n=26 follicles het 0.1IU FSH, n=13 follicles wt 0.01IU FSH and n=26 follicles het 0.01IU FSH).*

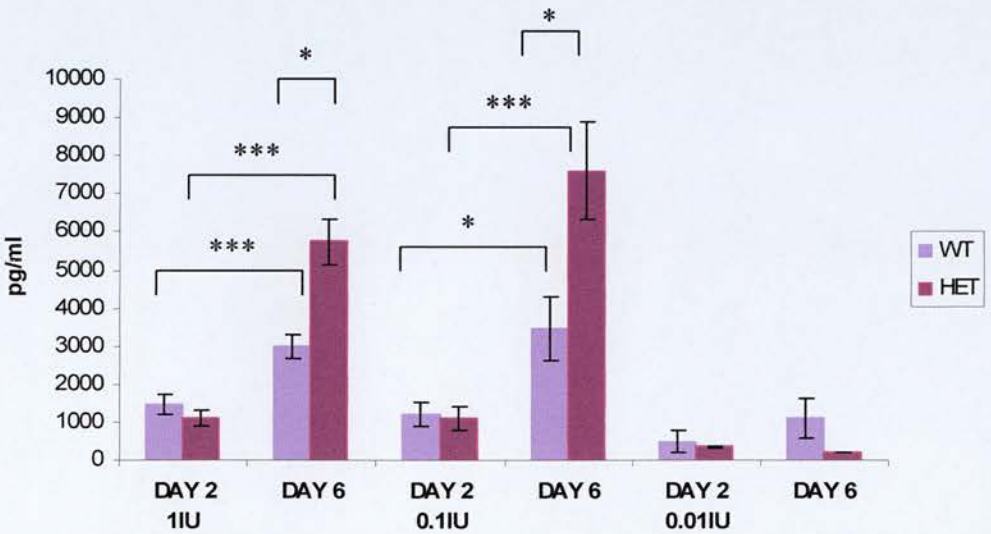


*Figure 4.8 Inhibin B secreted by follicles of wt and het mice cultured in 1IU FSH, 0.1IU FSH and 0.01IU FSH, on days 2 and 6. * p<0.05 and *** p<0.001. Values are means ± S.E.M (n= 48 follicles wt 1IU FSH, n=53 follicles het 1IU FSH, n=23 follicles wt 0.1IU FSH, n=17 follicles het 0.1IU FSH, n=17 follicles wt 0.01IU FSH and n=4 follicles 0.01IU FSH).*

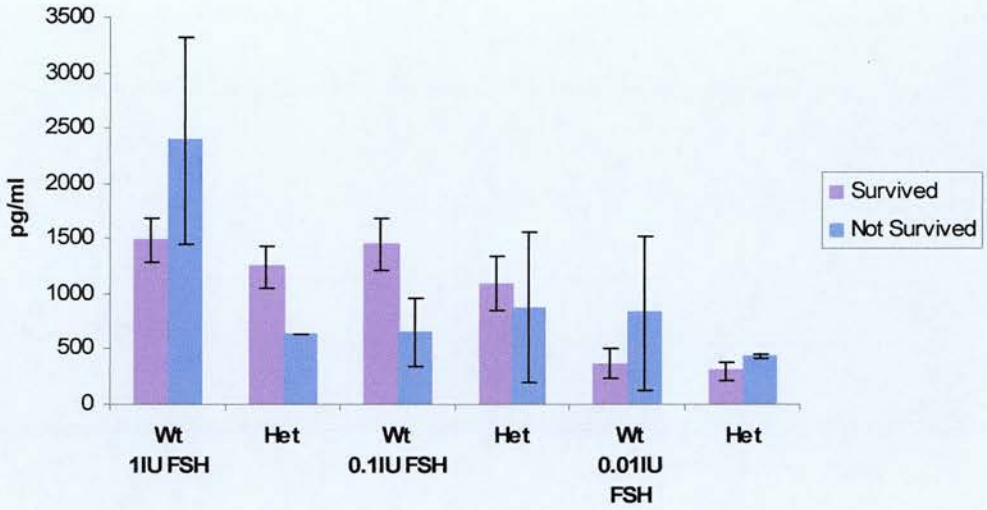


Figure 4.9 Inhibin B secreted on day 2 by follicles of wt and het mice that survived or did not survive in culture with 1IU FSH, 0.1IU FSH or 0.01IU FSH. Values are means ± S.E.M (n= 82 survived and 7 not survived follicles wt 1IU FSH, n= 61 survived and 1 not survived follicles het 1IU FSH, n= 49 survived and 5 not survived follicles wt 0.1 IU FSH, n= 22 survived and 2 not survived het follicles 0.1IU FSH, n= 37 survived and 7 not survived follicles wt 0.01IU FSH and n= 13 survived and 2 not survived follicles het 0.01IU FSH).

4.3.4 Steroid Secretion by Similarly Sized Follicles

Follicles from het mice cultured with all doses of FSH were significantly greater in size than follicles from wt mice (Figure 4.3). Oestradiol and progesterone secretion (Figure 4.10 (A) and (B) respectively) by follicles from wt and het mice did not differ although concentrations were much more variable in wt than in het follicle incubates. Similarly, follicles from wt and het animals secreted similar amounts of inhibin A and inhibin B on day six of the culture (Figure 4.10 (C)) and of inhibin B on day two of culture (Figure 4.10 (D)).

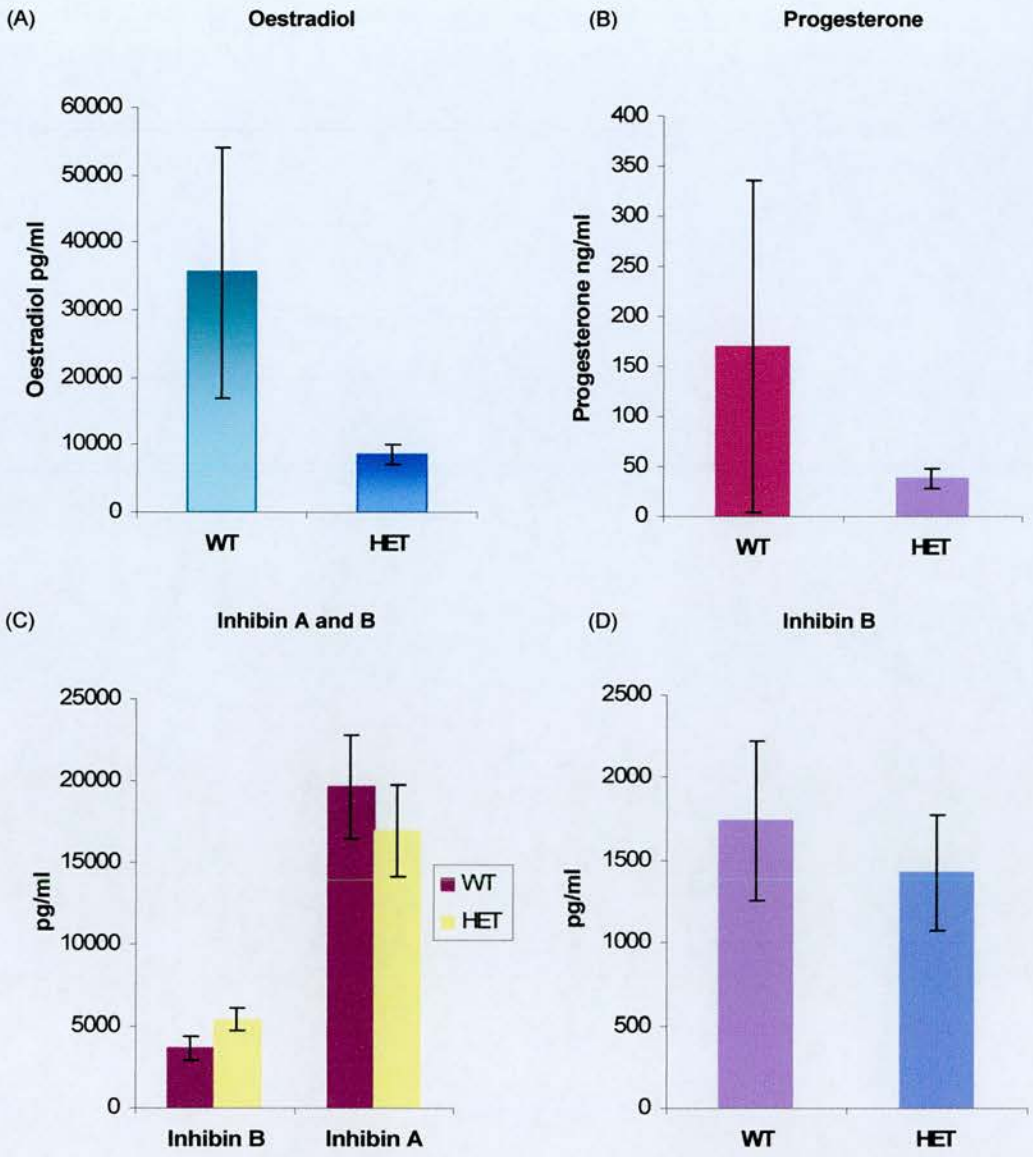


Figure 4.10 Secretion of (A) oestradiol (n=13 wt and n=18 het follicles), (B) progesterone (n=6 wt and n=9 het follicles), (C) inhibin B and A on day 6 of culture (wt and het inhibin B and A n=10 and 17 wt and n=19 and 21 het respectively and (D) inhibin B (n=17 wt and n=16 het follicles) on day two of culture by follicles of Dazl wt and het mice that were 368μm in diameter, cultured in the presence of 1IU FSH. Values are means ± S.E.M.

4.3.5 Zymography

The basement membrane of the follicle provides a separate micro-environment for surrounding somatic cells. ECM regulates fluid dynamics and controls proteins that enter and exit the follicle (Rodgers and Irving-Rodgers, 2002). The reduced incidence of bursting during culture observed in follicles from het mice may reflect ECM remodelling by MMP production. Zymography enabled the detection and quantification of active and latent forms of MMP-2 and MMP-9 (discussed in Section 2.9).

MMP-9 production was undetectable in both culture media and follicle extracts (Figure 4.11 (A), (B) and (C)). Latent MMP-2 expression was detected in culture media (Figure 4.12 (A)) but in wt and het samples levels did not vary (Figure 4.12 (A)). There was a marked decrease in the production of active MMP-2 compared to latent MMP-2 in both wt and het samples ($p<0.01$ and $p<0.001$, respectively). Levels in the follicle samples were almost undetectable and required further destaining (Figure 4.11 (B) and (C)). Only the latent form of MMP-2 was detectable in follicle samples and there was no difference in its production between follicles from wt and het mice (Figure 4.12 (B)). The levels of latent MMP-2 production were greater in media samples than in follicle samples for both wt and het mice ($p<0.001$; Figure 4.12 (B)). A third band with a slightly higher molecular weight compared to active MMP-2 was present in culture media. However, levels were too low to permit accurate quantification (Figure 4.11 (A)).

The size of follicles on day two of culture ranged from 161-241μm diameter. The levels of latent MMP-2 in both media and follicle samples of follicles with a similar size did not vary between wt and het mice (Figure 4.13). However, a higher level of latent MMP-2 production were found in media samples versus follicle samples for wt mice ($p<0.05$) but not for het mice (Figure 4.13). There was no correlation between latent MMP-2 expression and follicle diameter for either culture media or follicle samples of wt or het mice (Figure 4.14).

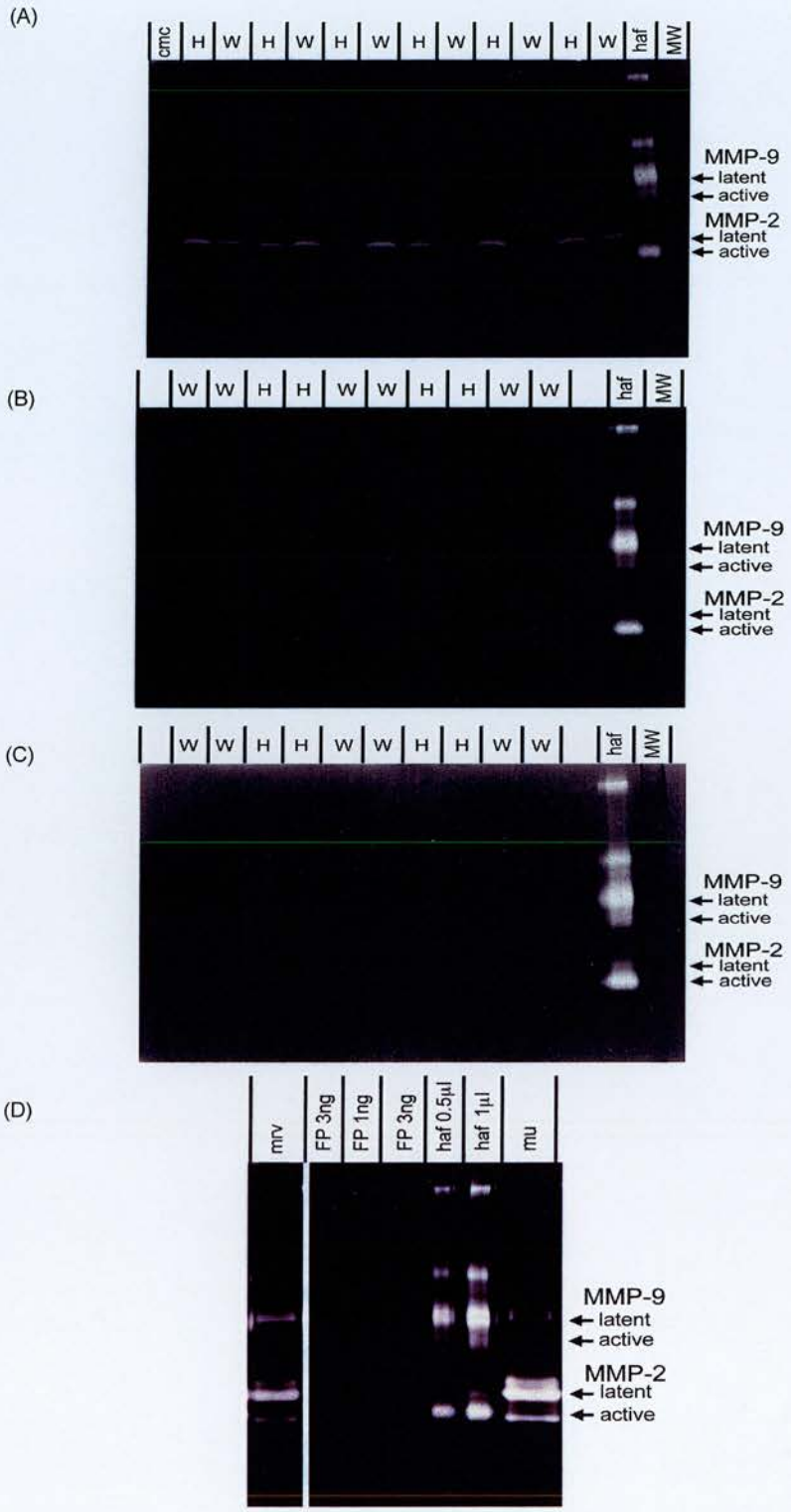


Figure 4.11 MMP production in zymograms for (A) culture media, (B) follicles prior to further destaining and (C) follicles after further destaining showing latent and active forms of MMP-2 and MMP-9. A control gel was run with previous characterised samples (D). No MMP-9 expression is present in any of the samples ((A)-(C)). Human amniotic fluid (haf) was a positive control for MMP-2 and MMP-9 expression. cmc= culture media control, mrv = mouse right ventricle, FP = follicles pooled, mu = mouse uterus and MW = molecular weight marker. The haf used was diluted to two concentrations to determine the quantity which demonstrated the clearest result.

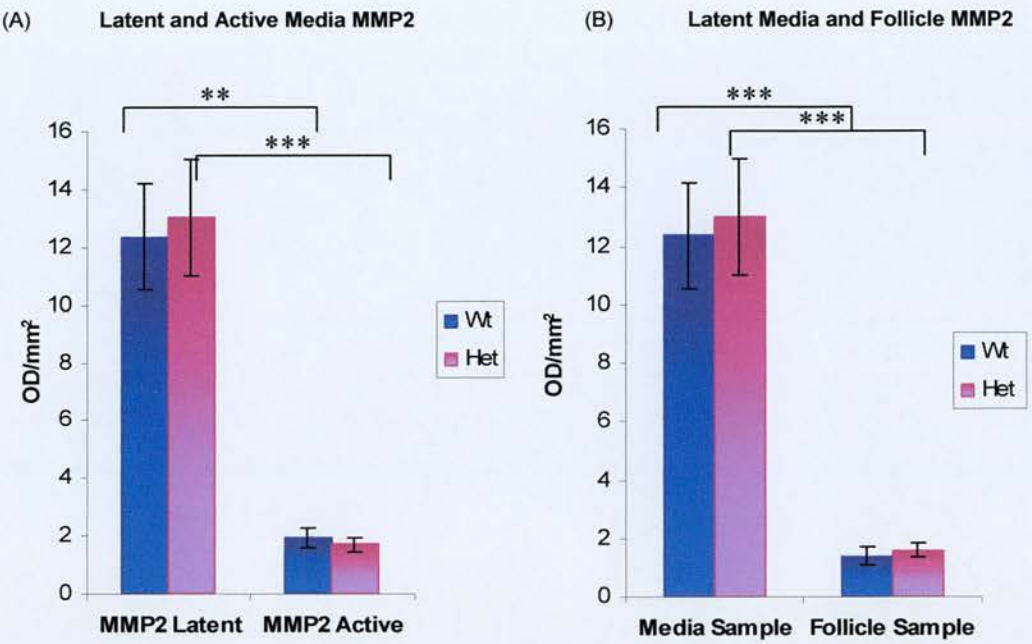


*Figure 4.12 Secretion of (A) latent and active MMP-2 in culture media (n=12 samples from Dazl wt and het mice) and (B) latent MMP-2 in culture media and follicle tissue on day 2 of culture by follicles of wt and het mice (n=12 media samples and 10 follicle samples from Dazl wt and het mice). ** p<0.01 and *** p<0.001. Values are means ± S.E.M.*

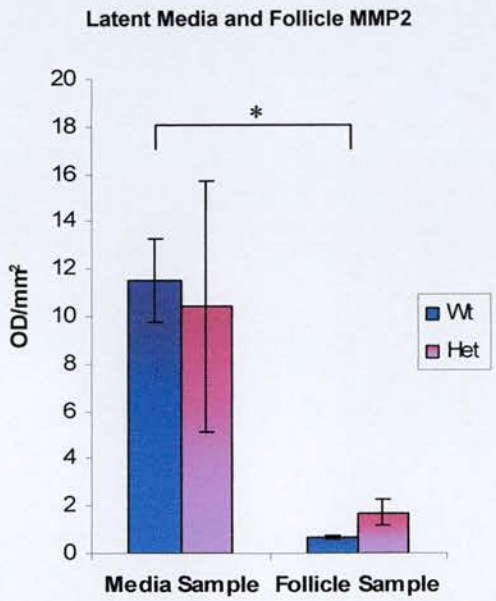


*Figure 4.13 Latent MMP-2 secretion in culture media and follicle tissue from follicles from Dazl wt and het mice (sized 207μm in diameter) on day 2 of culture. * p<0.05. Values are means ± S.E.M (n=4 and 3 samples from wt and het mice respectively).*

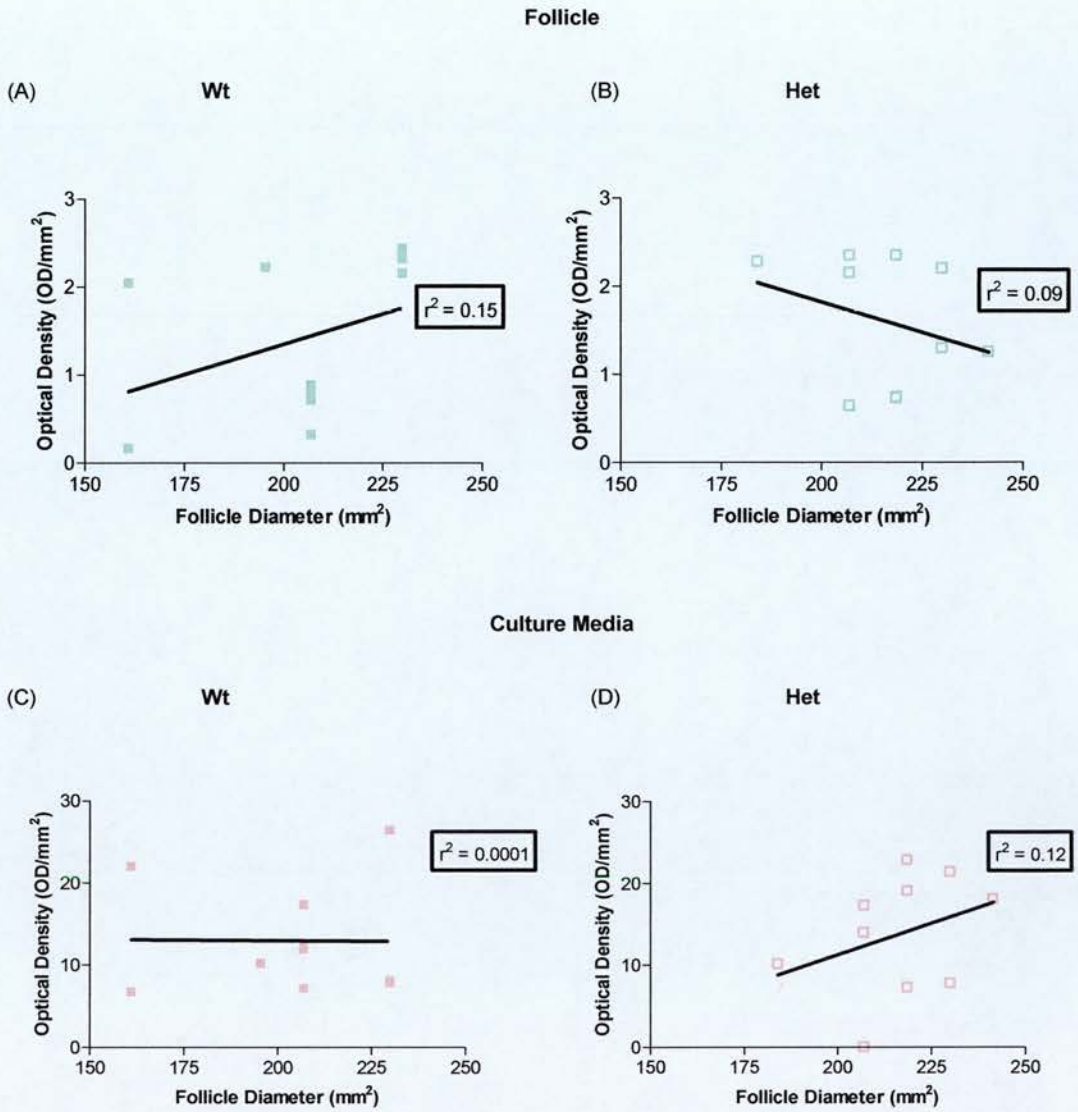


Figure 4.14 Lack of correlation between secretion of latent MMP-2 in follicle tissue and follicle diameter ((A) and (B)) and between culture media latent MMP-2 and follicle diameter ((C) and (D)) for ((A) and (C)) wt and ((B) and (D)) het mice.

4.4 Discussion

4.4.1 FSH Batch

The data presented in this chapter demonstrates that there was little variation between the two batches of FSH and their overall effects on follicle growth and development (Figure 4.1 (A-C)). Previous studies demonstrated that there could be great variation between FSH batches and their effects in stimulating follicle growth (Spears, Personal Communication). There was significant variation between the old and new FSH batches at only one time point in the follicle cultures (1IU FSH day 4 of culture) from wt mice, the new FSH stimulating follicle growth significantly more than the old FSH batch. However to limit any variation observed and to ensure consistency the new FSH batch was used for all further experiments.

4.4.2 Culture System

The follicle culture method used in this study was described by Murray *et al.* (2001) and enables follicle growth to be monitored in a controlled environment. This well-established culture method enables follicles cultured in 1IU FSH to develop successfully to the pre-ovulatory stage of development (Nayudu and Osborn, 1992; Spears *et al.*, 1998; Murray *et al.*, 2001). In addition, manipulation of the culture conditions (i.e. lowering FSH) allows their effects on follicle growth to be studied. Day 21 mice were used in this experiment as follicle isolation is easier at this age, when corpora lutea are absent (Spears, Personal Communication). The analysis of the inter-culture variation demonstrated that this was a robust culture system (Appendix E), with no significant variation observed between cultures of follicles from *Dazl* het mice at all doses of FSH.

The growth of follicles from wt mice cultured with 1IU FSH differed significantly ($p<0.01$) between cultures on day 3 though by the end of the culture this difference was no longer evident. The difference in growth rates between experiments was possibly due to follicles from *Dazl* wt mice being less stringently controlled than follicles from *Dazl* het mice. Previous studies have demonstrated that follicle growth varies considerably from one follicle to another within the same ovary (Hirshfield and Midgley, 1978a; Hirshfield and Midgley, 1978b). Together with the data in this study, this suggests that the follicles from *Dazl* het mice are more uniform in terms of growth compared to follicles from *Dazl* wt mice.

In the course of these studies an adaptation to the culture system was reported in which follicles were cultured inverted and no oil was thereby required to limit evaporation of culture media (Wycherley *et al.,* 2004). These modifications enhance follicle growth beyond that observed using the current method described in this chapter. This may be due to follicles in the inverted culture having a better supply of oxygen (Wycherley *et al.,* 2004). However, this new culture method was first published only when the experiments in this chapter were already in progress. Future studies would compare follicles growth and development using this methodology.

4.4.3 F1 vs. *Dazl* Follicle Growth

The results of the experiments demonstrated in this chapter showed that there was significant follicle growth variation between F1 and *Dazl* mice. Follicles from F1 mice grew significantly larger than follicles from both *Dazl* wt and het mice ($p<0.001$ and $p<0.01$ respectively; Figure 4.1 (D)). Previous studies demonstrated differences between strains in ovarian morphology, follicle numbers and in the rate at which the follicle reserve was exhausted (Canning *et al.,* 2003). These observations coupled with results presented in this thesis, suggest that the size to which follicles can develop is strain dependent. No studies to my knowledge have recorded this information and therefore a comparison with other studies is not possible. However, previous studies have demonstrated that follicle size can vary within different genotypes of animals of the same strain. *Gdf-9* and BMP-15 stimulate granulosa cell mitosis leading to variation in follicle size between genotypes (Erickson and Shimasaki, 2001; Otsuka *et al.,* 2000; McNatty *et al.*, 2004; Shimasaki *et al.,* 2004). Animals from the different *Gdf-9* and *Bmp-15* backgrounds have variations in ovulation rates (Yan *et al.,* 2001; McNatty *et al.*, 2004) as observed in the Dazl mice (McNeilly *et al.,* Unpublished; Appendix A) and the results in Chapter 3 confirmed similar variation in the *Dazl* strain. Follicles from *Dazl* het mice had larger follicles and granulosa cell mass than follicles from *Dazl* wt mice. Previous studies have shown that the *Dazl* het mice have larger litter sizes than wt mice (McNeilly *et al.,* 2000; McNeilly *et al.,* Unpublished; Appendix A). This suggests that (like *Gdf-9* and *Bmp-15* oocyte factors) *Dazl* also affects both follicles size and ovulation rates. The mechanisms by which it does so remain unknown, although increased FSH-sensitivity in follicles from het mice is likely to be responsible.

Analysis of follicles from F1 and *Dazl* wt and het mice initially used follicles from F1 mice as controls, as the laboratory group where these experiments were carried out had previously used the F1 strain (Murray *et al.,* 2001; Spears *et al.,* 2002). F1 mice were therefore used as controls when culturing follicles from the *Dazl* strain of mice. The experiments in this chapter compared follicles from *Dazl* wt and het mice, where any variation observed would be due to genotype, and not the strain of the mice.

Since FSH stimulates follicle growth and development, follicle size is used as a marker of follicle development (Hirshfield and Midgley, 1978b; Canipari, 2000; Sasson *et al.,* 2003a). The data in this chapter suggests that follicles from F1 mice have greater FSH-sensitivity compared to follicles from *Dazl* het and wt mice. However, *Dazl* het mice had larger litters compared to controls (McNeilly *et al.,* 2000; McNeilly *et al.,* Unpublished; Appendix A). Hyper-stimulation by FSH results in decreased FSHR activation and signalling (Simoni *et al.,* 1997; Amsterdam *et al.*, 2002; Amsterdam *et al.*, 2003). This may explain why the F1 mice (which seem more FSH-sensitive) had smaller litters compared to *Dazl* het mice. The *Dazl* wt mice had normal litter sizes similar to F1 mice (McNeilly, Personal Communication) but follicles from these mice were significantly smaller compared to F1 mice, suggesting that *Dazl* wt mice had insufficient FSH to stimulate follicle growth compared to F1 mice or that follicle maturation was possibly reduced in F1 mice due to FSH-desensitisation.

4.4.4 *Dazl* Wt and Het Follicles FSH Sensitivity

The experiments outlined in this chapter were designed to compare the FSH-sensitivity of follicles from *Dazl* het mice with those of *Dazl* wt mice. Follicles from *Dazl* het mice cultured with 1IU FSH and 0.1IU FSH were significantly larger than follicles from *Dazl* wt mice (Figure 4.3). These doses of FSH stimulated follicle growth and development resulting in follicles of greater than 300μm diameter. Follicle cultures using this strain of mouse have not been published to our knowledge but these doses of FSH have been demonstrated previously to stimulate follicle growth (Spears *et al.,* 2002). Reducing the level of FSH to 0.01IU stimulated follicle growth to a maximum of only 275μm in diameter (Figure 4.3 (C)), where-after follicle growth was either maintained (follicles from het mice) or follicles regressed (follicles from wt mice). Studies have demonstrated that follicle growth and development is reliant upon FSH *in vitro,* and without sufficient FSH, follicle growth is impaired (Spears *et al.,* 1998). Therefore, this data suggests that FSH levels below 0.1IU FSH do not stimulate follicle growth but can maintain the development that has already occurred. Furthermore, the follicles from the *Dazl* het mice were significantly larger than follicles from wt mice at all doses of FSH, and even at 0.01IU FSH, follicles from het mice were more responsive to FSH.

As mentioned previously, FSH-sensitivity is reliant upon several factors. These factors include FSHR expression, FSH binding and activation of the FSH signalling pathway (Simoni *et al.,* 1997; Themmen and Huhtaniemi, 2000; Amsterdam *et al.*, 2002; Amsterdam *et al.*, 2003). Furthermore, although FSH primarily signals via the PKA pathway, recent studies have suggested that other signalling pathways are activated by FSH (Zelenik *et al.,* 2003). Studies in the previous chapter demonstrated that oocytes from wt and het *Dazl* mice were of a similar size.

