IN VITRO GROWTH AND DEVELOPMENT OF OVARIAN FOLLICLES FOR FERTILITY PRESERVATION

LORNA EVELYN BLACKWELL

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The candidate confirms that the work submitted is his/her own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

A physiological culture system that supports the *in vitro* growth (IVG) and development of oocytes would be an invaluable tool for the development and optimisation of fertility preservation techniques. Markers of oocyte, follicle and ovarian stromal tissue normality need to be established to ensure *in vitro*-derived oocytes are healthy and developmentally competent.

A 2-step, physiological IVG system was developed that supported (1) activation, growth and development of ovine primordial follicles *in situ* over 16-23 days; yielding 37 secondary follicles over 31 repeat cultures and (2) development of *in vivo*- (n=85) and *in vitro*-derived secondary follicles to the early antral stage, 25% and 19%, respectively. Step (1) was compared to an accelerated system (n=24), which, unlike the physiological system, resulted in a significant increase ($p<0.05$) in the proportion of degenerating follicles and decrease ($p<0.05$) in stromal tissue integrity following 6 days culture compared to day 0 control tissue. In addition a significantly higher yield of transitional ($p<0.01$) and secondary ($p<0.05$) follicles resulted from the physiological vs. the accelerated system. The expression patterns of 20 genes key to oogenesis and folliculogenesis were established *in vivo* and compared to stage-matched samples derived using the physiological IVG system, revealing significant changes ($p<0.05$) in the expression of *AMH*, *IGF1*, *INHα*, *INHβA*, *FST*, *ZP2*, *GTSF1*, *BMP6*, *BMP15* and *MEST*. Step (1) was used to evaluate damage to oocyte and ovarian tissue health following the perfusion of 48 ovaries, with either 1.5M dimethyl sulphoxide (DMSO) or L-15 control medium, in combination with the use of NMR spectroscopy to determine the level of DMSO permeation. Perfusion times of 10 and 60 minutes were required permeate the pedicle and cortex tissue, respectively, however, 60 minutes perfusion resulted in a significant decrease in follicle number ($p<0.01$) and stromal tissue integrity ($p<0.05$).

The overall results indicate that the IVG of oocytes is a suitable tool to both assess the patency of fertility preservation systems and as a means to restore female fertility.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

~	approximately
√	square root
µg	microgram
µl	microlitre
µm	micrometre
<	less than
>	more than
1°	primary follicle
2°	secondary follicle
2°D	degenerating secondary follicle
2D	2 dimensional
3D	3 dimensional
A_4	androstenedione
AFC	accelerated follicle culture
AKT	thymona viral oncogene homolog
AMH	anti-müllerian hormone
ANCOVA	analysis of covariance
ANOVA	analysis of variance
Ar	androgen receptor
AREG	amphiregulin
ART(s)	assitive reproductive technologies
AS	Angelman syndrome
ATP	adenosine triphosphate
B_0	external magnetic field
B_1	second magnetic field
BAX	BCL2-associated X protein
BEGAIN	brain-enriched guanylate kinase-associated protein
bFGF	basic fibroblast growth factor
BCL	B-cell lymphoma/ leukaemia
BMP	bone morphogenetic protein
bp	base pairs
BSA	bovine serum albumin
BTC	betacellulin
BWS	Beckwith-Wiedemann syndrome
cAMP	cyclic adenosine monophosphate
Casp	caspase
cDNA	complementary DNA
cGMP	cyclic guanosine monophosphate
CL	corpus luteum
CO_2	carbon dioxide
COC	cumulus-oocyte complex
CPA(s)	cryoprotective agent(s)
Ct	cycle threshold

CV	coefficient of variation
Cx37	connexin 37
Cx43	connexin 43
D_2O	deuterium oxide
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferases
E_2	17β-oestradiol
EA	early antral
ECM	extracellular matrix
ET	endothelin
EG	Ethylene glycol
EGF	epidermal growth factor
EP	early primary
EREG	epiregulin
F	forward primer
FA-IPN	fibrin – alginate -interpenetrating network
FCS	fetal calf serum
FF	follicular fluid
FGF	fibroblast growth factor
FID	free induction decay
FIGα	factor in the germline α
FIM	follicle isolation medium
FOXO	forkhead transcription factor
FSH	follicle stimulating hormone
FST	follistatin
g	gram
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC(s)	granulosa cell(s)
GDF9	growth differentiation factor-9
GFP	green fluorescent protein
GH	growth hormone
GJ	gap junctional
GN	gonadotrophin
GnRH	gonadotrophin releasing hormone
GOI	gene of interest
Grb10	growth factor receptor-bound protein 10
GV	germinal vesicle
GVBD	germinal vesicle breakdown
H&E	haematoxylin and eosin staining
H2A	histone 2 α
H_2O	water
hCG	human chorionic gonadotrophin
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HGF	hepatocyte growth factor
HK	house keeping
HSA	human serum albumin

ICSI	intracytoplasmic sperm injection
IGF1	insulin-like growth factor 1
IGF1R	IGF1 receptor
IGF2	insulin-like growth factor 2
IGF2R	IGF2 receptor
IGFBP	IGF binding protein
IIF	intracellular ice formation
IL	interleukin
ITS	insulin, transferrin, selenium
IVF	*in vitro* fertilization
IVG	*in vitro* growth
IVM	*in vitro* maturation
KGF	keratinocyte growth factor
KL	kit ligand
l	litre
LDH	lactate dehydrogenase
LD	long distance
LH	luteinizing hormone
Lhcgr	luteinizing hormone/choriogonadotropin receptor
LHR	LH receptor
LHX8	LIM homeobox protein 8
LIF	leukemia inhibitory factor
LOS	large offspring syndrome
M	net magnetisation
MEM	minimum essential medium
mg	milligram
MI	metaphase I
MII	metaphase II
MAPK	mitogen activated protein kinase
mg	milligram
ml	millilitre
mm	millimetre
MMP	matrix metalloproteinase
MPF	maturation promoting factor
mRNA	messenger RNA
mTORC1	rapamycin complex 1
n	number of samples per group
nm	nanometre
NP	non-perfused
NAD(H)	nicotinamide adenine dinucleotide
ng	nanogram
NGF	nerve growth factor
NMR	nuclear magnetic resonance
NOBOX	Newborn ovary homeobox
NR	neutral red
°C	degrees centigrade
O_2	oxygen

Oct4	octamer-binding protein 4
oFSH	ovine FSH
OHSS	ovarian hyperstimulation syndrome
oLH	ovine LH
OWM	ovary wash media
p	pico
P	probability
P_4	progesterone
PA	preantral
PARP	poly(adenosine diphosphate-ribose) polymerase
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDE	phophodiesterase
PDK1	3-phosphoinositide-dependent protein kinase-1
p.f.	post fertilisation
PFA	paraformaldehyde
Pfkp	phosphofructokinase platelet
PGC(s)	primordial germ cell(s)
PI3K	phosphatidylinositol-3'-kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PIP_3	phosphatidylinositol (3,4,5)-trisphosphate
POF	premature ovarian failure
POI	primary ovary sufficiency
Prd	primordial
PROH	propane-1,2-diol
PTEN	phosphatase and tensin homologue deleted on chromosome ten
QC	quality control
qPCR	real time PCR
R	reverse primer
R^2	correlation coefficient
rFSH	recombinant FSH
RKWM	rozenburgh knife wash medium
rLH	recombinant LH
RNA(s)	ribonucleic acid(s)
rpm	revolutions per minute
RRBS	reduced representation bisulphite sequencing
SCID	severe combined immunodeficiency
SEM	standard error of the mean
Siglec	sialic acid-binding immunoglobulin superfamily lectin
snrpn	small nuclear ribonucleoprotein-associated protein N
SOHLH	spermatogenesis and oogenesis specific basic helix-loop-helix
Sostdc	sclerostin domain containing 1
SRS	Silver-Russell syndrome
Star	steroid acute regulatory protein
T	transitional stage follicle
TBE	trisaminomethane, borate, EDTA

TC	theca cell
TEM	transmission electron microscopy
TGF-β	transforming growth factor-β
TIMP	tissue inhibitors of metalloproteinases
TNF-α	tumour necrosis factor-α
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
U	units
VEGF	vascular endothelial growth factor
v/v	volume per volume
WOCP	whole ovary cryopreservation
w/v	weight per volume
YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxgenase activation protein, zeta polypeptide
ZP	zona pellucida

List of Presentations

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Blackwell, L.E., Jin, P., Hogg, J., Picton, H.M. (2012) The optimisation of a two-step serum-free culture system enabling the development of ovine oocytes from early preantral follicles. Poster presentation at the LIGHT graduate symposium. (Awarded student's choice poster prize).

1 General Introduction

Improvements in cancer treatments have resulted in increasing survival rates of patients. However, treatments used are often aggressive and can cause infertility. In addition patients suffering from premature ovarian failure (POF), abdominal trauma, Turner syndrome, or other autoimmune diseases that require chemotherapeutic treatment would benefit from the development of fertility preservation techniques. Current methods of fertility preservation, such as oocyte or embryo cryopreservation are not suitable for prepubertal girls or adolescents nor are they ideal for women of reproductive age who have not yet found their life partner. Recent advances in cryobiology have meant that it is now possible to freeze-store intact ovarian tissue for girls and young women requiring fertility preservation. At present fertility restoration may be achieved after ovarian freezing by autograft. However, there is a risk that malignant cells may be reintroduced. The ovary is not immunologically privileged so allografting of the tissue is not possible. The development of methods to enable the complete *in vitro* growth of follicles from the primordial to pre-ovulatory stages would overcome many of the problems posed by current fertility restoration methods. Although the complete *in vitro* growth and maturation and subsequent fertilisation of oocytes has resulted in the birth of live young in mice this has been much more difficult to replicate in larger species and humans. This is partly due to both the greater timescales of oogenesis and folliculogenesis and the size of oocytes and follicles in larger species, as well as decreased accessibility of tissue. However, progress has been made towards the optimisation of these systems.

Oogenesis and folliculogenesis are complex and protracted processes. Growth and development of follicles and oocytes *in vivo* are regulated by numerous peptides and growth factors as well as hormones such as the pituitary gonadotrophins: follicle stimulating hormone (FSH) and luteinizing hormone (LH) and ovarian steroid hormones including progesterone (P4), testosterone and oestrogen (E2). The development of oocytes within follicles from the primordial to the pre-ovulatory stage takes around 6-9 months in humans and is only completed once puberty has been reached.

A clear understanding of the processes of oogenesis and folliculogenesis, as well as the optimisation of *in vitro* growth and maturation systems are of great importance to fertility preservation strategies. Before these techniques are used as fertility restoration methods in

humans it is essential that the *in vitro*-derived oocytes are developmentally competent and that following fertilisation the health of the resultant offspring can be guaranteed. In order to ensure this goal is achieved a greater understanding of the expression patterns of genes during oocyte development *in vivo* is required, as well as the establishment of imprints that occurs during oogenesis. This review will therefore outline the current understanding of the processes of oogenesis and folliculogenesis *in vivo*, the current methods of fertility preservation, progress that has been made with respect to developing culture systems that support the *in vitro* growth and maturation of oocytes and the methods used to establish the developmental competence of these *in vitro*-derived oocytes.

1.1 Formation and colonization of the early human ovary

The human ovary is composed of germ and somatic cells. The somatic cell components are derived from primarily mesonephric cells, as well as coelomic epithelium and mesenchymal cells. The proliferation of these somatic cell components starts 4 weeks post fertilisation (p.f.) resulting in the formation of the fetal ovary (Byskov, 1986). Germ cells, however, are derived from an extragonadal region and must migrate to the genital ridge. The formation and migration of primordial germ cells (PGCs) and subsequent differentiation into oogonia and oocytes has been extensively reviewed by various authors (Motta et al., 1997a, Motta et al., 1997b, Pereda et al., 2006). PGCs can first be detected in the human embryo 3 weeks p.f., via staining for the enzyme, alkaline phosphatase. Bone morphogenetic proteins (BMPs) – 2 (endoderm-derived), 4 and 8b (ectoderm-derived) are required for PGC generation in mice (Ying et al., 2001, Ying and Zhao, 2001, Lawson et al., 1999). Early evidence suggests that these factors may also be required in human PGC specification (Hiller et al., 2011). Primordial germ cells are located in the endoderm of the dorsal wall of the yolk sac, close to the neighbouring allantois. To reach the genital ridge the PGCs first passively migrate to the connective tissue of the hindgut, this occurs around week 4 p.f. This movement is brought about by the rearrangement of the embryo into a tubular shape as it develops. Further migration of the PGCs from the hindgut, through the dorsal mesentery, to the genital ridge is an active process occurring in week 5-6 p.f.

The active migration of PGCs is enabled by a change in the morphology and cytoplasmic contents of the cells. Initially PGCs are rounded cells, with few organelles but rich in glycogen particles and lipid droplets, which may be sources of energy substrate once active migration has commenced (Funkuda, 1976). On leaving the hindgut PGCs become spindle-shaped. The

development of pseudopodia and the concentration of microtubules and microfilaments in the cytoplasm enable the PGCs to actively migrate via amoeboid-like movements to the genital ridge (Fujimoto et al., 1977). Migration is also aided by the interaction between the PGCs and somatic cells as the 2 cell components interact via cell contacts and chemotaxic signalling. Kit ligand (KL) exists in 2 forms, a soluble and a membrane-bound form, KL-1 and KL-2, respectively (see Section 1.5) (Huang et al., 1992). The interaction between the 2 forms of KL and their receptor, c-kit, stimulates PGC survival (KL-2) and migration (KL-1) to - and colonisation (KL-2) of - the genital ridge (Driancourt et al., 2000, Hoyer et al., 2005, Wehrle-Haller and Weston, 1995). Leukaemia inhibitory factor (LIF) and fibroblast growth factor (FGF) have been identified as cytokines which stimulate PGC survival and proliferation, respectively (Anderson et al., 2001). Members of the Transforming growth factor (TGF)-β superfamily have also been shown to contribute to the regulation of the migration process, for example TGF-β1 has been shown to negatively regulate PGC proliferation (Godin and Wylie, 1991). Active migration is further aided by the interaction between components on the surface of the PGCs and the extracellular matrix surrounding the somatic cells. During this migratory period the PGCs undergo division by mitosis (Garcia-Castro et al., 1997). Activin A has been implicated as a stimulatory factor with respect to PGC proliferation and survival (Martins da Silva et al., 2004).

1.2 Development of PGCs to primordial follicles

Upon reaching the genital ridge the PGCs lose their motility and become incorporated into germ cell nests. They are now termed oogonia. Within these nests, synchronous and rapid mitotic division of the oogonia, in which the cytoplasm is incompletely divided, results in the formation of intracellular bridges (Pepling and Spradling, 1998). In the human ovary mitotic division of oogonia continues until around 12 weeks p.f. when a transition to meiotic cell division begins (Gondos et al., 1986). Oogonia located in the inner cortex are the first to undergo this transition and are now termed primary oocytes. The commencement of meiosis is accompanied by both changes in the oocyte nucleus and cytoplasmic rearrangements, which are important for oocyte metabolism (Makabe et al., 1992). Meiosis progresses up to diplotene of prophase I (4 months p.f.) where it then arrests until oocyte maturity is complete. Mitotic divisions continue up to 7 months p.f. in the human ovary and the high rates observed in months 3-4 generate a maximum number of germ cells. Around 6.8 million have been observed at month 5 p.f. in humans. However, this number greatly declines to 2 million at birth as activation of oocyte growth occurs but is not sustained past the early antral (EA) stages

due to the absence of gonadotrophic stimulation. The stores are not re-established as mitosis ceases and germ cells degenerate within the ovary, and are lost via exfoliation from the ovarian surface (Baker, 1963, Motta and Makabe, 1986, Pepling and Spradling, 2001).

Oocyte survival is dependent upon germ cell incorporation into primordial follicles. Primordial follicles each consist of a diplotene oocyte encompassed by a single layer of squamous follicular somatic cells, termed pre-granulosa cells, which are derived from the ovarian surface epithelium (Eppig, 2001, Sawyer et al., 2002). Primordial follicle formation requires the breakdown of germ cell nests. This is achieved via the majority of oocytes undergoing apoptosis thus ensuring the surviving oocytes have increased access to pre-granulosa cells and intracellular organelles, which are actively transported via the aforementioned intracellular bridges (Gondos, 1973, Pepling and Spradling, 1998). Cytoplasmic processes originating from the surrounding somatic cells aid the breakdown of connections between germ cells in oocyte nests and once the pre-granulosa cells have secreted an outer membrane, the basal lamina, the primordial follicles become isolated from the surrounding stroma and exist as individual entities (Francavilla et al., 1990). The basal lamina is primarily composed of collagen IV, laminin and entactin/ nidogen, the exact composition, however, varies throughout follicular development (Rodgers et al., 2003).

Oocyte survival is dependent upon the maintenance of interactions with granulosa cell (GC) layers, and *vice versa*. The somatic cell compartment provides each oocyte with the necessary nutrients, amino acids, growth factors and oxygen to support growth and maturation. Bidirectional communication of regulatory factors between the germ cell and somatic compartments is required to regulate oocyte and follicular growth and development is achieved via the presence of heterologous and homologous gap junctions between the oocyte and somatic cells and between the GCs themselves (Anderson and Albertini, 1976, Eppig, 2001). As oocyte-derived factors play a key role in the regulation of paracrine factors produced by the GCs, the oocyte is effectively regulating its own microenvironment (Gilchrist et al., 2004). Furthermore, the production of factors by oocytes and the establishment of morphogen gradients results in a heterologous pattern of gene expression in GC populations in the later stages of development. The oocyte is held in meiotic arrest by the maintenance of gap junctional contacts which facilitate the provision of somatic cell-derived cyclic adenosine 3',5'-monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) to the oocyte (Anderson and Albertini, 1976, Gilchrist, 2010). Note that second messenger molecule cAMP is

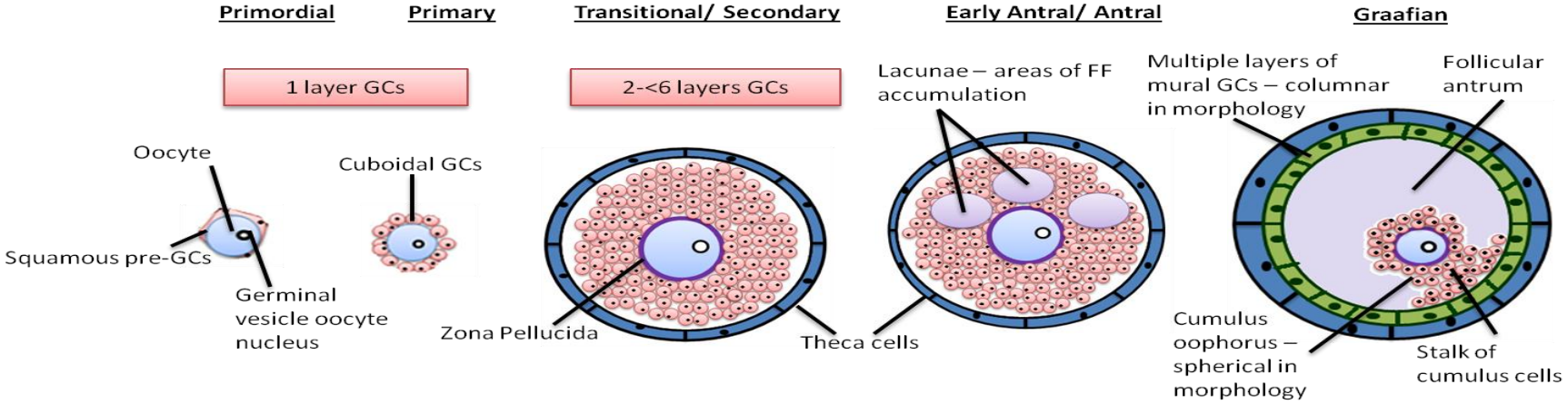
also produced by the oocyte. Maintenance of meiotic arrest by high cAMP levels is achieved via the promotion of cAMP-dependent protein kinase A, an enzyme that inhibits maturation promoting factor (Gilchrist, 2010). The supply of cGMP to the oocyte is also key as this inhibits phosphodiesterase 3, an enzyme that degrades cAMP, thus maintaining high levels of cAMP (Törnell et al., 1991, Tsafriri et al., 1996). Closure of gap junctions in response to the LH surge prevents the transfer of cAMP and cGMP from the somatic cells to the oocyte and thus results in the resumption of meiosis (see Section 1.5.4).

The transcription factor, Factor in the germline-α (Fig-α) has been implicated in the assembly of primordial follicles via its role in the regulation of the expression of oocyte-specific genes involved in folliculogenesis and the formation of the zona pellucida (ZP). Initially shown in mice prior to the identification of human homologues, Fig-α forms a heterodimer with ubiquitously expressed transcription factor, E12 and then binds an E-box in the promoter region of the zona genes (Huntriss et al., 2002, Bayne et al., 2004). Targeted mutagenesis of Fig-α in mice results in abnormal follicular development (Soyal et al., 2000).

1.3 Morphological aspects of preantral follicle development

Preantral follicle growth and development is characterised by the oocyte increasing in volume as it acquires the proteins needed for maturation, fertilisation and early development of the post-implantation embryo prior to embryonic genome activation. The activation of primordial follicle growth is accompanied by the pre-GCs becoming cuboidal and proliferating to form several avascular layers. The morphological changes occurring throughout follicle development and the approximate size of oocytes and follicles are summarised in Figure 1.1. At the primordial stage spheroidal/ elliptical follicles consist of a centrally located germinal vesicle (GV) oocyte surrounded by a continuous or discontinuous layer of squamous GCs all of which is enclosed by a thin basal lamina. These follicles are mainly located in the outer layer of ovarian cortex (Zamboni, 1974). Initiation of follicle growth can be divided into 2 stages. During the first stage GC proliferation results in the transition to a thicker single GC layer consisting of cuboidal/ columnar cells. During the second stage the follicle and oocyte diameter as well as GC number increase dramatically. The point at which the oocyte starts to grow at a higher rate can be linked to the number of GCs in the largest cross-section of the follicle. For example, this number is 14-15 in sheep and humans and 40 in the cow (Cahill and Mauleon, 1981, Gougeon and Chainy, 1987, Braw-Tal and Yossefi, 1997).

Species	Follicle Diameter (µm)				
Mouse [1,2]	17	~52	104-187	~ 250	~ 424
Sheep [3,4]	40.8	52-75	129-194	~ 330	2 – 6.5 mm
Human [1,5]	26 -40	40 –70	114-302	0.2 -10 mm	17 – 20 mm



Species	Oocyte Diameter (µm)				
Mouse [1,2]	12.6	28-54	54-67	~ 70	~ 72
Sheep [3,4]	34.6	40 -90	73-88	~ 120	~ 120
Human [1,5]	36	40 -173	73-120	~ 120	~ 120

Figure 1.1 A diagrammatic representation of the morphological aspects of follicular development from the primordial to the Graafian stage. Oocyte and follicle diameters are shown for mouse, sheep and human, information taken from 1 Griffin et al. (2006), 2 Pedersen (1970), 3 Lundy et al. (1999), 4 Gonzalez-Bulnes et al. (2001), 5 Himelstein-Braw et al. (1976)**.**

During the oocyte growth phase the deposition of a glycoprotein coat within the perivitelline space initiates, resulting in the formation of the ZP. In humans ZP deposition begins in the primary follicle and by the transitional stage these areas of deposition have coalesced to form a continuous layer around the oocyte. Cytoplasmic processes traverse the ZP allowing the maintenance of intercellular contact (Zamboni, 1974). The composition of the ZP is species-specific, ensuring that only sperm from the correct species can fertilise the oocyte. The mouse ZP is composed of 3 sulphated glycoproteins (ZP1, ZP2 and ZP3) and is permeable to relatively large macromolecules. A fourth ZP gene, ZP4, has been identified and shown to be expressed in the oocytes of humans, rats and hamsters (Lefievre et al., 2004, Gupta and Bhandari, 2010). Glycoproteins; ZP2 and ZP3 form heterodimers which are cross-linked by ZP1 (Wassarman et al., 1996). In the mouse, the sperm binds the ZP primarily via ZP3 resulting in the induction of the acrosome reaction. Whereas in the human 3 ZP proteins, ZP1, ZP3 and ZP4, facilitate the binding of sperm and induction of the acrosome reaction (Gupta and Bhandari, 2010). In both humans and mice the ZP2 protein acts as a secondary sperm receptor and is not involved in the acrosome reaction. Once the oocyte is fertilised hardening of the ZP provides a block to polyspermy (Wassarman, 1988). At the transitional stage of follicle development a theca layer begins to form. Theca cells are derived from fibroblast-like precursor cells present in the ovarian stroma. Once the follicle has developed to the secondary stage it will be enclosed by a full theca layer that will start to differentiate into 2 distinct populations. Surrounding the basal lamina, the theca interna is composed of highly vascularised steroidogenic cells, 1 or 2 arterioles provide vascularisation (Gougeon, 1996). Whereas the theca externa is composed of a loosely associated layer of connective tissue of non-steroidogenic cells (Magoffin, 2005).

Figure 1.1 contains information regarding oocyte and follicle diameter, respectively, during preantral development in the sheep, human and mouse. Development from the primordial follicle to the secondary stage takes around 18, 78 and 270 days in the mouse, sheep and human, respectively (Cahill and Mauleon, 1980, Himelstein-Braw et al., 1976, Gougeon, 1986, Picton, 2001, Pedersen, 1970). Throughout the process of follicle development, up to antral cavity formation, the oocyte increases dramatically in size (Cotterill et al., 2013) and acquires the components required to attain competence with respect to resumption of meiosis, germinal vesicle breakdown, chromatin decondensation and to block polyspermy (Picton et al., 1998). Oocyte growth is achieved via the accumulation of ions, water and lipids and due to the fact that the oocyte does not divide the volume of cytoplasm is maintained. During development the oocyte undergoes large-scale cytoplasmic rearrangement as organelles are redistributed and new organelles and vesicles containing substrates required for synthesis and

energy are acquired. The process of oocyte development has been reviewed in detail in numerous reviews (Picton et al., 1998, Li and Albertini, 2013, van den Hurk and Zhao, 2005).

1.4 Morphological aspects of antral follicle development

The major morphological changes that occur as follicles develop from the preantral stages onwards are related to growth. The manifestations of growth are marked by continued GC proliferation, followed by the formation of a follicular antrum (Lussier et al., 1987), and the differentiation of GCs into cumulus and mural populations. The process of follicle development is long and protracted, for example the development of follicles from the transitional stage up to the pre-ovulatory stage takes around 6 months in sheep (Cahill and Mauleon, 1980).

Antrum formation begins when the follicle reaches a species-specific size; 0.2-0.4mm in human (Gougeon, 1986, Gosden and Telfer, 1987a). Antral cavity formation is promoted by gonadotrophins (see Section 1.5.3). Figure 1.1 illustrates the process of antral cavity formation in which follicular fluid (FF) begins to accumulate in multiple lacunae, possibly in areas of reduced GC contact (Baca and Zamboni, 1967). As the accumulation of fluid continues these areas expand and coalesce forming 1 large antrum, which continues to grow (Baca and Zamboni, 1967). Fluid accumulation occurs as a result of glycoprotein production by the GCs. Glycoproteins, such as GC-derived hyaluranon, inter-α trypsin inhibitor and versican, are secreted into the FF then bind/ cross-link each other so are therefore retained (Clarke et al., 2006). The presence of these glycoproteins forms an osmotic gradient which causes fluid to enter the developing antral cavity from the thecal vasculature (Rodgers and Irving-Rodgers, 2010). This process requires the remodelling of cell-cell junctions, as well as the theca layer and ovarian stroma. The FF may be an important source of various blood and follicular cell-derived substances including gonadotrophins, steroids, growth factors, enzymes, proteoglycans and lipoproteins which are required for the regulation of folliculogenesis and oogenesis (van den Hurk and Zhao, 2005).

The division of the GCs into 2 morphologically distinct populations occurs as a consequence of the formation of the follicular antrum. The cumulus and mural GCs have a common origin but differentiate to become functionally distinct with respect to the production of secretory products and receptor expression (Telfer et al., 1988). For example, expression of LH receptors

is present to a much greater extent in the mural rather than cumulus GCs (Amsterdam et al., 1975). Follicular antrum expansion and GC proliferation continue until the pre-ovulatory follicle reaches a size of around 20mm (Gougeon, 1986). Progression from the secondary to pre-ovulatory follicle takes around 85 days in humans.

1.5 Factors controlling oogenesis and folliculogenesis

1.5.1 Primordial follicle activation

Until recently it was widely accepted that the total population of oocytes in the ovary – the ovarian reserve - was finite and that by birth there were no oogonial stem cells remaining, therefore explaining why when the ovarian reserve became depleted this resulted in ovarian failure and menopause in women (Gosden and Telfer, 1987a, Zuckerman, 1951). Based on this theory the Faddy-Gosden model, in which the rate of follicle activation increases with decreasing size of the remaining follicle reserve, was presented as a means of illustrating the rate of depletion of the follicle store (Faddy and Gosden, 1996). However, increasing evidence is emerging that a limited population of oogonial germ cells may exist in adult ovaries in both mice (Johnson et al., 2004, Johnson et al., 2005) and human (White et al., 2012) and that these cells are mitotically active and capable of forming oocytes and thus may contribute to the replenishment of the primordial follicle store. It remains unclear what contribution, if any, the small number of putative ovarian stem cells make to the reproductive longevity of an individual.

Locally produced growth factors are essential for the activation and development of follicles. From the EA stage onwards gonadotrophins are also essential (see Section 1.5.4). Both the oocyte and somatic cells of the follicle produce growth factors that have autocrine and paracrine actions. Gap junctional contacts which extend from the somatic cells, through the ZP to the oolemma enable the transfer of growth factors between the different cell types (Anderson and Albertini, 1976). The nature of these contacts alters throughout follicular development, with respect to their number and form, to meet the requirements of the follicle. The process of gap junction (GJ) alteration is mediated to an extent by external factors, such as FSH (Anderson et al., 2001).

Both oocyte and follicular somatic cell development depend upon the maintenance of signalling between the 2 compartments. Granulosa cell contact is essential to the oocyte to hold it in meiotic arrest until it has become competent to progress to metaphase II (MII) (Pincus and Enzmann, 1935, Eppig, 2001, Gilchrist et al., 2008). Although oocytes are usually able to resume meiosis once antrum formation has occurred they will not yet have acquired the molecular and cytoplasmic machinery needed to complete nuclear maturation and to progress to MII (Handel and Eppig, 1998). The oocyte will not acquire the machinery to enable vital processes such as 'sperm head penetration, decondensation of chromatin and polyspermy block until the final days of maturation prior to ovulation' (Picton et al., 1998). Furthermore, exposure to certain oocyte-secreted factors is essential to prevent the premature luteinisation of somatic cells (el-Fouly et al., 1970, Nekola and Nalbandov, 1971). In this section the roles of growth factors that are key to folliculogenesis and oogenesis will be summarised and the importance of oocyte-granulosa cell interactions discussed. Key species-specific differences and conflicting information will be highlighted. These are complex processes and conflicting evidence concerning the roles of various factors also exists, this may be partly due to differences in techniques used in different studies.

1.5.2 Oocyte-derived factors

The oocyte and somatic cell compartment of the follicle are mutually dependent upon each other with respect to the production of growth factors that promote development and differentiation and the provision and utilisation of nutrients. The oocyte was originally thought to be passive, however, evidence now shows that the oocyte is key in regulating its own microenvironment. The oocyte achieves this via the secretion of paracrine factors that promote the surrounding GCs to differentiate and develop in such a way as to provide the oocyte with the substrates required for normal continued growth and development.

Numerous oocyte-derived transcription factors that are key to PGC migration, oocyte survival, follicle assembly and oogenesis have been identified in mice, with some homologues also identified in humans, see Table 1.1.

Table 1.1 Oocyte-derived transcription factors; their expression pattern and experimental evidence to support their roles. References: 1 Kehler et al. (2004), 2 Yamaguchi et al. (2009), 3 Liu et al. (2007), 4 Castrillon et al. (2003), 5 Li et al. (2010), 6 John et al. (2008) 7 Biggs et al. (2001), 8 Pangas et al. (2006), 9 Choi et al. (2008b), 10 Huntriss et al. (2002), 11 Bayne et al. (2004), 12 Soyal et al. (2000), 13 Rajkovic et al. (2004), 14 Choi et al. (2008a).

Transcription Factor	Expression	Role	Evidence/ Extra Information
Octamer-binding protein 4 (OCT-4)	PGCs	Required for PGC survival	Decrease in PGC population in *Nanog and Oct4* knockdown mice [1, 2]
NANOG	PGCs	Required for PGC survival	
FOXO3a - mouse, FOXO2 - human	Primordial and Primary	Inhibition of primordial follicle activation	Inactivation of FOXO in mouse ovarian cortex *in vitro* results in the large scale activation of primordial follicles. Activation regulated by (PI3K)-Akt pathway; which, when active hyperphosphorylates FOXO3a/2 leading to deactivation and nuclear export [3, 4, 5, 6, 7]
Spermatogenesis and oogenesis specific basic helix-loop-helix 1 (SOHLH1)	Primordial and Primary	Required for oogenesis	Knockout of *Sohlh1* in mice results in down regulation of *Fig-α* and *Nobox* [8]
Spermatogenesis and oogenesis specific basic helix-loop-helix 2 (SOHLH2)	Primordial and Primary	Critical for regulation of early oogenesis	Misexpression of oocyte-specific genes in *Sohlh2* knockout mice. GCs are unable to differentiate past primordial phenotype in knockout mice [9]
FIG-α	PGC onwards	Primordial follicle assembly and zona formation via regulation of the expression of oocyte-specific genes	Targeted mutagenesis of Fig-α in mouse oocytes resulted in abnormal follicular development. Fig- α forms a heterodimer with ubiquitously expressed transcription factor, E12 and then binds an E-box in the promoter region of the zona genes [10,11,12]
Newborn ovary homeobox (NOBOX)	Primordial onwards	Regulates expression of oocyte-specific genes	Oocyte-specific genes are down-regulated in NOBOX deficient mice. Critical for primordial to primary follicle transition [13]
LIM homeobox protein 8 (LHX8)	Primordial onwards	Critical for regulation of early oogenesis	Misexpression of oocyte-specific genes in *Lhx8* knockout mice. Down regulates Nobox pathway [14]

One way in which the activation of primordial follicles is promoted is via the phosphatidylinositol-3'-kinase (PI3K)-Akt pathway, as shown in Figure 1.2 (Reddy et al., 2008). As mentioned in Table 1.1 the action of Forkhead transcription factor (FOXO) 3a/2 is regulated via PI3K-Akt (Castrillon et al., 2003). FOXO inhibits primordial follicle activation. Interaction of KL and its receptor, c-kit, results in the phosphorylation of PI3K, which then binds and phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP_2) to form phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) (Reddy et al., 2005). Once phosphorylated PIP_3 then phosphorylates and activates the protein kinase B, Akt, which can then phosphorylate FOXO. Hyperphosphorylation of FOXO by PI3K-Akt results in its exportation from the nucleus thus preventing its inhibitory action on follicle activation (Adhikari and Liu, 2009). In addition, the PI3K-Akt pathway promotes activation of follicles via the indirect activation of mammalian target of rapamycin complex 1 (mTORC1). The promotion of human primordial follicle activation by mTORC1 has been demonstrated in a study in which rapamycin, an inhibitor of mTORC1, reduced follicle activation rates (McLaughlin et al., 2011). The PI3K-Akt pathway and therefore primordial follicle activation is negatively regulated, in part, by phosphatase and tensin homologue deleted on chromosome ten (PTEN) (Reddy et al., 2008). The actions of PI3K are antagonised by PTEN as it dephosphorylates PIP_3 to PIP_2, thus inhibiting Akt. In a study in which PTEN was inhibited, large-scale activation of primordial follicles was observed resulting from the exportation of FOXO from the nucleus (Li et al., 2010). The follicle reserve is therefore partly maintained via the actions of PTEN. Maintenance of the follicle reserve is also achieved via the PI3K-pathway mediated activation of 3-phosphoinositide-dependent protein kinase-1 (PDK1), although rather than suppressing primordial follicle activation this is achieved by promoting the survival of dormant follicles (Reddy et al., 2009).

A number of important oocyte-specific growth factors that play key roles in the regulation of the development of the follicles have been identified, these include: growth differentiation factor 9 (GDF9) and bone morphogenetic protein (BMP)15 and BMP6. These factors are members of the TGF-β superfamily, which will be discussed in greater detail in the following section. There is also some evidence of TGF-β expression in the oocytes of rats, humans and cows, but not sheep (Juengel and McNatty, 2005). To this end, GDF9 is expressed by oocytes from the primary stage in mice and humans, and the primordial stage to the Graafian stage in sheep (Bodensteiner et al., 1999, McGrath et al., 1995, Bodensteiner et al., 2000, Teixeira Filho et al., 2002, Aaltonen et al., 1999). This expression patterns is unlike that of BMP15, which is expressed from the primary stage up to ovulation, in sheep, humans and mice (Aaltonen et al., 1999, Galloway et al., 2000, Dube et al., 1998). GDF9 and BMP15 may form

homo/heterodimers, however, unlike all other members of the TGF-β superfamily they lack the conserved 4th cysteine residue needed for dimerisation (Dube et al., 1998). BMP6 is expressed from the primordial to the antral stage in sheep (Juengel et al., 2006). Unlike GDF9 and BMP15, BMP6 expression has been detected in the GCs in mice, from the primary to antral stages (Elvin et al., 2000). The exact expression patterns of BMP6 with respect to follicular stage are ill-defined, in the mouse and human. Expression of BMP6 has been detected in primordial- and primary human follicles (Shi et al., 2009).

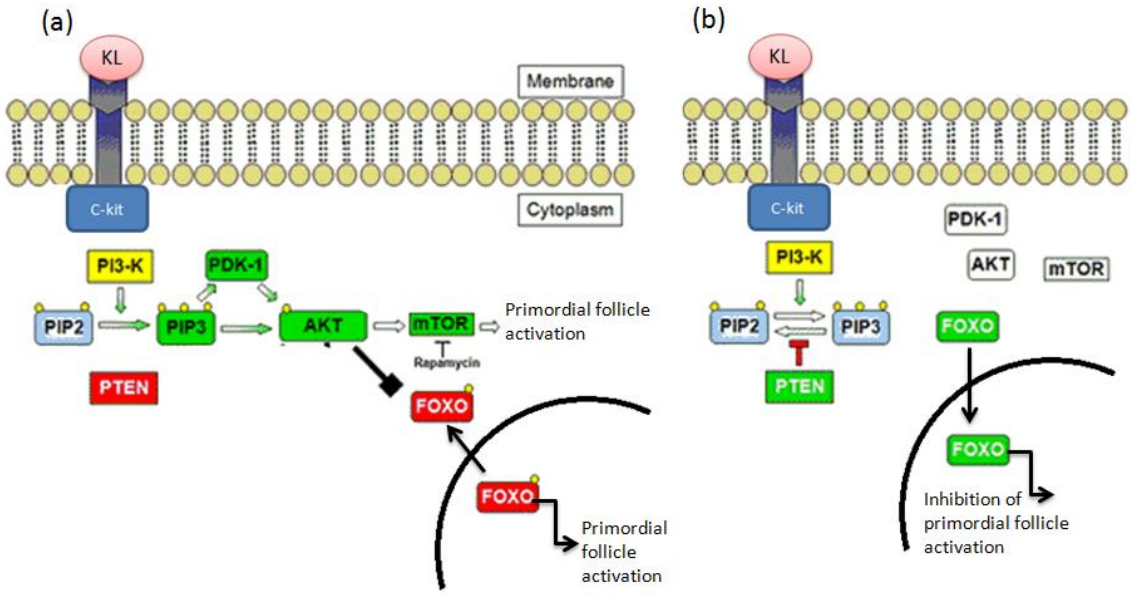


Figure 1.2 Schematic representation of process of primordial follicle activation via the PI3K-Akt pathway in the (a) absence and (b) presence of activated PTEN. Green and red boxes represent activation and deactivation, respectively. Yellow circles indicate phosphorylation.

The roles of GDF9, BMP15 and BMP6 are summarised in Table 1.2. As previously described the expression patterns of these oocyte-derived factors are species-specific. Similarly the actions of these factors vary between species. For example, in rodents GDF9 is sufficient to promote GC proliferation, whereas in sheep both GDF9 and BMP15 are required (Lin et al., 2012). In all species detailed in Table 1.2, however, the role of GDF9, BMP15 and BMP6 can be broadly summarised as regulation of the proliferation and differentiation of CCs in order to regulate and optimise the microenvironment experienced by the oocyte.

Table 1.2 Oocytes regulate the development and differentiation of granulosa cells to ensure that an optimised microenvironment is provided. This is achieved via secretion of oocyte-secreted factors (OSFs). The table below summarises key roles of the OSFs, the species in which there is evidence of this, the mechanism by which it is achieved and the evidence for this. Abbreviations: gonadotrophin (GN), mouse (M), rodent (R), porcine (P), bovine (B), ovine (O) and human (H). References: 1 Joyce et al. (2000), 2 Thomas and Vanderhyden (2006), 3 Vanderhyden et al. (1992), 4 Otsuka et al. (2001), 5 Gilchrist et al. (2004), 6 Vitt et al. (2000) 7 Gilchrist et al. (2008) 8 Otsuka et al. (2000), 9 (McNatty et al., 2005), 10 Su et al. (2009), 11 Sugiura et al. (2005), 12 Colonna and Mangia (1983), 13 Su et al. (2008), 14 Hussein et al. (2005), 15 Dragovic et al. (2005).

OSF-regulated process (species)	Mechanism	Evidence
Regulation GC differentiation e.g. KL expression (M)[1,2]	KL expression is suppressed by GDF9 (antral follicle) and stimulated by BMP15 (EA follicle). BMP15 is also regulated by FSH via KL signalling	*In vitro* studies revealed that GDF9 and fully grown, but not partially grown, oocytes inhibited GC KL expression
Promotion of GC proliferation via production of mitogens (R,O)[3,4,5,6]	GDF9 identified as OSF responsible but is not the sole contributor. BMP6 is has a weak or no mitogenic effect on GCs in the sheep and mouse, respectively	Culture of GCs with denuded rodent oocytes or GDF-9 increased GC number and thymidine incorporation
Inhibition of CC luteinisation (M, P, B, O)[6,7,8,9]	Via regulation of GC steroidogenesis - GDF9 and BMP15 inhibit FSH-induced GC differentiation and P4 production. GDF9 inhibits FSH-induced E2 production. BMP6 inhibits P4 production (O)	Removal of oocyte from COCs led to increased CC markers of luteinisation. This effect was reversed by oocyte co-culture. Effects of GDF9 and BMP15 on FSH-induced E2 and P4 assessed using GC culture
Regulation of metabolic cooperativity between CCs and oocyte (M, B, H)[10,11,12,13]	Production of oocyte-derived factors GDF9 and BMP15 stimulate CC production of metabolites (amino acids, cholesterol and oxidative phosphorylation substrates), which are then transferred to the oocyte via GJs	Glycolysis - oocytes unable to use glucose as an energy source as they lack the glycolytic machinery, which CCs possess allowing them to supply oocytes with substrates required for oxidative phosphorylation. Illustrated by removal of oocyte from COCs (M) Amino Acid and Cholesterol Biosynthesis - used radioactively labelled amino acids (eg. L-alanine) and acetate (cholesterol precursor) and tested levels of radioactivity in denuded oocytes *vs.* CC enclosed oocytes. Levels in the cumulus-enclosed group were higher than in denuded oocytes (M). Illustrated by oocyte removal from COCs
Promotion of CC survival (B)[14]	BMP15 and 6 implicated, whereas GDF9 is not. Effect via promotion and suppression of Bcl-2 (anti-apoptotic) and Bax (apoptotic) gene expression, respectively, in CCs	Removal of oocyte from COCs resulted in increased levels of apoptosis in CCs, this effect was reversed by co-culturing with oocytes
Promotion of CC mucification and expansion (M)[7,10,15]	GDF9 and BMP15 production enables CCs to respond to GN and EGF-like peptide stimulation to undergo expansion and mucification, via EGF receptor induction	Oocytectomy prevents cumulus expansion and mucification. This can be reversed by co-culture with oocytes and/or factors GDF9. Other OSFs also involved - shown using neutralising antibodies against GDF9

1.5.3 The transforming growth factor- β (TGF-β) subfamily and superfamily

Many of the factors involved in folliculogenesis and oogenesis are members of the TGF-β subfamily and superfamily. The cellular localisation and expression patterns in the mouse, sheep and human and roles of the members of the TGF-β subfamily and superfamily members are summarised in Table 1.3 and Table 1.4. The TGF-β superfamily members share common structural motifs and form homo-or heterodimers via covalent disulphide bonds between conserved cysteine residues (Massague, 2000). However, GDF9 and BMP15 are exceptions and form non-covalent bonds. With the exception of inhibins A and B (see below), the majority of TGF-β superfamily members bind to and signal via serine/ threonine kinase receptors via a common pathway, summarised in Figure 1.3 (Chang et al., 2002, Miyazawa et al., 2002, Massague, 2000). Oocyte-derived factors, GDF-9 and BMP-15, however, also signal via an alternative Mitogen-activated protein Kinase (MAPK) pathway. Once activated MAPK isoforms ERK1/2 interact with Smads, which is essential for the promotion of GC proliferation (Moore et al., 2003, Su et al., 2002).

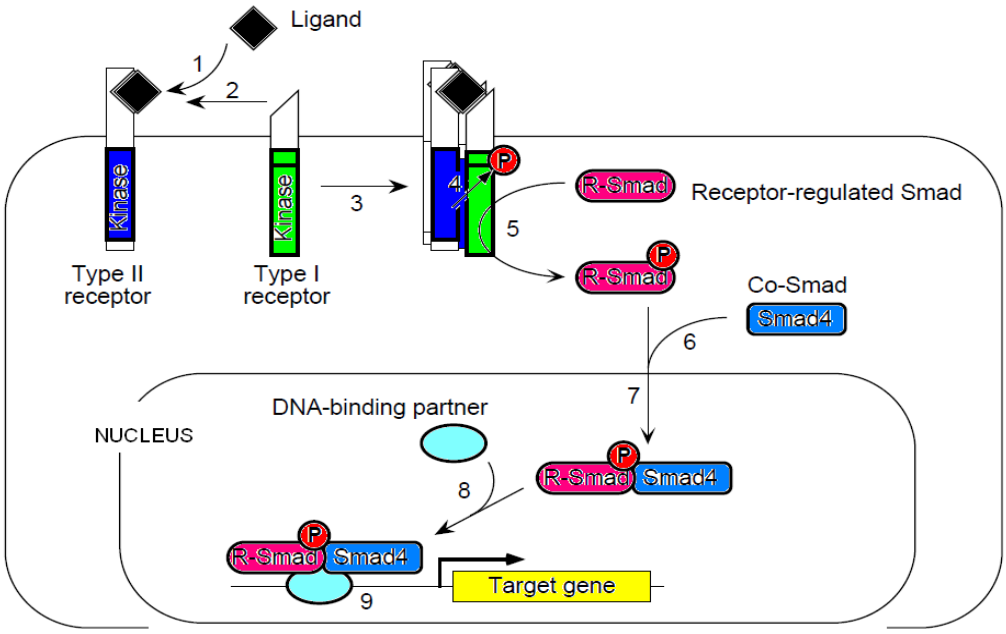


Figure 1.3 The TGF-β/SMAD pathway. A member of the TGF- β superfamily (1) binds a type II receptor (2) and a type I receptor (3) resulting in the formation of a receptor complex (4). This induces the phosphorylation and activation (5) of the type I receptor, which can then phosphorylate a receptor-regulated SMAD (R-Smad). Smad4 (a co-smad) (6) then forms a heteromeric complex with the active R-Smad (7) and translocates to the nucleus, where it binds a DNA-binding partner (8) and modulates target gene expression (9). These actions are differentially inhibited by Smads 6 and 7. Diagram adapted from (Massague, 1998).

Information regarding expression patterns shown in Table 1.3 suggests that the theca cells are the main site of action of members of the TGF-β subfamily. However, particularly as there is some disagreement regarding TGF-β receptor-II expression in GCs, the role of the TGF-β subfamily in this cell type requires further investigation (Qu et al., 2000, Roy and Kole, 1998). Note that the beta-glycan referred to in Table 1.3 facilitates the binding of TGF-β 2 to its receptor (Jaatinen et al., 2002).

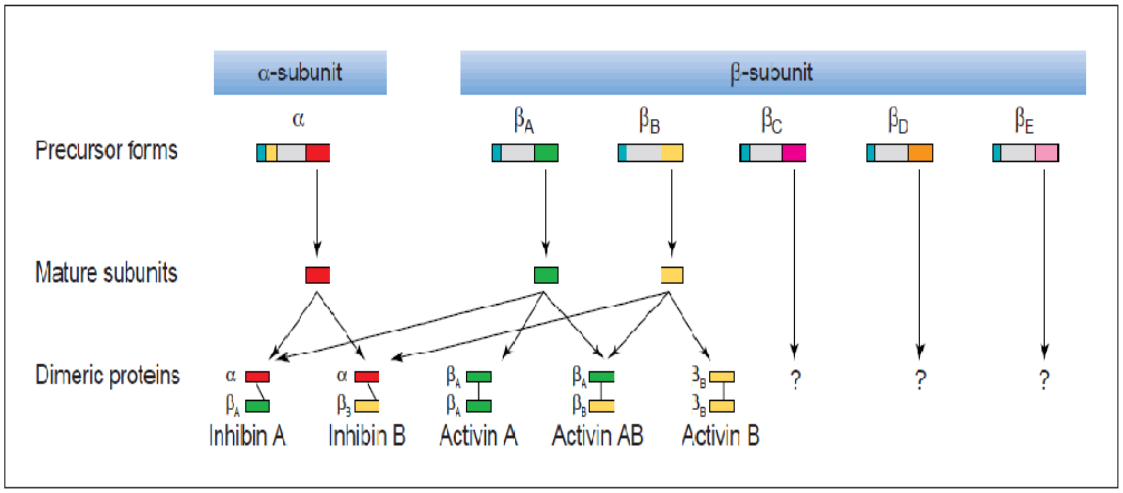


Figure 1.4 Subunit composition of known inhibin and activin forms.

As described in Table 1.4 the actions of inhibin and activin oppose each other. Inhibins exert their effect by blocking the actions of activins. As mentioned above the mechanism of inhibin A and B signalling is different to that of the other members of the TGF-β superfamily as binding of an inhibin β subunit to the activin type II receptor and binding of an inhibin α subunit to the receptor, ALK4, antagonises activin signalling thus bringing about the effects of inhibin (Zhu et al., 2012). TGF-β superfamily member activin A has been shown to accelerate the development of secondary follicles *in vitro* (Thomas et al., 2003, Telfer et al., 2008). Activin is a dimeric protein which exists in 3 forms; see Figure 1.4 (Pangas et al., 2002). When co-expressed with a distantly related α subunit the dimeric protein inhibin (see Figure 1.4) is formed. The effects of activin A on secondary follicle development are species-specific, for example this growth factor promotes antrum formation in human but not ovine follicles (Zhao et al., 2001). A summary of the key factors involved in folliculogenesis and oogenesis that are not part of the TGF-β subfamily and superfamily is provided in Table 1.5. Insulin and Insulin-like Growth Factors (IGF1 and IGF2) are considered separately in Section 1.5.4.

Table 1.3 The role, localisation and stage of follicular development during which TGF-β subfamily members, their receptors and binding proteins are expressed in sheep and humans. The + symbol indicates the factor's presence throughout follicular development/ a – symbol indicates its absence. Abbreviations: androstenedione (A4), steriodogenic acute regulatory protein (StAR).

Species	TGF-β subfamily member/ receptor/ binding protein	Site of Expression			Effect
		Oocytes	Granulosa Cells	Theca Cells	
Ovine (Juengel, 2004)	TGF-β1 and 2	-	-	Small preantral ->antral	Anti-differentiative role, inhibited P4 synthesis by granulosa cells. Possible role in regulation of follicular cell proliferation and apoptosis.
	TGF-β RI	Primordial -> antral	Small preantral -> antral	Small preantral -> antral	
	TGF-β RII	-	-	Small preantral -> antral	
	Beta-glycan	+	+	+	
Human (Chegini and Flanders, 1992, Qu et al., 2000, Pangas et al., 2002, Liu et al., 2003, Attia et al., 2000, Carr et al., 1996)	TGF-β1	+	+	+	TGF-β1 inhibits steroidogenesis in human theca cells. Production of A4 by theca cells is suppressed via the inhibitory effects of TGF-β1 on StAR.
	TGF-β2	-	All stages	Larger follicles only	
	TGF-β RI	Primordial -> antral	Primordial -> antral	Preantral -> antral	
	TGF-β RII	Primordial -> primary	-	All stages	
	Smad 2	Primordial -> primary	Preantral -> antral	Preantral -> antral	
	Beta-glycan	-	Antral -> pre-ovulatory	Antral -> pre-ovulatory	

Table 1.4 The origin, site of action and role of somatic-cell derived TGF-β superfamily members and the stage of follicle development at which they are expressed in the mouse (M), rat (R), sheep (S), cow (C) and human (H). Note the BMP4 and BMP7 expression was detected at very low levels in the sheep therefore these factors are unlikely to play a significant role in regulating ovine follicular development (Juengel et al., 2006).

TGF-β superfamily member	Origin	Site of action	Effect	Expression pattern	Extra information	References
BMP4	TC	GC/ oocyte	Promote primordial to primary transition[R]. Suppression of GC apoptosis via non-Smad pathways; BMP4 via the PI3K/PDK-1/PKC and BMP7 via the PI3K/PDK-1/Akt.	Primordial follicle[M] - may also have an effect later in development	Promotes primordial follicle survival	(Shimasaki et al., 1999, Lee et al., 2001, Nilsson and Skinner, 2003)
BMP7	TC	GC/ oocyte			Increases GC proliferation via the promotion of BMP15 expression	(Lee et al., 2004b, Shimizu et al., 2012)
Anti-mullerian hormone (AMH)	GC	GC	Inhibits follicle development	Primary -> antral[H,M,S]	Alters gene expression of stimulatory factors and, in the antral follicle, attenuates actions of FSH	(Durlinger et al., 1999, Weenen et al., 2004)
Activin A	GC	GC	Stimulates GC proliferation and differentiation - specifically steroidogenesis and FSH receptor expression. Inhibited by follistatin	Primordial stage onwards. However, activin SUs, receptors, and Smads not coexpressed until small antral stage [S,H]	Studies have shown that activin a accelerates secondary follicle growth *in vitro* - exact effects are species-specific	(Rabinovici et al., 1991, Findlay, 1993, Liu et al., 1999, McNatty et al., 2000, Zhao et al., 2001, Pangas et al., 2002, Thomas et al., 2003, Telfer et al., 2008)
Inhibin A and B	GC	GC/TC	Opposes effects of activin in order to promote the latter stages of follicle development	Detected in the antral follicle - increases during dominant follicle selection[C]		(Wrathall and Knight, 1995)

Table 1.5 The target, localisation and action of key somatic growth factors involved in folliculogenesis, in the mouse (M), rat (R), sheep (O) and human (H). Abbreviations: Oocyte (Ooc), primary (1°), Tyrosine Kinase (TyrK). References 1 Manova and Bachvarova (1991), 2 Huang et al. (1992), 3 Manova et al. (1993),4 Clark et al. (1996), 5 Tisdall et al. (1997), 6 Joyce et al. (1999), 7 Parrott and Skinner (1999), 8 (Driancourt et al., 2000), 9 Hoyer et al. (2005), 10 Anderson and Lee (1993), 11 Lavranos et al. (1994), 12 van Wezel et al. (1995), 13 Rodgers et al. (1996), 14 Yamamoto et al. (1997), 15 Quennell et al. (2004), 16 Arici et al. (1997), 17 Abir et al. (2004), 18 Nilsson et al. (2002), 19 Parrott et al. (1994), 20 Kezele et al. (2005), 21 Nilsson et al. (2006), 22 Yoon et al. (2006), 23 Pinkas et al. (2008).

Factor (species)	Target	Localisation	Action	Extra information
KL (M,O,H) [1,2,3,4,5,6,7,8,9,10]	c-kit (Ooc)	PGG c-kit expression until meiosis initiation. KL (GC) and c-kit expression primordial to antral	Promotes PGC migration, primordial follicle activation, development and survival	Exists in 2 trans membrane forms KL-1 and KL-2. Both of which can be cleaved to soluble forms, however, KL-2 lacks the cleavage site thus mainly exists in the membrane bound form and KL-1 in the soluble form
Basic Fibroblast Growth Factor (bFGF) (M,O,H) [11,121,3,14,15,16]	Receptor on GCs	Ooc - all stages, GC - 1° onwards, TC - preantral onwards (human)	Induces primordial follicle development via stimulation of basement membrane formation and GC proliferation and differentiation (mice)	Species-specific differences in terms of the role of bFGF in follicular development. Conflicting evidence regarding the role of bFGF in humans - inhibitory vs stimulatory
LIF (R,H) [17,18,19]	GC and TC	GC	Promotes primordial follicle development via promotion of KL expression	Potential role in ovulation as the concentration of LIF in FF increases in response to LH
Keratinocyte Growth Factor (KGF) (R) [20,21]	TC and GC	Pre-TCs	Stimulates development from the primordial to 1° stage via recruitment of theca cells and promotion of GC proliferation	Exists in a positive feedback loop with KL
Platelet-derived growth factor (PDGF) (R, H) [21,22,23]	TyrK receptor (GCs)	Ooc - all stages of development. GCs and TCs - growing follicles	Promotion of primordial to 1° follicle transition possibly via the stimulation of KL expression	Treatment of rat ovaries with PDGF led to a significant decrease in the number of primordial follicles and a concomitant increase in the number of growing follicles as well as an increase in KL expression

1.5.4 The role of Insulin, IGF1 and IGF2

Insulin and insulin-like growth factors, IGF1 and IGF2 are of particular interest as they are common constituents of IVG culture media (see Table 1.10). Insulin is an oocyte-derived growth factor that acts upon the GC and TC via its receptor. The actions of insulin, IGF1 and IGF2 in rodent, cow, sheep and human are similar in that they all stimulate mitosis, DNA synthesis, steroidogenesis in somatic cells as well as the nuclear maturation of the oocyte (Duleba et al., 1997, Duleba et al., 1998, Thomas et al., 2007, Walters et al., 2006). Additionally *in vitro* insulin increases both basal and LH-stimulated steroid hormone production by GCs (Willis et al., 1996). The actions of IGF1 are mediated predominantly via the IGF1R although it also binds IGF2R with very low affinity (Silva et al., 2009). The actions of IGF2 are mediated predominantly via the IGF2R although it binds IGF1R with relatively high affinity (Silva et al., 2009). Insulin also binds IGF1R, although with very low affinity, but does not bind IGF2R (Silva et al., 2009). The bioavailability of IGFs is regulated by 6 IGF binding proteins (IGFBPs), resulting in either the augmentation or inhibition of the actions of IGFs (Baxter, 2000, Silva et al., 2009). Levels of IGFBPs are themselves regulated by IGFBP proteases; for example, during dominant bovine follicle selection IGFBP4 protease is responsible for the proteolysis of IGFBP4 resulting in an increase in IGF bioavailability and subsequent establishment of follicular dominance (Rivera et al., 2001). In addition, the level of IGFBP expression is linked to the level of exposure to IGFs as well as the stage of follicular development (Walters et al., 2006). The importance of the regulation of IGF bioavailability was demonstrated in a study conducted by (Thomas et al., 2007) in which bovine preantral follicles were treated with the IGF1 analog-long-R3 IGF1, for which IGFBPs have a lower binding affinity for than for IGF1. Treatment resulted in follicles displaying greater increases in diameter but smaller oocyte: follicle ratios and higher levels of follicular degeneration than that observed in controls. Oocyte health in larger antral follicles, however, improved in response to incubation with high levels of IGF1, thus illustrating the fact that the effects of IGFs are stage dependent (Walters et al., 2006).

The thecal cell-derived factor IGF1 is not expressed until the EA follicular stage, whereas its receptor (IGF1R) is expressed from the primordial stage by oocytes and GCs, in the cow and human (el-Roeiy et al., 1993, Qu et al., 2000, Armstrong et al., 2002). Thecal-cell expression of IGF1R has been detected in large antral bovine follicles. The expression of IGF1R early in follicular development can be exploited *in vitro* to promote follicle development (el-Roeiy et al., 1993). In mice IGF1 has also been shown to stimulate FSHR expression (Zhou et al., 1997). In human follicles expression of IGF2 by GC and TC is detected during preantral follicle

development and increases with increasing follicle size (el-Roeiy et al., 1993). In addition IGF2 mediates the effects of FSH on E2 production in preantral follicles, thus promoting growth and steroidogenesis (Erickson et al., 1979, Voutilainen et al., 1996). *In vitro* IGF2 increases E2 production by GCs – this is augmented by insulin pre-incubation, possibly via increased expression of the IGF1R (Mason et al., 1994).

Research regarding the expression patterns of IGFs and their receptors in the ovine model has focused on mainly on the latter stages of development with all follicles <2-4mm diameter grouped together. Therefore the exact patterns of expression during ovine preantral follicle development remain to be elucidated (Perks et al., 1995, Monget and Bondy, 2000). Although IGF1 was not initially detected in ovine follicles (Perks et al., 1995) subsequent studies have detected low levels of IGF1 expression in GC and TC (Hastie and Haresign, 2006). Expression of IGF2 was detected in the GC, but not the TC layer. Expression of IGF1R and IGF2R was detected in both the GC and TC layer, but to a lesser extent in the latter, with levels increasing with follicle size (Hastie and Haresign, 2006).

1.5.5 The role of gonadotrophins

Gonadotrophins (GNs); FSH and LH are produced by the anterior pituitary gland in response to gonadotrophin releasing hormone (GnRH) stimulation, which is released by the hypothalamus in a pulsatile manner every 1-3 hours in humans (Johnson, 2000). Both FSH and LH are heterodimers, composed of a common α subunit and distinct β subunits (Pierce and Parsons, 1981). Oestrogen levels indirectly regulate FSH and LH release by the pituitary gland, via regulation of the frequency of GnRH pulsatile release by the hypothalamus (McNatty et al., 1975, Bouchard et al., 1988, Aerts and Bols). Oestrogen levels in the bloodstream determine the frequency of GnRH release which itself controls FSH and LH release by the pituitary. Low oestrogen levels promote a lower frequency of GnRH release, which promotes the production of FSH by the pituitary. Whereas high oestrogen levels result in a relatively faster frequency of GnRH release, which promotes LH release and suppresses FSH release by the pituitary gland (Wildt et al., 1981, Molter-Gerard et al., 1999). Progesterone also regulates LH release via regulation of GnRH frequency, in the presence of oestrogen (Bergfeld et al., 1996, McCartney et al., 2007). FSH production and release is also moderated by numerous members of the TGF-β superfamily. The rate-limiting step with respect to the overall production of FSH is the production of the FSH β subunit. The members of the TGF-β superfamily implicated in the regulation of FSH production are: inhibin A and B - which inhibit production and release;

activin A, AB and B - which promote production and release, BMP4 - which inhibits production as well as the effects of activin (Faure et al., 2005); and BMP6, BMP7 and BMP15 - which promote production (Rivier and Vale, 1981, Rivier and Vale, 1989, de Kretser et al., 2000, Huang et al., 2001, Otsuka and Shimasaki, 2002).

The pituitary-derived GNs FSH and LH are both required for follicular growth and development from the EA up to the pre-ovulatory stage. Furthermore, FSH and LH promote GC proliferation and steroidogenesis and are required for the selection of the dominant follicle (Gougeon, 1986, Gougeon, 1996, Webb et al., 2007). In the absence of these hormones, for example prior to puberty, later-staged antral follicles will become atretic and degenerate. FSH has been identified as the key survival factor as investigations carried out by (Chun et al., 1996) revealed that at the EA stage FSH reduced the levels of apoptosis to a greater extent than other previously identified anti-apoptotic factors. In these experiments IGF1 was identified as a possible mediator of the anti-apoptotic actions of FSH. Further experiments with pre-ovulatory follicles have shown that the level of suppression of apoptosis is stage and dose-dependent, with respect to various GNs and GFs (Chun et al., 1994). Prior to this stage GNs are not essential, however, FSH receptors are expressed from the primary stage in sheep and humans, thus FSH is able to play a permissive role in supporting follicle health as well as growth and development (Tisdall et al., 1995, Oktay et al., 1997a). FSH has been shown to promote the growth and differentiation of rat preantral follicles (McGee et al., 1997). In the sheep FSH has been shown to promote GC proliferation in small (2-3.5mm) and large follicles *in vitro* (Campbell et al., 1996), furthermore FSH has been shown to promote antral cavity formation in cultured secondary follicles (Newton et al., 1999b) and FSH has been shown to stimulate the growth of large antral follicles *in vivo* (Picton et al., 1990).

The hormones FSH and LH exert their effects on follicular development through binding to their cognate FSH and LH receptors, respectively. The GN receptors are members of the 7 transmembrane G-protein superfamily (Richards and Midgley, 1976, McFarland et al., 1989, Sprengel et al., 1990, Hillier, 2001). Post-receptor signalling then results in the activation of genes involved in proliferation, differentiation, steroidogenesis and the production of peptides regulating GN release, via adenylyl cyclase-mediated signalling and the stimulation of cAMP production, which acts as a secondary messenger (Richards, 1994, Hillier, 2001, Segaloff et al., 1990). FSH receptors are expressed only by GCs, whereas LH receptors are initially only expressed by theca cells, however, following FSH stimulation, at the mature, antral stage GCs

also express LH receptors (Zeleznik et al., 1974, Camp et al., 1991, Piquette et al., 1991). Prior to the expression of FSH and LH receptors on GCs and TCs, respectively, GNs are unable to directly stimulate follicle growth and development. As mentioned previously, it is initially FSH that stimulates follicle growth and development via the promotion of GC maturation and proliferation, FSHR production by GCs – thus increasing their sensitivity to FSH and augmenting its action, production of peptides involved in the regulation of GN production (inhibin and activin) and proteoglycan production resulting in FF formation (Yanagishita and Hascall, 1979, Yanagishita et al., 1981). Following LHR induction in the theca layer, FSH and LH become involved in a loop termed the 2-cell, 2-GN theory which promotes the production of oestrogens, as shown in Figure 1.5 (Armstrong and Dorrington, 1977). The production of oestrogens in GCs is achieved via the aromatization of thecal-derived androgens, which have been produced in response to LH stimulation. The aromatisation of androgens requires the induction of cytochrome aromatase ($P450_{arom}$) expression via FSH binding to the GCs (Hillier, 1994).

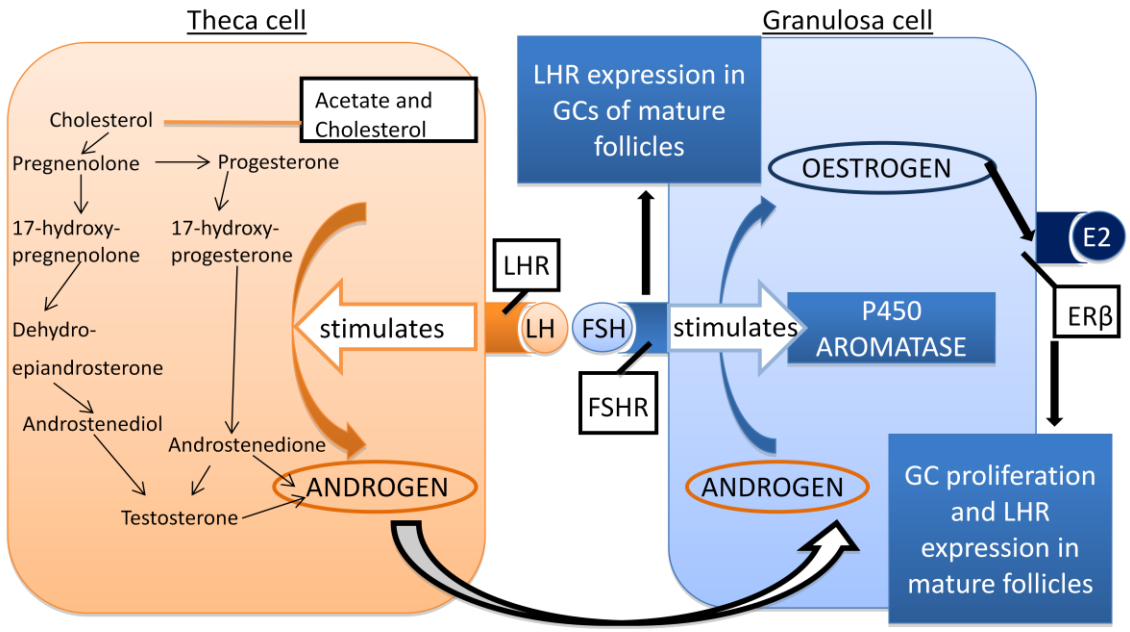


Figure 1.5 The 2 cell, 2 gonadotrophin theory. Androgens, produced in the theca layer as a result of LH stimulation, are used as substrates for oestrogen production in the GC layer. Oestrogens then bind receptors in the GC layer and promote GC proliferation thus resulting in increased production of oestrogen. Both FSH and oestrogen promote the expression of LHRs in the GC layer allowing LH to promote growth and development in a similar manner to FSH, once FSH levels start to fall (Zeleznik and Hillier, 1984, Taylor and Al-Azzawi, 2000). It should also be noted that in some species a small amount of oestrogen synthesis by the thecal cells is exhibited (Armstrong, 1981, Tsang et al., 1985).

As previously described E2 has a central effect on GnRH secretion but it also has a local, intra-ovarian effect. Oestrogen promotes GC proliferation and enhances aromatase activity; thus increasing its own production and amplifing the ability of GNs to stimulate the expression of FSHR and LHR (Richards and Midgley, 1976). Locally produced growth factors also promote GC differentiation and proliferation, enhance the actions of FSH with respect to: E2 production, LHR expression by GCs, stimulation of the conversion of cholesterol to androgens. For example, IGF1 amplifies the effect of FSH in GCs (Adashi, 1995). The effects of E2 are important in terms of the establishment of dominance.

Thus the development of follicles from the EA stage onwards involves a complex interaction of steroids, gonadotrophins and locally produced GFs. Development from the EA to preovulatory follicle stage can be described in 3 phases: recruitment, selection and dominance. Recruitment requires a transient increase in FSH levels to rescue a cohort of EA follicles from atresia. Such a rise in FSH levels requires a drop in E2 and inhibin levels, which occurs at the transition between the luteal and follicular phases when a corpus luteum (CL) is degenerating. Studies have shown that the decrease in E2 rather than in inhibin is the main cause of the FSH rise (le Nestour et al., 1993). At this stage the P4 to E2 production ratio has increased. In humans around 11 follicles per ovary are recruited to grow in each new reproductive cycle (Schipper et al., 1998). Follicles continue to develop to the Graafian stage and reach a diameter of 2-5mm, at which point selection occurs during the follicular phase of the cycle (McGee and Hsueh, 2000). As the follicles are developing E2 production increases, resulting in the negative feedback and hence suppression of FSH production and increasing LH release by the pituitary gonadotrophs. During the follicular phase E2 levels rise further resulting in a decrease in FSH levels, at this point the dominant follicle appears (van Santbrink et al., 1995). In monovular species one follicle becomes dominant and continues to grow, while the remaining follicles regress. The selected follicle has an early developmental advantage and is larger early in the cycle and is either the largest at the time of selection or the most oestrogenic, or both (Ginther et al., 1997). Studies in humans have shown that the dominant follicle can be identified around day 7 of the menstrual cycle. The dominant follicle continues to increase in size to reach a median diameter of 20mm in humans and 5-7 mm in sheep, at the pre-ovulatory stage (Fauser and Van Heusden, 1997, Hunter et al., 2004). This follicle has the highest levels of E2 and growth factors thus increasing its sensitivity to FSH and allowing its continued development in a low FSH level environment. Furthermore, this follicle will be the first to express LHRs on GCs thus allowing LH to take over the role of FSH in driving E2 production in the presence of declining peripheral FSH levels. This follicle will continue to

produce high levels of E2 thus further decreasing FSH levels to an extent to which the remaining recruited follicles are unable to continue their development and so then undergo atresia. The production of E2 in the late follicular phase of the cycle promotes LH production and so ultimately causes the LH surge. The LH surge ultimately results in the ovulation of the cumulus oocyte complex (COC) (Aerts and Bols, 2010). In order for ovulation to occur the CC mass must undergo a process known as expansion, in which GC-derived hyaluronic acid, secreted in response to the GN-surge, binds cumulus cells (CCs) and increases the space between them (Eppig, 2001, Schoenfelder and Einspanier, 2003). At this point activity of P450 aromatase is suppressed and the steroid production by the follicle switches from mainly E2 to P4 causing the resumption of meiosis, cellular and nuclear maturation, follicle wall rupture and COC release (Hillier, 2001). The reduction in the level of cAMP being transferred to the oocyte, as a result of the LH surge disrupting the CC-oocyte interactions also contributes to the resumption of meiosis (Dekel, 1988). A summary of the mechanism by which the LH surge occurs is provided below, adapted from Conti et al. (2012). Prior to the GN-surge high levels of cAMP maintain the phosphosphorylation and therefore the inhibition of maturation promoting factor, via the promotion of cAMP-dependent PKA, which promotes Wee1 kinases and inhibits Cdc25 phosphatase. Although high enough levels of cAMP are produced by the oocyte, connections between the oocyte and GCs are still essential for the maintenance of meiotic arrest, due to the provision of cGMP to the oocyte by the GCs. In response to the LH surge, the permeability of GJs between GCs is reduced resulting in cGMP levels in the GCs and subsequently the oocyte dropping. Reduced levels of cGMP in the oocyte cause the activation of phosphodiesterase 3A, an enzyme which hydrolyses cAMP, thus resulting in a decrease in cAMP levels. The exact mechanism of this is unknown, however, the EGF network has been implicated, as the levels of EGF-like GFs including: amphiregulin, epiregulin and betacellulin, in murine (Shimada et al., 2006), human (Zamah et al., 2010), ovine (Cotterill et al., 2012) and porcine (Procházka et al., 2011) follicular somatic cells rapidly and transiently increase in response to LH. This increase is proposed to be mediated via the phosphorylation and activation of serine/threonine kinase, ERK1/2 and p28 map kinase induction (Conti et al., 2012). The TGF-β superfamily have also been implicated in the regulation of ovulation and luteinisation as knock out of Smad4 in mice revealed that it was required for LH-induced cumulus expansion and expression of ovulation related-genes (Yu et al., 2013). The resumption of meiosis progresses as follows: the nuclear membrane degenerates and meiotic arrest ends; chromosomes complete their first meiotic division; the polar body is extruded and an MII oocyte is formed; nuclear progression is accompanied by cytoplasmic maturation and gap junctional breakdown with the cumulus cells. Following ovulation the somatic cells of the

follicle enter a phase of terminal differentiation in which the cells stop proliferating, they undergo cellular remodelling and they produce large amounts of P4 before eventually regressing. At this stage the follicle is referred to as a corpus luteum (Stocco et al., 2007). Note that in sheep, in contrast to humans, the large antral follicles continue to develop throughout the luteal phase, as sufficient levels of FSH and LH are present (Baird, 1983).

The responsiveness of the different follicular components to GNs are regulated by autocrine and paracrine actions, via IGFs and their binding proteins, activin and inhibin and various steroids including; androgens, E2, P4 and glycocortoids – the latter possibly being involved in injury repair post-ovulation. The oocyte will remain at the MII stage until fertilisation triggers the resumption and subsequent completion of meiosis and extrusion of the second polar body, this has been reviewed in (van den Hurk and Zhao, 2005, Li and Albertini, 2013).

The process of oogenesis and folliculogenesis is therefore highly complex and still not fully understood. A summary of the key factors involved and their role (if known) in sheep and human is provided in Table 1.6.

Table 1.6 The role of growth factors in human and ovine oogenesis and folliculogenesis. Abbreviations; human (H), ovine (O). References; (1) see Table 1.2; (2) see Table 1.5; (3) see Section 1.5.4; (4) Zeleznik et al. (1974); (5) Pangas et al. (2002) and Thomas et al. (2003); (6) see table 1.3; (7) Weenen et al. (2004); (8) Wrathall and Knight (1995); (9) McNatty et al. (2000).