Oocyte size is often used as a marker of oocyte maturation (Zuckerman, 1951). Coupled with the experiments in this current chapter, the data suggest that follicles from *Dazl* het mice are more FSH-sensitive than follicles from wt mice, although oocyte maturity is similar in both wt and het mice. This suggests that the *Dazl* gene has paracrine actions on the granulosa cells.

The exact mechanisms by which *Dazl* affects follicle growth are not known at present (Moore *et al.*, 2003a). *Dazl* action has been postulated to be linked to several proteins (34) but the extent to which these factors affect follicle development has not been studied (Venables *et al.*, 2001). Studies in this and the previous chapter suggest that follicles from the het mice are more FSH-sensitive compared to follicles from wt mice. In addition, these studies suggest that a single copy of the *Dazl* gene stimulates granulosa cell proliferation and increases FSH-sensitivity compared to wt mice. In order to determine how this affects follicle sensitivity to FSH future experiments should involve quantitative analysis of FSHR expression and assess that of down-stream signalling messengers (such as cAMP) to determine the activation of the signalling pathway utilised. The greater granulosa cell proliferation in the follicles from het mice compared to wt mice suggests that these follicles may have greater numbers of FSHRs explaining the increased FSH-sensitivity.

Although follicles are not reliant upon FSH until the latter stages of development, primordial follicles have been shown to express FSHRs (O'Shaughnessy *et al.*, 1996; Findlay and Drummond, 1999). It has been suggested that these receptors may not be fully functional. However when small follicles were treated with FSH, follicle growth and development was enhanced (Simoni *et al.*, 1997). The increased FSH-sensitivity observed in this study could simply be a result of longer exposure to FSH levels above the FSH threshold. Follicles which exceed the FSH threshold (or are stimulated for a longer duration) become more developmentally advanced, requiring stimulation by LH instead of FSH (Brown, 1978; Zeleznik *et al.*, 1985; Zeleznik, 2004).

Greater LHR expression is observed in mural granulosa cells of follicles which have been stimulated by FSH (Eppig *et al.*, 1997b; Hillier, 2001). Therefore greater sensitivity to FSH could result in more rapid maturation of the follicles from *Dazl* het mice compared to wt, and could explain the larger litters observed in het mice. The results presented in this chapter suggest that the *Dazl* gene works in ovarian follicles by affecting the granulosa cells, and implies a paracrine effect in the ovarian follicle.

4.4.5 Oestradiol, Inhibin A, Inhibin B and Progesterone

The endocrinology of follicles can be used as an index of the stage of follicle maturation (Adashi, 1994; Yong *et al.,* 1994). The experiments in this chapter examined oestradiol, inhibin A, inhibin B and progesterone secretion by cultured follicles from *Dazl* wt and het mice to determine whether the endocrinology of the follicles was affected by the number of copies of the *Dazl* gene. Since oestrogen, inhibin A and inhibin B secretion is FSH-dependent this would also confirm differences in FSH-sensitivity.

Oestradiol secretion on day 6 of culture was similar for both wt and het animals cultured with 1IU FSH or 0.1IU FSH, whereas follicles from het mice cultured with 0.01IU FSH secreted significantly less ($p<0.001$) oestradiol compared to follicles from wt mice (Figure 4.5). High levels of oestradiol were associated with mature follicles that have been stimulated by FSH, although levels started to decline in the final stages of follicle development when progesterone levels increased (Yong *et al.,* 1994; Rosenfeld *et al.*, 2001). Although oestradiol is an important factor for follicle development, it is not essential for growth *in vitro* (Spears *et al.,* 1998). Follicles from the het mice were larger in size. Therefore, follicles from het mice would be expected to have higher levels of oestradiol compared to wt mice, due to increased granulosa cell expression of P450 aromatase. However, no difference in oestradiol secretion was observed between genotypes from individual follicles on the final day of culture treated with 1IU and 0.1IU FSH.

McNeilly *et al.* (2000) also showed that plasma oestradiol levels in adult *Dazl* mice were similar between wt and het mice. In the final stages of follicle maturation oestradiol levels start to decline and progesterone levels increase (Valdez *et al.,* 2005). The results presented in this chapter, coupled with previous studies on this strain of mice, suggest that follicles from *Dazl* het mice were more developmentally advanced with levels of oestradiol declining compared to wt mice. Oestradiol secretion by follicles from het mice cultured with 0.01IU FSH were significantly ($p<0.001$) lower although these follicles were larger in size than follicles from wt mice (Figure 4.5). This again suggests that these follicles were more developmentally advanced than follicles from wt mice. In order to assess follicle maturity, progesterone levels should also be measured. However, due to sample volume limitations this was not possible.

As observed the oestradiol ELISA inter-assay variation (low= 35% and high= 33%) was high. Future studies would use a kit ELISA with lower coefficients of variation. Follicles isolated for culture required the presence of some theca cells for the follicles to survive (Murray, Personal Communication; Wycherley *et al.,* 2004). The theca layer present would vary from one follicle to another as there was no means of measuring this. This may explain the large standard error in oestradiol secretion observed in these experiments (Figure 4.5). Future studies would involve incubating follicles with androstenedione as a substrate for oestradiol synthesis (Knobil and Neill, 1994). In addition, analysis of oestrogen receptors within the ovary would also determine whether there were any differences in receptor expression.

Inhibin B secretion on day 6 of culture was similar from follicles of wt and het mice at all doses of FSH (Figure 4.7). Inhibin B is a marker of small follicles *in vivo,* but studies *in vitro* have demonstrated that levels continue to increase throughout the culture duration (Groome *et al.,* 1996; Smitz and Cortvrindt, 1998). *In vivo,* inhibin would reduce FSH secretion. However, *in vitro* inhibin B levels would continue to increase with FSH stimulation throughout the culture period. Previous studies on adult *Dazl* mice demonstrated that het mice had higher levels of inhibin B compared to wt mice and it was postulated that these mice would have more smaller follicles (McNeilly *et al.,* 2000). However, analyses of follicle counts showed that there were similar numbers of small follicles in both wt and het mice. Therefore, it would appear that follicles from het mice are capable of secreting more inhibin B per follicle compared to follicles from wt mice. Published data suggest that inhibin secretion is greatly affected by the culture method and may not be representative of the situation *in vivo* (Smitz and Cortvrindt, 1998).

Comparison of inhibin B secretion by follicles cultured with 1IU FSH and 0.1IU FSH on day 2 with that on day 6 showed that inhibin B levels were significantly greater on day 6 compared to day 2 for follicles from both wt and het mice (Figure 4.8). As mentioned previously, inhibin B levels in culture increased throughout the culture duration (Smitz and Cortvrindt, 1998). Therefore, it would be expected that inhibin B levels would increase with continual FSH stimulation and greater granulosa cell proliferation. Interestingly, analysis between genotypes demonstrated that on day 6 follicles from the het mice cultured with 1IU FSH and 0.1IU FSH secreted significantly ($p<0.05$) more inhibin B compared to wt mice (Figure 4.8). Previous studies on *Dazl* mice found similar findings *in vivo* (McNeilly *et al.,* 2000). Together, these findings suggest that follicles from het mice were capable of secreting greater levels of inhibin B per follicle than wt mice. This may also explain why FSH levels were lower in adult mice plasma in *Dazl* het mice (McNeilly *et al.,* 2000), since higher inhibin B levels would exert a greater negative feedback on FSH secretion.

In addition, follicles from het mice appear to be more advanced in terms of growth with greater granulosa cell proliferation and therefore inhibin B levels would be expected to be greater. The secretion of inhibin B by follicles cultured with 0.01IU FSH was similar for both wt and het mice on days 2 and 6 of culture (Figure 4.8). However, with those minimal FSH levels granulosa cell proliferation was also minimal, as observed by the small increase in growth (Figure 4.3 (C)) observed for these follicles, and perhaps indicating that inhibin B would be at basal levels (Hohmann *et al.*, 2005). Therefore, a similar secretion of inhibin B between follicles from wt and het mice would be expected. A study by Smitz and Cortvrindt (1998) suggested that levels of inhibin B could be used as a marker of follicle health, with low levels of inhibin B indicating a poor quality of follicle. Inhibin B was measured on day 2 of culture to determine whether inhibin B could be used to predict follicle outcome (Figure 4.9). The results presented here demonstrated no difference in inhibin B secretion by follicles that survived or did not survive in culture for either wt or het mice.

Inhibin A levels were similar in follicles from wt and het mice cultured with 1IU FSH or 0.1IU FSH, but follicles from het mice cultured with 0.01IU FSH secreted significantly ($p<0.05$) higher levels of inhibin A (Figure 4.7). Secretion of inhibin A was greatest in larger follicles (Smitz and Cortvrindt, 1998; Welt and Schneyer, 2001). Therefore, it would be expected that inhibin A levels would be greater in follicles from het mice at all doses of FSH. However, this could be masked by the culture system and the lack of negative feedback as would occur *in vivo*. Inhibin A (similar to oestradiol) levels begin to decline in the final stages of follicle development (Campbell and Baird, 2001). Therefore, in follicles from het animals, inhibin A secretion would be expected to start to decline by day 6 of culture. At 0.01IU FSH the inhibin A levels were as expected, with follicles from het mice secreting greater levels than from wt mice, as the follicles from the het mice were significantly larger than wt mice (Figure 4.3). Together this suggests that follicles from *Dazl* het mice were more developmentally advanced since inhibin A levels were starting to decline by day 6 when cultured in the presence of 1IU FSH or 0.1IU FSH, resulting in similar levels secreted by follicles from wt mice. Future studies should determine plasma inhibin A in the *Dazl* strain at day 21.

Inhibin A levels were greater than inhibin B levels at all does of FSH for both wt and het mice on day 6 (Figure 4.7). As mentioned previously, inhibin A is secreted in greater amounts in the final stages of follicle development (Smitz and Cortvrindt, 1998; Welt and Schneyer, 2001). This study supports such findings with follicles from wt and het *Dazl* mice secreting greater levels of inhibin A than inhibin B on day 6 of culture. Therefore, although the culture system used here does effect inhibin B secretion *in vitro* compared to *in vivo*, inhibin A levels demonstrated similar trends *in vitro* compared to those observed *in vivo*.

4.4.6 Burst Follicles

Follicles that demonstrated signs of atresia or that burst during culture were recorded. Although antrum formation was recorded, analysis of this data was not assessed. Since follicles from *Dazl* mice (wt and het) were darker than F1 mice, the analysis of antrum formation was difficult. Bursting due to damage to the follicles during follicle isolation usually occurs by day 2 of culture (Spears, Personal Communication). In experiments discussed in this chapter, the follicles that burst did so on day 3 or 4 of culture. When differences between genotypes were analysed it was observed that follicles from het mice were significantly less likely ($p<0.05$) to burst during culture than follicles from wt mice cultured with 1IU FSH or 0.1IU FSH (Figure 4.4). However, there was no difference in bursting rates between follicles from wt and het mice cultured with 0.01IU FSH. Various factors that affect the integrity of the basement membrane could cause this bursting during culture of these follicles. Bursting of follicles is not a phenomenon that occurs naturally in the ovary and may simply be an artefact of the culture system used (Spears, Personal Communication).

Previous studies using this culture system demonstrated that ascorbic acid supplemented to the culture media inhibits follicles bursting (Murray *et al.,* 2001). However, follicles in the cultures reported here were exposed to similar levels of ascorbic acid. Ascorbic acid is metabolised by the granulosa cells and follicles from het mice with their increased granulosa cell mass and larger follicles would be expected to have a higher uptake leading to increased protection against bursting of these follicles. However, this would not explain the lack of difference between bursting rates in follicles from the wt and het mice cultured with 0.01IU FSH although follicles from het mice were significantly larger ($p<0.01$) (Figures 4.3 (C) and 4.4). The data suggests that follicles from het mice may simply be more robust as a result of their increased FSH-sensitivity, as FSH is known to protect against atresia (Hirshfield, 1989) and could potentially protect against bursting *in vitro*.

Similar bursting rates of follicles cultured with 0.01IU FSH may be explained by the size of these follicles. At this dose the follicles are not growing, but are maintained at a pre-antral stage of development. Therefore bursting may only occur when follicles are larger and become antral and gonadotrophin-dependent. Osmotic pressure could affect the bursting of follicles (Wallace and Selman, 1985; Hess *et al.,* 1998; Rodgers *et al.*, 2000; McConnell *et al.,* 2002). However this does not explain why the follicles from het mice were less likely to burst as larger follicles would be expected to have a greater follicular pressure. Bursting was only observed at the higher doses of FSH, when follicle growth has been stimulated and intra-follicular pressure may be higher (Wallace and Selman, 1985; Rodgers *et al.*, 2000; McConnell *et al.,* 2002). Future studies should analyse the level of aquaporins in wt and het *Dazl* follicles. These proteins regulate water movement (McConnell *et al.,* 2002) and could alter pressure in the follicles.

Under the microscope, burst follicles demonstrated a basement membrane that was not intact (Bagavandoss, 1998). This led to experiments which analysed levels of MMP-2 and MMP-9 activity (by zymography) since these play an essential role in the degradation of the basement membrane (Riley *et al.,* 2004). Gelatin zymography is semi-quantifiable and detects gelatinases activity (Riley, Personal Communication). Analysis was carried out on day 2 of culture, just prior to the time when bursting of follicles occurred. However, there was no difference in the levels of latent and active forms of MMP-2 both in culture media or follicles (Figure 4.12). Furthermore, no MMP-9 (latent or active) could be detected in any of these samples (Figure 4.11). However, previous studies have demonstrated that MMP-2 is expressed in greater quantities compared to MMP-9 suggesting MMP-9 levels were below the detection limit of the method, and therefore providing a possible explanation for this result (Bagavandoss, 1998; Hess *et al.,* 1998; Smith *et al.,* 2002; Riley *et al.,* 2004). MMP-9 activity increases in the latter stages of follicle development, when follicles have been stimulated by gonadotrophins (Liu *et al.,* 1998; Ke *et al.,* 2004). This may also explain why samples in this study did not have detectable MMP-9 activity, as the follicles in this study were smaller than 250μm in diameter, and were therefore pre-antral. Little or no MMP-9 activity is observed at this stage (Bagavandoss, 1998).

The levels of latent MMP-2 detected in the culture media were significantly greater than the levels of active MMP-2 (Figure 4.12 (A)). Furthermore, only the latent form of MMP-2 was detected in follicle samples. Higher levels of latent than active MMPs were expected to be detected, as MMPs are not stored and are released once activated (Rawdanowicz *et al.,* 1994). This would explain why higher levels were detected in culture media. MMP activity in these follicle samples and culture media samples was very low. In pre-antral follicles, MMP activity is basal and increases during follicle growth when greater remodelling is required (Curry and Osteen, 2001; Curry and Osteen, 2003; Imai *et al.,* 2003). The data presented in this study suggest that follicles from *Dazl* wt and het mice had similar rates of follicle degradation by MMP-2 and MMP-9. However, follicle degradation by the MMPs is not solely reliant upon the activity of the MMPs themselves. The availability of substrate and levels of TIMPs also determine the rate of follicle degradation (Smith *et al.*, 1999; Brew *et al.,* 2000; Riley, Personal Communication). Therefore, to get a fuller picture of the extent of follicle degradation by MMPs, the levels of TIMPs also need to be analysed. Unfortunately, this study was restricted by low sample volumes which enabled only MMP-2 and MMP-9 to be analysed.

The follicles from het mice with larger follicles would be expected to have increased extracellular matrix turnover (Curry and Osteen, 2003). However, it is these follicles that are less likely to burst, despite having a similar MMP-2 activity. Although no differences were observed in the activity of the gelatinases, several other factors (such as laminin and collagen) are essential for the maintenance of the basement membrane. Future studies analysing such components in ovaries of these mice would be essential to determine variations in the basement membrane composition of follicles from *Dazl* wt and het mice.

A previous study examined gelatinase activity by the same methods used in this study, but studied rat granulosa cells which had been stimulated by equine chorionic gonadotrophin (eCG) and demonstrated gelatinase activity increased after stimulation (Ke *et al.,* 2004). Follicles from wt and het mice of a similar size were assessed for MMP-2 and MMP-9 activity (Figure 4.13). Hence, follicle size was not a variant that could skew results. Previous studies on MMPs demonstrated that FSH stimulated MMP-2 and MMP-9 activity (Ke *et al.,* 2004). However, this study found no correlation between MMP-2 activity and follicle size for either wt or het mice. This lack of difference could be species dependent or simply a result of the detection limits of this method. More likely this was due to the stage of follicle development as these follicles were pre-antral, and would have lower levels of active MMP-2 and MMP-9 (Bagavandoss, 1998; Curry and Osteen, 2003). However, since follicle bursting occurred on days 3-4 of culture, follicles could not be cultured for any longer under these conditions.

Very low levels (not quantifiable) of an unidentifiable MMP were detected in follicle culture media samples (Figure 4.11). As more research is carried out on the MMP family, more MMPs are identified (Kim *et al.,* 2001; Curry and Osteen, 2003). Recently a study by Kim *et al.* (2001) detected an isoform of MMP-2 in bovine follicles with an approximate molecular weight of 110kDa. The band observed in this study had a slightly higher molecular weight compared to active MMP-2. Although this differed in molecular weight to the protein observed by Kim *et al.* (2001), this could simply be due to species differences. The main difficulty in detecting MMPs in these samples was that the level of the MMPs present was at or below the detection level of this method. Furthermore, samples could not be pooled, since this would mask any differences between individual follicles.

4.4.7 Role of the Oocyte

The studies presented in this chapter suggest that a single copy of the *Dazl* gene enhances both follicle growth and maturation. However, the mechanisms by which it does this are unknown. The advanced maturation demonstrated by the follicles from het mice may be due to an increased sensitivity to FSH, resulting in stimulation of follicle growth and development (Vanderhyden *et al.,* 1992).

Oocytes are vital in regulating follicle growth and previous studies have shown that they can enhance or inhibit granulosa cell response to growth factors (Eppig *et al.,* 1997a; Eppig *et al.,* 1997b; Hirshfield, 1991). The oocyte plays a role in the regulation of the FSH-induced LHR expression that signals the final stages of follicle development and maturation (Eppig *et al.,* 1997b). Altering the number of copies of the *Dazl* genes could affect FSH-induced LHR expression. Further studies are required to determine whether the *Dazl* gene effects granulosa cell differentiation, and the mechanism involved in increasing follicle FSH-sensitivity.

Analysis of FSHRs and signalling pathways is also essential to determine why follicles from *Dazl* het mice are more FSH-sensitive. Previous studies have demonstrated the importance of the oocyte in follicle growth and development (Eppig, 2001). Both Gdf-9 and BMP-15 are oocyte factors that are known to stimulate follicle growth (Otsuka *et al.,* 2000; McNatty *et al.,* 2005). The present studies suggest that the number of *Dazl* gene copies present also alters paracrine signalling between the oocyte and granulosa cells, a single copy stimulating granulosa cell proliferation compared to two copies of the gene.

4.4.8 Conclusion

In conclusion, the studies presented in this chapter have shown that follicles from *Dazl* het mice are more responsive to FSH and grow larger than follicles from wt mice cultured with 1IU FSH, 0.1IU FSH or 0.01IU FSH. In addition, the follicles from these mice were also more advanced developmentally than follicles from wt mice, with higher inhibin A secretions. Although similar in both genotypes, follicles from het mice seem to be at a later stage of development, when oestradiol levels are declining. Follicles from the het mice were more robust and less likely to burst during culture. However, MMP-2 activity was similar in follicles from wt and het mice. This suggests that degradation of the ECM by the gelatinases was not affected by the *Dazl* gene, although several other factors that are involved in remodelling and degrading the ECM could be affected and explain the reduced bursting rate observed in follicles from het mice. The oocyte is fundamental in follicle growth (Eppig, 2001) and this can be observed by the significant differences observed in follicle maturation of *Dazl* wt and het mice. However, the exact mechanisms involved in how this gene effects follicle growth remain unknown. These studies suggest that one copy of the *Dazl* gene stimulates granulosa cell proliferation due to a greater response to FSH and, in turn, follicle maturation is more advanced in these follicles. A larger cohort of growing follicles would be associated with an increased ovulation rate (LaPolt *et al.,* 1992). The findings of this thesis so far led to experiments in the next chapter to determine how follicles from wt and het mice grew when co-cultured in contact with one another.

Chapter 5

Co-Cultured *Dazl* Follicles

5.1 Introduction

During follicle growth and development, ovarian follicles undergo three stages of regulation: recruitment, selection and dominance. Follicle dominance occurs when a selected follicle(s) begin to express LHRs within the granulosa cells, allowing them to respond to both FSH and LH, whilst the other follicles become atretic (Fortune, 1994; Armstrong and Webb, 1997; Baker and Spears, 1999; Webb *et al.*, 2003). These three stages of follicle maturation regulate the number of oocytes ovulated and, therefore, litter size. However, the exact mechanisms of recruitment and follicle dominance are still not fully understood (Armstrong and Webb, 1997; Baker and Spears, 1999).

5.1.1 Follicle Dominance

The dominant follicle is recognised as being significantly larger than the largest subordinate follicle (termed follicle deviation). This increase in follicle size is a result of increased growth compared to subordinate follicles in the growing cohort (Fortune *et al.*, 2001). All follicles within the growing cohort are thought to have the potential to reach ovulation prior to selection for dominance and following induction of super-ovulation, a greater number of follicles from the cohort reach ovulation (Baird, 1987; LaPolt *et al.,* 1992; Baker and Spears, 1999). When the dominant follicle was destroyed, a subordinate follicle assumed dominance. It was thought that the destruction of the dominant follicle results in a small increase in FSH, enabling the less mature follicle to respond and develop (Fortune, 1994; Fortune *et al.,* 2001; Webb *et al.,* 2003). This suggests that the dominant follicle secretes a factor(s) that inhibits the growth of the other follicles in the cohort without affecting their health.

As discussed in Chapter 1, in addition to endocrine hormones inter-follicular factors also regulate follicle dominance. Several studies have been undertaken to determine the factor(s) that affect follicle growth. However, these studies have provided conflicting results. These differences may have arisen as a result of differing experimental conditions used for follicle cultures and or analysis of follicle growth and development of different sized follicles (Zhao *et al.*, 2000; Spears *et al.*, 2002). One study concluded that co-culture of 4 or 6 pre-antral follicles without any physical contact stimulated growth in all follicles (Zhao *et al.*, 2000).

However, other studies demonstrated that culture of late pre-antral follicles of a similar size in contact resulted in one follicle becoming dominant (Spears *et al.*, 2002). Mizunuma *et al.* (1999) demonstrated that large pre-antral (secondary) follicles secreted a substance that inhibited growth of primary follicles and suggested that this inhibitory factor was activin. Follistatin acts by binding to activin and 'neutralising' its actions (Lin *et al.*, 2003). However, when follistatin was added to co-cultured follicles, dominance still occurred suggesting that activin was not the inhibitory factor (Spears *et al.*, 2002). However, the inhibitory action of activin on follicle growth was dependent on the age of the animal. Activin A stimulated follicle development in small pre-antral follicles of day 11 mice but opposed the action of FSH in small pre-antral follicles in adult mice (Mizunuma *et al.*, 1999). Baker *et al.* (2001) concluded that follicle dominance can be separated into two distinct phases; firstly, the establishment of dominance and secondly, the maintenance of dominance. However, the factors that regulate these distinct phases are still poorly understood.

Previous studies by Spears *et al.* (1996) demonstrated that when follicles were co-cultured one follicle became dominant, but although its growth and development was inhibited the 'health' of the subordinate follicle was not affected. Furthermore, when the dominant follicle was physically separated from the subordinate follicle, the subordinate follicle resumed growth (Baker *et al.*, 2001). When cultured in physical contact with each other in the presence of low levels of FSH, the dominant follicle not only retarded the growth of the subordinate follicle but also induced apoptosis (Baker *et al.*, 2001). Previous studies concluded that the factor(s) that result in dominant/subordinate follicle regulation required follicle-to-follicle contact, as follicle dominance was not observed when follicles were cultured together but not in physical contact with each other (Spears *et al.*, 1996).

Follicle dominance may result either from inhibitory factors retarding the growth of subordinate follicles or from stimulatory factors that accelerate the growth of the dominant follicle. Two mechanisms could influence the dominant/subordinate follicle relationship; inductive signalling or lateral specification. Inductive signalling occurs between two distinct cell populations where communication occurs and the outcome of one cell population is determined by the other, whereas lateral specification regulates the 'pattern' of differentiation of cell types (Baker and Spears, 1999). The accepted view is that follicle dominance occurs due to inductive signalling, as the dominant follicle inhibits the growth of subordinate follicles and leads to failure to reach ovulation (Baker and Spears, 1999). Little is known regarding the genes that are regulated by such mechanisms of communication between ovarian follicles (Baker and Spears, 1999). However, factors such as activin, inhibin, IGF and granulosa cell-inhibitory factor (GCIF) have all been proposed to be involved in regulating follicle dominance (Hynes *et al.*, 1996; Mizunuma *et al.*, 1999; Chada *et al.*, 2003; Webb *et al.*, 2003).

The method used to study follicle dominance in *Dazl* wt and het mice by co-culture of follicles in physical contact with each other was detailed by Spears *et al.* (2002). This method was adapted to analyse follicles that were cultured within the same culture well, but not in physical contact. Previous studies examining growth of follicles not in physical contact used a semi-permeable membrane to separate follicles physically but allow molecules in the media to pass through this barrier (Spears *et al.,* 2002). This method was not adopted for this study due to the fact that this was technically more demanding and due to time constraints with animal breeding.

5.1.2 Octadecyl Indocarbocyanine (DiI) and Oxacarbocyanines (DiO) Labelling

Octadecyl indocarbocyanines and oxacarbocyanines that penetrate into the lipid interior of the cell membrane are referred to by their generic names, DiI and DiO, respectively (Molecular Probes, Detection Technologies Information, Paisley, UK). DiI and DiO are fluorescent, lipophilic, membrane-staining agents of low toxicity levels that are frequently used to stain cells (Sparks *et al.*, 2000; Molecular Probes, Product Information). DiI emits a red colour at approximately 580nm, whereas DiO emits a green colour at approximately 500nm (Molecular Probes Product Information). DiI and DiO have been used previously in a limited number of follicle cultures and did not affect follicle growth and development (Spears, Personal Communication). These reagents are ideal for labelling cells *in vitro,* as they are non-toxic to cells and cells are uniformly labelled (Molecular Probes Product Information). Treating each genotype with different reagents enabled easy identification of genotype of co-cultured follicles from *Dazl* wt and het mice.

5.1.3 Aims of Study

Previous experiments in this thesis demonstrated that follicles from *Dazl* het mice were larger *in vivo* and *in vitro* compared to wt mice. Furthermore, follicles from het mice were less likely to burst when cultured in 1IU FSH or 0.1IU FSH. Previous studies on the *Dazl* strain demonstrated that adult het mice had significantly larger litters compared to wt mice (McNeilly *et al.,* Unpublished; Appendix A). In addition, studies on co-cultured follicles demonstrated that one follicle always became dominant and the subordinate follicle regressed (Spears *et al.,* 1996). These results, coupled with previous findings in this thesis, imply that there is a larger cohort of growing follicles in het mice compared to wt mice at a similar stage of development. With larger litter sizes (McNeilly *et al.,* Unpublished; Appendix A) follicles from het mice were hypothesized to not demonstrate follicle dominance to the extent of that of follicles from wt mice. It was expected that more follicles from het mice would be able to survive the selection process in a cohort of follicles of the same size possibly explaining the larger litter sizes.

The aims of the experiments described in this chapter were to determine whether dominance occurred when follicles from *Dazl* wt and het mice were co-cultured. This study enabled identification of the genotype of the follicle that was primed to become dominant. Follicles were also co-cultured with or without physical contact to determine whether the effects observed were the result of paracrine or secreted intra-follicle factors, and to determine whether follicles from *Dazl* het mice differed from follicles from *Dazl* wt mice in terms of follicle dominance.

5.2 Methods

5.2.1 Follicle Co-Culture

Ovaries were removed and follicles dissected as described in Sections 2.2 and 2.6. After dissection follicles were grouped according to their genotype (Section 2.3) and placed into a well of a (flat-bottomed) 96-well plate containing 1IU FSH in 200µl α-MEM (Section 2.6.3). Media were supplemented with 5µl per ml of either DiI or DiO (Molecular Probes) and covered with 100µl of silicon fluid (Merck). Follicles were incubated for 3 hours as described in Section 2.6.4 to allow the DiI and DiO to penetrate the theca layer, prior to washing in fresh α-MEM.

Follicles were measured and placed into the wells of a flat-bottomed, 96-well plate (Bibby Sterilin) containing follicles of the same size in the combinations set out in Table 5.1. Follicles were placed in physical contact with one another, or at least 500µm from one another (Figure 5.1). Follicles were cultured for two days with 100µl of α-MEM containing 1IU FSH and covered with 100µl of silicon oil, as described in Sections 2.6.3 and 2.6.4. Follicle sizes were measured daily. Media were not changed over this time period.

Follicle Co-Culture Combinations	DiI or DiO	Colour of Dye
Dazl Wt/Wt	DiI/DiI	Green/Green
Dazl Het/Het	DiO/DiO	Red/Red
Dazl Wt/Het	DiI/DiO	Green/Red

Table 5.1 Follicle co-culture combinations and dye used for identification.

5.2.2 Identification of *Dazl* Wt and Het Follicles

After culture, follicles were photographed using a fluorescent microscope (Leica) with a digital camera attached (Lambda Photometrics, Harpenden, UK). Follicles were identified by the fluorescent colour emitted by DiI or DiO staining (which demonstrated their genotype – Table 5.1). The size of the follicle was measured from the digital image and compared with the actual measurement made on the final day of culture.

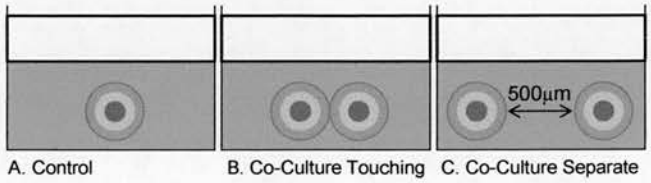


Figure 5.1 Illustration of the co-culture set up.

5.2.3. Statistical Analysis

Growth rates were analysed using one-way ANOVA (Parametric) followed by Tukey's multiple tests. Prior to analysis, data was Log10 transformed to assume Gaussian distribution. The incidence of follicle bursting, and the effect of genotype on the incidence of becoming the larger follicle were analysed using the Chi-square test.

5.3 Results

All co-culture experiments also monitored the growth of a control follicle. The control group consisted of individually cultured follicles of a similar size, stained with either DiI or DiO. This allowed the final growth rates of both follicles to be compared to follicles that were cultured individually.

5.3.1 Co-cultured Follicles from *Dazl* Wt Mice

This experiment analysed the growth of co-cultured follicles from wt mice. When follicles from *Dazl* wt mice were cultured in physical contact follicle dominance occurred on day 1 of culture. One follicle became significantly larger on day 1 ($p<0.05$) and this difference was even greater on day 2 of culture ($p<0.01$; Figure 5.2 (A)). There was no significant difference between the large co-cultured follicles and control follicles cultured alone. However, control follicles were significantly larger than the smaller co-cultured follicles ($p<0.01$).

When follicles were cultured within the same well but without physical contact, follicle dominance did not occur (Figure 5.2 (B)) and there was no difference in growth rate between control and co-cultured follicles.

5.3.2 Co-cultured Follicles from *Dazl* Het Mice

This experiment analysed the growth of co-cultured follicles from het mice. Follicles from *Dazl* het mice demonstrated similar findings when cultured in physical contact (Figure 5.3 (A)). Control follicles were significantly larger on day 1 of culture compared to the smaller co-cultured follicles ($p<0.01$) (Figure 5.3 (A)). However, there was no difference in size between the larger and smaller co-cultured follicles at this stage of culture. By day 2 of culture, follicle dominance was established ($p<0.05$). There was no difference between the growth of control follicles and the larger co-cultured follicles at this stage but control follicles remained significantly larger compared to the smaller co-cultured follicles ($p<0.001$).

Follicle dominance was not observed when follicles from *Dazl* het mice were cultured without physical contact (Figure 5.3 (B)). In addition, no difference in growth rates was observed between control and co-cultured follicles.

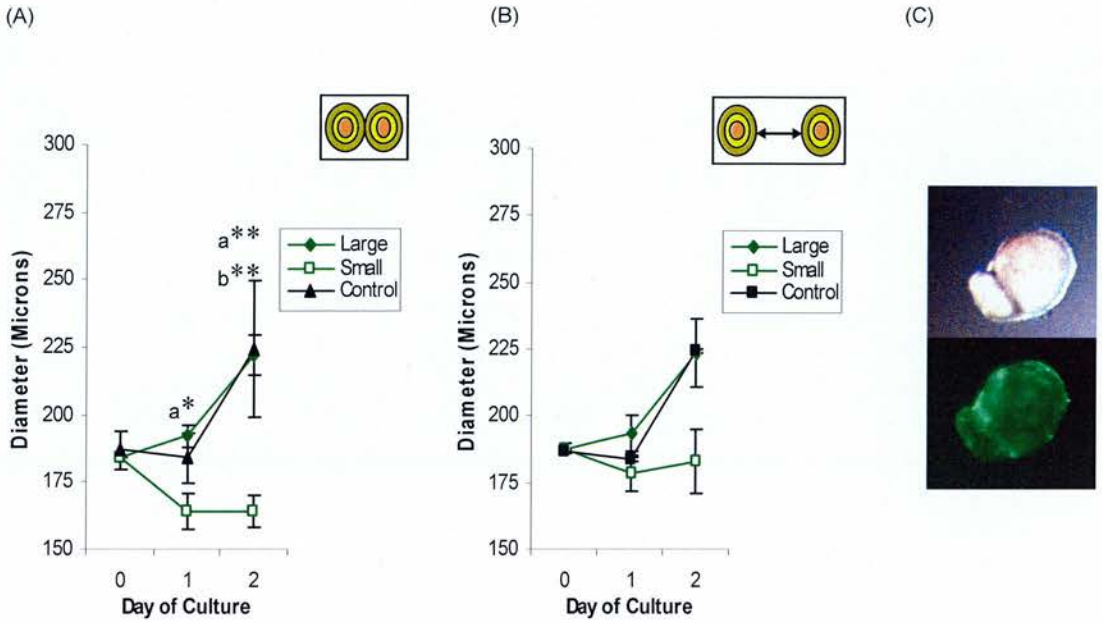


*Figure 5.2 Growth rates of follicles from Dazl wt mice co-cultured with 1IU FSH (A) in physical contact (n=7) or (B) physically separate (n=14). (C) Image of co-cultured follicles from wt mice stained with DiO. a= comparison of follicles from wt mice and b= comparison of subordinate follicles from wt mice to control follicles cultured alone. * p<0.05 and ** p<0.01. Values are mean ± S.E.M.*

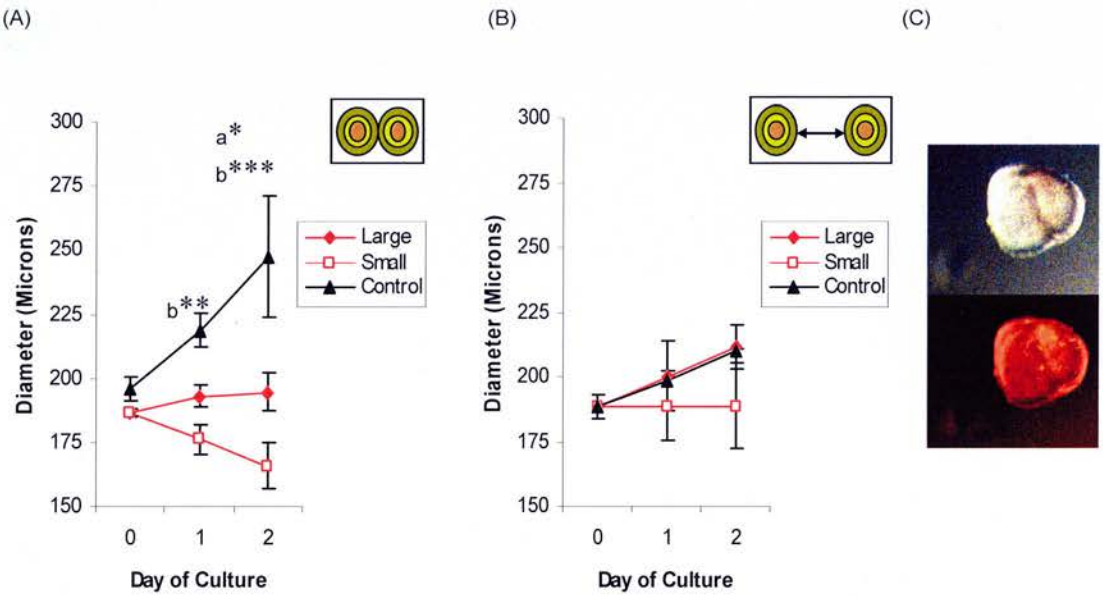


*Figure 5.3 Growth rates of follicles from Dazl het mice co-cultured with 1IU FSH (A) in physically contact (n= 14) or (B) physically separate (n=5). (C) Image of co-cultured follicles from het mice stained with DiI. a= comparison of follicles from het mice and b= comparison of subordinate follicles from het mice to control follicles cultured alone. * p<0.05, ** p<0.01 and *** p<0.001. Values are mean ± S.E.M.*

5.3.3 Co-cultured *Dazl* Wt and Het Follicles

When follicles from *Dazl* wt and het mice were co-cultured in physical contact, follicle dominance did not occur (Figure 5.4). However, when this experiment was repeated with follicles that were not physically touching, follicle dominance did occur (Figure 5.5). On day 2 of culture, follicles from het mice were significantly larger ($p< 0.05$) than wt follicles (Figure 5.5 (B)). However, there was no difference between the growth rate of control follicles and follicles from wt and het mice.

In these experiments one follicle always became larger than the other, although this did reach statistical significance. Analysis of which follicles became the larger follicle when wt and het follicles were co-cultured in physical contact, showed that follicles from het mice were more likely to become the larger follicle though this was not statistically significant (Figures 5.6 (A) and (C)). However, when follicles were cultured not touching each other, the het follicle was significantly more likely to become dominant ($p<0.01$) (Figures 5.6 (B) and (C)).

5.3.4 Incidence of Follicles Bursting

The incidence of follicles bursting was noted in all these experiments. There was no significant difference in the incidence of bursting and genotype in any of these cultures (Figure 5.7).

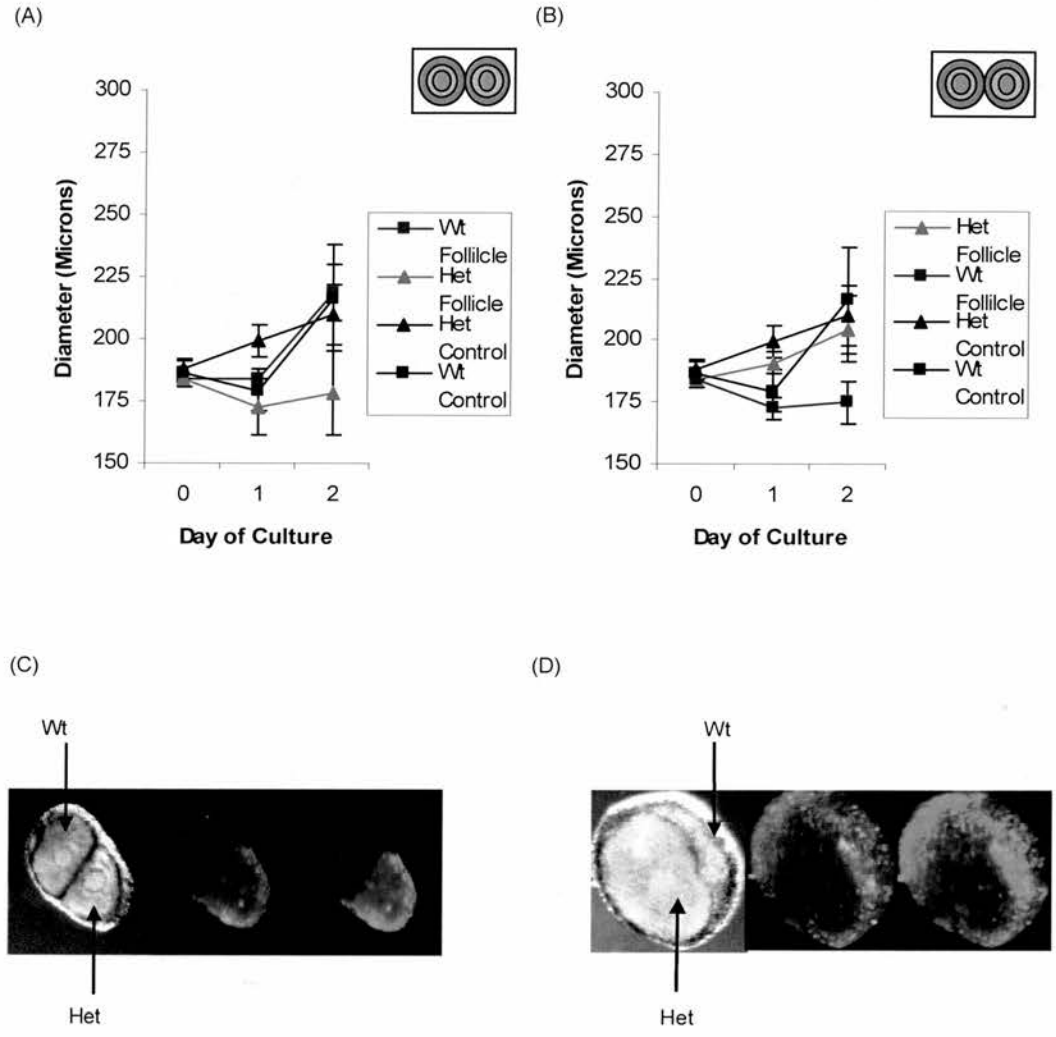


Figure 5.4 Growth rates of follicles from Dazl wt and het mice co-cultured in physical contact, treated with 1IU FSH (A) where follicles from wt mice were larger (n=2) or (B) where follicles from het mice were larger (n=5). (C) Images of follicles from wt and het mice co-cultured (wt larger follicle) and (D) images of follicles from wt and het mice co-cultured (het larger follicle) where follicles from wt mice are stained with DiO and follicles from het mice stained with DiI. Values are mean ± S.E.M.

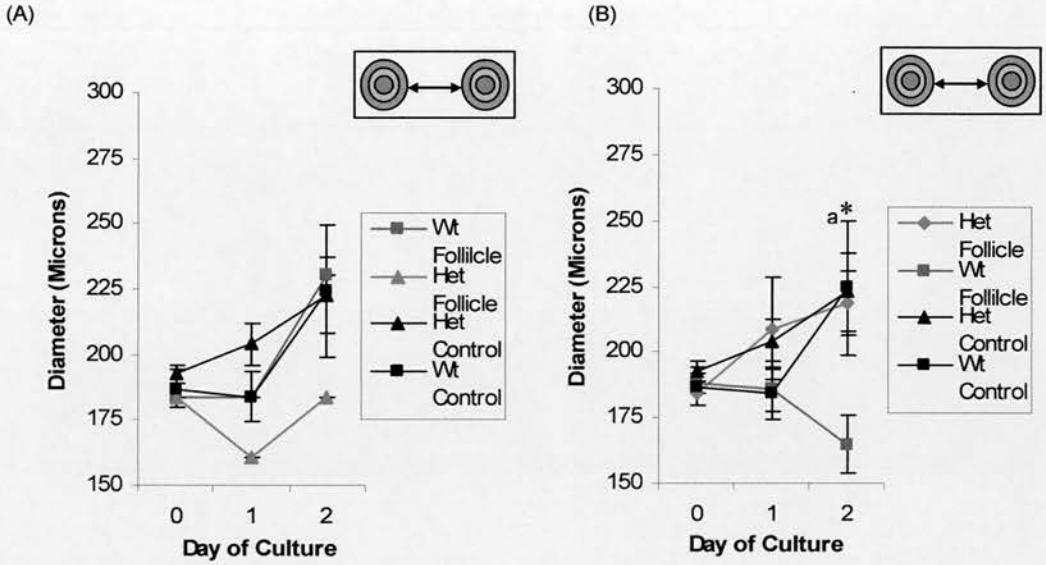
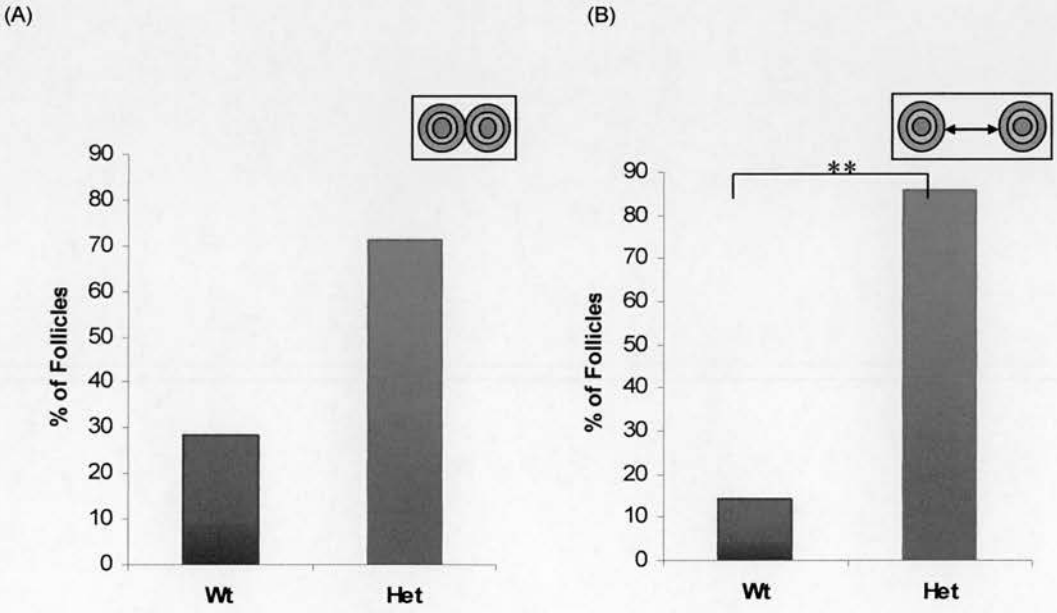


*Figure 5.5 Growth rates of follicles from Dazl wt and het mice co-cultured physically separate, treated with 1IU FSH (A) where follicles from wt mice were larger (n=1) or (B) where follicles from het mice were larger (n=6). a= comparison of follicles from het mice compared to wt mice. * p<0.05. Values are mean ± S.E.M.*



(C)

Physical Contact	Total Number	Wt>Het	Het>Wt	Significant
Yes	7	2	5	No
No	7	1	6	p<0.01

*Figure 5.6 Percentage of larger follicle from follicles from Dazl wt and het mice co-cultured together, treated with 1IU FSH (A) in physical contact or (B) physically separate. ** p<0.01. Values are mean percentages. (C) Summary of this data.*

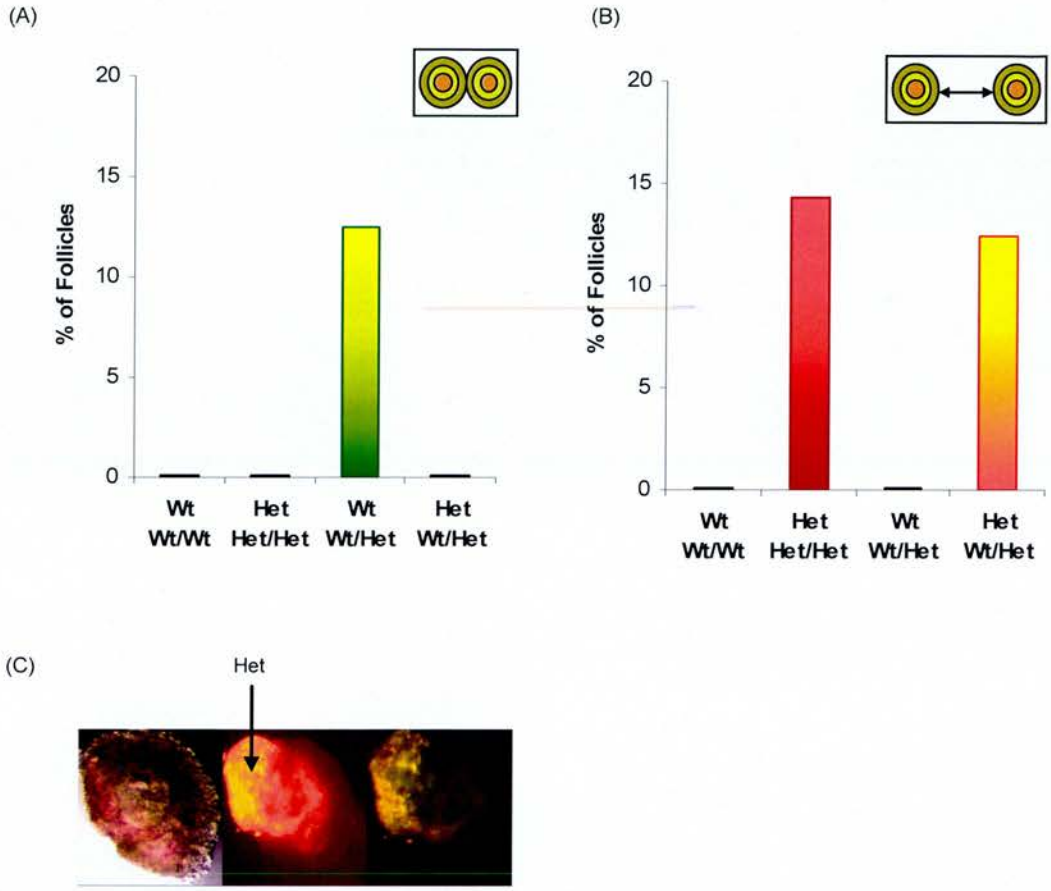


Figure 5.7 Percentage of burst follicles following co-culture of follicles from Dazl wt and het mice with 1IU FSH (A) either in physical contact or (B) physically separate. Wt/Wt= cultures of follicles from wt mice, Het/Het= cultures of follicles from het mice and Wt/Het= cultures of follicles from wt and het mice. (C) Images of co-cultured follicles from wt and het mice where the wt follicle burst. Follicles from wt mice are stained with DiO and follicles from het mice stained with DiI. Values are mean percentages.

5.4 Discussion

5.4.1 Co-Cultured Follicles

Follicles were co-cultured (in physical contact or separate) in the following combinations: follicles from wt mice with wt mice, from het mice with het mice or from wt mice with het mice. This study demonstrated that one follicle becomes dominant when follicles are co-cultured in physical contact for all follicle combinations (Figures 5.2, 5.3 and 5.4). The dominant follicle became significantly larger for wt/wt ($p<0.01$) and het/het combinations ($p<0.05$) but although a trend was observed for wt/het combinations, this failed to reach significance. This study confirms previous data which demonstrated that follicle dominance occurs when two or more follicles are cultured together (Nayudu and Osborn, 1992; Spears *et al.,* 1996; Mizunuma *et al.,* 1999; Zhao *et al.*, 2000). However, the factor(s) involved remain unknown. Interestingly the larger follicle grew to a similar size to that of control follicles. Previous studies using the same method have demonstrated that the dominant follicle grew larger than control follicles (Spears *et al.,* 2002). The variation between the two studies may be due to strain differences. Whereas the previous study examined follicle dominance in F1 mice, this study examined dominance in *Dazl* mice. Experiments (Chapter 4) demonstrated differences between these strains of mice, as follicles from F1 mice grow larger than follicles from *Dazl* mice.

The use of DiI and DiO to mark the genotype of co-cultured follicles has not been published previously. Previous studies have demonstrated that these reagents are not toxic and that follicle growth is not impaired when used to label follicles (Spears, Personal Communication; Molecular Probes Product Information). Dominant follicles treated with DiI and DiO grew to the same size as untreated control follicles confirming that these reagents are not toxic to ovarian follicles *in vitro.* Maximal staining of late pre-antral follicles with DiI and DiO occurred after a 3-hour incubation period. This incubation period enabled reagents to penetrate the theca layer of the follicle to allow easy identification.

When follicles from wt and het mice were co-cultured in physical contact one follicle always became dominant, although this difference in growth rate failed to reach statistical significance (Figure 5.4). This was most likely due to the low number of experiments, and further experiments are required to address this. Interestingly, when follicles from wt and het mice were co-cultured together, the larger follicle in 70% of cases was derived from the het mice, suggesting that follicles from het mice have a greater ability to establish follicle dominance than to those from wt mice (Figure 5.6).

Previous studies (Chapters 3 and 4) demonstrated that follicles from het mice matured at a faster rate. Follicle dominance occurs at the stage of antrum formation when FSH is falling and LHRs are beginning to be expressed (Xu *et al.,* 1995; Eppig *et al.,* 1997b; Webb *et al.,* 2003). Since follicles from het mice developed faster (due to increased FSH-sensitivity; Chapters 3 and 4) it is not perhaps surprising that follicles from het mice established dominance sooner than follicles from wt mice.

As demonstrated earlier (Chapters 3 and 4) follicles from het mice were more FSH-sensitive. Previous studies of co-cultured follicles demonstrated that lower FSH levels in the culture media resulted in increased apoptosis in the subordinate follicles; whilst there were adequate levels of FSH, apoptosis was at a minimal level (Baker *et al.,* 2001). Therefore, FSH can counteract the effects of the factor(s) necessary for establishment of follicle dominance (Baker *et al.*, 2001; Spears *et al.*, 2002). Since follicles from het mice were more sensitive to FSH, this may explain why *Dazl* het mice had increased ovulation rates as a result of a larger number of follicles surviving follicle dominance leading to larger litter sizes. Additionally, more follicles from het mice may be able to survive the drop in FSH levels and survive the follicle dominance process due to greater FSH-sensitivity.

Follicle dominance occurred when wt/wt, het/het and wt/het ($p<0.05$) follicles were co-cultured physically separate (Figures 5.2, 5.3 and 5.5). However, this failed to reach statistical significance for the wt/wt and het/het combinations, though differences were significant for wt/het. Previous studies (Spears *et al.,* 1996) have demonstrated that follicle dominance does not occur when follicles are placed physically separated in culture, suggesting that dominance occurs as a result of secreted paracrine factors. However, other studies have shown that culturing groups of follicles together but not physically touching each other led to some follicles with subordinate growth (Nayudu and Osborn, 1992). The experiments presented in this chapter suggest that factor(s) that inhibit the growth and development of the subordinate follicles were secreted. When follicles are physically separate, secreted factor(s) will be diluted to a greater extent to when follicles are physically touching each other (Figure 5.8). This may explain why follicle dominance was observed less often when follicles were cultured in physical contact.

Interestingly when follicles from het mice were co-cultured (either in physical contact or physically separate) follicle dominance took an extra day to reach significance compared to follicles from wt mice co-cultured. However, follicles from het mice matured faster (Chapters 3 and 4). Therefore, it would be expected that they would become dominant sooner. Indeed previous studies on *Dazl* mice have shown that het animals had larger litter sizes than wt animals (McNeilly *et al.,* 2000). This suggests that follicles from het mice may secrete lower levels of the inhibitory factor(s), explaining why het mice had larger litter sizes.

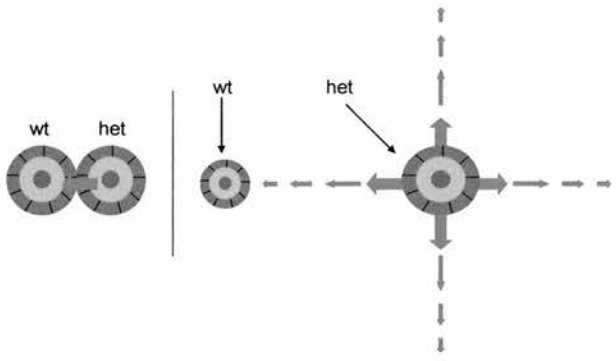


Figure 5.8 Possible communication mechanisms between follicles cultured together in vitro. The red arrows demonstrate that follicles physically touching each other are exposed to a greater signal than those cultured apart.

The follicles that grew larger when cultured within the same culture well but physically separated were significantly more likely to be follicles from het mice, suggesting that these follicles were developmentally advanced, enabling them to secrete factor(s) that inhibited the growth of follicles from wt mice (Figure 5.6). This supports previous studies (Chapter 4) which demonstrated follicles from het mice were developmentally advanced.

Although the factor(s) that cause follicle dominance remain unknown, most studies have proposed that inhibitory factors are responsible for dominance (Hynes *et al.,* 1996, Durlinger *et al.,* 2001; Gruijters *et al.,* 2003; Webb *et al.,* 2003). However, factor(s) could be secreted from follicles that stimulate growth of the dominant follicle. Although from research carried out on follicle dominance and the events that occur at follicle dominance such as a drop in FSH levels (Webb *et al.,* 2003) the factor(s) involved are most likely to be inhibitory.

One inhibitory factor that could be involved in this process is inhibin, as it is necessary for the drop in FSH (Campbell *et al.,* 1991) at the time that follicle dominance occurs. However, *Dazl* het mice had higher levels of plasma inhibin B which would result in fewer dominant follicles rather than the increased litters sizes observed in these mice. AMH has also been postulated to play a role in follicle dominance by regulating follicle development as FSH levels decline - the stage of development associated with follicle dominance (Durlinger *et al.,* 1999; Durlinger *et al.,* 2001; Gruijters *et al.*, 2003; Webb *et al.,* 2003). However, studies on *Dazl* mice (Chapter 6) demonstrated that Amh expression was similar in both wt and het mice and could not account for differences in establishing follicle dominance between wt and het mice.

5.4.2 Future Studies

Although the exact mechanisms of follicle dominance remain unknown, this study concluded that follicle dominance did occur in the *Dazl* strain of mice. However, further experiments are required to increase the confidence of these observations. It would be interesting to examine co-cultures with follicles of different starting sizes from wt and het mice. This would examine maintenance of follicle dominance and the factors involved in this process. Such experiments were not possible due to limitations in animal numbers. However, one experiment that examined co-culture of a large follicle from a wt animal with a small follicle from a het animal (data not presented) indicated that the follicle from the het animal (although subordinate at the start) became the dominant follicle, implying that follicles from het mice had the ability to both establish and maintain follicle dominance over follicles from wt mice. This may explain why more follicles from het mice ovulated compared to wt mice. In addition to further experiments with co-cultured follicles from *Dazl* wt and het mice, exchanging culture media would determine whether the factor(s) involved in follicle dominance is secreted, and if so whether it has the same effect on follicles from wt mice compared to het mice.

Numerous inhibitory factors have been suggested to be involved in follicle dominance (Hynes *et al.*, 1996; Durlinger *et al.,* 2001; Richards, 2001a; Gruijters *et al.*, 2003; Webb *et al.*, 2003). Further work is required to determine what factors are involved in this process in the *Dazl* mouse. Initially, immunocytochemistry of co-cultured follicles would be required to examine the expression of Bmps, Igfs and their binding proteins, inhibin and activin in dominant versus subordinate follicles. Additionally, microarrays of dominant and subordinate follicles would allow studies of gene expression in co-cultured follicles from these mice.

5.4.3 Follicle Bursting

Follicle bursting was observed in individual follicle cultures (Chapter 4) although follicles from het mice were less likely to burst at higher doses of FSH than follicles from wt mice. This chapter examined bursting rates in co-cultured follicles. As mentioned previously, bursting is not observed *in vivo* (Spears, Personal Communication). This study demonstrated no significant difference in bursting rates between genotypes under all co-culture combinations (Figure 5.7). In addition, the bursting rate was lower than that observed in individual cultures (Chapter 4). Follicles *in vivo* are often found in small clusters within the ovary. Therefore, this culture method is more representative of the *in vivo* situation, with follicles being in direct contact with each other. This may explain why co-cultured follicles are less likely to burst than follicles which are individually cultured. Follicles may also secrete factors that enhance the survival of other follicles, perhaps explaining why the subordinate follicles in follicle co-cultures were still deemed healthy, even though their growth was suppressed.

Follicles undergo continual remodelling during co-culture. Interestingly the theca layer of the dominant follicle encapsulated both follicles (Figure 5.9) and the theca of the follicle that encapsulates both follicles was derived from the dominant follicle. This was easily identified by DiI and DiO staining. In addition, follicles originally placed side by side become spherical in shape. Previous studies have demonstrated that the theca layer between the two follicles becomes a very thin layer, allowing growth factors to pass more readily between the two follicles (Baker *et al.*, 2001).

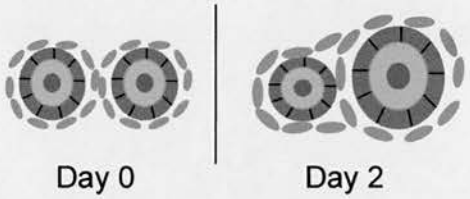


Figure 5.9 Image of follicle theca recruitment during follicle co-cultures.

These data suggest that the dominant follicle controls the development of both follicles. Cell-cell communication is known to be vital in follicle development, as detailed in Section 1.10. The finding of theca remodelling in follicle co-cultures demonstrates the importance of cell communication in determining the fate of the follicle. This could have further implications for follicle development and maturation, as some growth factors such as TGFβ, bind to the ECM of the follicle. Hence, since the theca of the larger follicle surrounds the smaller follicle, the larger follicle may have more growth factors binding to the ECM, enhancing the growth of the larger follicle (Stamenkovic, 2003). Such dramatic remodelling of the ECM could thereby alter the health and fate of the follicle.