Follicle stage	Stimulatory		Inhibitory	
	Factor (Origin) Species	Effect	Factor (Origin) Species	Effect
Primordial	GDF9 (Ooc) O[1]	Mitogenic	TGF-β2 (GC) H[6]	Anti-differentiative, inhibits steroidogenesis
	BMP6 (Ooc) H, O[1]	Possibly mitogenic		
	KL[2]	Promotes primordial follicle activation, growth and development		
	bFGF (Ooc) H,O[2]	Unknown		
	LIF (GC) H[2]	Promotes KL expression and thus follicle development		
	PDGF (Ooc) H[2]	Promotes KL expression and thus follicle development		
	IGF1R (Ooc, GC) H[3]	Mediates actions of insulin, IGF1 and IGF2		
Preantral	GDF9 (Ooc) H,O[1]	Mitogenic	TGF-β2 (GC) H, (TC) H, O[6]	Anti-differentiative, inhibits steroidogenesis
	PDGF (Ooc, GC, TC) H[2]	Promotes KL expression and thus follicle development	AMH (GC) H,O[7]	Inhibits primordial follicle activation and desensitises GC and TC to FSH and LH
	BMP15 (Ooc) H, O[1]	Mitogenic	Follistatin (GC) O,H[9]	Inhibits actions of activins
	BMP6 (Ooc) H, O[1]	Possibly mitogenic		
	FSHR (GC) H,O[4]	Mediates the actions of FSH		
	KL[2]	Promotes growth and development		
	bFGF (Ooc, GC, TC) H[2]	Unknown		
	IGF1R (Ooc, GC) H, O[3]	Mediates actions of insulin, IGF1 and IGF2		
	IGF1 (GC, TC) O?[3]	Promote GC and TC proliferation		
	IGF2 (GC, TC) H, O?[3]	Promotes growth and steroidogenesis		
	IGF2R (GC, TC) H, O[3]	Mediates actions of insulin, IGF1 and IGF2		

Table 1.6 (continued)

Follicle Stage	Stimulatory		Inhibitory	
	Factor (Origin) Species	Effect	Factor (Origin) Species	Effect
EA	GDF9 (Ooc) H, O[1]	Mitogenic	TGF-β2 (GC) H, (TC) H, O[6]	Anti-differentiative, inhibits steroidogenesis
	BMP15 (Ooc) H, O[1]	Stimulate KL expression	Follistatin (GC) O,H[9]	Inhibits actions of activins
	BMP6 (Ooc) H, O[1]	Mitogenic (O)	Activin A and B(GC) H,O[5]	Inhibit FSH-stimulated E2 and inhibin A production and LH-stimulated A4 production
	FSHR (GC) H,O[4]	Mediates the actions of FSH		
	IGF2R (GC, TC) H, O[3]	Expression patterns unclear - mediates actions of insulin, IGF1 and IGF2	AMH (GC) H,O[7]	Inhibits primordial follicle activation and desensitises GC and TC to FSH and LH
	PDGF (Ooc, GC, TC) H[2]	Promotes KL expression and thus follicle development	Inhibin A (GC) O[8]	Inhibits FSH release by pituitary and inhibit actions of activins
	Activin A and B (GC) H,O[5]	Promote GC proliferation, FSHR and LHR expression		
	KL[2]	Promotes growth and development		
	IGF1R (Ooc, GC) O, H[3]	Mediates actions of insulin, IGF1 and IGF2		
	IGF2 (GC, TC) H, O?[3]	Promotes growth and steroidogenesis		
	IGF1 (TC)[3]	Promotes GC and TC proliferation, FSHR expression		
Graafian	GDF9 (Ooc) H, O[1]	Mitogenic, promote CC mucification, inhibit luteinisation	GDF9 (Ooc) H, O[1]	Suppress KL expression and FSH-stimulated P4 and inhibin production by GCs
	BMP15 (Ooc) H, O[1]	Mitogenic, promote CC mucification and survival, inhibit luteinisation	BMP6 (Ooc) H, O[1]	Inhibits A4, E2 and inhibin A production by GCs and TCs
	BMP6 (Ooc) H, O[1]	Mitogenic (O), promote CC survival, inhibit luteinisation	AMH (GC) H,O[7]	Inhibits primordial follicle activation and desensitises GC and TC to FSH and LH
	FSHR (GC) H,O[4]	Mediates the actions of FSH	TGFβ2 (GC) H, (TC) H, O[6]	Anti-differentiative, inhibits steroidogenesis
	Activin A and B (GC) H,O[5]	Promote GC proliferation, FSHR and LHR expression	Activin A and B (GC) H,O[5]	Inhibit FSH-stimulated E2 and inhibin A production and LH-stimulated A4 production
	IGF1 and IGF1R[3]	Promote GC and TC proliferation and GC expression of FSHR	Inhibin A (GC) O[8]	Inhibits FSH release by pituitary and inhibit actions of activins
	KL[2]	Promotes growth and development	Follistatin (GC) O,H[9]	Inhibits actions of activins

1.6 Current methods of female fertility preservation

1.6.1 Introduction

A detailed understanding of the mechanisms regulating oogenesis and folliculogenesis is essential to support the development of strategies aimed at preserving the fertility of girls, adolescents and young women who are at risk of premature ovarian failure (POF). For example, improvements in cancer treatments have resulted in increasing survival rates of patients. The treatments used, however, are often aggressive and can cause infertility. In fact, cancer treatments are the most common cause of POF in girls (Jadoul et al., 2010). Other causes of POF include treatments used for autoimmune diseases as well as the diseases themselves, genetic abnormalities such as chromosome X defects: Turner syndrome and Fragile X syndrome, infections such as herpes zoster or isolated defects: for example those affecting GN receptors (Beck-Peccoz and Persani, 2006, Grynberg et al., 2012). Therefore, effective methods of fertility preservation are required. In the following sections fertility preservation techniques that are currently in use, or are under development, are summarised and discussed. All these techniques require the use of cryopreservation, therefore firstly the basic principles and methods of optimising cryopreservation techniques will be considered.

1.6.2 Basic principles of cryopreservation

The development of cryopreservation techniques has enabled the long-term storage of cells. In order to optimise cryopreservation techniques an understanding of the mechanisms of cellular freezing and the damage that this can cause is required. The level and nature of damage to cells when cryopreserved are dependent on a number of factors including cell type and size, external cooling and warming rates, and the presence/ absence of cryoprotective agents.

The major factors likely to cause damage during the cryopreservation process are intracellular ice formation and exposure of the cells to solute effects. The formation of ice in the extracellular environment can also cause cell damage. When ice forms extracellularly a series of channels are formed within which cells are held, the width of which decrease with decreasing temperature thus increasing the mechanical pressure on the cells (Rapatz, 1966). Ashwood-Smith et al. (1988) showed that the initial contact of the cell caused a change in cell shape and formed a weak spot where blebbing of the cytoplasm was seen during thawing. Gas

bubbles were also observed which were probably formed as dissolved gases come out of solution upon freezing (Carte, 1961). The mechanical stress from the formation of both intracellular and extracellular ice can damage the plasma membrane to an extent which is lethal to the cell. Furthermore, the formation of intracellular ice can lethally damage the membranes of intracellular organelles (Fujikawa, 1986, Karlsson and Toner, 1996). Fujikawa (1980) used freeze-fracture experiments and etching studies to assess the effect intracellular ice coming into direct contact with membranes, thus revealing the structural alterations and molecular disorganisation.

As ice forms in the extracellular environment this causes the concentration of electrolytes present to increase, thus raising the ionic strength of the medium and altering the osmolality of the system. This change in osmolality leads to water leaving the cell along the osmotic gradient resulting in an increase in electrolyte concentration in the cell. These changes can lethally damage cells (Lovelock, 1954). Ice formation alters the pH within and without the cells and causes substances, such as urea or dissolved gases that at normal physiological levels would not be damaging, to reach toxic levels (Lovelock, 1957). These 'solution effects' can lead to the denaturation of lipid-protein complexes of which membranes are composed, thus acting as another mechanism by which freezing can cause damage. Other mechanisms of intracellular ice formation (IIF) damage include cytoskeleton rearrangement, chromosome loss/gain, polyspermy, polygyny and tetraploidy, DNA fusion and rearrangements, dissolution and rearrangements and denaturation of proteins/enzymes (Shaw et al., 2000, Lane et al., 2000, Eroglu et al., 1998).

Cooling and warming rates used in cryopreservation procedures have long been recognised as important factors with respect to cell survival. This is mainly due to the relationship between cooling/ warming rates and IIF. When cells are cooled to a sufficiently low temperature, of between -5°C and –15°C, ice initially forms in the extracellular medium. As there are no intracellular nucleation centres present the protoplasm can theoretically become super-cooled down to -40°C - the temperature at which spontaneous ice formation occurs (Richard, 1995). Optimisation of cooling rates is of great importance. Slow, rather than rapid cooling rates have been shown to result in the highest cell survival rates. However, if the cooling rate used is too slow this can also result in cell damage. When a cell is cooled sufficiently slowly ice will firstly form in the extracellular environment and thus the vapour pressure of the extracellular medium will decrease rapidly to a level that is a lot lower than that of the protoplasm.

Furthermore the formation of ice will change the osmolality of the system. These changes will result in water leaving the cell along the vapour pressure differential and osmotic gradients. The rate at which water leaves a cell is dependent upon a number of factors: the surface area to volume ratio of the cell; the permeability of the membrane to water; and the temperature co-efficient of the permeability constant (Mazur, 1963). If the cooling rate is sufficiently slow this will allow water to leave the cell at a rate that keeps the protoplasm at its freezing point thus preventing ice from forming intracellularly. However, if the rate of cooling is more rapid then water will be unable to leave the cell quickly enough to maintain this equilibrium and ice will form intracellularly. Due to the absence of any nucleation centres in the intracellular compartment IIF should theoretically not be possible at temperatures above -40°C (Richard, 1995). Furthermore, the plasma membrane has been shown to act as an effective barrier against ice in the extracellular environment (Chambers and Hale, 1932). A number of possible mechanisms by which IIF could be induced at temperatures higher than –40°C have been suggested. Mazur (1960) hypothesised that the presence of aqueous pores in the plasma membrane provides a route through which ice crystals can grow and seed ice formation intracellularly. However, temperatures must be sufficiently low for ice crystals that are small enough to grow through these pores to exist before this is possible. A second mechanism by which IIF can occur has been proposed in which the frictional drag of water moving out of the cell is sufficient to rupture the extracellular membrane and allow extracellular ice crystals to enter the cell (Muldrew and McGann, 1994).

Therefore a slow cooling rate to facilitate cellular dehydration is preferable to a rapid cooling rate. However, if the cooling rate used is too slow this too can have detrimental effects. An excessively slow cooling rate can result in the cells being exposed to the high solute concentrations for an extended period thus causing cell damage (Schneider and Mazur, 1984). Conversely, rapid warming rates increase the survival rates of cells that have been cryopreserved. This is due to a process called recrystallisation, in which small crystals that have formed during the cooling process aggregate upon the increase in temperature to form larger crystals. Before the recrystallisation process these ice crystals are too small to cause any damage to the cell, whereas, after they are large enough to cause damage, which can cause cell death. If warming rates are sufficiently rapid the small crystals melt before recrystallisation is able to occur (Schneider and Mazur, 1984).

1.6.3 Cryoprotective agents (CPAs)

Some of the adverse effects of cryopreservation can be reduced with the use of cryoprotective agents (Lovelock, 1954). Agents used for cryoprotection can be divided into two categories: penetrating and non-penetrating CPAs. Penetrating CPAs are able to pass through the plasma membrane and therefore must be gradually added to the medium in which the cells are suspended to prevent excessive cell shrinkage prior to the establishment of an osmotic equilibrium (Meryman, 1971). Examples of penetrating CPAs include glycerol, dimethyl sulphoxide (DMSO), ethylene glycol and propene glycol (Newton et al., 1998). Non-penetrating CPAs are unable to pass through the cell's plasma membrane. Sucrose is a very effective and commonly used example of a non-penetrating CPA (Koshimoto and Mazur, 2002).

The use of CPAs protect against cryodamage partly via depression of the freezing point of water (Wolfe and Bryant 1999). Due to their relatively large molar volume the addition of CPAs reduces cell shrinkage when water leaves the cell by osmosis during the freezing procedure (Meryman, 2007). In addition, CPAs protect against cryodamage by stabilising proteins and membranes at low temperatures (Crowe, 1990). The penetrating CPA, DMSO is also a scavenger of free radicals (Yu and Quinn, 1994). The mode of protection against cryodamage by non-penetrating CPA is probably via the reduction of the extracellular medium freezing point. Non-penetrating CPAs are most valuable when used in combination with penetrating cryoprotectants by protecting against detrimentally large volume changes by acting as osmotic buffers (Fuller, 2004).

The use of CPAs can, however, be detrimental to cell viability as many commonly used CPAs have cytotoxic effects. For example, the use of CPAs in the cryopreservation of mature oocytes has been shown to result in damage to the internal structure of the cell. For example, the use of DMSO as a cryoprotectant when cryopreserving murine oocytes resulted in zona hardening and disorganisation of the cytoskeleton and plasma membrane (Trounson and Kirby, 1989). Furthermore, if CPAs are not used at the optimal concentration and/or applied/ removed at the correct rate their use can cause cell shrinkage prior to cryopreservation and the swelling of cells once they are thawed and returned to hypotonic solution possibly leading to cell lysis (Picton et al., 2000). A delicate balance must be struck between ensuring the CPA has fully penetrated the cell/ tissue and overexposure to cytotoxic effects (Meryman, 1971). Note that the degree of cytotoxicity varies between CPAs, for example glycerol is relatively

non-toxic, however its low penetration rate makes it less efficient than other more toxic CPAs with respect to cell survival following cryopreservation of larger cells and tissue.

Newton et al. (1998) found that adding the CPA at a lower temperature reduced the penetration rate and the level of cytotoxic damage. Reducing the rate of cooling also decreases the level of damage caused by CPAs however, there must be a compromise as too slow a rate increases the risk of intracellular freezing (Newton et al., 1999a). Although glycerol is a useful CPA when cryopreserving sperm, when used during the cryopreservation of ovarian tissue it produced a much lower survival rate (10%) than other CPAs; DMSO, propylene glycol and ethylene glycol (50-80%) (Newton et al., 1996). Therefore it is important to establish the optimal cryoprotectant for the type of cell/ tissue that is to be cryopreserved with respect to permeation rate, toxicity and ice crystal formation.

1.6.4 Cryopreservation techniques

Standard cryopreservation protocols utilise either slow freezing or vitrification techniques. 'Slow-cool' protocols are commonly used for the cryopreservation of embryos, oocytes, sperm and ovarian tissue (Karlsson and Toner, 1996, Newton et al., 1996, Royere et al., 1996, Edgar et al., 2005). The specifics of the freeze-thaw techniques, such as the type of CPAs used, warming and cooling rates and seeding temperatures vary with tissue type however the basic principles remain the same. It should be noted that every cell type has an optimal cooling rate that is determined by the cell's permeability and surface area to volume ratio (Schneider and Mazur, 1984). The cells/ tissue are first incubated in CPA for a sufficient period of time for equilibration to occur. Following this the samples are then cooled to a specific temperature at which manual seeding is carried out. Manual seeding is the process by which some form of instrument, such as forceps, is cooled in liquid nitrogen then applied to the straw containing the sample thus inducing ice formation. If the samples were left to become highly super-cooled ice formation would occur spontaneously which is more damaging to the cells and results in less predictable survival rates than manually inducing ice formation. This is because when ice forms the solution will briefly increase in temperature then cool again. The lower the temperature at which ice formation occurs the more rapid the subsequent cooling leading to rapid crystallisation thus increasing the risk of cell damage and IIF as cellular dehydration will not be able to occur at this rate (Isachenko et al., 2008). Research has shown that using a seeding temperature close to the melting point of the solution resulted in higher survival rates of the frozen cell samples (Trad et al., 1999). Once the crystallisation has spread throughout

the solution the cooling process can continue, at a slow rate to reduce IIF. Once a temperature of –40°C has been reached it is no longer necessary to cool the cells at this slow rate as the plasma membrane is no longer permeable to water. Thus the sample is rapidly cooled to a temperature of –196°C, at which it will be stored to prevent any chemical reactions due to the lack of thermal energy (Karlsson and Toner, 1996).

Unlike the freeze-thaw protocols, in which ice is allowed to form extracellularly, vitrification techniques avoid the formation of ice altogether. Vitrification is the process by which a liquid enters a glass-like solid state upon super-cooling (Rall and Fahy, 1985). The probability of vitrification can be roughly defined by the formula shown in Equation 1.1.

Equation 1.1

Probability of vitrification = (cooling rate x medium viscosity) / volume of media

Thus vitrification protocols require: a small sample volume; incubation with high CPA concentrations, which will raise the viscosity of the solution and therefore raise the glass transition temperature, thus lowering the probability of ice nucleation, and rapid cooling-achieved by plunging samples into liquid nitrogen or liquid nitrogen slush (Saragusty and Arav, 2011). The use of vitrification methods carry a number of advantages over the use of slow-freeze methods as there is no risk of ice formation or dehydration damage, the procedures are quicker, simpler and require less expensive equipment and the osmotic stress, solute effects and chilling sensitivity are all reduced in comparison. However, although using high concentrations of CPAs does allow exposure time to be reduced this means that the cells will be exposed to CPAs at more toxic levels. In both techniques rapid warming rates are used for reasons explained in Section 1.6.2. Although more research is needed to determine which method of cryopreservation is preferable there is increasing evidence that vitrification may be equally effective as slow-freezing for embryo cryopreservation (Loutradi et al., 2008, Wennerholm et al., 2009).

1.6.5 The use of cryopreservation for fertility preservation

Table 1.7 summarises the current techniques that are used to preserve fertility and highlights the advantages and disadvantages of each. The information in provided Table 1.7 shows that the use of ovarian tissue cryopreservation holds numerous advantages over the other methods

of fertility preservation. Earlier studies investigating cryopreservation of MII and GV oocytes utilised slow-freezing protocols, see Table 1.7. More recently vitrification is increasingly being investigated. Survival rates of cryopreserved human GV oocytes have been shown to be improved by the use of vitrification rather than slow-freeze protocols (Zhang et al., 2011). Investigations using ovine (Mo et al., 2014), human (Khalili et al., 2012), porcine (Rojas et al., 2004) and bovine (Men et al., 2002) oocytes have shown that MII oocytes are more tolerant to the vitrification procedure than GV oocytes, with respect to cleavage, blastocyst formation and survival rates. In addition the use of a cytoskeleton stabilisers such as Taxol, can also improve these rates (Mo et al., 2014).

Although cryopreservation of ovarian tissue was believed to be the only method suitable for fertility preservation in prepubertal girls there have been several investigations in which oocytes/ COCs from small antral follicles were retrieved from the ovaries of prepubertal girls and the oocytes matured *in vitro* (Revel et al., 2009, Fasano et al., 2011). While embryo and mature oocyte cryopreservation are viable means of preserving the fertility of young women, see Table 1.7 for details, the main focus of this review is on ovarian tissue cryopreservation.

1.6.6 Ovarian tissue cryopreservation

The use of ovarian tissue cryopreservation for fertility preservation in females of all age groups is preferable over the previously discussed methods for a number of reasons as detailed in Table 1.7. There are no major ethical issues associated with the collection and frozen-storage of ovarian tissues for either young girls or young women. The vast majority of follicles in ovarian tissue are at the primordial stage. Primordial follicles have a higher tolerance to both cryopreservation and autografting as a means of fertility restoration than follicles at later stages. This may be due to their small size, lack of zona pellucida and cortical granules, metabolic quiescence and undifferentiated state (Newton et al., 1996). In addition their low capillary density shows that they are more tolerant to hypoxic conditions than more developed follicles (Gosden et al., 2002). It has also been suggested that the meiotic stage at which primordial follicles are arrested (prophase) should result in fewer cytogenetic errors, and their prolonged growth phase allow more time to repair sublethal damage (Oktay et al., 1998, Kim et al., 2001).

Table 1.7 Utilisation of cryopreservation as a method of fertility preservation. This table considers the advantages and disadvantages of the various ways in which cryopreservation can be used to preserve fertility. Abbreviations: ovarian hyperstimulation syndrome (OHSS).

Cell type	Advantages	Disadvantages/ Problems	References
Embryo	• Most established and routinely used technique in assisted conception • High success rates (80-90%)	• Not suitable for prepubertal girls or adolescents • Women without life partners require the use of donor sperm • Ovulation induction can delay cancer treatment and cause OHSS • Risk associated with ovarian stimulation and breast cancer • Not allowed in certain countries • Ethical issues and risk of creating orphan embryos	(Testart et al., 1986, Gook and Edgar, 2007, Lahav-Baratz et al., 2003, Tiitinen et al., 1995)
MII oocytes	• Overcomes ethical issues associated with embryo cryopreservation by reducing the number of unused embryos • Suitable for use with egg donation programmes	• Low success rates with respect to survival, fertilisation and development can be attributed to the following factors; • Damage to oocyte cytoskeleton can result in disruption of syngamy and cytokinesis • Cryopreservation can induce parthenogenetic activation • Both the subzero temperatures and use of CPAs can irreversibly disrupt the MII spindle in humans; disrupts final meiotic division/ results in an anueuploid embryo • Disruption of the plasma membrane • Hardening of the ZP thus preventing sperm penetration (overcome by use of ICSI)	(Pickering and Johnson, 1987, Sathananthan et al., 1992, Gook et al., 1995, Kazem et al., 1995, Carroll et al., 1990, Paynter, 2000, Jones et al., 2004, Mullen et al., 2004, Koutlaki et al., 2006)
Germinal vesicle (GV) oocytes	• Less temperature-sensitive than MII oocytes as the microtubular spindle has not yet formed - chromosomes are packaged within the GV • GV oocyte collection would require patients to undergo less (or no) hormonal stimulation in comparison MII oocyte collection	• Although higher survival rates have been reported in more recent studies than earlier studies maturation and fertilization rates remain variable • Necessary to cryopreserve cumulus enclosed oocytes • Post-thaw GV oocytes must be matured *in vitro* - complex and made more difficult by the fact that cryopreservation results in CC loss • High incidence of chromosomal and spindle abnormalities	(Mandelbaum et al., 1988, Park et al., 1997, Son et al., 1996, Paynter, 2000, Koutlaki et al., 2006, Versieren et al., 2011)
Ovarian tissue	• Tissue collection by laparoscopy at any stage of menstrual cycle • No delay in cancer treatment • High primordial follicle survival rates • Fertility drugs not required • Ovarian tissue rich in primordial follicles • Suitable for prepubertal girls, adolescents	• Autotransplantation of tissue may carry the risk of reintroducing the cancer, in blood borne leukaemia and cancers with a high risk of ovarian metastasis, e.g. breast or ovarian cancer • Primordial follicle IVG and IVM techniques have not been optimised	(Newton et al., 1996, Oktay et al., 1997b, Oktay et al., 1998, Hovatta et al., 1996, Onions et al., 2008, Onions et al., 2009, Onions et al., 2013)

Early investigations have shown that primordial follicles are able to survive the freeze-thaw and autografting process. Histological comparisons of fresh and frozen-thawed tissue, and individual follicles before and after freeze-thaw have revealed relatively high survival rates (Newton et al., 1996, Hovatta et al., 1996, Gook et al., 1999). It should be noted that the survival rates of primordial follicles are highly dependent upon the CPA used in the cryopreservation process. Furthermore, studies in sheep and humans have shown primordial follicles within slices of cortical tissue are able to survive the grafting process (Newton et al., 1996, Gosden et al., 1994). More recently, the capacity of primordial follicles to survive the process of whole ovary cryopreservation (WOCP) and grafting has been demonstrated in ewes (Arav et al., 2010, Onions et al., 2008, Onions et al., 2009, Onions et al., 2013). Studies of the *in vitro* growth capacity of ovine secondary follicles post freeze-thaw have indicated that these follicles have the potential to develop to antral stages over 30 days *in vitro* (Newton et al 1999b). Secondary follicles recovered following WOCP were also observed to survive and exhibit signs of GC proliferation for 48 hours in culture following WOCP (Onions et al., 2008). The authors, however, draw attention to the fact that in studies in which the follicle population dynamics are monitored within the graft for an extended period of time growing preantral follicles show signs of irrevocable damage (Gosden et al., 1994, Baird et al., 1999). However, there is some evidence to suggest that although primordial follicles are morphologically normal following the freeze-thaw process there may be some alterations at the cellular level, with respect to metabolomics and developmental rate (Qu et al., 2000, Choi et al., 2007, Collado-Fernandez, 2013). Conventionally, slow-freeze methods are used to cryopreserve ovarian tissue, with penetrating CPAs: DMSO, ethylene gycol (EG) or propanediol (PROH), in combination with the non-penetrating CPA- sucrose (Hovatta, 2005). Human ovarian tissue cryopreservation protocols have been optimised via experimentation initially with *in vitro* culture of frozen-thawed tissue, then xenotransplantation in severe combined immunodeficiency (SCID) mice, and then heterotopic and orthotopic autotransplantation, to assess follicle, oocyte and stromal tissue health and survival rates (Picton et al., 2000). Optimisation of orthotopic autotransplantation techniques have resulted in numerous live births, which will be discussed in more detail below.

More recently research into the optimisation of vitrification methods for the cryopreservation of ovarian tissue has been conducted (Isachenko et al., 2009). Although relatively high follicle cell and oocyte survival rates have been reported, using slow-freeze protocols, the survival rate of the stromal cells may be improved using vitrification. Histological analysis of vitrified ovarian tissue using electron microscopy revealed improved preservation of ovarian tissue

morphology compared to tissue that had been frozen using slow-freeze protocols (Keros et al., 2009, Hreinsson et al., 2003). In a subsequent study the effects of the use of vitrification on follicle and oocyte ultrastructural morphology were assessed histologically using both light and electron microscopy revealing no differences when compared to non-vitrified control tissue (Sheikhi et al., 2011). Detailed investigation of the integrity and long-term developmental competence of vitrified ovarian tissue has yet to be rigorously assessed and proven using either xenografting or autotransplantation.

Another advantage of the use of ovarian tissue cryopreservation to restore fertility over other methods is the fact that tissue is relatively easy and safe to obtain. Tissue can be obtained laparascopically under anaesthetic; procedures that are of relatively low risk even in infants, over the age of 3 years old (Davidoff et al., 1996, Weintraub et al., 2007).

The main cause of follicle loss following freeze-thaw and autotransplantation is ischaemic damage during the grafting process, rather than the cryopreservation procedure itself (Liu et al., 2002). During the period of ischaemia the tissue is susceptible to damage by reactive oxygen species (Nugent et al., 1998). It should also be noted that the stromal tissue is more susceptible to cryo-injury than primordial follicles, although this can be reduced by the use of vitrification rather than slow-freeze protocols (Keros et al., 2009).

Xenografting, in which the recipient of the graft is of a different species to that of the donor, was first used to investigate the grafting of ovarian tissue. Xenografting, of both animal and human ovarian tissue into immunodeficient animal strains, such as SCID mice, has proved successful (Gosden et al., 1994, Candy et al., 1995, Van den Broecke et al., 2001, Gook et al., 2003, Kim et al., 2005). Although in the case of human tissue MII oocytes were able to develop but with microtubule and chromatin abnormalities. This technique cannot be used to restore fertility due to the risk of viral transfer across species. However, it has proved a useful experimental tool with respect to the use of different cryoprotectants, exogenous gonadotrophic stimulation and reducing ischaemic damage (Newton et al., 1996, Israely et al., 2006, Israely et al., 2004, Maltaris et al., 2007).

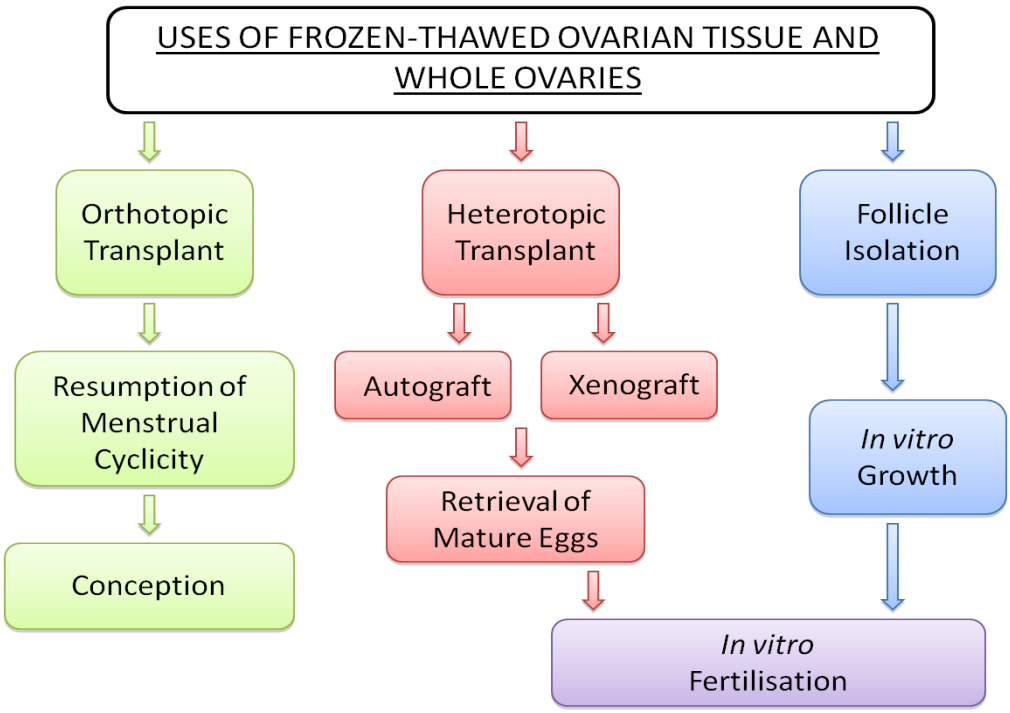


Figure 1.6 Diagrammatic summary of the uses of frozen-thawed ovarian tissue and whole ovaries. Adapted from (Oktay et al., 1998).

Figure 1.6 summarises the potential uses of ovarian tissue with respect to fertility preservation. There are 2 methods of autografting, in which the donor and recipient of the graft are the same individual - orthotopic and heterotopic grafting, which can be used either alone or in combination. Orthotopic transplantation is a procedure in which the ovarian tissue is transplanted to either the remaining ovary or the pelvic region of a patient. Whereas heterotopic transplantation is a procedure in which ovarian tissue is grafted to various other sites such as the abdominal wall or even subcutaneous regions, such as the forearm. In rodents it has been possible to orthotopically transplant frozen-thawed whole ovaries, however due to the larger size of the ovaries and lower follicular density this is more difficult in larger animals and humans, therefore small pieces of ovarian cortex are usually transplanted instead of the intact organ (von Wolff et al., 2009, Grynberg et al., 2012). Most of the cases reported have used slow-freezing cryopreservation techniques, however, more recently vitrification techniques have been utilised to preserve tissue. The success of ovarian tissue transplantation is not only dependent upon grafting site, freezing protocol and level of ischaemic damage, but also on the density and distribution of follicles within the frozen-thawed transplanted tissue, between patients, the age of the patient at the time of tissue cryopreservation and the size of the grafts transplanted (Newton, 1998).

Orthotopic autotransplantation has been more often favoured than heterotopic transplantation in the restoration of human fertility as it has the potential to enable natural conception to occur, although assisted reproduction is more commonly used as an adjunct therapy post grafting to restore fertility. Furthermore, the transplanted tissue will be exposed to optimal conditions in terms of locally produced factors and levels of mechanical stress and temperature. The first birth resulting from orthotopic transplantation of frozen-thawed ovarian tissue was reported by Donnez et al. (2004). Since then there have been numerous reports of births resulting from orthotopic transplantation of tissue (Meirow et al., 2005, Andersen et al., 2008, Revelli et al., 2013). The potential of this technique to restore fertility long-term was first demonstrated when a patient underwent orthoptopic transplantation in May 2006 and gave birth to two healthy children in June 2007 and November 2009 (Demeestere et al., 2007, Demeestere et al., 2010). In these cases patients have had sterilising treatment however it is still possible that the pregnancies could have resulted from surviving oocytes in the native ovary, rather than the graft. However, in these cases the patients have undergone ovarian failure and the timing of restoration of ovarian function following grafting corresponds with the time it takes for primordial follicles to initiate growth and reach maturation stage. Furthermore, a live birth has been reported following the transplantation of ovarian tissue to a patient who had undergone a bilateral oophorectomy, thus in this case the resumption of ovarian function had to be due to the grafted tissue (Donnez et al., 2012). However, Detti et al. (2012) argued that the many cases in which orthotopic transplantation is not successful go unreported, therefore the overall efficiency of the technology is unknown.

The nature of the heterotopic site used for autografting can affect oocyte quality, possibly due to suboptimal temperatures and increased exposure of the tissue to mechanical stress (Wolner-Hanssen et al., 2005, Yang et al., 2006). Autografting of fresh tissue to a subcutaneous site in primates resulted in the development of mature oocytes that were then aspirated and fertilised using ICSI, resulting in a live birth (Lee et al., 2004a). Furthermore, in humans, autografting frozen-thawed tissue to a heterotopic site resulted in the development of oocytes that were aspirated, matured *in vitro* and fertilised, resulting in development to the 4-cell embryo stage (Oktay et al., 2004). Recently a 26 week twin pregnancy has been reported following the transfer of 2 embryos resulting from the IVF of 2 oocytes derived from a heterotopic graft in the anterior abdominal wall of a patient who had undergone bilateral oophorectomy in response to ovarian stimulation (Stern et al., 2013). For the great majority of heterotopic transplants there is, however, evidence to suggest that grafting to a heterotopic site may affect oocyte development as shown by the fact that oocytes reach maturity at a

smaller size than when orthotopically grafted (Oktay et al., 1997a). Heterotopic transplantation has also been used to induce fertility in a young girl who received sterilising treatment for sickle-cell disease (Poirot et al., 2012). Heterotopic ovarian cortex transplantation may be useful in patients in which there is excessive scarring or radiation damage in the pelvic region.

Heterotopic and orthotopic transplantation are often used in combination and this has resulted in spontaneous pregnancies. The presence of a heterotopic graft may aid oocyte development in the orthotopic graft via the increased number of follicles transferred and the subsequent improvement in cycles (Demeestere et al., 2006). Similarly, heterotopic grafting in humans has also promoted oocyte development in a native, atrophic ovary following sterilising treatment, thus resulting in 4 spontaneous pregnancies (Oktay et al., 2011). There are a number of theories regarding the mechanism by which this occurred. Controversially, Oktay et al. (2011) proposed that the production of endocrine-paracrine factors by the grafted tissue promoted oogonial stem cells from either the bone marrow or native ovary to develop into functioning oocytes, or alternatively that the grafted tissue itself provided the oogonial stem cells. Although the theory that oogonial stem cells exist and are able to repopulate the ovary has been contested (Telfer et al., 2005, Byskov et al., 2011) the idea has been supported by more evidence from a study carried out by Lee et al. (2007) in which bone marrow-derived stem cells are taken up by native ovaries resulting in the restoration of fertility in mice. Indeed the presence of oocyte stem cells in the mouse and human ovary has been demonstrated via the use of green fluorescent protein (GFP) (Woods et al., 2013). Human oogonial stem cells engineered to express GFP were competent to develop into oocytes when injected into human cortical tissue and xenotransplanted into SCID mice (White et al., 2012).

Finally, ovarian tissue cryopreservation has proven successful in monozygotic twins discordant for POF. Transplantation of an ovary from the fertile twin to the twin with POF resulted in a number of live births or on-going pregnancies (Silber et al., 2008).

Concerns have been raised about the potential increased risks of cancer treatments to the offspring of cancer survivors. It was hypothesised that there would be an increased risk of offspring inheriting genetic abnormalities following the gonadotoxic treatment of their parents. However, the results of a large study conducted comparing the incidence of genetic abnormalities in offspring of cancer survivors and sibling controls suggested there was no increased risk (Boice et al., 2003). Another concern is that the offspring of cancer survivors will

be of increased risk of developing cancer themselves. However, a large epidemiological study failed to find a significant link, with the exception of diseases where there is a recognised familial link (Hawkins et al., 1989).

Autotransplantation of tissue back into a patient may in some circumstances carry the risk of re-establishing disease, therefore the technique would not be suitable for all patients (Shaw et al., 1996). This risk is particularly relevant to blood borne diseases such as leukaemia and also to cancers where there is a high risk of secondary metastasis in the ovary- such as breast cancer. In order to minimise the risk of reintroduction of malignant cells with the graft, techniques including histological and immunohistochemical analysis of the ovarian tissue have been developed to detect malignant cells prior to grafting (Meirow et al., 2008). In addition real time (RT) polymerase chain reaction (PCR) techniques were developed in the aforementioned study to enable testing for minimal residual disease. Grafting of isolated cryopreserved follicles is an alternative technique that may enable the restoration of fertility whilst avoiding re-introduction of malignant tissue. The successful freeze-thawing of isolated primordial follicles followed by aggregation into ovarian tissue and transplantation has resulted in the birth of live offspring in mice (Carroll and Gosden, 1993). The use of this technique in humans is currently under investigation, with isolated follicles being transferred to SCID mice (Dolmans et al., 2007, Dolmans et al., 2008). Finally, where there is a high risk of reintroducing malignant cells the only safe option for fertility restoration is to grow follicles to maturity *in vitro* to facilitate fertility restoration following IVF and embryo transfer (see Section 1.7).

1.6.7 Whole ovary cryopreservation

Due to the difficulties associated with whole ovary cryopreservation, in terms of optimizing cryoprotectant type and exposure time, ovarian tissue is usually stored as slices. Optimization of the type of CPA to use for whole ovary cryopreservation (WOCP) is challenging because it must be suitable for the stroma, follicular cells, oocyte and vasculature (Hovatta, 2005). However, when grafting, the slices of ovarian tissue are exposed to relatively long periods of ischaemia, as it takes 5-7 days in humans for the formation of new vasculature (Israely et al., 2004, Israely et al., 2006). This period of ischaemia is a greater factor in determining the number of surviving follicles than the cryopreservation or perfusion processes (Newton et al., 1996). In contrast when grafting whole ovaries the existing vasculature is used and microsurgical anastomosis carried out (Onions et al., 2009). There have been a number of

successful attempts at transferring whole fresh ovaries in sheep and humans, in terms of restoration of ovarian function (Jeremias et al., 2002, Courbiere et al., 2009, Mhatre et al., 2005, Imhof et al., 2006, Laufer et al., 2010, Onions et al., 2013). Furthermore, autotransplantation of frozen-thawed ovaries into ewes resulted in limited restoration of ovarian function and in one case a spontaneous live birth 545 days after transplantation (Bedaiwy et al., 2003, Imhof et al., 2006). However, these procedures have lead to a large loss of follicles, which can be attributed to the perfusion and cannulation techniques, as opposed to the cryopreservation techniques, as shown by the use of fresh ovary controls (Onions et al., 2009). The use of WOCP as a fertility restoration technique will be discussed in more detail in Chapter 6.

1.7 *In vitro* growth of oocytes as a method of fertility preservation

Cryopreservation of ovarian tissue and the subsequent *in vitro* growth and maturation of oocytes could potentially be used as a method of fertility preservation. This method would be suitable for both prepubertal girls, adolescents and young women (Picton et al., 2008). Development of culture systems able to support the complete *in vitro* growth (IVG) and *in vitro* maturation (IVM) of oocytes would also aid the understanding of the role of growth factors *in vivo* and the determination of acceptable candidates for this treatment, with respect to, for example, age (Smitz et al., 2010).

Eppig and O'Brien (1996) were the first to develop a culture system that was able to support the IVG and IVM of oocytes from the primordial stage in mice. Note that IVG refers to the development of the immature MI oocyte *in vitro*, whereas IVM refers to the process by which fully grown/competent oocytes undergo germinal vesicle breakdown (GVBD) and progression to the MII stage in response to GN stimulation *in vitro*. Fertilisation of the resultant mature oocytes resulted in the live birth of one pup, named Eggbert. Eggbert, however, exhibited a range of health problems, which were likely to result from epigenetic disruption induced by suboptimal culture conditions (Dennis, 2003). Furthermore, less than 2% of the 2-cell embryos so derived developed to the blastocyst stage and only 0.5% of embryos transferred resulted in a live birth. Subsequent alterations to the culture medium and protocol improvements resulted in improved success rates and a further 59 live births (O'Brien et al., 2003). Due to the larger size and longer growth period of oocytes in larger mammals and humans it has proved difficult to adapt these rodent culture systems to suit these species and work is still ongoing (Picton et al., 2008). Since the advances made by Epigg and O'Brien there has been a

lot of research into the optimisation of IVG and IVM systems. Details of investigations, which have provided the evidence and knowledge needed to continue this process of optimisation are shown in Table 1.8 and Table 1.9. It is important to note that the culture systems developed must be optimised for both fresh and frozen-thawed tissue, as the latter is more therapeutically-relevant for treatment of iatrogenic infertility (Smitz et al., 2010).

1.7.1 Optimisation of IVG culture systems

The optimisation of follicle culture systems is of high importance, not only to improve success rates but also to ensure the health of the resultant offspring. When developing a culture system the culture media composition, pH, temperature, oxygen tension as well as the method of follicle culture must be considered. There is conflicting evidence regarding the optimal oxygen tension for follicle culture. Some authors have advocated the use of low (5%) oxygen tension, presenting evidence that low oxygen tension improved growth and differentiation rates of sheep follicles *in vitro* (Cecconi et al., 1999). In contrast, studies performed by Jin et al. (2007) showed that low oxygen tension usage led to reduced rates of antral cavity formation compared to follicles grown in 20% oxygen. Similarly, the use of 5% oxygen in mouse preantral follicle culture prevented antral cavity formation (Smitz et al., 1996). Furthermore, culturing follicles in hypoxic environments increased the rates of chromosomal misalignment and precocious oocyte release and germinal vesicle breakdown in mice (Smitz et al., 1996, Hu et al., 2001).

Regulation of the temperatures to which ovarian follicles and oocytes are exposed, during transport to the laboratory, tissue processing, tissue culture and media changes, is important. Regulation of tissue temperature following collection and transport to the laboratory is often challenging. The temperature at which tissue is processed is often species-specific, with respect to body temperature (Telfer et al., 2008, McLaughlin and Telfer, 2010, Newton et al., 1999b). This applies to the temperature during follicle culture and media changes also. However, in some culture systems tissue is processed at lower temperatures (4°C). Processing the tissue at a lower temperature increases the efficiency of differential staining using the viability stain- neutral red dye (Chambers et al., 2010). Culture of ovine follicles following processing at 4°C did not have a detrimental effect on follicle growth and development. Furthermore, Lucci et al. (2004) showed that storing bovine preantral follicles at 4°C for 18 hours did not result in morphological damage, as assessed by histological analysis.

Table 1.8 Culture Systems for the *in vitro* growth of early preantral follicles. Details concerning the nature of the culture system, species and results are shown. Abbreviations; mouse (M), human (H), sheep (S), cow (C), non-human primate (P), primordial (prd), primary (1°), secondary (2°), preantral (PA), transitional (trans), insulin, transferrin, selenium (ITS+), proliferating cell nuclear antigen (PCNA).

Details of follicle culture system (species)	Follicle stage at start and end of culture	Culture length	Results/ extra information	References
Isolated follicle culture within alginate beads, serum-based (H)	Prd/EP->1°	7 days	Isolated using enzymes (Liberase and collagenase) -> large numbers of viable follicles Large numbers of follicles were able to survive and grow using this culture system	(Amorim et al., 2009, Vanacker et al., 2011)
Isolated follicles cultured on matrices composed of collagen, ECM or poly-L-lysine, serum-based (H)	Prd	24 hours	Follicles cultured within collagen gels grew over the 24 hour period	(Abir et al., 2001)
Cultured lectin-aggregated follicles, serum-free (S)	Prd	28 days	Follicles grew and exhibited a 50% survival rate	(Muruvi et al., 2005)
Cortical slices cultured on ECM coated inserts, serum-based (H)	Prd-2°-> late PA	≤21 days	Follicles cultured on inserts pre-coated with ECM showed increased rates of viability compared to uncoated inserts	(Hovatta et al., 1997)
Whole ovary, serum-based (M)	Prd->mature oocytes – fertilised ->birth of a live pup	8 days (organ culture), then 14 days (isolated COCs), IVM	One resultant live birth, Eggbert, however, <2% of 2-cell embryos developed to blastocyst stage and only 0.5% of embryos transferred resulted in a live birth. Eggbert exhibited health problems, including 'obesity and neurological abnormalities'.	(Eppig and O'Brien, 1996, Eppig and O'Brien, 1998)
Whole ovary, serum-free (M)	Prd->mature oocytes-> fertilised ->birth of a 59 live pups->adulthood	8 days (organ culture), further 6+8 days (change in media composition) (isolated COCs), IVM	Modifications to the culture medium used resulted in increased rates GVBD in oocytes, cleavage to 2-cell stage and live births	(O'Brien et al., 2003)
Cortical slice culture, serum-free (H)	Prd->transitional	6 days	Cortical pieces were cut into pyramid shapes and mechanically loosened. Follicles isolated after cortical culture were competent to develop up to the antral stage	(Telfer et al., 2008)

Table 1.8 (continued)

Details of follicle culture system (species)	Follicle stage at start and end of culture	Culture length	Extra information	References
Cortical slice culture, serum-free (P)	Prd->2°	≤20 days	Compared the use of ITS+ vs. serum. Cortical slices cultured in the absence of serum exhibited higher rates of primordial follicle activation	(Wandji et al., 1997)
Cortical slice culture, serum-free (C)	Prd->1°	≤7 days	Utilised PCNA staining to distinguish between quiescent and activated follicles	(Wandji et al., 1996b)
Cortical slice culture, serum-free (H)	Prd->2°	≤15 days	Compared the use of ITS+ vs. serum. Cortical slices cultured in the absence of serum exhibited significantly lower rates of atresia, grew significantly larger and were responsive to FSH	(Wright et al., 1999)
Cortical slice culture, serum-free (S)	Prd->PA	8 days	Investigated the use of NR dye as a viability stain	(Chambers et al., 2010)
Cortical slice culture, serum-free (S)	Prd->EA	18 days	Compared the use of a control, constant and sequential medium	(Peng et al., 2010)
Cortical slices cultured on ECM coated inserts, serum-free (H)	Prd->data not shown	≤14 days	Compared tissue preparation methods (slices vs. cubes), ECM density and ECM composition. Cubed tissue appeared optimal as the % of viable follicles was higher than in slices of tissue	(Scott et al., 2004a)
Cortical slice culture, serum-free (C)	Prd->Trans	6 days	Cortical pieces were cut into pyramid shapes and mechanically loosened. Following cortical culture and isolation follicles were competent to develop up to the antral stage	(McLaughlin and Telfer, 2010)

Table 1.9 Culture systems for the *in vitro* growth of preantral- antral stage follicles. Details of the nature of the culture system, species and results are shown. Abbreviations; mouse (M), human (H), ovine (S), bovine (C), non-human primate (P), primordial (prd), primary (1°), transitional (T) secondary (2°), preantral (PA).

System	Follicle stage at the start and end of culture	Culture length	Isolation method	Attachment- or non-attachment-based system	Results/ extra Information	References
Serum-based	1°/2°(85-145µm) (M)	16 days	Mechanical	Attached - lost 3D structure	Cultured in V-shaped wells in media covered by a layer of oil. The oocyte, GC and TC remained functional with respect to steroid hormone production	(Cortvrindt et al., 1996)
	PA->antral (S)	6 days	Mechanical	Non-attachment-based	Significant increases in size and antrum formation, enhanced by the addition of FSH and low oxygen tension	(Cecconi et al., 1999)
	PA (30-70µm) (C)	30 days	Mechanical (grating device)/ enzymatic (collagenase)	Attachment- based	Follicles co-cultured with bovine somatic cells. Follicles able to survive and grow when co-cultured with mesenchymal cells	(Itoh and Hoshi, 2000)
	1°-> Graafian, then fertilised (M)	5 days (IVG) 14hours IVM	Mechanical	Non-attachment-based	Fertilisation was only successful in IVG oocytes cultured in medium supplemented with FSH, transferrin and 5% serum alone. The addition of insulin, sodium pyruvate and glutamine slowed growth. Serum substitution for BSA-> ZP hardening	(Spears et al., 1994)
Serum-free	2°(150µm)-> antral (M)	6-7 days	Mechanical	Non-attachment-based (follicles cultured on Millicell inserts)	Group and individual culture of follicles was compared: individual culture resulted in improved growth rates. Formation of antral cavities and E2 production resulted when follicles were cultured with hFSH	(Nayudu and Osborn, 1992)
	Trans/2°(100-180µm)->antral (M)	8-12 days	Mechanically isolated	Non-attachment-based. Encapsulated follicles in sodium alginate matrix	Antral follicles were competent to resume meiotic maturation. The mechanical stiffness of the support matrix used needs to be optimized to allow differentiation of the follicles. Evidenced by the fact that the stiffness of the matrix altered the steroid hormone output of the follicles	(West et al., 2007)
	2° (COCs) 190-240µm-> antral (S)	30 days	Enzyme (collagenase and DNase)	Non-attachment-based	Follicles formed antral cavities - this was enhanced by GN supplementation of the medium	(Newton et al., 1999b)

Table 1.9 (continued)

System	Follicle stage at the start and end of culture	Culture length	Isolation method	Attachment- or non-attachment-based system	Extra information	References
Serum-free	2° (250-300µm) (P)	30 days	Mechanically isolated (using needles)	Non-attachment-based. Encapsulated follicles in alginate hydrogels	Supported the growth and steroidogenic function of follicles. Growth was enhanced by the addition of FSH alone, in comparison to FSH and LH	(Xu et al., 2009)
	2°(125-225µm) -> antral (P)	40 days	Enzymatically isolated (collagenase and DNase) then needles were used	Non-attachment-based. Encapsulated follicles in sodium alginate matrix	Antral follicles were competent to undergo oocyte meiotic maturation.	(Xu et al., 2011a)
	Trans (66-132µm) -> antral (H)	6 days (cortical) + 4 days (PA)	Mechanically isolated from cortical tissue following culture (using needles)	Non-attachment-based	Follicles cultured in the presence of activin A exhibited enhanced growth and survival rates. Furthermore the use of activin A resulted in antrum formation and normal morphology in 30% of the follicles	(Telfer et al., 2008)

Table 1.10 Commonly used additives in follicle culture systems. The mechanism by which the additives support and promote follicle and oocyte growth and development *in vitro* as well as the species in which these additives have been used are summarised. Abbreviations: human (H), ovine (O), mouse (M), bovine (B) progesterone (P4), basement membrane (BM), extracellular matrix (ECM).

Additive	Mechanism	Extra Information	Reference(s)
Insulin	Promotion of follicle activation and survival via the promotion of the uptake of glucose and amino acids by cells (H,O,B)	Commonly used in combination with transferrin and selenium. Supplementation of medium with TS+ alone did not result in follicle activation (B) but did provide a useful system to examine the effects of other growth factors, such as KL	(Czech, 1985, Louhio et al., 2000, Muruvi et al., 2009, Newton et al., 1999b)
IGF-1 and IGF-2	Enhance follicle survival and proliferation. Promote P4 secretion via promotion of FSHR expression (H,O)	When used in combination with insulin increased the number of growing follicles after 14 days culture. Effects are stage-specific	(Monniaux and Pisselet, 1992, Newton et al., 1999a, Louhio et al., 2000)
Transferrin	Facilitates transport of iron to cells in culture (H,O,R)		(Demeestere et al., 2005, Louhio et al., 2000, Newton et al., 1999b)
Sodium selenite	Protects against oxidative damage via its incorporation into seleno-enzymes (H,O,R)		(Hreinsson et al., 2002)
GDF9	Promotion of follicle viability, survival and development (H)		(Telfer et al., 2008, McLaughlin and Telfer, 2010, Thomas et al., 2003, McGee et al., 1997, Mizunuma et al., 1999, Smitz et al., 1998)
Activin	Promote follicle growth and development (H,O,B,R)	Effects are species and stage specific	
cGMP	Promotion of growth and development (H,R)	Anti-apoptotic, promotes AC formation in larger follicles	(McGee et al., 1997, Scott et al., 2004b)
cAMP	Promotion of follicle growth, development and survival (H,R)	Effects are species and stage specific	(Zhang et al., 2004, Hartshorne et al., 1994)
Ascorbic Acid	Promotion of follicle survival and enhancement of BM integrity (H,O,R)	Promotes BM formation by increasing production of Matrix Metalloproteinase-2 (MMP-2) and Tissue Inhibitor of Metalloproteinases-1 (TIMP-1), key enzymes for ECM remodelling	(Telfer et al., 2008, Peng et al., 2010, Murray et al., 2001)
Pyruvate	Energy source for the oocyte (H,O,R) via oxidative metabolism	Somatic cells preferentially use glucose (in basal medium) as an energy source	(Roberts et al., 2002, Harris et al., 2007, Harris et al., 2009)

1.7.2 Culture media composition

Basal culture media

Commonly used basal culture media include alpha-MEM (mouse: (Spears et al., 1994), ovine: (Newton et al., 1999b), human: (Abir et al., 1997, Hovatta et al., 1997, Scott et al., 2004a), Waymouth's medium (mouse: (Eppig and O'Brien, 1996, O'Brien et al., 2003), ovine: (Muruvi et al., 2009), human: (Wright et al., 1999) and McCoy's 5a medium (ovine: (Thomas et al., 2003, Telfer et al., 2008). A summary of additives commonly used in IVG systems is given in Table 1.10, some additives will be discussed in more detail later in the text.

Use of serum

In the early studies basal media was commonly supplemented with serum. Serum contains numerous components that aid follicle growth *in vitro* including albumin, precursors for steroid biosynthesis, 'proteins, amino acids, carbohydrates, hormones, growth factors and extracellular matrix components' (Picton et al., 2008). However, due to its complex composition it is difficult to assess the effects of different additives on follicle development. Furthermore, serum has been shown to have deleterious effects when used in both mouse and sheep embryo culture media leading to fetal overgrowth syndrome and alterations in behaviour, caused by aberrant imprinting (Young et al., 1998, Fernandez-Gonzalez et al., 2004). In addition, Wandji et al. (1997) suggested that serum contains components which are inhibitory to the activation of primate pre-granulosa cells. Culture systems in which serum has been replaced by insulin, transferrin and selenium (ITS) and a source of albumin (e.g. bovine or human serum aIbumin (BSA/HSA)) (ITS+) have been developed. In the aforementioned study although the use of serum resulted in a greater increase in follicle diameter after culture the rates of follicle activation and development to later stages were lower than when ITS+ was used as a substitute (Wandji et al., 1997). Furthermore, culture of primordial and primary human follicles in media supplemented with ITS and HSA were larger, more developed and showed fewer signs of atresia than those cultured in media supplemented with serum (Wright et al., 1999). In addition, culture of ovine secondary follicles in the presence of serum resulted in follicle collapse as opposed to those cultured in serum-free media in which follicles maintained their 3D structure (Newton et al., 1999b). Numerous studies have shown that follicles are able to survive and grow in serum-free culture systems, see Table 1.8 and Table 1.9. Early investigations conducted in mice revealed that use of serum-free media resulted in ZP hardening, a large decrease in both the percentage of inseminated ova that underwent

cleavage to the 2-cell stage and the percentage that progressed to the blastocyst stage. However, the addition of fetuin to the serum-free media reduced ZP hardening and improved cleavage rates (Eppig et al., 1992). Furthermore, supplementation of the media with ITS was necessary to maintain high levels of follicular integrity.

Activin A

Activin A is an additive of particular interest as the effect of activin A on follicle growth *in vitro* has been investigated in a number of mammalian species. Studies in rodents have provided evidence that activin A stimulates preantral follicle growth and differentiation, with respect to FSH-induced inhibin and E2 production, antrum formation and other aspects of maturation, *in vitro* (Smitz et al., 1998, Zhao et al., 2001, McGee et al., 2001). McGee et al. (2001) showed that the growth seen in the preantral follicles was due to an increase in cell number and that activin A did not appear to have any apoptosis-inducing effects. Whereas in the study conducted by (Smitz et al., 1998) activin A appeared to have a detrimental effect on follicle survival.

Activin A has also been shown to promote the growth and survival of caprine primordial follicles cultured within cortical tissue pieces, and isolated primary follicles (Silva et al., 2006). No effects were seen with respect to primordial follicle activation. Although the effects of activin A were blocked by the addition of follistatin in primary follicle culture the same effect was not seen with the primordial follicles. The authors suggest that this is likely to be due to the concentration of follistatin being too low resulting in incomplete blockage of activin A (Silva et al., 2006). More recently activin A has been shown to promote the growth and viability of feline preantral follicles *in vitro* (Wongbandue et al., 2013). In this study the effects of thyroxin were also investigated and found not to be beneficial.

Telfer et al. (2008) have developed an *in vitro* culture system in which human primordial follicles were activated within cortical tissue then isolated and cultured in the presence of activin A. The inclusion of activin A in the culture media promoted growth and antrum formation in preantral follicles. This group conducted a similar study using bovine ovarian tissue in which activin A and FSH inclusion increased oocyte and follicle growth, antrum formation and initially E2 secretion, although this final effect was not sustained after the ninth day of culture (McLaughlin and Telfer, 2010). However, in neither the bovine nor the human

study was follistatin used to determine whether these effects were reversed, which would have provided more convincing evidence that the aforementioned effects were due to the presence of activin A. Note also that in both of these studies neither activin A nor FSH was added during the initial cortical culture period, rather they were added following follicle isolation.

The effect of activin A with respect to the *in vitro* culture of ovine ovarian follicles has been studied (Thomas et al., 2003). Culture of isolated preantral follicles in the presence of activin A promoted follicle growth and increased oocyte diameter. As in the bovine investigation activin A initially increased E2 secretion; however this effect was not sustained after the second day of culture. No effect was seen on antrum formation or follicle survival. Thus activin A may promote follicle growth but not differentiation of ovine preantral follicles *in vitro*.

Gonadotrophins – FSH and LH

As previously mentioned gonadotrophic stimulation of follicle development is not essential until the EA stage, however, gonadotrophins play a permissive role at earlier follicle stages, as illustrated by numerous *in vitro* studies. FSH has been shown to promote the growth and differentiation of rat preantral follicles (McGee et al., 1997). In the sheep FSH promotes antral cavity formation in ovine secondary follicles *in vitro* (Newton et al., 1999b) and has been shown to promote GC proliferation in small (2-3.5mm) and large follicles *in vitro* (Campbell et al., 1996), FSH stimulates the growth of large ovine antral follicles *in vivo* also (Picton et al., 2000). In the aforementioned study in which *in vitro*-derived transitional bovine follicles were cultured to the EA stage, FSH was shown to promote follicle growth in the presence of activin A (McLaughlin and Telfer, 2010). Furthermore, survival rates of secondary, non-human primate follicles *in vitro* were increased when high or medium concentrations of FSH were used in the presence of fetuin and 5% oxygen (Xu et al., 2011a). In this study FSH levels were also shown to affect steroidogenesis, as high levels ($15ngml^{-1}$) increased the production of androstenedione (A4), E2 and P4. The authors suggested that this may be due to the premature differentiation of GCs.

More research is required to determine the optimal stage at which FSH should be added to the IVG system and also to define the optimal concentration to be used. These effects may be species-specific as well as being dependent upon other additives in the culture system. This is

evidenced by the fact that FSH has, in some studies, been shown to be detrimental to the IVG of follicles, at specific stages of development. For example, although the development of mouse secondary follicles is FSH-dependent, high levels of FSH, of greater than 25mIUml^{-1} led to a reduction in follicle survival rates, in serum-free culture media (Kreeger et al., 2005). Furthermore, the addition of either 10- or 100-mIUml^{-1} FSH to serum-based media, during the *in vitro* development of non-human primate follicles from the preantral to EA stage has been shown to have detrimental effects on the oocyte—GC interactions and the meiotic spindle structure (Xu et al., 2011b).

Evidence is emerging that a sequential culture system in which the concentrations of specific GNs and GFs are adjusted at specific points in the culture period may be optimal. Peng et al. (2010) investigated the effect of FSH on the *in vitro* development of ovine preantral follicles. In this study the use of a control, constant and sequential media was compared. In the sequential media increasing amounts of FSH and epidermal growth factor (EGF) and decreasing amounts of GDF-9 and bFGF were used. Use of the sequential media resulted in the progression of primordial follicles to the EA stage. Antral follicles were also found in the control and constant media but these were abnormal. Furthermore, the percentage of secondary follicles and the growth of primary and secondary follicles increased. Celestino et al. (2011) conducted a study which also supports the use of a sequential culture media. In this study caprine secondary follicles were cultured in the presence of increasing concentrations of FSH, this promoted follicle survival, development and subsequent rates of meiotic resumption.

The question of whether LH should be added in combination with FSH or in a sequential manner is one that remains to be answered. In the aforementioned study conducted by (Newton et al., 1999b) the addition of 5ngml^{-1} LH to the culture media, containing 0.1ngml^{-1} FSH, did not affect the rate of antrum formation in ovine secondary follicles. However, LH did affect the steroidogenic activity of non-human primate preantral follicles *in vitro* in that A4 and P4 production increased in response to its addition to the culture media (Xu et al., 2011a).

1.7.3 Follicle isolation method – mechanical vs. enzymatic

Follicle isolation can be achieved via enzymatic or mechanical means. Isolation of follicles by mechanical means requires the use of needles, scissors, forceps or mesh filters. Viable follicles competent to survive culture have been obtained from mouse, ovine and human ovaries using

these methods (Spears et al., 1994, Amorim et al., 2000, Abir et al., 1997). However, isolation of follicles using mechanical methods alone, particularly in larger mammals and humans in which ovarian stroma is tougher and more collagenous than that of rodents, can result in lower yields when compared to enzymatic methods (Telfer, 1996). The age of the animal from which the ovary is obtained can also affect how many follicles it is possible to isolate as follicle density reduces with advancing age and stromal tissue becomes increasingly tough. Enzyme isolation is therefore often used in combination with mechanical methods resulting in greater follicle yields however, the use of enzymes results in the loss of the basement membrane and theca cells, which could be avoided by using solely mechanical methods (Nayudu and Osborn, 1992, Telfer, 1996, Newton et al., 1999b).

Nicosia et al. (1975) were the first to show that structurally and functionally normal follicles could be enzymatically isolated from the ovaries of rabbits, using collagenase. Collagenase has been used in numerous studies and enabled the isolation of follicles competent to grow and develop in culture from both ovine (Newton et al., 1999b, Nandi et al., 2009) and human (Abir et al., 2001, Amorim et al., 2009) ovaries. However, it should be noted that in the latter mentioned study there was an increase in the number of lipid droplets in the granulosa cells relative to non-isolated controls, indicating that the health of the follicles may have been compromised (Abir et al., 2001).

Liberase, an enzyme originally shown to improve the quality of human pancreatic islet isolation with respect to their anatomical integrity and viability compared to collagenase, has also been used in follicle isolation protocols from human ovarian tissue (Linetsky et al., 1997). Dolmans et al. (2006) presented evidence that favoured the use of Liberase over collagenase. They showed that although the follicle yield following ovarian tissue digestion using Liberase was lower than when collagenase was used, the proportion of viable and morphologically normal follicles was higher. The authors attributed this to the fact that collagenase contains higher levels of endotoxins than Liberase. However, in a more recent study by the same group conflicting evidence is presented (Vanacker et al., 2011). In this study there was no significant difference in the percentage of viable follicles, isolated using either collagenase or Liberase, before or after 7 days of isolated follicle culture. As Liberase does not digest the tissue as effectively as collagenase and collagenase may cause more morphological damage a mixture of both enzymes may be preferable (Kristensen et al., 2011). It should be noted that the preparations of collagenase varied between studies. In conclusion, the type and concentration

of the enzyme(s) used and the length of exposure time must be optimised to ensure sufficient follicles can be isolated without irreversibly damaging the structural integrity of the follicles.

1.7.4 Isolated vs. *in situ* culture

Hovatta et al. (1999) showed that the isolation of primordial, primary and transitional follicles conferred no advantage over *in situ* culture (within slices of cortical tissue) of follicles at these developmental stages. However, at later developmental stages follicle growth is inhibited, possibly by the surrounding ovarian tissue and therefore follicle isolation is required (Hovatta et al., 1997). Indeed, numerous researchers advocate the use of a multi-step system in which the first phase of culture is carried out *in situ* (Eppig and O'Brien, 1996, Picton et al., 2000, Telfer et al., 2008, Picton et al., 2008). Culturing the primordial follicles *in situ* provides an environment that more closely resembles the ovary *in vivo*, in terms of the locally produced factors, and maintenance of oocyte-granulosa cell interactions which are important for their continued growth and maturation (Picton et al., 2000).

There are various methods by which primordial follicles can be cultured *in situ.* These include: chorioallantoic membrane grafts, whole ovary and cortical slice culture. It is possible to maintain the viability of baboon and cow primordial follicles using the chorioallantoic membrane of chick embryos, but there was no development past the primordial stage (Fortune et al., 2000). However, if the pieces were then transferred to serum-free media the follicles were activated and developed to the primary stage (Cushman et al., 2002). The use of this culture system led to the elucidation of the inhibitory role of AMH on follicle activation (Gigli et al., 2005). Therefore the *in ovo* method may be useful for studying regulators of primordial activation.

The most commonly used method of *in situ* culture is the use of whole ovary or ovarian cortical slice culture. Whole ovary culture is suitable for rodents (see Table 1.8 for examples), it is not suitable for larger mammals and humans as the ovaries are larger and therefore nutrients and metabolites cannot permeate the tissue sufficiently *in vitro*. This can be overcome via the use of thin cortical slices whilst still providing sufficient support to maintain the native architecture of the follicle (Gosden, 1998). Therefore in these species cortical slices are utilised, allowing the architecture of the follicles to be maintained during the culture period. Cortical slices have been utilised for the IVG of primordial follicles in various species

some of which are detailed in Table 1.8. Semi-permeable membrane inserts are utilised in some systems (e.g. Chambers et al. (2010)) to ensure that the cortical slices are covered in a sufficient volume of medium with respect to provision of growth factors and hormones without the diffusion distance for oxygen being too great (Devine et al., 2002). In the systems developed by Telfer's laboratory cortical tissue was mechanically loosened prior to culture to reduce the amount of medulla present, as the authors hypothesise this will reduce the concentration of medulla-derived inhibitory factors. As previously mentioned once follicles reach the transitional/ secondary stages of development further growth is inhibited, thus follicle isolation is required. Although it should be noted that Peng et al. (2010) reported that follicles were able to develop up to the EA stage in ovine cortical tissue culture. In order to increase the efficiency of IVG systems the tissue selected for culture should be relatively follicle-rich. Chambers et al. (2010) developed the use of neutral red dye as a method of identifying viable follicles prior to culture and of guesstimating viable follicle density without compromising the subsequent developmental competence of the follicles. Follicles derived *in vitro* using the system developed by Telfer's laboratory were competent to develop to the EA stage following isolation and individual culture (Telfer et al., 2008, McLaughlin and Telfer, 2010).

Use of extracellular matrices (ECMs) and supporting hydrogels

Although there may be no direct advantage to isolating small preantral, growing follicles rather than culturing them *in situ*, isolated culture enables further investigation of the optimisation of IVG systems as the system is more easily manipulated. The use of isolated culture of follicles at all stages is a useful tool for the investigation of growth factors and mechanisms involved in *in vivo* growth and development (Devine et al., 2002). Therefore research into the optimisation of IVG culture systems that are able to support the growth and development of isolated follicles at both the earlier and later preantral follicle stages is important. Isolation of follicles results in the loss of the physical support provided by the ovarian stroma. Therefore in order to overcome this; researchers have developed the use of ECMs and gels in IVG culture systems. Gels must provide sufficient rigidity to promote the maintenance of oocyte- GC interactions, but without being too rigid and inhibiting follicle growth (Smitz et al., 2010). The gel must also be permeable to the culture media and factors produced by the follicle. The gel must be supportive and adhesive to follicles, but not too adhesive or this may cause somatic cells to migrate away from the oocyte. The use of gels may be particularly relevant to the culture of follicles from larger mammals and humans as the time frame of follicle growth and

development is much longer than in rodents and therefore it is more difficult to prevent follicle attachment and resultant loss of integrity during these extended culture periods. Smitz et al. (2010) also suggests that pressures exerted on the follicle by the ECM *in vivo* as it grows may affect the release of paracrine factors via alterations to actin organisation.

Collagen and lectin have been used as ECM components (see Table 1.8). More recently, researchers have used alginates as a gel composite. Alginate hydrogels have been shown to maintain the ultrastructure of follicles *in vitro* with respect to the general morphology and the oocyte- GC interactions (Pangas et al., 2003). Xu et al. (2009) showed that alginate gels could be utilised for culture of non-human primate, secondary follicles allowing continued follicle growth and development and supporting steroidogenesis for up to 30 days. The development of this system has enabled further elucidation of the requirements of follicles in IVG systems, with respect to, for example, the effects of GNs and GFs and oxygen tension (Xu et al., 2010, Xu et al., 2011a). Furthermore, this system has been used to compare cryopreservation methods, revealing that vitrification methods may be a useful alternative to slow-freeze methods with respect to subsequent follicle survival rates in culture, as well as steroid hormone production and antral cavity formation (Ting et al., 2011).

The development of a hydrogel that is modulated, with respect to its composition and therefore its rigidity, by the follicles it encapsulates has been reported (Shikanov et al., 2009, Shikanov et al., 2011). In this system a fibrin – alginate -interpenetrating network (FA-IPN) is used. As the follicles grow the fibrin component of the hydrogel is degraded by plasmins produced by the follicle, thus allowing the follicle to increase in size. The alginate component, however, is biologically inert and therefore is retained and continues to support the follicle. The level of fibrin degradation can be regulated by the addition of plasmin inhibitor, aprotinin, to the culture media (Longstaff, 1994). The authors suggested that this would provide the follicles with a more physiologically relevant environment as it would mimic the reduction in rigidity experienced as developing follicles migrated from the tougher cortex to the less tough medullary interior of the ovary. The use of the FA-IPN hydrogel resulted in a significantly greater percentage of meiotically competent oocytes developing from secondary follicles in mice, than the use of a gel composed of solely alginate. It should be noted that gels have not only been used for isolated follicle culture but can also be utilised for cortical slice culture. Kedem et al. (2011) reported that the use of an alginate rather than Matrigel ECM for the culture of human primordial follicles within cortical slices and that this resulted in higher

activation rates. However, a recent study in non-human primates reported that the use of an alginate-fibrin gel was only optimal for follicles at the primary stage and conferred no advantage to the culture of secondary follicles in relation to 'growth, steroidogenesis, AMH/vascular endothelial growth factor (VEGF) production and oocyte maturation' (Xu et al., 2013). Conversely, a recent investigation has provided evidence that the use of alginate as an ECM may adversely affect the developmental competence of *in vitro*-derived oocytes as an increased incidence of spindle defects, chromosome misalignment and cortical granule biogenesis errors were observed when an alginate gel system was used to culture mice follicles (Mainigi et al., 2011). Therefore, the development of alternative ECMs may be necessary. Although it should be noted that Xu et al. (2011b) reported a normal meiotic spindle structure of baboon MII oocytes derived *in vitro*, as long as FSH was not present in the medium. Hyaluranon-based gels have been proposed as an alternative to alginate (Desai et al., 2012). Hyaluronan is a naturally occurring ECM component and is modifiable with respect to its rigidity. Although the use of hyaluronan alone and in combination with Matrigel has been shown to support the IVG of mouse follicles, the rate of progression to MII stage was less than that observed in control cultures. The authors suggest that a more dynamic environment, such as the FA-IPN described above, may be needed (Desai et al., 2012).

The aforementioned studies relate to the culture of transitional/ secondary- staged preantral follicles. More recently a culture system that supports the survival and growth of baboon primordial/ primary follicles *in vitro* has been reported (Hornick et al., 2012). In this system enzymatically isolated follicles are encapsulated in 0.5-2% alginate gels, with 2% alginate optimal with respect to the follicle survival and maintenance of follicle morphology. Use of this system for the culture of mouse primary follicles revealed the positive effects of the co-culture of follicles with respect to the growth and survival of follicles resulting in the formation of meiotically competent oocytes (Hornick et al., 2013). The authors suggest this is due to exposure of the follicles to paracrine factors secreted by the other follicles in co-culture.

At the later preantral stages it is possible to prevent attachment to the culture surface and maintain the 3D structure of the follicle without the use of gels (mouse; West et al. (2007), sheep; Newton et al. (1999b) and Picton (2002); human; Telfer et al. (2008). Initial work was carried out using rodents, however, as the GCs of ruminant and humans remain morphologically and steroidogenically similar to cells *in vivo* as opposed to forming fibroblastic phenotype, maintaining the 3D structure *in vitro* has proved easier in these species (Picton et

al., 2008). Examples of 3D, non-attachment-based systems are shown in Table 1.9 with details of the extra steps required to prevent attachment detailed if necessary.

An alternative approach to culturing isolated preantral follicles is the use of either 2D, attachment-based systems (see Table 1.9; mouse; Cortvrindt et al. (1996), bovine; Itoh and Hoshi (2000). Although there has been some success using 2D culture systems, the use of 3D systems confers many advantages. As previously mentioned the maintenance of oocyte-GC interactions is of great importance to the continued growth and development of both the oocyte and somatic components of the follicle. When a 2D culture system is used the follicle is allowed to attach to the culture surface thus losing its native architecture and potentially disrupting these interactions. Whereas, when 3D culture systems are used oocyte-GC interactions are more likely to be maintained. This is of particular importance in the IVG of larger mammals and humans as these follicles require a much longer period to attain maturity *in vitro*.

Culture timescales – accelerated vs. protracted

As detailed earlier in this chapter the process of oocyte and follicle development *in vivo* is long and protracted. Whether it is necessary for *in vitro* systems to follow a physiologically-relevant timescale or if a much more accelerated system would be suitable is a point of discussion between research groups. The growth and development of both bovine and human follicles from the primordial to the EA stage has been achieved using a two-step system after culture periods of 12 and 10 days, respectively (Telfer et al., 2008, McLaughlin and Telfer, 2010). Concerns have been raised regarding the health of oocytes resulting from systems in which follicle growth is so greatly accelerated (Picton et al., 1998). Although some researchers argue that the use of physiologically-relevant culture periods is key to producing healthy *in vitro*-derived oocytes, as this would allow sufficient time for the oocytes to develop the molecular machinery required for normal development (Picton et al., 1998). Others claim that extended culture periods are not necessary and may in fact be detrimental to oocyte health due to increased exposure to suboptimal conditions *in vitro*. Furthermore, accelerated culture systems would partly overcome the difficulties associated with maintaining oocyte-GC interactions long-term (McLaughlin and Telfer, 2010). In order to determine whether accelerated or protracted culture systems are optimal more research into the effect of each on the genetic health of the follicle, by comparing *in vivo*- and *in vitro*-derived counterparts, is required.

Significant progress has been made with regards to the optimisation of IVG systems, particularly with respect to developments in ruminants and non-human primates. A multi-step strategy in which primordial follicles are first cultured *in situ*, then isolated and cultured individually, with increasing concentrations of GNs present in the culture media, prior to IVM may support the *in vitro*-derivation of MII oocytes. However, much more research is needed to determine whether this would be best achieved using an accelerated or protracted culture system, using gels to maintain 3D structure, using a sequential or constant culture media, using enzymatic or mechanical isolation methods. In addition to the optimum concentrations and time points at which GFs and GNs should be added to the culture media.

In order to determine which culture methods are optimal there need to be clear methods of assessing *in vitro*-derived oocyte quality, not only with respect to follicle and oocyte morphology, survival and growth rates, progression through the various developmental stages, but also with respect to the genetic and epigenetic health (Picton et al., 2008).

1.8 Assessment of follicle and oocyte health and viability *in vitro* and *in vivo*

In order to ensure follicle and oocyte health both compartments must pass through the same check-points in terms of protein production and growth. Furthermore, conditions *in vitro* must mimic those *in vivo*. Various cellular markers can be used to aid assessment, a number of techniques are summarised in Table 1.11 however a more extensive knowledge of cellular markers and their expression patterns is required, in order to develop and validate IVG culture systems. Non-invasive methods of monitoring development may be used, such as monitoring the levels of secreted factors in spent media when the follicles are cultured individually (Telfer et al., 2000). For example, the levels matrix metalloproteinase MMP-9 and tissue inhibitors of metalloproteinases (TIMPs), important for basement membrane reconstruction, can be used as markers of follicle development (McCaffery et al., 2000). The levels of steroids – E2 and P4, can be monitored in spent media also, using Enzyme-linked immunosorbent assays. The concentration of E2 can be used to determine whether the aromatase enzyme complex has been induced in cultured cells (Newton et al., 1999b).

During ART procedures women are induced to superovulate and the resultant oocytes are collected. Although the majority of these are at the mature MII stage some immature oocytes are also retrieved (Jones et al., 2008). These immature oocytes can be matured *in vitro*.

However, the developmental competence of these oocytes is compromised following IVM as evidenced by the lower rates of zygotes developing to the blastocyst stage once fertilised compared to oocytes matured *in vivo*; 12% vs. ~50%, respectively (Chen et al., 2000, Jones, 2000). Furthermore, the percentage of embryos developing to full term is also lower in IVM oocytes than *in vivo* matured oocytes in humans; 14% vs. 35%, respectively (Blake et al., 2005, Jones et al., 2008). The compromised developmental competence of IVM oocytes has been linked to a number of factors including alterations in oocyte morphology, cytoplasmic and nuclear maturation and spindle positioning (de Loos et al., 1992, Combelles et al., 2002, Moon et al., 2005).

Table 1.11 Summary of various cellular markers which can be used to determine whether follicles and oocytes are developing normally and are healthy. The techniques by which these markers are assessed are also shown. The information was collated from data published in a number of different sources including (Newton et al., 1999b, Picton et al., 2000, Telfer et al., 2000, Picton et al., 2008).

Markers	Techniques used for assessment
Cell morphology	Oocyte diameter
	Zona pellucida thickness
Changes in cell number	Follicle diameter
Steroid production	Measure E2 and P4 levels in spent media
Basement membrane formation	Azan staining of collagen component of basement membrane
Somatic cell differentiation Oocyte development	Patterns of expression of cellular markers, eg. IGFs, activin, inhibin, GDF-9
DNA analysis	Fluorescence assays
	Incorporation of thymidine
Oocyte viability	Formation of antral cavity
	Diameter
	Staining with vital dye (e.g. neutral red dye)
Nuclear maturation	Production of maturation promoting factor
	Dissolution of nuclei
	Chromosomal condensation
	Formation of functional spindle apparatus

In numerous studies changes in gene expression have been linked to reductions in developmental competence of IVM oocytes. For example, in a study comparing *in vitro* and *in vivo* matured human oocytes many genes involved in 'transcription, the cell cycle and its regulation, transport and cellular protein metabolism' were expressed at a 2-fold higher level

in the IVM group (Jones et al., 2008). Furthermore, in a different study the expression patterns of numerous genes involved in nuclear maturation, homeostasis and cytoplasmic functions observed in IVM oocytes were more similar to those in immature GV oocytes than *in vivo* matured MII oocytes (Wells and Patrizio, 2008). Alterations in oocyte gene expression patterns resulting from *in vitro* maturation have been observed in the mouse and bovine species as well (Tesfaye et al., 2009, Kind et al., 2013). In addition, differences have been observed between human cumulus cells from oocytes matured *in vitro* and those matured *in vivo* with respect to expression of genes related to oocyte maturation, cumulus expansion and cell cycle regulation (Ouandaogo et al., 2012, Guzman et al., 2013). In the rhesus monkey IVM has resulted in differing expression patterns of genes involved in cumulus cell interactions between themselves and with the oocyte (Lee et al., 2008, Lee et al., 2011). Kyasari et al. (2012) demonstrated how altering IVM conditions can also alter gene expression patterns, illustrating the importance of optimizing IVM techniques. Interestingly, the use of slow-freeze or vitrification to cryopreserve human MII oocytes also affects gene expression patterns relative to those observed in fresh MII oocytes (Monzo et al., 2012).

The use of IVM has been shown to alter gene expression patterns in numerous studies using both animal and human oocytes. As IVG of oocytes is a developing technique there are fewer studies investigating the impact of IVG on gene expression and most of these have utilized murine follicle culture systems. However, these studies have revealed differences in expression patterns of genes between *in vivo* and *in vitro*-derived oocytes. In one such study oocytes were grown *in vitro* within secondary stage follicles up to the antral stages and the expression patterns of *Bmp*-15, *Mater*, *Zar-1* and *Fgf-8* were measured and compared to expression levels in *in vivo*-derived oocytes . This revealed significantly lower expression levels of these genes after 10 days of culture (Sanchez et al., 2009). In more recent studies the concentration of FSH and insulin in the IVG medium was shown to affect the expression of oocyte genes including- *Gdf9, BMP15* and *Fgf8*, as well as cumulus cell-specific genes including- luteinizing hormone/choriogonadotropin receptor (*Lhcgr*), *Amh*, androgen receptor (*Ar*), phosphofructokinase platelet (*Pfkp*) genes (Sanchez et al., 2010, Sanchez et al., 2011). Furthermore, in a study in which secondary follicles were cultured up to the antral stages using alginate gels as a support matrix the concentration of the gel was shown to affect the expression patterns of *Gdf9, Bmp15,* NOD-like receptor family pyrin domain containing 5, T cell leukemia/lymphoma 1, and *Zp3* (Jiao and Woodruff, 2013).

1.9 Epigenetics

Epigenetics is the study of the process by which gene expression patterns are regulated by modifications to the DNA as opposed to the underlying sequence (Qiu, 2006). Epigenetic information can be conferred in the form of DNA methylation, in which a methyl group is added to a cytosine residue allowing subsequent recognition and binding by regulatory factors (Reik et al., 2001). This form of epigenetic modification represses gene activity. Histone modifications, in the form of the attachment of proteins to the histone tails, are another form of epigenetic regulation, which can result in either the repression or activation of gene expression. A third mechanism of epigenetic regulation is the attachment of non-histone proteins to chromatin. Epigenetic modifications regulate the expression of imprinted genes (Qiu, 2006). Imprinted genes are expressed monoallelicallly. Monoallelic expression patterns are determined by the parental origin of the allele. This mode of regulation is key to embryonic growth and development, placental and postnatal growth, as well as the prevention of parthenogenesis. Imprinted genes are needed for the regulation of nutrient supply to the embryo. Maternally and paternally expressed genes have opposing effects with respect to nutrient supply to the embryo. Paternally expressed imprinted genes tend to promote mechanisms that increase nutrient supply to the embryo, whereas maternally expressed imprinted genes reduce nutrient supply. This helps maintain a balance in which the embryo is provided with sufficient nutrients without being detrimental to the mother.

Genomic imprints are erased at the stage of development that the PGCs are at when they reach the genital ridge. Imprints are then re-established in an asynchronous manner as the gametes develop. Imprint acquisition differs between sperm and oocytes, with respect to timing and is completed by day E18.5 in murine sperm and just prior to ovulation in oocytes, see Figure 1.7. Once genomic imprints have been established they are maintained, even following fertilisation when genome-wide de-methylation and re-establishment occurs (Morgan et al., 2005). DNA methyltransferases (DNMTs) are key enzymes with respect to the establishment of imprinting marks as well as the maintenance of these (Reik et al., 2001). The enzyme DNMT3a and a DNMT3-related protein, DNMT3L, are essential for the establishment of the maternal imprint (Hata et al., 2002). The enzyme DNMTb is also involved, however, may play more of a permissive role (Kaneda et al., 2004). Evidence suggests that DNMT3L is a key regulator of DNMT3a and DNMT3b activity via its co-localisation and establishment of the maternal imprint is not possible without this protein (Hata et al., 2002). Once established the maternal imprints must be maintained. DNMT1o is essential for the maintenance of the maternal imprint in oocytes and preimplantation embryos. DNMT1o homologues have been

identified in the mouse, sheep and human (Hayward et al., 2003, Taylor et al., 2009). As previously mentioned epigenetic reprogramming occurs at 2 points in development: during gametogenesis and following fertilisation. In the mouse, once fertilisation has occurred the paternal genome is actively demethylated, whereas demethylation of the maternal genome is passive. Once completed, *de novo* methylation of the embryonic genome occurs (Reik et al., 2001).

Due to the timing of epigenetic reprogramming, concerns have been raised that ARTs may result in epigenetic disruption. There is evidence to suggest that the use of ARTs may increase the risk of epigenetic errors. Furthermore increasing evidence of connections between imprinting disorders and the use of ARTs is emerging. The incidence of Beckwith Weidemann syndrome (BWS), an epigenetic disorder with symptoms such as childhood tumours, fetal and postnatal overgrowth, macroglossia and organomegaly (Amor and Halliday, 2008) has been linked the use of ARTs. Over 60 cases of BWS had been reported in ART-conceived children by 2008 (Amor and Halliday, 2008). In addition the incidence of ART in children with BWS is significantly higher (2.9%) than the general population (0.8%), which has been associated with both the use of ICSI and IVF (Maher et al., 2003b, Sutcliffe et al., 2006). In a study of 25 ART-conceived children with BWS 24 had hypomethylation of the differentially methylated region, KvDMR1 on chromosome 11p15 (Lim et al., 2009). Similarly the incidence of Angelman syndrome (AS) in children conceived using ARTs is higher than in the general population (Amor and Halliday, 2008). In addition the percentage of cases of AS resulting from an imprinting defect is very low (~3%) in the general population, however by 2003, 6 of the cases of AS reported following the use of ovarian hyperstimulation alone (1 case) or in combination with ICSI (5 cases) resulted from imprinting defects, which suggested that the use of these ARTs caused the defects (Cox et al., 2002, Orstavik et al., 2003). It has been suggested that subfertility may also be a cause of the increased incidence of AS resulting from epigenetic disorders (Ludwig et al., 2005, Sutcliffe et al., 2006).

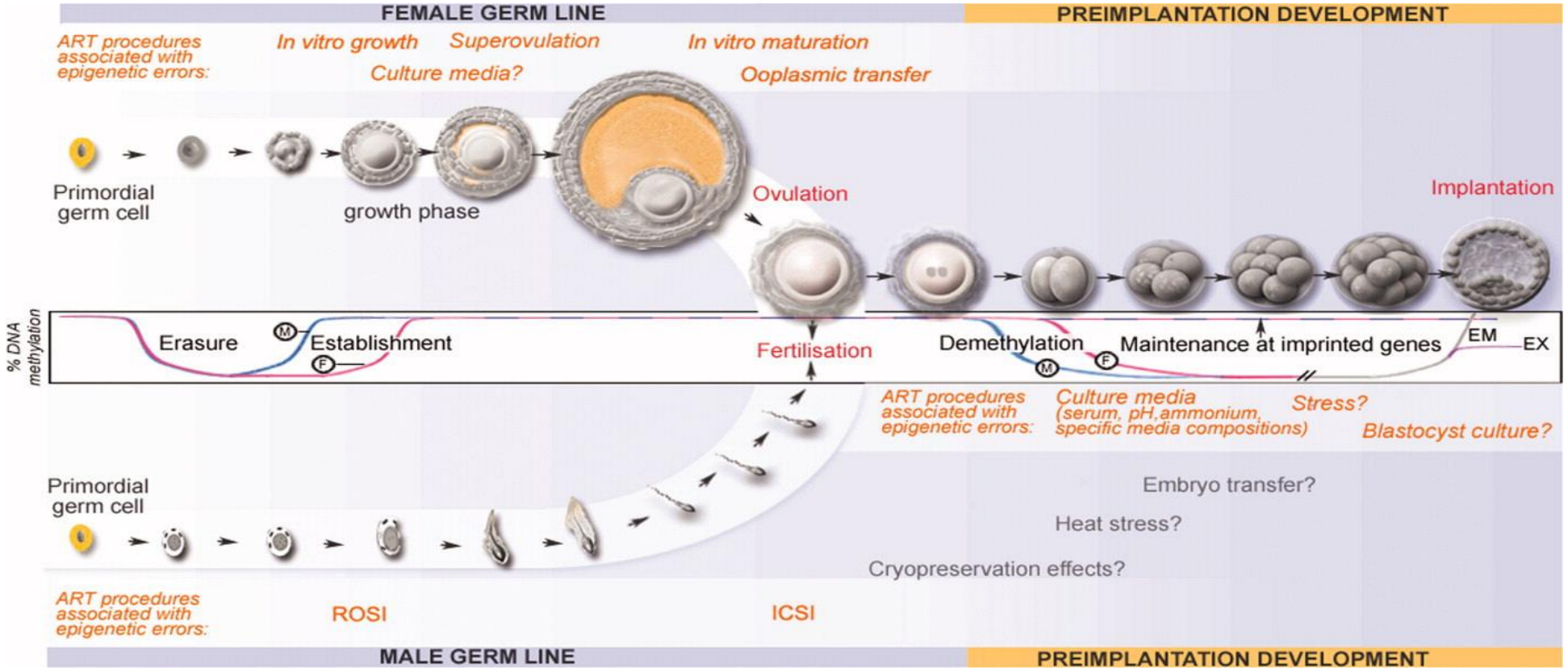


Figure 1.7 The methylation status of developing oocytes, spermatozoa and pre-implantation embryos. The maternal imprint is mostly erased in primordial germ cells and then gradually re-established throughout oocyte growth; throughout the primary to antral stages, as opposed to at one specific time point. The ARTs that have been implicated in the disruption of epigenetic reprogramming are highlighted (Huntriss and Picton, 2008).

Silver-Russell syndrome (SRS) is another epigenetic disorder that has been associated with the use of ARTs as SRS resulting from the hypomethylation of *H19/IGF2* gene has been observed in ICSI/IVF-conceived children (Chopra et al., 2010). In an isolated case the use of IVF was associated with the incidence of SRS resulting from the hypermethylation of the *PEG/MEST* gene, although the pattern was also observed to a lesser extent in the Father, therefore imprinting errors may have been related to genetic errors, possibly involved in the couple's subfertility (Kagami et al., 2007). Symptoms of SRS include low pre- and postnatal growth rates, delayed development and learning disabilities. A newly recognised fetal overgrowth syndrome that causes seizures and developmental delay has also been linked to the use of ICSI/IVF (Shah et al., 2006). More recently, Retinoblastoma- a form of eye cancer that usually affects children under the age of 5 years old, has been linked to the use of ARTs due to the fact that it is more common in IVF patients and that the retinoblastoma gene is imprinted (Kanber et al., 2009, Marees et al., 2010). Although the incidence of imprinting disorders following the use of ARTs is relatively low Maher et al. (2003a) suggests that many more epigenetic errors have not been identified and that these errors predispose children to certain conditions, such as cancer, later in life. In Table 1.12 the findings of investigations in which the mechanisms by which epigenetic errors, resulting from the use of ARTs, may have occurred are summarized. The results of these investigations reveal numerous inconsistencies that are probably due to inconsistencies in the media composition and techniques used. Therefore, it is difficult to directly compare each study and to ascertain the exact cause of the effects on epigenetics/imprinted genes. Note that the use of IVG culture systems is not included in Table 1.12 as this is considered in more detail below.

To further complicate matters both infertility and subfertility can increase the risk of conceiving a child with an imprinting defect. In the case of subfertile couples increasing time to pregnancy increased the relative risk. The risk is further increased in subfertile couples that conceive using ART (Ludwig et al., 2005). As briefly mentioned in Table 1.12 further evidence of a link between infertility and increased risk of imprinting errors is provided by a study in which the methylation patterns of normozoospermic and oligozoospermic patients were compared. The methylation pattern of maternally expressed imprinted gene *H19* was altered in the oligozoospermic group, changes ranging from moderate (17%) to severe (30%). Whereas the methylation pattern of *MEST*, a paternally expressed imprinted gene, was not affected (Marques et al., 2004). Similarly imprinting errors were observed in tetrazoospermic men, in the form of demethylation in either *IGF2* alone or *IGF2* and *H19* (Boissonnas et al., 2010). In addition hypomethylation of *IGF2/H19* gene was observed in men with low sperm

counts. Unlike the aforementioned study aberrant methylation of the *MEST* gene was also observed. Hypermethylation of *MEST* was not only observed in men with low sperm counts, but also men with bad sperm morphology and poor sperm motility (Poplinski et al., 2010).

A number of studies have been conducted which compared the epigenetic status of ART-derived children and spontaneously conceived children with slightly conflicting results. Studies have shown changes in methylation patterns of both imprinted and non-imprinted genes, resulting in changes in expression of certain imprinted genes (Palermo et al., 2008, Katari et al., 2009). However, in a more recent study methylation patterns at DMRs of imprinted genes were examined and no differences were found between children conceived spontaneously or via the use of IVF or ICSI, other than in the DMR of *MEST* (Tierling et al., 2010).

One of the overarching aims of this thesis is to define the culture conditions required to derive normal, healthy, developmentally competent oocytes *in vitro*. In order to achieve this goal, the parameters by which normality is to be assessed need to be established. One such parameter by which normality should be assessed is the establishment of epigenetic modifications equivalent to that observed *in vivo*. In more recent years the effect of culture conditions on the methylation patterns of important imprinted genes has been investigated. Studies in mice have shown that the methylation patterns of small nuclear ribonucleoprotein-associated protein N (*Snrpn*), *Igf2* or *H19* in MII oocytes grown *in vitro* from the secondary stage were not affected under normal culture conditions (Anckaert et al., 2009a). Furthermore, when follicles were subjected to suboptimal culture conditions such as the addition of high levels of FSH ($100iUL^{-1}$, compared to $10iUL^{-1}$) to the culture media, or exposure to increased levels of ammonium (up to ~1500µm following 12 days culture) and significantly reduced levels of oestrogen and progesterone, induced by culturing under mineral oil, this did not affect the methylation of the aforementioned genes (Anckaert et al., 2009a, Anckaert et al., 2009b, Trapphoff et al., 2010). Furthermore, embryos resulting from the fertilisation of *in vitro*-derived oocytes also exhibited normal methylation of the aforementioned genes (El Hajj et al., 2011). Similarly, ovine oocytes grown from the preantral to EA stages *in vitro* displayed normal methylation patterns of *IGF2R*, Brain-enriched guanylate kinase-associated protein (*BEGAIN*) and *H19* (Barboni et al., 2011). Nor were the methylation patterns of *IGF2R* or *H19* affected following the IVM of ovine oocytes (Colosimo et al., 2009). Conversely, although culturing in the presence of reduced levels of methyl donors did not affect the methylation levels of *Snrpn, Igf2* or *H19* in murine MII oocytes grown *in vitro* from the secondary stage, the

methylation levels of *Mest* decreased (Anckaert et al., 2010). Therefore, the overall methylation patterns observed in *in vitro*-derived oocytes were similar to that observed in *in vivo*-derived oocytes in animal models, suggesting that the mechanisms by which DNA methylation patterns are established are quite robust. Whereas studies conducted in humans in which oocyte are matured *in vitro* have resulted in aberrant methylation patterns of *H19* (Borghol et al., 2006) suggesting that these mechanisms may be less robust in humans.

A limited number of studies have been conducted regarding the epigenetic status of IVG oocytes that are derived from early preantral stages via the use of cortical slice culture. In a number of studies differences in the methylation statuses of *in vivo* and *in vitro*-derived follicles were observed (Lees-Murdock et al., 2008, Song et al., 2009). However, in these studies the oocytes were not competent to develop to the MII stage. Whereas in a study in which IVG oocytes were competent to develop to the antral stage the methylation status of all imprinted genes investigated was normal (Obata et al., 2002). As mentioned previously, Eggbert exhibited many health problems, which may have been due to aberrant epigenetic modifications (Eppig and O'Brien, 1998). However, as Eggbert was the only mouse resulting from that study the exact causes of his poor health were unclear. In a subsequent study in which alterations to the culture media composition, which promoted the normal acquisition of IVG oocyte developmental competence, were made 59 live offspring were born and survived to adulthood (Eppig et al., 2000, O'Brien et al., 2003).

A vast amount of research has been conducted to elucidate gene expression patterns during oogenesis and folliculogenesis *in vivo*, as covered in Section 1.5. However, minimal research has been conducted to determine whether these gene expression patterns are the same in *in vitro*-derived oocytes. Although some investigations have examined gene expression patterns in *in vitro*-matured oocytes, very little research has been conducted concerning IVG oocytes. Furthermore the majority of the research has been conducted using mice. It is essential to ensure that *in vitro*-derived oocytes are competent to be fertilized and develop into normal offspring. Therefore one of the main focuses of this thesis was the determination of expression patterns in *in vivo*-derived ovine oocytes and their stage-specific *in vitro*-derived counterparts.

Table 1.12 Table summarising the evidence linking the use of ARTs to the incidence of imprinting errors. Details of the species, the ART conditions implicated and the nature of the effect on imprinting are shown. Abbreviations; mouse (M), sheep (S), human (H), fetal calf serum (FCS), growth factor receptor-bound protein 10 (Grb10).

ART	Specific ART conditions (species)	Nature of effect on imprinting	Extra information	References
Embryo Culture	Culture media used was suboptimal (M)	Use of Whittens Medium resulted in loss of methylation leading to aberrant expression of the *H19* gene	Methylation status of *Snrpn* gene was unaffected therefore unlikely that the alterations to *H19* were via aberrant DNMTo activity	Doherty et al. (2000)
	In vitro culture (M)	Aberrant expression of *H19* gene in the extraembryonic tissue		Sasaki et al. (1995)
		Aberrant *Igf2* expression at the blastocyst stage	Biallelic expression rather than monoallelic	Ohno et al. (2001)
		Aberrant *H19* gene expression	Aberrant gene expression resulting from abnormal DNA and histone methylation patterns	Li et al. (2005)
	Exposure to high levels of ammonium in culture medium (M)	Aberrant *H19* gene expression	Ammonium level also affected 'blastocyst differentiation, metabolism, pH regulation [and] fetal development'	Lane and Gardner (2003)
	Addition of FCS to culture media of preimplantation embryos (M)	Gain of methylation of *Igf2* and *H19* led to decreased expression. Whereas *Grb10* expression decreased and *Mest* was unaffected	Lower birth weights probably resulted from aberrant gene expression	Khosla et al. (2001)
	Addition of FCS to culture media of blastocyts (M)	Increased expression of *Igf2*, *Grb10* and *Mest*. *H19* expression not affected	Effect of FCS on gene expression is not consistent with findings by Khosla et al. (2001), however, both studies report aberrant expression	Fernandez-Gonzalez et al. (2004)
	In vitro culture of pre-implantation embryos (S)	Aberrant methylation and expression of *IGF2* receptor gene	Linked to fetal overgrowth syndrome. This gene is not imprinted in humans	Young et al. (2001)
Ooplasmic transfer	Transfer of ooplasm from donor oocyte may compromise recipient oocyte (M)	Suggests there may be a risk with respect to the establishment of normal epigenetics due to the timing of ooplasmic transfer		Hawes et al. (2002)

Table 1.12 (continued)

ART	Specific ART conditions (species)	Nature of effect on imprinting	Extra information	References
Microinjection of immature Spermatids.	Use of immature gametes (H)	Aberrant DNA methylation patterns, due to immature gametes not having acquired epigenetic marks or the machinery required for their maintenance	Long-term health of the resultant zygotes and offspring may be affected	Kishigami et al. (2006)
	Round spermatid injection (M)	No effect on expression patterns of imprinted genes	Inconsistent findings compared to humans	Shamanski et al. (1999)
Superovulation	(H,M)	Disrupted methylation patterns of *H19* and *MEST* genes. Effects on *H19* and *SNRPN* methylation patterns seen trans-generationally	Disrupted imprinting patterns and methylation in superovulated eggs. Both imprinting establishment and factors required for maintenance are affected	Sato et al. (2007) and Stouder et al. (2009)
	(M)	Altered expression of imprinted genes in the placenta	Resulted in biallelic expression of *H19* and *Snrpn* genes	Fortier et al. (2008)
ICSI	Introduction of sperm acrosome and digestive enzymes into ooplasm may disrupt oocyte activation (H)	Link between use of ICSI and incidence of AS	There may be a link between imprinting disruption and infertility as sperm from oligozoospermic males exhibited aberrant methylation of *H19* gene	Cox et al. (2002) and Marques et al. (2004)
	Oocytes were matured *in vitro* prior to fertilisation	Aberrant imprinting of *H19* and *KCNQ1OT1*	Expression patterns of DNMTo unaltered. Unclear whether aberrant imprinting observed following IVM is maintained following ICSI	Borghol et al. (2006) and Khoueiry et al. (2013)
Embryo Transfer	(M)	Aberrant expression of imprinted genes in both the embryonic and placental tissues	Disruption to gene expression patterns was augmented by *in vitro* culture prior to embryo transfer	Rivera et al. (2008)
Embryo Vitrification	Embryo vitrification following *in vitro* culture (M)	Vitrification resulted in lower levels of *H19* expression	Aberrant gene expression patterns results from disrupted methylation patterns of the differentially methylated domains of both *H19* and *Igf2*	Wang et al. (2010)

1.10 Aims and objectives

The information presented in this literature review summarises the highly complex nature of oocyte and follicle development *in vivo*, it shows how this information has been used to underpin the development and advancement of systems to preserve female fertility and it highlights the complexities associated with trying to develop a culture system that supports these processes *in vitro*. The importance of establishing biologically relevant parameters for assessing the normality of follicle and oocyte development during IVG and maturation has also been highlighted. Development of techniques that facilitate the cryopreservation of ovarian tissue either as slices of cortex or in the form of whole ovaries and the subsequent *in vitro* growth and development of oocytes that are competent to undergo maturation and fertilisation *in vitro* to produce healthy offspring would be of great value to young girls, adolescents and women facing POF. Before these techniques can be used therapeutically in humans it is of vital importance that markers for follicle and oocyte normality are established following IVG and that these are tested for throughout oocyte and follicle development *in vitro* in order to ensure the health of the resultant offspring.

On the basis of the evidence presented in this review it can be hypothesised that the IVG of oocytes is suitable as a tool to both assess the patency of fertility preservation systems and as a means to restore female fertility.

The main aims of this thesis were:

i. To develop and validate a culture system that was able to support the *in vitro* activation, growth and normal development of ovine primordial follicles to the early antral stages within a physiologically-relevant timescale.
ii. To measure the impact of a slow physiologically-relevant follicle growth system on markers of follicle and oocyte survival, yield, health and normality, as well as stromal health, to compare these to similar parameters measured using an accelerated follicle culture system.
iii. To establish the normal expression patterns of important oocyte- and follicular somatic genes across oogenesis and folliculogenesis *in vivo and to* compare these to gene expression patterns in stage-matched oocytes and follicles grown *in vitro.*
iv. To utilise the ovarian cortex culture system at a tool to validate WOCP as a fertility preservation strategy, using sheep as a model for humans.

2 General Materials and Methods

Unless otherwise stated all reagents detailed in this thesis were purchased from Sigma-Aldrich Company Limited, (Sigma-Aldrich, Dorset, UK), all culture plastics were from Nunc® (Thermo Fisher Scientific, Hertfordshire, UK) and all pipette tips from Starlab Ltd (Milton Keynes, UK). All company addresses are provided in Appendix V.

2.1 Use of ovine ovarian tissue

The investigations regarding the validation and optimisation of culture techniques were performed using ovine ovarian tissue provided by a local abattoir (J Penny and Sons, Rawdon, Leeds, UK). The ovine model was selected because it is more similar to human with respect to the size of the ovaries and oocytes, the developmental time frame *in* vivo, the fibrous nature of the tissue and the body temperature, than rodent model species (Campbell et al., 1996, Griffin et al., 2006, Lundy et al., 1999). Furthermore, ovine tissue was relatively easy to obtain on a weekly basis. As both human and sheep are predominantly monovular the mechanisms regulating follicle and oocyte growth and maturation *in vivo* will be paralleled by that *in vitro.* The sheep has previously been used as a model system for the development of IVG systems (Muruvi et al., 2009, Newton et al., 1999b, Thomas et al., 2003).

2.2 Collection and preparation of ovarian tissue

Reproductive tracts were collected from a local abattoir and transferred to the lab at room temperature within one hour. Ovaries were dissected from the tracts using scissors, removing all excess tissue and fat and transferred to 200ml pre-warmed (37°C) Ovary Wash Media (OWM), which was comprised of: 500ml Milli-Q water, mixed with 1 phosphate buffered saline (PBS) tablet (Thermo Fisher Scientific) per 500ml solution, 100Uml^{-1} penicillin G, 0.1mgml^{-1} streptomycin sulphate, 0.25µgml^{-1} amphotericin B (antibiotic/ antimycotic). The OWM was made up in a sterile glass beaker. The ovaries were transferred to fresh 100ml OWM 2 – 3 times to ensure that all traces of blood and cellular debris were removed. The ovaries were then transferred to 50ml cold (4°C) Rozenburgh Knife Wash Medium (RKWM), which was comprised of: Hepes-modified minimum essential medium (MEM), supplemented with 100Uml^{-1} penicillin G/ 0.1mgml^{-1} streptomycin sulphate, 1mgml^{-1} bovine serum albumin (BSA

Fraction V; cell culture tested (Sigma A9418)), and stored on ice in a sterile 60ml universal (Scientific Laboratory Supplies (SLS), Nottingham UK).

2.3 Preparation of cortical slices

Under class I laminar flow conditions, using a skin-grafting knife (Ref 9942, Swann-Morton Ltd, Sheffield, UK), thin slices were carefully cut from the outer cortex of the ovary at a thickness of around 0.4-0.5mm (see Section 2.4). It is important to minimise the thickness of the tissue to allow sufficient gas and nutrients to diffuse through the tissue whilst maximising the number of follicles for culture (Avgoustiniatos and Colton, 1997). Furthermore, in early experiments it was found that if the tissue was too thin it was more likely to become necrotic, as perhaps the procedure had been too damaging. Dissections were carried out in RKWM at 4°C, in a sterile glass petri dish placed on a cold ice pack. Cortical slices were then transferred to a sterile universal (SLS) containing 5ml RKWM and left at 4°C until a sufficient number of slices had been obtained for culture. Cortical slices were briefly washed again in RKWM. Using a sterile scalpel blade and a clean glass petri dish the slices were carefully trimmed, under a stereo microscope (Olympus Industrial, Essex, UK), at room temperature. Tissue pieces were processed using a scalpel (handle size 7 with a size 15 blade) and 26G½ needles (Terumo Europe N.V., Belgium). The tissue pieces were trimmed to roughly $5mm^2$.

2.4 Validation of cortical thickness

In order to determine the exact thickness 5 samples of single tissue pieces were fixed (see Section 2.9) after they had been trimmed. The tissue pieces were sectioned to a thickness of 4µm and the total number of sections per piece was recorded. The tissue pieces covered around a mean number of 116.6 sections ± 5.3 (n=5, range: 98-130), therefore the mean tissue thickness was calculated as 0.47mm ± 0.02 (n=5, range: 0.39-0.52).

2.5 Quantification of viable follicle density – using neutral red (NR) dye

The use of neutral red (NR) dye enabled the identification of viable follicles in cortical tissue slices prior to culture, thus reducing the chance of culturing follicle depleted cortex and/or non viable cortex. Neutral red dye is water-soluble and non-toxic and it is able to pass through lipid membranes via non-ionic diffusion (Nemes et al., 1979). The dye is taken up by lysosomes, this process is more rapid in follicular cells and oocytes than stromal cells, due to

the higher metabolic rate of the former cell types (Babich and Borenfreund, 1987, Chambers et al., 2010). Viable tissue is stained red; this can be visualised using light microscopy. It is essential to perform dissections and initial NR staining reactions at 4°C as this reduces uptake of the dye by the stromal tissue, ensuring that the follicles are stained more strongly and are therefore identifiable (Chambers et al., 2010).

Table 2.1 Composition of cortical culture media. The volumes required to make 30ml solution are shown, with the stock and final concentrations. The solution was filtered and stored for up to 1 week at 4°C. See Appendix I for details of the preparation of α-MEM with bicarbonate basal culture media, with 1% (v/v) penicillin/streptomycin and 1mg ml^{-1} BSA fraction V (fatty acid free) and additive stock solutions.

Component	Volume (µl)	Stock Concentration	Final Concentration
α-MEM with bicarbonate basal culture medium	29.448 ml		
Bovine holo-transferrin	30	5mgml^{-1}	5µgml^{-1}
Sodium pyruvate	30	47mM	0.047mM
Sodium selenite	3	50µgml^{-1}	5ngml^{-1}
L-glutamine	450	200mM	3mM
Bovine insulin	30	10mgml^{-1}	10ngml^{-1}
Human long-R3 IGF-1	3	100µgml^{-1}	10ngml^{-1}
Ovine FSH	3	0.1iUml^{-1}	1 x 10^{-5}iUml^{-1}
Ovine LH	3	0.0023iUml^{-1}	2.3 x 10^{-7}iUml^{-1}

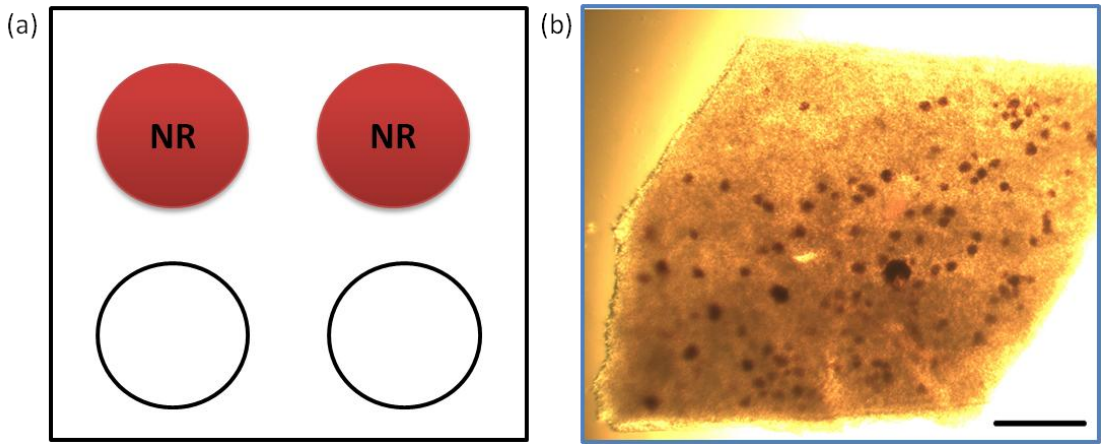


Figure 2.1 (a) Follicle assessment plate set-up.

Stock concentration NR dye (Sigma N2889) = 3300µgml^{-1}

Final concentration 50µgml^{-1}

Top 2 wells = 492.5µl culture media supplemented with 7.5µl NR dye

Bottom 2 wells = 500µl culture media

(b) Representative image of NR stained ovarian cortex. Scale bar = 1mm.

The composition of cortical culture media, used to prepare the NR assessment plates is detailed in Table 2.1. Two Nunc®, 4-well follicle assessment plates were set up before the ovaries arrived in the lab and placed into the incubator at 37°C and 5% CO_2 to pre-warm and gas for 3 hours prior to use as shown in Figure 2.1 (a). A representative image of tissue following NR staining is shown in Figure 2.1 (b).

2.6 Assessment of viable follicle density

The methods of assessment of viable follicle density within cortical slices have been previously described (Chambers et al., 2010). Once the cortical slices had been prepared for culture they were transferred to the follicle assessment plates (~25 slices per well). The plates were then returned to the humidified incubator (Galaxy B) at 37°C and 5% CO_2 in air and left for 2.5 - 3 hours. The NR staining of follicles *in situ*, within the fragments of ovarian cortex, was assessed using a stereo microscope (Olympus SZX16) fitted with a heated stage (Olympus Tokai hit) using 64 x magnification under sterile conditions, at 37°C. Bright- field microscopy was used to visualise the red colouration. At the end of the NR incubation period the plates were removed one at a time from the incubator. The cortical tissue slices were transferred from the wells containing NR to those containing culture media only to wash away any crystals of dye on the tissue surface as these interfered with the assessment of follicle density. Each tissue piece was assessed for the presence of red colouration and non-staining tissue pieces discarded.

The number of red structures (follicles) present within each of the positively staining tissue pieces was counted and recorded. The pieces were turned over with sterile forceps to ensure that all follicles had been counted. Follicles did not always stain completely; therefore only if over 50% of the follicle population had stained was the tissue used for future culture or analysis. Once the red colouration of all tissue pieces had been assessed they were divided into groups of 5 containing approximately equal numbers of follicles (which were recorded). The dry weight of each group of 5 tissue pieces was recorded. Excess liquid was removed from each tissue piece by blotting on sterile gauze (Richardson Healthcare, Borehamwood, UK) prior to placing on the lid of a sterile flat-cap microcentrifuge tube (Starlabs). A microbalance (Sartorius Stedim UK, Surrey, UK) was used to measure the weight of the empty tube which was subtracted from the combined weight of the universal and tissue pieces to obtain the dry tissue weight. Representative images were taken of spare tissue pieces following NR staining prior to culture using an inverted microscope (Olympus IX70) fitted with a camera (Olympus DP11) and heated stage (Linkam Scientific Instruments Ltd, Tadworth, UK). Any spare tissue

pieces were discarded. Representative images were also taken at the end of culture (see Section 2.8).

Up to 5 extra tissue pieces were fixed in 1ml of 4% (w/v) paraformaldehyde (PFA) (see Appendix II) overnight then transferred to 70% (v/v) ethanol at room temperature (~20°C). These pieces were used as time 0 controls to allow histological comparison with tissues fixed at the end of culture (see Section 2.9). Tissue pieces were stored in a plastic container, containing 70% (v/v) ethanol for several months prior to embedding, the ethanol was topped up at regular intervals.

2.7 Culture of cortical slices

Culture plates were set up prior to the ovaries arriving in the lab and transferred to the incubator to equilibrate for a minimum of 2.5 hours. Nunc® 24- well culture plates were used. Sterile, un-coated Millicell^{TM} culture plate inserts (0.4µm pore size, 12mm diameter, Millipore, Watford, UK) were carefully placed into the wells using forceps. To the well 500µl of culture media was then added, the composition of which is shown in Table 2.1. A maximum of 8 wells per plate were utilised for tissue culture to minimise the length of time required to perform a media change and thus avoid pH and temperature changes. Only the central wells were utilised to reduce the potential for culture media loss by evaporation, during extended culture. To further prevent culture media loss the surrounding channels were flooded with sterile embryo tested water supplemented with Penicillin G and Streptomycin sulphate at the same concentration as the OWM (see Section 2.2). Tissue slices (5 per well) were placed on the insert, and then a sterile gel-loading tip (Alpha Laboratories Ltd, Hampshire, UK; catalogue number LW1100) was used to adjust the level of the media to ensure the pieces were covered by a thin film. Note that this procedure was carried out on a stereo microscope fitted with heated stage set at 37°C. The plates were then returned to the humidified incubator and cultured at 37°C and 5% CO_2 in air until the first media change was due.

Media changes were carried out on a heated stage at 37°C; 50% of the media in each of the wells was removed and replenished during each change. Media changes were carried out every Monday, Wednesday and Friday until the completion of culture. This media change strategy prevented unnecessary disturbance of the cultured tissue pieces and ensured that locally produced factors were retained in the media. On the morning of the media change

sterile aliquots containing the appropriate volume of culture media required were placed in the incubator to equilibrate for 2-3 hours prior to use. A sterile gel-loading tip was used to carefully remove 250µl of the media from each well and replace it with 250µl of fresh media, ensuring that there were no bubbles under the insert and that it was not damaged; the plate was returned to the incubator.

2.8 Tissue and follicle viability assessment at the end of culture

At the end of the culture period the tissue pieces were removed from the culture plate one well at a time. The dry weight and the number of tissue pieces present were recorded (see Section 2.6), before returning them to the culture plate in the incubator. Tissue pieces were transferred to pre-warmed and gassed NR plates containing NR solution at 50 µg ml^{-1} (see Figure 2.1). The NR plates were then returned to the incubator and left for 15 – 30 minutes. The NR viability assay incubation time for tissue at the end of culture was reduced in comparison to the incubation time prior to the start of culture because the tissue pieces were warm and less dense in structure and therefore able to take up the dye much more readily than fresh, cold tissue. This made it more difficult to distinguish between stromal tissue and follicles, such that in many cases only global tissue viability could be recorded. However - where possible - viable follicle counts were noted. The tissue from one well of each sample group was then fixed in 1ml of 4% PFA for histological analysis (see Section 2.9). The tissue was kept in 4% PFA overnight before being transferred to 70% (v/v) ethanol (~5ml) at room temperature (~20°C). Representative images of tissue pieces at the end of culture were taken as described in Section 2.6.

2.9 Histology

Fixed tissue (see Section 2.8) collected before or after culture was removed from 70% (v/v) ethanol after a minimum of 2 hours, placed on a slide, covered and subsequently embedded in 2% (w/v) melted agar, to facilitate handling. The 2% (w/v) agar was prepared by dissolving 0.4g of agar granules in 20ml PBS, whilst heating in a microwave. Once the agar had set, after leaving it at room temperature, it was trimmed to ~0.5cm^2 using a scalpel blade (Swann-Morton) to minimise the amount of agar surrounding the tissue. The agar block was wrapped in tissue wrapping paper (Whatman; GE Healthcare, Buckinghamshire, UK), to prevent the tissue from being lost and placed inside a plastic embedding cassette (Simport, Beloeil, Canada) and then transferred to fresh 70% (v/v) ethanol. All tissue pieces from the same well

were fixed and embedded together. After a minimum of 24 hours the tissue pieces were manually embedded in pastillated paraffin wax (VWR; West Sussex, UK). The multiple steps of the embedding procedure are outlined in Table 2.2**,** including the order and timeframes in which the agar-surrounded tissue must be placed in the various reagents. These steps were carried out in an incubator (Stuart hybridisation oven/ shaker S130H) set at the temperatures detailed in Table 2.2, with agitation. The wax was melted on a hot plate at 60°C prior to its addition to the Histo-clear (National Diagnostics; East Riding of Yorkshire, HU139LX). Once the steps in Table 2.2 were complete the agar blocks containing the tissue were then transferred to a 16mm by 16mm by 6mm Tissue Tek stainless steel base mould (Sakura; AJ Alphen aan den Rijn, The Netherlands) and blocked in molten wax. The first 3 steps were carried out in a 150ml sterile plastic bottle (Sterilin Ltd; Newport, UK), after which the moulds were transferred to a pyrex beaker (Starlabs). The volume of each reagent used was sufficient to ensure all moulds were submerged. Once set the blocks were transferred to the – 80°C freezer for storage.

Table 2.2 Manual wax embedding protocol. Around 100ml of each reagent was used per step.

Reagent	Time	Temperature
90% (v/v) ethanol	1 hour (at least)	~20°C
100% (v/v) ethanol	1 hour	~20°C
100% (v/v) ethanol (fresh)	30 minutes	~20°C
100% Histo-clear	1 hour	~20°C
100% Histo-clear (fresh)	30 minutes	60°C
Add molten wax (25%) to Histo-clear (75%)	30 minutes	60°C
Add molten wax (50%) to Histo-clear (50%)	30 minutes	60°C
Fresh molten wax	30 minutes	60°C
Fresh molten wax	30 minutes	60°C

2.10 Tissue sectioning and staining

Using a microtome (Ergo star HM 200; Laborgeräte, Waldorf, Germany) fitted with MB35 premier microtome blades (Thermo Fisher Scientific; length; 80 by 8mm, thickness; 0.25mm, cutting angle; 37^{o}) the blocked tissue was sectioned at 4µm thickness, and ribbons of the cut block were transferred to a heated water bath at 40°C to remove creases, before being transferred onto Superfrost+ microscope slides (VWR). Slides were left to dry in a rack on a hot plate at ~40°C, for ~2-3 hours, after which they were stored at room temperature (~20°C)

prior to staining, for up to 2 months. To enable histological analysis of the tissue the sections were stained using haematoxylin and eosin (H&E) (Culling, 1974), details of the protocol are shown in Table 2.3. At the end of the staining procedures glass cover slips (VWR) were mounted onto the slides using Distrene, Plasticiser and Xylene (DPX) mountant (VWR).

Table 2.3 Haematoxylin and Eosin staining protocol.

Solution	Time
Xylene (Thermo-Fisher Scientific) (x 2)	10 minutes in each
100% (v/v) ethanol	5 minutes
90% (v/v) ethanol	2 minutes
70% (v/v) ethanol	2 minutes
Wash in tap water	2 minutes
Haematoxylin (100%)	75 seconds
Wash in tap water	5 minutes
Acid Alcohol (1% hydrochloric acid in 70% (v/v) ethanol)	~ 5 seconds
Wash in tap water	2 minutes
Scott's tap water (1% (w/v) Sodium Hydrogen Bicarbonate in distilled water (VWR))	30 seconds
Wash in tap water	6 minutes
Eosin (100%)	7 minutes
Wash in tap water	5 minutes
70% (v/v) ethanol (x 2)	~ 5 seconds
90% (v/v) ethanol	30 seconds
100% (v/v) ethanol	1 minute
Xylene	2 minutes

2.11 Histological assessment of cortical tissue

Tissue sections were examined under an inverted light microscope (Nikon, Melville, USA). As the number of follicles in tissue pieces was so high only the follicles in every fifth section were counted, classified and measurements of oocyte and follicle diameter were taken (Block, 1951, Chambers, 2002). Absolute follicle counts were determined by multiplying the number of follicles by 5. Note that in order to avoid duplication only oocytes in which a distinct nucleolus that was visible within the nucleus were counted (Lundy et al., 1999, Wright et al., 1999, Chambers, 2002). The number of follicles per mg of tissue was determined by dividing the absolute number of follicles by the dry weight of the tissue at the end of culture (Section 2.8).

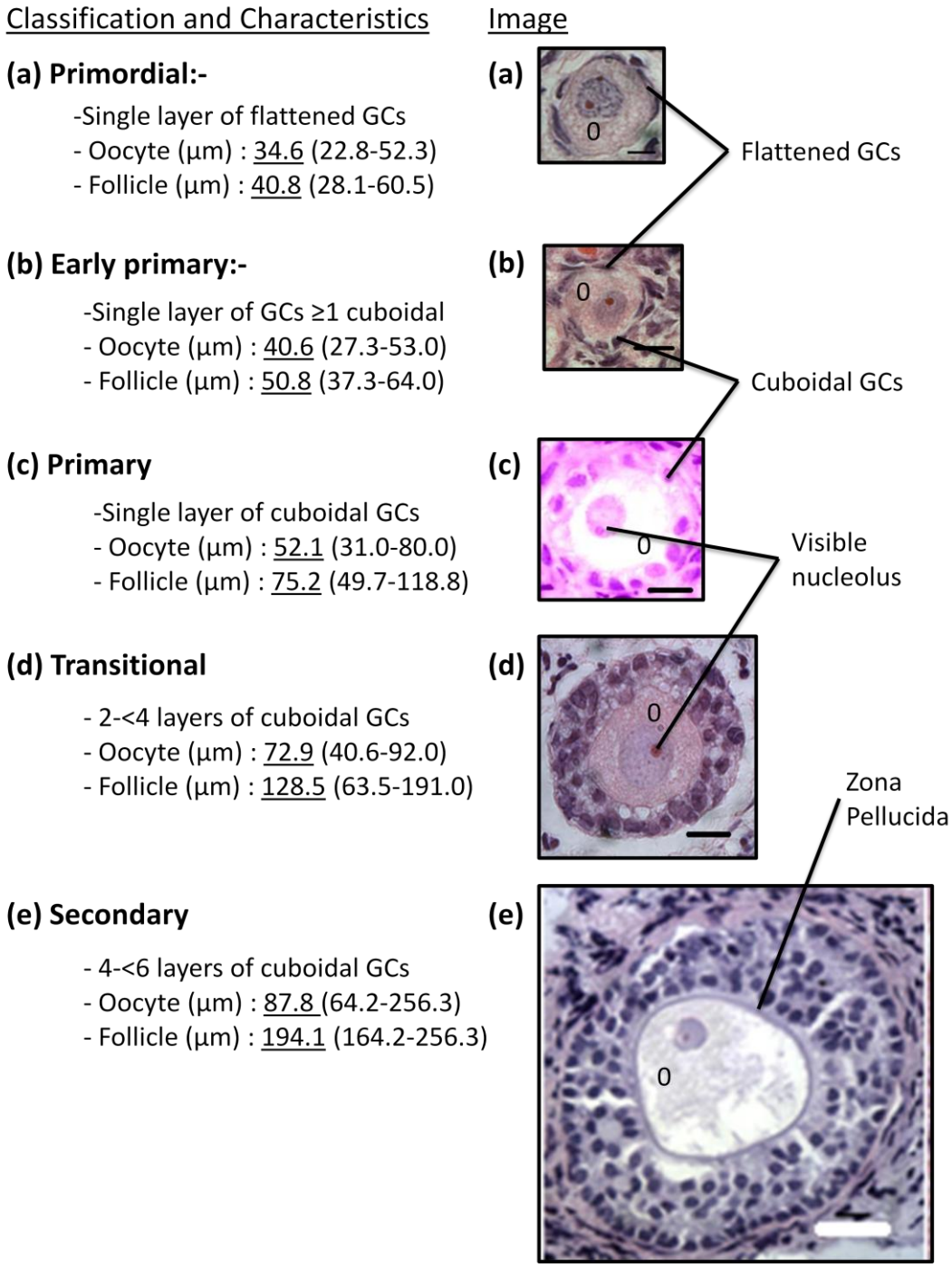


Figure 2.2 Characteristics of (a) primordial, (b) early primary, (c) primary, (d) transitional and (e) secondary follicles and representative histological images, taken following H&E staining. Mean (underlined) oocyte and follicle diameters and ranges (shown in parentheses) values taken from (Lundy et al., 1999). Oocyte is indicated on the image using 0. Scale bar = 20µm.

Follicle and oocyte diameters were calculated by taking two measurements, using a calibrated eyepiece graticule, at 90° angles to one another and averaging them. Note that the follicle diameter was measured up to the basement membrane surrounding the GC layer(s) (Wandji et al., 1997) and that the ZP was excluded when measuring oocyte diameter (Lundy et al., 1999). Follicles were classified according to the criteria presented in Figure 2.2.

The integrity of the stromal tissue was also analysed histologically. Only the middle third of histological sections for each sample was considered to avoid any processing artefacts as the outer sections may have been damaged during the fixing and blocking procedures. Every 4th section within the middle third of the sample was analysed. The stromal scores used were: good, adequate and poor which were converted into numerical scores, 3, 2 and 1, respectively. These stromal scores reflected the tissue and follicle integrity. Representative images of good, adequate and poor quality tissue are shown in the relevant chapters (Chapters 4 and 6). The poor quality tissue had a grainier, less even appearance than the adequate and good quality tissue. In the healthier and better quality tissue the cytoplasm of the oocytes was also smoother and more even than in the lower quality tissue and follicles. The scores were based on an estimate of the density of the tissue such that tissue was classified as: (i) poor if the stromal density appeared to be >40% absent/degenerate structure; (ii) adequate if 20-40% of the tissue demonstrated a poor/degenerate structure; or (iii) good if 80-100% of the tissue demonstrated a consistently healthy appearance with clearly defined nuclei and tissue structure. This stromal scoring system has previously been validated by Gunson (2009) when scoring ovarian stromal tissue in relation to the percentage positive terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining of the tissue.

2.12 Data analysis and statistical evaluation

Excel (Microsoft® Office Excel 2007) was used to process data and plot graphs. All data was analysed using Minitab version 16 (Minitab® 16.1, Minitab Limited, UK). Data was tested for normality using the Anderson-Darling test. Details of the statistical analyses carried out are described in the methods section of each experimental chapter, p values ≤ 0.05 were considered statistically significant.

3 Development Of A Two-Step, Long-Term Culture System To Support The Complete *In Vitro* Growth And Maturation Of Ovine Oocytes From The Primordial To The Early Antral Stage.

3.1 Introduction

Development of an IVG system that supports the complete *in vitro* growth and maturation of oocytes from the primordial follicle stage would increase the understanding of this process *in vivo* and may facilitate the use of IVG for fertility preservation in young girls and women (Picton et al., 1998, Smitz et al., 2010). Primordial follicles are present in the ovarian cortex of all pre-menopausal females. These follicles are present in relatively high abundance compared to other more advanced stages of follicular developmental (Gosden and Telfer, 1987b). Ovarian cortex can be relatively easily obtained from patients, via laparoscopy, and subsequently cryopreserved (Davidoff et al., 1996, Weintraub et al., 2007). Primordial follicles are relatively tolerant to the cryopreservation procedure (Newton et al., 1996, Oktay et al., 1998, Kim et al., 2001, Hovatta et al., 1996, Gook et al., 1999).

The activation, growth and development of oocytes and follicles from the primordial stage is a complex and protracted process *in vivo* and is not yet fully understood. During oocyte development the acquisition of RNAs and proteins required for meiotic progression to the MII stage, fertilisation and early embryo development is essential (Picton et al., 1998). Therefore replicating this process *in vitro* and providing the developing oocyte with a follicular micro-environment as similar to that experienced *in vivo*, to promote oocyte health, is extremely challenging. However, the IVG and subsequent IVM of oocytes has resulted in the birth of live offspring in mice (Eppig and O'Brien, 1996, O'Brien et al., 2003). Due to the larger size and longer growth period of oocytes in larger mammals and humans it has proved far more difficult to adapt the murine culture systems to suit these species (Picton et al., 2008). However, some progress has been made in larger species and primates.

3.1.1 Primordial follicle activation and development

As discussed in Chapter 1 many studies have investigated the optimal conditions for the activation and development of primordial follicles *in vitro*. The use of ECM- and serum-free culture media containing ITS and albumin substitutes is preferential to the use of serum-

containing culture media with respect to the activation and growth of oocytes *in vitro* as well as the health of the oocytes and potential resultant offspring (Wandji et al., 1997, Young et al., 1998, Newton et al., 1999b, Wright et al., 1999, Fernandez-Gonzalez et al., 2004, Fujihara et al., 2012). Although the major components of serum and ECM are known their exact compositions are complex and ill-defined (McGuire and Seeds, 1989, Picton et al., 2008). Interactions between components of the serum and ECM affecting follicle and oocyte growth, development and health would therefore be difficult to elucidate. Therefore, despite the fact that the use ECM has been shown to improve the survival of early preantral follicles in long-term cortical culture it will not be used in this experimental series (Hovatta et al., 1997). The use of serum in culture media of oocytes has been shown to result in reduced activation rates, decreased growth and development, increased levels of atresia and poor follicle morphology with respect to the collapse of 3D structure when compared to follicles and oocytes cultured in alternate serum-free media supplemented with ITS and albumin substitutes (Wandji et al., 1997, Wright et al., 1999, Newton et al., 1999b). The addition of serum has also been shown to inhibit the stimulatory effect of oocyte-derived factor, GDF9, on the IVG and development of human follicles during cortical slice culture (Hreinsson et al., 2002). Additionally serum has been shown to have deleterious effects when used in both sheep and mouse embryo culture media leading to fetal overgrowth syndrome and alterations in behaviour, caused by aberrant imprinting (Young et al., 1998). Therefore there are concerns that addition of serum to IVG media could result in aberrant imprinting as the maternal imprint is established during gametogenesis as well as following fertilisation (Reik et al., 2001).

The *in situ* culture of preantral follicles within slices of cortical tissue has been shown to support follicle survival and development in the mouse (Eppig and O'Brien, 1996, O'Brien et al., 2003), cat (Fujihara et al., 2012), dog (Fujihara et al., 2012), goat (Silva et al., 2004a, Magalhaes-Padilha et al., 2012), sheep (Chambers et al., 2010, Peng et al., 2010), cow (McLaughlin and Telfer, 2010, Wandji et al., 1996b), non-human primate (Wandji et al., 1997, Jin et al., 2010, Brito et al., 2013) and human (Wright et al., 1999, Hovatta et al., 1997, Scott et al., 2004a, Telfer et al., 2008, Parte et al., 2013, Liebenthron et al., 2013). Culturing *in situ* provides the follicles with sufficient mechanical support to maintain oocyte-GC interactions and so provides an environment very similar to that of the ovary *in vivo* (Gosden, 1998, Picton et al., 2000). Therefore in this experimental series a serum-free, *in situ* cortical culture system will be utilised to support the *in vitro* activation and development of primordial follicles.

Chambers (2002) described a serum- and ECM-free culture system in which ovine primordial follicles were cultured *in situ* within slices of cortical tissue for up to 30 days. This culture system was able to support the *in vitro* activation and subsequent growth and development of some primordial follicles at a physiologically relevant rate (Sawyer et al., 2002). Some researchers have reported the activation of the majority of primordial follicles present in the tissue within the first couple of days of cortical culture (Wandji et al., 1996b, Wandji et al., 1997, Telfer et al., 2008, McLaughlin and Telfer, 2010). The use of the system developed by Chambers (2002) resulted in a decrease in primordial follicle numbers accompanied by an increase in early primary (EP) follicle numbers following 15 days culture. Following 30 days of culture a 17% decrease in primordial follicle population was observed. The use of proliferating cell nuclear antigen (PCNA) and TUNEL staining showed that the levels of apoptosis remained constant throughout the IVG of follicles thus indicating the maintenance of follicular and stromal health. Additionally the weight of the tissue significantly increased following 15 days culture, compared to day 0, showing that the tissue was healthy and the cells able to proliferate.

The use of the culture system developed by Chambers (2002) showed that NR could be used to determine viable follicle counts and tissue viability in tissue without being detrimental the health of the follicles, oocytes or stromal tissue. Tissue pieces were incubated with NR dye at the start and end of an 8-day culture period showing that 61.5% tissue pieces remained viable after culture (Chambers et al., 2010). Only 25% of follicles from viable cultured tissue samples exhibited signs of apoptosis on day 8 of culture and the levels of GC and oocyte apoptosis were not significantly different from levels in day 0 tissue. The culture system developed by Chambers (2002) is able to support the growth, development and survival of ovine ovarian follicles for up to 30 days. The use of NR dye enables the determination of the follicle load in each cortical slice. Therefore this culture system will be adapted and used in combination with NR dye in this experimental series.

The hypothesis that oogenesis is a start-stop process resulting from the presence of inhibitory factors produced by follicles *in vivo* can be investigated using models in which either there are no, or very few growing follicles. Such models can be based around the culture of fetal ovaries which contain only primordial follicles, following ovarian tissue autografting, or when the number of growing follicles is very low, such as during menopause. In these situations the level of inhibitory factors being produced would be lower than in a premenopausal, adult

ovary therefore if the hypothesis is correct, rates of follicle and oocyte development should be accelerated and so become more similar to that observed *in vitro*. In support of this notion, declining levels of AMH have indeed been linked to increased levels of primordial follicle activation in the human ovary as they decline to undetectable levels around 5 years prior to menopause, as a result of the declining follicular reserve (Faddy et al., 1992, Sowers et al., 2008, Kelsey et al., 2011). Additionally increased rates of follicle growth and development have been observed *in vivo* in the absence of large, growing follicles. Development of follicles from the primordial to the secondary stage takes around 18 days in adult mice, however, in newborn mice secondary follicles and antral follicles are present after 8 and 12 days, respectively (Gigli et al., 2005, Kerr et al., 2006). Similarly in both ovine and human fetal ovaries increased rates of development of primordial follicles to the secondary stage have been observed as taking 45 days (Sawyer et al., 2002, Juengel et al., 2002b) and 133 days (Konishi et al., 1986), in contrast to 78 days (Cahill and Mauleon, 1981) and 270 days (Himelstein-Braw et al., 1976, Gougeon, 1986) in the adult ovary, respectively. Following grafting of frozen-thawed ovarian tissue only primordial follicles are present, thus making this a useful tool for studying follicle growth in the absence of growing follicles *in vivo* (Newton et al., 1996, Baird et al., 1999). In both the sheep and human, exposure of grafts containing only primordial follicles to increased levels of FSH resulted in increased levels of development (Oktay et al., 1998, Campbell et al., 2000). Exposure of autografted ovine ovarian tissue to increased levels of FSH resulting from decreased levels of inhibin and oestrogen in the absence of Graafian follicles resulted in the development of small and large antral follicles following only 3-4 months (Campbell et al., 2000). In contrast grafts that had been exposed to physiologically normal levels of FSH contained a lower number of small antral follicles over the same time frame. Similarly, human ovarian tissue xenografted to SCID mice and exposed to FSH stimulation resulted in development of follicles to the antral stage following only 129 days, whereas in the absence of FSH stimulation development past the secondary stage was not observed (Oktay et al., 1998). It should be noted that even in the absence of FSH stimulation increased rates of follicle development were observed as secondary follicles were present 77 days after grafting, compared to 270 days in the adult ovary *in vivo*. Therefore a reduction in the follicular reserve or the absence of growing follicles can result in increased levels of follicle activation and development.

3.1.2 Isolated preantral follicle culture

Although the earlier preantral stages of follicular development can be supported *in situ* once the transitional/ secondary stages are reached it is necessary to isolate the follicles to allow growth to continue (Abir et al., 1997, Hovatta et al., 1997, Telfer et al., 2008, Picton et al., 2008). Various systems have shown that it is possible to support the development of isolated preantral follicles up to the EA stage in the mouse (Nayudu and Osborn, 1992, Cortvrindt et al., 1996, West et al., 2007), sheep (Cecconi et al., 1999, Newton et al., 1999b) cow (Itoh and Hoshi, 2000, McLaughlin and Telfer, 2010), non-human primate (Xu et al., 2011a) and human (Telfer et al., 2008). As mentioned in Chapter 1 a large amount of research has been conducted to define the optimal conditions required to support preantral follicle development *in vitro*. A major challenge during isolated preantral follicle culture is the maintenance of vital oocyte-GC interactions (Picton et al., 2008, McLaughlin and Telfer, 2010, Smitz et al., 2010). As discussed in Chapter 1 approaches affecting the maintenance of these interactions include the use of 2D vs. 3D culture systems, enzymatic vs. mechanical isolation, the length of culture and the composition of the culture media.

Newton et al. (1999b) developed a serum-free culture system that supported the induction of antral cavity formation and development of isolated ovine oocyte-GC complexes, following a period of tissue digestion using collagenase. Follicle survival rates were dependent upon the size of the follicle at the time of isolation. Around 40% of follicles sized 190-240µm on the day of isolation maintained their 3D structure after 120 hours in culture. This was not however, observed in follicles <190µm on the day of isolation, as all follicles in this size range exhibited some degree of loss of 3D structure, as manifest by: extrusion of the oocyte; necrosis; collapse and adherence to the culture plate, after 120 hours. It should be noted that the maintenance of 3D structure was also dependent upon the length of exposure to collagenase, which was used to soften the tissue in order to facilitate follicle isolation. Inclusion of either FSH alone or in combination with LH, in the medium promoted antral cavity formation. Culture of follicles isolated from fresh tissue in the presence of gonadotrophins resulted in 25±9% forming antral cavities, around day 14 of culture. When the media was not supplemented with gonadotrophins only ~7% of follicles formed antral cavities. Furthermore, follicles that formed antral cavities and were cultured in the presence of testosterone produced levels of oestradiol indicative of the induction of aromatase in the cells and comparable to that of follicles isolated from fresh tissue (Picton et al., 1990). Progesterone production was not affected by the presence or absence of testosterone. Although oocytes grew to full size after 30 days culture,

follicles did not: probably to minimise the diffusion distance of required metabolites. This culture system is able to support the growth and development of secondary ovine follicles of 190-240µm at the time of isolation to the EA stage. Early antral follicles derived using this system exhibit normal steroidogenic function *in vitro* when incubated with testosterone. Therefore in this experimental series the preantral follicle culture system used will be based upon this system.

As with cortical culture, there is some disagreement regarding the optimal length of culture needed to support growth and development from the preantral to EA stage. The longer follicles remain in culture the more difficult it is to maintain oocyte-GC interaction integrity, thus in this respect accelerated culture is preferable to protracted culture (McLaughlin and Telfer, 2010). However, as with cortical culture, there are concerns that accelerated culture may affect the developmental competence and epigenetic health of the resultant oocytes (Picton et al., 2008). There are examples of the use of both long- and short-term preantral follicle culture periods. In the aforementioned study (Newton et al., 1999b) the timescale of ~14 days before antral cavity formation is observed is the same as that reported by Barboni et al. (2011), however, in the latter study the ovine preantral follicles were slightly smaller at the start of culture ~170µm. Additionally, antral cavity formation was observed in a considerably higher percentage of follicles than reported by Newton et al. (1999b) (70% vs. 30%), although it should be noted that in the Barboni et al. (2011) study the media was not serum-free and the follicles were mechanically isolated, which may have affected antral cavity formation rate. However, Cecconi et al. (1999) used a serum-free culture system and reported a high rate of antral cavity formation (80%) in isolated preantral follicles, 120-260µm diameter, after only 6 days *in vitro*, when cultured in 5% O_2 and 1µgml^{-1} FSH. McLaughlin and Telfer (2010) observed the development of transitional bovine follicles, ~110µm diameter, to the EA stage after only 12 days of isolated culture. In a separate study this group isolated more developed bovine preantral follicles (~160µm) from fresh tissue and reported >40% developing to the EA stage by day 8 of culture (McLaughlin et al., 2010). In both these studies the addition of a high concentration (100ngml^{-1}) of activin A to the culture medium appears to be responsible for the accelerated growth rate. In the absence of activin A the mean growth rates exhibited by the follicles was significantly lower (McLaughlin and Telfer, 2010, McLaughlin et al., 2010).

The composition of the culture media affects preantral follicle growth and survival *in vitro*. For example, in addition to the promotion of follicle growth *in vitro*, activin A has been shown to promote preantral follicle survival, potentially partly via the promotion of oocyte-GC

interactions (McLaughlin and Telfer, 2010, da Silva et al., 2013). Zhao et al. (2001) used transmission electron microscopy to assess the ultrastructure of rat preantral follicles *in vitro*. Follicles cultured in activin A- supplemented media had more developed oocyte-GC cell contacts than those cultured in activin A- free media. Connexin protein, Cx43, is a key component of gap junctions in bovine somatic follicular cells (Vozzi et al., 2001). Inclusion of activin A in the media of cultured bovine preantral follicles resulted in the promotion of Cx43 expression, when compared to follicles cultured in the absence of activin A (McLaughlin et al., 2010). Ascorbic acid is another culture media additive that has been identified as being able to promote preantral follicle survival, which it does via its anti-oxidative actions and its capacity to promote basement membrane production (Murray et al., 2001, Telfer et al., 2008, Andrade et al., 2012, Thomas et al., 2001). Arunakumari et al. (2010) carried out an extensive investigation into the determination of the optimal combination and concentrations of growth factors and hormones required to promote the development of secondary ovine follicles to the EA stage; 1% ITS, 10ngml^{-1} each of IGF1, and Insulin and 1mIUml^{-1} of GH. Under these conditions ~3% of follicles, 250-400µm diameter at the start of culture, were grown and matured up to the MII stage and then fertilised and were competent to develop to the morula stage, however, none were competent to develop to the blastocyst stage. Briefly, under these conditions, of the 250 preantral follicles cultured antral cavity formation was observed in 80% and 68% developed up to the MII stage. Of the 170 MII oocytes 26% reached the 2-cell stage following fertilisation and 16% reached the morula stage. The production of an embryo from an *in vitro*-derived oocyte, cultured for a total of 18 days from the secondary stage, has also been reported in the goat (Magalhaes et al., 2011). Secondary follicles were cultured in the presence of 50ngml^{-1} GH; resulting in antral cavity formation in 93% of follicles and 50% of the oocytes maturing to the MII stage. Following IVF of the 35 *in vitro*-derived MII oocytes one embryo was produced. In the current experimental series a serum-free, 3D preantral follicle culture system will be utilised in order to assess ovine follicle growth and development over a maximum of 30 days.

3.1.3 Multi-step IVG systems

Research suggests that the IVG of oocytes will be best achieved using a multistep system in which conditions are altered to meet the requirements of the oocyte and somatic cells at each developmental stage (Picton et al., 1998, Eppig and O'Brien, 1996, Telfer et al., 2008, Smitz et al., 2010). Although considerable research has been conducted with the aim of optimising each individual step of the IVG culture systems, limited research has been conducted in which

multiple steps have been linked, particularly in larger mammals and humans. The aim of this chapter was therefore to develop and link the first 2 phases of a multi-step culture system that supports oocyte development *in vitro*, from the primordial follicle stage up to the EA stage in sheep. The first step of this system will support the activation and development of primordial follicles to later preantral stages *in situ*. At the end of the first stage of culture the preantral follicles will then be isolated and cultured individually to the EA stage.

3.1.4 Aims

The first aim of this experimental series was to compare the utility of long- and short-term cortical culture systems with respect to the activation, survival, growth and development of ovine primordial follicles. In order to achieve this; follicles were cultured *in situ* for varying lengths of time, in order to determine optimal culture length. At the end of the cortical culture period follicle population dynamics were assessed both in the fresh tissue and histologically. The second aim of this experimental series was to determine whether follicles grown *in vitro* using this culture system were competent to develop up to the EA stages once they were isolated and cultured individually. The third aim of this experimental series was to compare the growth, development and survival rates of *in vitro*- and *in vivo*-derived follicles. Preantral follicles derived *in vitro* were isolated and put into individual culture and their developmental competence assessed with respect to survival and growth rates as well as their ability to develop to the EA stages, relative to follicles isolated from fresh tissue.

3.2 Materials and Methods

3.2.1 Experiment 1 – step 1 *In situ* cortical culture

Tissue culture was set up and carried out as detailed in Sections 2.1-2.7. A diagrammatic representation of the basic methodology used is shown in Figure 3.1. As described previously the pieces of cortical tissue were weighed at the start and end of culture. Cultures were run for 6, 16, 19 or 23 days. At the start and end of the culture period tissue pieces were transferred to Neutral Red dye to assess tissue viability, determine viable follicle counts and to aid follicle isolation, as described in Section 2.8, in preparation for Section 3.2.2, Experimental step 2 – preantral follicle culture. Pieces of cortex were fixed for histological evaluation on days 0, 6, 16, 19 and 23 of culture, then processed and analysed as described in Sections 2.12-2.14. The number of viable follicles in each classification and the number of degenerating

follicles per mg tissue at each time point was determined as well as the diameter of each follicle and oocyte. Five pieces of cortex were analysed per culture repeat and 4 culture repeats were performed for each time point.

3.2.2 Experiment 2 – step 2 preantral follicle culture

Follicles were isolated from both cultured cortical tissue on days 16, 19 and 23 of culture and from fresh day 0 tissue. A culture period of 6 days yielded only primordial and early primary (EP) follicles, therefore follicles isolated from this tissue were not put into culture. It was possible to isolate follicles from cultured tissue using mechanical means only, whereas follicle isolation from fresh tissue required the use of enzymes to digest and soften the tissue prior to mechanical isolation as this tissue was tougher.

The process of follicle isolation from fresh tissue will be described first, which as previously mentioned was achieved using enzymes to soften the tissue. The process of ovary isolation from the reproductive tracts was the same as described in Sections 2.1 and 2.2, except that the ovaries were stored in 50ml pre-warmed Follicle Isolation Media (FIM; see Appendix I) in a 60ml sterile universal, at 37^{o}C rather than in RKWM. All processing of tissue was carried out on a heated stage at 37^{o}C under laminar flow conditions. Each ovary was transferred to a sterile Petri dish then sliced in half along the line of connective tissue which had previously connected the ovary to the tract, using a sterile scalpel (handle size 4 with a size 22 blade). Using the same scalpel the stromal tissue on each half of the ovary was first loosened by making small incisions. Care was taken to not cut completely through the tissue thus avoiding any damage to the outer cortex. The stromal tissue was then removed by slicing at an angle with the scalpel to leave a piece of cortex <2mm thick. The pieces of cortex were then transferred to a 5ml sterile universal containing ~4ml pre-warmed FIM at 37^{o}C. This process was repeated using 4-6 ovaries. All of the pieces of cortex were then transferred to a new sterile Petri dish where the tissue was 'minced' into very small pieces (1-2mm^{-3}) using 2 sterile scalpel blades at a 180^{o} angle to each other to slice simultaneously. Care was taken to apply an adequate amount of pressure to ensure the tissue was sliced rather than torn apart. The tissue was then transferred to 10ml of pre-warmed 50-100% w/v collagenase (type 1A) containing 40µl DNase to give a final concentration of 2µgml^{-1} (see Appendix I) for 45-60 minutes with agitation, at 37^{o}C in a hybridisation oven/ shaker (Stuart; S130H). Following enzyme digestion tissue pieces were washed 3-4 times in cold L-15 medium containing 10% FCS v/v, at 4^{o}C.

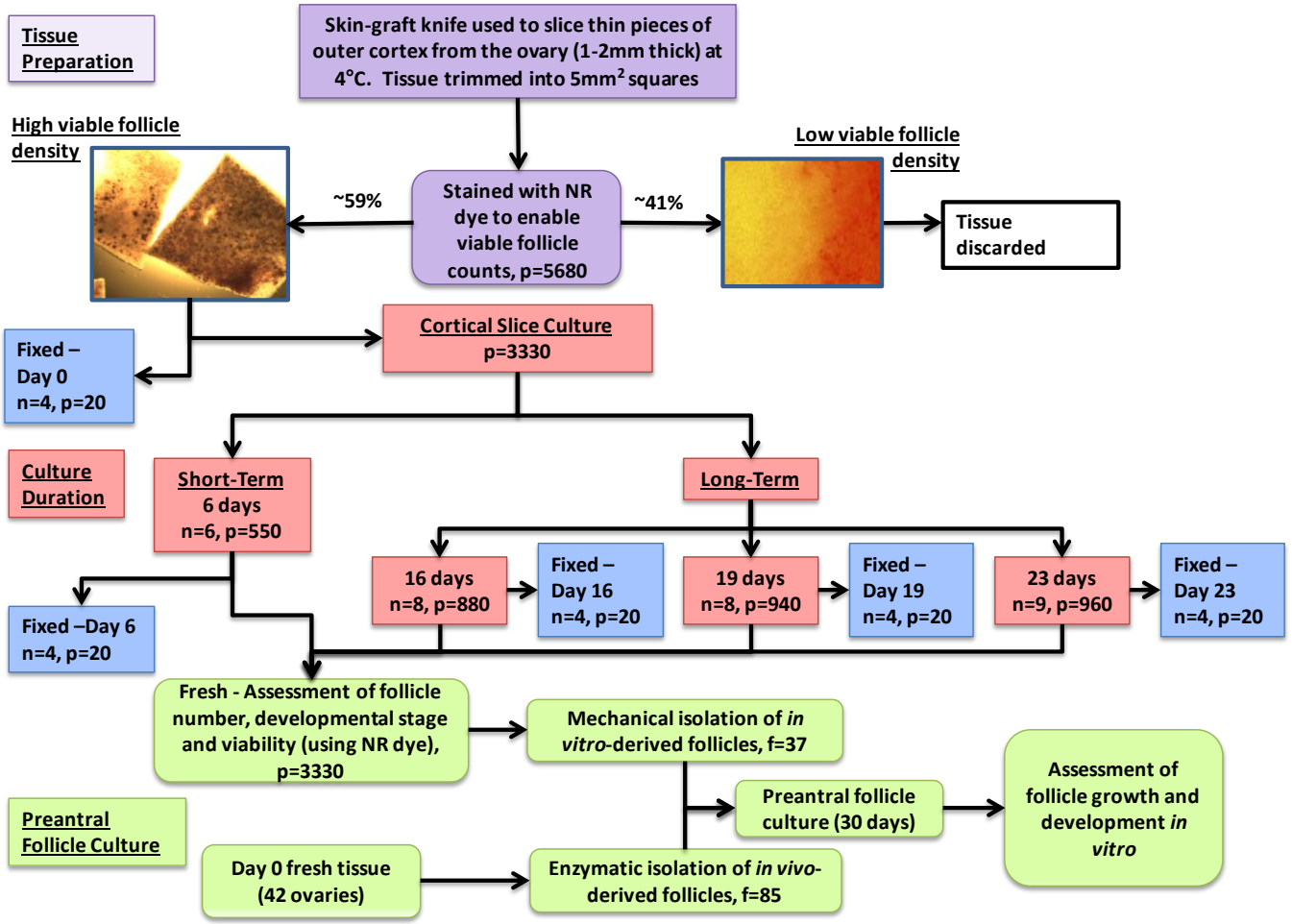


Figure 3.1 A flow diagram showing the basic methodology used in this experimental series. The number of pieces of cortical tissue (p) used in each step is indicated, as is the number of culture repeats (n) and the number of follicles (f) following follicle isolation.

Tissue pieces were then transferred to pre-warmed and gassed NR plates containing NR solution at 50µgml^{-1} (see Figure 2.2). The NR plates were then returned to the incubator and left for 40-60 minutes. As previously mentioned follicles were isolated from cultured tissue using mechanical means only, in the absence of enzymes therefore from this point the fresh day 0 tissue was processed in the same way as the tissue cultured for 16, 19 or 23 days, see below.

After incubation in NR solution the tissue was transferred to pre-warmed FIM and positively stained follicles were dissected in a glass bottom dish (WillCo Wells B.V., Willco Beheer BV, Amsterdam, Netherlands) using 30 gauge needles attached to 1ml syringe barrels. Care was taken to ensure the majority of stromal tissue was removed from each follicle.

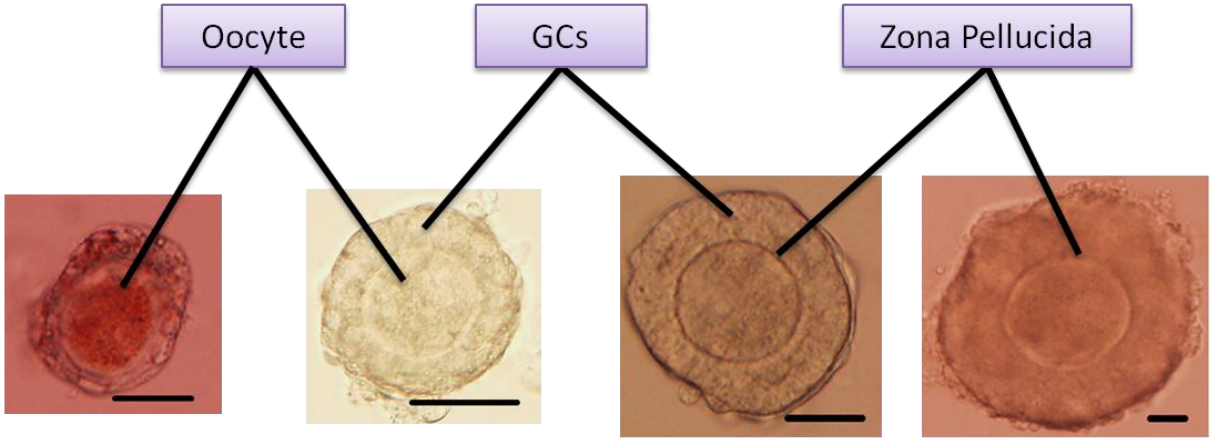
Table 3.1 Composition of preantral culture media. The volumes required to make a 30ml solution are shown, with the stock and final concentrations. The solution was filtered and stored for up to 1 week.

Additive	Volume (µl)	Stock Concentration	Final Concentration*
Basal culture media (see Appendix I)	9.716ml		
Bovine holo-transferrin	10	5mgml^{-1}	5µgml^{-1}
Sodium Pyruvate	10	47mM	0.047mM
Sodium Selenite	1	50µgml^{-1}	5ngml^{-1}
L-glutamine	150	200mM	3mM
Bovine Insulin	10	10µgml^{-1}	10ngml^{-1}
Human long-R3 IGF-1	1	100µgml^{-1}	10ngml-1
Ovine FSH	1	0.1iUml^{-1}	1 x 10^{-5}iUml^{-1}
Ovine LH	1	0.0023iUml^{-1}	2.3 x 10^{-7}iUml^{-1}

*The solution was filtered and stored for up to 1 week. See Appendix I for details of the preparation of basal culture medium and additives.

Following dissection, preantral follicles from EP stage onwards were transferred to a Nunc 4-well dish containing 500µl pre-warmed and gassed culture media to wash off any FIM. Note that only secondary follicles were isolated from fresh tissue. Isolated follicle culture was undertaken using 96-well sterile flat-bottomed plates (Nunclon, catalogue number: 167008) containing 200µl preantral culture media (see Table 3.1) per well. Note that the media used to culture follicles isolated mechanically also contained 50µgml^{-1} ascorbic acid, which was added immediately prior to use, to aid basement membrane remodelling (Murray et al., 2001,

Thomas et al., 2001). As *in vivo*-derived secondary follicles had been isolated using enzymatic means this removed the basement membrane, therefore the addition of ascorbic acid was not necessary. Plates were set-up 3 hours prior to use and placed in the incubator to equilibrate. Using a pipette, isolated follicles were carefully transferred to the 96 well plate in a minimal volume of media, one follicle was cultured per well. Care was taken to position the follicles as close to the centre of the well as possible. Follicle diameter (excluding any attached stromal cells) and oocyte diameter were measured using an inverted microscope (Olympus IX70) fitted with a calibrated eye graticule and a heated stage at 37°C. To calculate follicle and oocyte diameter 2 measurements were taken at 90° angles and then averaged. The number and classification of the follicles at the start of the second phase of culture were also recorded. The follicle classification system used in this experimental series is detailed in Figure 3.2.



Follicle Stage	Early Primary	Primary	Transitional	Secondary
Oocyte Diameter (µm)	40.6 (27.3-53.0)	52.1 (31.0-80.0)	72.9 (40.6-92.0)	87.8 (64.2-256.3)
Follicle Diameter (µm)	50.8 (37.3-64.0)	75.2 (49.7-118.8)	128.5 (63.5-191.0)	194.1 (164.2-256.3)
Number of GC Layers	1 (≥1 cuboidal)	1 (all cuboidal)	2 - <4	4 - <6

Figure 3.2 A diagrammatic representation and tabulated description of the classification and characterisation of EP, primary, transitional and secondary ovine follicles. The mean diameter of follicles and oocytes is shown with ranges in parentheses. Images are of *in vitro*-derived follicles, scale bar = 25µm. Data regarding follicle and oocyte diameters taken from Lundy et al. (1999).

Preantral follicle cultures were run for a maximum of 30 days. Media changes and follicle/ oocyte diameter measurements were carried out and photos were taken every Monday, Wednesday and Friday of culture. Media changes were conducted on a heated stage at 37°C; 50% of the media in each of the wells was replenished during each change. If the follicle diameter did not increase across 3 consecutive media changes/diameter measurements then culture of the follicle was terminated as it was deemed that this follicle was either dead or degenerating. Culture of the follicle was also terminated if the oocyte was completely extruded or the follicle had completely attached to the plate or was clearly necrotic. Representative images of follicles exhibiting these features are shown in Figure 3.3.

In order to determine whether follicles were viable either at the end of the 30 day period or when they were deemed to be degenerating or dead NR staining was used. A 3µl aliquot of NR dye was added to the culture media in the well to give a final concentration 50µgml^{-1} and the tissue was left for 15 minutes. The level of staining observed was recorded and photos taken using an Olympus DP11 attached digital camera. Samples of viable and degenerating follicles were snap frozen in lysis buffer (see Appendix III) for subsequent molecular analysis (Chapter 5).

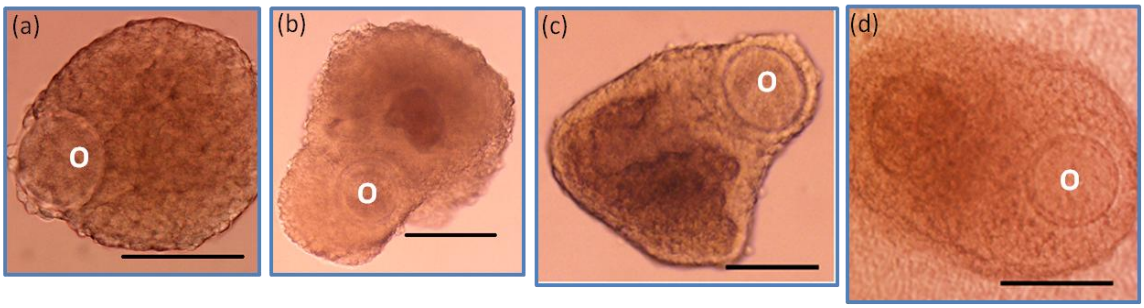


Figure 3.3 Representative images of secondary follicles exhibiting poor health in culture; partial oocyte extrusion (a), misshapen (b), early necrosis (c) and attachment of follicle to the culture surface (d). Scale bar = 100µm.

3.2.3 Statistical analysis

The distribution of data was analysed for normality using the Anderson-Darling test. Follicle counts in Experiment 1, step 1 – *in situ* cortical culture and increases and percentage increases in follicle diameter recorded in Experiment 2, step 2 were analysed using one-way ANOVA. Follicle proportions data in Experiment 1 was analysed using Chi-squared analysis.

3.3 Results

3.3.1 Experiment 1 Cortical Slice Culture – primordial follicle activation and development

In this experimental series 4 lengths of cortical culture were compared; 6, 16, 19 and 23 days over a total of 31 cultures, see Figure 3.1. The follicle population dynamics observed in fixed tissue are shown in Figures 3.6 and 3.7. Note that the term 'fixed tissue' refers to tissue that has been analysed histologically. The follicle population dynamics observed in fresh tissue are shown in Figure 3.9. Note that the term 'fresh tissue' refers to tissue that has been stained using NR dye either before or after culture.