5.4.4 Factors Involved in Follicle Dominance

Several factors are involved in paracrine regulation of follicle development that could play a role in follicle dominance. Amh inhibits follicle recruitment from the resting follicle pool (Durlinger *et al.,* 1999; Durlinger *et al.,* 2002; Gruijters *et al.,* 2003). Amh is expressed in the granulosa cells of growing follicles (primary – antral follicles) and in the follicular fluid of antral follicles (when follicle dominance occurs) and has been shown to inhibit activation of primordial follicles through 'paracrine' regulation (Durlinger *et al.,* 2001; Durlinger *et al.,* 2002; Pigny *et al.,* 2003; Themmen, 2005). Due to its inhibitory effect on follicle recruitment Amh has been proposed to be a factor that regulates follicle dominance. The follicle expressing the most Amh will thereby achieve follicle dominance (Durlinger *et al.,* 2001; Themmen, 2005).

GCIF is a low molecular weight factor that has been postulated to be involved in follicle dominance. It was detected in bovine follicular fluid and it inhibited granulosa cell proliferation *in vitro* and reduced the number of small-medium follicles *in vivo* (Hynes *et al.,* 1996). In addition, Igf is also important in rat follicle dominance which is expressed only in follicles that are deemed to be 'healthy' (Wang and Chard, 1999). IGF stimulates granulosa cell proliferation and differentiation and augments the effects of FSH, stimulating follicle development (Wang and Chard, 1999; Richards, 2001b; Quirk *et al.,* 2004). IGF action is regulated by insulin-like growth factor binding proteins (IGFBPs) which bind to IGF receptors. Low levels of IGFBP (especially IGFBP-2 and IGFBP-4) are expressed in dominant bovine follicles (Webb *et al.*, 2003), implying an important role for IGFs in follicle dominance.

Other factors thought to play a role in follicle dominance include the *BMP* family members. This family has demonstrated increased ovulation rates (McNatty *et al.,* 2001) suggesting an increased number of follicles become dominant. For example, the Booroola sheep have increased ovulations due to a mutation at the *BMPR1B* (Souza *et al.,* 2001).

Both inhibin and activin have been postulated to be necessary for the dominant follicle process (Campbell *et al.,* 1999; Chada *et al.,* 2003; Hohmann *et al.,* 2005). Both inhibin A and inhibin B are produced in the granulosa cells and secreted into follicular fluid (Groome *et al.,* 1996; Yokota *et al.,* 1997) and previous studies have demonstrated that treatment with inhibins suppresses follicle growth and development (Campbell *et al.,* 1999). Studies in the *Dazl* mouse (Chapter 3) demonstrated fewer antral follicles in day 21 mice treated with charcoal-stripped oFF. In addition, McNeilly *et al.* (2000) demonstrated that adult het mice had increased inhibin B levels compared to wt mice. The study presented in this thesis demonstrated follicles from *Dazl* het mice produced greater levels of inhibin B per follicle than follicles from wt mice (Chapter 4).

As follicles are stimulated by FSH, granulosa cell proliferation and inhibin production increase (Hirst *et al.,* 2004). Thus inhibin is secreted in greatest amounts from the largest follicles and inhibits smaller follicles from developing through paracrine mechanisms and enables follicle dominance to take place. If this factor is indeed involved in follicle dominance, follicles from het mice which secrete greater inhibin B levels per follicle should exert a stronger inhibition. However, larger litter sizes in het mice suggest that inhibin may not play an important role in follicle dominance.

Activin is expressed by the granulosa cells of healthy follicles (Kishi *et al.,* 1998) and stimulates FSHR expression and promotes follicle development (Xiao *et al.,* 1992; Kishi *et al.,* 1998). Previous studies have suggested that activin A inhibits the growth of smaller follicles. When follicles were cultured together, one follicle became dominant and this effect was not observed when follicles were cultured with follistatin, suggesting that activin may be the inhibitory factor involved in follicle dominance and that activin secreted by the dominant follicle causes subordinate follicles to remain in a 'dormant' state (Mizunuma *et al.,* 1999). However, other studies culturing two late pre-antral follicles of a similar size together have not been able to replicate these findings (Spears *et al.,* 2002). Since the studies did not use the same methods, a direct comparison is not possible. However, if activin is necessary for follicle dominance, then treatment of late pre-antral follicles with follistatin should suppress follicle dominance. However, follicle dominance did occur (Spears *et al.,* 2002). The experiments in this chapter used a similar method to the latter study. However, activin levels *in vivo* or *in vitro* were not examined in the experiments in this thesis, and previous studies of *Dazl* mice did not measure activin levels. Therefore, the role of activin in follicle dominance in the *Dazl* mice remains uncertain.

5.4.5 Conclusion

The studies described in this chapter examined follicle dominance in the *Dazl* mouse, and analysed this follicle process in follicle cultures of het mice compared to follicles from wt mice. The findings presented here demonstrate that when follicles are co-cultured in physical contact follicle dominance did occur with wt/wt, het/het and wt/het follicles although this was not statistically significant in the latter combination. Follicles cultured together but physically separate also showed dominance, though this was only significant for wt/het follicles. When follicles from wt and het mice were co-cultured in physical contact or when physically separate within the culture well, follicles from het mice were most likely to become dominant. This is most likely due to follicles from het mice being more responsive to FSH (as demonstrated in Chapters 3 and 4) and less susceptible to inhibition by this factor than follicles from wt mice.

Furthermore, this data suggests that the factor(s) involved in establishing follicle dominance is secreted, and its effects are diluted when follicles are not in physical contact. Moreover, the rate of burst follicles in co-cultures was lower than in individual follicle cultures, suggesting that this culture system reflects an *in vivo* environment more than individual cultures.

Further studies are required to determine the expression of factors thought to be involved in follicle dominance. However, these studies have stressed the importance of cell-cell communication and follicle-follicle communication in determining follicle development. Follicle dominance may be the stage of follicle development which differs between *Dazl* wt and het mice and explains the differences in litter sizes. Alternatively, differences in litter size could be due simply to variations in the ratio of healthy and atretic follicles. This led to a study to investigate proportions of healthy and atretic follicles in *Dazl* wt and het mice.

Chapter 6

Amh Expression and Cleaved Caspase-3 in Ovaries from *Dazl* Wt and Het Mice

6.1 Introduction

The ovulation rate and subsequent litter size of an animal is a tightly regulated species-specific process and this fine balance is critical for a 'normal' reproductive lifespan. There are several factors which are necessary for this balance to be maintained including: size of follicle reserve, follicle activation, follicle selection and follicle dominance (Durlinger *et al.,* 1999; Durlinger *et al.,* 2002; Webb *et al.,* 2003; Allan *et al.,* 2006), and a slight alteration to this balance can have drastic consequences with regard to litter sizes (Webb *et al.,* 2003). For example, treatment with recombinant FSH stimulates follicle growth and development and results in a larger cohort of growing follicles, increased ovulation rate and larger litter sizes (Galway *et al.,* 1990). Additionally, follicle atresia is fundamental in regulating ovulation rates by eliminating 99.98% of follicles (Hillier, 1994). The rate of follicle recruitment and the health status of follicles are crucial in regulating the depletion of the ovarian reserve and determining the ovulation rate (Manabe *et al.,* 2004; Yu *et al.,* 2004; Lo *et al.,* 2005). The experiments in this chapter will analyse Amh expression and Cleaved Caspase-3 activity to determine the health status and atresia rates of follicles from *Dazl* wt and het mice.

6.1.1 AMH and Follicle Development

AMH is a member of the TGFβ family and it is expressed postnatally within the ovary (Themmen, 2005). Amh has been postulated to be involved in inhibiting follicle activation from the ovarian reserve. Mice deficient in *Amh* demonstrated accelerated follicle activation and exhaustion of the ovarian reserve at an earlier age compared to wt mice (Durlinger *et al.,* 1999; McGee *et al.,* 2001). However, a recent study showed low expression of AMHRII in pre-antral follicles. AMHRII is the only receptor through which AMH signals and therefore this suggests that AMH does not play a fundamental role in inhibiting follicle growth in the early stages of development (Durlinger *et al.,* 2002; Rice *et al.,* 2006). In addition, Amh has also been proposed to be involved in regulating the number of follicles that are selected for dominance by reducing the response to FSH and, in turn, reducing ovulation rates (Durlinger *et al.,* 2002).

There is a high correlation between serum AMH levels and antral follicle numbers (Pigny *et al.,* 2003). Hence serum AMH levels have been proposed to be a marker of the size of the human ovarian reserve and antral follicle numbers (van Rooij *et al.,* 2002; La Marca *et al.,* 2005; Themmen, 2005; Meduri *et al.,* 2007). Serum AMH analysis is used to assess the human ovary during IVF treatment and after cancer therapy (Themmen, 2005).

Amh is produced solely by the granulosa cells of growing ovarian follicles (primary to antral stages of follicle development) and expression is greatest in late pre-antral follicles, whereafter its expression declines (Baarends *et al.,* 1995; Salmon *et al.*, 2004; La Marca *et al.*, 2005). Follicles that ovulate and those that are atretic do not express AMH (Baarends *et al.,* 1995; van Rooij *et al.*, 2002; La Marca *et al.,* 2005). Therefore, AMH has been identified as a marker for 'healthy' follicles (van Rooij *et al.*, 2002). In the mouse follicles, greater *Amh* levels have been found in cumulus granulosa cells compared to mural cells, suggesting a possible role of the oocyte in regulating *Amh* levels (Salmon *et al.*, 2004). Higher AMH levels would be predicted in larger follicles with greater numbers of granulosa cells. However, high levels of AMH are observed in patients with polycystic ovary syndrome (PCOS), even though these ovaries contain more small follicles (van Rooij *et al.*, 2002). Amh is postulated to suppress follicle recruitment in a paracrine manner, as it is secreted by larger growing follicles and prevents growth of primordial follicles (Salmon *et al.,* 2004).

6.1.2 Follicular Atresia

Apoptosis (as described in Section 1.12) is a cellular process involved in follicle atresia. Atresia regulates follicle numbers, and ensures that the correct number of follicles proceed through dominant follicle selection for that particular species. In addition, this process ensures that the follicle(s) selected are the healthiest follicle(s) and will in turn give rise to the healthiest embryo(s) (Amsterdam *et al.,* 2003). Previous studies have demonstrated that granulosa cell apoptosis is an essential part of follicular atresia (Krysko *et al.*, 2004). Apoptosis is characterised by the activation of Ca^{2+}/Mg^{2+} endonucleases which cleave DNA (Berardinelli *et al.,* 2004).

In the early stages of follicle development apoptosis originates in the oocyte and then progresses to the granulosa cells, whereas in mature follicles apoptosis is initiated in the granulosa cells, and oocyte death is the final stage, prior to follicle remains being cleared by phagocytosis or resorption (Matikainen *et al.*, 2001; Glamoclija *et al.*, 2005). Two pathways (extrinsic and intrinsic) are involved in apoptosis. The extrinsic pathway involves 'death' receptors, whereas the intrinsic pathway involves mitochondria mechanisms. Both of these pathways result in the activation of a caspase cascade (Figure 1.9; Yacobi *et al.*, 2004).

6.1.2.1 Cleaved Caspase-3 and Follicular Atresia

Follicle atresia is characterised by the activation of caspases (Marti *et al.,* 1999; Webb *et al.*, 2003). The main caspases involved in atresia are Caspase-2 (in primordial follicle atresia) and the caspase-3 subfamily (which consists of Caspases-3, -6, -7, -8 and -10) (Johnson and Bridgham, 2002; Glamoclija *et al.*, 2005). CASPASE-3 expression is found in granulosa cells of atretic follicles and in the theca cells of the 'healthy' human corpus luteum (Hussein, 2005). Activation of the caspase 'cascade' (Figure 1.9) causes rapid cell death (Manabe *et al.,* 2004). Mitochondria are a source of several pro-apoptotic factors (e.g. cytochrome c which is required for caspase-9 activation (initiator) which leads to activation of executor caspases (Caspase-3; Li *et al.,* 1997; Pru and Tilly, 2001).

Inactive Caspase-3 is expressed located in the cytosol of healthy follicles, whereas activated or Cleaved Caspase-3 is located in the nucleus of atretic follicles (Boone and Tsang, 1998; Johnson and Bridgham, 2002; Glamoclija *et al.*, 2005). Caspase-3 activates many pro-apoptotic factors as well as degrading many cellular components (Yacobi *et al.*, 2004). Once activated, CASPASE-3 stimulates DNase activity resulting in nucleosome DNA fragmentation (Glamoclija *et al.*, 2005). The cleavage of caspase-3 increases as the follicle develops and matures. Cleaved Caspase-3 is expressed in the oocyte and granulosa cells of mouse ovaries but not primordial follicles (Fulton *et al.*, 2005). Interestingly, *Caspase-3* KO mice have similar numbers of follicles to wt littermates (Matikainen *et al.*, 2001). Although Caspase-3 activation is required for granulosa cell apoptosis, it is not required for oocyte death (Matikainen *et al.,* 2001; Pru and Tilly, 2001). Activated Caspase-3 is also expressed in theca cells of both healthy and atretic follicles. However, theca cells do not usually undergo apoptosis (Boone and Tsang, 1998).

Antral follicles that are healthy and responsive to gonadotrophins do not express Cleaved Caspase-3, whereas atretic follicles do. This suggests that the cleavage of Caspase-3 is regulated to some degree by gonadotrophins (Boone and Tsang, 1998; Glamoclija *et al.*, 2005). Indeed, the LH surge is thought to prevent atresia in the pre-ovulatory follicle although the mechanism is not clear (Glamoclija *et al.*, 2005). Downstream the activation of a caspase cascade results in cleavage of apoptosis inhibitors and substrates that are essential for DNA repair (Lo *et al.*, 2005). Laminin, a fundamental component of the ECM, is a known caspase substrate. When cleaved it causes cell shrinkage and a loss of basement membrane integrity (Markstrom *et al.*, 2002).

6.1.3 Aims of Study

Chapter 3 demonstrated that *Dazl* wt and het mice had similar proportions of follicles at each stage of development in all treatment groups, except for untreated mice, where het mice had a larger percentage of antral follicles. The aim of this study was to determine whether the differences in the litter sizes between Dazl wt and het mice could be explained by differences in the numbers of growing healthy follicles and atretic follicles.

Variations in either the percentage of healthy or atretic follicles could provide another explanation for the increased litter sizes observed in het mice (Figure 6.1).Consecutive ovarian sections of untreated, FSH-treated and oFF-treated *Dazl* wt and het mice were stained for both Amh and Cleaved Caspase-3. Previous studies demonstrated that AMH is a marker of a 'healthy' follicle and Cleaved Caspase-3 is a marker of follicle atresia (Baarends *et al.,* 1995; Boone and Tsang, 1998; van Rooij *et al.,* 2002; Glamoclija *et al.*, 2005). This study analysed whether the follicle cohort activated from the resting pool and atresia levels differed in wt and het mice.

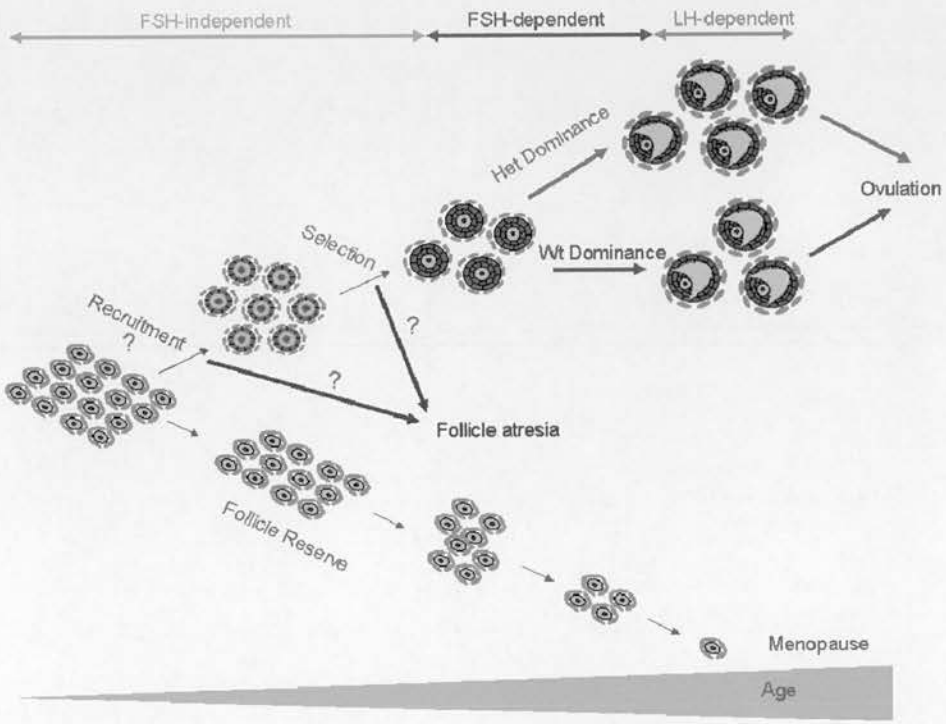


Figure 6.1 Diagram of stages of follicle recruitment, selection and dominance (adapted from Webb et al., 2003). Question-marks indicate processes that may influence numbers of follicles selected for ovulation.

6.2 Materials and Methods

6.2.1 Tissue Processing and Immunohistochemistry

Tissue was collected and processed as described in Section 2.2. One section per ovary from the central region of every ovary (animals and treatment groups are detailed in Table 6.1) was analysed for Amh expression and the subsequent section for Cleaved Caspase-3 activity. Immunohistochemistry was performed as described in Sections 2.4.3 to 2.4.8. The ovaries used were from the same animals on which follicle counts were carried out, and the genotype remained unknown until full analysis was completed.

Group	Number/Group
Untreated	5 het mice (10 ovaries) – 10 ovary sections 5 wt mice (10 ovaries) – 10 ovary sections
10 IU FSH-treated	3 het mice (6 ovaries) – 6 ovary sections 3 wt mice (6 ovaries) – 6 ovary sections
oFF-treated	3 het mice (6 ovaries) – 6 ovary sections 3 wt mice (6 ovaries) – 6 ovary sections

Table 6.1 Groups of Dazl ovaries analysed.

6.2.2 Analysis of Immunohistochemistry

Following immunohistochemistry of the selected sections, the sections were photographed (x 20) using a BH2 Olympus Microscope (Olympus, UK) with attached digital camera (Hitachi HV-C20A, Japan) using Image Pro Plus™ software and a Stereology 5.1 plug-in. Primary, secondary and early antral follicles were identified using criteria detailed in Section 2.5.1 by tracking each follicle on serial sections (from H&E stained sections used for follicle counts) until the nucleus of the oocyte could be observed and the follicle could be classified. As the sections were only 5µm thick, the follicle was usually present on the consecutive slide and could be analysed for expression of Amh and Cleaved Caspase-3 activity. If the follicle was not present on the consecutive slide, further analysis was not undertaken.

6.2.2.1 Analysis of AMH Expression

The sections were analysed for Amh using a 4-graded scaling system as described in Table 6.2 and examples of follicles demonstrating different levels of Amh staining are shown in Figure 6.2. A similar grading system was previously used to enable semi-quantification of ERα expression in epididymis (Atanassova *et al.*, 2001).

Level of Staining	Intensity of Staining
Negative (-)	No staining present in follicle granulosa cells.
Weakly Positive (+)	Some follicle granulosa cells were stained.
Positive (++)	All follicle granulosa cells were stained.
Strongly Positive (+++)	All follicle granulosa cells were stained with more intense brown stain.

Table 6.2 Amh analysis staining criteria

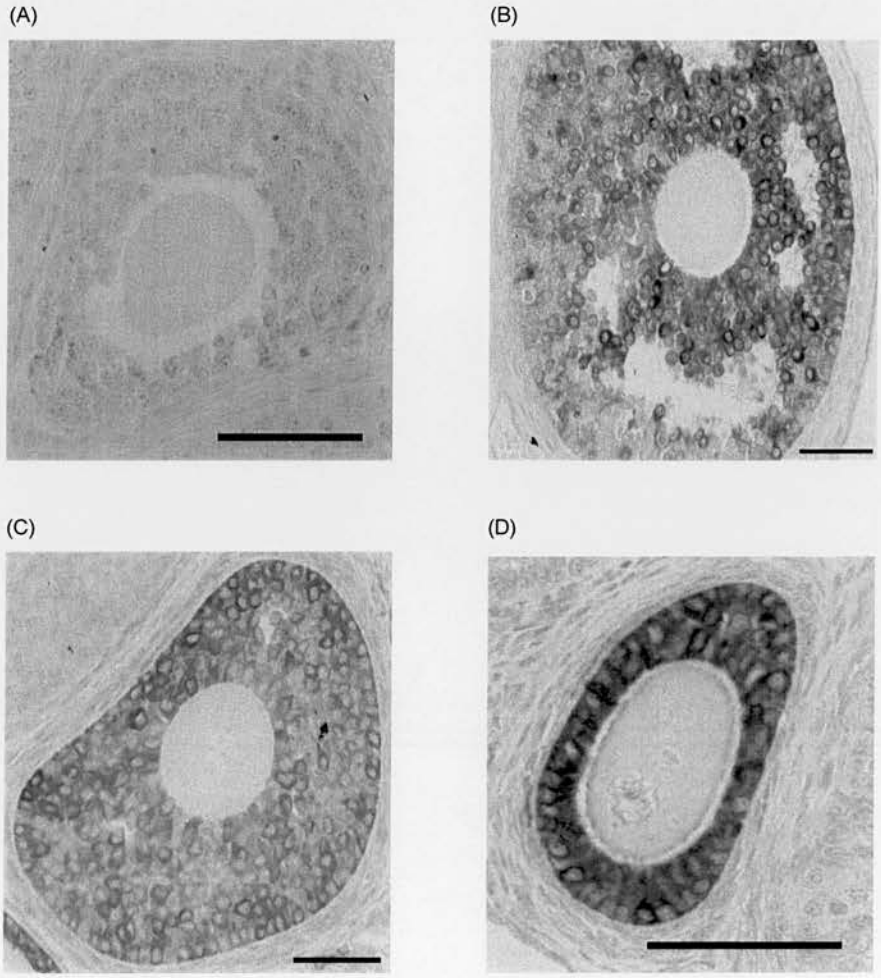


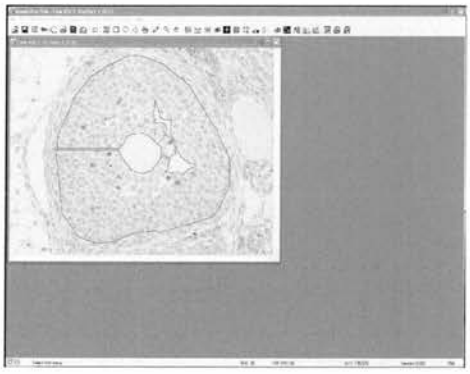
Figure 6.2 Images of levels of Amh staining (x40). The scales bars represent 50μm.
Where (A) is negative, (B) is weakly positive with not all cells stained positive, (C) is positive and (D) is strongly positive.

6.2.2.2 Analysis of Cleaved Caspase-3

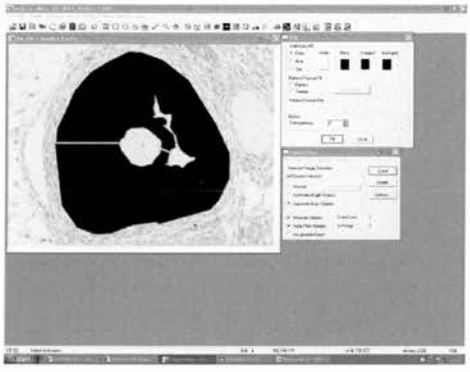
Low levels of Cleaved Caspase-3 staining required different analysis of these sections. Within any given follicle on each section, the most accurate means of quantifying this data was to count the number of granulosa cells stained positively compared to total numbers of granulosa cells. As detailed in Chapter 3 (Figure 3.9) granulosa cell area is representative of total granulosa cell number. Therefore the granulosa cell area was measured, positively stained cells for Cleaved Caspase-3 were counted and the relationship to granulosa cell area calculated.

Cleaved caspase-3 staining was quantified using the stereologer as described in Section 2.5. The granulosa cell area was calculated (Figures 6.3 (A) and (B)). The manual tag option was selected and the number of positive caspase-3 stained cells counted (Figure 6.3 (C)).

(A)



(B)



(C)

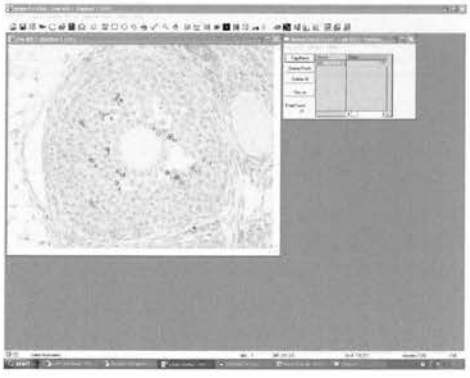


Figure 6.3 Stereological method for measuring granulosa cell area and positively-stained Cleaved Caspase-3 cells. (A) and (B) demonstrate measurement of granulosa cell area and (C) demonstrates counting of positively stained cells.

6.2.3 Statistical Analysis

To analyse Amh and Cleaved Caspase-3 staining between genotypes and across all treatment groups, one-way ANOVA was carried out followed by Tukey's Multiple Comparison tests (parametric). Data could not be log transformed, as in some categories there were values of zero. However, when this data was analysed it did not have a Gaussian distribution. When parametric analysis was performed, some results clearly differed by more than two standard errors yet were not marked as significant. This is because non-parametric analysis is less stringent. For this chapter, parametric testing was carried out on data that was not log transformed.

Furthermore, data comparing negative and positive staining for both Amh and Cleaved Caspase-3 in primary follicles was not amenable to analysis by one-way ANOVA. Results in these categories could only be analysed statistically if they had a standard error and this was the case, except for Amh data of untreated primary follicles. A Chi-square test was also not applicable as the test requires two numeric values above zero for at least one out of the two rows in the contingency table.

6.3 Results

6.3.1 Amh Expression

Intensity of Amh expression was similar for primary follicles from wt and het mice in all treatment groups (Figure 6.4). In both the untreated and oFF-treated animals there was a similar trend in Amh expression, with follicles from wt and het mice having greater levels of positive (++) Amh expression than any other levels of Amh staining (Figures 6.4 (A) and (C)). However, in the FSH-treated group most follicles from wt and het mice had positive (++) or strongly positive (+++) staining for Amh. There was no difference in Amh expression in primary follicles across treatment groups for wt and het mice.

Amh expression was similar for secondary follicles from wt and het mice in all treatment groups (Figure 6.5). In follicles from untreated and oFF-treated mice, the greatest percentage of secondary follicles expressed strongly positive (+++) levels of Amh whereas the greatest percentage of follicles in the FSH-treated group were either negatively (-) stained or strongly positively (+++) stained (Figure 6.5). There was no difference in Amh expression in secondary follicles across treatment groups in wt and het mice.

The expression of Amh in antral follicles did not differ between genotypes (Figure 6.6) in all treatment groups. Furthermore, at this stage of follicle development, the follicles from untreated and oFF-treated animals demonstrated a similar trend in Amh expression, with most follicles staining weakly positive (+) for Amh. For wt and het FSH-treated mice the greatest percentage of antral follicles were either negatively (-) or weakly positively (+) stained for Amh (Figure 6.6). FSH-treated mice had significantly greater percentages of follicles that were negative for Amh ($p<0.01$) than untreated and oFF-treated wt mice (Figure 6.6). *Dazl* FSH-treated het mice had significantly more negative Amh follicles ($p<0.05$) compared to untreated mice (Figure 6.6).

Primary, secondary and antral follicles with negative and positive expression of Amh were analysed (Figures 6.7 and 6.8). There was no difference in positive and negative Amh staining for follicles from wt and het mice in all treatment groups. Nearly all primary follicles (untreated mice) if not every (FSH-treated and oFF-treated mice) demonstrated positive expression of Amh (Figure 6.7 (A)). Untreated follicles from wt and het mice had significantly greater Amh staining ($p<0.0001$ for both wt and het mice) than negative Amh staining. In oFF-treated and FSH-treated mice, all primary follicles stained positive for Amh.

Secondary follicles from untreated and oFF-treated wt and het mice had significantly more positive ($p<0.001$) follicles expressing Amh than negative follicles (Figure 6.7 (B)). Follicles from FSH-treated het mice had significantly more positive than negative Amh expression of follicles ($p<0.01$), whereas follicles from wt mice had similar levels of negative and positive Amh expression. However, there was no significant difference of either negative or positive Amh expression between wt and het mice (Figures 6.7 (B) and 6.8).

Amh expression in antral follicles from untreated and oFF-treated wt and het mice showed a similar trend to secondary follicles in these groups. Antral follicles in the untreated group had significantly higher percentages of positively Amh-stained follicles ($p<0.001$ both wt and het mice) similar to follicles from oFF-treated wt and het mice ($p<0.001$ and $p<0.01$ respectively; Figure 6.7 (C)). However, there was no difference in the frequency of negative or positive Amh expression by antral follicles from wt and het FSH-treated mice (Figures 6.7 (C) and 6.8).

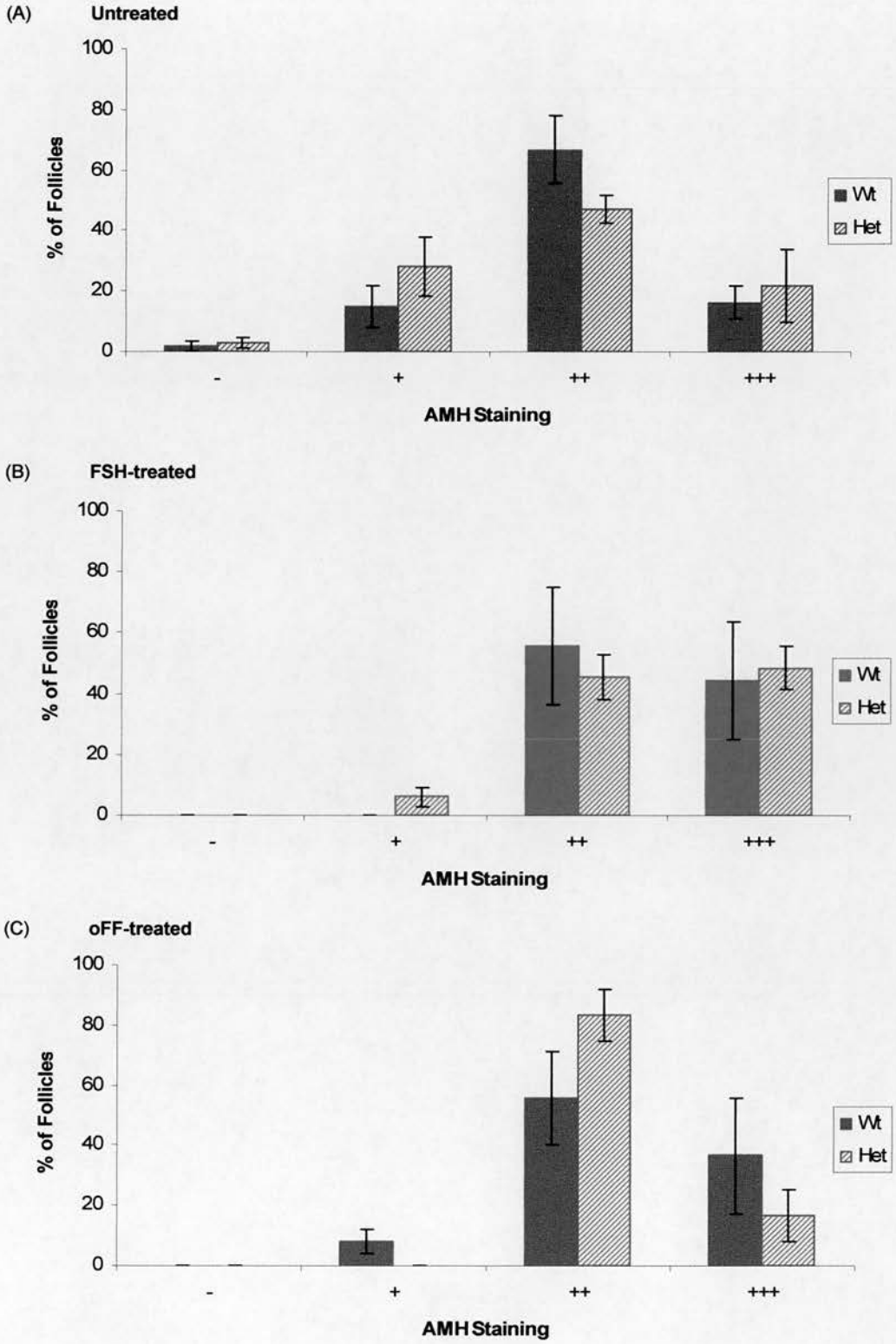


Figure 6.4 Amh expression of primary follicles from Dazl wt and het (A) Untreated n=5 mice, (B) 10IU FSH-treated n=3 mice and (C) oFF-treated n=3 mice. The percentages per animal were determined and the mean percentages ± S.E.M calculated.

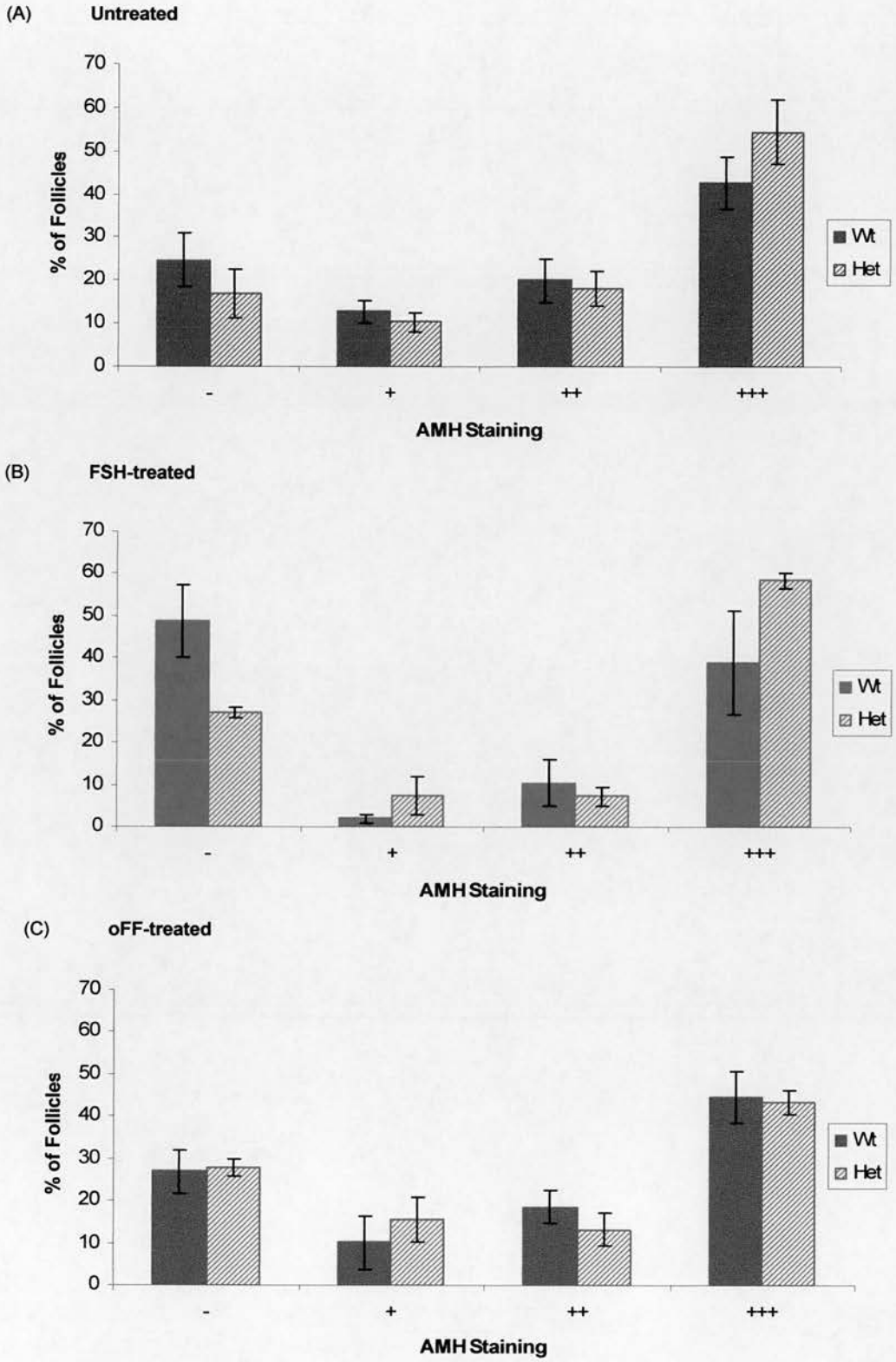


Figure 6.5 Amh expression of secondary follicles from Dazl wt and het (A) Untreated n=5 mice, (B) 10IU FSH-treated n=3 mice and (C) oFF-treated n=3 mice. The percentages per animal were determined and the mean percentages ± S.E.M calculated.

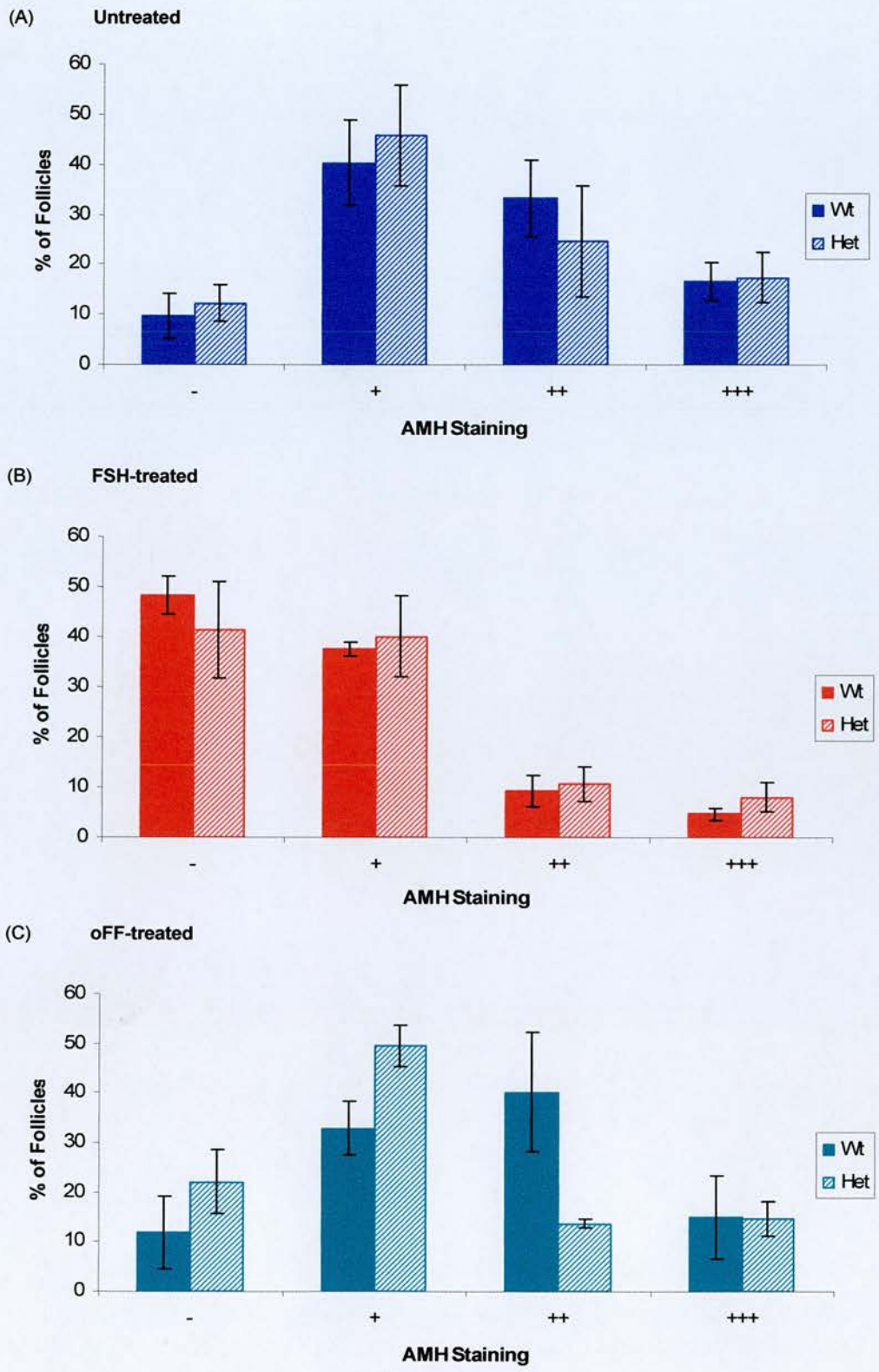


Figure 6.6 Amh expression of antral follicles from Dazl wt and het (A) Untreated n=5 mice, (B) 10IU FSH-treated n=3 mice and (C) oFF-treated n=3 mice. The percentages per animal were determined and the mean percentages ± S.E.M calculated.

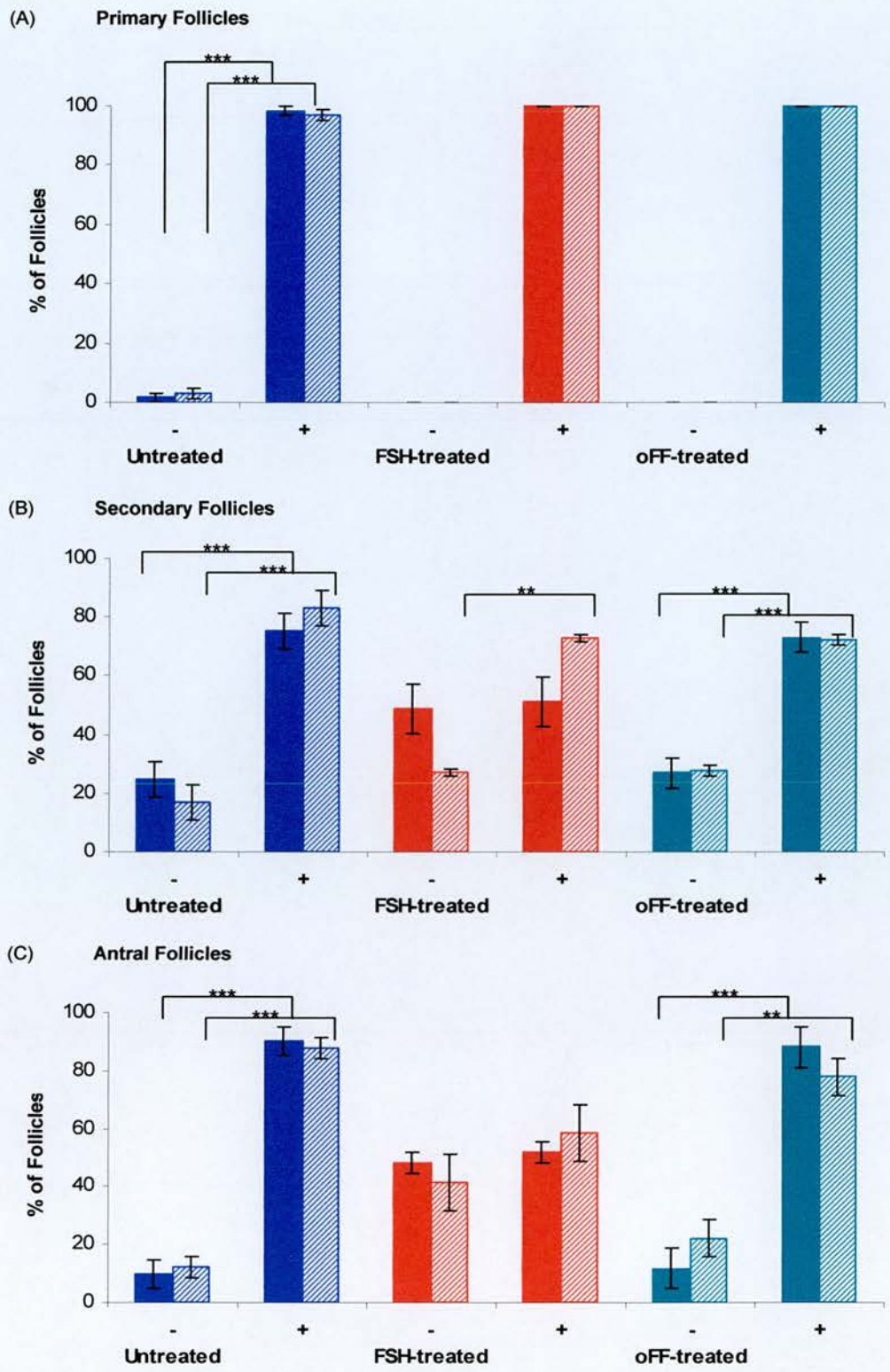


*Figure 6.7 Amh positive (+) and negative (-) expression by (A) primary, (B) secondary and (C) antral follicles from Dazl untreated (n=5 mice), 10IU FSH-treated (n=3 mice) and oFF-treated (n=3 mice) wt and het mice. Follicles with any positive AMH staining were grouped into the positive group. ** p=<0.01 and *** p=<0.001. The percentages per animal were determined and the mean percentages ± S.E.M calculated.* ■ *wt and* ▨ *het.*

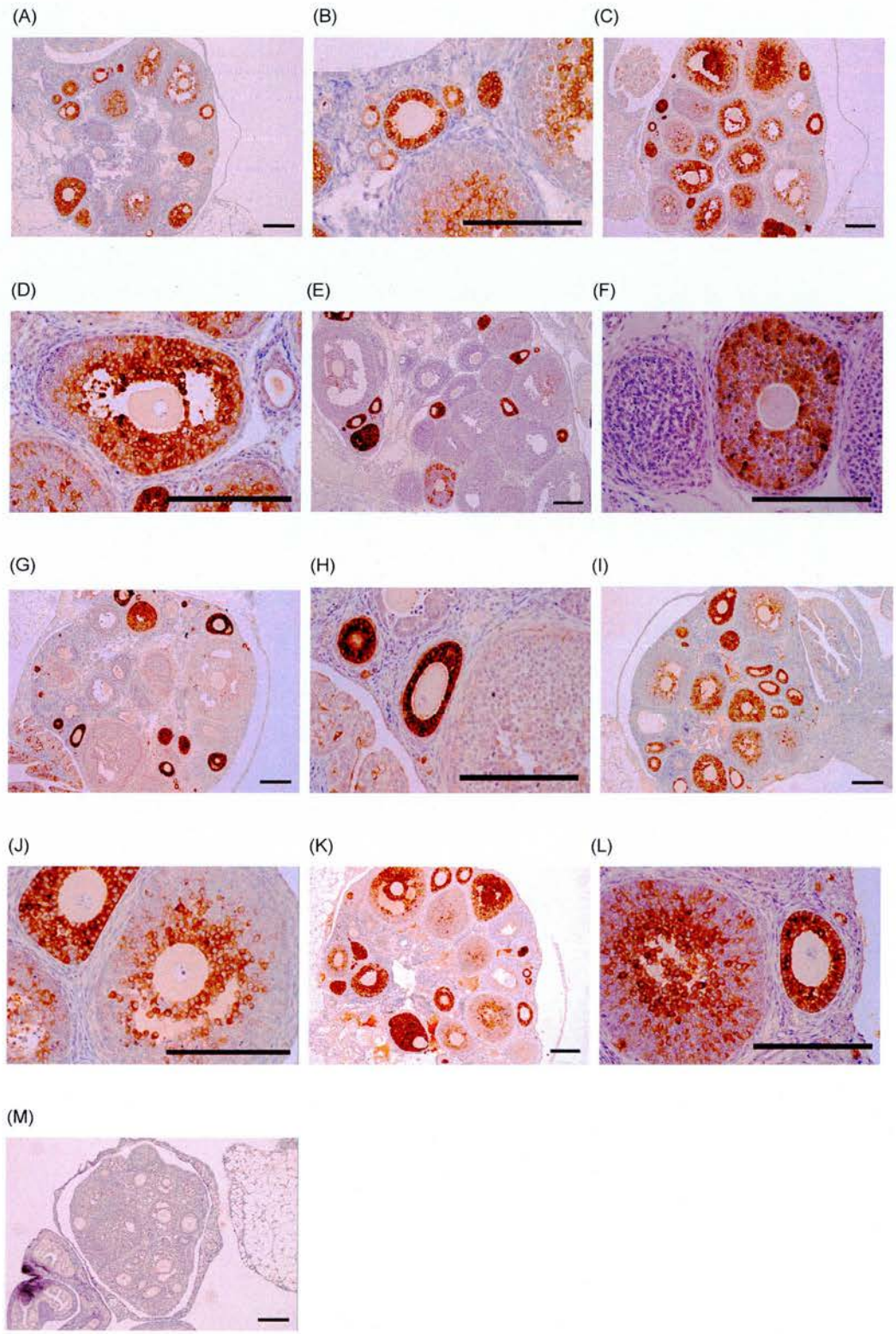


Figure 6.8 Immunohistochemistry images (x40) of Amh staining in ovaries from Dazl untreated ((A) to (D)), FSH-treated ((E) to (H)) and oFF-treated ((I) to (L)) mice for wt ((A), (B), (E), (F), (I) and (J)) or het ((C), (D), (G), (H), (K) and (L)) mice. (M) is a negative control. The scales bars represent 100μm.

6.3.2 Cleaved Caspase-3

All primary follicles from wt and het mice in all treatment groups stained negatively for Cleaved Caspase-3 (Figure 6.9 (A)). Cleaved Caspase-3 staining in secondary follicles demonstrated a significantly greater percentage of follicles with no Cleaved Caspase-3 in wt and het untreated ($p<0.05$ wt and <0.01 het) and in oFF-treated ($p<0.01$ wt and <0.05 het) animals (Figure 6.9 (B)). However, there was no difference in Cleaved Caspase-3 staining between wt and het mice for either of these treatment groups. FSH-treated wt mice had a similar percentage of Cleaved Caspase-3 negatively-stained secondary follicles to positively stained follicles (Figure 6.9 (B)), whereas, FSH-treated het mice had significantly fewer secondary follicles that stained positively for Cleaved Caspase-3 ($p<0.001$) (i.e. more healthy follicles) compared to wt mice. There was no difference in follicles with positive or negative Cleaved Caspase-3 activity in FSH-treated wt and het mice (Figure 6.10).

Significantly greater percentages of antral follicles that were positive for Cleaved Caspase-3 were observed in *Dazl* untreated ($p<0.001$ for both wt and het mice) and oFF-treated ($p<0.001$ (wt) and <0.05 (het)) mice (Figure 6.9 (C)). Animals treated with FSH had similar proportions of follicles with negative and positive staining for Cleaved Caspase-3 (Figure 6.9 (C)). There was no difference in the levels of Cleaved Caspase-3 staining for antral follicles from wt and het mice in any of the treatment groups (Figure 6.10).

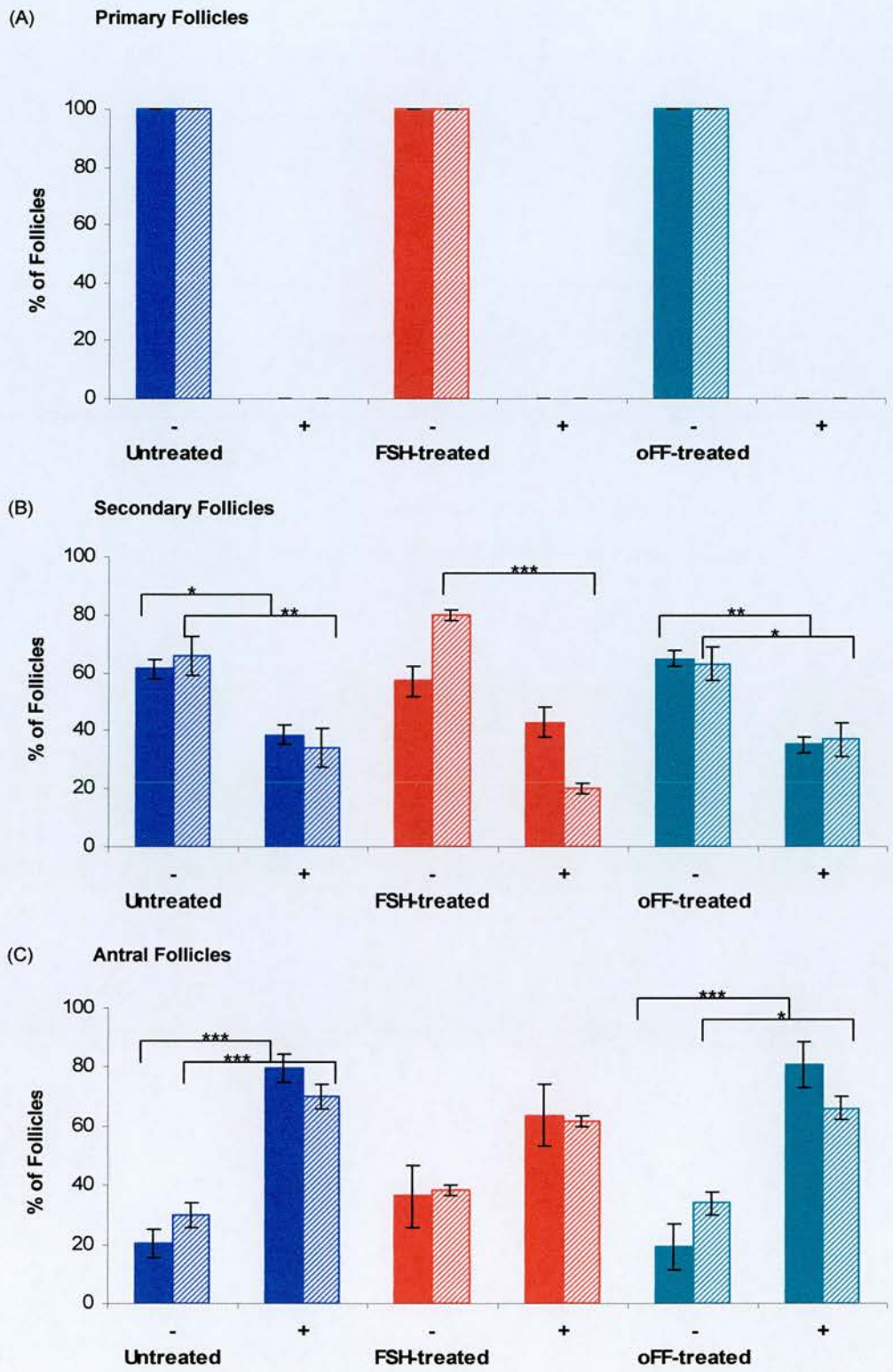


*Figure 6.9 Cleaved Caspase-3 positive (+) and negative (-) expression by (A) primary, (B) secondary and (C) antral follicles from Dazl untreated (n=5 mice), 10IU FSH-treated (n=3 mice) and oFF-treated (n=3 mice) wt and het mice. Follicles with any positive Cleaved Caspase-3 staining were grouped into the positive group. * p=<0.05, ** p=<0.01 and *** p=<0.001. The percentages per animal were determined and the mean percentages ± S.E.M calculated.*

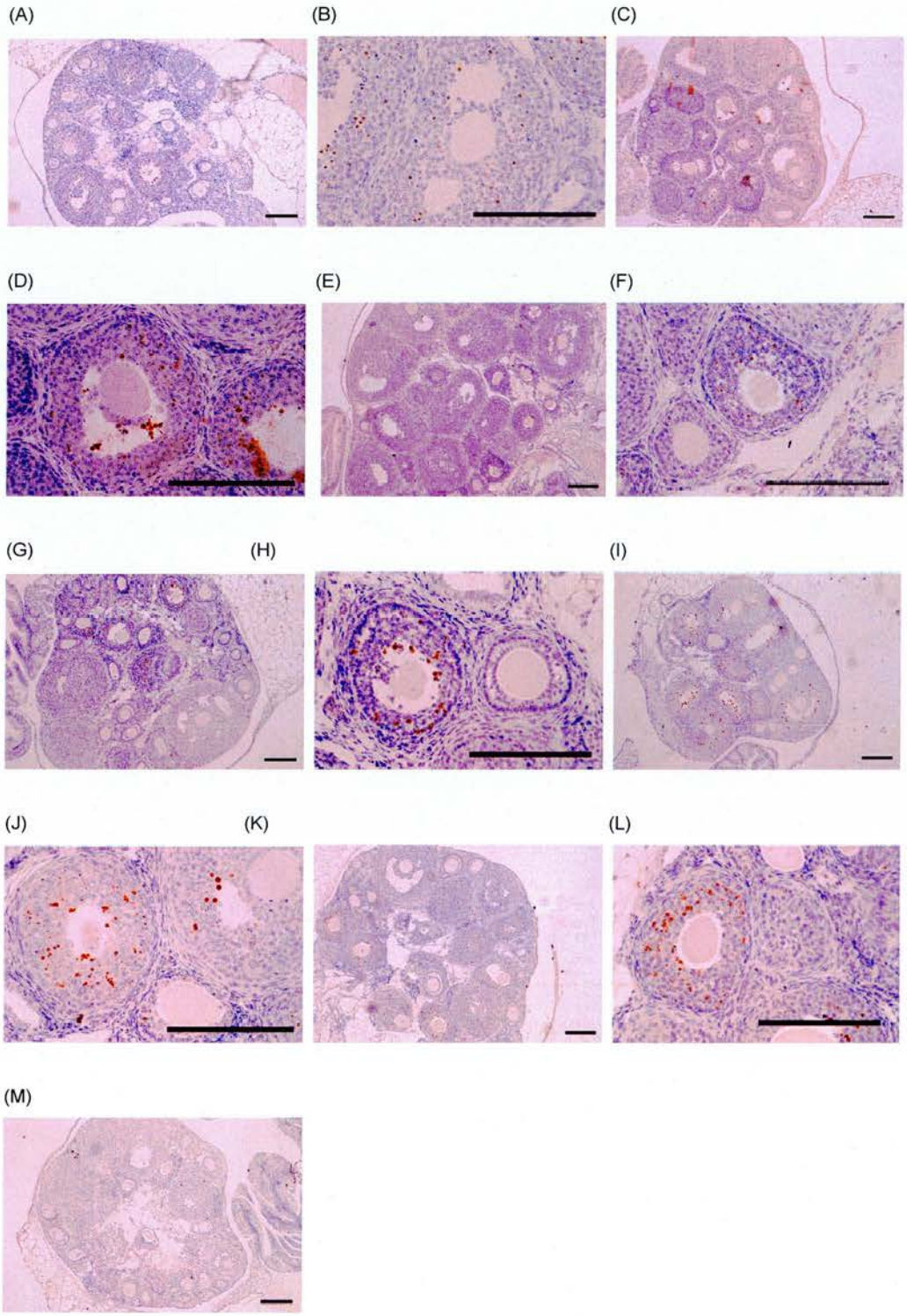


Figure 6.10 Immunohistochemistry images (x40) of Cleaved Caspase-3 staining in ovaries from Dazl untreated ((A) to (D)), FSH-treated ((E) to (H)) and oFF-treated ((I), to (L)) mice for wt ((A), (B), (E), (F), (I) and (J)) and het ((C), (D), (G), (H), (K) and (L)) mice. (M) is a negative control. The scales bars represent 100μm.

6.3.3 Amh Expression and Cleaved Caspase-3 Activity

Amh expression and Cleaved Caspase-3 activity was analysed within the same follicles. Primary follicles in every treatment group had the greatest percentage of follicles that stained positively for Amh and negatively for Cleaved Caspase-3 (Figure 6.11 (A)). In the untreated groups, a small percentage of primary follicles did not express either Amh or Cleaved Caspase-3. There was no difference in the expression of Amh and Cleaved Caspase-3 between wt and het mice in any of the treatment groups.

Secondary follicles in all treatment groups demonstrated a similar trend of Amh expression and Cleaved Caspase-3 activity (Figure 6.11 (B)) with most follicles at this stage of development expressing Amh but not Cleaved Caspase-3. However, in untreated and oFF-treated animals, the greatest percentage of follicles were Amh positive and Cleaved Caspase-3 negative, followed by: similar levels of Amh negative but Cleaved Caspase-3 positive follicles, and follicles positive for both Amh and Cleaved Caspase-3, and finally follicles negative for both Amh and Cleaved Caspase-3. The FSH-treated group had a larger percentage of follicles with Cleaved Caspase-3 activity but not expressing Amh. At this stage of development, there was no difference in Amh expression or Cleaved Caspase-3 activity between untreated and oFF-treated wt and het animals. In the FSH-treated group, het mice had a significantly lower percentage of follicles that were stained negative for Amh but positive for Cleaved Caspase-3 ($p<0.01$), and het mice had a significantly greater percentage of follicles that were positive for Amh but negative for Cleaved Caspase-3 ($p<0.01$).

The expression of Amh and Cleaved Caspase-3 in antral follicles did not differ between wt and het mice in any of the treatment groups (Figure 6.11 (C)). In untreated animals, the greatest percentage of antral follicles in both wt and het mice was positive for Cleaved Caspase-3 and negative for Amh. In FSH-treated animals, there were similar numbers of follicles positively expressing Amh and Cleaved Caspase-3, positive for Amh but negative for Cleaved Caspase-3, or positive for Cleaved Caspase-3 and negative for Amh. In oFF-treated animals, the greatest percentage of antral follicles were positive for Amh and Cleaved Caspase-3.

The intensity of Amh staining was examined in follicles that stained positive for both Amh and Cleaved Caspase-3 (Figure 6.12). There was no difference in the levels of Amh staining in secondary follicles of all treatment groups between wt and het mice (Figure 6.12 (A)). Follicles in this category had similar percentages of follicles within each of the Amh staining levels. There was no difference in Amh intensity between wt and het antral follicles in each of the treatment groups (Figure 6.12 (B)). A significantly greater percentage of Cleaved Caspase-3 positive follicles with low Amh expression was observed in wt and het untreated ($p<0.05$ (wt) and <0.001 (het)) and FSH-treated ($p<0.001$ for both wt and het) mice. However, only oFF-treated het animals had a significantly greater percentage of Cleaved Caspase-3 positive follicles with low Amh staining ($p<0.01$). The overall trend observed was that as Amh staining intensity decreased, the percentage of follicles staining positively for Cleaved Caspase-3 increased.