(i) Histological assessment of follicle population dynamics

Firstly, tissue fixed either on day 0 or after culture was used to determine the relationship between the diameter of oocytes and follicles derived either *in vivo* or *in vitro*, respectively, see Figure 3.4. Data regarding *in vitro*-derived follicle and oocyte diameters refers to that observed in tissue fixed after 6, 16, 19 and 23 days of culture. A strong positive correlation between oocyte and follicle diameter was observed in both *in vivo*- and *in vitro*-derived primordial, EP, primary and all follicle classifications ($p<0.001$). Transitional and secondary follicles were not considered separately as there were such low numbers of these classifications observed in fixed tissue samples, this data was therefore pooled before analysis.

The mean diameter of oocytes and follicles within each preantral follicle classification on days 0, 6, 16, 19 and 23 of culture are shown in Figure 3.5. The data were analysed both across 4 culture repeats (see (a) and (b)) and across all follicles recorded (see (c) and (d)). Similar patterns were observed in all groups with very little variation in follicle and oocyte diameter in the primordial and EP follicle categories at any of the culture lengths considered. Tissue fixed following 6 days of cortical culture did not contain follicles more advanced than the EP stages. In contrast in the more developed preantral follicle classifications more variation was observed. For example, the mean diameter of primary follicles and oocytes observed in day 0 tissue was significantly greater ($p<0.05$) than that observed on day 23. Whereas the mean diameter of transitional follicles present in day 23 tissue was significantly greater ($p<0.05$) than that observed in day 16 or 19 tissue. When the mean diameters of all oocytes and follicles were considered separately in Figures 3.5 (c) and (d) the overall patterns observed were similar.

Data regarding follicle population dynamics is shown in Figure 3.6 with respect to the mean number of follicles per mg of cultured tissue (a); determined using the tissue weight at the start of culture, and percentage (b) of total number of viable follicles, viable primordial and growing, and degenerating follicles in tissue fixed on days 0, 6, 16, 19 and 23 of culture. Both methods of presenting the data show a similar pattern of a decrease in the number of primordial follicles on days 16, 19 and 23 of culture, compared to day 0, accompanied by an increase in growing and degenerating follicles. None of these differences in the mean number of follicles (a) are statistically significant ($p>0.05$) except for the increase in number of degenerating follicles per mg tissue on day 16 (295), relative to days 0 and 6, 68 and 35, respectively ($p<0.05$). The distribution of follicles on days 0 and 6 was significantly different ($p<0.05$) from that on days 16, 19 and 23. The lower percentage of primordial follicles on days 16, 19 and 23 relative to days 0 and 6 was the biggest contributor. With respect to the day 6 tissue, although the mean number of follicles in all categories decreased compared to day 0 Figure 3.6 (a), the proportion of follicles remained similar Figure 3.6 (b). However, a slight decrease in the number of primordial follicles is apparent in the cultured tissue, compared to the day 0 tissue. Figure 3.6 (b) shows that the percentage of primordial follicles decreased on days 16, 19 and 23 of culture; 30%, 37% and 40% respectively, compared to days 0 and 6; 64%. This could be due to either follicle activation or atresia, or both. As the increase in the percentage of degenerating follicles is greater than that of growing follicles on days 16-23, this suggests that follicular atresia is the greater contributor towards the decrease in percentage of primordial follicles.

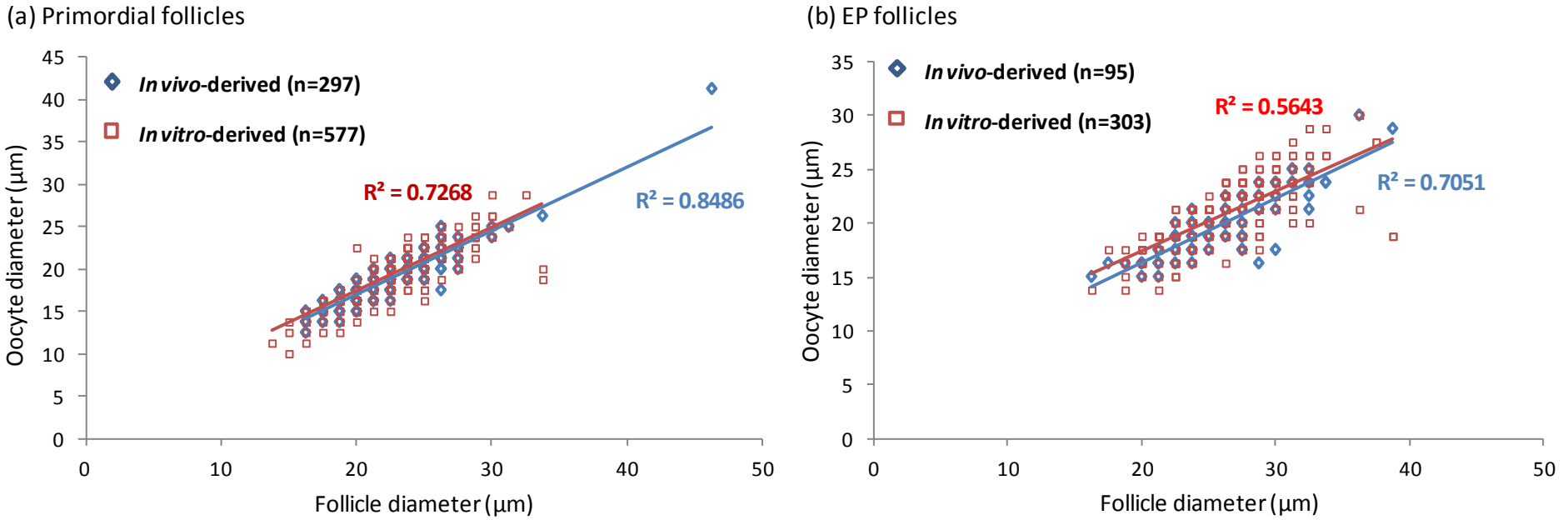
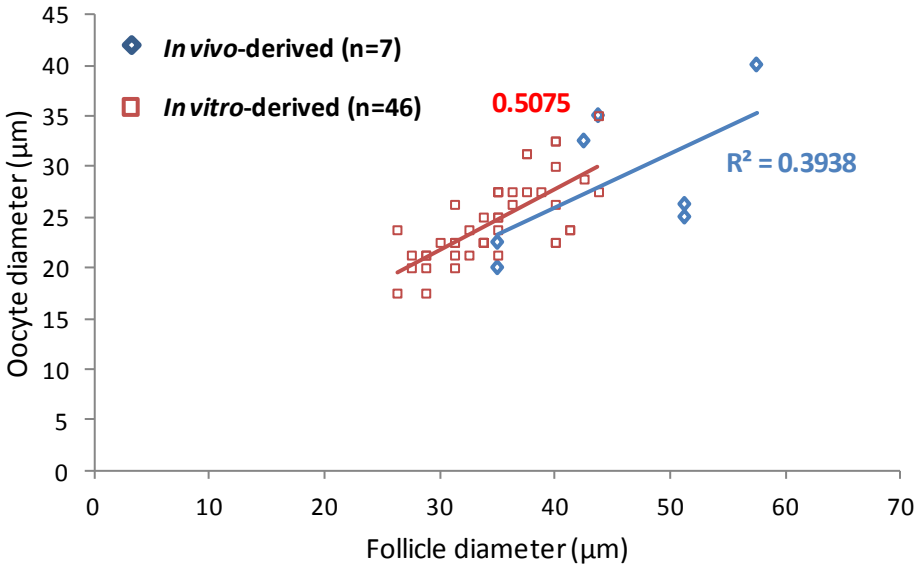


Figure 3.4 Relationship between follicle and oocyte diameter of *in vivo*- and *in vitro*-derived primordial (a), EP (b), primary (c) and all follicle classifications (d) in fixed tissue. A strong positive correlation ($p<0.001$) between follicle and oocyte diameter was observed in all data sets, analysed using analysis of covariance (ANCOVA). The number of follicles analysed in each data set is shown (n) as is the R^2 value for each trend line. Note that many of the points are overlaid.

(c) Primary follicles



(d) All classifications of follicles

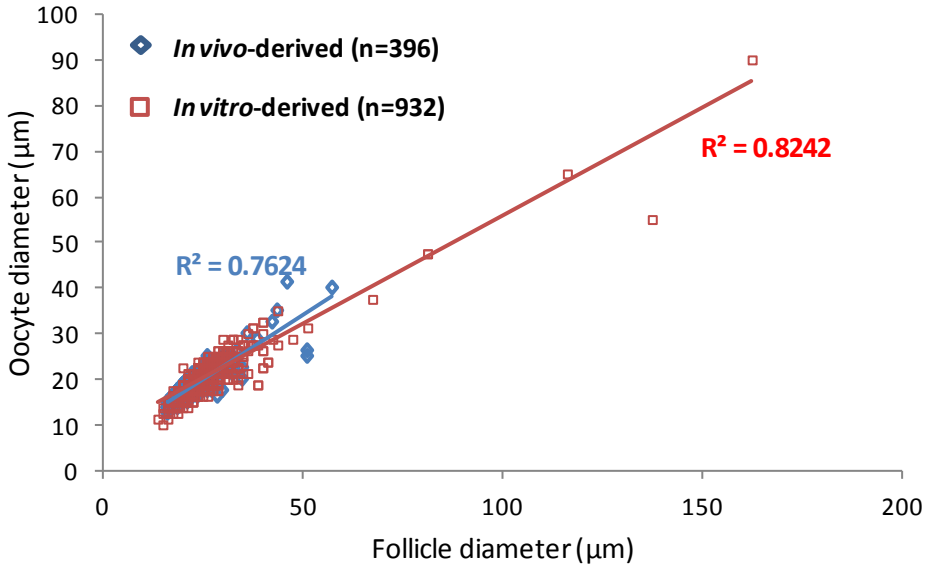


Figure 3.4 (contined)

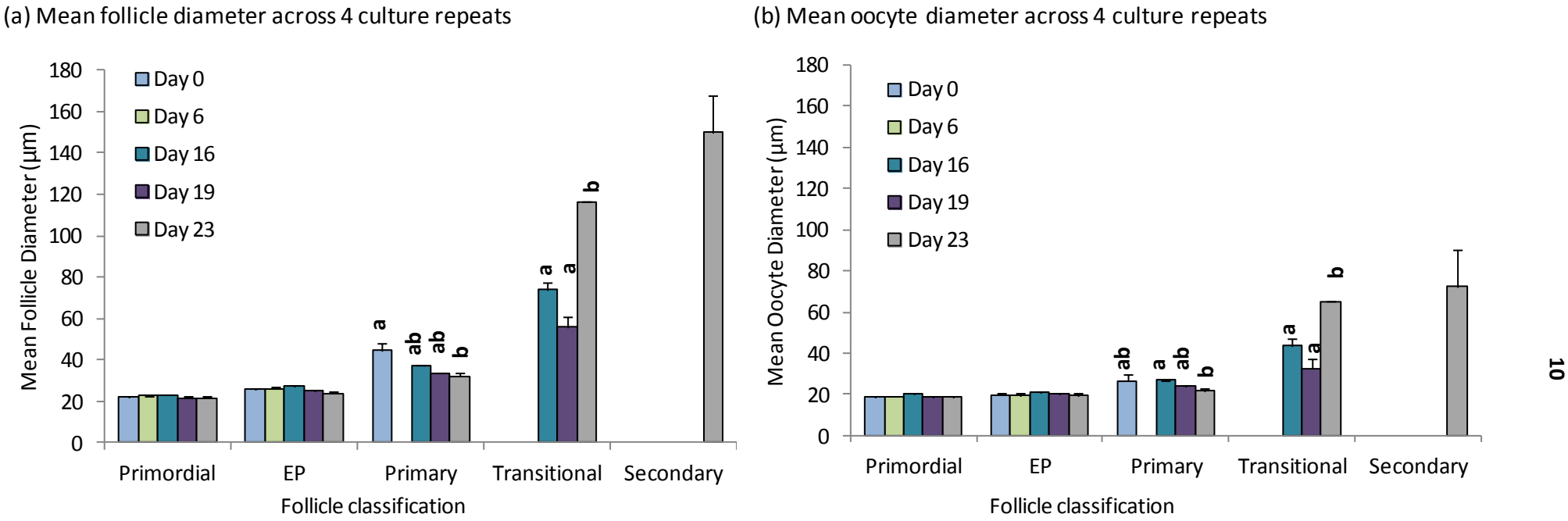
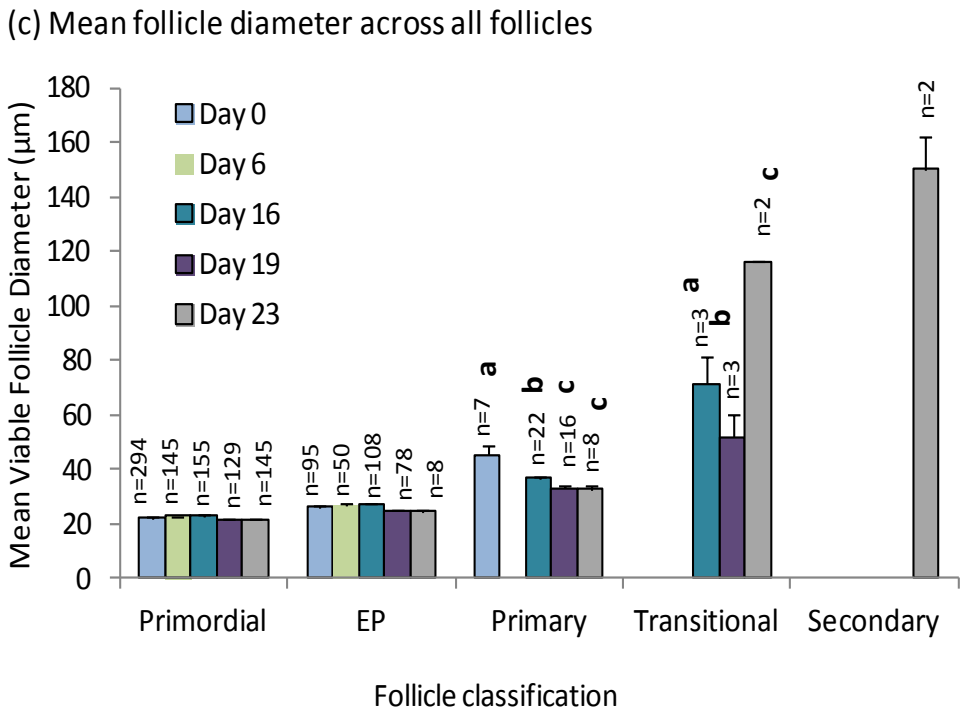


Figure 3.5 Mean follicle and oocyte diameter of primordial, EP, primary, transitional and secondary follicles in tissue fixed on days 0, 6, 16, 19 and 23 of culture. Data is presented as the mean follicle (a) and oocyte (b) diameter averaged over 4 culture repeats and as the mean follicle (c) and oocyte (d) diameter of all follicles (n) measured ± SEM. Mean diameters were compared across culture lengths within each follicle classification and statistically significant differences are indicated with the use of different letters ($p<0.05$). For example, in (a), the mean primary follicles diameter is significantly greater ($p<0.05$) in tissue fixed on day 0 than in tissue fixed on day 23. There is no significant difference ($p>0.05$) between the mean diameter of primary follicles fixed on days 0, 16 and 19 or between those fixed on days 16, 19 and 23. Similarly there is no significant difference ($p>0.05$) in the mean transitional follicle diameter in tissue fixed on days 16 and 19, however, the mean diameter of transitional follicles in tissue fixed on day 23 is significantly greater ($p<0.05$) than in tissue fixed on days 16 and 19. The same principle applies in (b), (c) and (d).



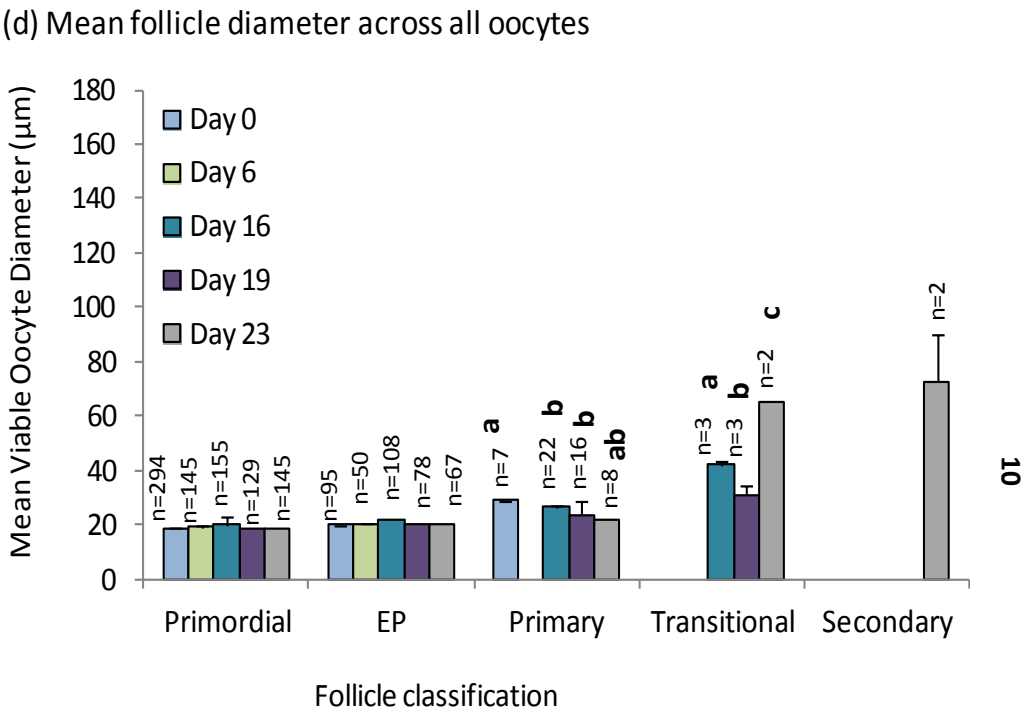


Figure 3.5 (continued)

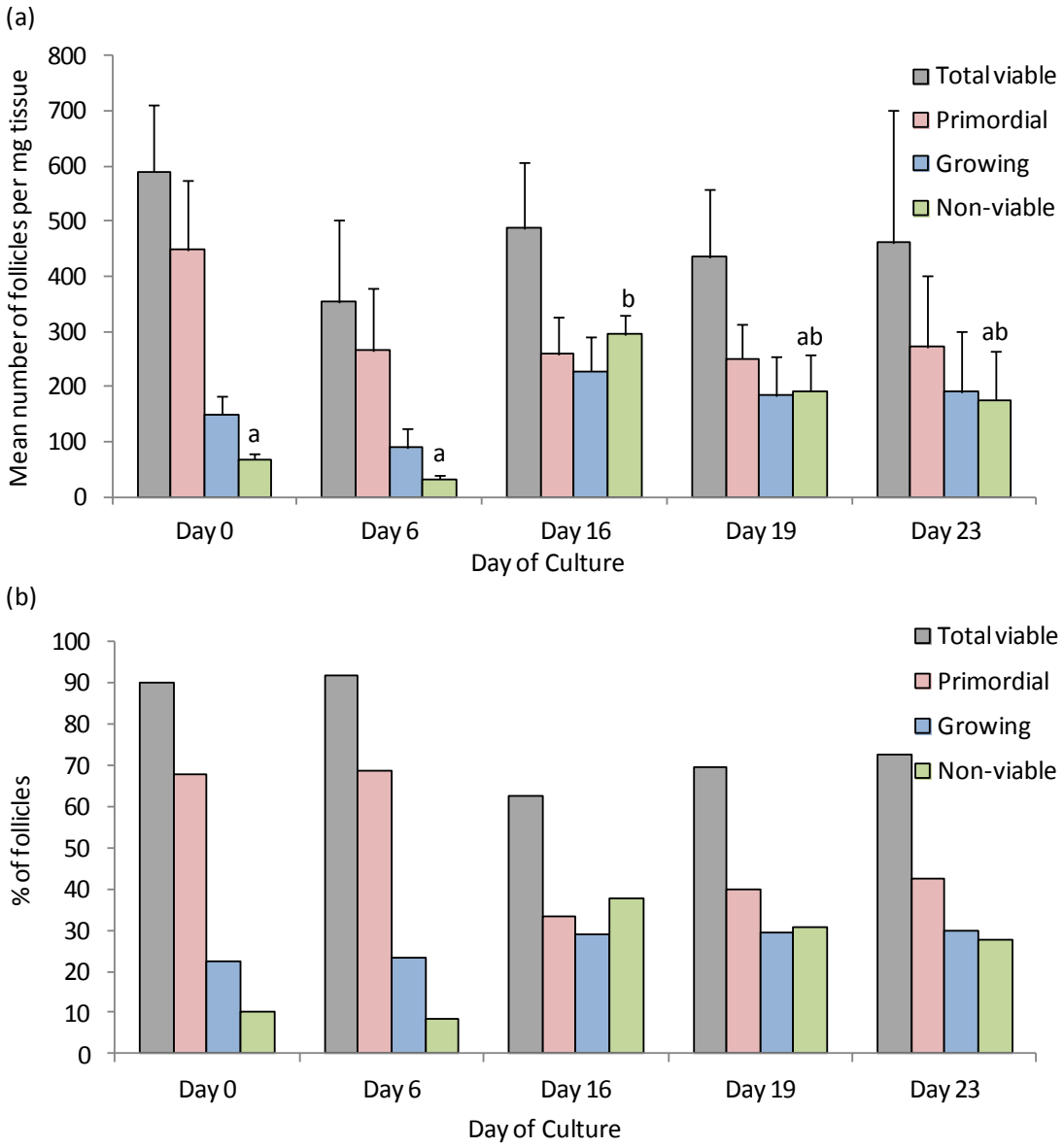


Figure 3.6 Follicle population dynamics in fixed tissue samples on days 0, 6, 16, 19 and 23 of culture. The total viable, viable primordial and growing and degenerating follicles are presented as mean number of follicles ± SEM per mg tissue at the start of culture for 4 culture repeats (a) and the % of follicles (b). The mean number of follicles within each category between days 0, 6, 16, 19 and 23 were analysed using one-way ANOVA, with Fisher's ad hoc testing. Statistical differences ($p<0.05$) are indicated using different letters. Statistically significant differences were only observed in the degenerating category. The distribution of the % of primordial, growing and degenerating follicles between days 0, 6, 16, 19 and 23 was analysed using Chi-squared analysis; there were no statistically significant differences.

The follicle population dynamics in fixed tissue samples were considered in more detail in Figure 3.7. In this figure only viable follicles were considered and the category of growing follicles was broken down into EP, primary and multi-layered (transitional and secondary). Data regarding tissue fixed on day 6 was not included as there were no follicles more developed than the EP stage at this culture point. The mean number of follicles per mg fixed

tissue is presented in Figure 3.7 (a). The number of EP follicles is similar before and after culture.

The number of primary follicles increased after long-term culture, although this increase was not statistically significant ($p>0.05$), see Figure 3.7. There were no multi-layered preantral follicles present in day 0 tissue, however, multi-layered follicles were present after 16-23 days of culture. There was no significant difference ($p>0.05$) in the number of multi-layered follicles on days 16, 19 and 23. The percentage of primordial, EP, primary and multi-layered follicles, in tissue fixed on days 0, 16, 19 and 23 is shown in Figure 3.7 (b). The percentage of primordial follicles decreased in the cultured tissue compared to the day 0 tissue, whereas the percentage of EP and primary follicles increased. The increase in EP follicles in cultured tissue appears more pronounced than in Figure 3.7 (a). Chi-squared analysis revealed that the distribution of the percentage of follicles in each classification on days 16, 19 and 23 was significantly different from the distribution on day 0 ($p<0.001$). On days 16 and 19 the higher percentage of primary follicles, compared to day 0 was the greatest contributor. In contrast, on day 23 the higher percentage of EP follicles, compared to day 0, was the greatest contributor ($p<0.001$). The greatest increase in the percentage of primary follicles was observed on day 19, 4.0%, vs. 1.8% observed on day 0. As there were no primary follicles observed in the day 6 tissue this suggests that the primary follicles present in the day 0 tissue had degenerated, thus the primary follicles observed on days 16-23 have resulted from the activation and growth of primordial or EP follicles *in vitro*. It should be noted that secondary follicles were only present on day 23 of culture; 0.7%.

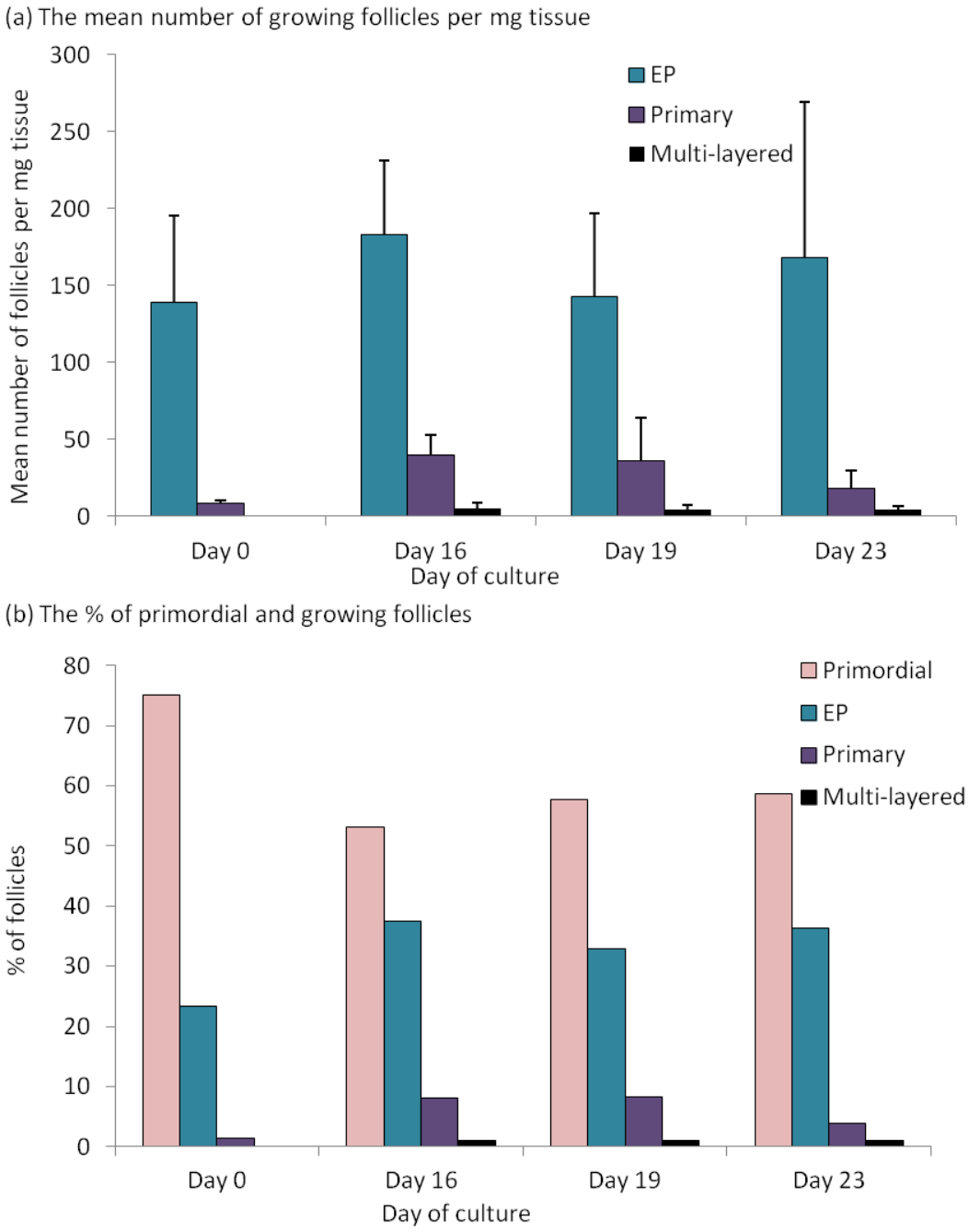


Figure 3.7 Preantral follicle population dynamics in fixed tissue samples on days 0, 16, 19 and 23 of culture. The total EP, primary and multi-layered follicles are presented as mean number of follicles ± SEM per mg tissue for 4 culture repeats (a) and the % of viable follicles of all preantral classifications (b). The mean number of primordial follicles was not shown in (a) as this was a much larger figure than that of the multi-layered follicles and therefore made the graph difficult to interpret. Furthermore, this data has already been presented in Figure 3.6. The mean number of follicles were analysed using one-way ANOVA, with Fisher's ad hoc testing. No statistical differences were observed when follicle numbers within each classification were compared at the different time points. The distribution of the % of viable follicles was analysed using Chi-squared analysis.

(ii) Isolation of follicles from cultured tissue

The mean weight of the tissue was measured and calculated at the start and end of each culture period, as shown in Figure 3.8. Following 6, 16 and 19 days of culture there were very small increases in mean tissue weight compared to the start of culture. Following 23 days of culture, however, there was a larger statistically significant ($p<0.05$) increase in mean tissue weight, from 0.0109±0.0011g to 0.0198±0.0014g for 9 culture repeats. The approximate mean follicle density in cortical slices selected for culture on day 0, determined using NR staining, was 320±209 follicles per mm^3.

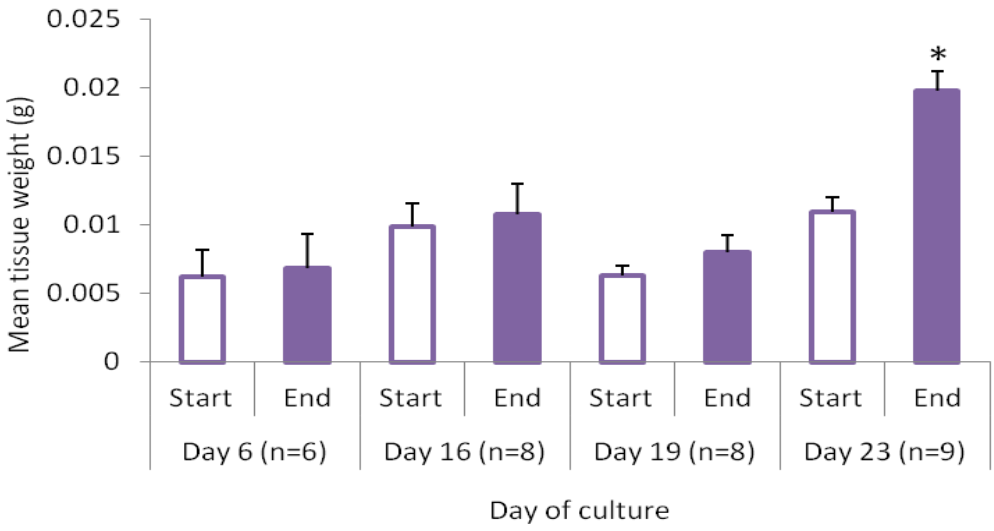


Figure 3.8 Histogram showing the mean weight of tissue at the start and end of each culture period. Data is shown as the mean weight ± SEM for the number of culture repeats indicated. Data was analysed using paired t-test to compared tissue weight at the start and end of culture within each culture duration tested. Statistical differences are denoted using *$p<0.05$. Only on day 23 was a statistically significant change in tissue weight detected.

Follicle population dynamics were also assessed following isolation from NR-stained cultured tissue, as shown in Figure 3.9. Note that in Figure 3.9 follicle counts are presented as the mean number per g, rather than mg, of tissue as the number of secondary follicles present at the end of culture per mg of tissue was <1. Therefore rather than present a proportion of a follicle, which is not very biologically relevant, follicle counts were considered per g. The primordial/ EP follicle counts are not shown for fresh tissue. This is because the tissue was warm after culture and therefore stained rapidly with NR dye such that it was difficult to discriminate between cortex staining and follicle staining. Therefore it was not possible to accurately count the number of viable primordial/ EP follicles *in situ* in tissue immediately at

the end of culture. For this reason it was not therefore possible to present the percentage of follicles present in fresh, cultured tissue. The mean number of primary, transitional and secondary follicles present on days 16, 19 and 23 in fresh tissue is shown. There were no follicles more developed than EP on days 0 and 6 of culture. There was no difference in the mean number of primary, transitional or secondary follicles after each culture duration. There were fewer primary follicles per g of tissue present on day 23, (87±31 follicles) compared to days 16 and 19, (371±101 and 272±106 follicles, respectively), although this difference was not statistically significant ($p>0.05$). This slight decrease in primary follicle numbers may have been due to either the follicles developing further or degenerating. As the transitional and secondary follicle counts are very similar on days 16, 19 and 23 of culture, it is most likely that the observed decrease in primary follicles on day 23 is most probably due to follicle degeneration.

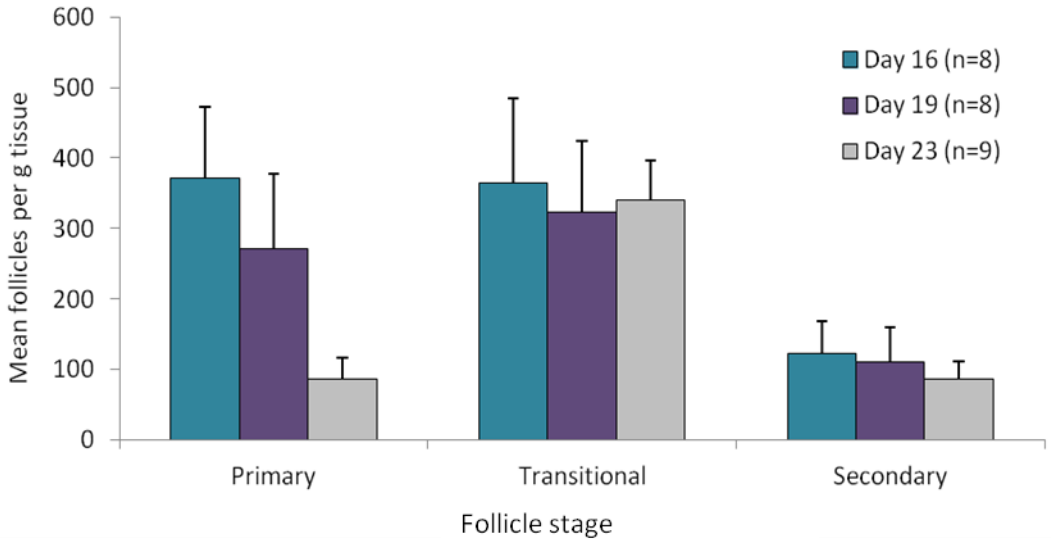


Figure 3.9 Mean number of primary, transitional and secondary follicles isolated per g of tissue cultured for 16, 19 and 23 days. Values are shown as mean ± SEM per culture repeat for the number of repeat cultures (n) shown in the key. Note that in this figure the mean number of follicles is presented per g rather than per mg of tissue. The mean number of follicles were analysed using one-way ANOVA, with Fisher's ad hoc testing. No statistical differences were observed when follicle numbers within each classification were compared at the different time points

3.3.2 Experiment 2: Preantral follicle culture using *in vitro*-derived follicles

The growth rates of *in vitro*-derived isolated EP, primary, transitional and secondary follicles over a 30 day period are shown in Figure 3.10. The percentage of follicles of each classification

surviving culture is shown in Table 3.2. In this context follicle survival was determined by the criteria for terminating culture detailed in Section 3.2.2. Secondary follicles had a mean diameter of 219.5±10.5μm on the day of isolation, i.e. day 0 of preantral culture, and reached a maximal diameter of 984.8μm (n=1) after a total of 43 days of culture (19 days cortical culture plus 24 days preantral culture). Of the 16 secondary follicles cultured 18.8% developed antral cavities. Secondary follicles exhibited the highest percentage of follicles surviving culture on day 21 of culture, although this was only 12.5%, and both attachment of the follicle to the culture surface and oocyte extrusion were very common.

Table 3.2 A summary of the percentage of follicles actively growing in preantral culture on days 7, 14 and 21.

Classification	Percentage of surviving follicles		
	Day 7	Day 14	Day 21
EP	40.0	0.0	0.0
Primary	25.6	15.4	10.3
Transitional	31.6	10.5	8.8
Secondary	43.8	12.5	12.5

The *in vitro*-derived transitional follicles (diameter 96.8±3.9μm, n=57 at the start of culture) developed to the secondary stage following isolation, however, they failed to form antral cavities after a total of 42-49 days in culture. The transitional follicles reached a maximum diameter of 207.1±49.5μm after 24 days of preantral culture, see Figure 3.10. Transitional follicles survived for a maximum of 26 days in culture (n=3). However, both attachment of the follicle to the culture surface and oocyte extrusion were more common in this classification of follicle than in primary follicles.

The *in vitro*-derived primary follicles did not develop past the primary stage in preantral culture, and only a small increase in size from 72.5±2.2μm (n=39) to 103.5μm (n=1) was observed, see Figure 3.10. Similarly the *in vitro*-derived EP follicles did not develop past the EP stage and only a small increase in diameter was observed, 53.0±2.8μm (n=5) to 68.2±10.1μm (n=2). Furthermore, EP follicles only survived to day 7 of culture, although this could be partially due to the very small number of EP follicles cultured following isolation, see Figure 3.10. Only 1 primary follicle was evident in culture after 28 days, whereas 10.3% of primary follicles survived culture up to day 21. It is worth noting that the termination of primary follicle culture was usually due to the fact that follicles stopped growing as indicated by increase in diameter rather than exhibiting increased signs of ill health.

The level of growth of *in vitro*-derived follicles after 7 days of culture is shown in Figure 3.11. Only follicles that were still viable on day 7 of culture have been included in these calculations. All 4 classifications of follicle increased in diameter after 7 days of culture. The mean increase in diameter of secondary follicles is significantly greater than that of EP, primary and transitional follicles (Figure 3.11 (a)). The mean increase in diameter of transitional follicles *in vitro* was only 17.1±3.4µm compared to a mean increase of 76.4±7.5µm exhibited by secondary follicles, which is a 4.5 fold greater increase. It is to be expected that a greater rate of growth would be observed in secondary follicles than follicles at an early stage of development due to their larger starting size and the fact that there are more somatic cells to proliferate. Therefore the percentage increase in diameter was also taken into consideration. The percentage increase in diameter of secondary follicles, 36.0±4.0%, was significantly greater ($p<0.05$) than that of the primary and transitional follicles, 16.3±4.5% and 17.0±2.9%, respectively. However, the percentage increase in diameter of EP follicles, 19.5±4.5%, was not significantly different ($p>0.05$) from that of any other follicle classification. This is likely to be due to the very low sample number (n=2) of EP follicles, rather than the EP follicles growing at as high a rate as the secondary follicles. These results show that the isolated secondary follicles exhibited the highest growth rates *in vitro*.

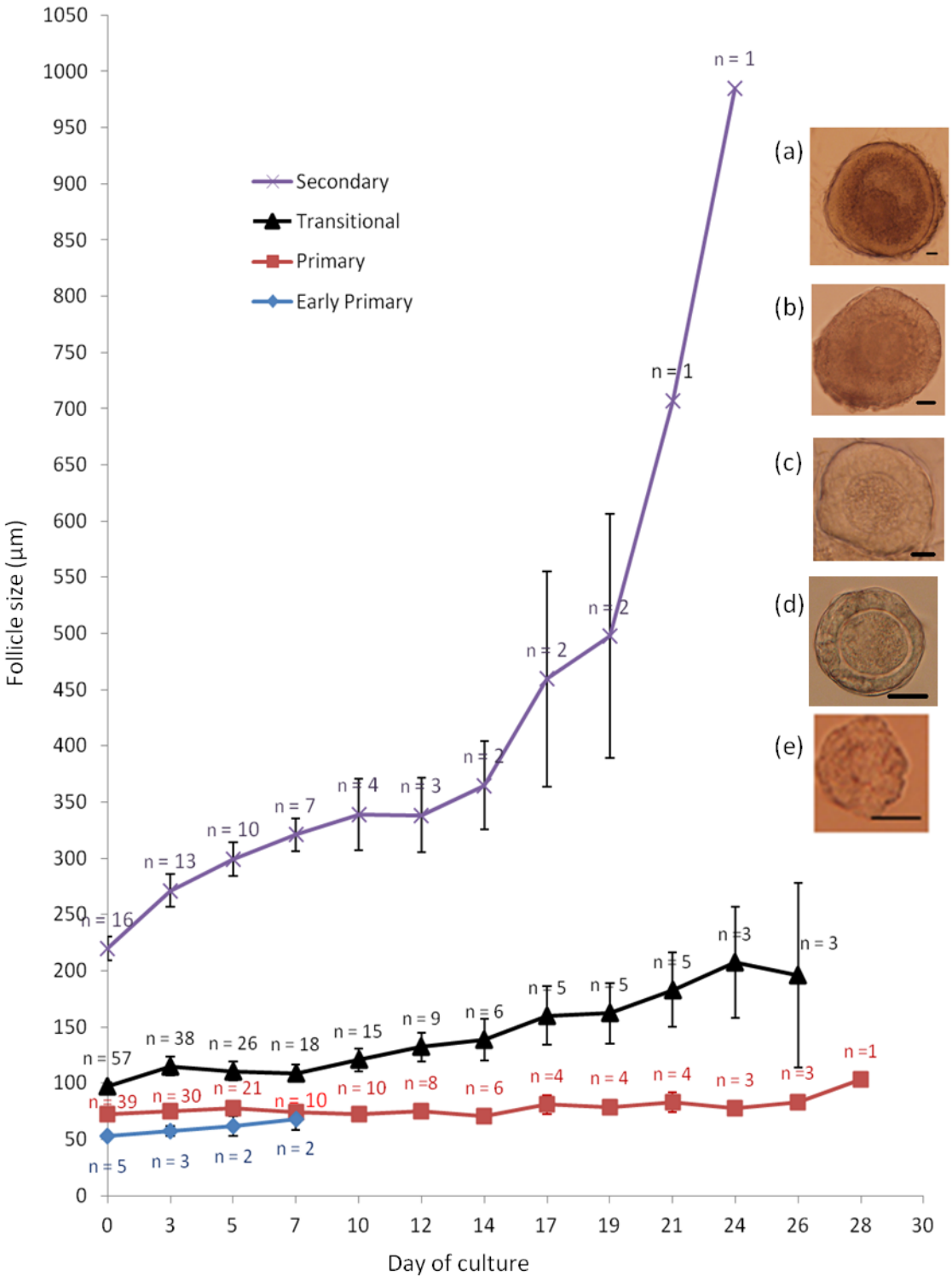


Figure 3.10 Growth rates of *in vitro*-derived preantral follicles in step 2 isolated culture over a period of 30 days. Values are presented as the mean diameter ± SEM for the number of follicles (n) shown. Representative images of an EA follicle (a) which developed from an *in vitro*-derived secondary follicle (b) following isolation and individual culture are shown, as well as images of *in vitro*-derived transitional (c), primary (d) and EP (e) follicles. Scale bar = 25µm.

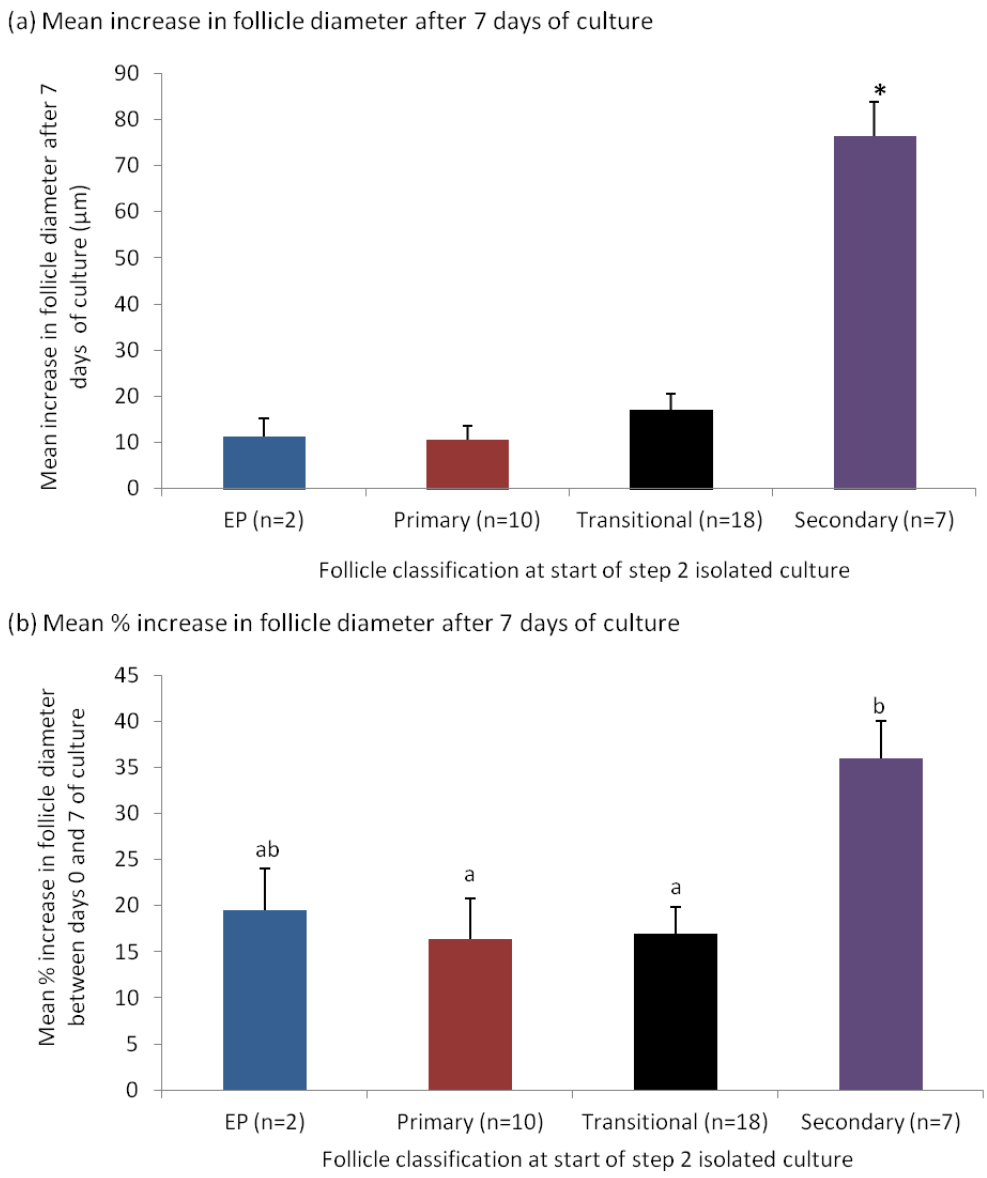


Figure 3.11 Growth of *in vitro*-derived follicles in preantral culture after 7 days of culture. (a) The mean increase in EP, primary, transitional and secondary follicle diameter after 7 days preantral culture. Values are presented as the mean diameter ± SEM for the number of cultured follicles shown. Statistical differences in the mean increase in diameter between different follicle classifications are denoted using *$p<0.05$. (b) The % increase in EP, primary, transitional and secondary follicle diameter after 7 days preantral culture. Values are presented as the mean % increase in diameter ± SEM for the number of cultured follicles shown. Statistical differences in the % increase in follicle diameter between follicle classification are denoted using different letters ($p<0.05$).

3.3.3 Preantral follicle culture using *in vitro*- vs. *in vivo*-derived follicles

In this experimental series the growth and development of *in vitro*- and *in vivo*-derived follicles was compared. As *in vitro*-derived secondary follicles exhibited the highest growth rates and were the only classification of follicle to develop to the next developmental stage over the 30

day isolated culture period then only follicles of this classification were considered in more detail. Representative images of *in vitro*- and *in vivo*-derived secondary follicles that developed to the EA stage are shown in Figure 3.12. The growth of isolated *in vitro*- and *in vivo*-derived secondary follicles over a maximum of 30 days culture is shown in Figure 3.13. Both sets of secondary follicles followed similar patterns of growth over the first 19 days of culture. On the day of isolation the mean diameters of *in vitro*-and *in vivo*-derived secondary follicles were not statistically significantly different ($p>0.05$), 219.5±10.5µm (n=16) and 217.7±2.6µm (n=85), respectively. This increased to 497.4±108.6µm (n=2) and 445.0±14.9µm (n=49), respectively on day 19 of culture; these mean diameters were not statistically significantly different ($p>0.05$). The terminal diameter of *in vitro*-derived secondary follicles was almost twice that of the *in vivo*-derived follicles, 984.8µm on day 24 (n=1) and 557.4±29.3µm on day 30 (n=24), respectively. This discrepancy, however, could be due to the fact that there was only one surviving follicle in the *in vitro*-derived group. Analysis of growth rates using ANCOVA revealed that the effect of culture length is highly significant with respect to follicle diameter ($p<0.001$). Comparison of growth rates of *in vivo*- and *in vitro*-derived secondary follicles revealed that the growth rate exhibited by the *in vitro*-derived follicles significantly higher than that exhibited by the *in vivo*-derived follicles ($p<0.05$).

As previously stated, 18.8% of the *in vitro*-derived secondary follicles formed antral cavities, whereas only 7.1% of *in vivo*-derived secondary follicles formed antral cavities. Analysis of antral cavity formation data using Chi-squared test revealed that the difference between *in vitro*- and *in vivo*-derived secondary follicles was not statistically significantly different ($p=0.132$). The slightly higher percentage of *in vitro*-derived follicles forming antral cavities, compared to *in vivo*-derived could be due to the fact that the tissue was digested using collagenase in the case of the second group of follicles, thus damaging the follicles. Alternatively the follicles isolated from fresh uncultured tissue may have already been degenerating, although they showed no morphological signs of this. Whereas the *in vitro*-derived follicles had all started to grow within the last 23 days (maximum) and therefore may have been healthier at the start of preantral culture. Although this may not be the case as the survival rate of *in vivo*-derived follicles up to day 24 is 35.3% compared to 6.3% of *in vitro*-derived follicles.

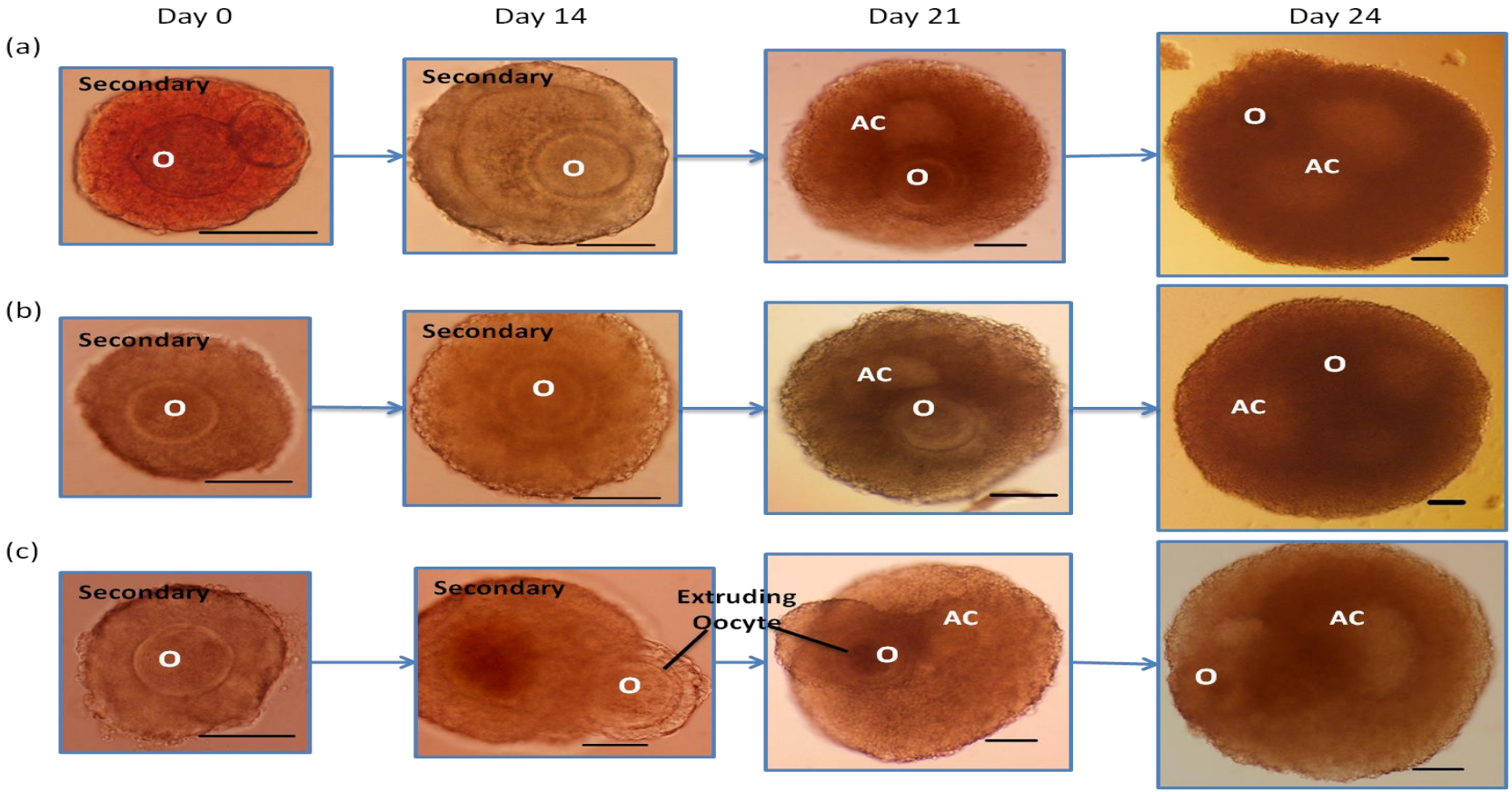


Figure 3.12 Representative images of (a) *in vitro*-derived and (b and c) *in vivo*-derived secondary follicles on day 0, the day of isolation and after 14, 21 and 24 days of culture. Follicle (b) appeared healthy throughout culture, whereas (c) began to extrude its oocyte, however, it was still competent to develop to the EA stage. The location of the oocyte and, if present, the antral cavity are indicated using the letters O and AC, respectively. Scale bar = 50µm.

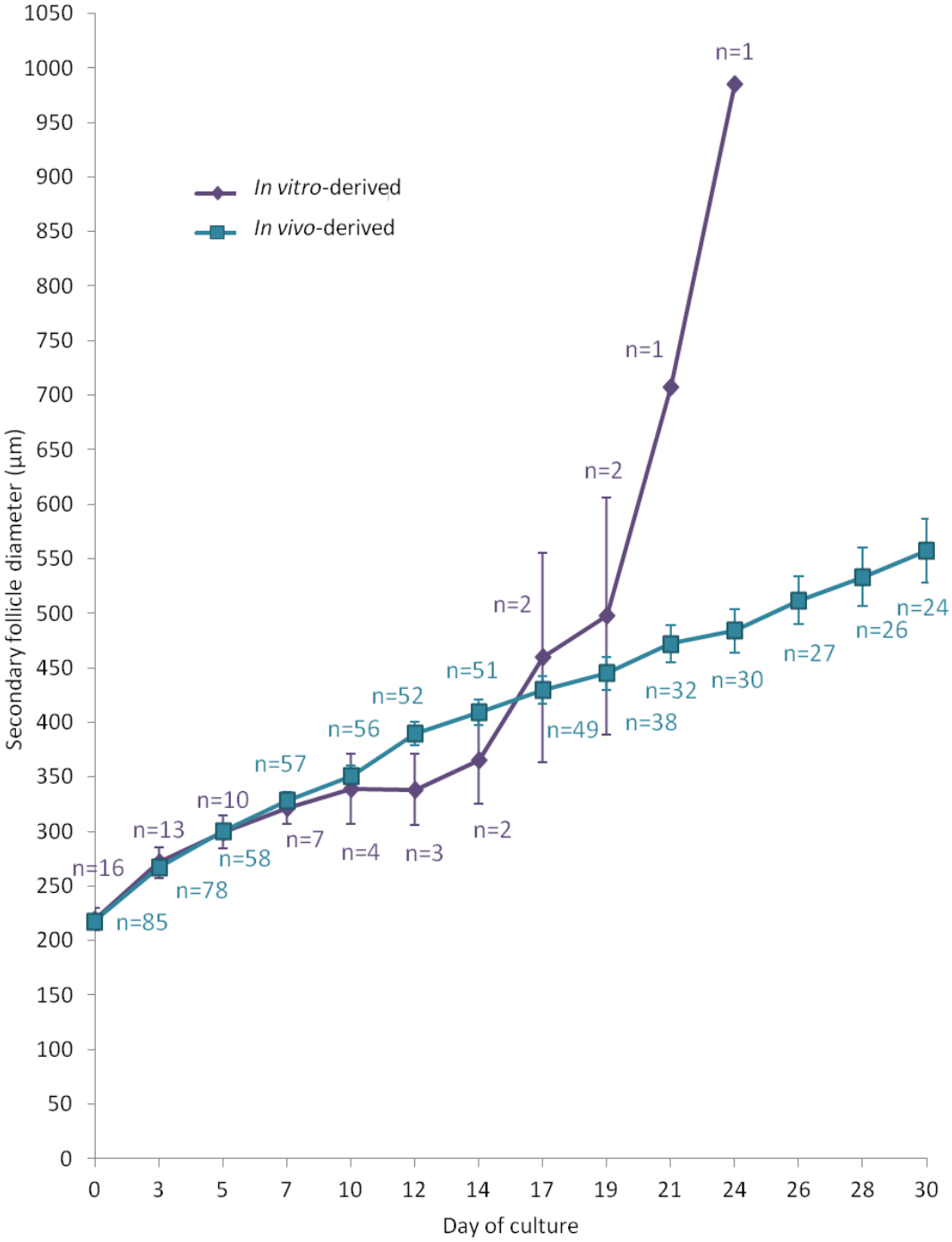


Figure 3.13 Growth rates of *in vitro*- and *in vivo*-derived secondary follicles in isolated culture for a maximum of 30 days. Values are presented as the mean diameter ± SEM for the number of follicles (n) shown. Growth rates were compared using ANCOVA; differences were not statistically significantly different ($p>0.05$).

The growth of *in vivo*- and *in vitro*-derived secondary follicles in isolated culture is shown in Figure 3.14. The mean increase in diameter Figure 3.14 (a) and the mean percentage increase in diameter relative to day 0 values Figure 3.14 (b) are shown. Day 19 was selected as the last point at which to compare growth as only one *in vitro*-derived follicle survived past this date.

There was no statistical difference ($p<0.05$) in the mean increase in diameter or the percentage mean increase in diameter between the *in vitro*- and *in vivo*-derived follicles, determined using one-way ANOVA, on days 7, 14 and 19 of culture.

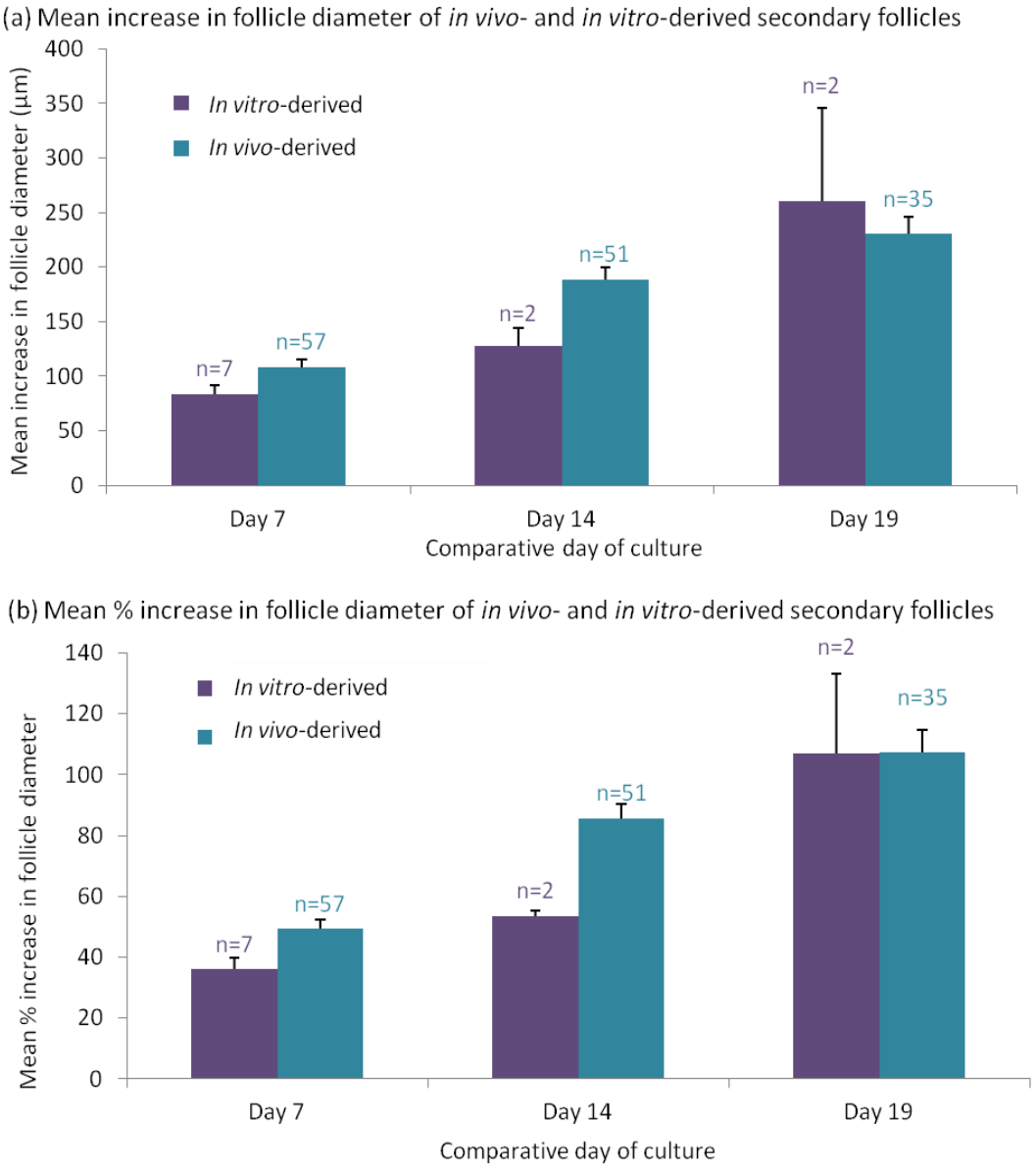


Figure 3.14 Growth of *in vivo*- and *in vitro*-derived secondary follicles in isolated culture. (a) The mean increase in follicle diameter compared to day 0 on days 7, 14 and 19 of culture. (b) The mean % increase in follicle diameter compared to day 0 on days 7, 14 and 19 of culture. Values shown as mean ± SEM for the number of follicles (n) shown on the graphs. The mean increase in follicle diameter was compared between *in vitro*- and *in vivo*-derived secondary follicles on days 7, 14 and 19, using one-way ANOVA. No statistically significant differences were observed ($p<0.05$). Similarly the % increase in follicle diameter was compared between *in vitro*- and *in vivo*-derived secondary follicles on days 7, 14 and 19, using one-way ANOVA. No statistically significant differences were observed ($p<0.05$).

3.4 Discussion

This experimental series has shown that it is possible to grow ovine oocytes from the primordial/ EP stage up to the EA stage using a two-step, serum-free culture system. This study differs from previous studies in which primordial ovine oocytes have been grown to the EA stage *in vitro* in that follicles were isolated at the secondary stage rather than being cultured up to the EA stage within cortical slices (Peng et al., 2010). A short-term culture period of 6 days was not sufficient to support the development of early preantral follicles up to the primary stage, whereas, a period of 16-23 days was sufficient to grow follicles up to the secondary stage, see Figures 3.6, 3.7 and 3.9. However, a higher number and proportion of follicles degenerated in the longer culture periods (16-23 days) than during the 6-day culture period, see Figure 3.6. Secondary follicles derived following long-term cortical culture were competent to develop up to the EA stage when isolated and cultured individually, see Figures 3.10, 3.11 and 3.12, at a rate comparable to that of *in vivo*-derived follicles.

3.4.1 Cortical slice culture – primordial follicle activation and development

The first aim of this experimental series was to compare the use of long- and short-term cortical culture systems with respect to the activation, survival, growth and development of primordial follicles. A long-term culture period of 16 days was selected initially as Chambers (2002) reported an increase in the number of primary and transitional follicles following 15 days of cortical culture. Extended culture lengths of 19 and 23 days were then tested to determine whether increasing the length of culture would increase the yield of secondary follicles, as Chambers (2002) has previously reported the presence of secondary follicles following 20 days of culture. Culture lengths were not extended past 23 days as Chambers found that increasing culture length from 20 to 25 days did not increase secondary follicle yield. The short-term culture period of 6 days was selected as development of transitional follicles from the primordial stage has been reported within this timeframe in human and cow (Telfer et al., 2008, McLaughlin and Telfer, 2010). Primordial follicle activation rates were investigated using histological evaluation of the tissue, revealing that after 6 days of culture the proportion of primordial to growing follicles was very similar to that observed in day 0 tissue (Figure 3.6 (a)). This observation is in agreement with what has been reported previously in ovine ovarian tissue in which growing follicles are not present *in vivo* (Juengel et al., 2002b, Sawyer et al., 2002). A much more accelerated rate of activation and development has been reported in some studies. For example, following the *in situ* culture of oocytes within whole newborn mouse ovaries significantly increased levels of primordial follicle activation

were observed compared to equivalent *in vivo* models (Gigli et al., 2005). Similarly in large mammal models higher levels of primordial follicle activation were observed following short-term culture periods compared to levels observed *in vivo*. Following 6 days of cortical culture the percentage of primordial/ EP follicles was reported to have decreased from around 90% to 60% in human tissue and <40% in bovine tissue (Telfer et al., 2008, McLaughlin and Telfer, 2010). The increased levels of follicular activation relative to those observed in the present study could be due to the method of cortical tissue preparation, which differed in that the tissue, prepared according to methods developed in Edinburgh (Telfer et al., 2008, McLaughlin and Telfer, 2010), was teased apart prior to culture. Culturing ovarian tissue in fragments, rather than as a whole ovary has previously been shown to result in higher rates of primordial follicle activation, possibly via the removal of inhibitory factors, such as AMH and activin A (see Section 3.1.1) (Cushman et al., 2002). Alternatively, manipulation of the tissue may result in increased exposure to stimulatory factors thus resulting in the increased levels of activation and growth observed (see Section 3.1.1). For example, teasing the tissue apart may increase the level of exposure to insulin and IGFs *in vitro*, which may promote early preantral follicle development (el-Roeiy et al., 1993). Furthermore, the shape of the tissue fragments has been linked to the level of follicular activation, with tissue trimmed into cubes exhibiting lower rates than tissue trimmed into slices (Hovatta et al., 1997, Scott et al., 2004a, Telfer et al., 2008). Therefore the effect of teasing the tissue apart may occur via a similar mechanism, but to a larger extent. Large-scale activation of primordial follicles has been reported within 2 days of cortical slice culture of bovine and baboon ovarian tissue, in studies in which the tissue has not been teased apart, however, very few follicles progressed to multi-layered stages (Wandji et al., 1996b, Wandji et al., 1997). The differences in activation rates observed in the various studies *in vitro* may also be due to species-specific differences. The number of primordial follicles activating each day *in vivo* differs between species; 1-4 in the ovine ovary (Turnbull et al., 1977, Driancourt et al., 1985); around 6 in the bovine ovary (Scaramuzzi et al., 1980); and 2-30 in the human ovary, depending on the age of the woman (Faddy and Gosden, 1995).

In the current study the total number of viable follicles present in tissue fixed on day 6 had decreased slightly, compared to that observed in tissue fixed on day 0 (Figure 3.6 (b)). This was not, however, accompanied by an increase in the number of degenerating follicles, suggesting that the day 6 tissue samples used for analysis were less follicle rich at the start of culture than the day 0 samples and not that follicle degeneration was the main cause of the decrease in the number of viable follicles. The low levels of follicular degeneration exhibited in this study, after 6 days of culture, are in agreement with those previously reported by

Chambers et al. (2010) and suggest that the culture conditions in the current study promote follicle survival to a greater extent to those used by Telfer et al. (2008) as in the latter study the percentage of degenerating human follicles had almost doubled on day 6 of culture. This could also be linked to the method of tissue preparation as this has previously been linked to follicle survival in short-term culture of human ovarian tissue, as Scott et al. (2004a) showed that culturing follicles within cubes of tissue resulted in higher rates of follicle survival after 7 days of culture. Therefore teasing tissue apart may increase levels of follicle degeneration relative to standard cortical slice preparation.

The follicle population dynamics in tissue fixed on days 16, 19 and 23 significantly differed from those observed in day 0 tissue ($p<0.001$), with respect to follicle proportions (Figure 3.6 (b)). Tissue fixed at the end of long-term culture contained a higher proportion of degenerating follicles and lower proportion of primordial follicles than tissue fixed on day 0 and 6. An increase in the proportion of growing follicles could also be observed indicating that some degree of follicle activation had occurred and therefore the decrease in proportion of primordial follicles was not solely due to follicular degeneration. However, the fact that the number of growing follicles per mg tissue was not significantly different from that observed in day 0 tissue, whereas the percentage of degenerating follicles was significantly higher suggests that follicular degeneration was a greater contributor to the decrease in primordial follicle number than primordial follicle activation (Figure 3.6 (a)). Following long-term culture the percentage of viable follicles decreased to ~60% which is similar to that reported by Peng et al. (2010). In the latter study the percentage of viable follicles decreased from 85% to ~50% in constant FSH-supplemented media, after 18 days cortical culture. When a sequential culture media was used the percentage of viable follicles on day 18 was slightly higher; ~60%. Similarly after 16 days of culture the percentage of viable follicles decreased from 90% to ~60% in caprine ovarian tissue, when FSH and growth hormone were present in the culture media (Magalhaes-Padilha et al., 2012). However, in this study a larger increase in the percentage of growing follicles was observed than in the current study, following both 8 and 16 days of culture, even in the control, gonadotrophin-free media. In this study the sequential addition of FSH and/or GH to the culture media was shown to affect follicle activation and survival. Therefore modifications to the culture media used in the current system may increase follicle activation and growth rates. In conclusion in order to increase the proportion and yield of growing follicles either a culture period longer than 6 days and/or changes to the culture conditions are required.

The effect of culture conditions on the growth and development of activated follicles was also considered. The number and proportion of EP, primary and multi-layered viable follicles, in tissue fixed on days 0, 16, 19 and 23 of culture, are presented in Figure 3.7. Both the number and proportion of primary follicles increased after long-term culture, compared to day 0 tissue. It should be noted that there were no primary follicles present in day 6 tissue suggesting that primary follicles present in tissue fixed on days 16-23 must have developed *in vitro*. Additionally, multi-layered follicles developed during long-term culture. Increasing culture length therefore increased the yield of multi-layered follicles. Increasing the duration of culture has previously been shown to increase multi-layered follicle yield in the sheep (Peng et al., 2010), goat (Magalhaes-Padilha et al., 2012), non-human primate (Wandji et al., 1997) and human (Scott et al., 2004a). Both the number and proportion of multi-layered follicles were very similar in tissue fixed on days 16, 19 and 23. Secondary follicles were, however, only present in tissue fixed on day 23 of culture, although this percentage was very low (0.7%), in comparison to the long-term culture studies in goat (23%) (Magalhaes-Padilha et al., 2012). The highest mean number of primary and multilayered follicles per mg fixed tissue was observed in tissue fixed on day 16 of culture; 40±13 (n=4) and 5±3 (n=4), respectively (Figure 3.7 (a)). The proportion of primary and multilayered follicles was similar in tissue cultured for 16 and 19 days, ~8% and 1%, respectively (Figure 3.7 (b)).

Although the speed of development of primordial follicles to the secondary stage exhibited in this study is much higher than that observed in the adult ovine ovary *in vivo* (75 days), it is similar to that observed in ovine ovarian tissue *in vivo* in the absence of growing follicles, for example in the fetal ovary (45 days), as discussed in the previous section on primordial follicle activation and development (Section 3.1.1) (Cahill and Mauleon, 1981, Juengel et al., 2002b, Sawyer et al., 2002). As the method of tissue preparation resulted in reduction in the proportion of growing follicles relative to primordial follicles this therefore resulted in an environment more similar to that experienced by the follicles in the fetal ovine ovary *in vivo*, than the adult ovary. Levels of inhibitory factors, such as AMH and activin A produced by growing follicles would be lower. In addition the low levels of primordial follicle activation and slow, physiologically relevant rate of growth observed during cortical culture in the current study are similar those which were observed when the system was developed by Chambers (2002). The development of secondary follicles, however, was not observed following 30 days in the Chambers (2002) study, unlike the current investigation. In addition Chambers (2002) found that increasing culture length past 20 days did not increase the yield of secondary follicles. These differences could be due to the use of the NR dye, which ensured all cortical

slices cultured contained a high number of primordial follicles, thus increasing the number of follicles able to develop to the secondary stage. In addition in the previous study tissue containing growing follicles, including secondary and EA follicles, was not excluded from culture. Inhibitory factors, detailed above, released by the growing follicles may have inhibited the activation and subsequent development of primordial follicles slightly. In conclusion the slightly increased rates of development relative to those observed in the adult ovine ovary, exhibited in the current study can be considered physiologically normal.

Numerous culture systems that support the activation and growth of primordial follicles at a physiological rate have been reported. Equivalent rates of follicle development from the primordial to the secondary stage *in situ* and *in vivo* in the absence of growing follicles have been observed in the culture of whole mouse ovaries, in which a period of 8 days was required (O'Brien et al., 2003). Isolated oocyte-GC complexes derived following 8 days of *in situ* culture were competent to develop and to undergo IVM, and subsequent IVF resulted in the birth of live offspring. Similarly secondary follicles derived via the *in situ* culture of primary follicles within whole mouse ovaries over a physiologically relevant timeframe of 4 days exhibited high survival and antrum formation rates of 74% and 72%, respectively, following isolation and 12 days culture within a fibrin-alginate hydrogel (Jin et al., 2010). Subsequently 88% of *in vitro*-derived secondary follicles developed to the MII stage and were fertilised resulting in 54% reaching the endpoint 2-cell embryo stage. Hornick et al. (2012) reported the use of alginate hydrogels to support the survival and growth of early preantral follicles, within a physiologically relevant timeframe. Higher concentrations of alginate (2%) were shown to be optimal with respect to follicle survival, as has also been demonstrated with human primordial follicles (Amorim et al., 2009). Alginate has also been used as scaffold for human cortical slices in a system that supported the activation and growth of primordial follicles to the transitional stage after 2 weeks culture (Kedem et al., 2011).

A previously mentioned a 2-step serum-free culture system has been developed which supports the *in vitro* growth of oocytes from the primordial to the EA follicle stage in both the bovine and human (Telfer et al., 2008, McLaughlin and Telfer, 2010). Step 1 is described above. In step two *in vitro*-derived transitional follicles were then isolated and cultured individually. The yield of multi-layered follicles achieved in the current investigation is much lower than has been reported in studies utilising accelerated culture systems in which the percentage of multi-layered follicles increased from 0% to 10% in bovine (McLaughlin and

Telfer, 2010); and 15% in human (Telfer et al., 2008), in 6 days. Note that this rate of development is highly accelerated even compared to that observed *in vivo* in the fetal bovine ovary in which development from the primordial to the primary stage takes 68 days (Nilsson and Skinner, 2009). It is however, difficult to directly compare the results in these studies as the published data presented only relates to the proportion of viable follicles (Telfer et al., 2008, McLaughlin and Telfer, 2010). Therefore it is difficult to determine whether the apparent larger increase in the proportion of growing follicles compared to that reported in the current experimental series, is due to the primordial follicles activating or a large number of primordial follicles degenerating. In the current experimental series the health of the stromal tissue was assessed using tissue weight as a marker. Tissue weight had consistently increased at the end of the cortical culture period, to a significant extent by day 23 ($p<0.05$), see Figure 3.8. Maintenance of the health and integrity of stromal tissue is of great importance to the continued development of follicles and oocytes as it provides a scaffold, without which oocyte-somatic cell interactions would be compromised. In addition receptors and growth factors expressed by ovarian stromal cells are required for primordial follicle activation, such as c-kit, and development, such as BMP4 and BMP7 (Knight and Glister, 2006). The health of the stromal tissue following accelerated culture was not reported in the aforementioned studies (Telfer et al., 2008, McLaughlin and Telfer, 2010). Therefore one of the aims of the future work conducted in this thesis will be to compare the use of accelerated and physiological culture systems with respect to the health of stromal tissue.

The composition of culture media used has been shown to affect follicle yield (Peng et al., 2010, Scott et al., 2004a). For example, addition of GDF-9 to the media increased the yield of human primary follicles from 13% on day 0 to 53% after 7 days, compared to 31% in the GDF-9-free media (Hreinsson et al., 2002). It is important, however, to note that the effect of culture media composition is species-specific. Therefore it may be possible to increase follicle yield by preparing the tissue using a different method and/ or by the addition of growth additives to the culture media. However, as discussed previously, large-scale activation of primordial follicles and accelerated growth rates may be detrimental to the health of *in vitro*-derived oocytes.

In contrast to the fixed tissue, secondary follicles were detected in the tissue used to isolate follicles after 16, 19 and 23 days of culture (see Figure 3.9). When follicle population dynamics were examined in fresh tissue the numbers of transitional and secondary follicles per g tissue

were similar on days 16, 19 and 23. A slight decrease in primary follicles was observed on day 23 and as this was not accompanied by an increase in the number of transitional and secondary follicles this was probably due to follicular degeneration, rather than progression of primary follicles to later preantral stages.

The results of the current experimental series show that this serum-free culture system is able to support the activation and development of primordial follicles up to the secondary stage within a culture period of 16-23 days. In addition this culture system is able to support the growth and survival of the ovarian stromal tissue as shown by the increase in tissue weight following culture (see Figure 3.8).

3.4.2 Preantral follicle culture using *in vitro*-derived follicles

The second aim of this experimental series was to determine whether follicles grown *in situ* within ovarian cortex, in physiological, ECM- and serum-free media, were competent to develop up to the EA stage once they were isolated and cultured individually. As mentioned earlier 18.8% of *in vitro*-derived secondary follicles were competent to develop to the EA stage. A similar proportion (~25%) of *in vivo*-derived secondary follicles were previously reported to have formed antral cavities in the presence of FSH and LH, or FSH alone (Newton et al., 1999b). This shows that the health of the *in vitro*-derived follicles was not affected by the cortical culture conditions enough to prevent development to the EA stage. Developmental competence was shown to be related to the developmental stage at the start of isolated culture, in that the more developed follicles were more likely to survive isolated culture and develop to later stages than the less developed follicles, in agreement with former studies in mouse (Kreeger et al., 2005); sheep (Newton et al., 1999b); and human (Hovatta et al., 1997). Antral cavity formation in *in vitro*-derived secondary follicles occurred around day 14 of isolated culture. This is also comparable to that reported in a previous studies in which *in vivo*-derived secondary follicles formed antral cavities around day 14 of culture (Newton et al., 1999b). These results are in agreement with those observed in the ovine fetal ovary as development from the secondary to EA stage has been reported after 15 days (Juengel et al., 2002a). The increased rates of development from the secondary to the EA stage, compared to that observed in the adult ovine ovary *in vivo* may be a result of the addition of FSH to the media as increased rates of development have also been observed in the autografted ovine and human ovarian tissue, as detailed in Section 3.1.1 (Oktay et al., 1998, Campbell et al., 2000).

In the current study although *in vitro*-derived transitional follicles were competent to develop to the secondary stage in isolated culture, these follicles degenerated prior to progression to the EA stage. This is in agreement with previous findings that there is no advantage to isolating follicles prior to the secondary stage of development and that smaller follicles require some form of support matrix in order to survive and develop in isolated culture (Hovatta et al., 1997, Hovatta et al., 1999). In contrast, the development of *in vitro*-derived transitional follicles to the EA stage has been reported in both human (Telfer et al., 2008) and bovine (McLaughlin and Telfer, 2010) after 4 and 12 days, respectively. The rate of development reported in human follicles *in vitro* is highly accelerated compared even to that observed *in vivo* in the adult human ovary in the presence of increased FSH stimulation, in which development from the primordial to the EA stage required 17 weeks (Oktay et al., 1998). The accelerated growth timeframe reported in these papers and the fact that the culture system was able to support follicular development from the transitional stage is probably due to the addition of activin A to the culture media. In the current study *in vitro*-derived secondary follicles also exhibited a significantly greater increase in diameter than EP, primary and transitional follicles, after 7 days isolated culture (see Figure 3.11).

In vitro-derived secondary follicles exhibited the highest survival rates in this study, with 12.5% still actively growing on day 21 of step 2, isolated culture (see Table 3.2). Loss of normal follicle morphology was often observed with follicles attaching to the culture surface and/or extruding their oocytes (see Figure 3.3), in transitional and secondary follicles, although, interestingly these phenomena were not observed in EP and primary follicles. As mentioned above the termination of culture of *in vitro*-derived EP and primary follicles was mainly due to follicles deemed not be growing when no increase in diameter, over 3 consecutive media changes, was observed. Researchers that advocate the use of accelerated culture systems suggest that shorter culture duration allows less time for the breakdown of oocyte-GC interactions to occur, therefore the maintenance of normal morphology is easier (McLaughlin et al., 2010). Follicle attachment and oocyte extrusion, however, were most commonly observed during the first 5 days of culture, and follicles that had maintained a normal morphology up to that point continued to do so. Alternatively, as shown in Figure 3.12 (c), follicles that initially exhibited signs of oocyte extrusion were occasionally able to 'repair' themselves as somatic cells continued to proliferate. This suggests that altering the culture length would not improve follicle survival rates rather it is more likely that damage caused during the mechanical isolation process resulted in the loss of normal follicle morphology and

therefore altering techniques used at the isolation stage may be more beneficial. Therefore the current preantral follicle system is best able to support the development of secondary follicles *in vitro* rather than early stages of follicle development.

3.4.3 Preantral follicle culture using *in vitro*- vs. *in vivo*-derived follicles

The third aim of this experimental series was to compare the growth, development and survival rates of *in vitro*- and *in vivo*-derived follicles. Only secondary follicles were used in this comparison as these were the only follicles competent to develop to the EA stage. The growth rates exhibited by *in vitro*- and *in vivo*-derived secondary follicles were similar, as shown in Figures 3.13 and 3.14. The average terminal diameter of *in vitro*-derived secondary follicles was much larger than that of *in vivo*-derived secondary follicles however this was probably due to the very low sample number of *in vitro*-derived follicles. As previously stated the rate of antral cavity formation was higher but the survival rate lower in *in vitro*-derived follicles than *in vivo*-derived follicles. The lower rate of antral cavity formation in *in vivo*-derived follicles may be due to damage caused by collagenase digestion of the tissue prior to isolation (Telfer, 1996, Abir et al., 2001).

In the current study both oocyte extrusion and attachment of follicles to the culture surface were frequently observed. One way in which to avoid the loss of normal follicular morphology during preantral follicle culture is to use hydro or alginate gels as a matrix to support the follicles. Gels have successfully been utilised to support the maintenance of the morphology of isolated preantral follicle *in vitro* in the mouse (Kreeger et al., 2005, Kreeger et al., 2006, Jin et al., 2010) and in larger mammalian species including; dog (Songsasen et al., 2011); pig (Hirao et al., 1994); cow (Harada et al., 1997, Itoh et al., 2002); buffalo, (Sharma et al., 2009); non-human primate (Xu et al., 2009, Xu et al., 2011a, Xu et al., 2011b, Hornick et al., 2012); and human (Amorim et al., 2009). The exact effect of the use of gels, however, has not been entirely elucidated, and therefore may be detrimental to oocyte health and developmental competence. Indeed although embedding murine secondary follicles in a gel resulted in the maintenance of normal follicular morphology, with respect to a centrally located oocyte and GC-oocyte interactions the composition of the gel was shown to affect the incidence of antral cavity formation which was delayed when gels were composed of laminin and fibronectin, compared to alginate (Kreeger et al., 2006). Additionally in this study components of the laminin and fibronectin gel were shown to block oestrogen and inhibin secretion. Detrimental effects of using alginate gels to culture murine secondary follicles have also been observed as

the *in vitro*-derived MII oocytes exhibited increased rates of spindle formation and chromosomal alignment defects as well as compromised developmental competence compared to controls (Mainigi et al., 2011). At the end of culture removal of follicles from the gel can also be problematic and result in the loss of healthy follicles (Desai et al., 2010). An alternative to the use of gels is the use of a rotation system to prevent follicular attachment, however, the stress placed on the follicles in this system can result in mechanical damage resulting in the loss of GC-oocyte contacts (Rowghani et al., 2004).

In the current investigation the culture system utilised was able to support the growth and development of both *in vivo*- and *in vitro*-derived ovine secondary follicles up to the EA stage at a physiologically relevant rate. Barboni et al. (2011) have recently reported a system capable of supporting the *in vitro* growth of ovine preantral follicles from 170µm to 340µm and the development of 70% to the EA stage within a physiologically relevant timeframe of 14 days *in vitro*. The mean diameter of oocytes derived using this system was found to be comparable to that of those derived *in vivo* (~120µm). Although the rate of antral cavity was higher than reported in the current study with respect to secondary follicles derived both *in vivo* and *in vitro* serum was included in the culture media and therefore may have been detrimental to the health of the oocytes. Physiologically relevant rates of preantral follicle development have also been reported following mouse (Jin et al., 2010); cow (Gutierrez et al., 2000); non-human primate (Xu et al., 2009, Xu et al., 2011a); and human (Abir et al., 1997) preantral follicle culture. The continuous growth of non-human primate preantral follicles from ~250µm to ~1mm and normal steroidogenic function were supported *in vitro* for 30 days (Xu et al., 2009). Some of the examples given above will be compared to accelerated culture systems below.

Acceleration of preantral follicle growth *in vitro* has been achieved via the inclusion of high concentrations of FSH in the follicle culture media. This was demonstrated using baboon preantral follicles (~285µm), which were cultured within an alginate gel in the presence of 10miUml^{-1} FSH and subsequently required only 10 days to reach the same diameter as follicles cultured in the absence of FSH for 14 days (Xu et al., 2011b). The addition of FSH was, however, shown to be detrimental to oocyte health as upon removal from the COCs oocytes derived in the presence of FSH were smaller than those derived in the absence FSH. Additionally oocytes in the latter group had at least 1 layer of cumulus cells, unlike those in the former group, which were denuded. Whereas 13 of the 16 COCs derived in the absence of FSH

were competent to undergo GVBD, 2 of which formed MII oocytes displaying normal spindle and chromosome alignment, none of the COCs derived in the presence of FSH were competent to resume meiosis. Addition of high levels of FSH (≥20ngml^{-1}) to the culture media of mouse COCs resulted in significantly higher levels of aneuploidy in IVM MII oocytes, compared to oocytes matured in the presence of lower concentrations of FSH (Roberts et al., 2005). Although FSH was added to the preantral culture media in the current study this was at a concentration 1000 fold lower than that used by Xu et al. (2011b), this more physiologically relevant concentration of FSH resulted in more physiologically relevant rates of growth and may also result in oocytes displaying higher rates of meiotic competence. Furthermore, in the study conducted by (Xu et al., 2011b) follicles were encapsulated within an alginate gel and therefore there may be components of the gel interacting with the high concentrations of FSH that resulted in the detrimental effects on oocyte health. In a study by the same group macaque preantral follicles (125-225µm) were cultured within an alginate gel and the effect of FSH concentration on follicle growth and survival demonstrated (Xu et al., 2011a). Although survival rates of follicles were highest when high concentrations of FSH, in the presence of fetuin, were used, growth rates were highest in the presence of low FSH, without fetuin. As the concentration of FSH is not presented in international units in this study it is unclear how the concentrations compare to those used in the aforementioned study (Xu et al., 2011a, Xu et al., 2011b). The level of steroid hormone production by follicles increased with increased FSH concentration in the culture media. Of the *in vitro*-derived oocytes 12.5% were competent to develop to the MII stage following IVM, whereas no follicles cultured in the presence of high FSH concentrations were competent to do so, unless fetuin was also included in the media, in which case 4% of oocytes developed to the MII stage. Therefore the use of high FSH concentrations to accelerate follicle growth and development may be detrimental to oocyte developmental competence.

The rate of antral cavity formation can also be accelerated via the inclusion of FSH or growth factors in the culture media. The addition of FSH, EGF1 or IGF1 to the culture media of mechanically isolated bovine preantral follicles (~165µm) increased the probability of antral cavity formation from 20% to 50% during 28 days culture (Gutierrez et al., 2000). In the presence of FSH 68% of follicles formed full antral cavities and only 16% degenerated following 28 days culture. Antral cavity formation is observed when preantral follicles reach 120-160µm *in vivo* and the whole process from start to full antral cavity formation takes 42 days (Lussier et al., 1987). Therefore the high rates of antral cavity formation observed in the aforementioned study, conducted by Gutierrez et al. (2000), could be due to the fact that at the start of culture

the preantral follicles had reached the maximal diameter prior to antral cavity formation. In a subsequent study in which bovine preantral follicles were cultured within a collagen gel highly accelerated rates of development were reported upon the addition of FSH, LH and insulin to the culture media with antral cavity formation observed following 3 days (7%) and 5 days (30%) of culture (Itoh and Hoshi, 2000). Accelerated rates of antral cavity formation have also been reported following the *in vitro* growth of mechanically isolated porcine preantral follicles, which increased in size from 300µm to 500µm, as 90% exhibited antral cavity formation following only 3 days culture, in the presence of serum, FSH and LH (Wu et al., 2007). Additionally >50% of oocytes derived from the COCs were competent to develop to the MII stage of which 5-10% developed into blastocysts following IVF. Comparable rates of meiotic maturation have been observed in porcine preantral follicles grown in collagen gels *in vitro* within a more physiologically relevant timescale of 16 days, with 60% of oocytes that reached 110µm maturing to the MII stage following IVM (Hirao et al., 1994). The number of oocytes which reached 110µm following 16 days culture was, however, very low, only 15/486 follicles cultured, although 19% of follicles formed antral cavities. As discussed in detail in the Section 3.1.2 the inclusion of activin A in the culture media has also been shown to promote follicle survival and accelerate the rate of preantral follicle development to the EA stage in the cow (McLaughlin et al., 2010); goat (da Silva et al., 2013); and human (Telfer et al., 2008).

Survival and antral cavity formation rates in this study are lower than those that have been previously reported for *in vitro*-derived bovine and human preantral follicles (Telfer et al., 2008, McLaughlin et al., 2010). Telfer et al. (2008) hypothesised that the high rates of follicle survival compared to other studies could be due to the homogeneity of the follicular population at the end of cortical culture, with respect to the health of the follicles. When isolating follicles from fresh tissue some follicles may have started to degenerate but are not yet displaying morphological signs of this and therefore are selected for culture. Follicles that have activated within the last 6 days are less likely to have started to degenerate. Similarly follicles that have activated during long-term culture may be more likely to have started to degenerate.

The results of this experimental series show that the rate of growth and antral cavity formation of *in vitro*-derived secondary follicles is comparable to that of *in vivo*-derived counterparts. These results suggest that in order to increase survival, growth and antral cavity formation rates culture conditions, rather than the derivation of the follicles need to be

altered. However, as mentioned earlier, a higher proportion of follicles derived from fresh tissue or following long-term culture may be degenerating, compared to short-term culture. Therefore if cortical culture conditions can be altered to support follicle development to the secondary stage within an accelerated timeframe of 6 days this hypothesis can be tested. This will be the aim of Chapter 4.

A physiologically relevant culture system is a useful tool for the study of follicle population dynamics and has the potential to advance the therapeutic application of follicle culture as a means to both treat female infertility and to conserve the fertility of young cancer patients. Indeed, Chambers (2002) utilised her system to investigate the effects of ovine ovarian tissue cryopreservation on subsequent follicle survival and development. In addition this system has been utilised to investigate the effects of the cytotoxic chemotherapeutic chemical, cisplatin on ovine follicle population dynamics (Gunson, 2009). As the culture system used does not support the mass activation of primordial follicles it was possible to evaluate the effect of cisplatin on the follicular and stromal health revealing detrimental effects on both as exposure to high levels of cisplatin resulted in increased levels of both follicle degeneration and lactate dehydrogenase (LDH) production. The development of this system also enabled the investigation of the use of viability dye, NR. One of the major obstacles of developing a cortical culture system is the fact that follicles are not evenly distributed throughout the ovary. Although the population of primordial follicles is most dense in the ovarian cortex the distribution is still highly uneven therefore without any way of visualising the follicles this can often lead to the culture of 'empty' tissue containing very low numbers of follicles, or no follicles.

Oocyte and follicle development are long and protracted processes *in vivo*. Whether it is necessary for *in vitro* systems to follow a physiologically relevant timescale or if a much more accelerated system would be suitable is a point of discussion amongst research groups working in this field. As mentioned previously the growth and development of both bovine and human primordial follicles, from the primordial to the transitional stage has been achieved after a culture period of 6 days (Telfer et al., 2008, McLaughlin and Telfer, 2010). In these studies researchers claim that physiologically relevant, 'extended' culture periods are not necessary, and that they may in fact be detrimental to oocyte health due to increased exposure to suboptimal conditions *in vitro*. Telfer et al. (2008) suggest that the use of a short-term culture period is preferable to extended culture periods as this may result in lower proportion of

atretic follicles at the end of culture. Additionally in an extended culture system the resultant preantral follicles may have activated at different times, the use of an accelerated culture system would reduce the difference in the length of time for which follicles had been developing thus resulting in a more homogenous follicle population.

Concerns have been raised regarding the health of oocytes derived from systems in which follicle growth is so greatly accelerated relative to the normal developmental time frames *in vivo* (Picton et al., 1998). Some researchers argue that the use of physiologically-relevant culture periods is key to producing healthy *in vitro*-derived oocytes, as this would allow sufficient time for the oocytes to develop the molecular machinery required for normal development (Picton et al., 1998). Research into the nuclear maturational competence of nuclei obtained from non-growing oocytes has shown that following transfer to a fully-grown GV oocyte the nuclei are competent to complete meiotic maturation, be fertilised, develop up to the blastocyst stage and implant (Kono et al., 1996). Following implantation however, embryos were not competent to develop to term. Subsequent studies have revealed that this is due to the absence of epigenetic modifications, which are established prior to the oocyte's acquisition of competence to resume meiosis I, and are essential for the establishment of the maternal imprint and for subsequent normal embryonic development (Obata et al., 1998, Bao et al., 2000, Obata and Kono, 2002). These epigenetic modifications are established gradually throughout specific stages of oogenesis and they are thought to differ in juvenile and adult mice (Bao et al., 2000). Therefore there are concerns that the use of an accelerated culture system may result in oocytes with aberrant imprinting as there will be insufficient time for the correct establishment of epigenetic modifications and this may result in resultant embryos being incompetent to complete normal development.

Smitz et al. (2010) have recently introduced the hypothesis that the process of oocyte development *in vivo* is not as protracted as originally thought, and that development may not be a continuous process, rather a start-stop process. These authors suggest that pauses in follicle development *in vivo* may be caused by presence of inhibitory factors, such as those expressed by follicles either at the primordial (Da Silva-Buttkus et al., 2009), secondary (Mizunuma et al., 1999) or Graafian stage. Spatial analysis of follicle activation dynamics in mice has led to the conclusion that primordial follicles produce a diffusible factor or factors that inhibit the activation of neighbouring primordial follicles (Da Silva-Buttkus et al., 2009). This was evidenced by the fact that follicles with neighbouring primordial follicles were less

likely to activate compared to those without. Although the identity of these factors is yet to be elucidated the authors have proposed the following possible candidates: TGF-β1 and TGF-β2; or BMP binding proteins/ antagonists of stimulatory factors such as: follistatin, noggin, gremlin and myostatin. Subsequent research has ruled out follistatin and noggin as candidates, but highlighted the possible roles of chordin and Sclerostin domain containing 1 (Sostdc1) as paracrine factors capable of inhibiting follicle activation (Fenwick et al., 2011).

It is well established that factors produced by growing follicles inhibit the growth of neighbouring follicles. The GC-derived factor, AMH, is produced by the granulosa cells of growing follicles and has been shown to be an inhibitor of the activation of surrounding primordial follicles (Durlinger et al., 1999). Co-culture of small preantral follicles with secondary follicles revealed that activin A, produced by secondary follicles caused small preantral follicles to become dormant, a state that could be reversed by the removal of either the secondary follicles and/or activin A, or the addition of activin binding protein, follistatin (Mizunuma et al., 1999). Therefore follicles that have become dormant can continue their development upon removal of inhibitory factors. Inhibin and oestrogen are also examples of systemic inhibitory factors produced by growing follicles as these factors inhibit FSH release by the pituitary gland (Wildt et al., 1981, Rivier and Vale, 1989, Molter-Gerard et al., 1999). The increased levels of inhibitory factors present in the ovary *in vivo* compared to *in vitro* may explain why the process of follicle development is a more prolonged process *in vivo*. The removal of these inhibitory influences *in vitro* may result in large-scale primordial follicle activation followed by continuous growth and development rather than the start-stop process proposed to occur *in vitro* (Smitz et al., 2010). The pauses in follicle development may, however, be vital to ensure the health of resultant oocytes. Evidence that this may not be the case was presented in a study in which the rate of murine follicle development was accelerated *in vivo*, via the suppression of PTEN and subsequent promotion of the PI3K pathway resulted in an increased litter size (Reddy et al., 2008). As discussed in Chapter 1, PTEN is a negative regulator of primordial follicle activation via its antagonism of the PI3K-Akt inhibition of FOXO3 action (Castrillon et al., 2003). This supports the theory that accelerating the process of follicle development does not affect the competence of resultant oocytes with respect to their ability to be fertilised and subsequently develop into live offspring (Fan et al., 2008).

Comparisons of murine oocyte culture lengths have shown that increased culture length; 21 days compared to 11 days, resulted in increased birth weights of the resultant pups, which authors suggest could be due to epimutations (Obata et al., 2007). It is important to note that in this study the follicles cultured for 21 days were at the primordial stage at the start of culture, whereas the follicles cultured for 11 days were at the secondary stage at the start of culture. Therefore although the authors attribute both the increased birth weights as well as the decreased maturation and fertilisation rates of the IVG oocytes to the use of an extended length of culture, the combined period of growth *in vitro* and *in vivo* in both groups is the same therefore it is likely that the increased length of exposure of oocytes to suboptimal culture conditions rather than the overall time period of follicle growth was the cause of these problems. Minimal research has been conducted directly comparing the effects of altering the duration of culture on the developmental IVG oocytes, in large mammals and humans. Therefore in this experimental series the length of *in situ* cortical culture period was varied and the effect of the duration of culture on the yield, health and subsequent developmental competence of the resultant oocytes compared.

3.4.4 Future work

In order to further investigate the health and developmental competence of the *in vitro*-derived follicles it would be interesting to measure and compare the amount of oestrogen and progesterone produced by antral follicles grown to the amount produced by those derived i*n vivo*, in order to determine whether the induction of normal steroidogenic activity is supported following incubation with testosterone, as has been shown previously by Newton et al. (1999b). In addition, the expression patterns of key genes required for the growth and development of oocytes should be compared between *in vivo*-derived EA follicles and EA follicles derived from secondary follicles *in vitro.* Further work should be conducted to facilitate analysis of the gene expression patterns of EA follicles derived from the primordial stage *in vitro*. Although it was possible to isolate secondary follicles from cortical tissue following 16-23 days culture it may be interesting to examine the effects of briefly incubating the tissue with low levels of collagenase to soften the tissue more as this may reduce the level of damage caused by follicle isolation. It is, however, possible that this method may be more detrimental to follicle health than using solely mechanical means of isolation. Alternatively, different types of enzyme, such as Liberase, may be less aggressive and detrimental to oocyte health (see Section 1.7.2), and can be used either alone or in combination with collagenase, to

aid the isolation of *in vivo*- and *in vitro*-derived secondary follicles and determine the effect on their subsequent survival and growth rates (Dolmans et al., 2006, Kristensen et al., 2011).

In some of the aforementioned studies follicle count data is only presented in terms of proportions (Telfer et al., 2008, McLaughlin and Telfer, 2010), however, this can be misleading and can largely exaggerate small changes in follicle population dynamics, particularly when total follicle counts are low. Therefore in the current study follicle counts were presented using two different methods; the data presented as either mean follicle number per mg tissue ± SEM or as a percentage. This is an accurate and informative way of displaying follicle counts as the values obtained using each method can be compared. The method of cortical tissue preparation used can result in the culture of tissue pieces of varying sizes and thicknesses and therefore follicle number can be highly variable this is why it was necessary to standardise follicle counts per mg tissue culture, rather than presenting absolute follicle numbers, which varied widely between cultures.

3.5 Conclusions

This experimental series has shown for the first time that it is possible to grow ovine oocytes from the primordial/ EP stage up to the EA stage using two-steps of a serum-free culture system, within a physiologically relevant timeframe. In order for *in vitro*-derived follicles to develop to the EA stage they had to be at the secondary stage at the start of preantral culture. In this physiological system a cortical culture period of 6 days was not sufficient to support the development of follicles from the primordial to the secondary stage. However, if the cortical culture period was extended to 16-23 days it was possible to isolate secondary follicles from the tissue. Although the percentage of degenerating follicles was greater following long-term than short-term cortical culture, the follicles that survived and developed to the secondary stage were competent to continue to develop to the EA stage. However, it may be possible to increase the survival and antral cavity formation rates by deriving follicles from short-term culture as the population of follicles would potentially be more homogenous, with respect to health, at the end of culture. Previous research suggests that alterations to the culture media composition and method of tissue preparation may facilitate accelerated follicle development. Further optimisation of the cortical culture method used for ovine tissue is therefore necessary and subsequent experiments should test the efficacy of published short-term, 6 day, accelerated growth systems that have been shown to support the development of early preantral follicles to the secondary stage in bovine and human. Finally, the ability of *in vitro*-

derived secondary follicles to develop to the EA stage cannot be used as the sole marker of follicular health. Detailed molecular analysis of gene expression of *in vivo*- and *in vitro*-derived follicles is essential in future studies to confirm the developmental normality of *in vitro* derived follicles.

4 Comparison Of The Effects Of A Physiological, Slow-Growth System And An Accelerated, Rapid-Growth System On The Rates Of Primordial Follicle Activation And Preantral Follicle Growth And Development *In Situ* In Ovine Cortical Tissue.

4.1 Introduction

In Chapter 3 the first 2 steps of a serum-free, long-term, multistep culture system that supported the development of ovine primordial follicles to the EA stage were evaluated. This system consisted of a minimum of 30 days culture in which 16 days of cortical culture and 14 days of isolated preantral culture were required to grow follicles to the EA stage. Development of primordial follicles to the EA stage has been achieved in much shorter time periods in the bovine and human, 18 and 10 days, respectively, using a rapid growth system as reported by Telfer et al. (2008) and McLaughlin and Telfer (2010). In these studies it was possible to develop primordial follicles to the transitional stage in 6 days. The culture system presented in Chapter 3 was not able to support development at this accelerated rate.

Whether an accelerated or a more physiologically relevant developmental timescale *in vitro* is optimal is a source of debate, as discussed in Chapter 3. It can be argued that an accelerated culture period would result in a more homogenous population of healthy, developing follicles at the end of cortical culture (Telfer et al., 2008). Only a small percentage of *in vitro*-derived secondary follicles reported in Chapter 3 were competent to develop onto the EA stage following isolation, possibly due to the fact that these follicles had started to degenerate. Further research is therefore required to test the functional significance of speed of follicle and oocyte development *in vitro* on developmental competence. To define the culture conditions needed to produce healthy follicles and oocytes from ovine cortex *in vitro* and to test the impact on the culture environment on follicle and oocyte growth it is necessary to compare the developmental competence of multi-layered follicles derived using the long-term-cortical culture system reported in Chapter 3 with a rapid short-term- growth system that has supported follicle growth and activation in the cow and human within an accelerated timeframe. The rapid growth system which will be adapted to support sheep follicle growth in cortex tissue *in vitro* is that based upon that previously published by Telfer et al. (2008) and McLaughlin and Telfer (2010). To aid clarity from here on in this rapid growth system will be referred to as the 'Accelerated Follicle Culture (AFC) system'; the slow cortical culture system detailed in Chapter 3 will be referred to as the 'Leeds system'.

4.1.1 Comparison of AFC vs. Leeds cortical culture system

Just as has been reported for the Leeds system, in Chapter 3, the AFC system is a 2 step method that supports the *in vitro* growth of oocytes from the primordial to the early antral follicle stage in both bovine and human (Telfer et al., 2008, McLaughlin and Telfer, 2010). In the first step of the AFC system oocytes are cultured *in situ* within pieces of ovarian cortical tissue that had been mechanically teased apart and trimmed prior to culture in such a way as to reduce the amount of medullary tissue present, for 6 days. Follicles were reported to activate and develop from the primordial/ early primary (EP) to the transitional stage *in situ*. In the second step, *in vitro*-derived transitional follicles are isolated and cultured individually, some of which were competent to develop to the EA stage. Histological analysis of the tissue has revealed low levels of follicle degeneration/ atresia on day 6 of cortical slice culture as assessed by morphological analysis in both human tissue (14%) and bovine tissue (<10%). Furthermore, primordial follicle activation in bovine tissue was demonstrated by a significant increase in the proportion of growing follicles relative to control day 0 tissue and a concurrent significant decrease in the proportion of quiescent follicles with respect to viable follicles, as well as an increase in mean follicle diameter (McLaughlin and Telfer, 2010). Similarly, in human tissue the percentage of quiescent follicles decreased from 90% (day 0) to 60% (day 6), with a subsequent increase in primary (day 0; 10%, day 6; 25%) and transitional/ secondary follicles (day 0; 0%, day 6; 15%), with respect to viable follicle counts (Telfer et al., 2008). As described in Chapter 3 the Leeds culture system is based on a serum-free system in which ovine primordial follicles are cultured *in situ* in slices of cortical tissue for up to 30 days as developed initially by Chambers (2002) and subsequently repeated to evaluate the efficacy of NR dye as a marker of follicle viability (Chambers et al., 2010).

There are a number of fundamental differences between the AFC and Leeds culture systems. The Leeds culture system is designed to support the physiological activation of some, but not all, primordial follicles and the growth of EP, primary and transitional follicles at a similar growth rate to that observed *in vivo* in the absence of large preantral follicles (Juengel et al., 2002b). In contrast the AFC system is designed to support the activation and development of the greater majority of primordial follicles up to the EA stage in an accelerated timeframe. Both the AFC and Leeds systems utilise a serum-free strategy in which primordial/ EP follicles are cultured within slices of cortical tissue although the basal culture media and additives used are different between the 2 approaches. The major differences being that the AFC media does not contain FSH, LH or a source of pyruvate, but it does contain 50µgml^{-1} of ascorbic acid, unlike the Leeds system. In addition the basal media used in the two systems are different; α-

MEM and McCoy's 5a medium with bicarbonate buffering are used in the Leeds and AFC systems, respectively, the compositions of these 2 base media are detailed in Appendix I. Furthermore, in the Leeds culture system the cortex tissue fragments are placed upon an insert in order to minimise the oxygen diffusion distance between the tissue and the surface of the media, whilst ensuring the tissue pieces are submerged in a great enough volume of media to provide sufficient growth factors and hormones (Devine et al., 2002, Chambers, 2002). A supportive membrane is not used in the AFC system and for this reason a lower volume of media is used and only 1 tissue piece is cultured per well, whereas 5 tissue pieces are cultured per well in the Leeds system. The tissue pieces used in the AFC system are also smaller than those cultured in the Leeds system, see below.

In addition, the tissue preparation protocol differs slightly between the 2 culture strategies. In both systems a thin layer of cortex is removed from the ovary. In the Leeds system the cortex is then cut into a small square see Figure 4.1 (a). Whereas in the AFC system the tissue is cut into a pyramid shape to remove excess stromal/ medullary tissue. Preparing the tissue as in Figure 4.1 (b and c) increases the ratio of cortex: medullary tissue cultured, and therefore is proposed to increase the primordial follicle density of the tissue cultured as these follicle stages are located at the greatest density in the cortex, whereas more developed follicles are located in the medullary region of the ovary. Furthermore, as discussed in Chapter 3 the shape and volume of the cortical tissue fragments has been linked to the level of follicle activation observed *in vitro* (Hovatta et al., 1997, Scott et al., 2004a, Telfer et al., 2008). Reducing the amount of medullary and stromal tissue may increase the rate of follicle activation as these tissues may contain higher concentrations of growth factors that inhibit follicle growth, potentially released by growing follicles (Wandji et al., 1996b).

4.1.2 Aims

The aim of this study was therefore to determine whether the use of the physiological, slow-growth system reported in Chapter 3 (Leeds system) is optimal for the sustained development of ovine follicles *in situ* compared to an accelerated growth system (AFC system). Cortical culture systems were compared with respect to primordial follicle activation and preantral follicle growth, development and survival *in situ* for a period of 6 days.

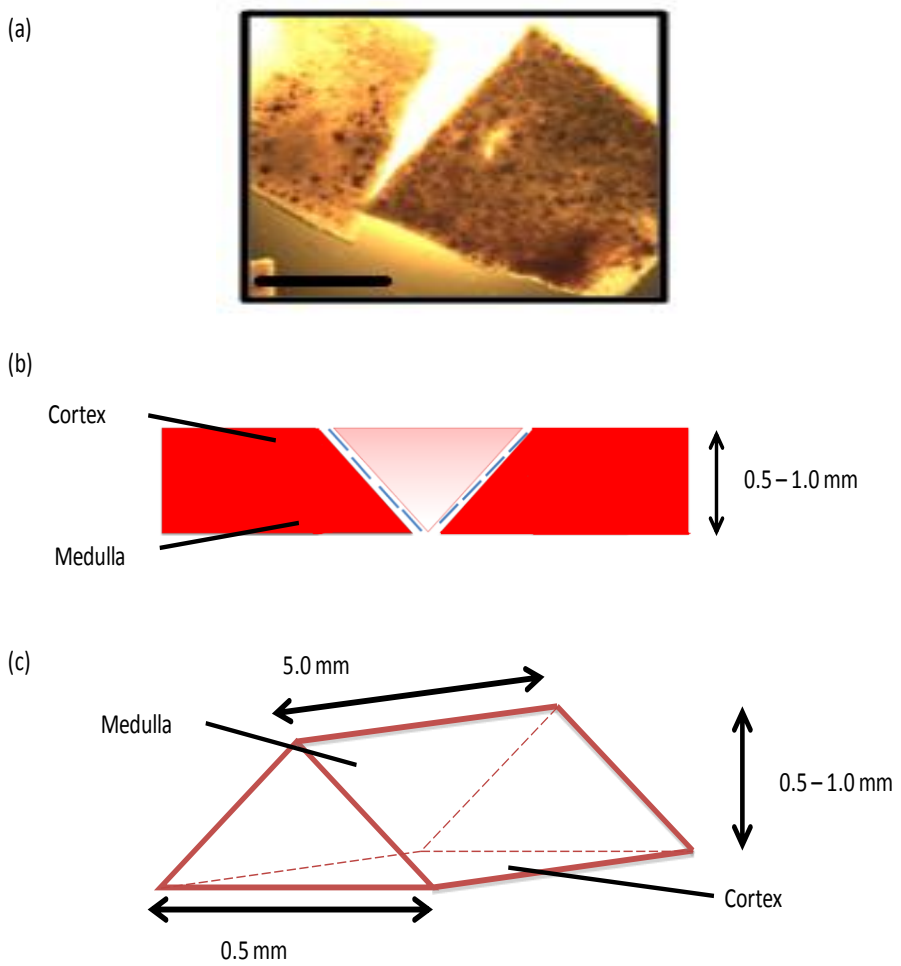


Figure 4.1 Preparation of cortical tissue pieces in the (a) Leeds and (b and c) AFC systems. In the Leeds system (a) cortical slices of ~ 2mm thickness were trimmed into 5mm^2 squares. Scale bar = 2mm. In the AFC system cortical slices were prepared as shown in (b) resulting in a pyramid shaped tissue piece as shown in (c).

4.2 Materials and Methods

4.2.1 Cortical culture

This experimental series was conducted to compare 2 serum-free IVG culture systems developed by research groups based at Leeds University (Chambers, 2002, Chambers et al., 2010) and Edinburgh University (Telfer et al., 2008, McLaughlin and Telfer, 2010) to evaluate the impact of tissue preparation techniques and culture environments on parameters of follicle and oocyte health and growth *in situ* in ovarian cortex. In order to compare these systems 4 different culture treatments were tested. The 4 treatment groups were: (1) Leeds, (2) AFC, (3) Leeds modified and (4) AFC modified. The composition of the Leeds and AFC culture media and the methodological differences between the 4 culture treatments are

summarised in Table 4.1 and Table 4.2, respectively. The unmodified Leeds system was as previously reported by Chambers (2002) and Chambers et al. (2010) and the unmodified AFC system was as previously reported by Telfer et al. (2008) and McLaughlin and Telfer (2010). Slight modifications were made to both the Leeds and AFC systems to further test and optimise the culture conditions within each system. This strategy evaluated the relative importance of the basal culture media composition, volume and additives and compared 2 methods of cortex preparation on the developmental potential of ovine follicles. All other culture conditions: temperature (37°C) and 5% CO_2 in the air were kept constant.

Table 4.1 Composition of Leeds and AFC culture media.

Medium Composition		Leeds	AFC
		Final concentration	
Basal medium composition	Basal medium	α-MEM medium	McCoy's 5a with bicarbonate
	Penicillin/ Streptomycin	1% (v/v)	1% (v/v)
	BSA fraction V, fatty acid free	1mgml^{-1}	0.1mgml^{-1}
	Hepes	-	2% (v/v)
Additives	Bovine holo-transferrin	5µgml^{-1}	5µgml^{-1}
	Sodium selenite	4ngml^{-1}	5ngml^{-1}
	Sodium pyruvate	0.047mM	-
	L-glutamine	3mM	3mM
	Bovine insulin	10ngml^{-1}	10ngml^{-1}
	Human long R3 IGF1	10ngml^{-1}	-
	Ovine FSH	1 x 10^{-5}iUml^{-1}	-
	Ovine LH	2.3 x 10^{-5} iUml^{-1}	-
	Ascorbic acid	-	50µgml^{-1}

*Note that – indicates the absence of the additive.

Cortical slice cultures were set up according to methodologies detailed in Sections 2.2 and 2.5-2.7. The tissue pieces in the Leeds and Leeds modified systems were prepared as described in Section 2.3, see Figure 4.1 (a). Whereas the tissue slices in the AFC and AFC modified systems were prepared, as shown in Figure 4.1(b and c). Briefly, cortical slices were prepared as described in Section 2.2, then cut using a scalpel (handle size 7 with a size 15 blade) as shown in Figure 4.1 (b), resulting in a pyramid shaped tissue piece as shown in Figure 4.1 (c). The tissue was then gently teased apart using 26½G needles (Terumo Europe N.V., Belgium).

Culture plates were set up as described in Section 2.8 although there were modifications to the composition and volume of culture media used and inserts were not used in all culture systems (see Table 4.2). The cortical tissue was cultured for 6 days, with media changes performed on days 2 and 5 of culture. The dry weight of the tissue was recorded on day 0 and day 6 of culture as described in Section 2.6. A total of 24 replicate cultures were conducted; 6 for each treatment group. Within each replicate culture of treatment group (1), (2), (3) and (4) an average of 104±8, 20±4, 82±6 and 18±4 tissue pieces were cultured.

Table 4.2 Summary of the differences in the 4 culture treatments used.

Treatment group	Culture treatment	Culture media	Volume media per well (µl)	Insert used?	Growing follicles excluded?	Tissue shape	Number of tissue pieces per well
1	Leeds	Leeds	500	Yes	Yes	Square	5
2	AFC	AFC	300	No	No	Pyramid	1
3	Leeds modified	Leeds	500	Yes	No	Square	5
4	AFC modified	Leeds	300	No	No	Pyramid	1

4.2.2 Analysis of follicle population dynamics

On days 0 and 6 of culture cortical pieces were stained with NR dye enabling viable follicle counts to be taken in fresh tissue as described in Section 2.6-2.7. Following NR staining tissue pieces were examined and any pieces that appeared to contain growing follicles were excluded from the Leeds treatment group (1). The developmental stage of the follicles was estimated *in situ* by size and morphology. Samples of day 0 and day 6 tissue were fixed and analysed histologically with respect to follicle population dynamics and follicle and oocyte size, as described in Sections 2.7 and 2.12-2.14.

4.2.3 Stromal tissue scoring

The effect of culture treatments on stromal health was analysed in fresh/cultured tissue and using a histological stromal tissue scoring system. As described earlier the tissue pieces were stained using NR dye before and after culture. Uptake of NR dye is indicative of cell viability and therefore is not only useful for assessing follicle counts but also stromal tissue health. Prior to culture the tissue was processed at 4°C to ensure that the NR dye uptake was primarily

by the follicles rather than the surrounding stroma. Whereas at the end of culture the tissue was at 37°C, and the follicles and stroma both took up the NR dye at a similar rate. Therefore, the pattern of NR dye uptake was very different between day 0 and day 6 tissue and could not be directly compared. However, day 6 tissue from the various culture treatments can be compared with respect to NR dye uptake and this can be used as an indicator of tissue health and viability. The percentage of the tissue pieces that stained red was recorded for all tissue pieces at the end of culture, for 6 culture repeats.

The integrity of the stromal tissue was assessed as described in Section 2.11. The stromal scores used were: good, adequate and poor which were converted into numerical scores, 3, 2 and 1, respectively. These stromal scores reflected the tissue and follicle integrity. Representative images of 'good, adequate and poor' quality tissue are shown in Figure 4.2.

4.2.4 Statistical analysis

Statistics were performed using the Minitab 16.1 statistical programme. The Anderson-Darling test was used to determine whether the data was normally distributed. Follicle population dynamics are presented as means of follicle counts per mg tissue ± SEM per culture repeat and mean percentage follicles ± SEM per culture repeat. Follicle population dynamics were analysed within each culture system, i.e. on days 0 and 6 of culture, if the data was normally distributed using either paired t-test for the analysis of data from fresh tissue data, the Student's t-test for fixed tissue, or Wilcoxon signed ranks test for data that was not normally distributed. Follicle population dynamics were also compared between the 4 culture treatments using one-way ANOVA with post hoc Fisher's test. If the data was not normally distributed Kruskal-Wallis test was used. Follicle population data which are presented as proportions of total follicle counts were analysed using Chi Squared statistical analysis. In all analyses $p<0.05$ was considered to be statistically significant. Where required data was normalised using the following transformations, for example data on the number of degenerating follicles per mg of tissue was transformed by base 10 logarithmic transformation. Unless otherwise stated all graphs are presented are the arithmetic means ± SEMs for the number of replicates shown.

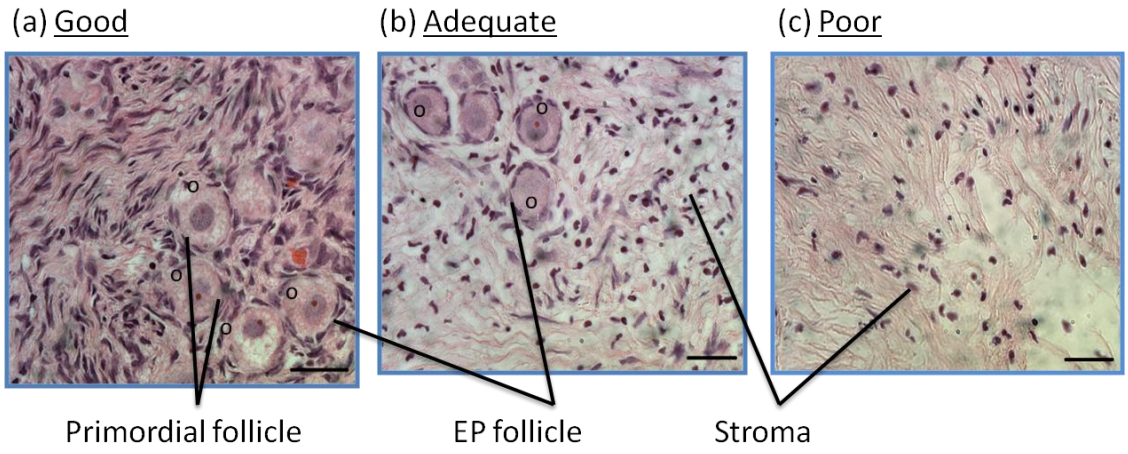


Figure 4.2 Representative histological images of poor (a), adequate (b) and good (c) quality stromal tissue. All follicles are at the primordial or EP stage of development. During histological analysis only follicles with a visible nucleolus were counted – the oocyte of these follicles is labelled 'O'. Scale bar = 20µm..

4.3 Results

4.3.1 Follicle population dynamics – primordial *vs*. non-growing

Four cortical tissue culture treatments were compared over 6 days. The mean number of viable primordial and growing follicles, per mg of tissue cultured, present on days 0 and 6 of culture, in fresh and fixed tissue samples are shown in Figure 4.4 and Figure 4.5, respectively. As in Chapter 3, follicle counts were standardised per mg tissue due to the differences in follicle load resulting from variability in tissue size at the start of culture, particularly in the current study due to differences in tissue preparation methods in the different systems. Tissue weights before and after culture are shown in Figure 4.3. There were no statistically significant changes ($p>0.05$) in tissue weight per well following culture in any treatment group, however, tissue weight increased slightly in the Leeds and Leeds modified treatment groups and decreased slightly in the AFC and AFC modified treatment groups following culture. Mean tissue weight per well was significantly lower in the AFC and AFC modified treatment groups than Leeds and Leeds modified groups before and after culture as would be expected as there were more pieces per well in the latter 2 treatments groups (5 *versus* 1). Fresh tissue refers to tissue that has been stained using NR dye either before or after culture. Note that tissue that did not stain with NR dye prior to culture was discarded as only tissue containing follicles was cultured. Fixed tissue refers to tissue that has been analysed histologically.

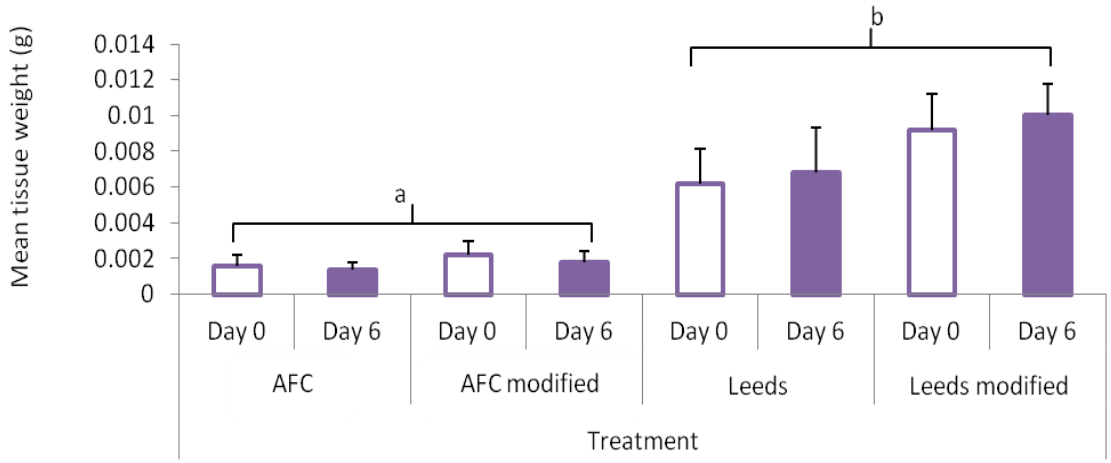


Figure 4.3 Mean tissue weight per well ± SEM for 6 repeat cultures on day 0 and 6 of cortical tissue culture using AFC, AFC modified, Leeds and Leeds modified treatments. Analyses of tissue weights within treatments using paired t-test revealed no statistically significant differences ($p>0.05$). Analysis of tissue weight between treatments using ANOVA revealed significantly lower weights in the AFC and AFC modified than the Leeds and Leeds modified treatment groups irrespective of day of culture ($p<0.05$). Different letters indicate significant differences at $p<0.05$.

Follicles were classified as primordial or growing based on size and appearance following NR dye staining *in situ*, see Figure 4.4. This is a rather crude method of establishing follicle classification and was therefore primarily used to determine which tissue pieces contained growing follicles in order to exclude them from the Leeds treatment group. Follicle population dynamics were analysed more accurately in fixed tissue (see Figure 4.5). The results presented in Figure 4.4 (a) show that in all 4 culture treatments the mean number of primordial follicles per mg of tissue had significantly decreased ($p<0.05$) after 6 days of culture. This could be due to follicles activating or degenerating. The results in Figure 4.4 (b) show that after 6 days of culture there were significantly fewer growing follicles in both the AFC and the AFC modified culture treatments ($p<0.05$). Therefore, it is unlikely that the decrease in the number of primordial follicles was due to follicle activation alone but rather the numbers of both primordial and growing follicles also decreased as a result of follicular atresia. The number of growing follicles did not change significantly in the Leeds modified culture treatment. After 6 days of culture the mean number of growing follicles present was 0.17 per mg tissue cultured ± 0.09 for 6 culture repeats. This evidence supports the idea that primordial follicles may be activating in the Leeds culture treatment.

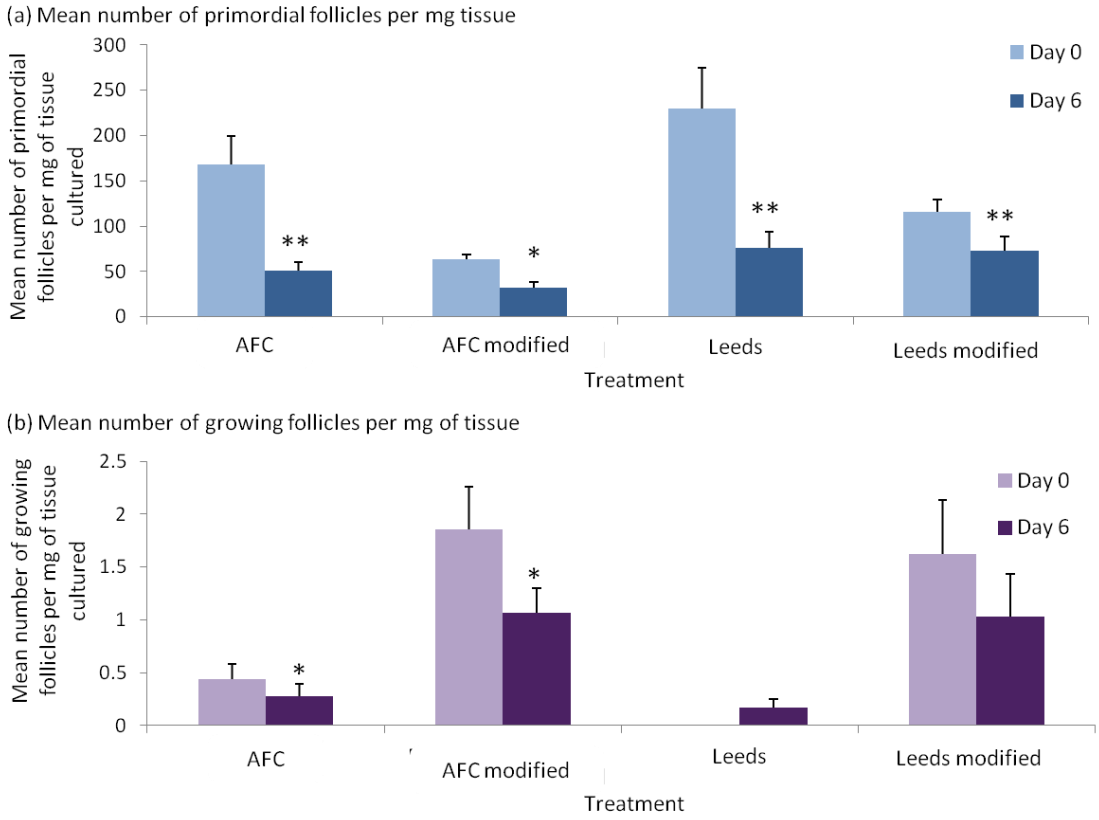


Figure 4.4 Comparison of the effect of different short-term culture treatments on follicle population dynamics in fresh tissue stained with NR dye on days 0 and 6 of culture. The mean number of viable (a) primordial and (b) growing follicles present on days 0 and 6 of culture using AFC, AFC modified, Leeds and Leeds modified culture treatments ± SEM per mg tissue at the start of culture, for 6 repeat cultures are shown. Statistical differences in the mean number of viable follicles on days 0 and 6 of culture within treatment groups are denoted as * $p<0.05$, ** $p<0.01$.

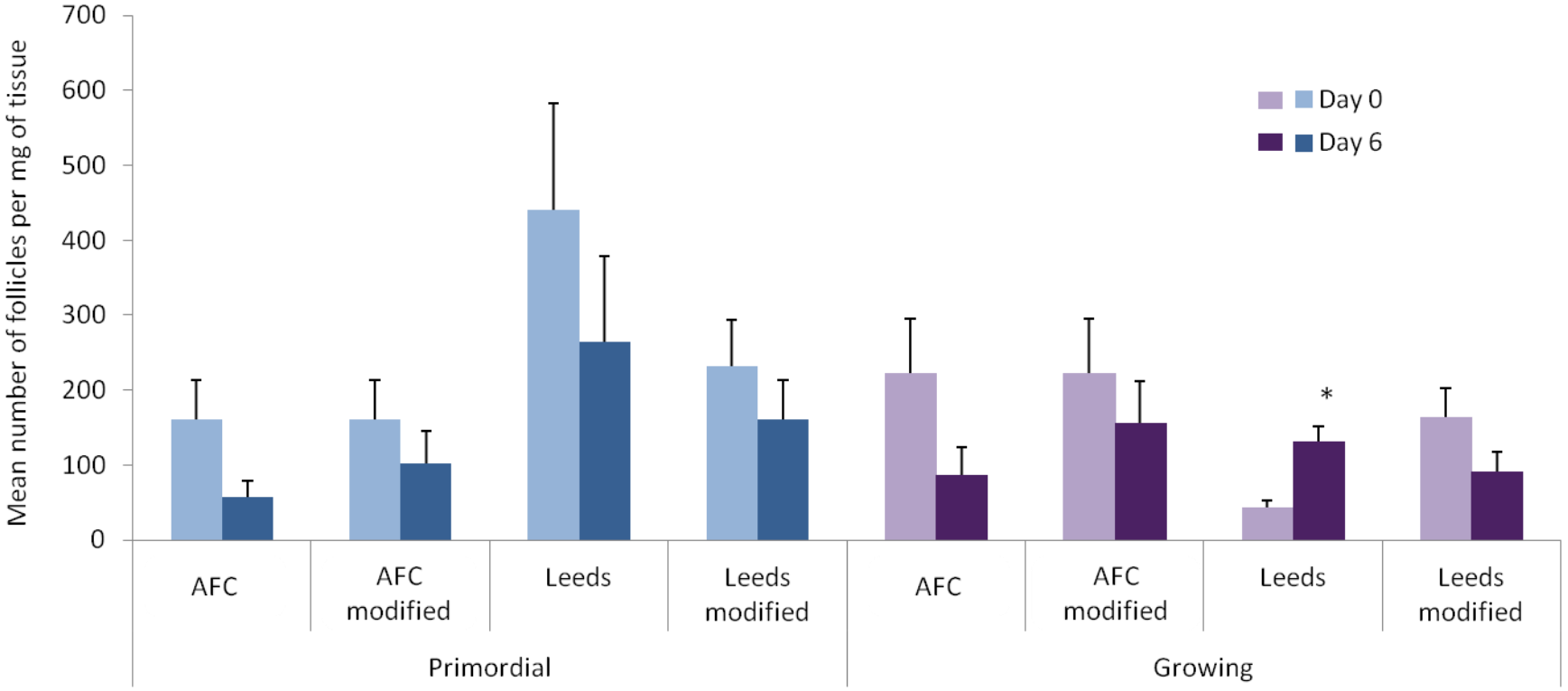


Figure 4.5 Histological comparison of short-term culture treatments on follicle population dynamics in tissue fixed on days 0 and 6 of culture. The mean number of viable primordial and growing follicles present on days 0 and 6 of culture using AFC, AFC modified, Leeds and Leeds modified culture treatments is shown. Values presented as mean ± SEM per mg of tissue at the start of culture for 4 repeat cultures. Statistical differences in the mean number of viable follicles on days 0 and 6 of culture within treatment groups are denoted using *p<0.05.

The tissue was also analysed histologically; the data presented in Figure 4.5 shows that the mean number of primordial and growing follicles did not change significantly in any culture treatment except for treatment (1)-the Leeds treatment, where tissue contained only primordial follicles as classified by NR staining on day 0, but which contained a significantly increased mean number of growing follicles after 6 days of culture. Although the Leeds method aimed to exclude any growing follicles on day 0 this was not always achieved as EP and primary follicles were detected histologically, however, no more advanced follicles were detected and the mean number of growing follicles was lower than any of the other treatment groups on the first day of culture. In all culture treatments the histological data mirrored the fresh tissue results as the mean number of primordial follicles decreased after 6 days of culture, although not significantly. In all treatments groups other than Leeds treatment the mean number of growing follicles decreased after 6 days of culture, however due to variability in the data and the limited number of culture repeats, when expressed on a per culture basis these differences were not significant ($p>0.05$). In addition data regarding degenerating follicle counts per mg fixed tissue is presented in Figure 4.6. Follicles were classified as degenerating if they either lacked a complete layer of GCs or if the oocytes had become detached from the GCs and appeared to be shrinking. There was no significant change in the number of degenerating follicles on days 0 and 6 of culture in any of the culture treatments.

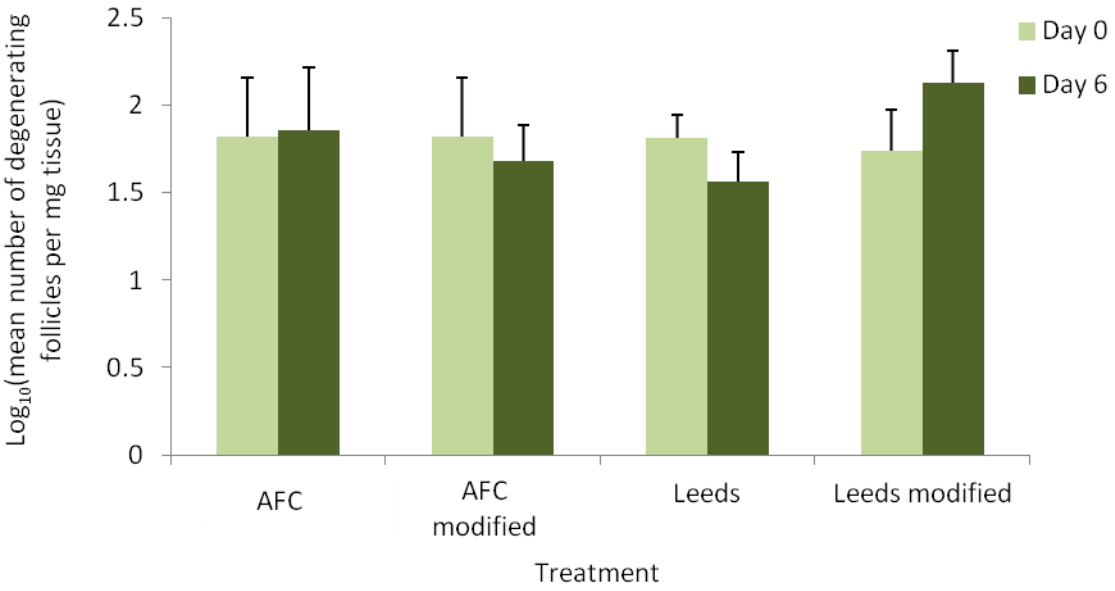


Figure 4.6 Comparison of the mean number of degenerating follicles per mg of fixed tissue on days 0 and 6 of culture. Values presented as $\log_{10}$(mean ± SEM) for 4 repeat cultures. Follicle counts were not statistically different ($p>0.05$) on days 0 and 6 of culture in any of the culture treatments, as determined using Student's t-test.

Follicle survival rates following the use of different culture treatments were analysed by considering the percentage of primordial or growing follicles that were still viable after culture. The data from fresh tissue is presented in Figure 4.7. Statistical analysis of the data presented in Figure 4.7 (a) showed no significant difference in the mean percentage of primordial follicles which survived culture between the four different treatments. In Figure 4.7 (b) only the data for the AFC and AFC modified culture systems is presented as only in these two groups did the mean percentage of follicles decrease after culture. There was no statistical difference ($p>0.05$) in the percentage of viable growing follicles between these two culture groups. In the Leeds modified culture treatment the mean percentage of viable growing follicles increased by 156.4±117.8% (n=6 cultures). The SEM indicates a large degree of variability in the data. As previously stated tissue pieces containing more advanced growing follicles were not used in the Leeds treatment therefore it was not possible to calculate the percentage increase for this group.

Figure 4.8 shows data regarding the percentage of viable (a) primordial and (b) growing follicles present in tissue fixed on day 6 relative to day 0. The data concerning the percentage of primordial follicles (Figure 4.8 (a)) is shown for the AFC, Leeds and Leeds modified treatments. In these systems the percentage of primordial follicles decreased on day 6 relative to day 0, however, there was no statistical difference ($p>0.05$) in the degree to which the percentage decreased. Data regarding the AFC modified method is not shown in Figure 4.8 (a). This is because the mean percentage of primordial follicles was greater on day 6 of culture (20.3±73.9%, n=4 cultures). This is probably due to the uneven distribution of follicles in the ovarian cortex and between ovaries. The SEMs are very large which indicates substantial variety in the data and the need for a higher number of culture repeats.

The data concerning the percentage increase in growing follicles (Figure 4.8 (b)) is shown for the AFC, AFC modified and Leeds treatments. Note that the data is presented differently from the fresh tissue data in Figure 4.7 (a) as the mean percentage of growing follicles increased following culture in fixed tissue in the AFC, AFC modified and Leeds treatment groups, unlike in the fresh tissue where it decreased. The percentage of growing follicles following culture was, however, calculated in the same way as the percentage of follicles surviving culture. In these systems the percentage of growing follicles has increased on day 6 relative to day 0, however, there was no statistical difference in the degree to which the percentage increased. Data regarding the Leeds modified method has been excluded from Figure 4.8 (b) because the mean

percentage of growing follicles was lower on day 6 of culture, a mean decrease of 32.2±20.6% for 4 culture repeats.

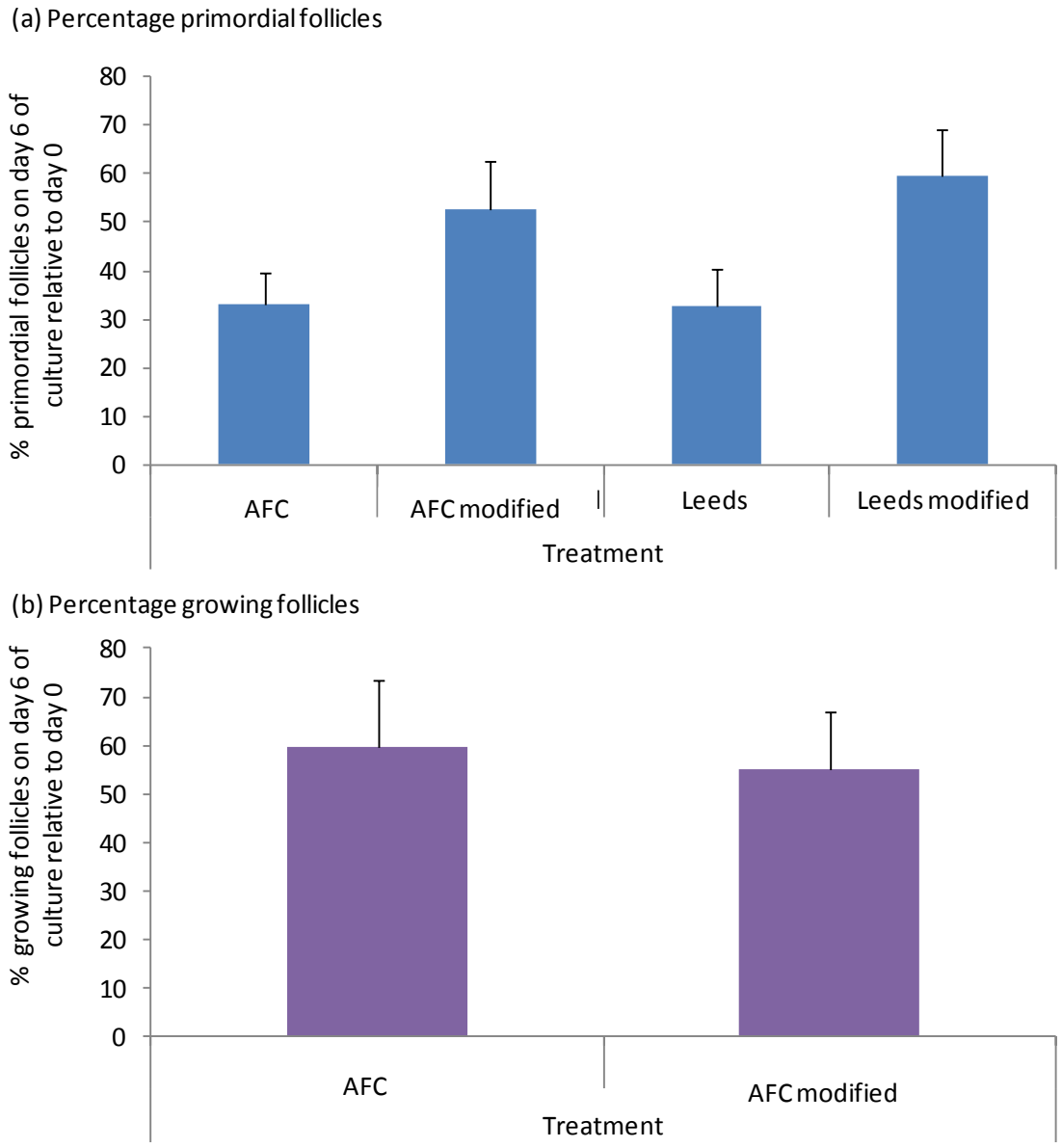


Figure 4.7 Fresh tissue comparison of short-term culture systems with respect to follicle survival rates in tissue stained with NR dye on days 0 and 6 of culture. The mean percentage of (a) primordial and (b) growing follicles present on day 6 relative to day 0 of culture using AFC, AFC modified, Leeds and Leeds modified culture treatments. Values presented are mean ± SEM for 6 cultures.

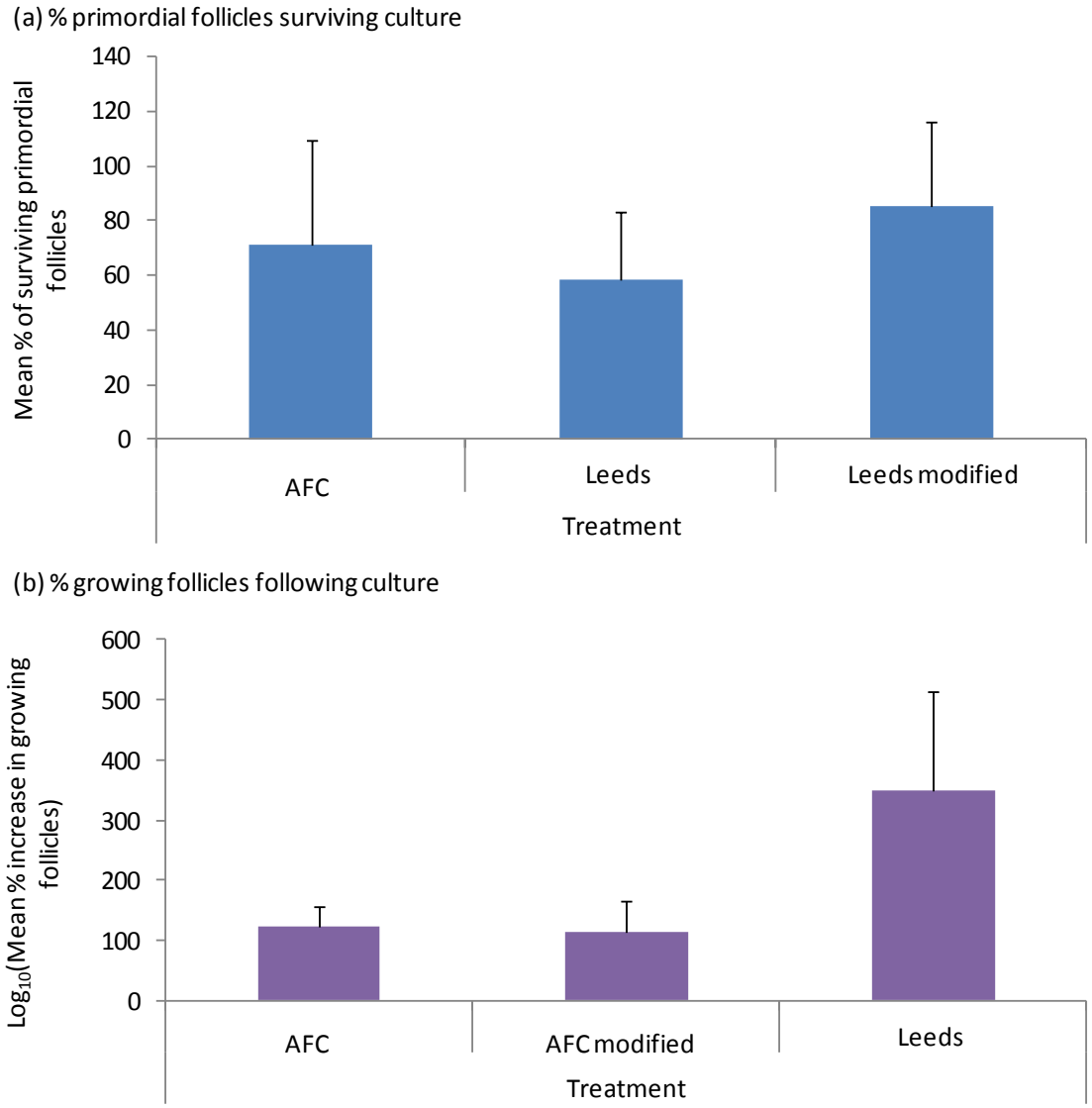


Figure 4.8 Histological comparison of short-term culture systems with respect to the mean percentage of viable follicles present on day 6 compared to day 0 of culture. Figure (a) shows the mean percentage of viable primordial follicles present after 6 days of culture in the AFC, Leeds and Leeds modified culture treatments. Figure (b) shows the $\log_{10}$ of the mean percentage increase in growing follicles present using AFC, AFC modified and Leeds treatment groups. Values presented as the mean ± SEM for 4 cultures.

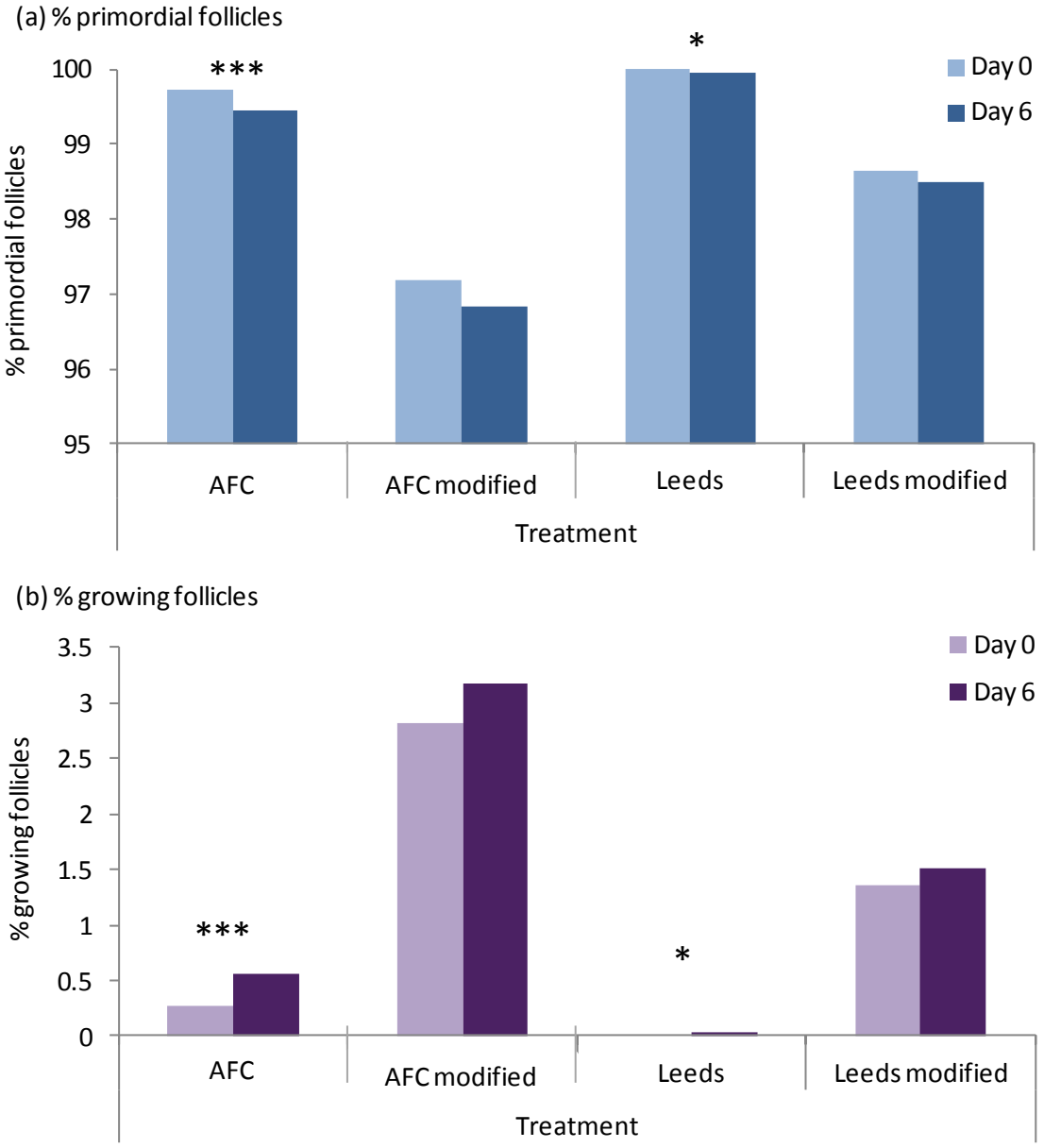


Figure 4.9 Percentage of (a) primordial and (b) growing follicles in fresh tissue – assessed by NR staining - on days 0 and 6 of culture in AFC, AFC modified, Leeds and Leeds modified treatment groups. 6 culture repeats were performed for each system and the total number of follicles recorded was used to calculate follicle proportions. Statistical differences in the proportion of follicles on day 0 and 6 of culture, within each treatment group, as denoted using: *p<0.05. **p<0.01, ***p<0.001, were determined using Chi-squared analysis.

In Figure 4.9 the percentage of viable primordial and growing follicles in fresh tissue on days 0 and 6 of culture are shown. Percentages were calculated from the total number of follicles across all 6 culture repeats for each treatment group. Total viable follicle numbers in fresh tissue, on days 0 and 6 of culture, are shown in Table 4.3. The percentage of primordial follicles significantly decreased after 6 days of culture following both the AFC and Leeds treatment. Conversely, a significant increase in the percentage of growing follicles was seen

following culture using the AFC and Leeds treatments. In both the AFC modified and Leeds modified systems the percentage of growing follicles relative to primordial follicles present in the tissue increased on day 6 relative to day 0, although this change was not statistically significant ($p>0.05$). This method of presentation is the standard format used by numerous research groups to present follicle dynamics data (Hovatta et al., 1999, O'Brien et al., 2003, Telfer et al., 2008, Morgan et al., 2013). Presentation of data in this way can be misleading as the percentages are calculated as the number of either primordial or growing follicles of the total follicle numbers on the day of culture. There are far fewer follicles present in the tissue on day 6 of culture due to high levels of follicular atresia therefore although the total number of growing follicles on day 6 of culture has decreased, in all groups other than the Leeds treatment, the percentage relative to primordial follicles has increased. Therefore if data is presented in this manner information regarding survival rates should also be shown to give a clearer indication of why the follicle population dynamics have changed, i.e. to determine whether it is due to follicle activation or atresia.

Table 4.3 Total number of primordial, growing and total viable follicles in fresh tissue. Numbers refer to the summation of the total follicles numbers across 6 repeat cultures, in each culture treatment, on days 0 and 6 of culture.

Culture treatment	AFC		AFC modified		Leeds		Leeds modified	
Day	0	6	0	6	0	6	0	6
Primordial	11774	3426	6570	3481	14519	2651	19895	12576
Growing	31	19	190	114	0	1	272	193
Total	11805	3445	6760	3595	14519	2652	20167	12769

In Figure 4.10 the percentage of viable primordial and growing follicles in fixed tissue on days 0 and 6 of culture are shown. Percentages were calculated from the total number of follicles across 4 replicate cultures for each treatment. Total viable follicle numbers in fixed tissue, on days 0 and 6 of culture, are shown in Table 4.4. In the AFC modified and Leeds treatments there was no change in the percentage of primordial and growing follicles on days 0 and 6 of culture. Whereas in the Leeds modified system the percentage of primordial follicles had decreased significantly ($p<0.001$) by day 6, relative to day 0. This was matched to a subsequent significant increase ($p<0.001$) in the percentage of growing follicles. However, following the AFC treatment there was a significant increase in the percentage of primordial follicles on day 0 relative to day 6 of culture and a subsequent significant decrease ($p<0.001$) in the percentage of growing follicles.

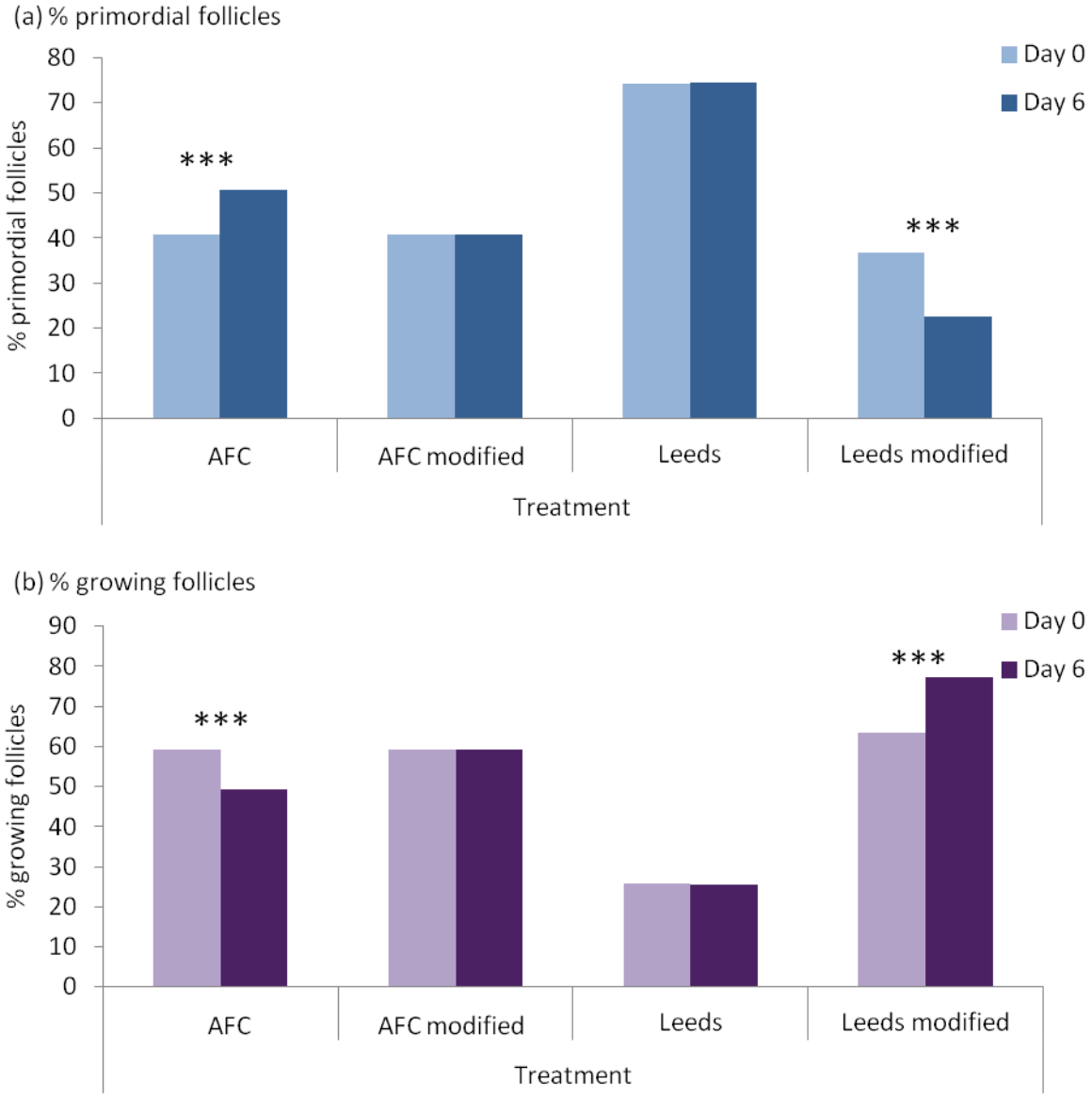


Figure 4.10 Percentage of (a) primordial and (b) growing follicles in fixed tissue on days 0 and 6 of culture as assessed in AFC, AFC modified, Leeds and Leeds modified treatment groups. Data from 4 replicate cultures were analysed for each treatment and the total number of follicles used to calculate follicle proportions. Statistical differences in the proportion of follicles on day 0 and 6 of culture, within each treatment group, are denoted using: *$p<0.05$, **$p<0.01$, ***$p<0.001$, with statistical significance being determined using Chi-squared analysis.