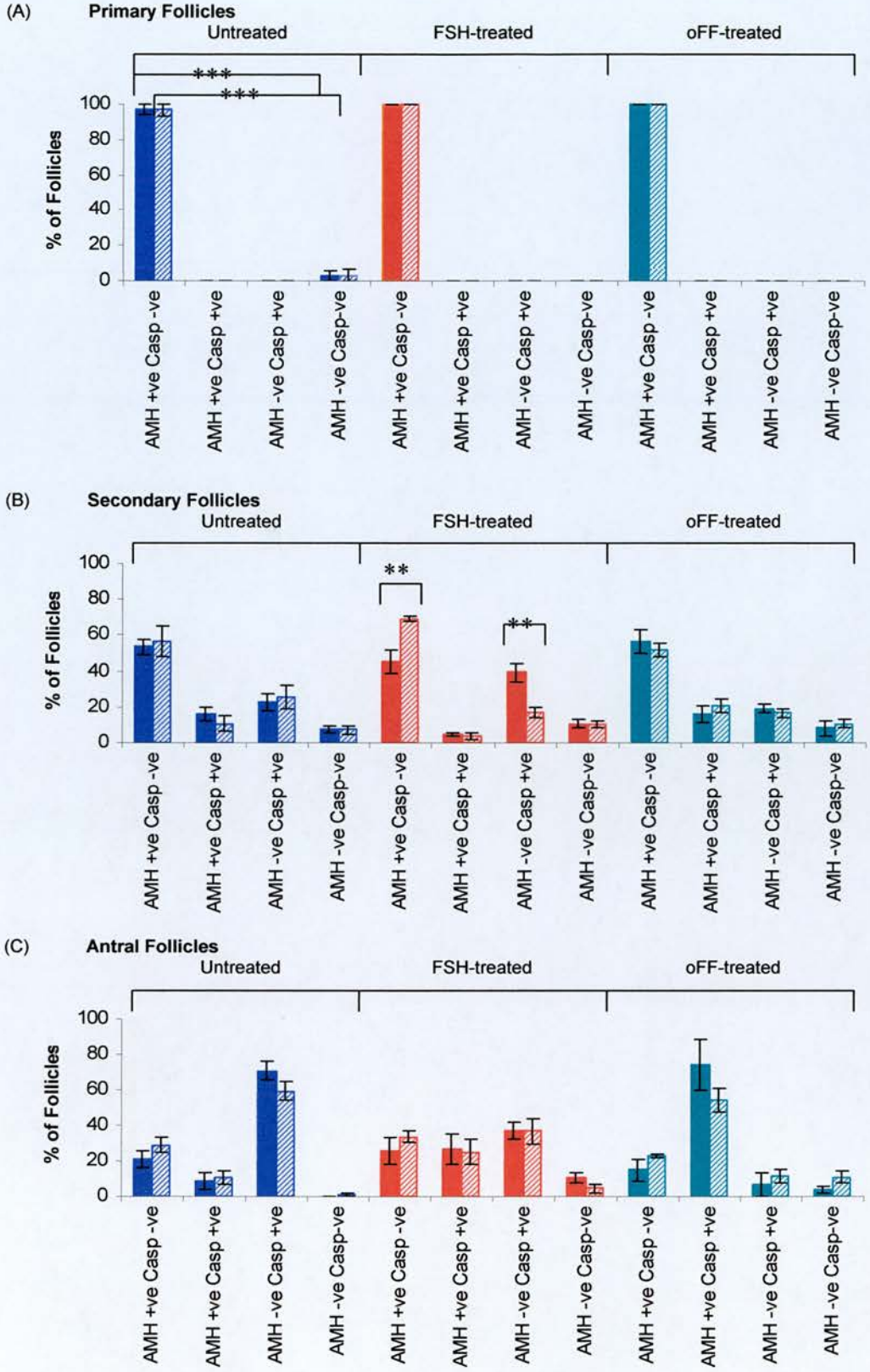


*Figure 6.11 The percentage of follicles expressing different levels of Amh and Cleaved Caspase-3 in (A) primary (B) secondary and (C) antral follicles from Untreated (n=5 mice), 10IU FSH-treated (n=3 mice) and oFF-treated (n=3 mice) wt and het mice. ** p=<0.01 and *** p=<0.001. The percentages per animal were determined and the mean percentages ± S.E.M calculated. wt and het.*

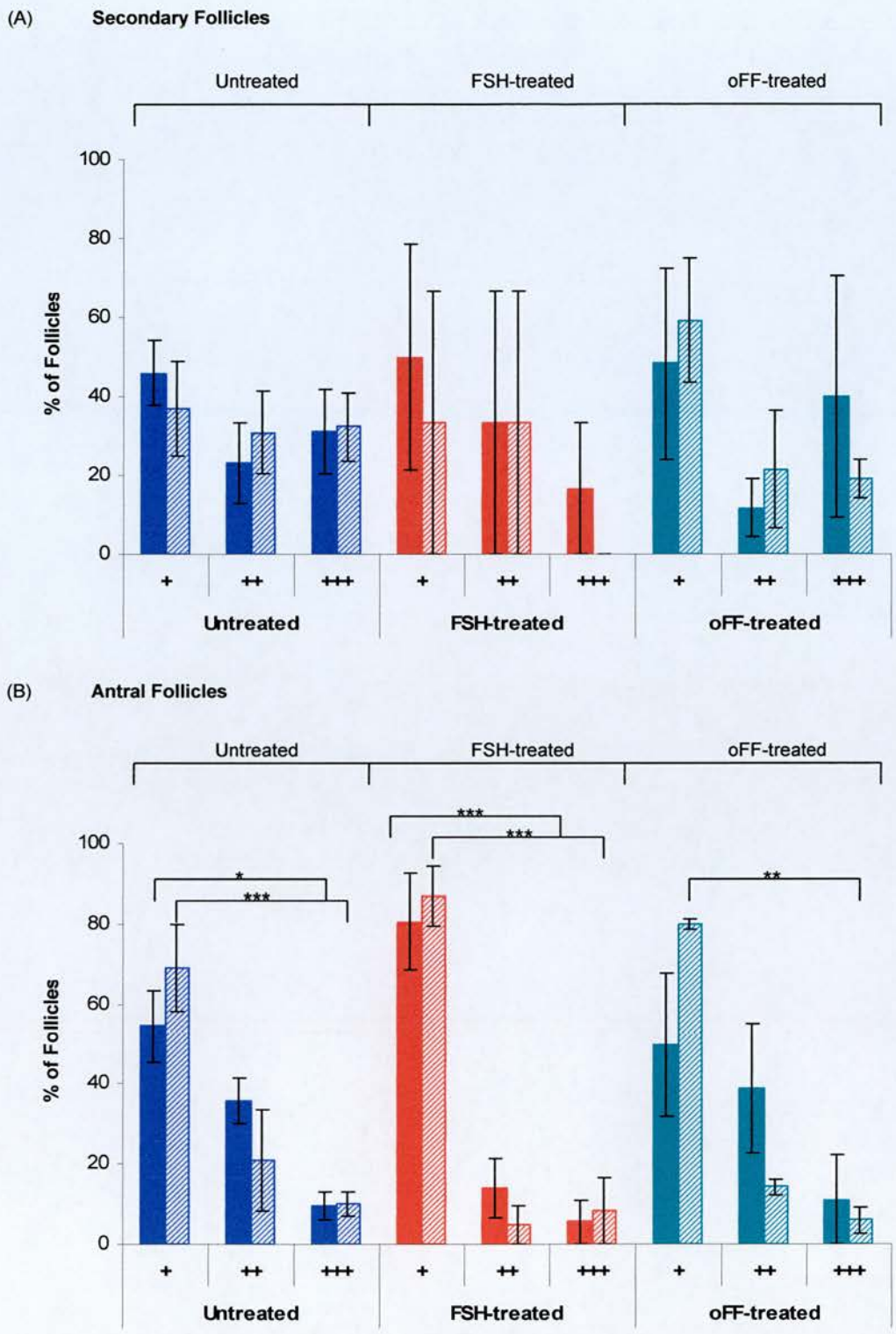


*Figure 6.12 Amh expression by follicles that were positive for Cleaved Caspase-3 activity. (A) secondary and (B) antral follicles from Untreated (n=5 mice), 10IU FSH-treated (n=3 mice) and oFF-treated (n=3 mice) wt and het mice. * p=<0.05, ** p=<0.01 and *** p=<0.001. The percentages per animal were determined and the mean percentages ± S.E.M calculated. wt and het.*

6.3.4 Amh Expression and Cleaved Caspase-3 Relative to Granulosa Cell Area

This analysis examined the percentage of follicles in oFF-treated mice that were negative (Figures 6.13 (A), (B) and (D)) or positive (Figures 6.13 (C) and (E)) for Cleaved Caspase-3 in relation to the granulosa cell area. Primary follicles were negative for Cleaved Caspase-3 staining, and there was no difference in distribution of granulosa cell area for follicles from wt and het mice (Figure 6.13 (A)). Secondary follicles that were negatively or positively stained for Cleaved Caspase-3 had similar granulosa cell area distributions (Figures 6.13 (B) and (C)). There was no difference in the distribution of granulosa cell area for antral follicles that stained positive with Cleaved Caspase-3 compared to those that were negative for Cleaved Caspase-3 (Figures 6.13 (D) and (E)). In addition, there was no difference in area for either positive or negative Cleaved Caspase-3 stained secondary or antral follicles in het compared to wt mice.

The percentage of follicles that were positive (Figures 6.14 (A), (B) and (D)) or negative (Figures 6.14 (C) and (E)) for Amh was not related to granulosa cell area in oFF-treated mice. Primary follicles showed positive Amh staining, and there was no difference between granulosa cell area distribution and Amh staining in follicles from wt and het mice (Figure 6.14 (A)). There was no difference in the range of granulosa cell area for follicles that stained positive with Amh compared to those that were negative for Amh staining (Figures 6.14 (B), (C), (D) and (E)) for both secondary and antral follicles, and no difference in follicles that were positive or negative stained for Amh from het mice compared to wt mice.

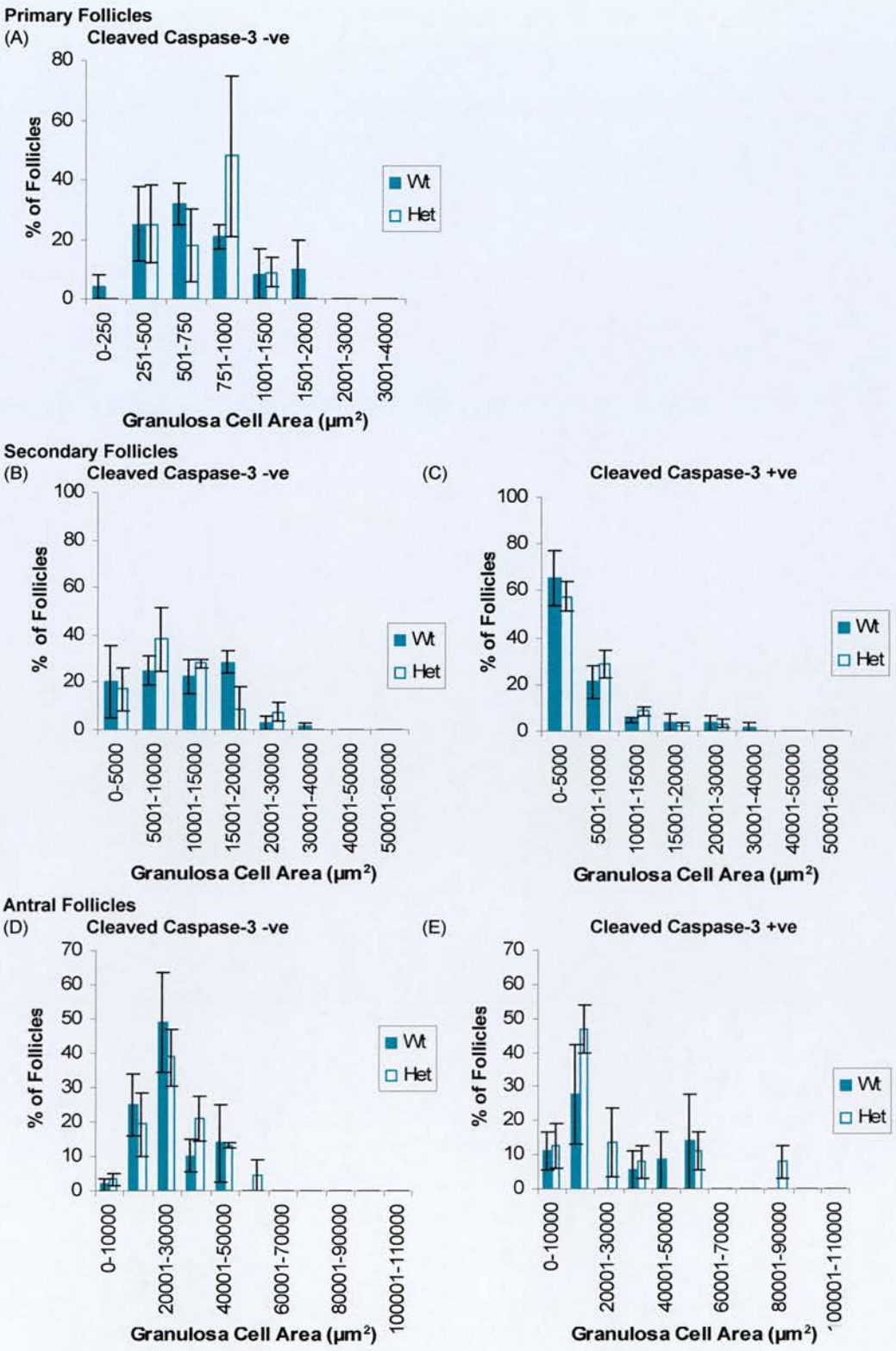


Figure 6.13 The percentage of (A) primary follicles, (B and C) secondary follicles and ((D) and (E)) antral follicles from Dazl oFF-treated wt and het mice (n=3 mice) that were Cleaved Caspase-3 negative ((A), (B) and (D)) or positive (C and E) relative to granulosa cell area. The mean per animal was calculated and the values are mean percentages ± S.E.M.

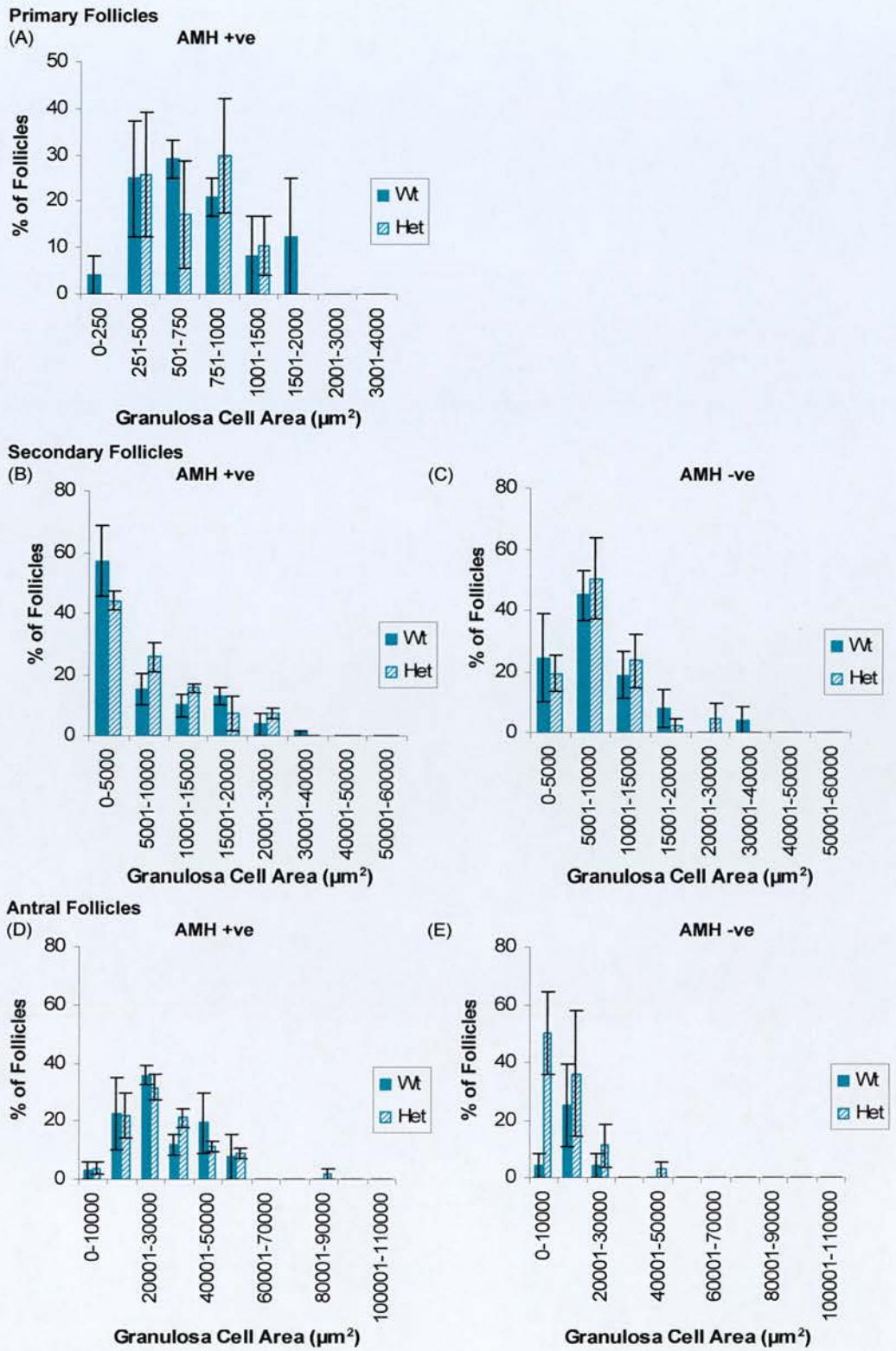


Figure 6.14 The percentage of (A) primary follicles, (B and C) secondary follicles and ((D) and (E)) antral follicles from Dazl oFF-treated wt and het mice (n=3 mice) that were AMH positive ((A), (B) and (D)) or negative (C and E) relative to granulosa cell area. The mean per animal was calculated and the values are mean percentages ± S.E.M.

6.4 Discussion

6.4.1 Amh Expression

The findings of the study presented in this chapter demonstrated that follicles from both *Dazl* wt and het mice showed similar levels of Amh expression in all treatment groups. Interestingly, untreated and oFF-treated groups demonstrated a similar trend of Amh expression in primary, secondary, and antral follicles (Figures 6.4, 6.5, and 6.6). Both treatments groups had primary follicles that were positive (++), secondary follicles that were strongly positive (+++) and antral follicles that were (+) weakly positive, whereas follicles from animals treated with FSH had positive (++) primary follicles, negative or strongly positive (-/+++) secondary follicles and negative or weakly positive (-/+) antral follicles. Further analysis of Amh (positive or negative) *Dazl* wt and het follicles (Figure 6.7) showed there was no difference in the percentage of follicles from *Dazl* wt and het mice in all treatment groups that stained positive or negative for Amh. In the untreated and oFF-treated wt and het animals, a greater percentage of primary, secondary and antral follicles expressed Amh compared to follicles with no expression, whereas, FSH-treated wt and het animals had a larger percentage of primary follicles which stained positive for Amh and similar levels of secondary and antral follicles that stained positive and negative for Amh.

Previous studies examining *Amh* in mice have shown increased levels as follicles develop, with maximal levels in late pre-antral follicles, at which point expression begins to decline (Salmon *et al.*, 2004). Similar expression of Amh was expected in untreated and oFF-treated *Dazl* mice as these (day 21) mice are pre-pubertal and therefore FSH levels would be low in untreated mice, similar to levels in oFF-treated mice. In addition, previous studies on *Dazl* mice (Chapters 3 and 4) have demonstrated that follicle development in these treatment groups is similar and therefore Amh expression would be expected to be similar.

FSH stimulates follicle development via the cAMP and PKA pathway and has also been linked to increasing *Amh* transcription in the testis (Galway *et al.,* 1990; Lukas-Croisier *et al.,* 2003). FSH-treated ovaries of *Dazl* mice had greater Amh expression in primary follicles, and this started to decline in secondary and antral follicles of both wt and het mice. The accelerated development of these follicles in FSH-treated mice compared to untreated or oFF-treated mice supports previous studies which showed a decline in AMH in antral follicles, and showed FSH stimulates follicle growth to a stage where this decline is observed (Galway *et al.,* 1990; Hirshfield, 1991; Baarends *et al.,* 1995).

Although the exact mechanism(s) by which Amh acts in the ovary is unknown, *Amh* transcription in the mouse testis is stimulated by FSH via the cAMP and PKA pathways (Lukas-Croisier *et al.,* 2003). FSH and oestrogen are proposed to regulate *Amh* expression in the ovary (Baarends *et al.,* 1995). FSH signals through the PKA pathway in the ovary though it can activate other pathways (Flores *et al.,* 1992; Richards, 2001b; Zeleznik *et al.,* 2003). Amh is thought to inhibit follicle activation through paracrine signalling (Salmon *et al.,* 2004), suggesting that *Dazl* does not affect paracrine communication between follicles by Amh signalling.

Recent data suggesting that oocyte renewal occurs in the murine ovary propose that this could expand the reproductive lifespan of an animal (Johnson *et al.,* 2004; Johnson *et al.,* 2005a; Johnson *et al.,* 2005b). However, this work has been met with much criticism and the authors have themselves changed the explanation of some of their initial findings (Johnson *et al.,* 2005a; Johnson *et al.,* 2005b). If oocyte renewal occurs then Amh levels would be expected to be greater for a longer period of time. However, studies examining human serum AMH levels have demonstrated that AMH levels begin to decline at 30 years (Tremellen *et al.,* 2005).

The results of this study conclude that *Dazl* wt and het mice follicles have a similar activation rate from the resting pool. Amh is thought to be involved in the regulation of follicle dominant selection (Durlinger *et al.,* 1999; Durlinger *et al.,* 2002). This suggests that the *Dazl* gene does not affect the number of follicles selected for ovulation by altering Amh action. Previous studies have demonstrated that *Amh* KO mice have lower plasma FSH levels, and that lower levels of Amh are associated with increased FSH-sensitivity (Durlinger *et al.,* 1999; Durlinger *et al.,* 2002). Previous studies of adult *Dazl* mice which demonstrated that *Dazl* het mice also had lower plasma FSH levels (McNeilly *et al.,* 2000), would imply that *Dazl* het mice would have reduced Amh expression with greater FSH-sensitivity. However, this was not observed. The previous study was performed on adult mice, whereas this study used day 21 mice, in which FSH levels were at basal levels. Therefore the effects of Amh on FSH and follicle development may not be observed at this age.

Salmon *et al.* (2004) observed that *Amh* was limited to the cumulus granulosa cells in mouse antral follicles. Amh expression in this study showed a higher intensity of Amh staining within the cumulus granulosa cells with less expression in mural granulosa cells (Figure 6.8) suggesting that the oocyte may play a role in regulating expression. The findings of this thesis suggest that the oocyte specific *Dazl* factor does not regulate Amh expression. In addition, unpublished work by McNeilly *et al.* (Appendix B) demonstrated that *Dazl* het mice had significantly lower ($p<0.05$) total follicle numbers and fewer primordial follicles. However, these mice had significantly larger litters ($p<0.001$; Appendix A), suggesting that *Dazl* het mice experienced greater follicle activation.

However, similar levels of Amh expression in day 21 *Dazl* wt and het mice suggest similar levels of follicle activation. Increased activation in adult mice could simply be a result of increased FSH-sensitivity whereby increased numbers of follicles are stimulated from the ovarian reserve (Allan *et al.,* 2006). This would explain why no difference was observed in day 21 mice, as FSH levels are at basal levels in pre-pubertal mice.

6.4.2 Cleaved Caspase-3

The rate of atresia can drastically affect the duration of the reproductive lifespan of an animal, and increased atresia rates accelerate the reduction in follicle reserve (Hillier, 1994). The rates of atresia in *Dazl* wt and het mice were investigated to determine whether a reduced atresia rate in het animals could explain the increased litter sizes observed in *Dazl* het mice (McNeilly *et al.,* 2000). The atresia rates were assessed in *Dazl* wt and het mice by analysing Cleaved Caspase-3 levels. This method of quantifying Cleaved Caspase-3 activity was adapted from Fulton *et al.* (2005) who counted positive stained cells in human fetal ovaries. However, the method used by Fulton *et al.* (2005) examined positive stained cells in a 10,000μm^2 area whereas this present study examined positive stained cells per follicle.

The activation of Caspase-3 precipitates cell death and once activated, cell death occurs rapidly (Manabe *et al.,* 2004). Rodent follicles that do not express activated Caspase-3 are classified as 'healthy' (Boone and Tsang, 1998). The experiments in this chapter demonstrate that the levels of Cleaved Caspase-3 were similar in primary, secondary and antral follicles in untreated, oFF-treated and FSH-treated *Dazl* wt and het mice (Figure 6.9). Furthermore, all treatment groups demonstrated a similar trend in Cleaved Caspase-3 activity (Figure 6.9).

Primary follicles in all treatment groups from wt and het mice did not stain for Cleaved Caspase-3, supporting previous studies that demonstrated that follicle atresia was a process that only occurred in the latter stages of follicle development (Irving-Rodgers *et al.,* 2001). Similarly, a greater percentage of secondary follicles did not express Cleaved Caspase-3. However, this was significant for untreated and oFF-treated wt and het mice, and for follicles from het FSH-treated mice. Interestingly, a larger percentage of antral follicles were positive for Cleaved Caspase-3 in untreated and oFF-treated mice (both wt and het), whereas, FSH-treated mice had similar percentages of follicles that stained for Cleaved Caspase-3 to those that did not stain for Cleaved Caspase-3.

As mentioned previously follicle atresia increases with follicle development through dominant follicle selection (Irving-Rodgers *et al.,* 2001; Quirk *et al.,* 2004). Therefore, Cleaved Caspase-3 levels would be expected to increase in antral follicles, as observed in this study. However, FSH is known to delay the onset of atresia (Hirshfield, 1989), and treatment of mice with 10IU FSH would be expected to reduce atresia rates in these mice compared to untreated and oFF-treated mice. The studies in Chapters 3 and 4 demonstrated that follicles from *Dazl* het mice were larger and more developmentally advanced than those from wt mice. Therefore, it would be expected that follicles from het mice would have higher levels of atresia. However, increased FSH-sensitivity at the stage of dominant follicle selection could prevent follicles from succumbing to atresia, thus accounting for the greater litter size observed in *Dazl* het mice (Appendix A). In addition, previous studies have proven that expression of Cleaved Caspase-3 does not regulate atresia. Rather it is the activity of the Cleaved Caspase-3 (Uma *et al.,* 2003). These results suggest that the *Dazl* gene does not affect atresia rates by affecting Cleaved Caspase-3. However, this is only one factor out of a potential list of more than sixty factors that have been associated with effects on follicle atresia (Pru and Tilly, 2001). Therefore, *Dazl* may affect atresia rates by altering the activity of any one of these other potential factors.

The oestrogen:progesterone ratio is also important in determining the health of a follicle. Dominant follicles have an oestrogen:progesterone ratio greater than one (Yu *et al.,* 2003). However, previous studies in *Dazl* adult mice concluded that wt and het mice had similar levels of oestrogen (McNeilly *et al.,* 2000). Furthermore, studies in Chapter 4 demonstrated that follicles from het mice cultured with sufficient FSH although larger in size, suggesting they were more developmentally advanced, secreted similar levels of oestradiol. However, progesterone levels were not assessed in this study and further experiments are required to assess *in vivo* and *in vitro* effects of oestradiol and progesterone levels in the *Dazl* wt and het mice.

Both FSH and LH can prevent follicle atresia (Hirshfield, 1989). Although both have a protective role, FSH only prevents atresia for 3 days (Hughes and Gorospe, 1991; Yu *et al.,* 2003). In addition, other studies have demonstrated that Caspase-3 activity was increased after FSH treatment even though atresia rates declined (Yacobi *et al.,* 2004). *Dazl* het mice had lower plasma FSH levels (McNeilly *et al.,* 2000), and atresia rates would therefore be expected to be higher in these mice. However, *Dazl* het mice had follicles which were more FSH-sensitive (Chapters 3 and 4). The present study showing similar atresia rates in both wt and het mice suggests that follicles from het mice with lower FSH and similar atresia rates were more resistant to atresia as a result of increased FSH-sensitivity.

6.4.3 Amh and Cleaved Caspase-3 Co-Localisation

Interestingly when consecutive slides were analysed for Amh and Cleaved Caspase-3 some follicles stained positive for both factors (Figure 6.11). Co-localisation was not possible due to time restraints. However, staining of consecutive sections enabled Amh and Cleaved Caspase-3 levels to be assessed in the same follicles. Previous studies concluded that atretic follicles do not express AMH (van Rooij *et al.,* 2002). However, studies in rodents demonstrated that Amh mRNA expression is occasionally detected in pre-antral follicles that are succumbing to atresia, whereas this is seldom observed in antral follicles (Baarends *et al.,* 1995).

Further analysis of Cleaved Caspase-3 and Amh positive follicles revealed that Cleaved Caspase-3 positive secondary follicles of untreated, oFF-treated and FSH-treated mice expressed all levels of Amh expression (from weakly (+) to strongly positive (+++); Figure 6.12). This suggests that these secondary follicles are in the early stages of atresia, explaining why Amh levels varied markedly from follicle-follicle, and suggests that follicles still have the potential to develop further until succumbing fully to atresia. Similar levels of Amh staining were observed in follicles from wt and het mice. However, the majority of antral follicles that were positive for Cleaved Caspase-3 and Amh expression showed weakly positive (+) levels of Amh expression (Figure 6.12). Again, a similar level of expression was observed in follicles from wt and het untreated, oFF-treated and FSH-treated mice.

The results presented in this study suggest that Amh is still expressed during atresia. Although previous studies support this finding, they state that Amh expression is not observed in antral follicles (Baarends *et al.,* 1995). However, Amh expression was observed in antral follicles from *Dazl* wt and het mice. These current findings suggest that Amh is expressed in the latter stages of follicle development, and the process of atresia is gradual with Amh levels declining gradually as the follicle becomes increasingly atretic (Figure 6.15). Previous studies support this since it has been shown that in follicles showing signs of atresia, some granulosa cells still function normally (Hirshfield, 1989; Quirk *et al.,* 2004).