The results shown in Figures 4.4-4.8 illustrate the advantages and disadvantages of analysing both fresh and fixed tissue and therefore the necessity of using both these methods. The analysis of fresh tissue with respect to follicle counts may be less accurate than that of fixed tissue, due to the difficulty in determining exact numbers of primordial follicles, particularly after culture when the tissue is at 37 °C when both the stroma and follicles rapidly take up the NR dye making it more difficult to distinguish between the 2 tissue compartments. However, repeated NR staining of fresh and cultured tissue allows the same tissue to be assessed on

both day 0 and day 6, which is not possible with the fixed tissue. The use of different fixed tissue samples on days 0 and 6 of culture is probably the cause of the increase in primordial follicle numbers seen after culture in the AFC modified culture group. Furthermore, the low number of culture repeats (n=4) analysed to generate the histological data will have contributed to data variability and hence the lack of statistical significance.

Table 4.4 The total number of primordial, growing and total viable follicles in fixed tissue. Numbers refer to the summation of the total follicles from 4 culture repeats, in each culture system, on days 0 and 6 of culture.

Culture treatment	AFC		AFC modified		Leeds		Leeds modified	
Day	0	6	0	6	0	6	0	6
Primordial	1355	660	1355	1360	1470	725	1470	510
Growing	1970	640	1970	1970	510	250	2535	1740
Total	3325	1300	3325	3330	1980	975	4005	2250

The data on total follicle numbers in fresh and fixed tissue, presented in Tables 4.3 and 4.4, were used to determine the level of agreement between the follicle counts in fresh and fixed tissue, see Figure 4.11. Regarding the total number of primordial and all classifications of follicles there was a weak positive correlation between the counts obtained from fresh and fixed tissue, which was not statistically significant ($p>0.05$). Whereas with regard to the total number of growing follicles there was a stronger, statistically significant ($p<0.05$) correlation between counts obtained from fresh and fixed tissue. This highlights the difficultly of using NR staining to accurately determine follicle loading and why it remains essential to also quantify follicle population dynamics in fixed tissue.

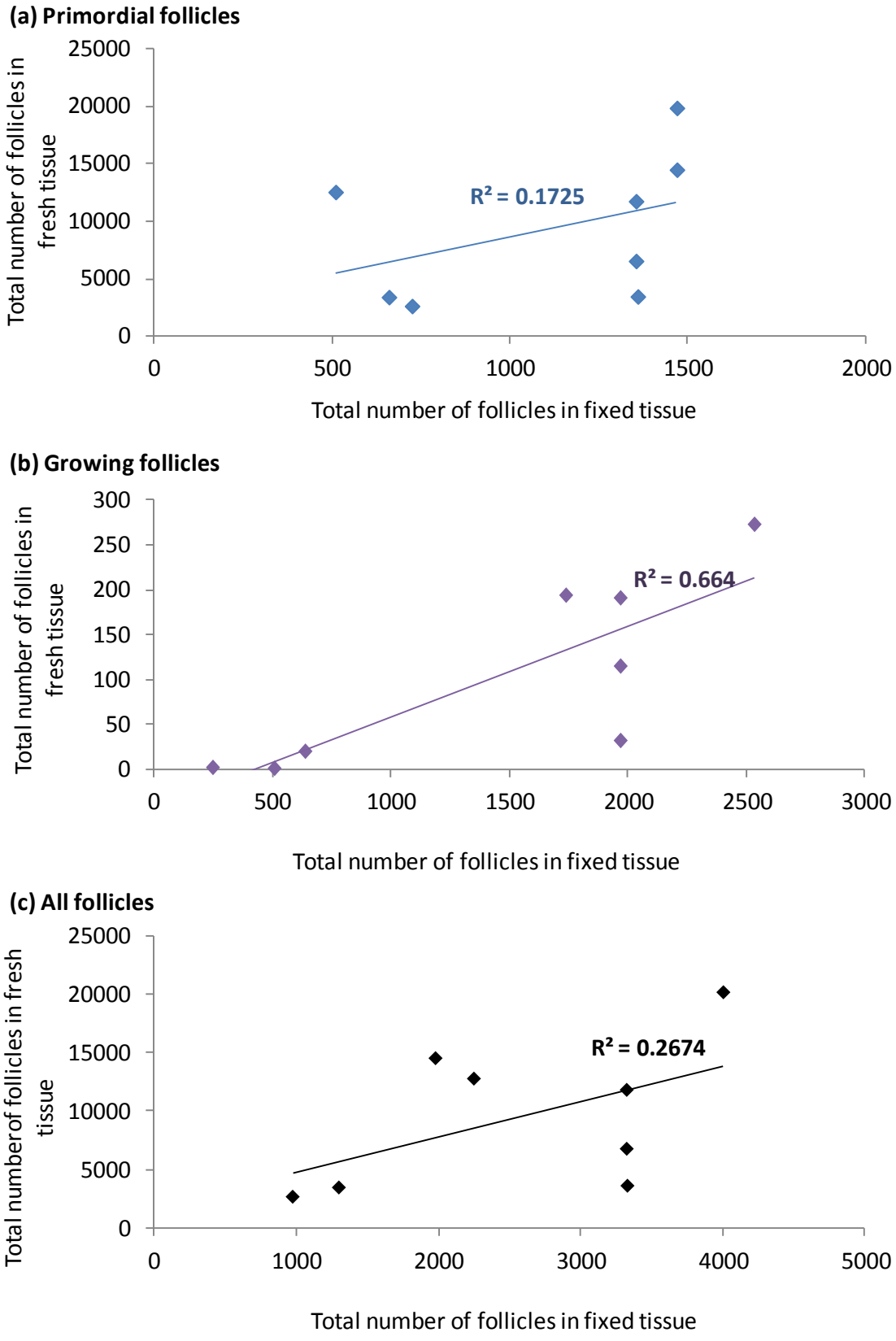


Figure 4.11 The relationship between the total number of (a) primordial, (b) growing and (c) all follicles in fresh and fixed tissue. A weak positive correlation was observed ($p<0.05$) between follicle count in fixed and fresh tissue in (a) primordial and (c) all follicle data sets, analysed using Spearman's rank order correlation. A stronger statistically significant ($p<0.05$) positive correlation was observed between the number of growing follicles present in fresh and fixed tissue. Data is presented for 8 repeats and the R^2 value is shown for each line of best fit.

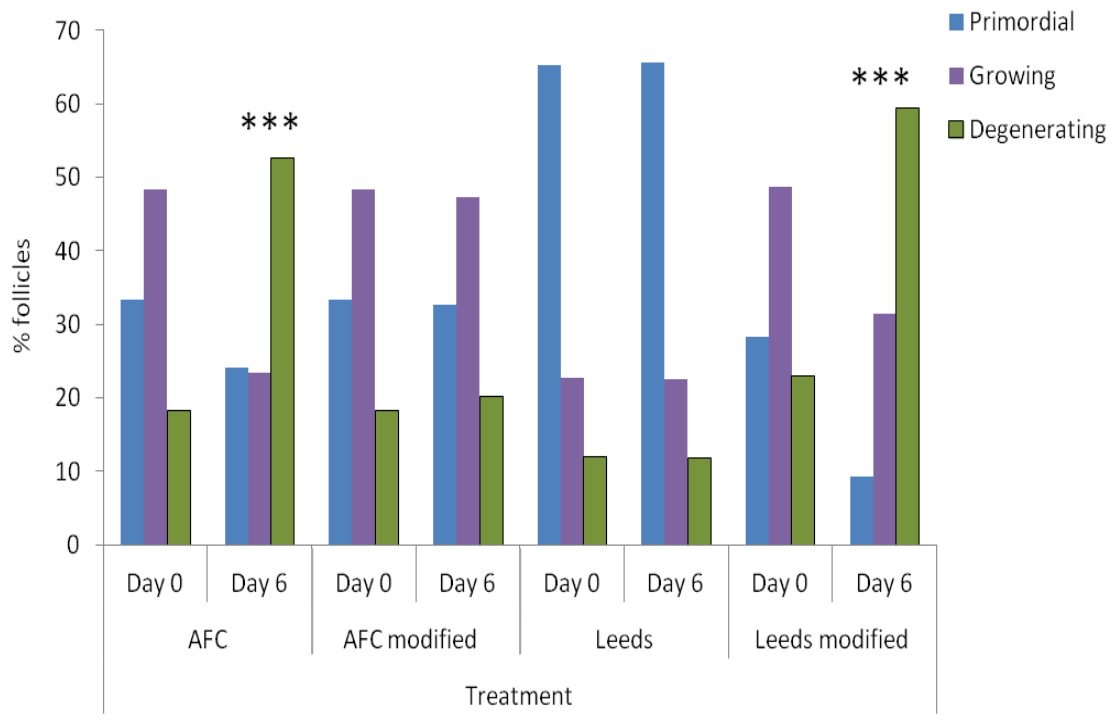


Figure 4.12 Percentages of viable primordial and growing follicles, and degenerating follicles present in tissue fixed on days 0 and 6 of culture in AFC, AFC modified, Leeds and Leeds modified culture treatments. 4 culture repeats were analysed for each system and the total number of follicles used to calculate follicle percentages. Statistical differences in the percentage of follicles between days 0 and 6 of culture within each treatment group, are denoted using *p<0.05, **p<0.01, ***p<0.001, determined using Chi-squared analysis.

Figure 4.12 shows the percentage of follicles present in tissue fixed on days 0 and 6 of culture. In Figure 4.10 similar data was presented, however, only viable follicles were included in the analysis. In Figure 4.12 counts of the number of degenerating follicle were used to calculate the relative percentages of viable primordial and growing follicles, and degenerating follicles in each culture system. Presenting data in this manner allows for a much clearer representation of why follicle percentages have changed. When the data presented in Figure 4.10 is considered the percentage of growing follicles in the AFC and Leeds modified treatment groups increased significantly ($p<0.001$) on day 6 relative to day 0. One may conclude that this is due to the activation of primordial follicles in culture, supported by the fact that the percentage of primordial follicles significantly decreased ($p<0.001$). However, when the counts of degenerating follicle numbers are also considered, as in Figure 4.12, the percentage of both primordial and growing follicles significantly decreased ($p<0.001$) on day 6 relative to day 0, in both the AFC and Leeds modified treatment groups. In contrast the percentage of degenerating follicles significantly increased on day 6, following culture using these treatments. This suggests that the decrease in the proportion of primordial and growing follicles is due to follicle loss by atresia.

Similarly, inclusion of the data on follicle degeneration in Figure 4.12 has aided interpretation of the data presented in Figure 4.9 with respect to the AFC system. In Figure 4.9 the percentage of primordial follicles increases, relative to the percentage of growing follicles, on day 6 of culture. In this figure the data is presented in a way that suggests the percentage of primordial follicles has increased on day 6 relative to day 0. When the data on follicular degeneration is included in Figure 4.12, however, it is clear that both the percentages of primordial and growing follicles have significantly decreased ($p<0.001$) on day 6 relative to day 0. The degree to which the percentage of primordial follicles has decreased is lower than that of the percentage of growing follicles. The percentage of degenerating follicles has significantly increased on day 6 of culture relative to day 0. Analysis of the AFC modified and Leeds systems presented in Figures 4.90 and 4.12 show that the percentage of primordial, growing and degenerating follicles did not change on day 6 relative to day 0.

Both the presentation of data as mean total follicles per mg of tissue (Figure 4.4 and 4.5) and as follicle proportions (Figure 4.9 and Figure 4.10) enables the comparison of follicle population dynamics before and after culture. However, presenting the data per mg of tissue is much more informative as tissue weights varied largely and therefore it is important to standardise the data using this method. It is also useful to present the data as the percentage change in follicles per mg of tissue (Figure 4.7 and Figure 4.8) as there are large variations in the numbers of follicles present on day 0 of culture therefore the percentage change data provided a clearer indication of the extent to which the follicle populations had changed. Based on these conclusions the remainder of the data regarding follicle population dynamics will be presented as the mean number of follicles per mg tissue in tabulated form and the percentage of follicles per mg tissue in graphical form.

4.3.2 Follicle population dynamics – follicle classifications

The follicle population dynamics on days 0 and 6 of culture are now considered in respect to follicle classification. Table 4.5 summarises both the total and mean number of primordial and EP, primary, transitional and secondary follicles per gram of fresh tissue on days 0 and 6 of culture.

Table 4.5 The total number and mean number of follicles ± SEM per g of fresh tissue on days 0 and 6 of culture in the AFC, AFC modified, Leeds and Leeds modified treatment groups, as determined using NR staining. The total number of follicles was not analysed statistically as this data is presented in terms of proportions in Figure 4.13. The mean number of follicles data was analysed using the paired t-test and statistically significant decreases in follicle number on day 6 of culture, compared to day 0 are denoted using *p<0.05 and **p<0.01.

	Day	Culture treatment	Follicle classification				
			Total	Primordial and EP	1°	Trans	2°
Total number of follicles per g tissue cultured	0	AFC	1008269	1005657	2013	599	0
		AFC modified	390490	379335	7005	4150	0
		Leeds	1299945	1299945	0	0	0
		Leeds modified	705260	695531	7003	2727	0
	6	AFC	304505	302823	1148	451	84
		AFC modified	200045	193675	4098	2150	121
		Leeds	609420	608586	833	0	0
		Leeds modified	440142	433951	5443	613	135
Mean number of follicles per g tissue cultured ± SEM	0	AFC	167440±32170	167000±32000	340±110	100±60	0
		AFC modified	64860±5520	63000±5000	1170±380	690±140	0
		Leeds	217000±42000	217000±42000	0	0	0
		Leeds modified	117620±13550	116000±13000	1170±490	450±60	0
	6	AFC	50750±10140	50470±10030**	190±70*	80±30	10±10
		AFC modified	33340±6500	32280±6190*	680±180	360±120*	20±10
		Leeds	101570±31860*	101430±31720*	140±140	0	0
		Leeds modified	73360±33420**	72330±16500**	910±370	100±40**	20±10

The data for the total number of follicles per g of cultured tissue, expressed as percentages is presented in Figure 4.13. Following all 4 culture treatments the mean number of primordial and EP follicles significantly ($p<0.001$) decreased on day 6 of culture, compared to day 0 (see Table 4.5). Only in the AFC, AFC modified and Leeds modified culture treatments were there primary and transitional follicles present on day 0. In all these systems the number of follicles decreased by day 6 of culture. Secondary follicles were not present in any culture system on day 0 however secondary follicles had developed by day 6 of culture in the AFC, AFC modified and Leeds modified culture systems. The absence of secondary follicles from the Leeds treatment tissue, which contained only primordial follicles at the start of culture indicates that the secondary follicles present on day 6 of culture in the other treatment groups were probably not derived from primordial follicles that activated *in vitro* but rather they developed from more advanced follicles that were already growing at the start of culture.

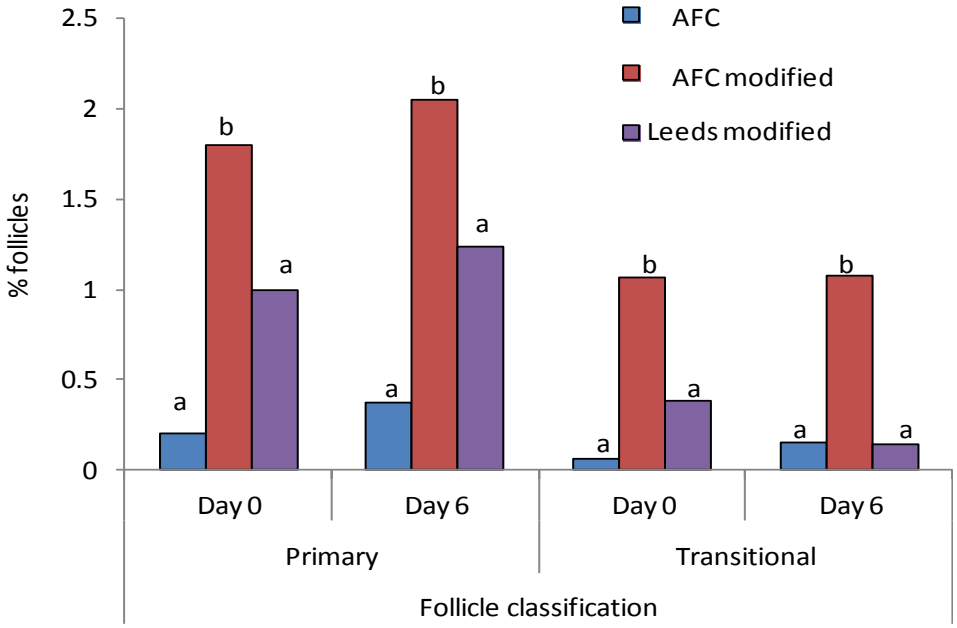


Figure 4.13 The percentage of primary and transitional follicles on days 0 and 6 of culture in fresh tissue following culture in the AFC, AFC modified and Leeds modified treatment groups. Chi-squared analysis of the data revealed statistically significant differences ($p<0.001$), indicated using different letters, both between and within treatment groups with respect to the percentage of follicles on days 0 and 6 of culture. Note that the total numbers of all follicles from all classifications present per g of tissue were considered when calculating the percentage of follicles and when performing the statistical analysis, for 6 culture repeats.

Follicle population dynamics are considered in terms of the percentage of follicles present in fresh tissue on days 0 and 6 of culture in Figure 4.13. Only follicles at the primary and transitional stages are considered here, data for the Leeds treatment group is not therefore shown as neither of these follicle classifications were present in Leeds day 0 tissue. Analysis of the data revealed that the percentage of follicles was significantly different ($p<0.001$) on day 0

and 6 of culture both within and between all treatment groups presented. Examination of the data also revealed a significantly higher ($p<0.001$) percentage of both primary and transitional follicles on both day 0 and day 6 of culture in the AFC modified system than in the AFC and Leeds modified systems. The higher percentage of follicles seen in the AFC modified system on day 6 is likely to be due to the higher percentage of follicles at these stages being present in the day 0 tissue. In the 3 culture treatment groups presented in Figure 4.13 secondary follicles were present in the tissue on day 6 of culture; AFC: 0.022%, AFC modified: 0.172% and Leeds modified: 0.021%. It should also be noted that a small number of primary follicles (0.17%) were present in the day 6 tissue following culture using the Leeds treatment.

Follicle population dynamics with respect to follicle classification were considered in fixed tissue samples. Table 4.6 summarises both the total and mean number of primordial and EP, primary, transitional and secondary follicles per g of fresh tissue on days 0 and 6 of culture. There were no statistically significant ($p>0.05$) changes within the follicle classifications between days 0 and 6 of culture in any of the culture systems. Follicle population dynamics were considered in terms of the percentage of follicles present in fixed tissue on days 0 and 6 of culture, see Figure 4.14. Only follicles at the primary, transitional and secondary stages were considered here and the data for the Leeds treatment group is not shown as none of these classifications of follicle was present in Leeds day 6 tissue. Analysis of the data revealed that the percentage of follicles was significantly different ($p<0.001$) on day 0 and 6 of culture both within and between all treatment groups presented. The proportion of primary and transitional follicles increased in all treatment groups presented in Figure 4.14. A large increase in the percentage of primary follicles was observed in the AFC treatment group on day 6 of culture. Examination of the data in Table 4.6 showed that the large increase in the percentage of primary follicles was accompanied by a large decrease in the percentage of primordial and EP follicles, suggesting that the increase was due to follicles activating and developing *in vitro*, however, the increase in number of primary follicles is much smaller (~30 times lower) than the decrease in number of primordial and EP follicles. Therefore the large increase in the percentage of primary follicles is more likely a result of high levels of follicle atresia than activation and development. Although a small percentage of secondary follicles were present in the tissue on day 0 of culture none were present on day 6, therefore these culture treatments were not able to support the survival of secondary follicles *in vitro*. To aid clarity the histological data regarding percentage of transitional and secondary follicles on days 0 and 6 of culture is shown in Table 4.7. The data was not normally distributed and was therefore analysed using non-parametric tests.

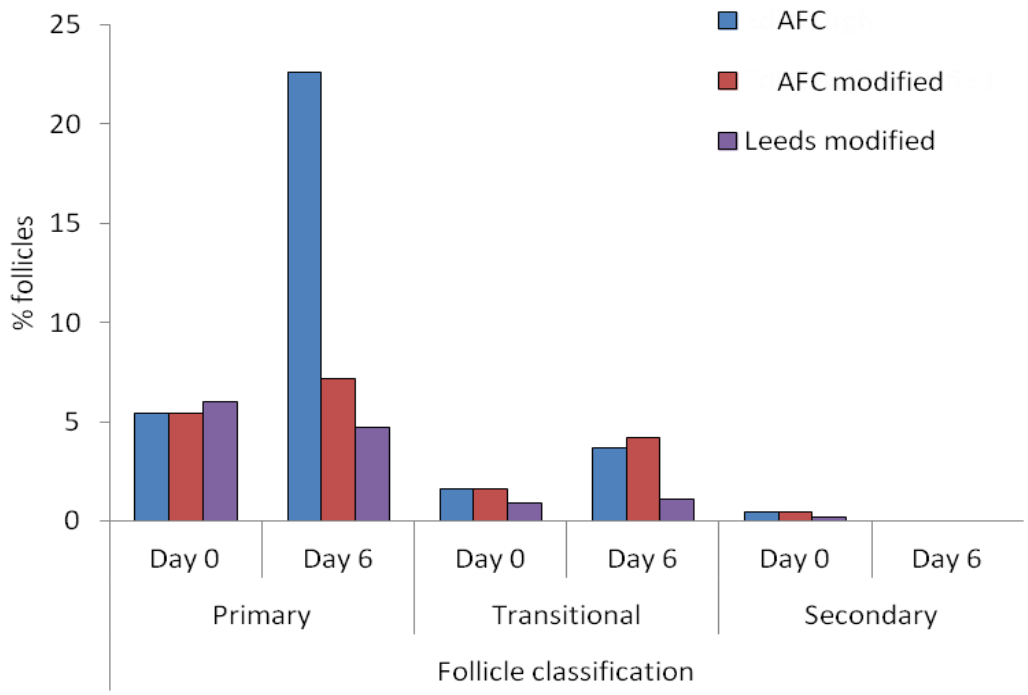


Figure 4.14 The percentage of primary and transitional follicles on days 0 and 6 of culture in fixed tissue following culture in the AFC, AFC modified and Leeds modified treatment groups. Chi-squared analysis of the data revealed statistically significant differences ($p<0.001$) both between and within treatment groups with respect to the percentage of follicles on days 0 and 6 of culture. Note that the total numbers of all follicles of all classifications present per g of tissue were considered when calculating the percentage of follicles and when performing the statistical analysis, for 4 culture repeats.

The mean diameters of follicles and oocytes in tissue on days 0 and 6 of culture in each treatment system are presented in Table 4.8. In all treatment groups and classifications follicle and oocyte diameter increased on day 6 of culture relative to day 0, with the exception of transitional follicles in the AFC modified treatment group in which the diameter decreased. Statistically significant ($p<0.05$) increases in diameter were observed in primordial follicles and oocytes and EP follicles in the AFC modified and Leeds modified treatment groups as well EP oocytes in the Leeds modified treatment group.

Table 4.6 The total number and mean number of follicles ± SEM per g of fixed tissue on days 0 and 6 of culture in the AFC, AFC modified, Leeds and Leeds modified treatment groups, as determined using NR staining. The total number of follicles was not analysed statistically as this data is presented in terms of proportions in Figure 4.14. The mean number of follicles data was analysed using t-test and changes in follicle number were not statistically significant ($p<0.05$).

	Day	Culture treatment	Follicle classification					
			Total	Primordial	EP	1°	Trans	2°
Total number of follicles per g tissue cultured	0	AFC	1534704	645559	774013	83333	24507	7292
		AFC modified	1534704	645559	774013	83333	24507	7292
		Leeds	2375758	1785985	556061	33712	0	0
		Leeds modified	1580245	927169	539300	95344	14728	3704
	6	AFC	573366	227393	194884	129798	21291	0
		AFC modified	1043110	418075	506229	74969	43838	0
		Leeds	1416250	1059464	356786	0	0	0
		Leeds modified	1008472	645046	304280	47617	11529	0
Mean number of follicles per g tissue cultured ± SEM	0	AFC	383280±128510	161390±51270	193500±64940	20830±8960	6130±2270	1820±1070
		AFC modified	383280±128510	161390±51270	193500±64940	20830±8960	6130±2270	1820±1070
		Leeds	593950±274700	446500±216510	139020±56570	8430±1620	0	0
		Leeds modified	395070±105040	231790±61330	134830±30510	23840±9070	3680±3200	930±930
	6	AFC	143340±63210	56850±22410	48720±16710	32450±21620	5320±2470	0
		AFC modified	260780±149080	104520±55850	126560±82160	18740±6150	10960±4920	0
		Leeds	354070±149690	264870±114280	89200±35410	0	0	0
		Leeds modified	252110±79950	161260±15652	76070±18100	11900±7050	2880±2540	0

Table 4.7 A histological comparison of the mean % of transitional and secondary follicles present per g of tissue fixed on days 0 and 6 of culture in the 4 systems tested. Means ± SEMs are shown for 4 culture repeats. There were no statistical differences within culture systems and follicle classifications on days 0 and 6 of culture, determined using either a paired t-test or Wilcoxon signed ranks test. Nor were there any statistically significant differences in the mean percentage of follicles within each follicle classification between culture systems on days 0 and 6 of culture as determined using the Kruskal-Wallis analysis.

		Mean % per g tissue fixed			
	Follicle Classification	Transitional		Secondary	
	Day of Culture	Day 0	Day 6	Day 0	Day 6
Culture treatment	AFC	3.2±1.8	6.4±3.9	0.3±0.2	0
	AFC modified	3.2±1.8	6.3±3.7	0.3±0.2	0
	Leeds modified	0.7±0.5	0.8±0.7	0.2±0.2	0

Table 4.8 The mean diameter of primordial, EP, primary, transitional and secondary follicles and oocytes ± SEM in tissue samples across 4 culture repeats on days 0 and 6 of culture in each treatment group. Blanks in the table indicate that there were no follicles of that classification present in tissue cultured within that system at that day of culture. Data was not normally distributed and therefore was analysed using the Mann-Whitney U test. Mean follicle and oocyte diameters were compared between days 0 and 6 of culture within each follicle classification and treatment group. Statistically significant differences are denoted using *($p<0.05$).

Culture treatment	Day	Primordial		EP		Primary		Transitional		Secondary	
		Follicle	Oocyte	Follicle	Oocyte	Follicle	Oocyte	Follicle	Oocyte	Follicle	Oocyte
AFC	0	23.9±0.5	20.3±0.4	27.0±0.8	21.3±0.6	35.4±1.9	25.1±1.6	80.4±4.6	41.8±2.1	140.9±13.4	56.6±11.6
	6	27.3±2	23.1±1.8	29.9±2.1	23.2±1.5	38.2±2.8	26.3±2.1	86.8±2.3	49.4±2.3		
AFC modified	0	23.9±0.5	20.3±0.4	27.0±0.8	21.3±0.6	35.4±1.9	25.1±1.6	80.4±4.6	41.8±2.1	140.9±13.4	56.6±11.6
	6	27.1±0.4*	23.4±0.5*	30.4±0.6*	23.6±0.3	43.6±2.6	30.9±2.7	71.7±5.3	42.6±4		
Leeds	0	22.0±0.4	18.6±0.3	25.8±0.6	19.7±0.6	44.3±3.5	26.7±3.1				
	6	22.4±0.2	18.9±0.3	26.0±1.2	19.4±0.9						
Leeds modified	0	23.2±0.4	19.6±0.4	26.7±0.4	20.9±0.3	38.3±1.5	26.5±0.7	79.7±0.9	33.4±2.2		
	6	28.2±1*	23.6±0.6*	37.9±3.6*	27.3±1.8*	39.8±2.2	27.9±1.3	80.3±18.4	52.4±16.3		

4.3.3 Stromal tissue scoring

On day 8 of culture tissue pieces were incubated in NR dye. Following this incubation period tissue pieces were assessed with respect to the percentage of the tissue that positively stained with NR dye. The results are shown in Figure 4.15. On day 8 of culture the tissue in the Leeds culture system had the highest percentage positively stained stroma (84.3±1.6%, n=6). Although this was not significantly different ($p>0.05$) from the tissue grown in the Leeds modified culture system, it was significantly greater ($p<0.05$) than the stroma cultured in both the AFC and AFC modified culture systems. The tissue in the AFC culture system has the lowest percentage positively stained stroma (31.3±5.3%, n=6).

The integrity of the stromal tissue was assessed histologically, as shown in Figure 4.16. The difference in the integrity of the control day 0 tissue was not statistically significantly different ($p>0.05$) from the Leeds day 6 tissue. However, the stromal tissue score of the tissue in the Leeds modified, AFC and AFC modified culture systems was significantly lower ($p<0.05$) than that of the day 0 control tissue. The tissue in the AFC culture system had the lowest stromal tissue score. The stromal tissue score of the Leeds and Leeds modified groups was not statistically significantly different ($p>0.05$).

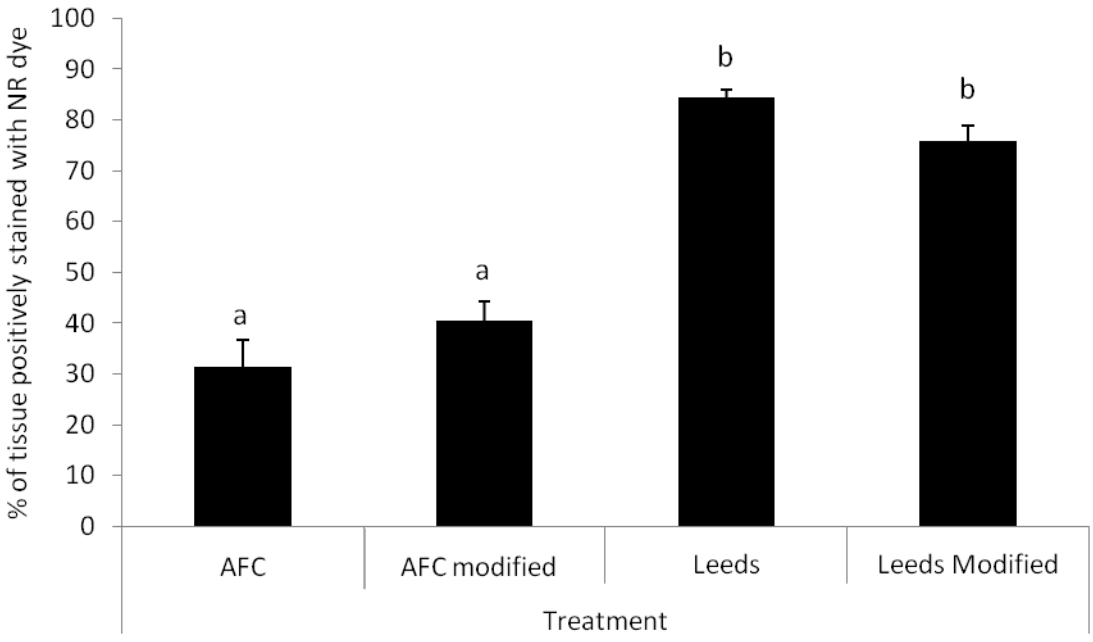


Figure 4.15 Analysis of tissue viability as assessed in fresh tissue by NR staining on day 8 of culture in all 4 culture systems. The percentage of the tissue that stained positively is shown as mean ± SEM for 6 culture repeats. Different letters represent statistically significant differences ($p<0.05$).

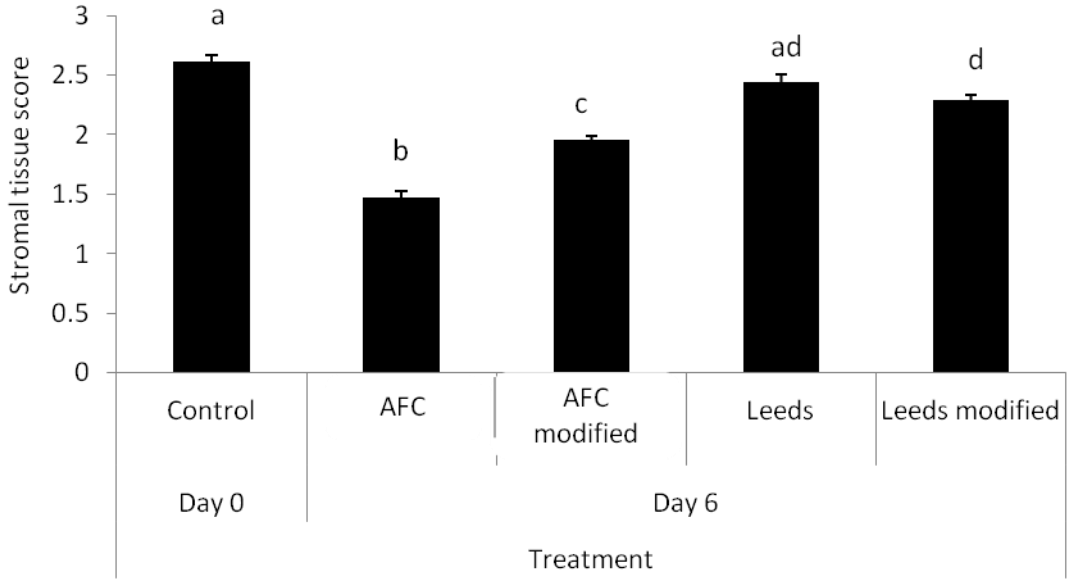


Figure 4.16 Histological analysis of stromal tissue and follicle integrity. The overall appearance of the tissue and follicles in the section of tissue was rated as good (3), adequate (2) or poor (1). Values are shown as mean ± SEM for 4 culture repeats. Different letters represent statistically significant differences ($p<0.05$).

4.4 Discussion

The results show that primordial follicle activation, development and survival are supported in the short-term by the Leeds culture system. The extent to which primordial follicle activation is supported in the AFC, AFC modified and Leeds modified systems is unclear, as it is uncertain as to whether primordial follicle counts and proportions decreased as a result of follicle activation or atresia or a combination of both. Both primordial and growing follicle counts decreased following all culture treatments, except when using the Leeds system, showing that even if primordial follicles are activating this occurred at a lower rate than atresia was induced in growing follicles. The data indicates however, that the decrease in growing follicles was lower in the Leeds modified system than the AFC and AFC modified systems. This could either be due to a higher rate of follicle activation in the Leeds modified group, or a lower rate of atresia of growing follicles, or both. This evidence suggests that the conditions in the Leeds and Leeds modified treatments may be preferential to those used in the AFC and AFC modified treatment groups. As previously mentioned the culture systems differ in terms of media composition, tissue preparation, use of insert and media volume as well as the number of cortex pieces per well. The potential effects of these differences will be discussed below.

4.4.1 Media composition

One of the major differences with respect to media composition was the addition of gonadotrophins, FSH and LH to the Leeds, Leeds modified and AFC modified culture media. LH receptors are not expressed until the development of the thecal cell layer in ovine follicles and therefore the addition of LH to the media is unlikely to affect preantral follicle growth or development (Carson et al., 1979). This constituent is included to support the later stages of development should these be present in tissue on day 0 or be induced in culture. Similarly, although FSH receptors are not expressed prior to the primary follicle stage in sheep (Tisdall et al., 1995) this gonadotrophin was added to the media from day 0, when the Leeds tissue should have only contained primordial follicles to support the progression of development. Primary follicles were present in the Leeds modified, AFC and AFC modified tissue on day 0.

Research also suggests that FSH promotes follicle survival *in vitro*, this has been demonstrated in numerous species including rodents (Chun et al., 1996), goat (Matos et al., 2007a, Matos et al., 2007b), sheep (Costa et al., 2010, Peng et al., 2010), cow (Yang and Rajamahendran, 2000) and human (Wright et al., 1999). Early studies by Chun et al. (1996) demonstrated the promotion of follicle survival via the anti-apoptotic actions of FSH in rodent early antral follicles. Further evidence of FSH as a survival factor has been produced by more recent studies in various species. Numerous studies have been conducted regarding the effect of FSH on caprine preantral follicles *in vitro*. Earlier studies provided evidence of the promotion of preantral follicle survival by FSH in cortical slice culture (Matos et al., 2007a). Prior to these studies research suggested that there was no link between the inclusion of FSH in the culture media and caprine follicle viability in short-term cortical culture (Silva et al., 2004b). However, subsequent research by the same group has lead authors to suggest that FSH is actually essential for the maintenance of caprine follicle viability *in vitro* (Matos et al., 2007b). Differences in these findings probably arose from the differences in concentration of FSH added to the culture media 50ngml^{-1} (Matos et al., 2007b) vs. 100ngml^{-1} (Silva et al., 2004b). Studies have also been conducted to elucidate the effects of FSH inclusion in *in situ* ovine follicle culture. In a study conducted by Costa et al. (2010) the inclusion of FSH in the media did not appear to promote ovine follicle survival compared to the control. However, when indole-3-acetic acid (IAA) was included in the media at deleterious concentrations that induced apoptosis, FSH was able to reduce these effects. One mechanism by which FSH has been shown to promote follicle survival in rodents is via the promotion of glutathione production by follicles, which suppresses reactive oxygen species production, thus reducing the level of apoptosis occurring due to oxidative damage (Tsai-Turton and Luderer, 2006). This is likely to

be the mechanism by which FSH counteracted the effects of IAA in the aforementioned study (Costa et al., 2010). Furthermore, Peng et al. (2010) showed that FSH promoted preantral follicle survival in ovine cortical culture when added to the culture media with epidermal growth factor (EGF) and ascorbic acid. In addition, the use of FSH combined with EGF was also shown to promote ovine primordial follicle viability *in vitro* when assessed using transmission electron microscopy (Andrade et al., 2010).

Addition of FSH to the culture media has also been shown to promote follicle growth and development. Inclusion of FSH in media promoted the *in situ* growth of caprine preantral follicles (Matos et al., 2011). When combined with fibroblast growth factor-2 (FGF2) FSH was also shown to promote caprine primordial follicle activation (Matos et al., 2007a, Matos et al., 2007b). In ovine cortical culture FSH was shown to increase follicle size and promote GC proliferation, when used in combination with ascorbic acid or EGF (Peng et al., 2010). Addition of FSH alone to the culture media promoted the development of ovine primary follicles to the secondary stage *in situ* (Costa et al., 2010). Furthermore, inclusion of FSH increased the percentage of developing follicles present in cultured ovine cortical tissue, although not significantly when compared to the FSH-free control media (Andrade et al., 2005). FSH inclusion in the culture media also promoted the growth of human preantral follicles *in situ* (Wright et al., 1999). There is conflicting evidence regarding the ability of FSH to promote growth and development of bovine preantral follicles *in vitro*. With respect to bovine follicles, FSH receptors are expressed from the primary stage (Wandji et al., 1992). However, FSH did not promote follicle development from the primary to secondary stage or follicle growth in short-term bovine cortical tissue culture, nor did FSH promote primordial follicle activation (Fortune et al., 1998, Derrar et al., 2000). Whereas, inclusion of FSH during isolated preantral follicle culture, has been shown to promote preantral follicle growth and proliferation of GCs (Wandji et al., 1996a, Saha et al., 2000). It should also be noted that in the isolated follicle cultures mentioned above the media contained serum, whereas the media in the cortical tissue cultures was serum-free. Therefore, there may have been some component present in the serum that interacted with FSH and resulted in the promotion of follicle development. Also the effects of FSH may differ between ovine and bovine preantral follicle development *in vitro*. Thus the inclusion of FSH in the culture media of the cortical culture system developed by McLaughlin and Telfer (2010) may not promote bovine follicle growth. However, when the culture system was adapted for use with ovine tissue the inclusion of FSH may be optimal to promote follicle survival, growth and development.

Therefore the impact of inclusion of FSH in the culture media could partly explain the data presented in Figure 4.4 (b) and Figure 4.9 which revealed a lower significant decrease in number of growing follicles observed in the Leeds modified group, compared to the AFC and AFC modified groups as well as a concomitant increase in the percentage of growing follicles rather than a decrease. As previously mentioned this may be due to either higher levels of follicle activation in this culture system, or higher growing follicle survival rates, or a combination of both. It is possible that the inclusion of FSH in the culture media may have promoted preantral follicle survival, growth and development. However, as FSH was included in the culture media used in the AFC modified system, the results observed cannot be due to FSH inclusion alone. The histology data presented in Figure 4.5 shows there was a significant increase in the number of growing follicles per mg tissue on day 6 of culture, relative to day 0 ($p<0.05$). Furthermore, the number of non-viable follicles decreased after culture in the Leeds system, see Figure 4.12.

Pyruvate

Both types of basal media, α-MEM and McCoy's 5a, used in this experimental series contain glucose as an energy source. In addition, sodium pyruvate was added as an additional energy source in the Leeds, Leeds modified and AFC modified culture media. Pyruvate was absent from the AFC culture media. Previous research has shown that follicles utilise both glucose and pyruvate (Boland et al., 1994b, Boland et al., 1994a, Biggers et al., 1967, Harris et al., 2007). Energy production from glucose is achieved both via glycolysis, which is non-oxidative as demonstrated experimentally in mice by assaying for lactate production and via the pentose phosphate pathway, an oxidative process (Boland et al., 1993, Harris et al., 2007). More specifically glucose metabolism is favoured by the somatic cells of the follicle, whereas pyruvate is utilised as the primary energy source during oocyte metabolism. Oocytes can be supplied with pyruvate by their supporting somatic cells however experimental evidence has also shown that pyruvate is taken up from the culture media during *in vitro* follicle culture (Leese and Barton, 1985, Harris et al., 2007, Harris et al., 2009). Experimental studies have shown that pyruvate consumption from the culture media by primordial follicles in culture is at a high level, such that it is 2-fold higher than glucose consumption (Harris et al., 2009). This could be due to the high energy demands of the oocyte at this stage of development, or indicative of the reduced ability of the somatic cells to provide sufficient pyruvate. Therefore, although it is not essential to add pyruvate to the culture media as it can be provided by the somatic cells, and/or glucose can be used as an alternative energy source the lack of pyruvate

supplementation may be detrimental to primordial follicle survival. This could partly explain the decrease in number of primordial follicles observed in the AFC culture system. Furthermore, when oocyte cellular volume is accounted for, pyruvate consumption by the oocyte is highest at the primary stage of follicular development (Harris et al., 2009). Authors suggest that this may be due to the increased energy requirements as the follicle goes through this transitory stage.

In addition, pyruvate acts as a free radical scavenger thus it is able to protect against oxidative damage caused by reactive oxygen species produced as a result of mitochondrial energy production (O'Donnell-Tormey et al., 1987, Van Blerkom, 2004, Picton and Hemmings, 2013). Therefore, although pyruvate supplementation in the culture media is not essential evidence suggests that its inclusion may provide more optimal culture conditions for follicle growth and development. This could further explain the decrease in numbers of early stage follicles observed in the AFC culture system and explain why this occurred to a greater extent than in the Leeds system.

IGF1

The media used in all systems except the AFC culture conditions reported here, contains a synthetic analogue of IGF1- Long R3 IGF1. This analogue does not bind to the IGF binding proteins so remains biologically active *in vitro.* IGF1 has been shown to promote GC proliferation in small ovine follicles (Monniaux and Pisselet, 1992). However, in bovine preantral follicle culture although the addition of recombinant human IGF1 and Long R3 IGF1 increased follicle diameter the rates of oocyte degeneration were also increased (Thomas et al., 2007). Therefore species-specific differences may exist and this may somewhat explain the discrepancies in the results reported for the AFC system in this experimental series and by McLaughlin and Telfer (2010). The major active IGF species is, however, the same in both cow and sheep, IGF2, the actions of which are mediated predominantly through IGF2R (Armstrong et al., 2000, Armstrong et al., 2002, Monget et al., 2002, Silva et al., 2009). It is unclear whether the inclusion of Long R3 IGF1 had any effect on the promotion of follicular growth in this experimental series as GC proliferation was not directly measured.

4.4.2 Tissue preparation

A major difference between the Leeds and AFC culture systems is the method of cortical tissue preparation, see Figure 4.1. Telfer and McLaughlin (2012) suggest that by teasing the cortical tissue apart and cutting the tissue in such a way as to reduce the amount of medulla present promotes primordial follicle activation, potentially via the reduction in concentration of inhibitory substances. This hypothesis is not supported by work conducted by Derrar et al. (2000) in which levels of primordial follicle activation and preantral follicle growth and development were not affected by the co-culture of bovine cortical slices with medullary slices or cortico-medullary slices. In the current experimental series the method of cortical tissue preparation advocated by Telfer and McLaughlin (2012) appears to have affected the health of the tissue, as shown by the data presented in Figures 4.3, 4.15 and 4.16. The strategy of cutting the tissue pieces into squares, as utilised in the Leeds and Leeds modified treatments resulted in the highest rates of viability and integrity of the tissue on day 6 of culture compared to that observed in the AFC and AFC modified systems, in which the tissue was cut into pyramid shapes and teased apart with needles. It is likely that the increased manipulation of the tissue caused mechanical damage resulting in higher rates of necrosis in the surrounding stroma and the follicles (Benaglia et al., 2009).

As discussed in Chapter 3 maintenance of the health and integrity of ovarian stromal tissue during cortical culture is of great importance to the health of the follicles and their continued growth and development (Knight and Glister, 2006). In the previous studies - on which the current AFC system was based - stromal health and integrity was not assessed (Telfer et al., 2008, McLaughlin and Telfer, 2010). Recognition of the importance of the health of stromal tissue is becoming increasingly evident, however, most of the studies in which the health of ovarian stromal tissue has been assessed focus on the effects of exposure to CPAs and cryopreservation (Faustino et al., 2010, Oskam et al., 2010, Oskam et al., 2011, Maffei et al., 2013). The latter will be discussed in more detail in Chapter 6. In the current chapter, changes in tissue weight following culture, level of NR staining and histological assessment of stromal tissue integrity were used as markers of stromal health. Therefore this is the first study to directly report on the effect of short-term accelerated cortical culture system on stromal tissue health. The results of all these parameters indicate that the method of cortical tissue preparation prior to culture in the Leeds system is more optimised with respect to the health of the stromal tissue than the AFC system. Note that the method of tissue preparation was the same in the Leeds and Leeds modified treatment groups, in which tissue was trimmed into squares, and the AFC and AFC modified treatment groups, in which tissue was trimmed into

pyramid shapes and teased apart using needles (see Table 4.2). Although changes in tissue weight following culture were not significant ($p>0.05$), the fact that tissue weight increased in the Leeds and Leeds modified treatment groups indicates some level of stromal and follicular growth in contrast to the decrease in tissue weight observed in the AFC and AFC modified treatment groups which indicates some level of degeneration of both cellular compartments (see Figure 4.3). This hypothesis is supported by the results of NR staining and stromal health scores following culture in which the score for both parameters was significantly higher in treatments in which tissue had been trimmed into squares at the start of culture compared to pyramids (see Figures 4.15 and 4.17). This is not unexpected as the reason for teasing the pyramid shaped tissue apart was to reduce the density of the tissue thus reducing the concentration of inhibitory factors present in the medullary tissue, which has been proposed to promote global primordial follicle activation (Telfer and McLaughlin, 2012) and also facilitate gas and nutrient penetration into the tissue during culture. In the current chapter, however, this did not appear to promote follicle activation, growth and survival and was in fact detrimental to both these processes and stromal health thus resulting in increased levels of follicle apoptosis and stromal death. When follicle and oocyte diameters were analysed, however, an increase was observed on day 6 relative to day 0 in all treatment groups, indicating that they were healthy and able to grow (see Table 4.8). Therefore, the detrimental effects of teasing the tissue apart are not completely preventing follicle survival and growth. The development of additional methods of quantifying follicle, oocyte and stromal health would be helpful to determine the exact effects of the different methods of tissue preparation (see Section 4.4.5).

4.4.3 Use of NR dye

In all culture treatments NR staining was used to assess the number of viable follicles present in the tissue at the start of culture. The use of this technique ensures that all tissue cultured contained viable follicles. Following NR incubation ~72% of all tissue pieces stained positively (data not shown) and tissue that did not stain positively was discarded. This result confirms the utility of NR staining as a crude means of screening viable follicle loading density in ovarian cortex prior to culture such that follicle depleted tissue can be discarded. However the data also show that NR staining of follicles *in situ* overestimated the number of primordial follicles and the total number of follicles present in the tissue when compared to the accurate follicle counts provided following detailed histological analysis of fixed tissue after culture, as follicle counts were ~5 and ~10 orders of magnitude lower, respectively. In contrast, the use of NR

dye lead to an underestimation of the number of growing follicles as the number in fixed tissue was ~10 orders of magnitude higher than that recorded using fresh tissue. This evidence highlights the importance of examining follicle population dynamics in both fresh tissue and histologically. Although the results showed that it was difficult to distinguish between primordial and growing follicles in fresh NR stained tissue, there was however, always a positive correlation between the number of follicles recorded following NR staining and histological analysis. Therefore, the results presented in this chapter are in agreement with previous studies in that the use of NR dye facilitates the rapid estimation of the number of follicles prior to culture so that the follicle rich tissue can be selected for culture (Chambers, 2002, Chambers et al., 2010, Kristensen et al., 2011). The histological examination of the tissue then enables a more accurate assessment of the follicle population dynamics. The results presented in this chapter also show that the use of NR dye does not appear to have any detrimental effects on the subsequent health and development of follicles and stromal tissue *in vitro*, as has been shown in previous studies using both ovine and human ovarian tissue (Chambers, 2002, Chambers et al., 2010, Kristensen et al., 2011).

4.4.4 Use of culture inserts

Use of a culture insert allows the tissue pieces to remain fully hydrated and covered with a film of culture media which facilitates gaseous diffusion and the transfer of nutrients, growth factors and gonadotrophins into and out of the tissue *in vitro*. If an insert is not used the tissue pieces must sit at the bottom of the culture dish resulting in an increased diffusion distance for gases between the surface of the media and the tissue. Therefore it is more likely that tissue pieces will experience hypoxic conditions if a culture insert is not used. The process of pyruvate metabolism requires high levels of oxygen therefore it is possible that this metabolic process and subsequently oocyte developmental competence may be compromised in systems where the tissue is potentially experiencing hypoxic conditions (Van Blerkom, 1998, Harris et al., 2007). This could further explain the decrease in numbers of early stage follicles observed in the AFC culture system and explain why this occurred to a greater extent than in the Leeds system. In order to investigate this hypothesis the expression levels of genes and/or proteins associated with apoptosis – *Bax, Bcl2* and Caspase 6 (*Casp6*) and hypoxia - thrombospondin 1 (*THBS1*), Ki67, endothelins (*ET*)-*1* and *ET-2* could be monitored (Onions et al., 2013). In addition to assessing gene expression profiles to determine whether the tissue encountered hypoxic conditions the levels of metabolites in spent culture media collected following perfusion could be measured. An enzyme-linked ultramicrofluorescence assay has previously

been used to assess the consumption and/ or production of glucose, pyruvate and lactate by murine oocytes and embryos (Leese and Barton, 1984, Harris et al., 2005, Harris et al., 2009). This method could be developed to assess the levels of metabolites in spent media on days 0, 2, 5 and 8 of culture using each of the culture treatments. The level of LDH, a cytoplasmic enzyme, in spent culture media can be used as a measure of cell damage as LDH present in the cell cytoplasm will be released into the media upon damage to the cell membrane (Decker and Lohmann-Matthes, 1988, Koukourakis et al., 2003). Therefore LDH assays could also be used to compare the levels of damage caused by the various culture treatments.

4.4.5 Future work

In order to extend this study it would be interesting to use a wider variety of markers to assess both follicle and stromal health and normality. For example, the process of assaying for the production of steroid hormones, such as oestradiol and progesterone has previously been used to assess the health and normality of ovine, bovine, porcine and human follicles (Newton et al., 1999b, McCaffery et al., 2000, Oktay, 2001). Similarly assaying for the levels of peptides, such as MMPs and TIMPs, in the spent media during *in vitro* culture of bovine follicles has been used to assess normality (Telfer et al., 2000). In addition levels of AMH in the spent media could be used to assess the number of growing follicles in cortical tissue during culture (Kelsey et al., 2011, Nelson et al., 2012, Morgan et al., 2012). Additionally, levels of steroid hormones, such as progesterone and factors such as interleukin-1 and prolactin in the FF can be indicative of oocyte developmental competence (Mendoza et al., 1999). Therefore it would be interesting to repeat this experimental series and assess the levels of key steroid hormones and peptides to determine whether the culture systems used have an effect on these parameters. The level of thymidine incorporation is often used as a marker of somatic cell proliferation (Oktay et al., 1995), it would be interesting to assess TUNEL staining to determine which culture system is optimal for somatic cell proliferation. Similarly immunohistochemical assessment of PCNA or Ki-67, which are both markers of early follicle growth, could be used (Oktay et al., 1995).

In future studies it would also be interesting and informative to assess ovarian tissue, follicle and oocyte quality using markers of apoptosis. For example, the use TUNEL is common, however, is better used in combination with other apoptotic markers as this method detects DNA fragmentation, which is not solely observed in apoptotic nuclei (Fujino et al., 1996, Kockx et al., 1998). Assessment of the expression of apoptotic genes, such as anti-apoptotic *BCL2*

and pro-apoptotic *BAX* and *FAS* and its ligand, *FAS-LIGAND* can also be used to determine oocyte normality (Liu et al., 2000). Another hallmark of apoptosis is the translocation of phosphotidylserine from the inner to the outer layer of the cell membrane and this can be detected using annexin V staining (Van Engeland et al., 1998). Fluorescence analysis of pro-apoptotic caspase activity can also be used as an apoptotic marker in oocytes (Perez et al., 1999). Assessment of the levels of poly(adenosine diphosphate-ribose) polymerase-1 (PARP) cleavage, a process indicative of apoptotic cell death in response to major breaks in DNA detected in cells, have previously been used to examine the effects of chemotherapeutic agents, such as cisplatin, on ovarian tissue via protein extraction and western blotting methods (Morgan et al., 2013).

The vast majority of research conducted regarding the development of IVG culture systems has focussed on the health and survival of follicles and oocytes *in vitro*. Recent work conducted by Morgan et al. (2013), however, has highlighted the importance of determining the mechanisms by which follicle loss occurs *in vitro*. Potential mechanisms of follicle loss include damage, induced by the culture conditions, to the either the oocyte alone or to the follicular somatic cells and subsequently the oocyte, resulting in follicular atresia (Morgan et al., 2012). It is also possible that damage to the ovarian stroma could result in follicle loss, therefore the current study could be extended by investigating this possibility. Follicle loss may also occur as a result of the induction of atresia in all classes of follicles, in primarily primordial follicles or alternatively primarily in growing follicles thus resulting in increased levels of primordial follicle activation in response to decreasing levels of inhibitory factors released by growing follicles, such as AMH (Morgan et al., 2012, Morgan et al., 2013). Follicle loss in ovarian tissue can be induced by follicle culture, as evidenced in the current chapter, by exposure to chemotherapeutic drugs, such as cisplatin (Gunson, 2009, Morgan et al., 2013) and by ovary perfusion and cryopreservation protocols, as evidenced in Chapter 6. It is unlikely that the same mechanisms of follicle loss are operating in these incidences. In order to determine the mechanisms of follicle loss, levels of apoptosis and the specific cell types as well follicle classifications affected must be identified. In order to determine the different mechanisms of follicle loss operating in murine ovarian tissue as a result of exposure to chemotherapeutic agents cisplatin and doxocyclin Morgan et al. (2013) assessed levels of apoptosis in follicles of different stages using TUNEL staining and assessing levels of PARP cleavage. This revealed that primordial follicle loss occurred as a result of damage to and subsequent loss of growing follicles thus inducing an increase in primordial follicle activation. It would be interesting to use these techniques to compare the mechanisms of follicle loss exhibited in response the use

of the AFC and Leeds systems. This study could be further extended by using similar methods assessing the levels of apoptosis exhibited by the stromal tissue in order to determine whether this was having a major effect on follicle loss. Gunson (2009) developed and validated methods of isolating stromal tissue then assessing levels of damage in response to cisplatin exposure via the histological assessment of the tissue to determine the level of TUNEL staining as well as the level of LDH production in culture. In order to ensure the stromal tissue was not contaminated with ovarian surface epithelium antibodies specific to vimentin and cytokeratin, expressed by the stroma and ovarian surface epithelium, respectively, were used *in situ*. In order to further investigate the effects of stromal health it would be useful to indentify factors expressed solely by the ovarian stromal cells. For example, the expression of Sialic acid-binding Immunoglobulin Superfamily Lectin (Siglec)-11 by human ovarian stromal cells has been detected and identified as a regulator of the expression levels of cytokines, IL-7 and -10, TGF-β and TNF-α, all of which are important regulators of folliculogenesis (Wang et al., 2011). Therefore the expression levels of Siglec-11 could be used as a marker for the health and normality of stromal tissue *in vitro* when cultured using the Leeds and AFC systems. Unfortunately, Siglec-11 expression was not detected when ovarian tissue was put into culture, however, it is possible that it would be detected using a different culture system or species. The identification of various markers of stromal normality is essential for the assessment of stromal health and the effect of follicle survival.

Alternatively levels of expression of genes encoding key oocyte and somatic cell specific proteins could be analysed. In order to achieve this, normal patterns of gene expression *in vivo* would need to be established to which the patterns observed *in vitro* could be compared. Similarly analysis of the levels of expression of key imprinted genes encoding proteins that have been implicated as regulators of folliculogenesis, oogenesis and embryogenesis as well genes encoding enzymes responsible for the establishment and maintenance of the methylation patterns of imprinted genes, would be a useful method of examining whether folliculogenesis and oogenesis are progressing normally *in vitro*. In Chapter 5 patterns of normal gene expression *in vivo* are established and compared to those observed in follicles derived from the long-term, physiological cortical culture system and isolated preantral follicle culture system described in Chapter 3. In the future it would be useful to perform the same analyses to determine whether follicles derived from a short-term cortical culture system are displaying normal patterns of gene expression.

4.5 Conclusion

The results reported in this experimental series show that it is possible to activate and grow ovine primordial follicles *in vitro* up to the primary stage using the Leeds cortical culture system in the short-term. However, the yield of growing follicles after 6 days of culture using the Leeds culture system was very low, 0.17±0.09 follicles per mg tissue cultured for 6 culture repeats. Furthermore, over this timeframe follicles only reached the primary stage of development. As was shown in Chapter 3 *in vitro*-derived primary follicles were not competent to develop to the EA stage in isolated culture. Therefore when the sheep is used as a model species, short-term accelerated growth systems, such as the AFC system tested here are suboptimal for the activation and sustained growth of healthy follicles whereas a long-term, more physiological, gentle growth environment better supports growth activation and the needs of growing oocytes and somatic cell division during cortical culture.

5 Comparison of the Expression Patterns of Key Genes Implicated in the Regulation of Folliculogenesis, Oogenesis, and Imprint Establishment and Maintenance in Ovine Follicles and Oocytes Grown *In Vivo* and *In Vitro*.

5.1 Introduction

The development of multiphase culture systems that enable the complete *in vitro* growth and maturation of oocytes would be hugely beneficial with respect to developing methods of fertility preservation and restoration. As discussed in Chapter 1 although the IVG, IVM and subsequent IVF of oocytes has successfully resulted in the birth of live offspring in rodents, the resultant offspring exhibited health problems (Eppig and O'Brien, 1996). Therefore prior to use in humans it is vital that *in vitro*-derived oocytes are tested for markers of normality that reflect gamete health and developmental competence and confirm the safety of the technology. In addition, testing for normality during oocyte development *in vitro* may facilitate further improvement of the culture conditions.

Chapter 3 documents the development of step 1 of a multistep culture strategy and recorded the development and testing of a long-term, cortical culture system that supported the activation and development of primordial follicles and facilitated the *in vitro*-derivation of primary, transitional and secondary follicles. The health of oocytes, follicles and stromal tissue was assessed both in fresh tissue and histologically. In fresh tissue NR staining, tissue weight and follicle population dynamics before and after culture were used as preliminary indicators of normal development *in vitro*. Follicle and oocyte diameter as well as follicle population dynamics relative to *in vivo*-derived controls at time 0 were used as histological indices of normality. Additionally, stromal health was assessed histologically. The step 1 long-term *in situ* cortex cultures generated *in vitro*-derived secondary follicles which could be isolated and cultured in the second step system up to the EA stage. The health of the oocytes and follicles generated in steps 1 and 2 were analysed during culture using NR viability staining, by morphological assessment with respect to the presence of a ZP, antral cavity and oocyte colour (dark patches indicating necrosis), and growth and somatic cell proliferation were quantified via the measurement of follicle and oocyte diameter. In the current chapter the health of the *in vitro*-derived primary, transitional, secondary and EA follicles documented and archived in Chapter 3 as well as GV and MII oocytes and somatic CCs will be evaluated in greater depth by the analysis of molecular markers of normality relative to stage matched *in vivo*-derived

controls. The expression patterns of key genes implicated in the regulation of folliculogenesis, oogenesis, embryogenesis and imprint establishment and maintenance will be used to investigate the normality of *in vitro*-derived samples. Genes encoding key oocyte- and somatic cell-specific proteins will also be considered. The third group of genes to be investigated include key imprinted genes encoding proteins that have been implicated as regulators of folliculogenesis, oogenesis and embryogenesis as well genes encoding enzymes responsible for the establishment and maintenance of the methylation patterns of imprinted genes. The expression patterns of some of the candidate genes have previously been established *in vivo* in ovine follicles and oocytes as will be discussed in detail below.

Commonly used markers of normality *in vitro* include assaying for the production of steroid hormones, such as E2 and P4, and peptides such as MMPs and TIMPs in the spent media (Newton et al., 1999b, McCaffery et al., 2000, Telfer et al., 2000). Additionally, levels of steroid hormones, such as P4 and factors such as IL-1 and prolactin in the FF can be indicative of oocyte developmental competence (Mendoza et al., 1999). The level of thymidine incorporation is often used as a marker of somatic cell proliferation (Oktay et al., 1995). Tissue, follicle and oocyte quality can also be assessed using markers of apoptosis. The use of TUNEL is common, however, is better used in combination with other apoptotic markers as this method detects DNA fragmentation, which is not solely observed in apoptotic nuclei (Fujino et al., 1996, Kockx et al., 1998). Assessment of the expression of apoptotic genes, such as anti-apoptotic *BCL2* and pro-apoptotic *BAX* and *FAS* and its ligand, *FAS-LIGAND* can also be used to determine oocyte normality (Liu et al., 2000). Another hallmark of apoptosis is the translocation of phosphotidylserine from the inner to the outer layer of the cell membrane and this can be detected using annexin V staining (Van Engeland et al., 1998). Fluorescence analysis of pro-apoptotic caspase activity can also be used as an apoptotic marker in oocytes (Perez et al., 1999).

As discussed in Chapter 1 a number of studies have investigated the impact of IVG on gene expression, the results of which have shown that culture conditions can affect the expression of important regulatory genes (Sanchez et al., 2009, Sanchez et al., 2010, Sanchez et al., 2011, Jiao and Woodruff, 2013).

5.1.1 Somatic cell-specific genes

The expression patterns of 8 primarily somatic cell-specific genes thought to be important in oogenesis and folliculogenesis have been determined throughout ovine follicular development *in vitro* and *in vivo*. The justifications for selecting these genes as candidates for developmental normality analysis are detailed below.

The GC-derived factor- AMH, exerts an inhibitory effect on follicle development via its autocrine and paracrine actions on GCs and TCs, respectively. AMH is expressed from the primary to the antral stage in mice, sheep and humans (Bezard et al., 1987, Durlinger et al., 1999, Weenen et al., 2004, Bonnet et al., 2011). The inhibitory effects of AMH have been well characterized in rodents with respect to the inhibition of follicle activation via suppression of the expression of genes encoding stimulatory factors (Durlinger et al., 1999, Weenen et al., 2004). Additionally, AMH decreases the sensitivity of antral follicles to FSH thus weakening the gonadotrophins's effects. In contrast, in sheep, AMH does not appear to inhibit primordial follicle recruitment, but rather the inhibitory effects of AMH are observed at later stages of follicle development with respect to the desensitisation of GCs and TCs to FSH and LH, respectively (Campbell et al., 2012).

The actions of FSH are mediated through its transmembrane receptor, FSHR, expressed by GCs (Meduri et al., 2008). The FSHR is a G-protein coupled receptor (Meduri et al., 2008). Binding of FSH to its receptor promotes follicular development and the dependence on FSH for the continued development of antral follicles has been demonstrated in sheep (Campbell et al., 1999). Expression of the FSHR has been detected from the primary follicle stage onwards in sheep (Tisdall et al., 1995).

One of the aims of the experiments in this chapter was to investigate the expression patterns of inhibins, activins and activin inhibitor – follistatin (FST) during ovine folliculogenesis. Studying the expression patterns of inhibins and activins is complex as these proteins are each composed of two subunits (Vale et al., 1988). Inhibin A and B are composed of an α subunit and a βA or βB subunit, respectively. Whereas activins are composed of only β subunits: activin A (βAβA), activin AB (βAβB) and activin B (βBβB). Furthermore glycosylation of the subunits determines expression patterns and association of subunits (Antenos et al., 2007). The subunits comprising activin and inhibin as well as FST are expressed by GCs and act upon

the oocyte, GC and TC. With respect to expression patterns in sheep, *FST* mRNA has been detected in GCs of preantral and antral follicles, the CL and the pre-ovulatory follicle, but at a reduced level in the latter (Tisdall et al., 1994). Whereas α and βA subunit mRNA has been detected only in antral follicles. Co-expression of these subunits in sheep forms inhibin A. Inhibin A production by the dominant follicle inhibits FSH release thus inhibiting the continued development of the subordinate follicles, as evidenced by a study in which the ablation of the dominant follicle leads to an acute decrease in inhibin A and a subsequent increase in FSH levels (Evans et al., 2002). As inhibin B has not been detected in ovine FF the expression of the inhibin subunits in this experimental series will indicate the levels of inhibin A, activin A and activin B expression (McNeilly et al., 2002). Briefly, activins in humans and rodents act to: increase GC proliferation and FSHR expression in both preantral and antral follicles; increase LHR expression; and to decrease both FSH-stimulated E2 and inhibin A production and LH-stimulated androgen production in antral follicles (Knight et al., 2012). In contrast, the role of inhibin is to promote androgen production indirectly, via the suppression of activin action (Knight et al., 2012). Similarly, in sheep activin inhibits androgen production, whereas inhibin promotes it via inhibition of activin action (Young et al., 2012). Follistatin blocks the actions of activin with respect to the inhibition of androgen production. As follicle development progresses in humans and rodents the ratio of α subunit to β subunit expression increases thus the concentrations of activin relative to inhibin is higher in earlier stage follicles than in larger, antral follicles. This is thought to prevent production of androgens in smaller follicles that may lead to premature differentiation of somatic cells (Knight et al., 2012). In this chapter the expression pattern of the α, βA and βB subunits were analysed throughout ovine follicular development. Determination of the expression pattern of activin is of particular interest as it has been used as a media supplement in a number of follicle culture systems and has been shown to promote follicle growth *in vitro* in bovine and human, see Chapter 1 (Thomas et al., 2003, McLaughlin et al., 2010).

The expression patterns of *IGF1* and its receptor, *IGF1R* in ovine follicles were selected for analysis in this chapter. The binding of TC-derived IGF1 to its receptor on follicular somatic cells promotes follicle development via the promotion of somatic cell proliferation and GC differentiation, with respect to *FSHR* expression (see Chapter 1) (Perks et al., 1995, Monget and Bondy, 2000). The exact expression patterns of IGF1 and its cognate receptor in the early stages of preantral follicle development are yet to be elucidated as most research conducted has been concerning only the later stages of development, with follicles smaller than 2-4mm

grouped together (Perks et al., 1995, Spicer et al., 1995, Monget and Bondy, 2000). The activity and bioavailability of both IGF1 and IGF2 are regulated by IGFBPs (Monget et al., 1993).

5.1.2 Oocyte-specific genes

As detailed in Chapter 1 oocyte-GC interactions are of vital importance to the continued growth and development of germ cells. Although previously thought to play a passive role in its own development, increasing information is emerging that supports the oocyte's role in regulating its own microenvironment and therefore its own development. Numerous studies have been conducted into the role of 3 important oocyte-derived growth factors: GDF9, BMP15 and BMP6. Briefly the role of these factors is to regulate the differentiation and proliferation of the somatic cells to ensure that this occurs at a rate that compliments oocyte growth and so ensures that the gamete is able to mature and achieve competence. Much research has been conducted in a number of species concerning the roles of these growth factors, however, species-specific differences have been highlighted thus hindering the elucidation of the exact roles (Lin et al., 2012, Rouhollahi Varnosfaderani et al., 2013). To summarise, in sheep both *GDF9* and *BMP6* are expressed by oocytes from the primordial stage and throughout antral follicle development, whereas *BMP15* expression starts at the primary stage and progresses until ovulation.

Briefly GDF9, BMP15 and BMP6 promote GC proliferation and regulate GC differentiation in the mouse, pig, cow sheep and human (Colonna and Mangia, 1983, Vanderhyden et al., 1992, Joyce et al., 2000, Otsuka et al., 2000, Vitt et al., 2000, Otsuka et al., 2001, Gilchrist et al., 2004, Dragovic et al., 2005, Hussein et al., 2005, Sugiura et al., 2005, Thomas and Vanderhyden, 2006, Gilchrist et al., 2008, Su et al., 2008, Su et al., 2009). In sheep GDF9, BMP15 and possibly to a lesser extent BMP6 have mitogenic effects on GCs. Unlike in rodents, in which GDF9 alone is sufficient, both GDF9 and BMP15 are required to promote GC proliferation in sheep, as evidenced by the level of thymidine incorporation (Lin et al., 2012). It is interesting to note that GDF9 derived from rodents is sufficient to increase thymidine incorporation in ovine GCs whereas ovine GDF9 is not. Conflicting evidence exists regarding the mitogenic effects of BMP6. In a study conducted by Juengel et al. (2006) BMP6 did not appear to promote GC proliferation. The authors, however, suggested that this might be due to the timing, culture length or conditions and highlight the fact that localization of BMP6 suggests that this factor does play a mitogenic role. Furthermore, in a study conducted by Campbell et al. (2009) BMP6 did appear to have a mildly mitogenic effect on GCs.

In mice GDF9 has been shown to promote CC mucification and expansion, however, in a recent study exogenous GDF9 did not affect the expression of genes, such as *HAS2* linked to the aforementioned processes (Rouhollahi Varnosfaderani et al., 2013). Nor was the expression of apoptotic genes greatly affected by the addition of exogenous GDF9. Authors suggest that although GDF9 may play a minor role in these processes it is likely that other factors, such as BMP15, play a more important role. Indeed in a recent study ewes were immunized against BMP15 resulting in increased levels of cAMP, which were associated with increased levels of CC luteinisation, suggesting that BMP15 inhibits CC luteinisation (Juengel et al., 2011).

Oocyte-derived factors GDF9 and BMP6 in sheep regulate GC differentiation with respect to the production of steroid hormones and inhibin. Ovine GDF9 has been shown to both inhibit the FSH-stimulated production of P4 and to stimulate inhibin A production by GCs (McNatty et al., 2005). Whereas ovine BMP15, either alone or in combination with GDF9, did not affect P4 or inhibin production by GCs (McNatty et al., 2005). The mechanism of BMP6 action on GC differentiation has not been fully elucidated in sheep. That fact that BMP6 regulates follicle development has been evidenced by its regulation of ovarian steroid hormones, A4, P4 and E2, and inhibin A production. In sheep BMP6 inhibits P4 production but promotes E2 and inhibin A production by GCs (Juengel et al., 2006, Campbell et al., 2009). The effect of BMP6 on A4 production by TCs is dose-dependent and is stimulatory at low concentrations but inhibitory at high concentrations (Campbell et al., 2009, Webb and Campbell, 2006). The promotion of A4 production at low BMP6 levels may occur via the promotion of TC proliferation, thus increasing the number of cells producing this hormone. It is also important to note that the effects of BMP6 on the production of E2, A4 and inhibin A are transitory (Campbell et al., 2009). In addition, the actions of BMP6 are also dependent upon the concentrations of IGF1 with respect to the promotion of the differentiative actions of FSH (Webb and Campbell, 2006).

The transcription factor *FOXO3* is expressed by the oocytes of primordial and primary stage follicles in mice (Castrillon et al., 2003). Less information exists regarding the exact expression patterns of *FOXO* in sheep and pigs however, it has been shown to be expressed by the oocytes of preantral follicles in both species (Baillet et al., 2008, Ding et al., 2010). As FOXO is an inhibitor of primordial follicle activation elucidation of its expression patterns in ovine follicles both *in vivo* and *in vitro* may indicate why higher levels of follicular activation are observed following cortical culture (Liu and Lehmann, 2006). Expression of *GTSF1* in human, ovine, bovine and murine oocytes has been detected throughout oogenesis, with the highest

levels of expression in secondary GV oocyte (Krotz et al., 2009, Lu et al., 2009, Liperis et al., 2013, Liperis, 2014). The localization of GTSF1 has implicated it as a potential regulator of oogenesis and folliculogenesis. In contrast to male murine *Gtsf1* mutants which are infertile, the fertility of female mutants does not seem to be affected (Yoshimura et al., 2009). Knockdown of *GTSF1* in ovine oocytes however, resulted in the genes associated with processes such as post-translational modification and cytoskeleton organisation being down-regulated, which led authors to conclude that *GTSF1* may be involved in 'post-transcriptional control of RNA processing, translational regulation and RNA storage' (Liperis et al., 2013, Liperis, 2014).

The composition of the ZP has been well characterized in a range of species including human and mouse (Wassarman, 1988, Lefievre et al., 2004, Gupta and Bhandari, 2010). Zona pellucida formation occurs at the primary stage in mice and humans (Zamboni, 1974, Epifano et al., 1995), see Chapter 1 for the composition and expression patterns of the ZP proteins. Information regarding the composition of the ovine ZP is however, scarce. Therefore the expression patterns of ZP proteins by the ovine oocyte are of value as an index of oocyte development.

5.1.3 Imprinted genes and the regulation of epigenetic modifications

The expression of imprinted genes is regulated by epigenetic modifications to the DNA (Qiu, 2006). The establishment of these modifications occurs during gametogenesis and following fertilisation. Therefore determining the *in vivo* expression patterns of imprinted genes known to be important for oogenesis and folliculogenesis and comparing these to patterns observed in follicles derived *in vitro* is of particular interest as there are concerns (discussed in Chapter 1) that long-term culture and/or the use of IVG and micromanipulation techniques could result in epigenetic disruption (Amor and Halliday, 2008). One form of epigenetic modification is the methylation of cytosine residues (Reik et al., 2001). The establishment and maintenance of DNA methylation requires DNA methyltransferases (DNMTs), therefore the expression patterns of 2 forms of these key enzymes identified in sheep will also be analysed, *DNMT1* and its splice variant *DNMT1(12b)*. DNA methyltransferase 1 (DNMT1) was first detected in mice. It was found to maintain methylation patterns in hemimethylated regions, and also to exhibit some *de novo* activity (Goll and Bestor, 2005). Disruption of *DNMT1* expression in the mouse has lead to *H19* expression by both alleles and *IGF2* and *IGF2R* expression by neither allele (Li et al., 1993). The expression patterns of *H19*, *IGF2* and *IGF2R* will also be analysed in this

chapter. An oocyte-specific form, *DNMTo* was identified in mice and found to be vital for embryogenesis. Similarly both somatic- and oocyte-specific forms of *DNMT1* have been identified in humans (Bestor, 2000, Hayward et al., 2003). In the porcine model 3 *DNMT1* isoforms have been identified – *DNMT1o1, DNTMT1o2* and *DNMT1s*, all of which are expressed by the oocyte, but only *DNMTs* is expressed by somatic cells (Giraldo et al., 2013). In the ovine model two *DNMT1* isoforms- *DNMT1s* and *DNMT1(12b)* have been identified, both of which are expressed by somatic cells and oocytes, unlike that observed in humans and mice (Taylor et al., 2009). Disruption of *DNMT1(12b)*- a splice variant of *DNMT1*, caused arrest of embryonic development thus indicating that it is likely to be the main isoform present which is required to maintain methylation patterns. In addition, higher levels of *DNMT1(12b)* were detected in GV oocytes than at later embryonic stages. This led authors to conclude that *DNMT1(12b)* is the main DNMT1 isoform in the oocyte, therefore from here on in it will be referred to as *DNMT1o*. One of the aims of this experimental series is to determine the expression patterns of ovine *DNMT1s* and *DNMT1o* throughout follicle development *in vivo* and *in vitro*.

As alterations to the methylation status of imprinted genes can be caused by ARTs and the fact that imprints are established during gametogenesis suggests that the use of IVG may potentially alter oocyte methylation patterns and therefore the expression of imprinted genes, see Chapter 1. Differences in expression levels of imprinted genes between *in vivo* and *in vitro* derived oocytes may indicate aberrations in methylation patterns. The expression patterns of paternally imprinted *H19* and *IGF2R*, and maternally imprinted *MEST* and *IGF2* during ovine folliculogenesis were therefore analysed.

Alterations in the methylation status of these 4 imprinted genes have been associated in epigenetic disorders SRS and BWS as well as male sub/infertility (Chapter 1 and Chang et al. (2005). The use of mouse models has enabled the elucidation of 2 roles for *H19*. It is both a tumour suppressor gene and a regulator of the expression of other imprinted genes, including *IGF2* and its receptor (Gabory et al., 2010). The methylation status of a DMR between *IGF2* and *H19* determines which gene is expressed on that allele, as both use the same set of tissue-specific enhancers and the genes are therefore co-ordinately transcribed throughout embryonic development. In the maternal allele the DMR is demethylated therefore the enhancers favour the expression of *H19* over *IGF2* (Cerrato et al., 2003). The methylation status of the DMR is established during oogenesis. Binding of IGF2 to its receptor elicits a

response required for normal fetal growth (Obata et al., 1998). The actions of IGF2 are reviewed in detail in Chapter 1. Briefly IGF2 stimulates mitosis, DNA synthesis, steroidogenesis in GCs and TCs and nuclear maturation of the oocyte.

5.1.4 Aims

The first aim of this experimental series was therefore to map the normal expression patterns of key genes implicated in the regulation of folliculogenesis, oogenesis, embryogenesis and imprinting establishment and maintenance in ovine follicles and oocytes grown *in vivo*. This work established the normal pattern of gene expression as a marker of oocyte and follicle development *in vivo* which could be used as a bench mark for gene expression following growth *in vitro*. Secondly, the expression patterns of the same genes were investigated in *in vitro*-derived follicle somatic cells and oocyte samples in order to determine the impact of extended serum-free culture on gene expression. The third aim of the experiment was to directly compare stage-matched *in vivo*- and *in vitro*-derived samples to determine whether the *in vitro* growth of oocytes lead to changes in gene expression, within specific follicle classifications. The IVG-derived follicles and oocytes analysed here were archived from the studies reported in Chapter 3.

5.2 Materials and Methods

This experimental series was conducted to compare the expression patterns of genes key to oocyte and follicle growth, development and health in *in vivo*- and *in vitro*-derived follicles and oocytes. A summary of the classifications of follicles analysed is shown in Table 5.1. It was not possible to obtain *in vivo*- and *in vitro*-derived counterparts for all follicle classifications studied as the starting point for all the proposed studies were *in vivo*-derived primordial follicles. Similarly, MII oocytes and cumulus cells were obtained following 24 hours of maturation *in vitro*. No *in vivo*-derived MII oocytes were available for study. The *in vitro*-derived primary, transitional and secondary follicles refer to follicles isolated following long-term (16-23 day) cortical culture as detailed in Chapter 3. The term secondary degenerating refers to follicles that were isolated from fresh tissue then put into isolated preantral follicle culture where they then started to degenerate as evidenced by either a decrease in diameter or no increase in diameter after 3 consecutive media changes, as described in Chapter 3. Early antral follicles were derived *in vitro* from *in vivo*-derived secondary follicles as detailed in Chapter 3.