Analysis of Amh and Cleaved Caspase-3 demonstrated that a significantly greater percentage ($p<0.01$) of follicles from FSH-treated het mice had staining for Amh but negative Cleaved Caspase-3 staining compared to wt mice (Figure 6.11). Furthermore, follicles from FSH-treated wt mice had a significantly greater percentage ($p<0.01$) of follicles that were negative for Amh but positive for Cleaved Caspase-3 compared to follicles from het mice (Figure 6.11). These findings suggest that follicles from het mice are healthier after stimulation from FSH, and supports previous findings from Chapters 3 and 4 which concluded that follicles from het mice were more FSH-sensitive.

These results have demonstrated that follicles from wt and het mice express similar levels of Amh and Cleaved Caspase-3. Therefore, it would be expected that follicles from het mice should have more healthy follicles or reduced atresia rates, and that would explain the larger litter sizes. However, several other factors (e.g. *Nobox* and *Gdf-9*) are involved in follicle activation and any of these factors that are known to affect follicle activation rate could be important in the *Dazl* mice (Rajkovic *et al.,* 2004; Shimizu, 2006). These results only examined one section of the ovary and therefore may not be fully representative of the entire ovary. Furthermore, other studies have concluded that Cleaved Caspase-3 is not necessary for atresia; follicle atresia can occur in its absence through the cleavage of Caspase-7 (Matikainen *et al.*, 2001).

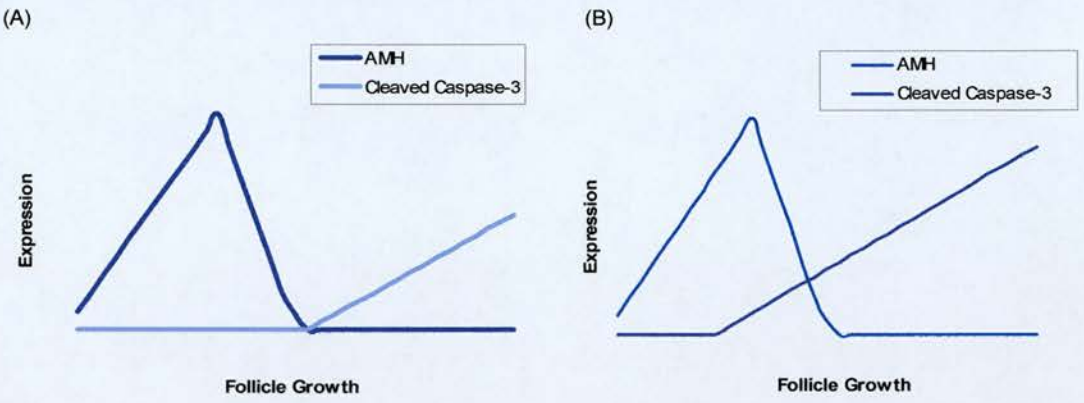


Figure 6.15 AMH expression and Cleaved Caspase-3 activity (A) suggested from previous studies and (B) proposed from this current study.

6.4.4 Relationship of AMH and Cleaved Caspase-3 Staining to Granulosa Cell Area

There was a high positive correlation between the rate of granulosa cell proliferation (diameter) and follicle atresia (Hirshfield and Midgely, 1978a; Lussier *et al.,* 1987). Analysis of Amh, Cleaved Caspase-3 and granulosa cell mass was carried out in oFF-treated follicles. Chapter 3 showed a high correlation between follicle diameter and follicle area. The oFF-treated group was selected as this group had reduced FSH levels, and would therefore be predicted to have higher rates of atresia. We also determined whether atretic follicles varied in size to those that were deemed to be 'healthy'. However, there was no difference in sizes of follicles that stained positive or negative for Amh (Figure 6.14). A similar observation was found for Cleaved Caspase-3 activity. Follicles positive for Cleaved Caspase-3 were similar in size to those that stained negative (Figure 6.13), and a similar trend was observed for both follicles from wt and het mice.

Although, larger follicles have been associated with increased atresia, granulosa cell area was similar for both Amh positive and Cleaved Caspase-3 positive follicles. This observation could simply be a result of the method used in this experiment, as only one section of ovary was analysed. However, the granulosa cell mass of follicles of all classes has similar AMH and Cleaved Caspase-3 levels in both wt and het mice, again confirming that the *Dazl* gene does not affect Amh expression or Cleaved Caspase-3 activity.

6.4.5 Future Work

As mentioned there were some limitations to this study, and many factors can affect follicle activation and atresia rates. The method used here was semi-quantitative. However, a strict grading scale, blind scoring, and analysis of the data by the same person ensured that the analysis was consistent. Furthermore, as mentioned previously, only one section of the ovary was analysed and therefore may not have been fully representative of the entire ovary. However, this did enable an initial analysis of Amh expression and Cleaved Caspase-3 activity in *Dazl* wt and het mice to be carried out. However, further studies of other potential apoptotic factors are required in *Dazl* wt and het mice.

As *Dazl* is an oocyte factor analysis of Cleaved Caspase-2 activity would be essential as this Caspase is associated with oocyte death (Bergeron *et al.,* 1998), and may be involved in regulating follicle atresia rates. However, studies have demonstrated that Cleaved Caspase-7 activity is independent of Caspase-3 and increases as apoptosis increases (Matikainen *et al.*, 2001). Analysis of expression of other members of the Caspase family would also be important in studying atresia rates of follicles from wt and het mice.

Bi-directional communication between oocyte and granulosa cells and between granulosa cell and theca cells is essential to suppress atresia (Tajima *et al.,* 2002), and it will be essential to determine the importance of this communication in atresia prevention (Takai *et al.,* 2003). Previous studies have demonstrated that theca cells secrete anti-apoptotic factors that prevent follicle atresia originating in the granulosa cells (Tajima *et al.,* 2002). In addition, gap junctions (involving Connexon-43) are essential for bi-directional communication and the overall fate of the follicle (Ackert *et al.,* 2001). Hence examining pro-survival factors as well as pro-apoptotic factors will be necessary to determine the cause of variations in atresia rates between follicles from wt and het mice. Co-culture of granulosa cells and theca cells, and examining levels of pro-apoptotic factors (such as *Bax*) and pro-survival factors (such as Survivin) will also be important (Takai *et al.,* 2003; Hussein, 2005).

Although Amh has been found to be important in follicle activation other factors such as *Nobox, Smad 3, Gdf-9* and *Kit* Ligand are all involved in follicle activation (Durlinger *et al.*, 2001; Tomic *et al.*, 2002; Rajkovic *et al.*, 2004; Hutt *et al.*, 2006a; Hutt *et al.*, 2006b; Kaivo-oja *et al.*, 2006; Shimizu, 2006). Smad-3 has been found to regulate pro-apoptotic factors such as *Bax* (Tomic *et al.*, 2002; Takai *et al.*, 2003). Analysis of these components in the *Dazl* mouse strain will be essential to determine the differences in follicle activation in wt and het mice, and may illuminate the signalling pathways through which *Dazl* functions.

6.4.6 Conclusion

In conclusion, the studies in this chapter have demonstrated that a single copy of the *Dazl* gene does not alter Amh expression or Cleaved Caspase-3 activity compared to two copies of this gene, suggesting that follicle activation, follicle health and the rate of follicle atresia were similar in het and wt mice. In addition, follicles that were deemed healthy by the positive expression of Amh could also show early evidence of atresia, supporting previous studies that concluded that atretic follicles could still function (Quirk *et al.*, 2004). The results presented in this chapter also support previous studies that suggest that atresia is a gradual process (Hirshfield, 1989), as in this study, more Cleaved Caspase-3 positive antral follicles had weaker levels of Amh compared to secondary follicles which had all levels of Amh staining. This study also concluded that granulosa cell mass was not affected by atresia, as Cleaved Caspase-3 positive follicles from oFF-treated mice were similar in size to those with no expression. A similar finding was found for Amh expression in oFF-treated mice.

Previous studies have demonstrated that the litter size is regulated by both follicle activation from the resting pool and the rate of atresia (Manabe *et al.*, 2004). Several factors are involved in promoting and inhibiting the survival rate of follicles (Pru and Tilly, 2001). Although the mechanism(s) by which Dazl works remains unknown at present, from this study *Dazl* does not alter Amh expression and Cleaved Caspase-3 activity. Further analysis will be required in the *Dazl* mouse to determine any atretic factors that regulate ovulation rate and lead to the larger litters experienced by het mice. However, since the *Dazl* gene does not affect Amh expression and Cleaved Caspase-3 activity these factors do not seem to affect ovulation rates.

Chapter 7

General Discussion

7.1 Findings of this Thesis

The experiments in this thesis were designed to examine the effect of a single copy of the *Dazl* gene on follicle growth and development, compared to two copies of this gene, and attempt to uncover the mechanisms behind the observed differences in litter sizes of *Dazl* wt and het mice. Initial studies examined follicle counts and follicle areas in day 21 *Dazl* wt and het mice that were untreated, oFF-treated or FSH-treated. The use of oFF and FSH in treatment of animals examined the impact of FSH levels on follicle development in the *Dazl* mouse strain (Hirshfield and Midgley, 1978b; Hirshfield, 1986; McLeod and McNeilly, 1987). Untreated, oFF-treated and FSH-treated day 21 *Dazl* wt and het mice had similar numbers of primordial, transitionary, primary, secondary and unclassifiable follicles (Figures 3.1 and 3.3) demonstrating that ovarian reserve and follicle activation were similar in these mice suggesting that the number of copies (i.e. one or two) of the *Dazl* gene has no effect on follicle numbers up to day 21. Interestingly, adult *Dazl* het mice have larger ovaries and greater litter sizes than to *Dazl* wt mice (McNeilly *et al.*, 2000; McNeilly *et al.,* Unpublished; Appendix A). Furthermore, adult *Dazl* het mice have increased plasma inhibin B levels, lower FSH levels, and similar levels of oestrogen and inhibin A compared to *Dazl* wt mice (McNeilly *et al.*, 2000). In addition, in adult *Dazl* het mice have fewer total follicles, a lower percentage of pre-antral follicles, but a greater percentage of antral follicles, whereas *Dazl* KO mice have no oocytes (McNeilly *et al.,* Unpublished; Appendix A and B).

Untreated *Dazl* het mice had a significantly greater percentage of antral follicles compared to wt mice ($p < 0.05$; Figure 3.3), confirming a previous study on *Dazl* adult mice (McNeilly *et al.,* Unpublished; Appendix B). This suggests that the action of the *Dazl* gene affects antral development and may explain why wt and het mice at day 21 are very similar, as these mice are pre-pubertal, a stage associated with little antral development. In addition, the follicles from untreated *Dazl* het mice were larger, with greater granulosa cell areas than follicles from wt mice. No oocyte renewal was observed in any ovaries. However, this study concluded that oocyte areas were similar for both wt and het mice in all treatment groups (Figures 3.18, 3.19 and 3.20). Oocyte size increases with maturity (Eppig *et al.,* 2002). Since the oocytes in wt and het mice are at a similar stage of development this data suggests that the *Dazl* gene does not alter oocyte growth.

A single copy of this oocyte gene stimulates granulosa cell proliferation to a greater extent than two *Dazl* genes and enhances FSH-sensitivity resulting in larger follicles that are more advanced and increased litters sizes in *Dazl* het mice. These results have shown that follicle growth is advanced in het mice and suggest that these follicles reach the FSH threshold earlier than follicles from wt mice (Figure 7.1). The duration of the FSH window has a direct effect on ovulation rates (Brown, 1978; Macklon and Fauser, 2001). Whether *Dazl* affects granulosa cell differentiation remains unknown. However, future studies on this are essential. Since FSH-sensitivity is enhanced by the presence of a single copy of the gene compared to two copies, the differences in follicle reserve observed in *Dazl* adult mice could simply be due to their increased FSH-sensitivity.

The oocyte is fundamental in regulating follicle development (Buccione *et al.,* 1990; Salustri *et al.,* 1990) and the studies presented in this thesis also conclude the oocyte is essential in granulosa cell development. The results presented in this thesis suggest that there is no gene dosage effect of *Dazl* on follicle reserve, although studies of adult wt and het mice suggest that the full effect of *Dazl* is not observed in pre-pubertal mice. Although at day 21 these mice are pre-pubertal the ovaries still contain antral follicles, the different trends observed in follicle numbers between day 21 *Dazl* wt and het mice and adult *Dazl* wt and het mice suggest that the *Dazl* gene could effect follicle development in different ways depending on the age of the animal. Mizunuma *et al.*, (1999) concluded that activin inhibited pre-antral follicle development in adult mice, however, stimulated pre-antral development in day 11 mice.

Increased FSH levels are associated with an increase in follicle activation (Hirshfield and Midgley, 1978b; LaPolt *et al.,* 1992) and must therefore be coupled with accelerated loss of ovarian reserve. Treatment with oFF inhibits FSH levels (McLeod and McNeilly, 1987). Follicle numbers and areas from oFF-treated and FSH-treated mice were similar for both genotypes in all these treatment groups and the differences in litter sizes observed in *Dazl* mice could simply be a result of increased FSH-sensitivity. This would explain why the observed differences in untreated mice are diminished in oFF-treated and FSH-treated mice when FSH levels are altered. Previous work has demonstrated that there is a narrow range of FSH levels required for full follicle maturation (Brown, 1978). This threshold of FSH needs to be exceeded for folliculogenesis to be reached yet excessive FSH levels will not stimulate growth and development beyond that of lower levels (Brown, 1978). Treatment with 10IU FSH exceeds the threshold and is sufficient for full follicle maturation in both wt and het mice, resulting in similar growth and development in follicles from wt and het mice.

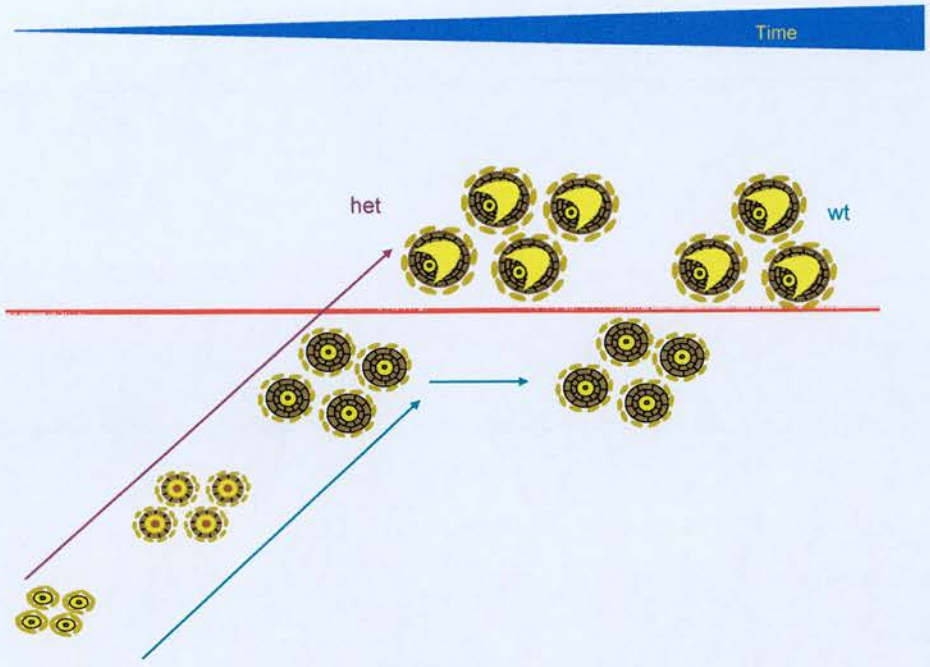


Figure 7.1 Illustration of follicles from Dazl wt and het mice reaching the FSH threshold. From the studies in this thesis it is proposed that the follicles from the het mice exceed the FSH threshold at a faster rate than follicles from the wt mice.

The work in this thesis also demonstrated that *Dazl* het mice have similar ovarian areas compared to wt mice in all treatments groups (Figure 3.8). Furthermore, treatment with FSH resulted in larger ovaries in both wt and het mice although there were no differences observed between genotypes in this treatment group. This contrasts with previous studies which observed larger ovaries in adult *Dazl* het mice (McNeilly *et al.*, 2000). However, this difference could simply be due to differences in the age of the mice.

The mice in this study were day 21 and would have basal levels of FSH, supporting the theory that the full effect on the *Dazl* strain will not be observed until basal levels of FSH increase in adults. As expected, FSH-treated mice had larger ovaries than ovaries from untreated or oFF-treated mice (Figure 3.8). FSH treatment stimulates granulosa cell proliferation and follicle growth. Hence, larger ovaries would be expected in these mice (Hirshfield and Midgley, 1978b; Hirshfield, 1986). Interestingly, although the follicles from het mice were larger and there were a significantly greater percentage of antral follicles in *Dazl* het mice, their ovaries were of a similar size (Figure 3.8). However, a slight increase in follicle numbers and size is unlikely to alter the overall size of the ovary significantly.

Further studies examined follicle FSH-sensitivity by culturing individual follicles with different doses of FSH (1IU, 0.1IU or 0.01IU FSH). This study demonstrated that follicles from *Dazl* het mice were more FSH-sensitive than follicles from wt mice, and had significantly greater follicle growth when cultured with all doses of FSH (Figure 4.3). Previous studies had suggested a greater level of sensitivity to FSH in het mice (McNeilly *et al.*, 2000; McNeilly *et al.*, Unpublished; Appendix A). However, these studies have not determined the mechanisms which a single copy of the *Dazl* gene stimulates follicle development through increased FSH-sensitivity. Increased FSH-sensitivity could be due to increased numbers of FSHRs, enhanced binding to the FSHRs or increased stimulation of downstream signalling (Simoni *et al.*, 1997; Zhou *et al.*, 1997). The larger follicles observed in this study and their greater granulosa cell areas (Chapter 3) suggests that follicles from het mice may have greater numbers of FSHRs as a result of increased granulosa cell proliferation. Antral follicle development *in vitro* required 0.1IU FSH, confirming previous studies (Spears *et al.*, 1998; Spears *et al.*, 2002) and confirming that the growth and development of follicles from *Dazl* mice were similar to other F1 mice. However, follicles from het mice were responsive to 0.01IU FSH with significantly larger follicles compared to those from wt mice ($p<0.01$; Figure 4.3).

The follicles from the *Dazl* wt and het mice had similar levels of oestradiol when cultured with both 1IU and 0.1IU FSH, although follicles from het mice were larger. Inhibin A levels were also similar with 1IU and 0.1IU FSH treatments, but with 0.01IU FSH, the follicles from het mice secreted significantly greater ($p<0.05$) levels of inhibin A (Figure 4.7). These results suggest that follicles from het mice were developmentally advanced to a stage where higher FSH levels (1IU and 0.1IU FSH) were beginning to cause oestradiol secretion to decline (Valdez *et al.*, 2005). However, inhibin A levels were significantly greater ($p<0.05$) in follicles from het mice with 0.01IU FSH when follicles are still not fully developed suggesting even at this low level of FSH, follicles from *Dazl* het mice were more developmentally advanced (Figure 4.7). Inhibin B levels increased throughout the culture period (Figure 4.8). Previous studies have also demonstrated similar increases in murine inhibin B levels during *in vitro* follicle growth (Smitz and Cortvrindt, 1998). This increase is likely to be due to the lack of a negative feedback system *in vitro*.

Smitz and Cortvrindt (1998) demonstrated that inhibin B is a marker of a healthy follicle. Analysis of inhibin B on day 2 of culture demonstrated no difference in levels from follicles that survived culture compared to those that did not survive culture in this strain of mouse (Figure 4.9). However, the culture system and the assay method used by Smitz and Cortvrindt (1998) differed to those used in this thesis. The findings of this thesis suggest inhibin B is not a good marker for ovarian follicle survival at this stage of culture. However, inhibin B may be a marker of a healthy follicle *in vivo* or at a later time-point in the culture.

On days 2 and 6 of culture follicles from het mice cultured with 1IU and 0.1IU FSH secreted significantly ($p<0.05$) greater levels of inhibin B than follicles from wt mice (Figure 4.8). This supports previous findings on *Dazl* mice which demonstrated that het mice have elevated levels of plasma inhibin B compared to wt mice (McNeilly *et al.,* 2000). Inhibin B levels have been used as a measurement of the number of small follicles present (Groome *et al.*, 1996; Hohmann *et al.,* 2005). However, follicles from *Dazl* het mice have the ability to secrete greater levels of inhibin B. This is most likely due to the fact that the larger follicles in het mice have greater numbers of granulosa cells (Figures 3.12 and 3.16) and therefore a greater potential to produce higher levels of inhibin B. Interestingly, follicles from *Dazl* wt and het mice were both less FSH-sensitive than follicles from F1 mice (Figure 4.1(D)). This suggests that the *Dazl* gene affects other factors such as growth and survival, explaining why *Dazl* het mice although having smaller follicles also have larger litter sizes than F1 mice. Alternatively, these mice may have optimal level of FSH-sensitivity (within the FSH threshold range) resulting in larger litter sizes.

Several follicles burst on days 3 and 4 of the culture period. Follicles from het mice were less likely to burst when cultured with 1IU and 0.1IU FSH, suggesting these follicles were more robust ($p<0.05$; Figure 4.4). MMP-2 and MMP-9 activity was analysed in follicles and culture media from wt and het mice. MMP-2 and MMP-9 are the major gelatinases that degrade collagen, a major component of the follicle basement membrane (Bagavandoss, 1998; Smith *et al.,* 2002; Curry and Osteen, 2003; Rodgers *et al.,* 2003). However, MMPs activity was at the detection limit of the method used, and only MMP-2 activity could be reliably detected. MMP-2 was the most abundant gelatinase during the early stages of follicle development (Riley *et al.,* 2004). Furthermore, higher levels of latent MMP-2 were detected in all samples (Figure 4.12). This was expected, as active MMPs are not stored (Rawdanowicz *et al.,* 1994). Interestingly, all MMP-2 activities were similar in follicle and culture media samples from *Dazl* wt and het mice, suggesting that basement membrane degradation is not due primarily to gelatinase activity. This phenomenon was not observed *in vivo* and could simply be an artefact of the culture system. However, the gelatinases are only one component of ECM remodelling, and analysis of other factors that degrade the ECM needs to be assessed further.

Additional studies were carried out to assess the establishment of follicle dominance in the *Dazl* mice in co-culture. Follicle dominance occurred for all follicle combinations (wt/wt, het/het and wt/het) when cultured either in physical contact or physically separate (Figures 5.2, 5.3, 5.4 and 5.5) although the effect was more apparent when cultured in physical contact. This suggests that the factor that enables follicle dominance to be achieved is secreted, and that the effect of the factor is diluted out when follicles are not in contact.

Various inhibitory factors have been proposed to be involved in regulating follicle dominance such as GCIF and activin (Hynes *et al.,* 1996; Mizunuma *et al.*, 1999) though the factor(s) that regulates follicle dominance remains unknown and require the actions of several factors working together. It will be important to determine the effects of these factors in the *Dazl* mice.

Follicle dominance occurs at a stage when the largest follicle(s) begins to express LHRs and can still continue growing in the face of lower FSH levels (Fortune *et al.*, 2001; Webb *et al.,* 2003; Otsuka *et al.,* 2005). Interestingly, when follicles from *Dazl* wt and het mice were co-cultured in physical contact the follicles from het mice were more likely to become dominant (Figure 5 6), possibly due to their increased FSH-sensitivity and ability to continue to grow with low plasma FSH levels. As shown previously (Chapters 3 and 4) follicles from het mice were larger and more developmentally advanced, supporting the suggestion that these follicles were more likely to establish follicle dominance. Follicle bursting was only observed *in vitro* and was possibly an artefact of the culture system (Spears, Personal Communication). However, the bursting rate of follicles from both wt and het mice was reduced in co-culture experiments, suggesting that follicles were healthier when in an environment surrounded by other follicles. Interestingly, follicles in the early stages of development are found in small clusters (Peters, 1969; Picton, 2001).

The majority of studies have concluded that the follicle reserve is finite (Zuckerman, 1951; Baker, 1963; Faddy *et al.,* 1987; Eggan *et al.*, 2006). The rates of follicle activation and atresia are main factors in determining the reproductive lifespan of an animal (Baker, 1963; Faddy *et al.,* 1976; Yu *et al.,* 2004; Lo *et al.,* 2005). Amh expression and Cleaved Caspase-3 activity was analysed to examine activation, health status of a follicle and follicle atresia rates in *Dazl* mice. Amh expression was similar in wt and het mice suggesting that follicle activation and the number of healthy follicles were similar in both genotypes (Figures 6.4 – 6.7). However, studies by Durlinger *et al.* (2001) demonstrated that increased FSH-sensitivity was associated with low Amh expression. Therefore it would be expected that follicles from het mice with increased FSH-sensitivity would demonstrate lower levels of Amh. However, there was no difference between *Dazl* genotypes suggesting that the *Dazl* gene does not alter Amh expression. Analysis of other factors involved in regulating follicle activation such as *Nobox* and *Smad-3* will be essential to determine differences in follicle activation levels between genotypes (Tomic *et al.,* 2002; Rajkovic *et al.,* 2004).

Similarly, Cleaved Caspase-3 activity was similar in follicles from *Dazl* wt and het mice (Figure 6.9). This suggests that atresia rates were similar in wt and het mice. However, in excess of sixty factors have been proposed to be involved in follicle atresia, and alterations in any one of these factors could influence atresia rates in these mice (Pru and Tilly, 2001). The follicles with increased FSH-sensitivity from *Dazl* het mice would be expected to have a lower atresia rate, as FSH has been shown to prevent follicles from undergoing atresia (Hirshfield, 1989). However, the lower plasma levels of FSH in adult mice (McNeilly *et al.,* 2000) and similar levels of atresia in day 21 mice suggest that follicles from *Dazl* het mice are more resistant to atresia.

7.2 Future Studies

This thesis has shown that untreated het mice have a greater percentage of antral follicles compared to wt mice and this was not observed in mice treated *in vivo* with 10IU FSH or oFF. The studies reported here have shown that antral follicles from untreated *Dazl* het mice are larger both *in vitro* and *in vivo*. Furthermore, the follicles from het mice contained a larger cell mass of granulosa cells compared to untreated wt mice but this was not observed when animals were treated with oFF or 10IU FSH.

The ovarian reserve can be assessed from blood hormone levels, mathematical calculations or ovarian follicle counts (Faddy *et al.,* 1976; Faddy *et al.,* 1992; Groome *et al.,* 1996; Kumar *et al.,* 1997; de Vet *et al.,* 2002; Ficicioglu *et al.,* 2003; Yong *et al.,* 2003). Although the latter method is a very labour intensive technique (Zuckerman, 1951) ovarian follicle counts are the most accurate means of assessing follicle numbers. There are various means of classifying follicles from oocyte or follicle diameter to granulosa cell morphology (Zuckerman, 1951; Hirshfield and Midgley 1978b; Flaws *et al.*, 2001a; Myers *et al.,* 2004). In this thesis follicles were classified into primordial, transitionary, primary, secondary, antral and unclassifiable follicles according to granulosa cell morphology. The method used in this study is a robust system that is widely used to calculate follicle numbers (Zuckerman, 1951; Bucci *et al.*, 1997; Flaws *et al.*, 2001a; Tilly, 2003). However, recently a modified version of this technique (the Fractionator/Optical or Fractionator/Physical Disector method) that allows analysis of the entire ovary and is more likely to represent actual follicle numbers was carried out by Britt *et al.* (2000) and Myers *et al.* (2004). Future analysis of the ovarian follicle reserve would compare the Fractionator/Physical Disector method to the method of ovarian counts utilised in this thesis to determine the most accurate system.

Future studies would determine the FSH level at which follicles from het mice become more FSH sensitive. Follicle counts and antral follicle sizes of *Dazl* wt and het mice treated with increasing concentrations of FSH (0–10IU) would determine the FSH threshold for these mice, and establish the endogenous FSH level that produces similar numbers of antral follicles in both wt and het mice, and the dose at which *Dazl* het mice have increased numbers of antral follicles. However, in addition to analysing follicle sizes and numbers under various doses of FSH, the ovarian weight should be measured. This current study did not examine the weights of the ovaries, although this would have given data on other aspects of ovarian morphology. In addition, measuring FSH, oestradiol, progesterone and inhibin levels *in vivo* in untreated pre-pubertal wt and het mice would enable direct comparisons of follicle endocrinology both between genotypes and with *Dazl* adult mice.

The culture system utilised in this study was a very robust system that enables full follicle maturation (Spears *et al.,* 1998) and allowed the effects of FSH dose on follicle growth and development to be monitored in a controlled environment. However, Wycherley *et al.* (2004) have adapted the method utilised in this study by growing the follicle in inverted culture, without the requirement for oil to cover the follicles. This method enhanced follicle growth through increased oxygen supply (Wycherley *et al.,* 2004). However, although the latter method results in larger follicles this study did not examine the viability of the oocytes. Therefore, further studies are required to determine if this new system is more beneficial for follicle maturation.

The cause of increased FSH-sensitivity in the present studies could not be determined. The mechanism by which follicles from *Dazl* het mice acquire increased FSH-sensitivity requires further examination. Analysis of FSHR numbers, binding affinity and signalling pathways need to be explored in follicles from *Dazl* wt and het mice. Real-time PCR measurements of FSHR and LHR expression in follicles from het mice and wt mice at all stages of development may provide an explanation for the advanced growth of follicles from het mice, and would also determine whether increased FSH-sensitivity is a result of increased FSHR expression or whether more advanced follicle maturity correlates with increased LHR expression. In addition, assaying cAMP levels in follicles would show whether the FSHR signalling pathway is more effective in follicles from het mice than wt mice, and could explain the increased FSH-sensitivity of follicles from het mice. *Smad-3* is essential for follicle development (Tomic *et al.,* 2002), therefore expression of Smads in the *Dazl wt* and *het* mice ovaries should be examined to determine if *Dazl* signals through this pathway.

Hormone measurements were limited by the volume of the samples from follicle cultures, and the high coefficient of variation for oestradiol measurements. The assays used in this study were in-house assays. Unfortunately the inter-assay variation correlation was high for the oestradiol ELISA. A kit assay may reduce the inter-assay variation observed. To allow for variations in the theca layer of cultured follicles, culturing follicles in the presence of androstenedione may give a more representative measure of oestradiol secretion. Initially plasma levels of oestradiol, progesterone, inhibin A and B in day 21 mice should be examined. This data, together with follicle counts and follicle sizes would give a further insight into follicle growth and development *in vivo,* and would also enable comparison of day 21 with adult mice (McNeilly *et al.*, 2000). This would allow a more detailed analysis in to how *Dazl* affects follicle development throughout life. Previous studies have demonstrated that androgens sensitise granulosa cells' response to FSH (Hillier, 2001). Similarly, activin also increases FSH mRNA expression (Inoue *et al.,* 2003). Therefore, assessing androgen and activin levels in *Dazl* wt and het mice would determine whether these factors play a role in the increased FSH-sensitivity of follicles from *Dazl* het mice.