Table 5.1 A summary of *in vivo*- and *in vitro*-derived samples analysed in this experimental series. The total number of oocytes and follicles used and the number of cells pooled per sample is shown. With respect to the number of CC samples this refers to the number of COCs from which samples were obtained. Samples which could not be obtained are denoted using -.

Follicle Classification	***In vivo*- derived**		***In vitro*- derived**	
	Total	**Number per sample (Number of replicate samples/stage)**	**Total**	**Number per sample (Number of replicate samples/stage)**
Primordial	120	40 (3)	-	-
Primary	40	10 (4)	24	10,10, 4 (3)
Transitional	20	5 (4)	24	10, 5, 5, 4 (4)
Secondary	40	10 (4)	17	10, 5, 1, 1 (4)
Secondary degenerating	-	-	21	9, 5, 4, 3 (4)
Whole EA follicle	4	1 (4)	4	1 (4)
GV Oocyte	12	3 (4)	x	x
GV CC	12	3 (4)	x	x
MII Oocyte	x	x	20	5
MII CC	x	x	4	1

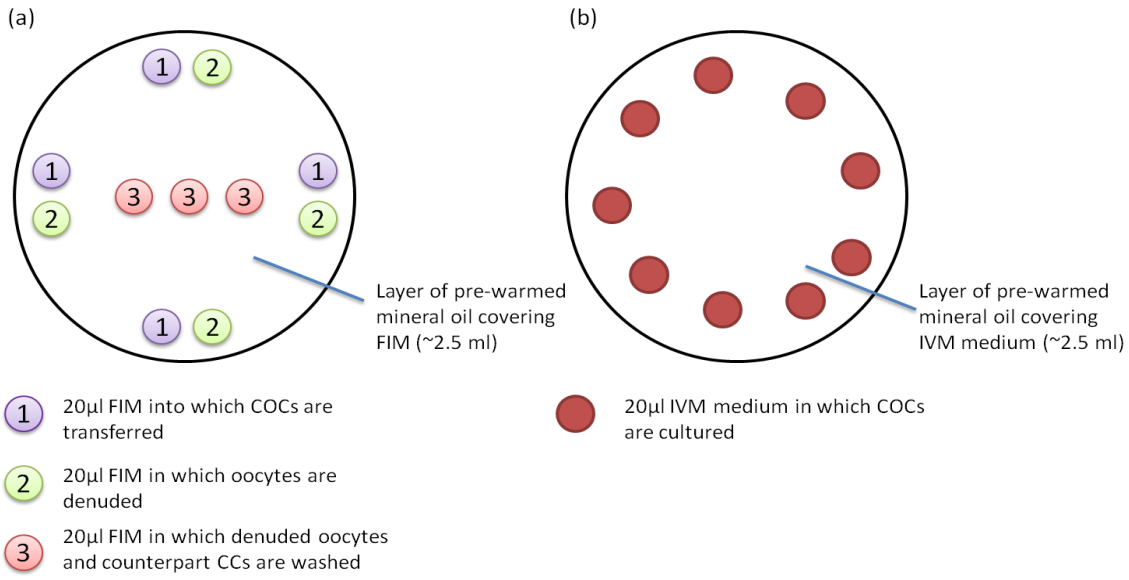


Figure 5.1 A diagrammatic representation of the set-up of the plates used (a) to denude COCs from GV and MII oocytes and (b) for IVM. All media in the plates was pre-warmed to 37°C and pre-gassed. In plate (a) the COCs were first transferred to an outer drop and rinsed in fresh medium, then transferred to a second drop in which a Flexipet with a 130µm tip attached was used to denude the oocyte, by repeatedly pipetting until all somatic cells had been removed. Denuded oocytes and counterparts were then washed a further 3 times in the central wells.

5.2.1 Collection of samples for molecular analysis

All *in vivo*-grown, preantral follicle isolations were carried out in pre-warmed FIM (37°C), as described in Chapter 3, Section 3.2.2, with care being taken to remove all stromal tissue. In order to obtain denuded GV oocytes and their CC counterparts 21G needles attached to 5ml syringe where used to trim GCs from the EA follicle. Follicles were then transferred to a sterile Petri dish, set up as shown in Figure 5.1 (a), denuded and washed.

5.2.2 *In vitro*-maturation of oocytes

GV oocytes, *in vitro*-derived MII oocytes and their counterpart unexpanded and expanded CCs, respectively, were obtained as follows. Ovaries were obtained as described in Sections 2.1 and 2.2. Ovaries were transferred to pre-warmed FIM (see Appendix I). All processing of the tissue was carried out under laminar flow conditions and on a heated stage (37°C). Antral follicles of approximately 2-5mm diameter were aspirated using a sterile 20ml syringe and 19G needle. The entire contents of the aspirate, i.e. the cumulus-oocyte complexes (COCs) and follicular fluid, was transferred to a sterile 90mm Petri dish. In order to obtain the GV and counterpart GV-CC samples ~20-30 ovaries were used per collection, for 4 replicate collections.

Table 5.2 Composition of IVM culture media. The volumes required to make a 10ml solution are shown, with the stock and final concentrations as detailed. The solution was filtered and stored for up to 1 week. See Appendix I for details of the preparation of the basal culture media and additive stocks.

Additive	Volume (µl)	Stock Concentration	Final Concentration
Basal culture media (see Appendix I)	9.768ml		
Bovine holo-transferrin	10	5 $mgml^{-1}$	5 μgml^{-1}
Sodium Pyruvate	100	47mM	0.47mM
Sodium Selenite	1	50μgml^{-1}	5$ngml^{-1}$
L-glutamine	100	200mM	2mM
Bovine Insulin	10	10μgml^{-1}	10$ngml^{-1}$
Human Long-R3 IGF-1	1	100μgml^{-1}	10$ngml^{-1}$
Ovine FSH	5	2$iUml^{-1}$	0.001$iUml^{-1}$
Ovine LH	5	2$iUml^{-1}$	0.001$iUml^{-1}$

For the *in vitro*-derived MII oocytes this was repeated for ~30-50 ovaries per IVM culture for 2 replicate cultures. Cumulus-oocyte complexes with homogenous cytoplasm and 3-4 layers of

cumulus cells were selected and transferred to a 4-well Nunc dish containing fresh, pre-warmed FIM, before being washed a third time in fresh FIM. Finally COCs were transferred to a second, 4-well dish containing ~50µl of pre-warmed and gassed *in vitro*-maturation (IVM) medium per COC, the composition of which is shown in Table 5.2.

A 90mm Petri dish was set-up as shown in Figure 5.1(b). Five COCs were added to each 20µl drop of IVM media. Plates were transferred to an incubator set at 38.6°C for 22-24 hours. Following this incubation period COCs were transferred to plates set up as shown in Figure 5.1 (a). In the first well needles (21G) were used to trim CCs from the oocyte. The oocytes were then transferred to a separate 90mm Petri dish containing 20µl drops of pre-warmed (37°C) 80IUml^{-1} hyaluronidase (see Appendix III) overlaid with pre-warmed mineral oil, using a Flexipet with a 140µm tip attached. The COCs were pipetted up and down 5-10 times for a maximum of 60 seconds to remove all CCs. The fully denuded oocyte was then transferred to the second well on the original plate (Figure 5.1). The oocytes were then examined to establish their meiotic stage, which was indicated by the presence or absence of a polar body. If a polar body was observed the oocyte and counterpart CCs were used for molecular analysis. Following isolation, and if relevant, denudation samples were washed 3 times in Dulbecco's PBS (Gibco® 14190-136, Invitrogen), snap frozen using liquid nitrogen in 10µl lysis buffer (see Appendix I), in thin-walled Eppendorf tubes, then stored at -80°C until analysed.

5.2.3 Gene specific primer design

The National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/) was used to obtain sequence information required to design gene specific primers. A search for the mRNA sequence information of each gene of interest for sheep was carried out, which could then be used to design primers within an appropriate coding region. If sequence information for a particular gene of interest did not exist for sheep, the information for bovine, human or mouse was used as appropriate. Primers pairs of around 20 base pairs (bp) in length, as this length is sufficient to ensure only the target gene is amplified due to the level of homology, were selected manually to ensure similar annealing temperatures. Primer pairs were then ordered from Invitrogen Ltd (http://www.lifetechnologies.com/uk/en/home.html). The 3 housekeeping genes selected, see Table 5.3 were as recommended by O'Connor et al. (2012) to ensure accurate, relative quantification of transcript abundance in ovine oocytes/follicles. The housekeeping genes were glyceraldehyde-3-phosphate dehydrogenase

(*GAPDH*), tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, zeta polypeptide (*YWHAZ*) and histone 2 alpha (*H2A*) (Radonić et al., 2004, Nygard et al., 2007).

Table 5.3 Real time housekeeping primer sequences.

Gene	Primer sequence	Size (base pairs)	Reference/ Species
GAPDH	F: 5' TGATTCCACCCATGGCAAGT 3'	93	AF030943 (ovine)
	R: 5' CGCTCCTGGAAGATGGTGAT 3'		
H2A	F: 5' GAGTAGGCGGCTGGTTCTC 3'	109	AY074805 (ovine)
	R: 5' GGAGTCCTTCCCAGCCTTAC 3'		
YWHAZ	F: 5'ACTGGGTCTGGCCCTTAACT3'	98	NM_001267887.1 (ovine)
	R: 5'TGGCTTCATCAAATGCTGTC3'		

Table 5.4 Real time somatic cell-specific gene primer sequences.

Gene	Primer sequence	Size (base pairs)	Reference/ Species
AMH	F: 5' TGGTGCTGCTGCTAAAGATG 3'	99	NM173890 (bovine)
	R: 5' GACAGGCTGATGAGGAGCTT 3'		
FSHR	F: 5' CAGGACAGCAAGGTGACAGA 3'	188	NM1009289 (ovine)
	R: 5' GGCAGGTTGGAGAACACATT 3'		
IGF1	F: 5'GATGTACTGTGCGCCTCTCA3'	120	NM_001009774.3 (ovine)
	R: 5'GCACTCCCTCTGCTTGTGTT3'		
IGF1R	F: 5'AGCCCGAGAACATGGAGAG3'	91	AF025303.1 (ovine)
	R: 5'TCAGCCTTGTGTCCTGAGTG		
INHα	F: 5'GAGCCCGAGGACCAAGATGTCTCC3'	91	NM_174094.3 (bovine)
	R: 5'CCTCAGCCTCTCCAGCATCTGGC3'		
INHβA	F: 5'ACCCTCCCAAAGGATGTACC3'	94	NM_174363.1 (bovine)
	R: 5'CGGGTCTCTTCTTCAAGTGC3'		
INHβB	F: 5'ATGGCCTGGCCTCCTCCCG3'	101	NM_176852.1 (bovine)
	R: 5'CTTCAGGTAGAGCCACAGGCTGGC3'		
FST	F: 5'CAACACGCTCTTCAAGTGGA3'	85	NM_001257093.1 (ovine)
	R: 5'ACAGTCCACGTTCTCACACG3'		

Table 5.5 Real time oocyte-specific gene primer sequences.

Gene	Primer sequence	Size (base pairs)	Reference/ Species
ZP2	F: 5'GCCCCAGTTGTCTCTCACTT3'	113	XM_00402082 7.1 (ovine)
	R: 5'CTGCTTCTGGATGACCCAAG3'		
GDF9	F: 5' AGTAAGCTGGAACCGGAATCG 3'	95	AF078545 (ovine)
	R: 5' GGTGGCCGACAAGAGAAGTCT 3'		
GTSF1	F:5'CTCTGGACCCTGAAAAGCTG3'	120	NM_00103427 3.2 (bovine)
	5'GTTTGTTTGCGACATCAGGA3'		
BMP6	F: 5'TGTGACGGAGAATGTTCGTT3'	83	XM_00401936 0.1 (ovine)
	R: 5'ATGAGGTGAACCAGGGTCTG3'		
BMP15	F: 5'GGCAAAAGCTCTGGAATCACA3'	102	NM_00111476 7.1 (ovine)
	R: 5'TGCCATGCCACCAGAACTC3'		
FOXO3	F: 5'ATGGGAGCTTGGAATGTGAC3'	120	NM_00126788 9.1 (ovine)
	R: 5'CCACGTTCAAACCAACAACA3'		

Table 5.6 Real time imprinted genes primer sequences.

Gene	Primer sequence	Size (base pairs)	Reference/ Species
IGF2	F: 5'TTCTGCCAAGTGACACCATC3'	87	NM_00100931 1.1 (ovine)
	R: 5'GGACGGTACAGGGATTTCAG3'		
IGF2R	F: 5'ATGCTTTCATCATCCGCTTC3'	91	AF353513.1 (ovine)
	R: 5'ATCCCCAGGCTAGAGTCGAT3'		
MEST	F: 5'AGCCTCTGTGCCAGAGATTTA3'	113	XM_00400807 0.1 (ovine)
	R: 5'CTAGCTCAGGAGGGACATGC3'		
H19	F: 5'CCCTCCTTTCCCTCTTTTTC3'	83	AF105429.1 (ovine)
	R: 5'GCTCTGGACGCTCTGAGTCT3'		
DNMT1o	F: 5'CAATTCACATATCAAAGTACCAGCA3'	83	FJ976676.1 (ovine)
	R: 5'CGAGTCATTTTCTTCTCAGTTAGTTC3'		
DNMT1s	F: 5'AGCCTGAAAGAGCAAAACCA3'	110	EF601875.1 (ovine)
	R: 5'CGAGTCATTTTCTTCTCAGTTAGTTC3'		

5.2.4 mRNA isolation

Procedures from previously described protocols were modified and used as these methods enabled the creation of cDNA libraries from very small numbers of cells (Huntriss et al., 2002, Huntriss et al., 2004). Isolation of mRNA from the *in vivo*- and *in vitro*-derived samples was achieved as follows. Cells were completely lysed by heating samples from -80°C to 80°C for 20 minutes, using an Applied Biosystems® 60 well Thermal Cycler (Life Technologies, Paisley, UK). Reagents from Dynabeads® mRNA DIRECT™ kit (Invitrogen) were warmed to room temperature prior to use. A 20µl volume of Dynabeads® Oligo$(dT)_{25}$ per sample was transferred to a sterile microcentrifuge tube in a Dynal-MPC®, Magnetic Particle Concentrator (Invitrogen, Ltd), resulting in the beads concentrating against the back of the tube thus allowing the supernatant to be removed using a pipette. The beads were then washed twice in a volume of Dynal® Lysis Buffer equal to that of the volume of beads originally used. A final volume of Dynal® Lysis Buffer was then added and the beads re-suspended then 20µl added to each sample following lysis. The tubes were then transferred to a blood wheel (Baird and Tatlock (London) Ltd, Romford, UK) and rotated for 20-30 minutes at room temperature. This allowed the oligo$(dT)_{25}$ on the magnetic beads to bind to the poly(A) tail of the mRNA sample. Samples were then briefly centrifuged then washed 3 times with 100µl of buffer A, then 100µl of buffer B (supplied by the Dynabeads® mRNA DIRECT™ kit), prior to resuspension in 3µl of sterile RNAse/DNase free water. Samples were then stored at -80°C until future use.

5.2.5 cDNA synthesis

Procedures previously described were modified and used to design primers (see Table 5.7) and create cDNA libraries (Eberwine et al., 1992, Wang et al., 2000). A 20µl volume of reaction mix per sample containing; 4µl (5x) first strand reaction buffer, 1µl diothiothreitol (DTT;0.1M), 1µl dNTP mix (10mM), 1µl RNAse out™, 1µl Superscript reverse transcriptase RNase-H ($200U\mu l^{-1}$) and 10µl RNase/DNase-free water, plus 1µl of both the Oligo$(dT)_{24}$ primer and template switching primer to give a final concentration of 10pM, was prepared at room temperature, then added to the sample. All reagents were supplied by Invitrogen Ltd. The sample with the added reaction mix was then heated to 42°C for 90 minutes, then cooled to 4°C for 2 minutes, using an Applied Biosystems® 60 well Thermal Cycler.

Table 5.7 The primer sequences used for cDNA synthesis. The oligo(dT)$_{24}$ primer binds the mRNA poly(A) tail and the template switching primer ensures the transcription of full length transcripts only.

Primer	Sequence
Oligo(dT)$_{24}$ primer	AAACGACGGCCAGTGAATTGTAATACGACTCACTATAGGGCGCT TTTTTTTTTTTTTTTTTTTTTT
Template switching primer	AAGCAGTGGTATCAACGCAGAGTACGCGGG

5.2.6 Amplification of cDNA using long distance PCR

As the initial amount of cDNA in the samples was very low, amplification using long distance (LD) PCR was required. A volume of 30µl LD reaction mix containing: 5µl 10x Advantage 2 PCR buffer, 1µl 50x Advantage 2 Polymerase Mix (both Takara Bio Europe/ Clontech), 1µl dNTP mix (10mM), 21µl DNase/RNase free water (Invitrogen) plus 1µl of both the Oligo(dT)$_{24}$ primer and template switching primer to give a final concentration of 10pM, was prepared at room temperature, then added to the 20µl cDNA library. Using an Applied Biosystems® 60 well Thermal Cycler samples were heated to 95°C for 2 minutes, followed by 35 cycles of 95°C for 30 seconds and 6 minutes at 65°C, before cooling and holding at 4°C overnight. Samples were then stored at -20°C until future use.

5.2.7 Verification PCR

Housekeeping gene primers for *GAPDH* and *YWHAZ* genes were used to ensure the successful creation of a cDNA library. All reagents were supplied by Invitrogen Ltd unless specified otherwise. A 1µl sample of the LD PCR product was added to a verification PCR reaction mix containing 14.9µl DNase/RNase free water, 2µl 10x NH_4 Reaction Buffer, 0.6µl $MgCl_2$ (50mM), 0.8µl dNTP mix (10mM), 0.1µl of both the forward and reverse housekeeping primers plus 1µl Taq Polymerase source. A positive control was set up in which a 1µl sample of an ovine ovarian cortex cDNA library containing a range of follicles and somatic tissue was added to the reaction mix. A negative control was also set-up which consisted of the verification PCR reaction mix with no added cDNA. Using an Applied Biosystems® 96 well Thermal Cycler samples were heated to 95°C for 5 minutes, followed by 40 cycles of 95°C, 60°C, 72°C for 30 seconds each, then holding at 72°C for 10 minutes. Samples were then stored at -20°C until future use.

5.2.8 Agarose gel electrophoresis

A 1% (w/v) agarose gel was prepared as detailed in Appendix III. Following verification PCR 4µl gel loading buffer (see Appendix III) was added to each 20µl cDNA sample and mixed, then 12µl of each was added to the individual wells of the sample. A 5µl volume of Quick-Load® 100bp DNA ladder (New England Bio Labs Ltd., Hitchin, UK) was added to one well to aid the determination of fragment size in the cDNA samples. Gels were run in a Sub Cell GT connected to a Powerpac 300 (Bio-rad Laboratories Ltd, Hemel Hempstead, UK), through 1x trisaminomethane, borate, EDTA (TBE) electrophoresis buffer (see Appendix III) at 90V for 60 minutes. The gel was transferred to the molecular imager Gel Doc™ XR+ imaging system (Bio-Rad; CA, USA) to check for the presence of bands corresponding to either *GAPDH* or *YWHAZ* expression, and an absence of bands for the negative control. If bands of the correct size were present then the cDNA samples were used as libraries. If bands were not present where appropriate the cDNA samples were discarded. All gel images were captured using Image Lab™ Software version 2.0 (Bio-Rad).

5.2.9 Real time PCR (RT PCR) analysis

A 480 Lightcycler (Roche, West Sussex, UK) and SYBR green technology (Applied Biosystems®) were used to perform real time PCR (RT PCR) analysis. Amplification of the cDNA in each sample for 40 PCR cycles was used to increase the amount of cDNA per sample exponentially. The SYBR green assay enabled the quantification of the amount of double stranded nucleic acids present relative to housekeeping genes. The web-based programme, primer3, (http://primer3.sourceforge.net/) was used for primer design (Rozen and Skaletsky, 1999). Primers with a melting temperature of around 60°C and around 20 base pairs in length were selected. The primer information for somatic cell-specific and oocyte-specific genes, genes involved in imprinting are shown in Tables 5.4, 5.5 and 5.6. Forward and reverse primers for each gene were dissolved in RNase/DNase free water (Invitrogen) to give a final concentration of 100µM. The forward (F) and reverse (R) primers were then combined and diluted to a final concentration of 0.25µM using RNase/DNase free water in a 1.5ml microcentrifuge tube. A number of primers were designed which successfully and specifically amplified cDNA however, when used for RT PCR analysis of the melting curve along with high levels of expression in negative samples lead to these genes being excluded from the study: primer design detailed in Appendix III.

The reaction mix used for the RT PCR was composed of 12.5µl SYBR green master mix (Applied Biosystems), 5µl of 50 times diluted test template, 5.5µl DNase/RNase free water and 2µl of 0.25µM primer. The reaction mix was loaded into 96 well PCR plates for 480 Lightcycler (Roche) then the plates were covered in sealing foil (Roche). Three replicates were analysed for each primer set and sample combination on the same 96 well plate. Four staged libraries were analysed for each follicle/ oocyte/ somatic cell classification. A representative image of the output data following a completed RT PCR is shown in Figure 5.2. Melting curve analysis was performed following the completion of RT PCR to ensure that unspecific amplification had not taken place. The presence of multiple peaks indicated that non-specific amplification had occurred as the mixture was heated and cooled allowing the release of SYBR green dye from the PCR products, therefore if products were present at different melting temperature this indicated the presence of multiple products. A clear single peak on the melting curve indicated the presence of a single PCR product and therefore a specific amplification as shown in Figure 5.3. The efficiency of every primer pair was not checked individually as there were so many; however, as the same primer pairs were used in all samples then they should exhibit the standard level of efficiency each time.

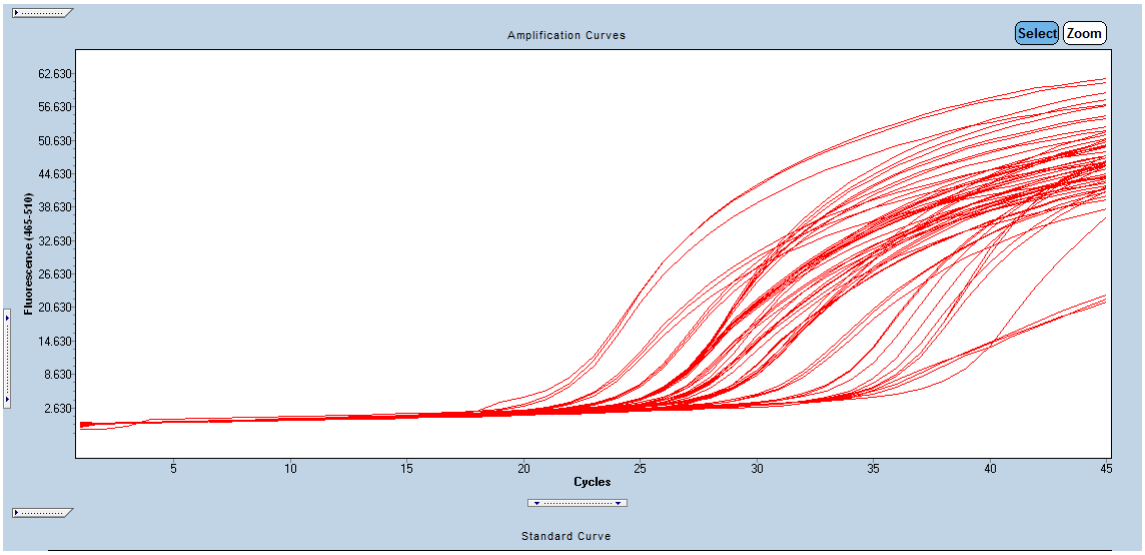


Figure 5.2 A representative image of the output data obtained for *GAPDH* following a RT PCR experiment, with each line representing a separate sample.

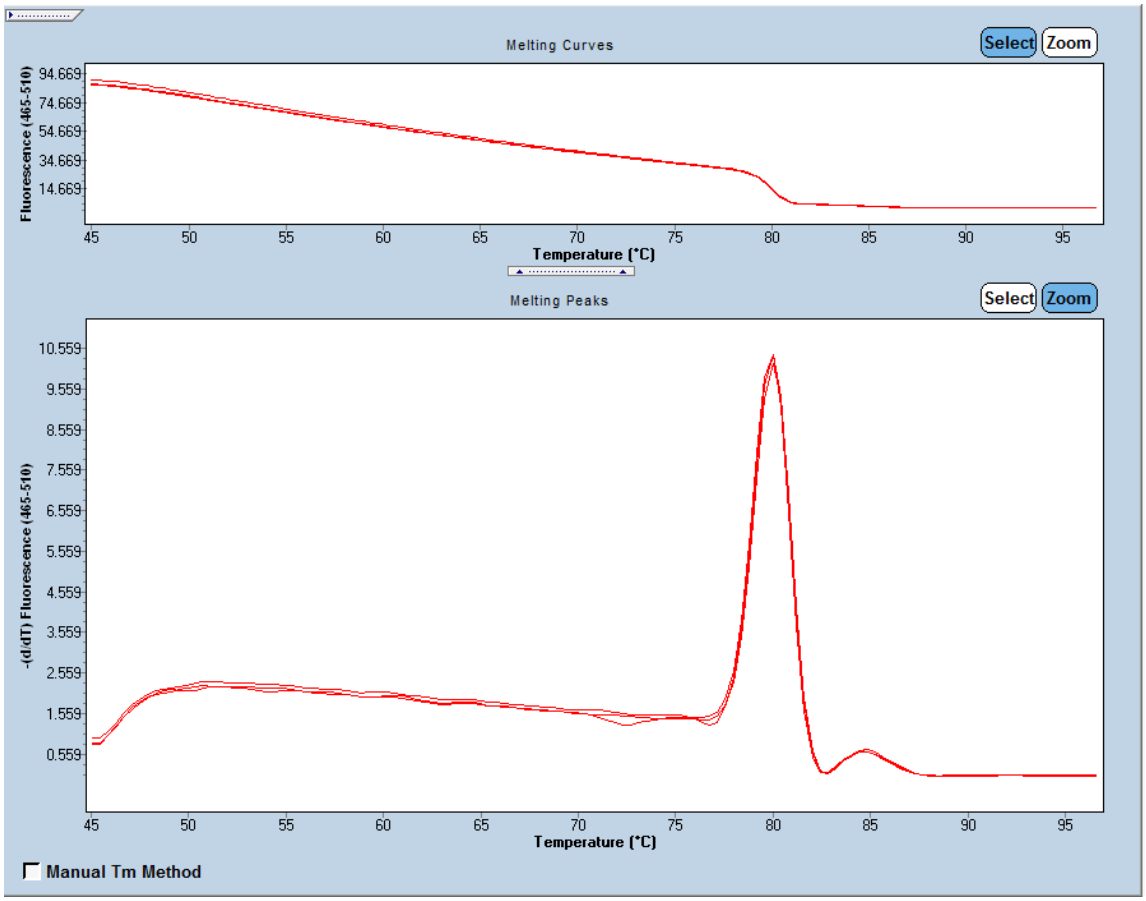


Figure 5.3 A representative image of the melting curve obtained for 3 repeats of a sample in which the primers showed high specificity. The absorbance value at a given temperature is shown for each sample. In this image the amplified gene is *GAPDH*.

5.2.10 Normalisation of data using RT PCR

Due to the nature of the study regarding the collection of *in vitro*-derived samples being dependent upon the follicle yield of each culture, which was very variable, the number of follicles and oocytes collected in each sample and therefore the amount of transcript in each sample varied. The 3 house keeping genes that were selected - GAPDH, H2A and YWHAZ (see Table 5.3) were used to standardise samples on the basis that the transcript levels of each of these should be similar within each oocyte or follicular somatic cell. The cycle threshold (Ct) value, the number of RT PCR cycles at which the levels of fluorescence crosses the threshold was determined for all genes for all samples. Three repeats were conducted for each sample and the mean calculated per sample. The geometric mean of the mean Ct values of the house keeping genes was calculated for each sample as shown in Equation 1.

Equation 1

$$\text{Geometric mean}_{(\text{HK genes})} = (\text{mean Ct}_{(\text{GAPDH})} * \text{mean Ct}_{(\text{H2A})} * \text{mean Ct}_{(\text{YWHAZ})})^{(1/3)}$$

Using the geometric mean of the housekeeping genes for each sample the change in Ct value (ΔCt) for each gene of interest (GOI) was calculated as shown in Equation 2, then the value of $2^{(\Delta Ct)}$ was calculated for each GOI. Where HK = housekeeping gene, mean = arithmetic mean and * = multiply.

Equation 2

$$\Delta Ct_{GOI-HK} = \text{mean Ct}_{(GOI)} - \text{geometric mean}_{(\text{HK genes})}$$

A reference sample with relatively low levels of variability within all genes tested was selected and used as a standard from which the $\Delta\Delta Ct_{(GOI)}$ could be calculated, as shown in Equation 3. The sample selected was sample 1 of *in vivo*-derived secondary follicles, against which all other samples, including the 3 other *in vivo*-derived samples, were compared. The ratio of *INHα*:*INHβA* expression was also calculated for each sample.

Equation 3

$$\Delta\Delta Ct_{GOI-HK} \text{ relative to } \textit{in vivo} \text{ secondary 1} = 2^{(\Delta Ct\ GOI-HK)} / 2^{(\Delta Ct\ Reference-HK)}$$

5.2.11 Statistical analysis

Statistics were performed using the Minitab 15 statistical programme (Minitab limited, UK). The Anderson-Darling test was used to determine whether the data was normally distributed. As the majority of the gene expression data was not normally distributed it was necessary to transform the data using $\log_{10}(1+\Delta\Delta Ct_{GOI-HK}$ relative to *in vivo* secondary 1) and present data in this way ± SEM per sample repeat. The ratio of *INHα*:*INHβA* expression is presented as the mean ± SEM per sample repeat. Levels of gene expression were compared between samples using one-way ANOVA. When data yielded significance, comparisons between samples were conducted using Fisher's post hoc test. In all analyses $p<0.05$ was considered to be statistically significant.

5.3 Results

In this experimental series the expression patterns of candidate genes that have been identified as important regulators of follicle and oocyte growth and development were established *in vivo* and *in vitro*, at various stages of follicle and oocyte development. Genes were classified as somatic cell-specific, oocyte-specific or imprinted, based on results from previous publications. The gene expression patterns in *in vivo*-derived samples were used to determine patterns of normality across oocyte and follicle development. Expression patterns in *in vitro*-derived samples were then analysed. Note that the samples differ in the *in vivo*- and *in vitro*-derived groups, as it was not always possible to obtain equivalent sample numbers, see Section 5.2. Classifications of follicles for which equivalent *in vitro*- and *in vivo*-derived samples had been obtained were directly compared, with respect to the expression patterns of the selected genes. Changes in the relative patterns of gene expression across the different developmental stages were also investigated. The validation of all primer sets used is shown in Figure 5.4.

5.3.1 Expression patterns of key somatic cell-specific genes

Firstly, the expression patterns of key somatic cell-specific genes were considered; see Figures 5.5, 5.6 and 5.7. Levels of *AMH* expression varied *in vivo* with statistically higher (p=0.015) levels of expression being detected in transitional follicles and GV CCs, than primordial and EA follicles or GV oocyte samples, see Figure 5.5. These discrete variations in expression pattern were not seen to such a great an extent in the *in vitro*-derived samples, however, there was a trend towards significance (p=0.083), with MII oocytes and CCs being the biggest contributors as the level of expression of *AMH* in these groups was much lower than in the other samples analysed, see Figure 5.6. Direct comparison of *AMH* levels, in stage matched *in vivo*- vs. *in vitro*-derived samples, revealed that in levels of *AMH* expression in EA follicles were significantly higher (p=0.007) in the *in vitro*-derived samples, see Figure 5.7. Note that the higher expression levels of *AMH* in *in vivo*-derived primary and transitional samples approached significance (p=0.082 and p=0.065, respectively).

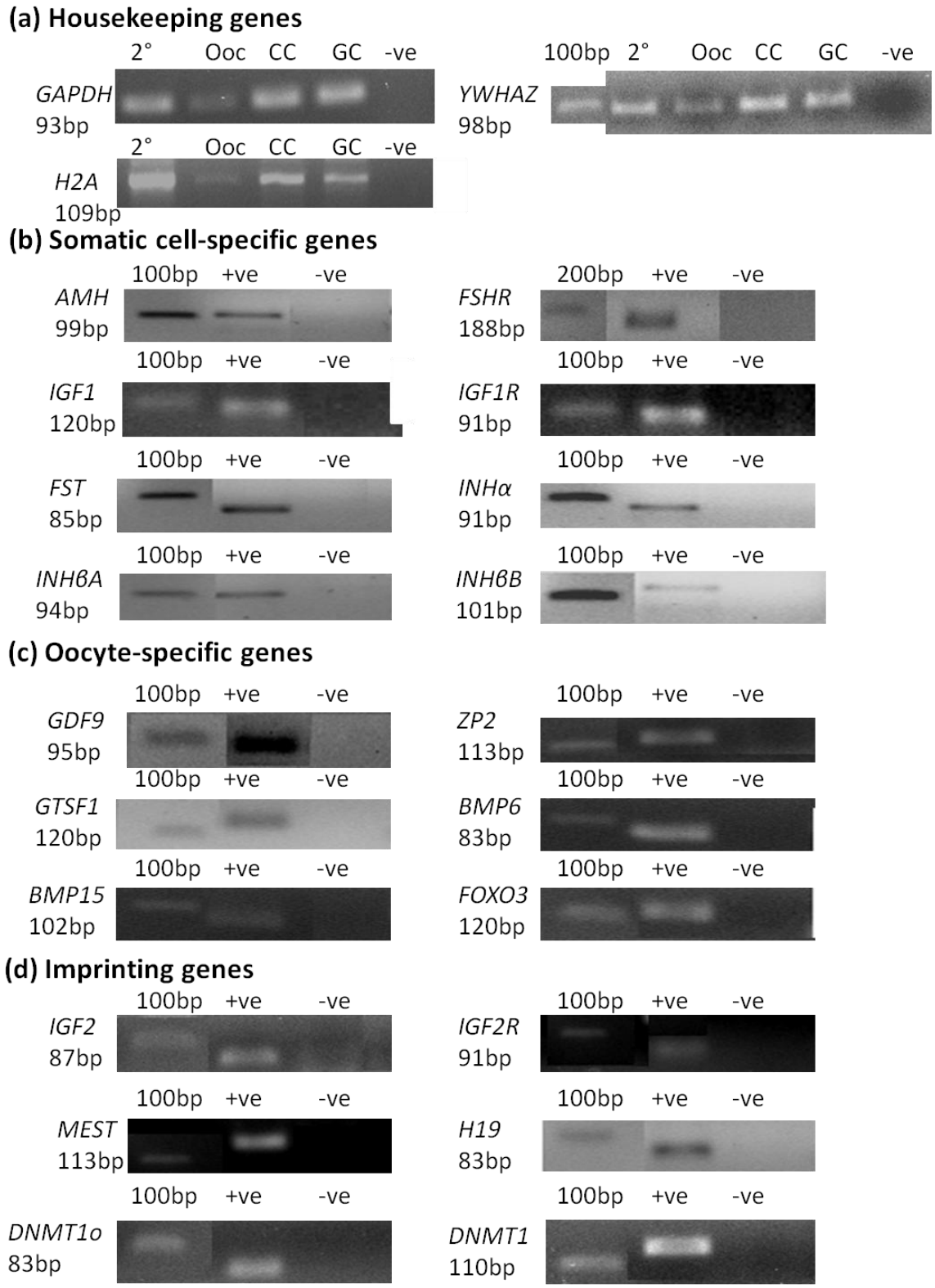


Figure 5.4 Validation of primers. Representative images of each primer set with a positive and negative control and the position of the band with respect to the DNA ladder. Positive controls contained cDNA from a range cell types in cortical tissue, including oocytes and follicular somatic cells. Negative controls contained water in place of cDNA.

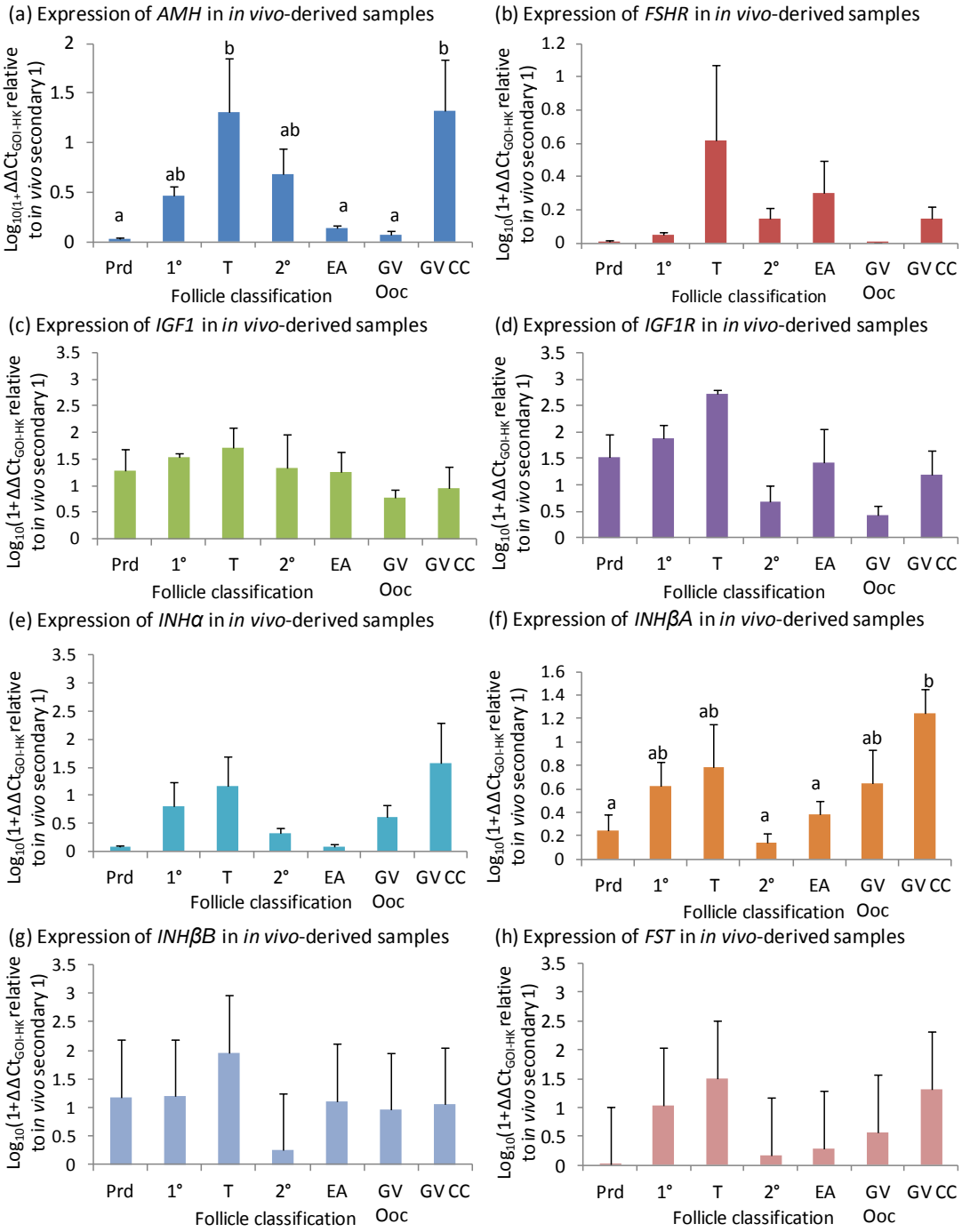


Figure 5.5 Histograms mapping the changing expression patterns of key somatic cell-specific genes- *AMH, FSHR, IGF1, IGF1R, INHα, INHβA, INHβB* and *FST* in *in vivo*-derived samples. Data is presented as $\log_{10}$ (mean expression relative to the level of expression in the control *in vivo*-derived secondary sample) ± SEM for 4 independent samples. Levels of expression were compared using one-way ANOVA. Statistically significant differences ($p<0.05$) are indicated using different letters. For example, in (a) the values obtained for primordial, primary, secondary, EA and GV oocyte samples are not significantly different ($p>0.05$), nor are the values for primary, transitional, secondary and GV CC samples. The values for primordial, EA and GV oocyte samples are, however, significantly different ($p<0.05$) from those obtained for transitional and GV CC samples. This applies to all data presented in this way. Note that the scales used for *AMH*, *FSHR* and *INHβA* are different from those used for all other genes due to lower levels of expression. Abbreviations: primordial (Prd), primary (1°), transitional (T), secondary (2°), early antral (EA), GV oocyte (GV) and CCs from GV oocytes (GV CC).

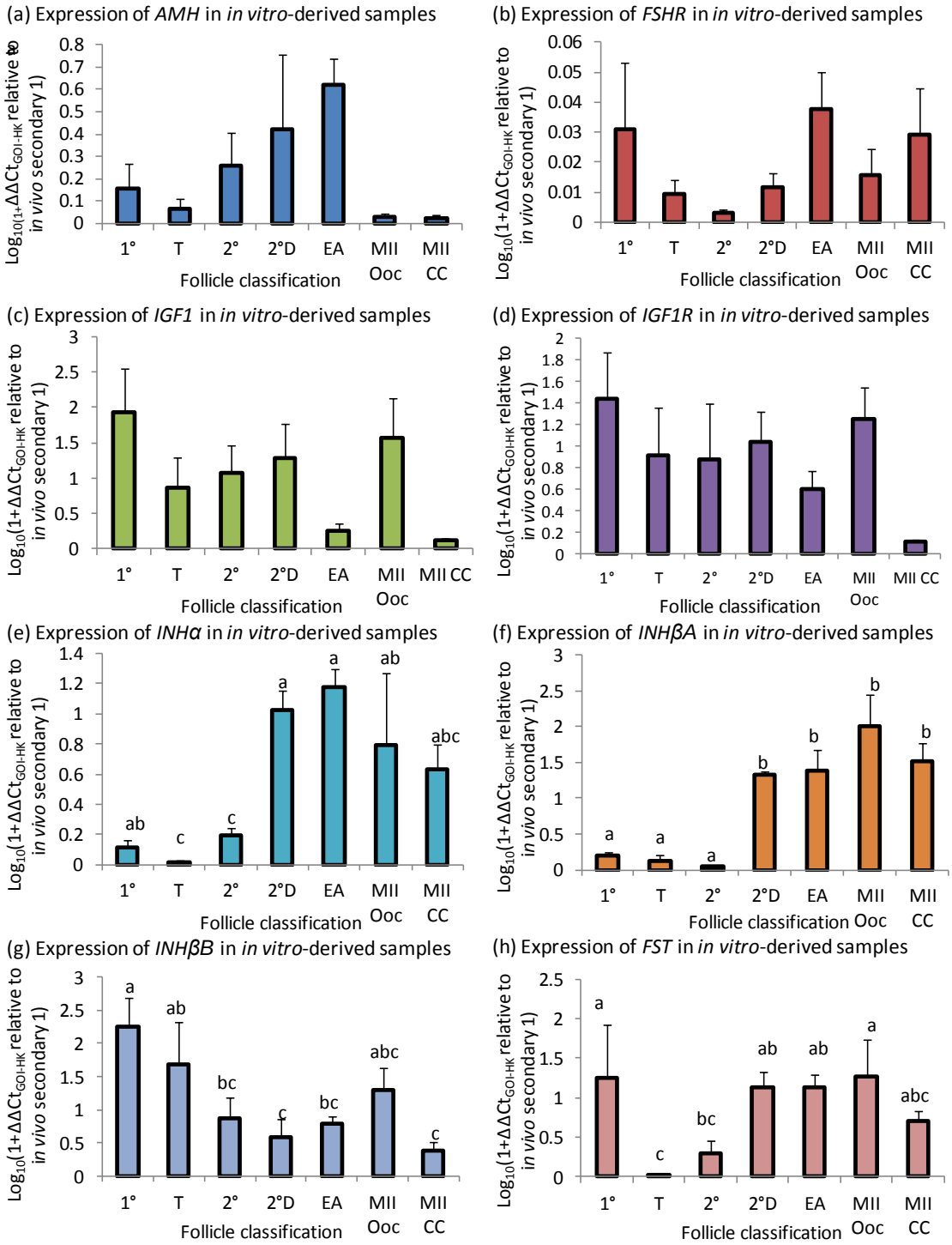


Figure 5.6 Histograms mapping the changing expression patterns of key somatic cell-specific genes- *AMH, FSHR, IGF1, IGF1R, INHα, INHβA, INHβB* and *FST* in *in vitro*-derived samples. Data is presented as $\log_{10}$ (mean expression relative to the level of expression in the control *in vivo*-derived secondary sample) ± SEM for 4 independent samples with the exception of primary stage follicles of which there were only 3 repeats. Levels of expression were compared using one-way ANOVA. Statistically significant differences ($p<0.05$) are indicated using different letters. Note that it was necessary to use different scales because of the large variations in expression levels of the genes analysed in this series. Abbreviations: primary (1°), transitional (T), secondary (2°), secondary degenerating (2D°), early antral (EA), MII oocyte (MII) and CCs from MII oocytes (MII CC).

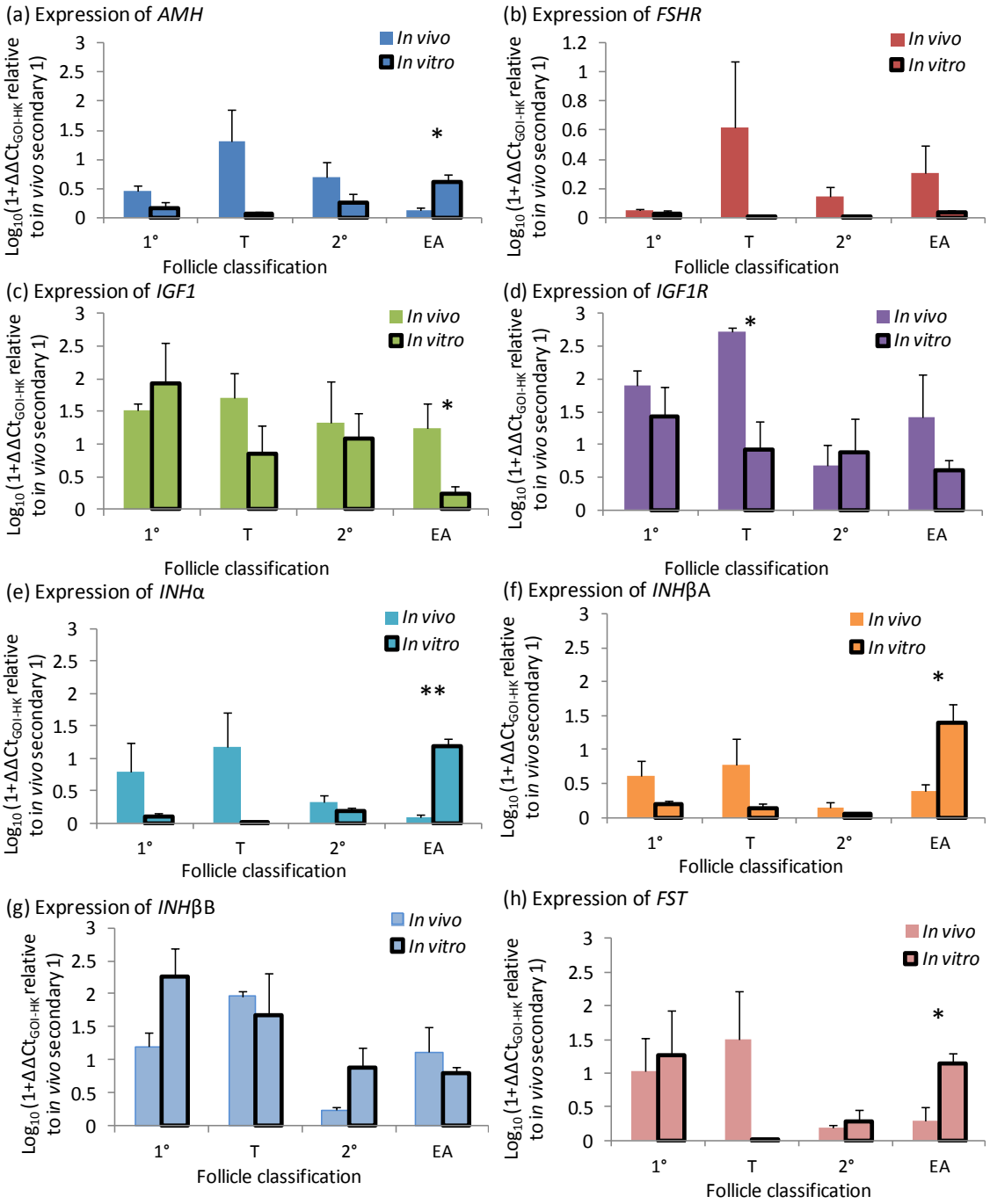


Figure 5.7 Histograms comparing the expression patterns of key somatic cell-specific genes: *AMH, FSHR, IGF1, IGF1R, INHα, INHβA, INHβB* and *FST* in *in vivo*- and *in vitro*-derived samples. Data is presented as $\log_{10}$ (mean expression relative to the level of expression in the control *in vivo*-derived secondary sample) ± SEM for 4 independent samples with the exception of *in vitro*-derived primary stage follicles of which there were only 3 repeats. Levels of expression were compared using one-way ANOVA. Statistically significant differences between *in vivo*- and *in vitro*-derived follicles of the same classification are indicated using * ($p<0.05$) and ** ($p<0.01$). Note that it was necessary to use a different scale for *FSHR* due to its low expression levels compared to the other genes analysed in this series. Abbreviations: primary (1°), transitional (T), secondary (2°), early antral (EA).

Levels of *INHβA* were significantly higher (p=0.037) in CCs from GV oocytes than in primordial, secondary and EA follicles, *in vivo*, see Figure 5.5. The expression pattern of *INHβA* observed *in vitro* was slightly different, with significantly higher (p<0.001) levels of expression in secondary degenerating, EA and MII oocyte and cumulus cell samples, than primary, transitional and secondary follicle samples, see Figure 5.6. Expression of *INHβA* in *in vitro*-derived EA follicle samples was significantly higher (p=0.016) than that observed in *in vivo*-derived samples, see Figure 5.7.

Levels of expression of *FSHR*, *IGF1*, *IGF1R*, *INHα*, *INHβB* and *FST* remained relatively consistent across all *in vivo*-derived samples tested, with no statistically significant differences (p>0.05) found, see Figure 5.5. Levels of *INHα* expression were lowest in primordial, EA and possibly secondary follicle samples. Similarly *FST* expression levels were lowest in primordial, EA and secondary follicles however, there were large variations in the data. Conversely, in the *in vitro*-derived samples expression levels of *INHα*, *INHβB* and *FST* in different follicle classification differed significantly, see Figure 5.6. For *INHα,* the levels of expression were significantly lower in transitional and secondary follicle samples, than primary, degenerating secondary, EA and MII oocyte samples (p=0.003). Levels of *INHβB* expression were highest in the *in vitro*-derived primary follicles, decreasing slightly at the transitional stage, although not significantly (p>0.05). Levels of expression were however significantly lower (p<0.001) than that observed in the primary, secondary, secondary degenerating, EA and MII CC samples. Levels of expression of *FST* were also significantly lower (p=0.032) in transitional and secondary follicle samples, although not secondary degenerating samples, than the other *in vitro*-derived samples.

In 5 out of the 8 key somatic cell-specific genes analysed, expression levels in the *in vitro*-derived EA follicle samples differed significantly from those observed in the *in vivo*-derived samples, see Figure 5.7. Expression of *AMH* and *INHβA* were significantly higher (p<0.05) in the *in vitro*-derived EA follicle samples. This was also observed with respect to levels of *INHα* and *FST* expression (p=0.001 and p=0.016, respectively). Whereas levels of *IGF1* expression in EA follicles were significantly lower (p=0.045) in the *in vitro*-derived samples, compared to the *in vivo*-derived samples, only the levels of *IGF1R* expression differed significantly in a follicle classification other than EA, as levels of expression were significantly higher (p=0.018) in *in vivo*-derived transitional follicle samples when compared to their *in vitro*-derived counterparts. It is worth noting that in a number of genes different trends in the expression levels between

in vitro- and *in vivo*-derived samples were observed such that higher levels of *FSHR* expression were observed in *in vivo*-derived transitional, secondary and EA samples. Higher levels of *INHα* expression were also observed in the *in vitro*-derived primary and transitional follicle samples, than in their *in vivo*-derived counterparts. The difference in expression patterns of *INHβB in vivo* and *in vitro* showed no clear pattern, however, expression was higher *in vitro* in primary follicle samples, to a level approaching statistical significance (p=0.06). In all follicle classifications analysed *FST* expression was consistently higher *in vitro* than *in vivo*, with the exception of transitional follicles in which the converse was true, although variability in the data meant these differences were not significant.

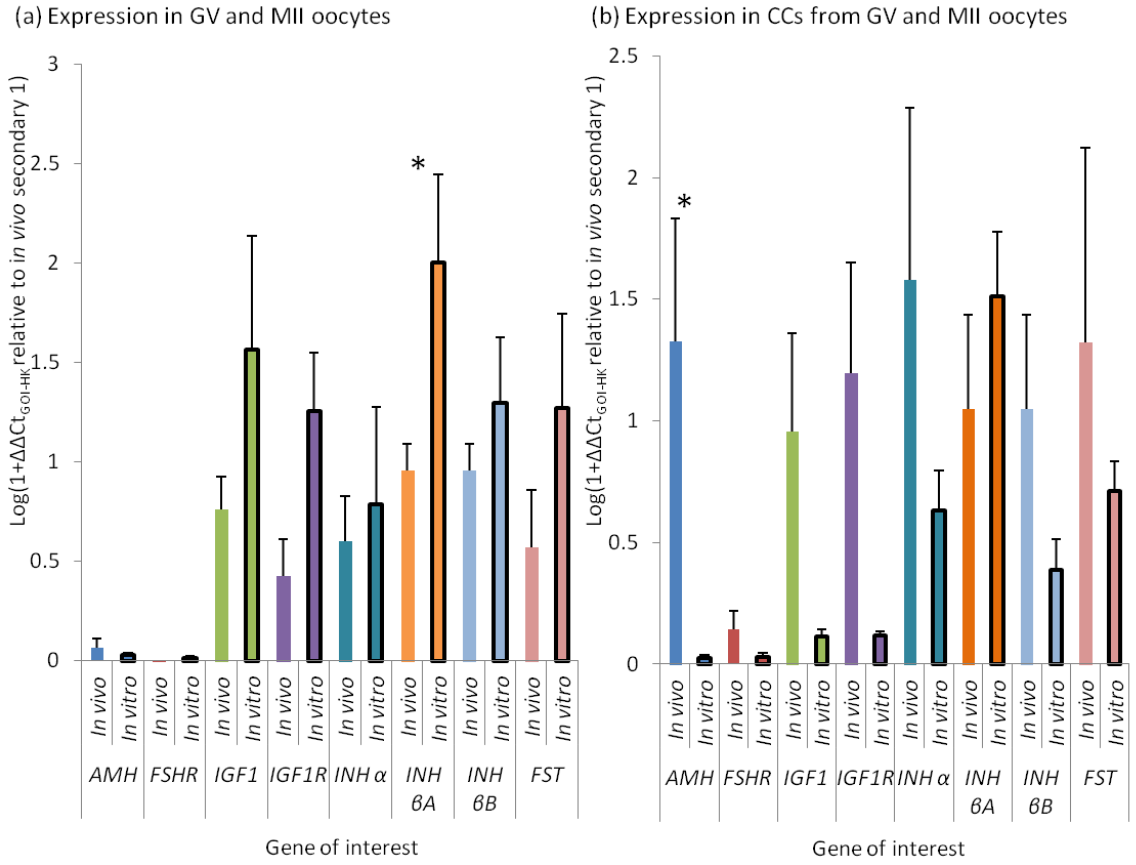


Figure 5.8 Histograms comparing the expression patterns of key somatic cell-specific genes: *AMH, FSHR, IGF1, IGF1R, INHα, INHβA, INHβB* and *FST* in samples of (a) *in vivo*-derived GV oocytes and *in vitro*-derived MII oocytes (black lines) and (b) *in vivo*-derived CCs from GV oocytes and *in vitro*-derived CCs (black lines) from MII oocytes. Data is presented as $\log_{10}$ (mean expression relative to the level of expression in the control *in vivo*-derived secondary sample) ± SEM for 4 independent samples. Levels of expression were compared using one-way ANOVA. Statistically significant differences between *in vivo*- and *in vitro*-derived samples are indicated using * ($p<0.05$).

Expression levels of key somatic-cell genes were compared between GV and MII oocytes and GV and MII oocyte-derived CCs, in Figure 5.8. Levels of *INHβA* expression were significantly higher ($p<0.05$) in MII oocytes than GV oocytes. Levels of expression of all genes except *INHβA* was lower in CCs derived from MII oocytes than those derived from GV oocytes, but due to variability in the data this was only significant ($p<0.05$) in the case of *AMH*.

The ratio of the relative expression levels of *INHα*:*INHβA* subunits were analysed to indicate the levels of inhibin A and activin A (see Figure 5.9). The ratio of *INHα*:*INHβA* expression *in vivo* was lowest in primordial follicles; the ratio then increased throughout preantral follicle development prior to decreasing in EA follicle samples and remaining low in GV oocyte and CC samples (see Figure 5.9 (a)). The ratio of *INHα*:*INHβA* expression was similar in primary, transitional and secondary follicles and there were no statistically significant differences ($p>0.05$) between any of the samples. The pattern of the ratio of *INHα*:*INHβA* expression observed *in vitro* was slightly different to that observed *in vivo* (see Figure 5.9 (b)), in that a significantly higher level of expression ($p=0.0009$) was observed in secondary follicles than all other samples, although interestingly not in secondary degenerating follicles. The lowest levels of expression were observed in transitional and MII CC samples. When the *in vivo*- and *in vitro*-derived samples were directly compared (Figure 5.9 (c)) the greatest differences in the ratio of *INHα*:*INHβA* expression were observed in the primary and transitional samples with lower levels observed *in vitro*, statistical significance was approached in the latter ($p=0.059$).

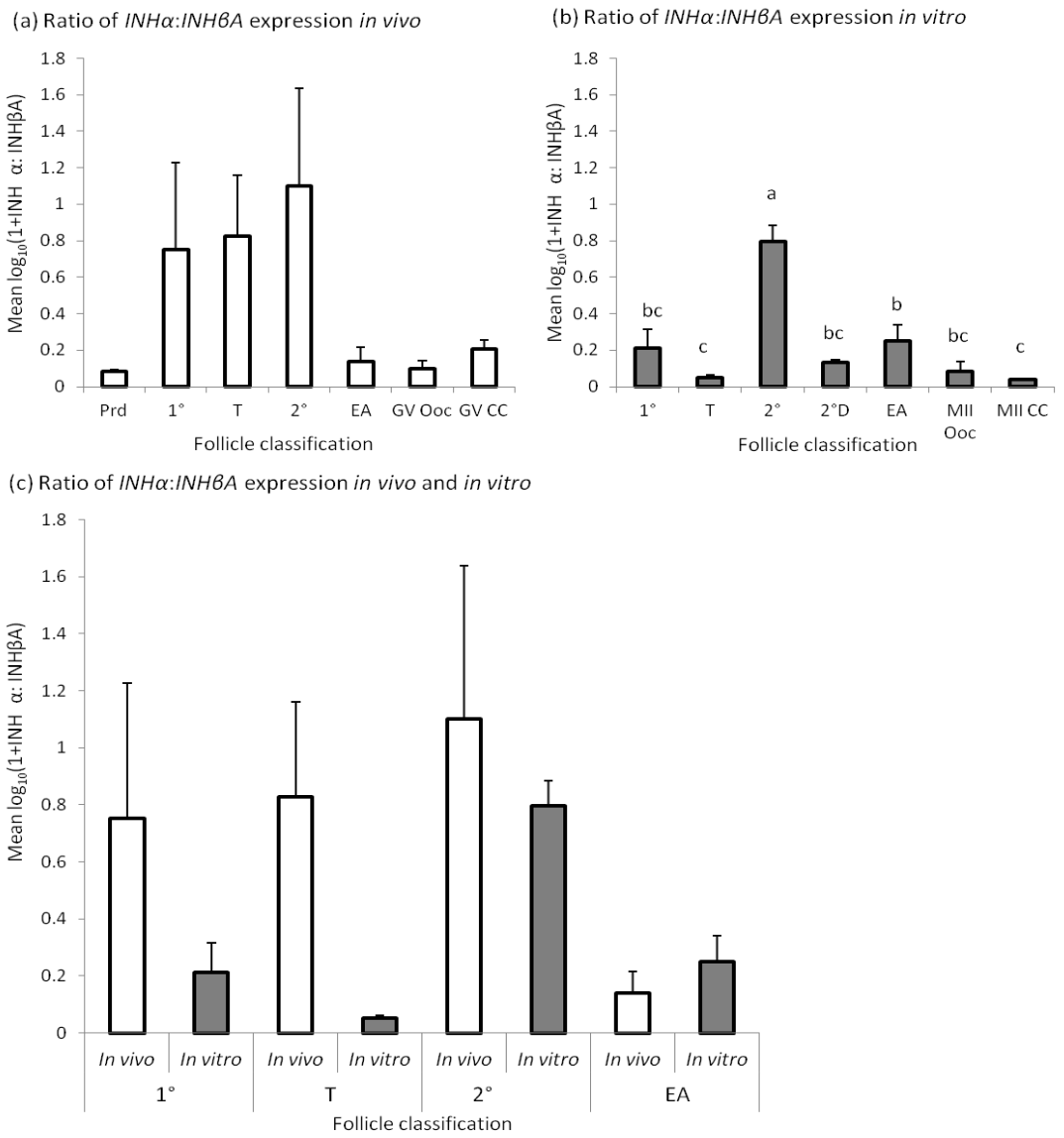
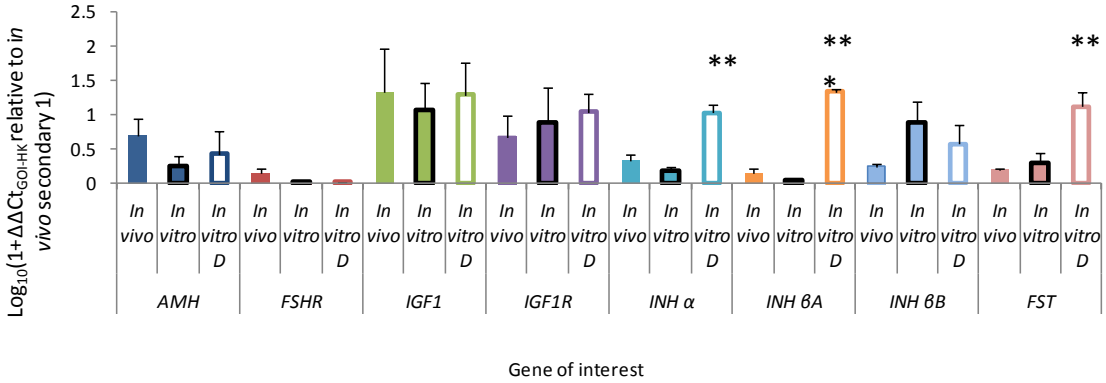


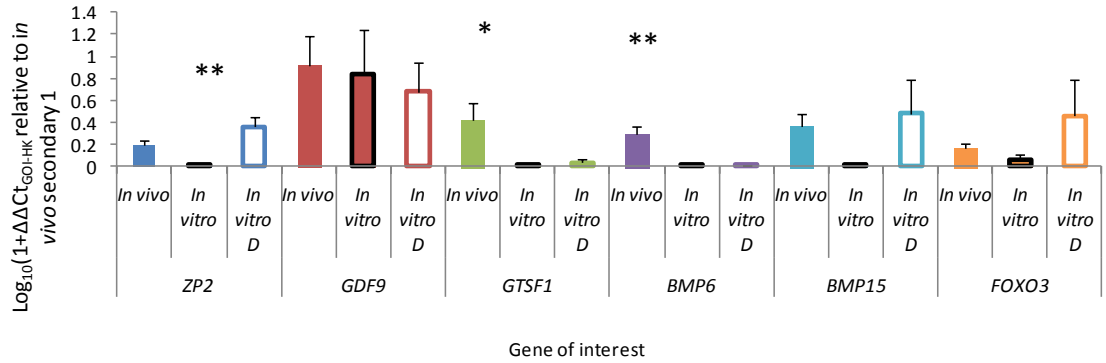
Figure 5.9 The ratio of *INHα:INHβA* expression in (a) *in vivo*-derived samples, (b) *in vitro*-derived samples and (c) *in vivo*- and *in vitro*-derived samples. Data is presented as mean $\log_{10}$(1+ *INHα*:*INHβA*) ± SEM for 4 independent samples. Ratios were compared using one-way ANOVA and statistically significant differences indicated with the use of different letters ($p<0.05$). Abbreviations: primary (1°), transitional (T), secondary (2°), early antral (EA).

Finally in Figure 5.10 (a) levels of expression of key somatic cell-specific genes in growing *in vivo*- and *in vitro*-derived secondary follicles, as well *in vitro*-derived degenerating follicles, were analysed. Interestingly, expression levels of *INHα*, *INHβA* and *FST* were significantly higher in the samples of degenerating secondary follicles compared to either the *in vivo*- or *in vitro*-derived growing secondary follicles ($p=0.001$, $p=0.0009$, $p=0.003$, respectively). Higher levels of *FSHR* expression were also observed in degenerating secondary follicles compared to growing follicles, to a degree approaching statistical significance ($p=0.057$).

(a) Expression of key somatic cell-derived genes in growing and degenerating *in vitro*- and *in vivo*-derived secondary follicles



(b) Expression of key oocyte-derived genes in growing and degenerating *in vitro*- and *in vivo*-derived secondary follicles



(c) Expression of key somatic genes in in growing and degenerating *in vitro*- and *in vivo*-derived secondary follicles

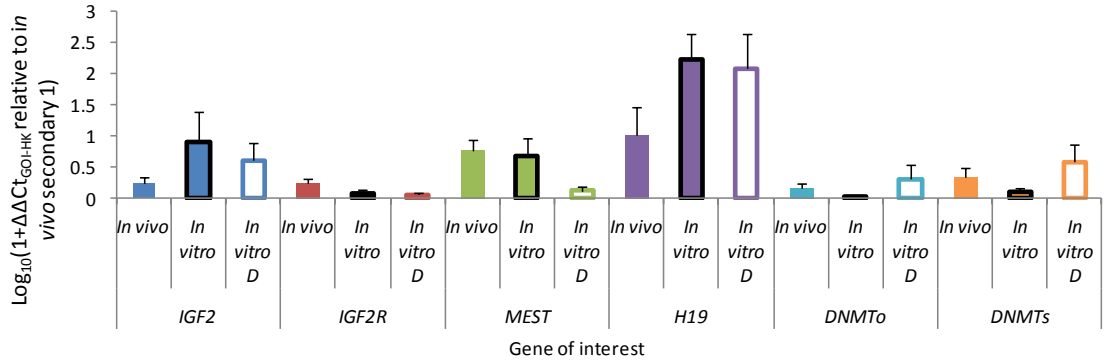


Figure 5.10 Summary comparison of the expression patterns of key (a) somatic cell- (b) oocyte-derived and (c) imprinted genes in *in vivo*- and growing and degenerating *in vitro*-derived secondary follicle samples. Data is presented as $\log_{10}$ (mean expression relative to the level of expression in the control *in vivo*-derived secondary sample) ± SEM for 4 independent samples with the exception of *in vitro*-derived primary stage follicles of which there were only 3 repeats. Levels of expression were compared using one-way ANOVA. Statistically significant differences between *in vivo*- and *in vitro*-derived follicles of the same classification are indicated using * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$). Note that *in vitro D* refers to secondary follicles derived i*n vivo*, then cultured in isolated culture *in vitro*, where they started to degenerate.

5.3.2 Expression patterns of key oocyte-specific genes

Expression patterns of key oocyte-specific genes *in vivo* and *in vitro* were considered in Figure 5.11, Figure 5.12 and Figure 5.13. In all oocyte-specific genes analysed expression levels were highest in the GV oocyte samples *in vivo*, with the exception of *GDF9*, and MII oocyte samples *in vitro*, see Figure 5.11 and Figure 5.12.

The level of expression of *ZP2* was significantly higher in GV oocytes than all other *in vivo*-derived samples, except primary follicles, see Figure 5.11. The level of *ZP2* expression in primary and transitional follicles *in vivo* was significantly higher ($p<0.001$) than all other samples considered. Similarly, *ZP2* expression *in vitro* was significantly higher ($p<0.001$) in MII oocyte samples, however, other than this the levels of expression remained relatively consistent across all samples compared to that observed *in vivo*, see Figure 5.12. Direct comparison of *in vivo*- and *in vitro*-derived samples in Figure 5.13, with respect to *ZP2* expression, revealed consistently higher levels of expression in the *in vivo*-derived samples. This difference was statistically significant in primary and secondary follicle samples ($p=0.001$ and $p=0.002$, respectively). Interestingly levels of *ZP2* expression in degenerating secondary follicles were not significantly different from those observed *in vivo*, however they were statistically significantly different ($p=0.006$) from those observed *in vitro*, see Figure 5.10. Therefore this suggests that the reduced levels of expression observed in the *in vitro*-derived samples occur earlier in development, possibly as a result of suboptimal cortical culture conditions.

Expression of oocyte *GDF9* was highest in transitional follicles *in vivo* and *in vitro*, this observation was statistically significant in the former ($p=0.014$), see Figure 5.11 and Figure 5.12. Levels of *GDF9* expression in EA follicle samples were significantly higher ($p<0.05$) than those observed in GV oocyte and CC samples. These high levels of *GDF9* expression were not observed in the *in vitro* derived samples, as shown in Figure 5.13, as expression levels were significantly higher *in vivo* ($p=0.021$).

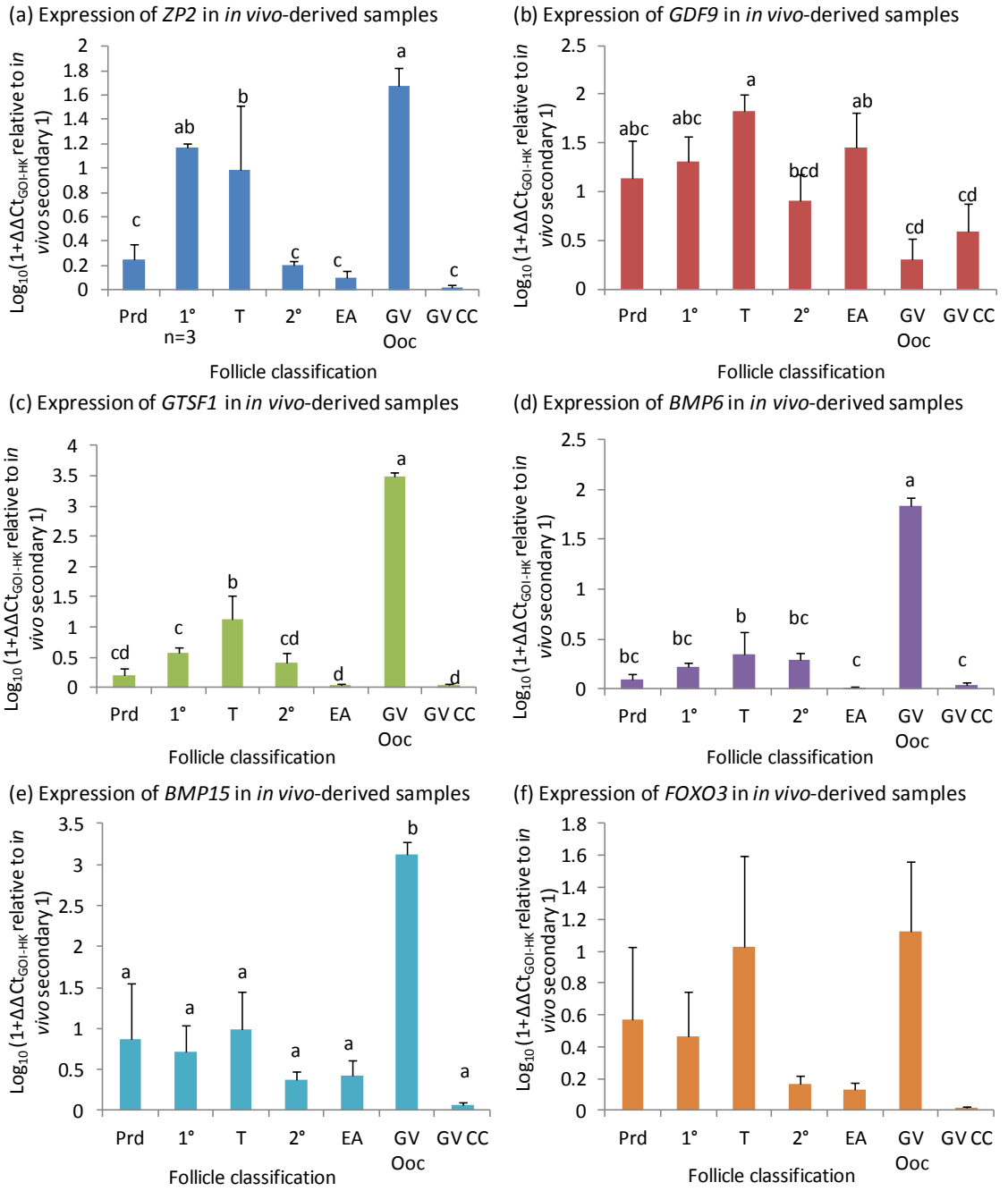


Figure 5.11 Histograms mapping the changes in expression patterns of key oocyte-specific genes; *ZP2, GDF9, GTSF1, BMP6, BMP15* and *FOXO3* in *in vivo*-derived samples. Data is presented as $\log_{10}$ (mean expression relative to the level of expression in the control *in vivo*-derived secondary sample) ± SEM for 4 independent samples, except where indicated. Levels of expression were compared using one-way ANOVA. Statistically significant differences ($p<0.05$) are indicated using different letters. Note that the scales used for *AMH*, *FSHR* and *INHβA* are different from those used for all other genes due to lower levels of expression. Abbreviations: primordial (Prd), primary (1°), transitional (T), secondary (2°), early antral (EA), MII oocyte (MII) and CCs from MII oocytes (MII CC).

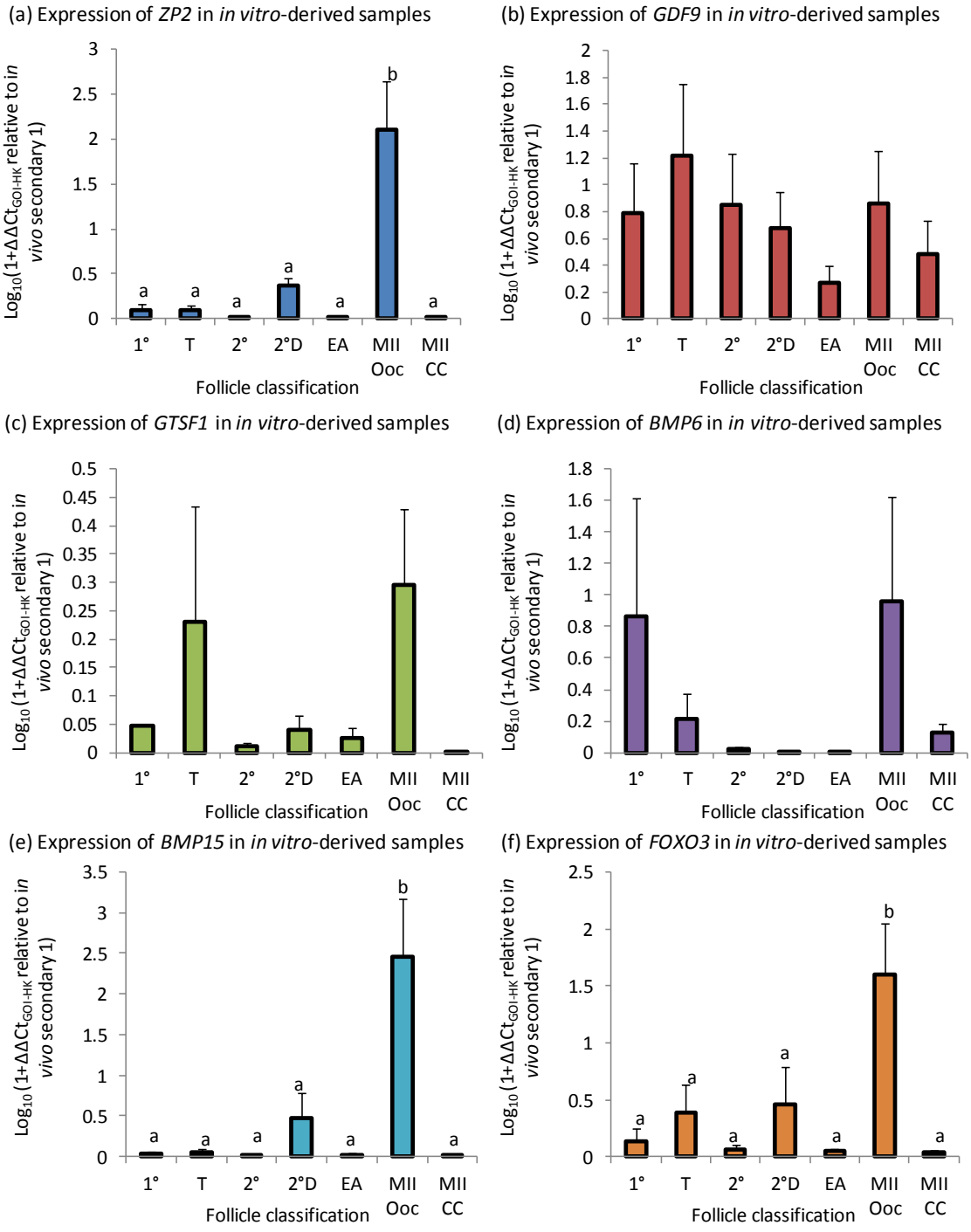


Figure 5.12 Histograms mapping the changes in expression patterns of key oocyte-specific genes; *ZP2, GDF9, GTSF1, BMP6, BMP15* and *FOXO3* in *in vitro*-derived samples. Data is presented as $\log_{10}$ (mean expression relative to the level of expression in the control *in vivo*-derived secondary sample) ± SEM for 4 independent samples with the exception of primary stage follicles of which there were only 3 repeats. Levels of expression were compared using one-way ANOVA. Statistically significant differences ($p<0.05$) are indicated using different letters. Note that it was necessary to use different scales because of the large variations in expression levels of the genes analysed in this series. Abbreviations: primary (1°), transitional (T), secondary (2°), secondary degenerating (2D°), early antral (EA), MII oocyte (MII) and CCs from MII oocytes (MII CC).