Follicles from *Dazl* het mice were more robust, as demonstrated by the reduced levels of burst follicles *in vitro.* However, MMP-2 and MMP-9 activities were similar for both genotypes. Zymography enables the semi-quantitative analysis of latent and active forms of MMPs in culture media and tissue samples. However, the small sample volumes used in this study meant that MMP activity was at the detection limit of this technique. Analysis of MMP levels in culture media from separate granulosa cell and theca cell cultures would give larger volumes for analysis and a more accurate detection of MMP activity. In addition, zymography and reverse zymography analysis (with larger sample volumes) would allow measurements of MMP activity and levels of TIMPs, giving a more complete picture of the overall rate of ECM degradation. Further studies would include immunohistochemical analysis of key components of the ECM (such as collagen IV) to examine the basement membrane structure and integrity (Bagavandoss *et al.,* 1983; Rodgers *et al.,* 2003).

The analysis of both Amh and Cleaved Caspase-3 expression demonstrated similar levels of expression in follicles from wt and het mice. Ideally, this study would have examined their co-localisation. This was not possible. In addition, there were other limitations to this study. Future analysis of the expression of other growth or atretic factors would analyse a greater proportion of the ovary to achieve a better representation. Although, the studies on the Cleaved Caspase-3 and Amh were not extensive, the data did not show any significant differences between follicles from wt and het mice, suggesting that there was no difference between the ratio of Amh-positive and Cleaved Caspase-3-positive follicles in wt compared to het mice. However, although the number of animals examined for Amh expression and Cleaved Caspase-3 activity were low, there were sufficient data to determine any trends.

Nevertheless, since Cleaved Caspase-3 is only one factor of more than sixty that are thought to be involved in follicle atresia (Pru and Tilly, 2001), a wider analysis of follicle atresia by means of micro-array would enable a broader quantitative measure of follicle atresia.

The previously published culture method used to examine follicle dominance demonstrated that follicle dominance did occur (Spears *et al.,* 1996). However, the number of co-cultured follicles was low. Repeats of these experiments will be required to obtain a more accurate reflection of the patterns of follicle dominance in the *Dazl* strain. Furthermore, variation of the culture method may be required. Studies by Baker *et al.* (2001) demonstrated that this culture system enables the establishment and maintenance of follicle dominance to be examined. However, this present study only examined establishment of follicle dominance. The maintenance of dominance is also an essential aspect of the entire process of follicle dominance. Co-culturing follicles of different starting sizes (Baker *et al.,* 2001) would demonstrate differences between follicles from wt and het mice, as well as differences in establishing dominance. Granulosa cell and theca cell co-cultures would enable identification of autocrine/paracrine factors that are involved in cell growth and differentiation. In addition, separate cultures with increased media volume would enable the measurement of MMP and TIMP activity.

Previous studies have suggested that follicle growth is determined by the oocyte (Buccione *et al.,* 1990; Salustri *et al.,* 1990; Eppig *et al.,* 1997b; Eppig *et al.,* 2002). The oocytes from wt and het mice did not differ in size in this study suggesting the *Dazl* gene, although being oocyte-specific, did not determine the activity and maturation of the oocytes. Culturing oocytes from *Dazl* het mice with granulosa cells from wt mice (and vice versa) and assessing granulosa cell proliferation would confirm this. Granulosa cell proliferation could be assessed by measuring tritiated thymidine uptake or by BrDU incorporation. This would show whether one copy of the *Dazl* gene in the oocyte can influence granulosa cell growth and development, regardless of background of the granulosa cells. It would also determine whether proliferation is driven solely by the oocyte and what effect one copy of the *Dazl* gene has on this proliferation rate. Finally, as the mouse is a poly-ovulatory animal, future work needs to determine the effects of *Dazl* in mono-ovulatory species.

7.3 Conclusions

In conclusion, the studies in this thesis have demonstrated that untreated day 21 *Dazl* het mice have an increased percentage of antral follicles which are larger compared to wt mice. This effect was diminished in the absence of FSH and in animals treated with high (10IU) FSH. Follicles from het mice were more FSH-sensitive than those from wt mice as demonstrated by their ability to grow larger and they were more developmentally advanced compared to follicles from wt mice following treatment with 1IU, 0.1IU and 0.01IU FSH *in vitro.*

These results show that a single copy of the *Dazl* gene enhances follicle growth stimulating granulosa cell proliferation through paracrine regulation. This increased FSH-sensitivity explains why *Dazl* het mice had increased litters sizes compared to wt mice (McNeilly *et al.*, Unpublished). The mechanism through which a single copy of the *Dazl* gene increases follicle FSH-sensitivity remains unknown. Future studies will focus on elucidating the mechanisms involved and identifying the threshold for FSH stimulation of follicle growth.

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Appendix A

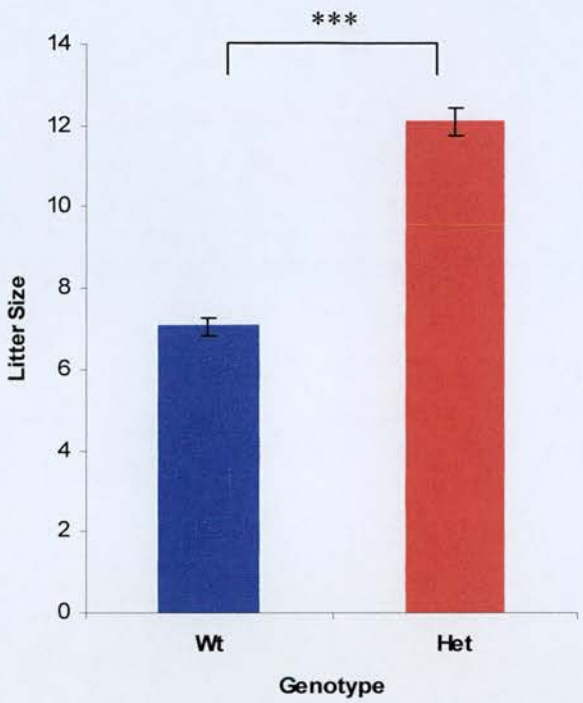


*Figure A.1 Data of Dazl litter sizes from wt (n-5) and het (n=5) mice where *** p= <0.001 (McNeilly, J. R. et al., Unpublished).*

Appendix B

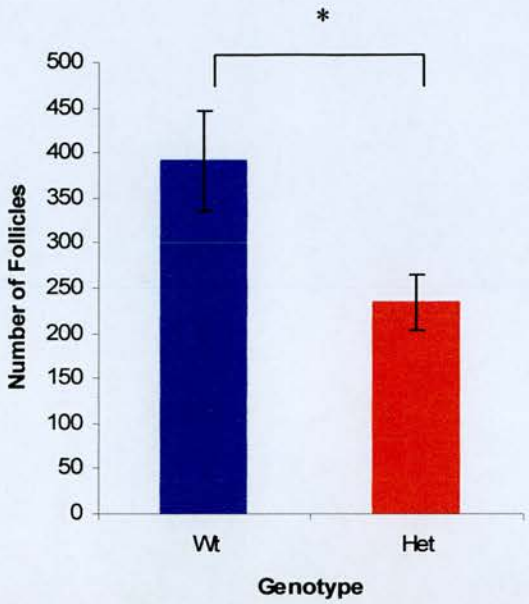


*Figure B.1 Total raw follicle counts for Dazl wt (n=5) and het (n=7) mice where * p=<0.05 (McNeilly, J. R. et al., Unpublished).*

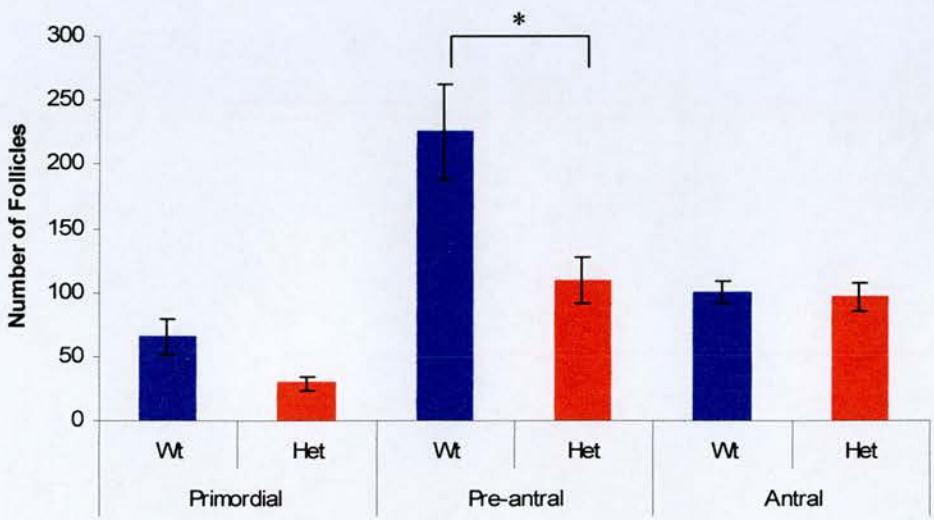


*Figure B.2 Total number of primordial, pre-antral and antral follicle counts for Dazl wt (n=5) and het (n=7) mice where * p=<0.05 (McNeilly, J. R. et al., Unpublished).*

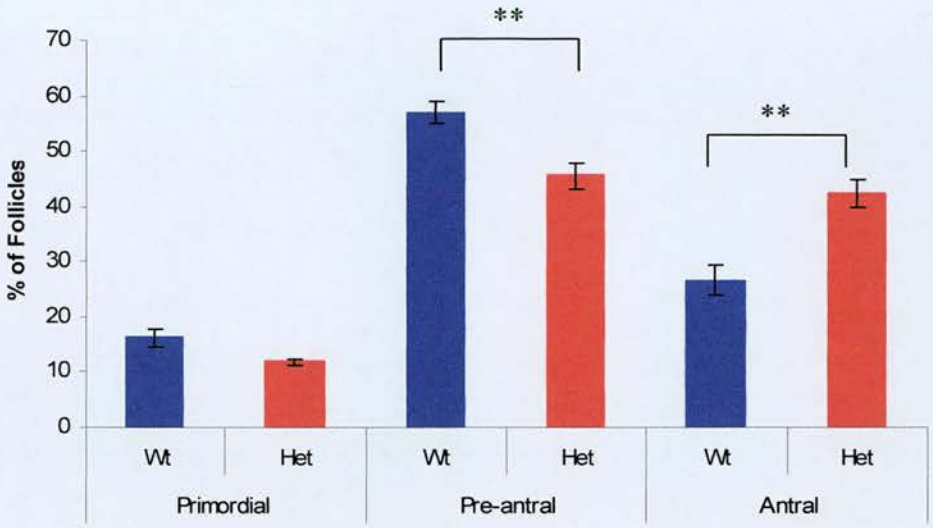


*Figure B.3 Percentage of primordial, pre-antral and antral follicles in Dazl wt (n=5) and het (n=7) mice where ** p=<0.01(McNeilly, J. R. et al., Unpublished).*

Appendix C

Address of Suppliers

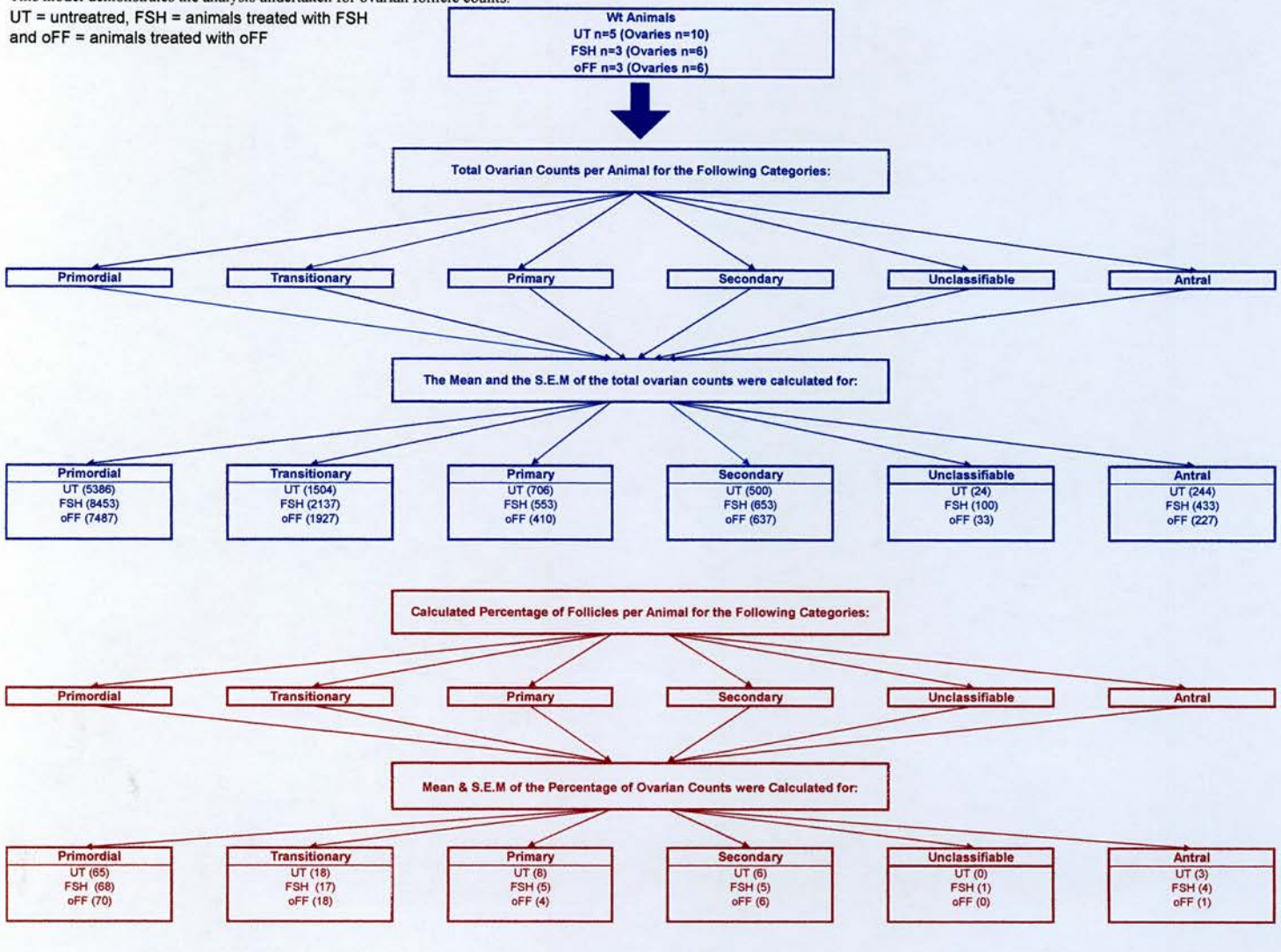
ABgene	ABgene House, Blenheim Road, Epsom, KT19 9AP, UK
Acumedic	101-105 Camden High Street, London, NW1 7JN, UK
Autogen Bioclear	Autogen Bioclear UK Ltd., Holly Ditch Farm, Mile Elm Calne, Wiltshire, SN11 OPY, UK
Baxter	TPS Healthcare Ltd, Baxter Distributor and Unicare, 27-35 Napier Place, WardPark North, Cumberland, Glasgow, G68 0EF, UK
Bayer Diagnostics	Bayer House, Strawberry Hill, Newbury, RG14 2JA, UK
BD Bioscience	BD Biosciences, 21 Between Towns Road, Cowley, Oxford, OX4 3LY, UK
BDH	BDH Laboratory Supplies, Poole, Dorset, BH15 1TD, UK
Bibby-Sterlin	Beacon Road, Stone, Staffordshire, ST15 0SA, UK
Biohit	Biohit Ltd., Unit 1 Barton Hill Way, Torquay, Devon, TQ2 8JG, UK
Bio-Rad	Bio-Rad Laboratories Ltd., Haylands Avenue, Hemel Hempstead, Hertfordshire, HP2 7TD, UK
Bios	Bios Europe Ltd., Unit 11, Pit Hey Place, West Pimbo, Skelmersdale, Lancashire, WN8 9PS, UK
CellPath	Unit 66, Mochdre Industrial Estate, Newtown, Powys, SY16 4LE, UK
Cell Signalling	Knowl Piece, Wilbury Way, Hitchin, Hertfordshire, SG4 OTY, UK
DAKO	Denmark House, Angel Drove, Ely, Cambridgeshire, CB7 4ET, UK
Diagnostic Scotland	Ellen's Glen Road, Edinburgh, EH17 7QT, UK
E.C. Apparatus Inc.	Colin Drive, Holbrook, New York
Forma Scientific	Marietta, Ohio, USA

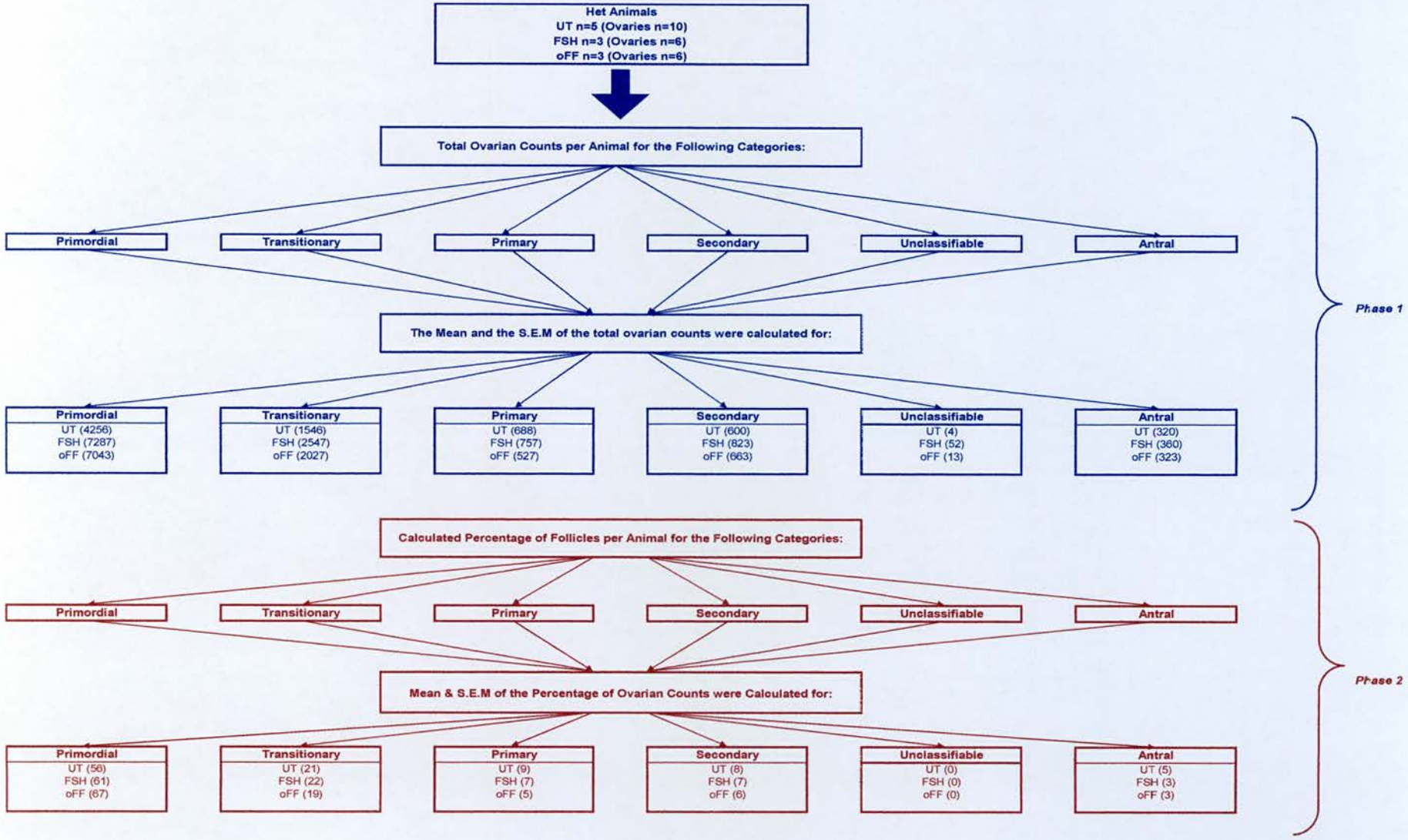
Fermentas	Fermentas UK, Sheriff House, Sheriff Hutton Industrial Park, York, YO6 0RZ, UK
Fischer Scientific	Bishop Meadow Road, Loughborough, Leicestershire, LE11 5RG, UK
GraphPad	GraphPad Prism Software Inc., 11452 El Camino Real, 215, San Diego, California, 92130, USA
Greiner Bio-one	Greiner Bio-one Ltd., Brunel Way, Stroudwater Business Park, GL10 3SX, UK
Hitachi	14-24 Nishi-Shimbasi, 1-Chrome Minato-ku, Tokyo, 105-8717, Japan
Invitrogen	Invitrogen Ltd., 3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF, UK
Iwaki	Beacon Road, Stone, Staffordshire, ST15 0SA, UK
Lambda Photometrics	Lambda House, Batford Mill, Harpenden, Hertfordshire, AL5 5BZ, UK
Larova	Larova GmbH, Rheinstr. 17a, 14513 Teltow, Germany
Leica	Leica UK Ltd., Davy Avenue, Knowlhill, Milton Keynes, MK5 8LB, UK
Linkam	Linkam Scientific Instruments, 8 Epsom Downs Metro Center, Waterfield, Tadworth, Surrey, KT20 5HT, UK
Life Technologies	Invitrogen Life Technologies Ltd., PO Box 35, Trident House, Renfrew Road, Paisley, PA34 4EF, UK
Media Cybernetics	Cyber House, Molly Millars Lane, Wokingham, Berkshire, RG41 2PX, UK
Merck	Merck Bioscience Ltd, Padge Road, Beeston, Nottingham, NG9 2JR, UK

Microsoft	Phoenix Software Ltd., Blenheim House, York Road, Pocklington, York, North Yorkshire, YO42 1NS, UK
Millipore	Millipore (UK) Ltd., Units 3&5, The Courtyards, Hatters Lane, Watford, WD18 8YH, UK
MJ Research	MJ Research Inc., 590 Lincoln Street, Waltham. MA 02451, USA
Molecular Probes	Invitrogen Ltd., 3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF, UK
MP Biomedicals	Stretton Scientific Ltd., Stretton House, Highstairs Lane, Stretton, DE55 6FD, UK
MWG Biotech	MWG Biotech UK Ltd., Mill Court, Featherstone-road, Wolverton Mill South, Milton Keynes, UK
Puregon	Organon Laboratories Ltd., Cambridge Science Park, Milton Road, Cambridge, CB4 0FL, UK
Roche Diagnostic	Roche Diagnostic Ltd., Bell Lane, Lewes, East Sussex, BN7 1LG, UK
Roebling	Berlin, Germany
Santa Cruz	Autogen Bioclear UK Ltd., Holly Ditch Farm, Mile Elm, Calne, Wiltshire, SN11 0PY, UK
Savant Instruments	Colin Drive, Holbrook, New York
Sharp	John Lewis, St. James Centre, Edinburgh, EH1 3SP, UK
Sherwood	Gosport, UK
Sigma	Sigma-Aldrich Company Ltd., The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT, UK
SPSS	SPSS Inc., 11th Floor, 233 S. Wacker Drive, Chicago, Illinois, 60606-6397, USA
Stuart Scientific	Beacon Road, Stone, Staffordshire, ST15 0SA, UK

Syngene, Bio Imaging	Beacon House, Nuffield Road, Cambridge, CB4 1TF, UK
The Binding Site	The Binding Site Ltd., PO Box 11712, Birmingham, B14 4ZB, UK
Thermo Hybaid	Action Ct., Ashford Road, Ashford, Middx, TW15 1XB, UK.
Vector	Vector Laboratories Ltd., 3 Accent Park, Bakewell Road, Orton Southgate, Peterborough, PE2 6XS, UK
VWR	VWR International, Hunter Boulevard, Magna Park, Lutterworth, Leicestershire, LE17 4XN, UK
Wallac	PerkinElmer Life and Analytical Sciences, Via Tiepolo, 24 20052 Monza (Milano), Italy
Zeiss	17-20 Woodfield Road, PO Box 78, Welwyn Garden City, Hertfordshire, AL7 1LU, UK

This model demonstrates the analysis undertaken for ovarian follicle counts.
UT = untreatred, FSH = animals treated with FSH
and oFF = animals treated with oFF




Het Animals
UT n=5 (Ovaries n=10)
FSH n=3 (Ovaries n=6)
oFF n=3 (Ovaries n=6)
Total Ovarian Counts per Animal for the Following Categories:
Primordial
Transitionary
Primary
Secondary
Unclassifiable
Antral
The Mean and the S.E.M of the total ovarian counts were calculated for:
Primordial
UT (4256)
FSH (7287)
oFF (7043)
Transitionary
UT (1546)
FSH (2547)
oFF (2027)
Primary
UT (688)
FSH (757)
oFF (527)
Secondary
UT (600)
FSH (823)
oFF (663)
Unclassifiable
UT (4)
FSH (52)
oFF (13)
Antral
UT (320)
FSH (360)
oFF (323)
Phase 1
Calculated Percentage of Follicles per Animal for the Following Categories:
Primordial
Transitionary
Primary
Secondary
Unclassifiable
Antral
Mean & S.E.M of the Percentage of Ovarian Counts were Calculated for:
Primordial
UT (56)
FSH (61)
oFF (67)
Transitionary
UT (21)
FSH (22)
oFF (19)
Primary
UT (9)
FSH (7)
oFF (5)
Secondary
UT (8)
FSH (7)
oFF (6)
Unclassifiable
UT (0)
FSH (0)
oFF (0)
Antral
UT (5)
FSH (3)
oFF (3)
Phase 2

Appendix E

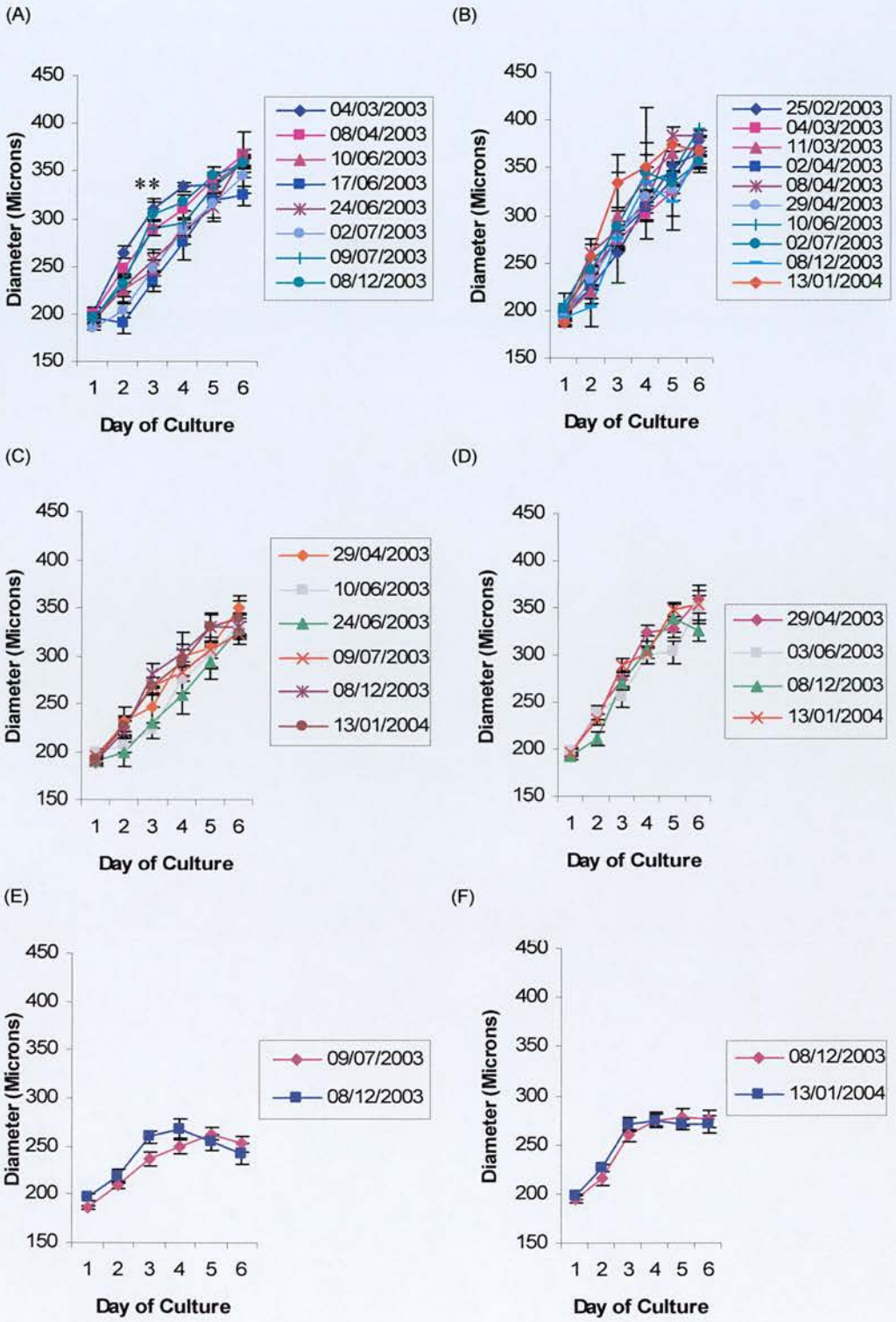


*Figure E.1 Inter-culture follicle growth rates in vitro for follicles from Dazl wt (A,C,E) or het mice (B,D,F) cultured in the presence of 1IU FSH (A,B), 0.1IU FSH (C,D), or 0.01IU FSH (E,F). ** p<0.01. Values are means ± S.E.M of the total number of follicles in every culture.*

Genotype	Date	FSH Dose	Number of Follicles
Het	25 Feb 03	1IU	14
Wt	04 March 03	1IU	4
Het	04 March 03	1IU	9
Het	11 March 03	1IU	10
Het	02 April 03	1IU	15
Wt	08 April 03	1IU	9
Het	08 April 03	1IU	5
Het	29 April 03	1IU	15
Wt	29 April 03	0.1IU	5
Het	29 April 03	0.1IU	9
Het	03 June 03	0.1IU	8
Wt	10 June 03	1IU	7
Het	10 June 03	1IU	1
Wt	10 June 03	0.1IU	9
Wt	17 June 03	1IU	5
Wt	24 June 03	1IU	7
Wt	24 June 03	0.1IU	4
Wt	02 July 03	1IU	7
Het	02 July 03	1IU	2
Wt	09 July 03	1IU	21
Wt	09 July 03	0.1IU	21
Wt	09 July 03	0.01IU	20
Wt	08 Dec 03	1IU	15
Het	08 Dec 03	1IU	3
Wt	08 Dec 03	0.1IU	11
Het	08 Dec 03	0.1IU	8
Wt	08 Dec 03	0.01IU	12
Het	08 Dec 03	0.01IU	13
Het	13 Jan 04	1IU	8
Wt	13 Jan 04	0.1IU	8
Het	13 Jan 04	0.1IU	20
Het	13 Jan 04	0.01IU	26

Table E.1 Number of follicles cultured in every experiment for Dazl wt and het follicles.