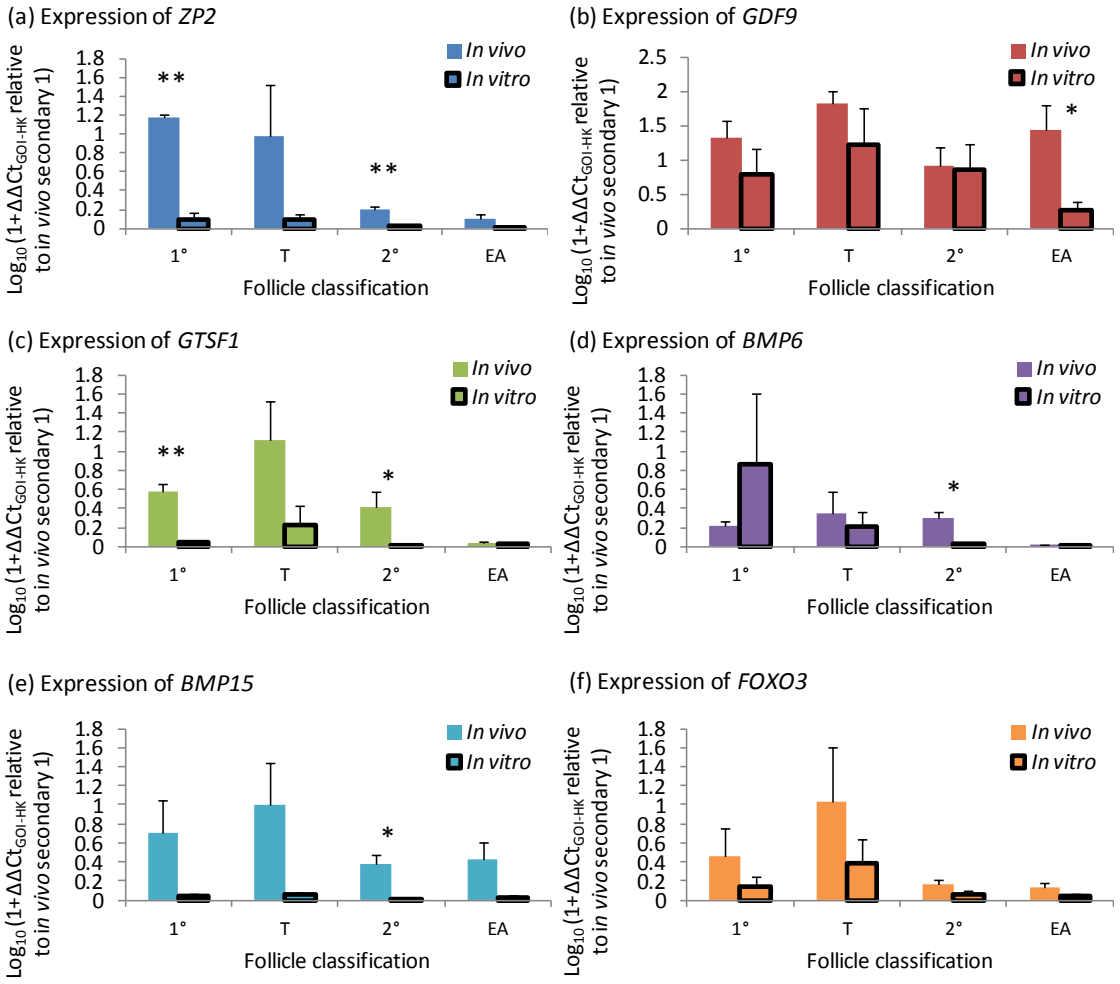


Figure 5.13 Histograms comparing the expression patterns of key oocyte-specific genes; *ZP2, GDF9, GTSF1, BMP6, BMP15* and *FOXO3* in *in vivo*- and *in vitro*-derived samples. Data is presented as $\log_{10}$ (mean expression relative to the level of expression in the control *in vivo*-derived secondary sample) ± SEM for 4 independent samples with the exception of *in vitro*-derived primary stage follicles of which there were only 3 repeats. Levels of expression were compared using one-way ANOVA. Statistically significant differences between *in vivo*- and *in vitro*-derived follicles of the same classification are indicated using * ($p<0.05$) and ** ($p<0.01$). Note that it was necessary to use a different scale for *GDF9* due to its high expression levels compared to the other genes analysed in this series. Abbreviations: primary (1°), transitional (T), secondary (2°), early antral (EA)

With the exception of GV oocytes, expression of *GTSF1 in vivo* was significantly higher ($p=0.0009$) in transitional follicle samples than all other samples considered, see Figure 5.11. Similarly, with the exception of MII oocyte samples, expression was highest in transitional follicles *in vitro,* however, this was not statistically significant ($p<0.05$), see Figure 5.12. Expression levels of *GTSF1* in primary follicles *in vivo* were significantly higher than those observed in EA and GV CC samples, whereas *in vitro* expression levels were similar in primary and EA samples; see Figure 5.11 and Figure 5.12. In addition, when *in vitro*- and *in vivo*-

derived samples were directly compared expression levels of *GTSF1* were significantly higher *in vivo*-derived primary follicle samples (p=0.005) as well as in secondary follicle samples (p=0.047). Similarly, expression of *GTSF1* was significantly higher (p=0.025) in *in vivo*-derived secondary follicles than degenerating secondary follicles, see Figure 5.10.

With respect to levels of *BMP6* expression *in vivo* the highest levels were observed in GV oocytes (p<0.001), see Figure 5.11. In addition, expression of *BMP6* was significantly higher in transitional follicle samples than EA and GV CC samples. Whereas *in vitro* expression of *BMP6* was lower in transitional follicle samples than in primary follicle samples, although this was not significant, see Figure 5.12. The levels of expression *in vivo* and *in vitro* did not differ in primary and transitional samples (p>0.05), when directly compared, see Figure 5.13. However, *BMP6* expression in secondary follicles was significantly higher (p=0.016) in the *in vivo*- than *in vitro*- follicles. Significantly lower (p=0.004) levels of *BMP6* expression were also observed in degenerating secondary follicles than those observed *in vivo*, see Figure 5.10.

In the *in vivo*- and *in vitro*-derived samples expression of *BMP15* was significantly higher (p<0.001) in GV oocyte and MII oocyte samples, respectively, whereas levels remained relatively consistent in all other classifications considered, see Figure 5.11 and Figure 5.12. Expression of *BMP15* was consistently higher *in vivo* than that observed *in vitro*, reaching statistical significance in secondary follicle samples (p=0.033) and a trend for higher expression was observed in EA samples, see Figure 5.13. However, levels of expression in degenerating secondary follicles did not differ from that observed in growing follicles (Figure 5.10).

Expression of *FOXO3 in vivo* did not vary significantly across development although the highest levels observed were from primordial to transitional stages and in GV oocyte samples, see Figure 5.11. There were, however, large variations within follicle stages, and more repeats, possibly using different primers are required to give some more convincing results. Expression of *FOXO3 in vitro* grown cells were significantly higher (p<0.001) in MII oocyte samples than all other classifications analysed, see Figure 5.12. Similarly, expression of *FOXO3* was slightly higher in *in vivo*- than *in vitro*- derived cells. These differences were not however, statistically significant, see Figure 5.13.

Expression patterns of key oocyte-specific genes were compared between GV and MII oocytes and GV and MII oocyte-derived CCs, in Figure 5.14. Oocyte-specific gene expression was similar in GV and MII oocyte samples, with the exception of *GTSF1* in that a significantly higher ($p<0.001$) level of expression was detected in the GV oocytes, than the MII oocytes. With respect to the CC samples, levels of expression of all oocyte-specific genes were similar with no significant differences ($p>0.05$) observed between GV and MII oocyte-derived samples, with the exception of *BMP15* expression, which was significantly lower ($p<0.05$) in GV oocyte samples.

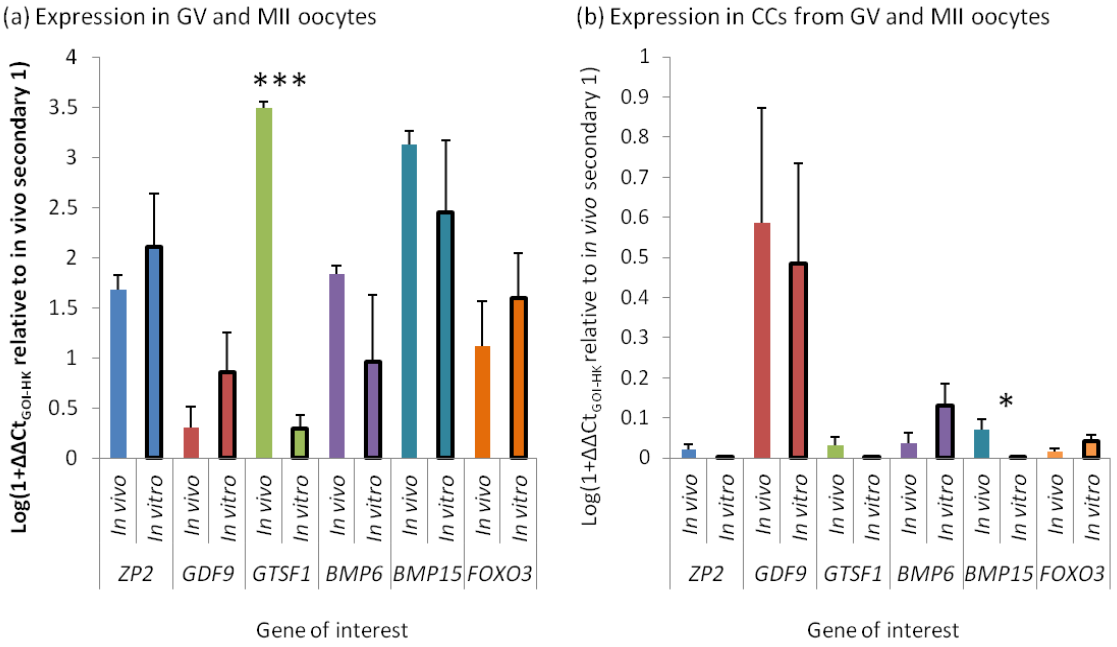


Figure 5.14 Histograms comparing the expression patterns of key oocyte-specific genes; *ZP2, GDF9, GTSF1, BMP6, BMP15* and *FOXO3* in samples of (a) *in vivo*-derived GV oocytes and *in vitro*-derived MII oocytes and (b) *in vivo*-derived CCs from GV oocytes and *in vitro*-derived CCs from MII oocytes. Data is presented as $\log_{10}$ (mean expression relative to the level of expression in the control *in vivo*-derived secondary sample) ± SEM for 4 independent samples. Levels of expression were compared using one-way ANOVA. Statistically significant differences between *in vivo*- and *in vitro*-derived samples are indicated using *** ($p<0.001$).

5.3.3 Expression patterns of key imprinted genes and genes involved in the regulation of epigenetic modifications

Finally the expression patterns of key imprinted genes *in vivo* and *in vitro* were considered, see Figures 5.15, 5.16 and 5.17. Expression of *IGF2* was highest in preantral follicles both *in vivo* and *in vitro* although differences were not significant ($p>0.05$), see Figures 5.15 and 5.16. Expression of its receptor, *IGF2R*, was relatively consistent both *in vivo* and *in vitro*, with no

statistically significant differences (p>0.05) observed, see Figures 5.15 and 5.16. Expression of *IGF2R* was highest in GV and MII oocyte samples. Direct comparison of *in vivo*- and *in vitro*-derived samples did not reveal any significant differences (p>0.05), with respect to either *IGF2* or its receptor, see Figure 5.17.

Similar patterns of *MEST* expression were observed *in vivo* and *in vitro*, see Figures 5.15 and 5.16, in that a transient increase was observed during preantral follicle development, followed by a slight decrease as follicle development progressed. This increase occurred at the transitional stage *in vivo* and appeared to be delayed to the secondary stages in the *in vitro*-derived follicles. Additionally, the level of *MEST* expression was significantly higher in *in* vitro-derived primary and transitional follicle samples, p=0.009 and p=0.017, respectively (see Figure 5.17). Similarly in EA follicle samples expression of *MEST* was higher *in vitro*, but this difference did not reach significance (p>0.05) due to sample variability.

The level of *H19* expression *in vivo* increased from the primordial stage to the transitional stage of development, before decreasing transiently at the secondary stage, and increasing again at the EA stage (see Figure 5.15). Expression was lowest in the GV oocyte and CC somatic samples. The difference in level of expression of *H19* between transitional and primordial, secondary, GV oocyte and CC samples was statistically significant (p<0.001). The expression pattern observed *in vitro* was very different to that observed *in vivo*. Relatively high levels of expression were consistently observed across all preantral follicle samples *in vivo* and *in vitro*, although there was a slight and non-statistically significant increase (p>0.05) in expression in transitional follicle samples. In the *in vitro*-derived EA, MII oocyte and CC samples expression of *H19* was significantly lower (p<0.001) than that observed in the preantral follicle samples, see Figure 5.16. This discrepancy between the *in vivo*- and *in vitro*-derived EA samples became even more apparent when the 2 were directly compared in Figure 5.17, showing a significantly lower level of *H19* expression *in vitro* (p=0.001). A lower level of expression *in vitro* was observed across all follicular classifications analysed, especially secondary follicles however, the differences were not significant due to variability between samples.

The highest level of expression of oocyte-specific *DNMT1o in vivo* was observed in GV oocytes (p=0.0009) compared to other developmental stages, see Figure 5.15. Expression levels in GV oocytes were over 9-fold higher than that observed in all other samples. Whereas in the *in*

vitro-derived samples there were no significant differences in the level of expression, see Figure 5.16. Unfortunately there were no *in vitro*-derived GV oocytes to enable comparison. Comparison of *DNMT1o* expression levels in *in vivo*- and *in vitro*-derived samples revealed similar levels of expression ($p>0.05$), although a higher level of *DNMT1o* expression was recorded for *in vivo* transitional follicle samples, however, this difference was not significant due to variability between samples. Expression of somatic cell-specific gene *DNMT1s* progressively increased from the primordial to the transitional stage, then progressively decreased up to the EA stage, *in vivo*, see Figure 5.15. Expression *in vivo* was significantly higher ($p=0.007$) in the transitional follicle and GV oocyte samples, than all other stages. Similarly a small increase in *DNMT1s* expression from the primary to the transitional stage, followed by a decrease at the secondary stage was observed *in vitro*, although this difference was not significant ($p>0.05$), see Figure 5.16 (f). However, unlike that observed *in vivo* expression levels of *DNMT1s* were significantly higher ($p<0.05$) in *in vitro*-derived EA follicles than those observed in all preantral follicle stages. This can also be observed in Figure 5.17 (f) as expression of *DNMT1s* was significantly higher ($p=0.011$) *in vitro* than *in vivo*. Interestingly in degenerating secondary follicles expression of *DNMT1s* was also higher than that observed in transitional follicles, although these differences were not significant.

The data shown in Figure 5.10 shows that expression of the key imprinted genes analysed remained relatively consistent across *in vivo*- and *in vitro*-derived growing follicles and *in vitro*-derived degenerating follicles.

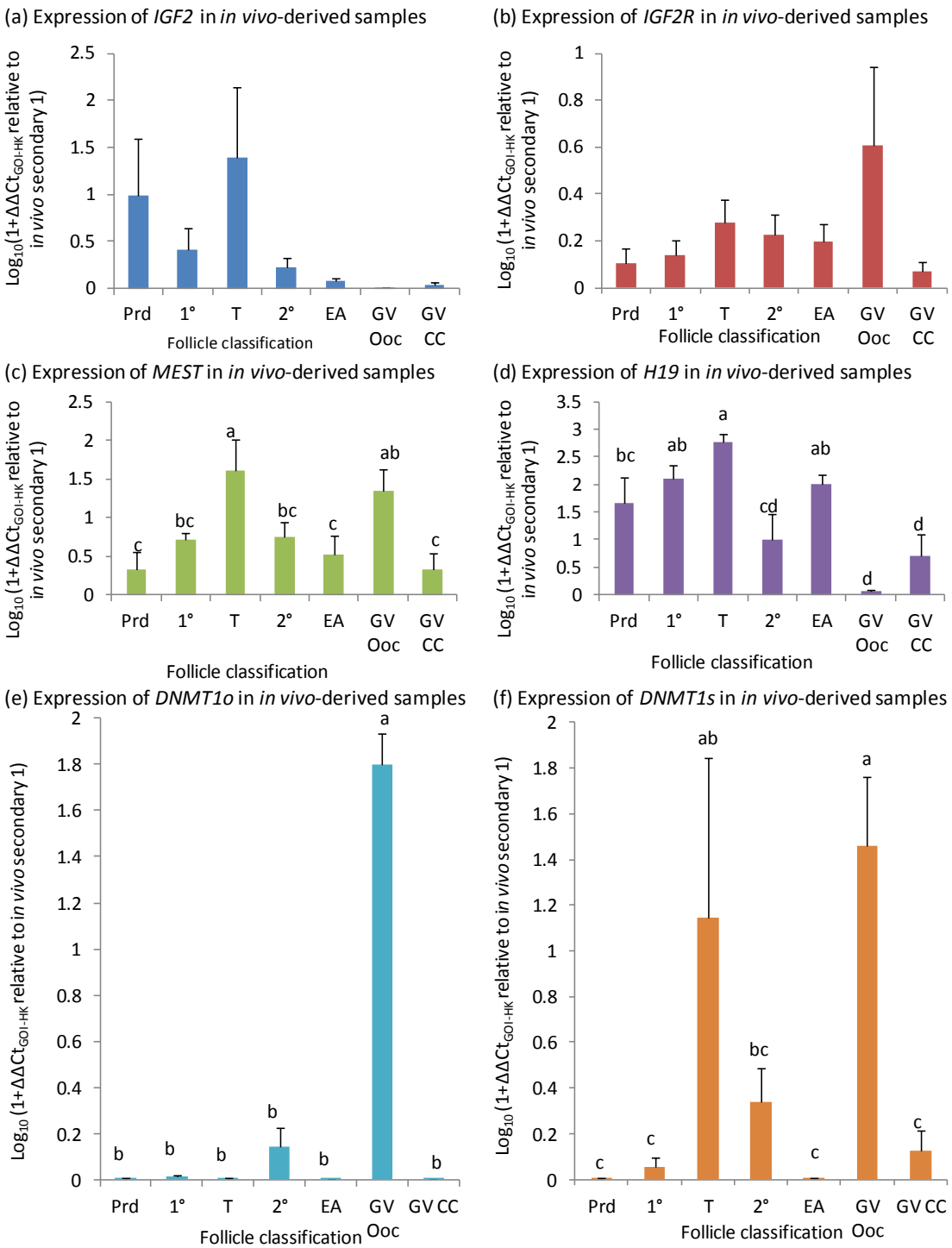


Figure 5.15 Histograms mapping the changes in expression patterns of key imprinted genes; *IGF2, IGF2R, MEST, H19, DNMT1o* and *DNMT1s* in *in vivo*-derived samples. Data is presented as $\log_{10}$ (mean expression relative to the level of expression in the control *in vivo*-derived secondary sample) ± SEM for 4 independent samples. Levels of expression were compared using one-way ANOVA. Statistically significant differences ($p<0.05$) are indicated using different letters. Note that it was necessary to use different scales because of the large variations in expression levels of the genes analysed in this series. Abbreviations: primordial (Prd), primary (1°), transitional (T), secondary (2°), early antral (EA), GV oocyte (GV) and CCs from GV oocytes (GV CC).

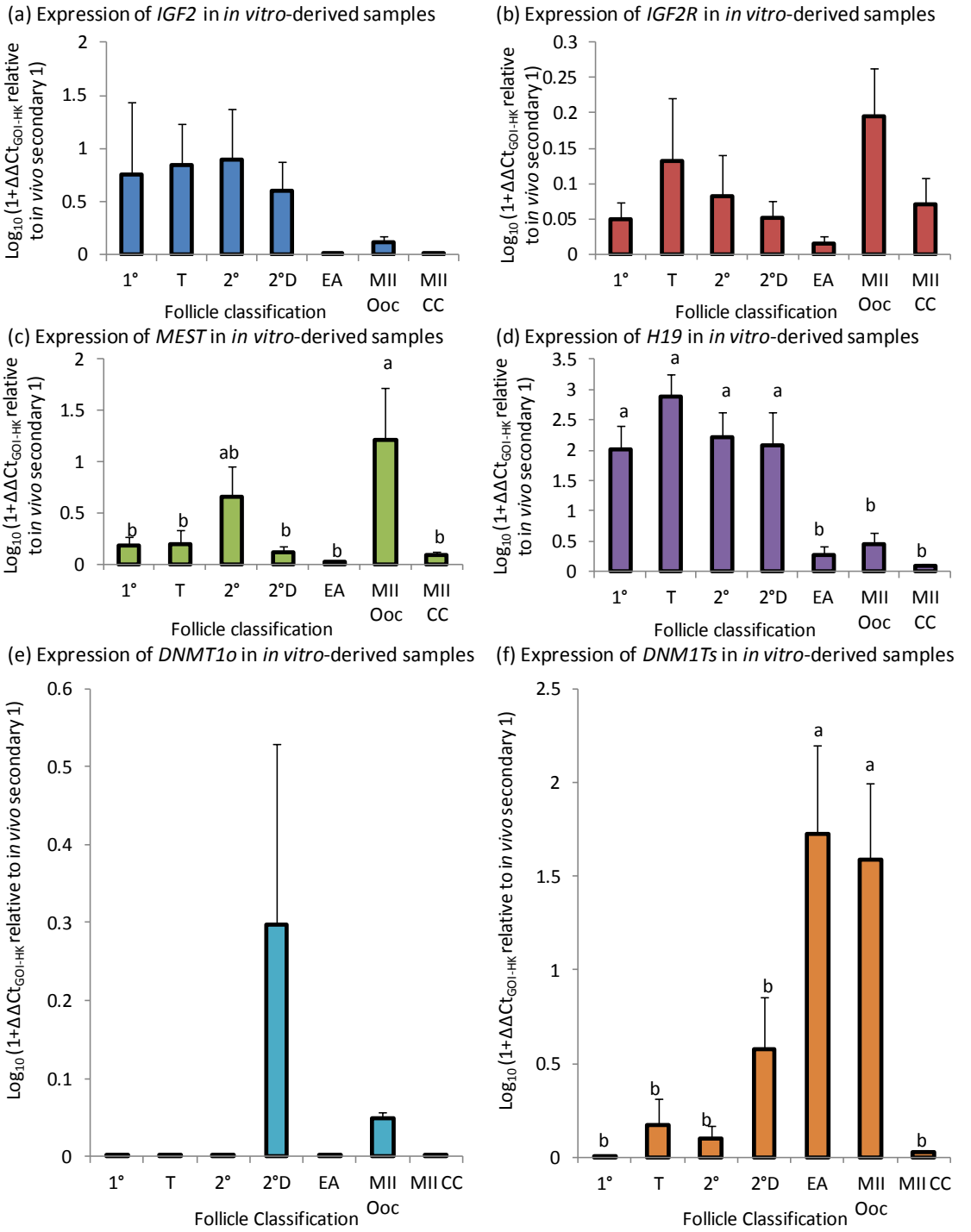


Figure 5.16 Histograms mapping the changes in expression patterns of key imprinted genes; *IGF2, IGF2R, MEST, H19, DNMT1o* and *DNMT1s* in *in vivo*-derived samples. Data is presented as $\log_{10}$ (mean expression relative to the level of expression in the control *in vivo*-derived secondary sample) ± SEM for 4 independent samples with the exception of primary stage follicles of which there were only 3 repeats. Levels of expression were compared using one-way ANOVA. Statistically significant differences ($p<0.05$) are indicated using different letters. Note that it was necessary to use different scales because of the large variations in expression levels of the genes analysed in this series. Abbreviations: primary (1°), transitional (T), secondary (2°), secondary degenerating (2D°) early antral (EA), MII oocyte (MII) and CCs from MII oocytes (MII CC).

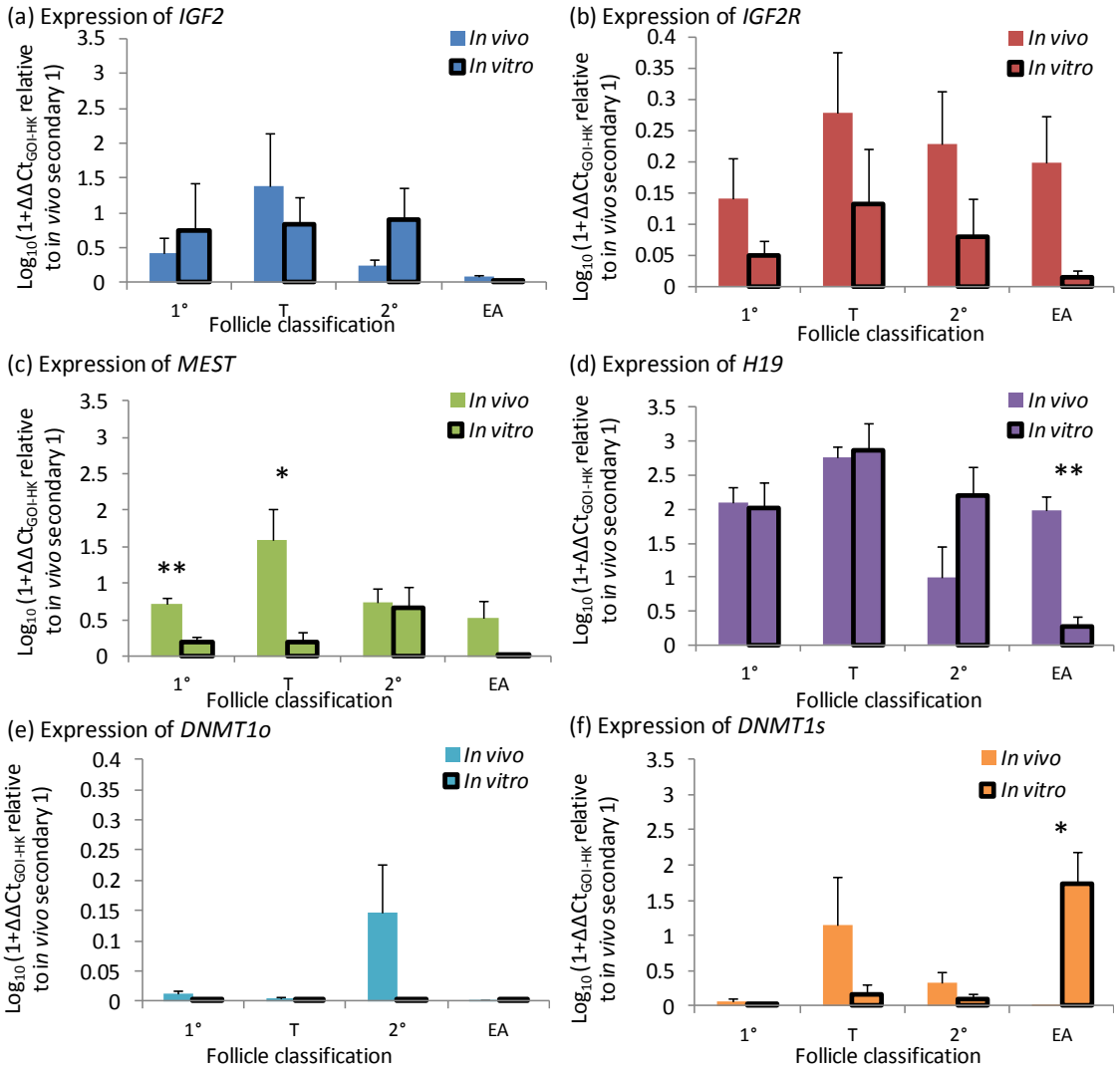


Figure 5.17 Histograms comparing the expression patterns of key imprinted genes; *IGF2, IGF2R, MEST, H19, DNMT1o* and *DNMT1s* in *in vivo*- and *in vitro*-derived samples. Data is presented as $\log_{10}$ (mean expression relative to the level of expression in the control *in vivo*-derived secondary sample) ± SEM for 4 independent samples with the exception of *in vitro*-derived primary stage follicles of which there were only 3 repeats. Levels of expression were compared using one-way ANOVA. Statistically significant differences between *in vivo*- and *in vitro*-derived follicles of the same classification are indicated using * ($p<0.05$) and ** ($p<0.01$). Note that it was necessary to use a different scale for *IGF2R* and *DNMT1o* due to their low expression levels compared to the other genes analysed in this series.

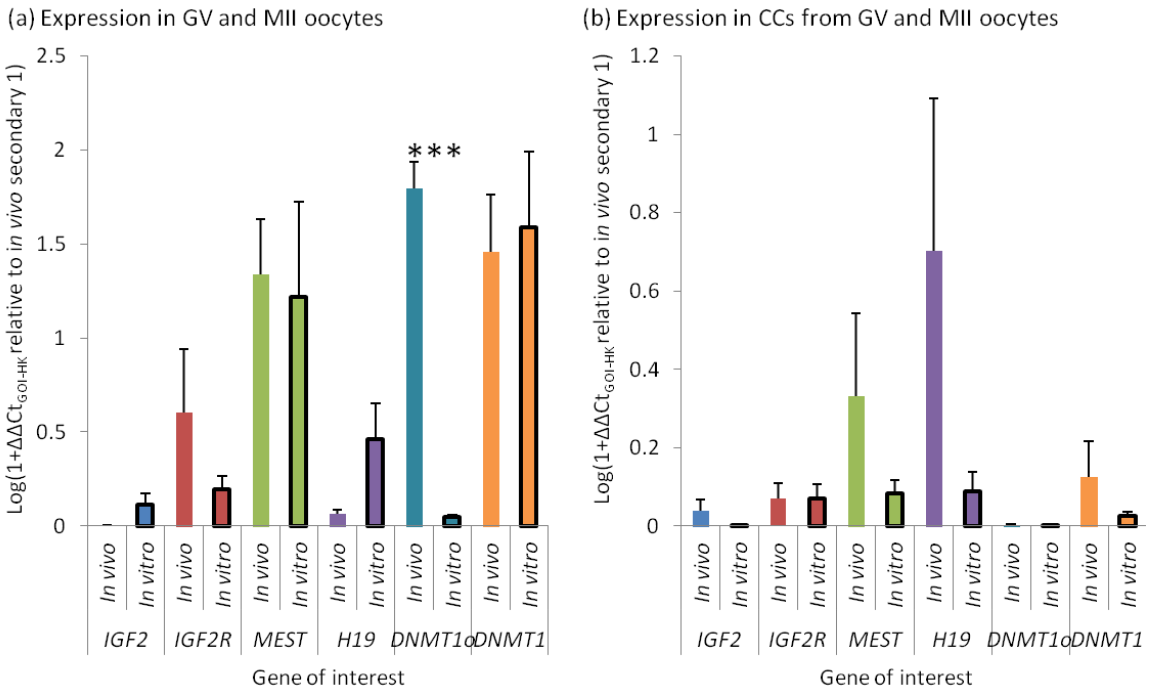


Figure 5.18 Histograms comparing the expression patterns of key imprinting genes; *IGF2, IGF2R, MEST, H19, DNMT1o* and *DNMT1s* in samples of (a) *in vivo*-derived GV oocytes and *in vitro*-derived MII oocytes and (b) *in vivo*-derived CCs from GV oocytes and *in vitro*-derived CCs from MII oocytes. Data is presented as $\log_{10}$ (mean expression relative to the level of expression in the control *in vivo*-derived secondary sample) ± SEM for 4 independent samples. Levels of expression were compared using one-way ANOVA. Statistically significant differences between *in vivo*- and *in vitro*-derived samples are indicated using *** ($p<0.001$).

Expression levels of key imprinting genes were compared between GV and MII oocytes and GV and MII oocyte-derived CCs, in Figure 5.18. Higher levels of expression of both *IGF2* and *H19* were observed in the MII oocytes than the GV oocytes, however, due to variability in the data these differences were not significant ($p>0.05$). Levels of both *MEST* and *DNMT1s* were similar in MII and GV oocyte samples. Expression of both *IGF2R* and *DNMT1o* is higher in GV oocytes than MII oocytes although only statistically significantly in the latter, ($p>0.05$) and ($p<0.001$), respectively. There were no statistically significant differences ($p>0.05$) observed in the levels of expression of imprinting genes between GV and MII oocyte-derived CC samples.

Levels of expression of all oocyte-specific genes were similar in GV and MII oocyte samples, with the exception of *GTSF1* in that a significantly higher ($p<0.001$) level of expression was detected in the GV oocytes, than the MII oocytes. With respect to the CC samples levels of expression of all oocyte-specific genes were similar with no significant differences ($p>0.05$) observed between GV and MII oocyte-derived samples.

5.4 Discussion

In this experimental series the expression patterns of 20 key genes implicated in the regulation of folliculogenesis, oogenesis, embryogenesis and imprint establishment and maintenance *in vivo* were elucidated and compared to that observed in oocytes and follicles derived *in vitro*.

5.4.1 Somatic cell-specific genes

Firstly, the expression patterns of key somatic cell-specific genes were considered. In this study very low levels of *AMH* expression by ovine primordial follicles were detected *in vivo* in contrast to that reported in previous studies, in which *AMH* expression was detected from the early primary stage (Bezard et al., 1987, Bonnet et al., 2011). In addition low levels of *AMH* expression were detected in the GV oocytes. This was unexpected as *AMH* is somatic cell-derived. It is possible that the primordial follicle and GV oocyte samples were contaminated with early primary follicles and GV CCs, respectively. It is also possible that the high sensitivity of the analytical methods used in the present study compared to published works resulted in the amplification and detection of very small amounts of cDNA present in the samples which had hitherto been undetectable. Alternatively the observed discrepancy in the *AMH* results for primordial follicles as reported here is due to a species-specific difference in gene expression. For example, the study conducted by Bonnet et al. (2011) used a bovine array to analyse gene expression profiles of ovine oocytes and somatic cells. It is possible that the use of a heterologous array meant that low levels of *AMH* expression were not detected, however Bezard et al. (1987) also reported the absence of AMH protein in ovine primordial follicles when analysed using immunohistological techniques. In the aforementioned studies increasing levels of *AMH* expression were detected with the progression of follicle development. This was observed to an extent in the current experimental series however levels of *AMH* expression detected in the EA follicle samples were very low. Interestingly, the expression pattern of *AMH in vitro* was more similar to that reported previously with respect to increasing levels of expression with progression of follicular development. When the expression patterns of *AMH in vivo* and *in vitro* were directly compared lower levels were detected *in vitro* across all stage-matched samples. This may partly explain the increased levels of primordial follicle activation exhibited *in vitro*, as AMH is an inhibitor of this process, although evidence suggests this may be species-specific and not the case in the ovine model (Campbell et al., 2012). Furthermore, the levels were not significantly lower until the EA stage. It is interesting to note that the expression of *Amh* by CCs in mouse preantral follicles, cultured

in the presence of 'low' levels of FSH (minimum 3.6 miUml^{-1}) were significantly lower than those *in vivo*, and in the presence of high levels FSH (25miUml^{-1}), expression stopped (Sanchez et al., 2010). It is therefore possible that exposure to FSH, although at a much lower concentration (1x10^{-5}iUml^{-1}), in the current investigation may have resulted in the decreased levels of *AMH* expression. In addition comparison of *Amh* expression levels by CCs from mouse preantral follicles cultured in the presence of low (5ngml^{-1}) and high (5µgml^{-1}) insulin concentration resulted in a significantly higher level of expression in the former group (Sanchez et al., 2011). It is possible that the slightly higher concentration of insulin used in the current study (10ngml^{-1}) resulted in the decreased levels of *AMH* observed *in vitro* compared to *in vivo*. The lower levels of *AMH* expression observed in *in vitro*-derived EA suggest that these follicles may be more responsive to gonadotrophin-stimulation, which may result in precocious luteinisation if follicle culture was continued (Campbell et al., 2012).

It would be interesting to assess the levels of AMH expression in accelerated culture systems, particularly those using tissue derived from species in which AMH has been shown to inhibit activation, such as human, in order to determine whether this could be a contributing factor. As discussed above *Amh* expression in mouse preantral follicles in suppressed by the addition of FSH to the culture media (Sanchez et al., 2010). In Chapter 4 an accelerated system developed in Edinburgh was tested, in this system FSH is not added to the cortical culture media therefore it is possible that levels of *AMH* expression by the ovine somatic cells may have been higher than in those cultured using the Leeds system. As AMH inhibits follicle activation lower levels of primordial follicle activation could be expected, however, this is contrary to what was observed in the AFC system (Chapter 4) and in previous studies by the Edinburgh group (Telfer et al., 2008, McLaughlin and Telfer, 2010), in which higher levels, compared to the Leeds physiological system were reported. This may be due to species-specific differences with respect to the actions of AMH (Campbell et al., 2012). Alternatively the method of tissue preparation or the effect of the culture system on the expression levels and/or bioavailability of other regulatory factors may also have resulted in increased levels of follicle activation. The findings in this study could be extended by monitoring *AMH* expression levels in somatic cells of ovine follicles grown *in vitro* using the AFC system (see Chapter 4) if it is possible to obtain sufficient numbers of follicles.

The pattern of *FSHR* expression observed in this study was similar to that reported previously in sheep, in that it was clearly evident from the primary stage onwards (Tisdall et al., 1995).

Very low levels of expression were also detected in the primordial follicle samples. Levels of expression were consistently higher in *in vivo* follicles than *in vitro*, although not significantly, particularly in secondary follicles. Lower levels of *FSHR* expression may hinder the development of IVG follicles once they become dependent upon gonadotrophin-stimulation if culture is continued (Campbell et al., 1999).

This study is the first to report the exact patterns of *IGF1* and *IGF1R* expression during *in vivo* growth of ovine oocytes from the primordial to the antral stage. Expression of both *IGF1* and its receptor remained relatively consistent throughout follicle development *in vivo* and *in vitro* (see Figures 5.5 and 5.6). When directly compared the levels of *IGF1* and *IGF1R* expression *in vitro* and *in vivo* fluctuated. Levels of *IGF1R* expression in transitional follicles were significantly lower *in vitro*. This may have had an effect on somatic cell proliferation and therefore follicle development (Perks et al., 1995, Monget and Bondy, 2000). The significantly lower levels of *IGF1* and non-significantly lower levels of *IGF1R* expression observed in EA follicles *in vitro* compared to *in vivo* may have more of a detrimental effect with respect to follicle development. This is particularly because IGF1 promotes *FSHR* expression and at this developmental stage follicles are becoming dependent upon gonadotrophin-stimulation. Therefore this may need to be taken into account when developing IVG systems, although IGF1 and its analogues are often included in the culture media (see Chapter 1). Although it should also be noted that increased levels of *IGF1* expression in small antral ovine follicles has been linked to increased levels of atresia. Therefore there might have been a greater proportion of atretic follicles derived *in vivo* (Hastie and Haresign, 2006).

Interestingly, the *in vivo* patterns of expression exhibited by *INHα*, *INHβA* and *FST* reported here are quite similar in that expression increased from the primordial to the transitional stage, decreased at the secondary stage then subsequently increased following cumulus cell differentiation as demonstrated by the GV CC stage, except in the case of *INHα* where a further decrease occurred at the EA stage (see Figure 5.5). The expression pattern of *INHβB in vivo* is more constant; however there also appeared to be a transient decrease in the level of expression at the secondary stage, although there was a lot of variability in the data. The fact that relatively high levels of expression of all inhibin subunits and *FST* were detected in GV oocytes suggests the methods used may have been too sensitive. It is possible that somatic cell gene contamination of both the GV and MII oocyte samples could have arisen from 2 sources: the first explanation is technical and could be accounted for by the failure to remove

all CCs during the denudation process; whereas the second explanation is biological transfer and is due to the potential to transfer cumulus expressed genes to the oocytes via transzonal processes and gap junctions which link the 2 cellular compartments *in vivo* and *in vitro* (Bortvin et al., 2003). In addition, although expression of *FST* has been detected throughout the development of ovine follicles, with a slight decrease at the pre-ovulatory stage, similar to that observed in Figure 5.6, the expression of inhibin subunits has not been detected in preantral follicles (Tisdall et al., 1994). During antral follicle development as increasing amount of inhibin A production relative to activin A was expected (Young et al., 2012). When the ratio of *INHα*: *INHβA* expression was analysed in Figure 5.9 this was not observed. The pattern of expression of inhibin subunits is complex and dependent upon the stage of the oestrous cycle and the level of E2 produced by the follicle, thus hindering the elucidation of expression patterns (Young et al., 2012). The ratio of *INHα*: *INHβA* expression in *in vivo*-derived EA follicles, shown in Figure 5.9, was most similar to that observed previously in GCs of small ovine antral follicles with low E2 concentration in the FF (Young et al., 2012). Therefore perhaps it would be useful to repeat the current experiment and determine the levels of E2 present in the FF or spent culture media. Within the current experimental series the methods of processing the follicles differed as *in vivo*-derived samples and *in vitro*-derived degenerating secondary and EA follicle samples were isolated enzymatically and so lacked a basement membrane and theca layer (Newton et al., 1999b), whereas the *in vitro*-derived preantral follicle samples were isolated mechanically and so contained a basement membrane and theca layer. This could possibly account for the differences in expression patterns of *INHα* observed between *in vitro*-derived secondary and degenerating secondary follicles, although this may not be the case as expression of neither *INHα* nor *INHβA* has been detected in the thecal layer of ovine follicles <1cm diameter (Nagamine et al., 1998). Note that the presence or absence of a theca layer may have also affected the apparent levels of expression of *IGF1* and its receptor, as these are expressed by theca cells (el-Roeiy et al., 1993, Qu et al., 2000, Armstrong et al., 2002).

The similarity in expression patterns exhibited by *INHα*, *INHβA* and *FST* both *in vivo* and *in vitro* suggests that some form of common regulatory mechanism exists (see Figure 5.5 and 5.6). This may explain why significantly higher levels of expression of *INHα*, *INHβA* and *FST* were observed in *in vitro*-derived EA follicles, compared to *in vivo*-derived (see Figure 5.7). As inhibin A production is promoted by both FSH and GDF9 it was possible that a link between the expression patterns of *INHα* and *FSHR* and ratio of *INHα* and *GDF9*. Significantly lower levels of *FSHR* and *GDF9* expression in *in vitro*-derived EA follicles did not however affect *INHα*

expression as this was significantly higher in *in vitro*-derived EA follicles, compared to those derived *in vivo* (see Figure 5.7 and 5.13).

It is interesting to note that when stage-matched *in vitro*- and *in vivo*-derived follicles were compared the gene expression patterns displayed by follicles derived from isolated preantral culture were more likely to be affected than follicles derived using cortical slice culture (see Figure 5.7). Of the somatic genes analysed, 5 out of 8 (*AMH*, *IGF1*, *INHα*, *INHβA* and *FST*) were statistically different ($p<0.05$) when the *in vitro*-derived EA follicles, grown from the secondary stage in isolated culture were compared to *in vivo*-derived EA follicles. This may be because by isolating follicles and culturing individually rather than *in situ* the follicles are provided with an environment that is even more different from that *in vivo*. Alternatively removal of the theca layer when enzymatically isolating secondary follicles may have affected the levels of gene expression in *in vitro*-derived EA follicles compared to *in vivo*-derived EA follicles. As discussed in Chapter 1, theca cells possess receptors for inhibin, activin and IGF1 therefore it is possible that in 'theca-free' follicles production of these factors by the GCs was down-regulated. The expression of *FST* by GCs may also have been affected as this factor is involved in the regulation of the actions of activin.

5.4.2 Oocyte-specific genes

Expression of *GDF9* and *BMP6* were observed *in vivo* from the primordial to the antral stage in agreement with previous reports (Bodensteiner et al., 1999, Bodensteiner et al., 2000, Juengel et al., 2006). The expression of *BMP15* was also detected from the primordial stage *in vivo* rather than from the primary stage as has been previously reported (Galloway et al., 2000). Direct comparison of the expression levels of these oocyte-derived growth factors *in vivo* and *in vitro* was quite revealing. The significantly lower levels of *BMP15* and possibly *BMP6* in *in vitro*-derived secondary follicles may have resulted in lower levels of GC proliferation (Campbell et al., 2009, Lin et al., 2012). Although the levels of *GDF9* expression observed *in vitro* were very similar to those observed *in vivo* in secondary follicles this is unlikely to have counteracted the potential effects of low *BMP15* and *BMP6* expression on GC proliferation as ovine GDF9 without BMP15 is not mitogenic (Lin et al., 2012). In addition levels of *BMP15* expression in EA follicles were lower, although not significantly, *in vitro* and were accompanied by a decrease in *GDF9* expression levels compared to those observed *in vivo* which may have a largely detrimental effect on GC proliferation. Although *in vitro*-matured MII oocytes - derived following 24 hours of IVM culture of *in vivo* grown GV oocytes - exhibited high levels of *BMP15* expression this might not be the case when mature MII gametes are derived from IVG EA

follicles as the levels of *BMP15* expression are already lower at this stage than *in vivo*-derived equivalent staged follicles. Therefore, this may be an important consideration when developing IVG culture systems as low levels of BMP15 could result in premature follicular luteinisation (Juengel et al., 2011). Similarly, lower levels of *GDF9* expression in *in vitro*-derived EA follicles may lead to prematurely increased levels of P4 and therefore precocious differentiation of the follicle (McNatty et al., 2005). In a number of species GDF9 has been shown to be important in terms of the regulation of CC metabolism in mouse, cow and human (Colonna and Mangia, 1983, Sugiura et al., 2005, Su et al., 2008, Su et al., 2009) and potentially mucification in mice (Gilchrist et al., 2008), therefore compromised levels of GDF9 *in vitro* may adversely affect these processes. This may be of particular importance with respect to energy requiring processes such as meiotic progression and cytoplasmic maturation, which involve both the oocyte and CCs, as these may potentially be compromised in *in vitro*-derived oocytes (Picton et al., 1998, Conti et al., 2012).

Expression of *Gdf9* and *Bmp15* by mouse oocytes *in vitro* has been shown to increase in response to increasing levels of FSH (from 10- to 25miUml^{-1}), whereas they were unaffected by changes in insulin concentrations (10ngml^{-1} vs. 10µgml^{-1}) (Sanchez et al., 2010, Sanchez et al., 2011). In order to extend the current investigation and compare the physiological slow growth system developed in Chapter 3 with the accelerated growth system used in Chapter 4 it would be interesting to monitor changes in expression levels of *GDF9* and *BMP15* in order to determine whether they are affected by the presence or absence of FSH in the culture media. Levels of *Gdf9* and *Bmp15* expression by secondary mouse oocytes *in vitro* are affected by the concentration of alginate gel used as a scaffold, with expression decreasing with increasing alginate concentration (0.25% vs. 1.5%) (Jiao and Woodruff, 2013). This suggests that the use of gel matrices may suppress the expression of oocyte-specific genes key to folliculogenesis and oogenesis. It would be interesting to test this theory by comparing gene expression patterns of ovine secondary follicles cultured in the absence of support matrices, as in Chapter 3 vs. those encapsulated in a gel, *in vitro*.

Although the levels of *BMP6* expression in EA follicles were similar *in vivo* and *in vitro* the lower levels of *IGF1* expression in *in vitro*-derived follicles of this classification may affect the promotion of the differentiative actions of FSH by BMP6 (Webb and Campbell, 2006). Levels of *BMP15* and *GDF9* expression in degenerating secondary follicles did not differ from those observed *in vivo*, whereas levels of *BMP6* were lower although no different from levels

observed *in vitro*. In contrast, relatively high levels of *GDF9* expression were detected in GV CC and MII CC samples. Although *BMP6* expression in mice has been previously detected in GCs, expression of neither *BMP15*, nor *GDF9* has been detected in the somatic follicular cells in any specie (Elvin et al., 2000). Low levels of *BMP15* and *BMP6* expression relative to that observed in other samples were detected in both GV CC and MII CC samples. In addition to this the detection of *BMP15* expression in primordial follicles calls into question the validity of these results.

Zona pellucida formation occurs at the primary stage in mice and humans (Zamboni, 1974, Epifano et al., 1995), however, in this experimental series expression of *ZP2* was detected in ovine primordial follicles, indicating a possible species-specific difference. The expression of *ZP2* has, however previously been detected in primordial human follicles analysed using similar methods of sample preparation as used here suggesting that the previously reported expression patterns may be due to differences in sensitivities of the methods used (Huntriss et al., 2002). Levels of *ZP2* expression *in vivo* significantly increased at the primary and transitional stages suggesting that these are the main stages at which ZP formation occurs in sheep. As the level of *ZP2* expression is very high in primary follicles it is possible that *ZP2* was detected in primordial samples because they were contaminated with primary follicles. As the expression of a number of other genes, including *BMP15*, *IGF1*, *IGF1R* and *IGF2R*, has been detected in primordial follicles when previous reports have evidenced their expression from the primary stage at the earliest (see Chapter 1), this further suggests that there may have been some contamination of the primordial follicle samples with primary or EP follicles as the primordial and EP follicle populations are the most difficult to classify accurately. Levels of *ZP2* expression *in vitro* were consistently lower than those observed *in vivo*, to a statistically significant level in the primary and secondary follicle samples ($p<0.01$). Therefore *in vitro*-derived ovine oocytes may not exhibit adequate ZP formation and this may lead to fertilisation problems. In mice and humans ZP2 acts as a secondary sperm receptor and is not involved in the acrosome reaction (Wassarman, 1988). Furthermore, the use of ICSI may overcome any potential problems. Despite this alterations in gene expression levels by the oocyte indicate that culture conditions are not optimal.

Details regarding the expression patterns of *FOXO3* during folliculogenesis in sheep have not previously been reported. In the current experimental series *FOXO3* expression was observed throughout follicle development *in vivo* (see Figure 5.11) and high levels of expression were

observed in MII oocytes (see Figure 5.12). Consistently higher levels of *FOXO3* expression were observed in *in vivo*- compared to *in vitro*-derived follicles. This could be one of the factors that results in higher levels of primordial follicle activation *in vitro* compared to *in vivo* (Liu and Lehmann, 2006). The expression pattern of *GTST1 in vivo* (see Figure 5.11) is similar to that reported in murine, human, bovine and ovine ovarian follicles in that expression was detected from the primordial stage then onwards throughout development, with the highest levels of expression observed in the GV oocyte (Krotz et al., 2009, Lu et al., 2009, Liperis et al., 2013, Liperis, 2014). The fact that lower levels of *GTST1* were consistently observed *in vitro* compared to *in vivo*, particularly in primary ($p<0.01$) and secondary ($p<0.05$) follicle samples indicates that *in vitro* culture conditions were not optimal resulting in aberrant oocyte-specific gene expression. As *GTSF1* has been implicated as being involved in the post-transcriptional control of RNA processing and storage as well as translational regulation these processes may be disrupted in *in vitro*-derived oocytes (Liperis et al., 2013, Liperis, 2014).

In contrast to that observed with somatic cell-specific genes the *in situ* culture of preantral follicles appeared to affect the expression of oocyte-specific genes. Significant decreases ($p<0.05$) in *ZP2, GTSF1, BMP6* and *BMP15* expression were observed in *in vitro*-derived secondary follicles and in *ZP2* and *GTSF1* expression in *in vitro*-derived primary follicles compared to their *in vivo*-derived counterparts (see Figure 5.13). This suggests that the oocyte is more sensitive to changes in culture conditions in the early stage of follicular development than the somatic-cells of the follicle.

5.4.3 Imprinted genes and genes involved in the regulation of epigenetic modifications

In this experimental series the expression patterns of ovine *DNMT1o* and *DNMT1s in vivo* were determined (see Figure 5.15). In agreement with previous research expression of both genes was detected in both ovine oocyte and somatic cell samples (Taylor et al., 2009). Expression of both genes was also detected throughout follicle development with highest levels of expression being recorded for GV oocytes. Levels of expression of both genes were also slightly elevated in secondary follicles and, of *DNMT1s* only in the transitional and GV CC cells. The expression of *DNMT1o* in all *in vitro*-derived samples was relatively low, except in degenerating secondary follicles, although as could be expected there were high levels of variation in this group (see Figure 5.16). Slightly elevated levels were observed in MII oocytes. In contrast, the level of expression of *DNMT1s* was highest in EA and MII oocyte *in vitro*-derived samples. When directly compared levels of *DNMT1o* expression were similar *in vivo*

and *in vitro* with the exception of the secondary follicle samples in which higher levels of expression were recorded *in vitro,* albeit the differences were not significant ($p>0.05$). Levels of *DNMT1s* expression were significantly higher ($p<0.05$) in EA follicle samples *in vivo* compared to *in vitro*.

As discussed in Chapter 1, DNA methyltransferases are key enzymes with respect to the establishment of imprinting marks as well as the maintenance of these (Reik et al., 2001). Increased levels of expression of *Dnmt1* have previously been reported in *in vitro*-derived mouse embryos, compared to *in vivo*-derived (Wang et al., 2005). The enzyme DNMT1 is essential for the maintenance of the maternal imprint in oocytes and preimplantation embryos (Hayward et al., 2003, Taylor et al., 2009). Therefore the differences in expression levels observed in the current study may lead to alterations in methylation patterns as the lower levels of *DNMT1s* expression levels may result in the maternal imprint not being adequately maintained thus resulting in aberrant expression of imprinted genes throughout oogenesis and embryonic development (Reik et al., 2001, Goll and Bestor, 2005). It is possible that the decrease in levels of *DNMT1o* expression may be embryonic lethal as this has previously been observed in mutant *Dnmt1* mice (Li et al., 1992). It is interesting to note that the higher levels of *DNMT1s* expression in *in vitro*-derived EA follicles were accompanied by lower levels of expression of all imprinted genes analysed *in vitro* than *in vivo*, to a level approaching significance with respect to *IGF2* ($p=0.076$), *IGF2R* ($p=0.133$) and *MEST* (0.090) and significantly with respect to *H19* ($p=0.001$). Therefore, it is possible that aberrant *DNMT1s* expression affected the expression of these other imprinted genes *in vitro*, which is concerning as these genes are important regulators of normal oogenesis, folliculogenesis and embryogenesis (Li et al., 1993).

This is the first investigation to document the exact pattern of *IGF2* and *IGF2R* expression in preantral ovine follicles. Expression of both genes was detected throughout *in vivo* follicle development, shown in Figure 5.15. Expression of *IGF2* decreases with progressive follicle development as has been described previously (Hastie and Haresign, 2006). The levels of *IGF2R* expression remained relatively constant throughout follicle development. Similar patterns of expression of both genes were observed *in vitro*, shown in Figure 5.16. Surprisingly high levels of *IGF2* and *IGF2R* were detected in the GV and MII oocyte. When the levels of *IGF2* expression *in vivo* and *in vitro* were directly compared differences fluctuated in the preantral stage, however, at the EA stage expression was higher *in vivo* to a level approaching

significance (p=0.076). As significantly higher levels of *IGF2* expression have been detected in healthy compared to atretic small ovine follicles (<2mm) this suggests that there was a higher proportion of atretic EA follicles in the *in vitro*-derived samples (Hastie and Haresign, 2006). Throughout development levels of *IGF2R* expression were consistently higher *in vivo*- than *in vitro*-derived follicles however not to a statistically significant extent. Changes in expression patterns of *Ifg2* and *Igf2r* have been detected in numerous studies as a result of embryo culture in the mouse (Khosla et al., 2001, Ohno et al., 2001, Fernandez-Gonzalez et al., 2004) and sheep (Young et al., 2001). Reduced levels of *IGF2R* expression, resulting from aberrant imprinting, have previously been associated with large offspring syndrome (LOS) in ovine foetuses, therefore it is possible that, as techniques progress, the slightly reduced levels of *IGF2R* expression observed in *in vitro*-derived oocytes may subsequently result in LOS if the oocytes are successfully fertilised (Young et al., 2001). One of the roles of the IGF2R is to regulate IGF2 levels via the endocytosis and degradation of excess extracellular IGF2 (Ludwig et al., 1995), therefore the reduced levels of *IGF2R* expression may have resulted in increased circulating levels of IGF2 – it would be interesting to repeat the process of IVG and assay for levels of IGF2 to determine whether this is the case. As discussed in Chapter 1, it has also been postulated that BWS in humans may result from excess IGF2 levels during human embryogenesis (Eggenschwiler et al., 1997). Studies in mice suggest that the increase in IGF2 levels may be a result of reduced *Igf2r* and *H19* expression (Eggenschwiler et al., 1997). Abnormal patterns of methylation of the *IGF2R* gene have also been observed in patients with BWS that were conceived using ARTs (Rossignol et al., 2006). Therefore the reduced levels of *IGF2R* expression observed in the current experimental series are concerning in terms of the potential subsequent effect on the health of embryos derived from IVG oocytes.

The expression of *MEST* was highest in transitional follicles *in vivo*, and was also high in GV oocytes *in vivo*, as shown in Figure 5.15. A similar pattern of expression was observed *in vitro* except that highest levels of expression were observed in secondary follicles. High levels were also observed in MII oocytes (see Figure 5.16). Direct comparison of expression levels in *in vivo*- and *in vitro*-derived samples revealed significantly higher levels of expression in *in vivo*-derived primary ($p<0.01$) and transitional ($p<0.05$) follicles. Previous work concerning the methylation patterns of *in vitro*-derived humans embryos has shown a considerable degree of variation in *MEST* methylation patterns which is of concern as aberrant expression has been linked to SRS, altered growth patterns and cancers, including colorectal and lung (Huntriss et al., 2013, Koza et al., 2006). In the aforementioned study authors propose that the variation in methylation patterns may also be observed in *in vivo*-derived embryos (Huntriss et al., 2013).

Therefore it is interesting that in the current study differences in patterns of *MEST* expression were observed between the *in vivo*- and *in vitro*-derived samples.

Subfertility/infertility and the use of ARTs have been linked to alterations in *H19* methylation and expression. Alterations in expression patterns of *H19* have been observed in numerous studies as a result of embryo culture in mice (Doherty et al., 2000, Khosla et al., 2001, Lane and Gardner, 2003). Additionally gain of methylation of the *H19* gene has been linked to both ovarian stimulation and subfertility/ infertility in humans (Borghol et al., 2006, Al-Khtib et al., 2011). Observations regarding the effect of IVF and ICSI on the methylation status of *H19* have not been consistent with gain of methylation, loss of methylation and no change in methylation being observed in different studies (Denomme and Mann, 2012). In the current investigation the expression of *H19* was highest in transitional follicles *in vivo* whereas *in vitro* levels were consistently high throughout preantral follicle development (see Figures 5.15 and 5.16). Significantly lower levels of expression were observed in EA follicles and MII oocytes and CCs than preantral follicles *in vitro* ($p<0.05$). When compared directly to levels of expression *in vivo*, shown in Figure 5.17, the level of expression in EA follicles was significantly lower than that observed *in vivo* ($p<0.01$). As both MEST and H19 have been implicated as important regulators of embryonic development and aberrant expression has been linked to the incidence of epigenetic disorders the fact that *in vitro*-derived follicles exhibit altered expression patterns is a potential cause for concern (Kagami et al., 2007, Chopra et al., 2010). Additionally, as *H19* regulates the expression of *IGF2* and *IGF2R* it is possible that the significantly lower levels of expression of *H19 in vitro* are the cause of the lower levels of *IGF2* and *IGF2R* observed *in vitro*.

As discussed above it may be a concern that the expression patterns of imprinted genes - that have been linked to the incidence of epigenetic diseases and LOS when aberrantly expressed - differed in numerous *in vivo*- and *in vitro*-derived equivalent follicle samples. It is possible that the changes in expression patterns of these genes may be a result of aberrant imprinting. However, as discussed in Chapter 1, previous research suggests that the mechanisms by which DNA methylation patterns are established are quite robust in murine and ovine models. Studies in mice have shown that the methylation patterns of *Igf2* and *H19* in MII oocytes grown *in vitro* from the secondary stage were not affected under normal culture conditions (Anckaert et al., 2009a). Furthermore, when follicles were subjected to suboptimal culture conditions such as the addition of high levels of FSH ($100 iUL^{-1}$, compared to $10 iUL^{-1}$) to the culture media, or exposure to increased levels of ammonium (up to ~1500µm following 12 days culture) and significantly reduced levels of E2 and P4, induced by culturing under mineral

oil, this did not affect the methylation of the aforementioned genes (Anckaert et al., 2009a, Anckaert et al., 2009b, Trapphoff et al., 2010). In addition embryos resulting from the fertilisation of *in vitro*-derived oocytes also exhibited normal methylation of the aforementioned genes (El Hajj et al., 2011). Similarly, ovine oocytes grown from the preantral to EA stages *in vitro* displayed normal methylation patterns of *IGF2R* and *H19* (Barboni et al., 2011). Nor were the methylation patterns of *IGF2R* or *H19* affected following the IVM of ovine oocytes (Colosimo et al., 2009). Conversely, although culturing in the presence of reduced levels of methyl donors did not affect the methylation levels of *Igf2* or *H19* in murine MII oocytes grown *in vitro* from the secondary stage, the methylation levels of *Mest* decreased (Anckaert et al., 2010). In order to establish whether the changes in gene expression observed *in vitro* relative to *in vivo* were due to aberrations in the imprinting process it would be interesting to extend this investigation in order to determine and compare the methylation patterns of these genes in stage-matched *in vitro*- and *in vivo*-derived follicles.

When the expression patterns of imprinted genes were compared between *in vivo*- and *in vitro*-derived counterparts only *MEST* was significantly affected by the *in situ* cortical culture conditions, as lower levels of expression were observed in *in vitro*-derived primary ($p<0.01$) and transitional ($p<0.05$) follicles compared to *in vivo*-derived counterparts (see Figure 5.17). Isolated preantral culture of follicles resulted in a significant decrease ($p<0.01$) in *H19* expression and a significant increase ($p<0.05$) in *DNMT1s* expression.

5.4.4 Future work

Due to the small number of replicates there was a large amount of variability in much of the data presented here– therefore in order to determine whether the differences in expression patterns observed between follicular stages and *in vivo*- and *in vitro*-derived samples were significant it would be necessary to increase the number of independent replicate samples analysed at all stages of development as well as and in the *in vivo*- vs. *in vitro*-derived follicle/oocytes analyses. The quality of the data could also be improved by altering the sequences of some of the more sensitive primers to ensure expression patterns are being interpreted correctly. As mentioned above the expression of numerous genes at the primordial stage contradicted previous reports, suggesting that the methods used in this chapter may have resulted in the amplification and detection of cDNA that did not result from the target gene. Reducing the number of RT PCR cycles from 40 to 35 may reduce the risk of this in the future. Furthermore, repetition of samples in which contamination may have

occurred would be necessary. In addition it would be interesting to extend the culture series in order to generate *in vitro*-derived samples of follicles and oocytes of all stages which have developed from the primordial stage *in vitro* i.e. to complete the growth trajectory of *in vitro* derived follicles and oocytes up to mature stages and then to progress the gametes so derived through IVM. In addition, the comparison of IVG MII oocytes with both IVM MII oocytes and *in vivo* matured MII oocytes would be very valuable in order to extend this field of research.

Ultimately, the comparison of full gene expression patterns (for example, using next generation sequencing techniques such as RNA-seq (Yan et al., 2013) as well as DNA methylation analysis (for example, using pyrosequencing techniques) (El Hajj et al., 2011, Huntriss et al., 2011) between embryos derived from i) IVG oocytes, ii) IVM oocytes, iii) *in vivo*-derived oocytes matured *in vitro*, iv) *in vivo*-derived MII oocytes fertilised *in vitro* as compared to v) *in vivo*-derived ovine blastocysts would be conducted in future experiments. Whole methylome analysis is extremely challenging in oocytes and preimplantation embryos due to the very small amount of genetic material present. Progress has been made using reduced representation bisulphite sequencing (RRBS) in murine oocytes and preimplantation embryos, as it is easier to obtain much larger numbers of murine oocytes/embryos (Smallwood et al., 2011). Some progress has been made regarding the use of this technique to analyse the whole methylome of small numbers of pooled and even single human and bovine blastocysts (Huntriss and Picton, unpublished data), the use of this technique to analyse the methylome of single or small numbers of oocytes of larger mammals and humans is highly challenging, due to the low amount of DNA available. Adaptations of the RRBS technique have however been used recently to assess the methylome of single embryonic stem cells, suggesting oocyte whole methylome analysis is feasible (Guo et al., 2013). In addition it would be a great benefit to perform a more detailed experiment by extending the studies to compare the full gene expression patterns and complete methylome of stage matched *in vivo*- and *in vitro*-derived ovine oocytes. It would also be essential to extend this work to the analysis of *in vitro*-derived human oocytes before these gametes can be used therapeutically.

It is interesting to note that the expression of the majority of genes (*AMH*, *FSHR*, *IGF1*, *IGF1R*, *INHα, INHβA, ZP2, GDF9, GTSF1, BMP15, FOXO3, IGF2R, MEST, DNMT1o* and *DNMT1s*) was higher *in vivo* than *in vitro* in stage-matched follicles. One possible explanation for this could be a difference in mean follicle size, and therefore a difference in the number of follicular somatic cells, between the *in vivo*- and *in vitro*-derived samples. Unfortunately the diameters

of the follicles samples collected were not recorded in this chapter. The diameters of *in vivo*- and *in vitro*-derived secondary follicles were recorded in Chapter 3, however, and therefore could be used as an indicator of whether there was likely to be a difference in size dependent on derivation. There was, however, no statistical difference ($p>0.05$) in the mean follicle diameter of *in vivo*- and *in vitro*-derived secondary follicles, 217.7±2.6µm (n=85) and 219.5±10.5µm (n=16), respectively. In addition there was no statistical difference ($p>0.05$) in the mean oocyte diameter, 101.8±3.4µm (n=85) and 106.9±2.0µm (n=16), of *in vivo*- and *in vitro*-derived secondary follicles, respectively. Therefore the possibility that the similarities in follicle size may be concealing a difference in follicular somatic cell number due to differences in the oocyte diameter can be ruled out. It is, however, possible that the *in vivo*- and *in vitro*-derived follicles used for the current chapter did differ in size, therefore it may be worth repeating the experiments and measuring the follicle and oocyte diameter of all samples.

One of the aims of the current chapter was to determine which genes would be most useful as molecular markers of normality. Genes that display discrete patterns of expression throughout follicle development would be useful – in the current investigation these included; *AMH, INHα, INHβA, FSHR, IGF1R, FST, ZP2, GDF9, GTSF1, BMP6, BMP15, MEST, H19, DNMT1o* and *DNMT1s*. Genes that display interesting differences when *in vivo*- and *in vitro*-derived stage-matched samples are compared are also likely to be useful markers as these are the genes that are sensitive to changes in the environment in which they are grown – in the current investigation these included; *AMH, IGF1, IGF1R, INHα, INHβA, FST, ZP2, GDF9, GTSF1, BMP6, BMP15, MEST, H19, DNMT1o* and *DNMT1s*. Although the expression patterns of a number of genes (*AMH INHα, INHβA, FST, INHβB, GDF9, BMP6* and *BMP15*) vary a little from what has previously been reported alterations to the techniques used (discussed above) may alleviate these problems somewhat. Therefore, in conclusion, the genes identified as the most useful as molecular markers of normality are *AMH, IGF1R, INHα, INHβA, ZP2, GDF9, GTSF1, BMP6, BMP15, MEST, H19, DNMT1o* and *DNMT1s*.

5.5 Conclusion

The first aim of this experimental series was to define molecular markers of normal follicle and oocyte development by examining the expression patterns of 20 candidate genes implicated in the regulation of folliculogenesis, oogenesis, embryogenesis and imprinting establishment and maintenance *in vivo*-derived ovine oocytes and follicles. For a number of the genes analysed the expression patterns had previously been reported, however, this is the first report of the

pattern of *IGF1*, *IGF1R*, *INHα*, *INHβA*, *INHβB, ZP2, FOXO3, GTSF1, DNMT1o, DNMT1s, IGF2, IGF2R, H19* and *MEST* expression throughout preantral and antral follicle development in the sheep. As expression patterns of some genes, such as *AMH* differed from previous reports it is possible that the methods used in this experimental series were more sensitive than those used previously. It is also possible that the methods used were too sensitive. The expression patterns of these genes in oocytes and follicles derived *in vitro* using a long-term, physiological culture system is also reported. The direct comparison of *in vivo*- and *in vitro*-derived samples revealed significant changes in the expression patterns of *AMH, IGF1, IGF1R, INHα*, *INHβA, FST, ZP2, GDF9, GTSF1, BMP6, BMP15, DNMT1o, DNMT1s, H19* and *MEST* in at least one developmental stage although further replicate analyses are needed it would appear the expression of a large proportion of the genes analysed was adversely affected by the culture conditions to which the oocytes and follicles were exposed. Different stages of follicle culture appeared to have different effects on gene expression patterns as *in situ* culture mainly affected the expression patterns of oocyte-specific genes, whereas isolated preantral follicle culture mainly affected the expression patterns of somatic cell-specific genes.

6 Use of Follicle Culture as a Tool to Quantify the Impact of Cryoprotectant Perfusion for Whole Ovary Perfusion.

6.1 Introduction

The development and optimisation of IVG techniques as a means of fertility preservation has been investigated in the first 3 experimental chapters of this thesis. It is important to note that underpinning these techniques is the cryopreservation of ovarian tissue, without which these techniques would be of no use. In some patients the cryopreservation and subsequent autotransplantation of thawed ovarian tissue without the combined use of IVG techniques is a potential suitable means of fertility preservation for women with primary ovary insufficiency (POI) or women requiring sterilising treatment (Donnez et al., 2013, Onions et al., 2013). In some patients, however, it is not possible to autotransplant the ovarian tissue; therefore in these cases the development of IVG techniques would be of great value. For example, fertility preservation by ovarian cryopreservation and autografting is not suitable for patients in which transplantation of tissue back to the body following treatment risks reintroducing the disease (Shaw et al., 1996). Ovarian tissue can be easily obtained via laparoscopy and cryopreserved then, when appropriate, the tissue can then be thawed and transplanted back into the patient when she wishes to start or complete her family (Newton et al., 1996, Davidoff et al., 1996). The vast majority of follicles present in the ovary are at the primordial stage and follicles at this stage exhibit a high survival rate following cryopreservation, due to the fact that they are relatively tolerant to the procedure (Newton et al., 1996, Oktay et al., 1998, Kim et al., 2001). Although when secondary follicles are present they can also survive the freeze-thaw process (Newton et al., 1996). Therefore this technique is preferable to the cryopreservation of GV and MII oocytes, which are less tolerant to the cryopreservation procedure, and are unsuitable for fertility preservation strategies in prepubertal girls. The development and optimisation of WOCP techniques would be a highly beneficial alternative to the traditional methods of cryopreserving then transplanting fragments of ovarian tissue as this can be wasteful and result in the loss of a significant proportion of the ovarian follicle reserve.

6.1.1 Ovarian tissue vs. whole ovary cryopreservation

The use of ovarian tissue cryopreservation and orthotopic transplantation has resulted in the transient restoration of ovarian function in numerous patients, in some cases enabling pregnancy and live birth as discussed in Chapter 1 (Meirow et al., 2005, Demeestere et al., 2007, Andersen et al., 2008, Demeestere et al., 2010, Jadoul et al., 2010, Donnez et al., 2012,

Revelli et al., 2013). However, long-term restoration of ovarian function using this technique is unlikely due to the low number of follicles transplanted back into the patient compared to the follicle reserve prior to treatment (Baird et al., 1999, Salle et al., 2003). The follicle reserve is initially reduced simply because only the follicles present within the slice of cryopreserved tissue are transplanted back into the patient. The follicle reserve is further reduced by the cryopreservation and transplantation processes. A histological comparison of fresh and frozen-thawed slices of human ovarian tissue revealed no significant differences in the number or proportion of primordial and primary follicles, until the tissue was transplanted into SCID mice when the follicle density in the frozen-thawed tissue decreased significantly compared to the fresh tissue grafts (Nisolle et al., 2000). These findings support the hypothesis that the period of ischaemia to which the ovarian tissue is subjected following transplantation of the graft and prior to revascularisation is the major cause of follicular reserve depletion (Newton et al., 1996, Liu et al., 2002, Israely et al., 2004, Israely et al., 2006). For these reasons transplantation of frozen-thawed tissue is only a viable option, with respect to fertility preservation, for younger women, as the follicle reserve in the ovaries of women over 35 years has already been depleted - due to reproductive ageing - to such an extent that there would be too few follicles present in slices of ovarian tissue to restore ovarian function for a significant period of time (Onions et al., 2013). In contrast the use of WOCP techniques overcomes some of the problems associated with ovarian tissue slice cryopreservation as the entire follicular reserve is cryopreserved and then transplanted back to the patient. Therefore although follicular loss may still occur following freezing and thawing and transplantation a greater number of follicles are still transferred and fewer follicles are lost as a result of ischaemia following transplantation. Indeed, if both the whole ovary and its vasculature is cryopreserved the level follicular depletion as a result of ischaemia is significantly reduced as the graft can be immediately revascularised via surgical anastomosis (Onions et al., 2009).

There are a number of challenges associated with WOCP. Whole ovary cryopreservation requires protection of the tissue via the use of a CPA suitable for the range of cell types comprising both the ovary and its vasculature. DMSO is often used for whole ovary cryopreservation (Onions et al., 2009, Onions et al., 2013), and will be used in this experimental series, as it has a relatively high permeation rate at low temperatures and is suitable for the range of ovarian cell types (Newton et al., 1996, Newton et al., 1998). Histological evaluation of ovine ovarian tissue, with respect to the proportions of healthy and degenerating oocytes present following exposure to various CPAs including; DMSO, PROH, EG and glycerol at 1.5M and 3M concentrations; and subsequent cryopreservation, revealed that

1.5M DMSO exposure was the least detrimental to follicle and oocyte health (Santos et al., 2006). Additionally whereas EG and PROH were found to be genotoxic to Chinese hamster ovarian cells, in that exposure caused 'DNA damage leading to chromosomal mutations' (Aye et al., 2010). Prior to cortex cryopreservation slices of ovarian tissue are soaked in CPA to allow tissue permeation via diffusion. This CPA has also been utilised successfully during WOCP in rodents where orthotopic transplantation of frozen-thawed ovaries has resulted in the restoration of fertility, although decreases in the follicle reserve resulting from ischaemic damage were reported (Harp et al., 1994, Candy et al., 2000, Yin et al., 2003). However, this method of permeating the ovary with CPA by diffusion is not suitable for larger mammals as their ovaries are too large and would therefore have to be exposed to the cytotoxic CPA for a long period of time to ensure full permeation. This could be highly detrimental to the tissue. Partial permeation would also be highly detrimental as this may allow ice formation to occur during the cryopreservation procedure (Wolfe and Bryant, 1999, Meryman, 2007). This can be overcome by perfusing the ovary with CPA via the cannulation of the native vasculature supplying the ovary resulting in a shorter CPA exposure time. This was first carried out in sheep by Bedaiwy et al. (2003), and has subsequently been used by (Onions et al., 2008, Onions et al., 2009, Onions et al., 2013, Arav et al., 2010). The ovine model will be used in the current investigation as it similar to human with respect to the size of the ovaries in girls and the fibrous nature of the human ovary (Gosden et al., 1994, Onions et al., 2008).

There have been a number of successful attempts at transferring whole fresh ovaries in sheep and humans, resulting in the restoration of ovarian function (Jeremias et al., 2002, Courbiere et al., 2009, Mhatre et al., 2005, Imhof et al., 2006, Laufer et al., 2010). Furthermore, autotransplantation of ovaries that had been cryopreserved using slow-freezing methods then thawed, into ewes resulted in limited restoration of ovarian function and in one case a spontaneous live birth 545 days after transplantation (Bedaiwy et al., 2003, Imhof et al., 2006). However, major depletion of the follicle reserves was observed in the frozen-thawed ovaries. Following transplantation a comparison of perfused fresh and frozen-thawed ovine ovaries revealed 'similar follicle losses and endocrine disruption' in both (Onions et al., 2009). In this study the fresh ovaries were perfused with L-15 medium plus 10% FCS, not CPA. This suggests that the large loss of follicles can be attributed to the perfusion and cannulation techniques, as opposed to the cryopreservation techniques and use of CPA *per se*, as shown by the use of fresh ovary controls. Although long-term patency following cryopreserved whole ovary grafting has been achieved, damage to the microvasculature, caused by the perfusion process, is probably a major contributor to the high levels of follicle depletion (Onions et al., 2009).

Indeed, a recent study has shown that whole ovine ovary cryopreservation and perfusion procedures can lead to alterations in the expression levels of numerous endothelial cell-related genes, including those associated with vascular tone regulatory pathways (e.g endothelial nitric oxide synthase), endothelin (*ET*)-1 and *ET-2* as well as alterations to expression levels of genes which lead to increased levels of apoptosis, e.g. up-regulation of *Bax* (pro-apoptotic) and down-regulation of *Bcl2* (anti-apoptotic) (Onions et al., 2013). However, ovarian function and full vascular patency has been reported in ewes 6 years after the orthotopic transplantation of whole frozen-thawed ovaries, using slow-freeze protocols (Arav et al., 2010). In this study the ovary was perfused with 1.3M DMSO for only 3 minutes. This may explain the long-term restoration of fertility, although it should be noted that a novel multithermal gradient freezing methodology was utilised in this study, unlike in the aforementioned examples. In conclusion it is necessary to minimise the length of time spent perfusing the ovary with CPA in order to reduce the level of damage to the vasculature during preservation. One of the aims of the current experimental series was therefore to use follicle culture as a tool to determine the minimum perfusion period required to fully permeate the ovary with CPA prior to WOCP by slow freezing whilst retaining maximal follicle and oocyte viability. Nuclear magnetic resonance (NMR) spectroscopy was used to directly quantify CPA permeation. The basic principles of NMR spectroscopy are covered in Section 6.1.2.

6.1.2 Basic principles of NMR spectroscopy

NMR spectroscopy enables the determination of the composition of a sample via the measurement of energy absorption and emission of nuclei within the sample when a magnetic field is applied externally. The information regarding NMR spectroscopy provided in this section has been adapted from Pavia et al. (2001) and Lambert and Mazzola (2004). Some atomic nuclei process a property called 'spin' and can occupy a specific number of spin states. In the absence of a magnetic field all the spin states of a given nucleus are of equal energy with an equal number of atoms occupying each of the spin states. Charged nuclei generate a magnetic field when spinning and possess a magnetic moment. When an external magnetic field (B_0) is applied, in the direction of the z axis, the magnetic moments of the nuclei will precess in either the same or opposite direction as B_0, +z or –z, respectively. The nuclei opposed to B_0 (-z) will be in a higher energy state and the nuclei aligned with B_0 (+z) in a low energy state. The greater the external magnetic field the greater the energy difference between the spin states. Nuclei have a tendency towards the +z precession in the presence of B_0 therefore more nuclei will be in a lower energy state. The processional motion of the

magnetic moment around B_0 occurs with angular frequency, known as Larmor frequency. Note that Larmor frequency is directly proportional to B_0. If a second magnetic field (B_1) is applied at a radio frequency equal to that of the Larmor frequency of the nuclei this results in a spin state change. Nuclei aligned with B_0 absorb energy and change to the opposing spin state, whereas nuclei opposed to B_0 emit energy and change to the aligned spin state. As there is an excess of nuclei in the spin state aligned with B_0 there is a net absorbance of energy, known as resonance, which can be detected electronically. Nuclei in different environments will absorb radio waves at different frequencies; this allows the determination of the structure of a compound.

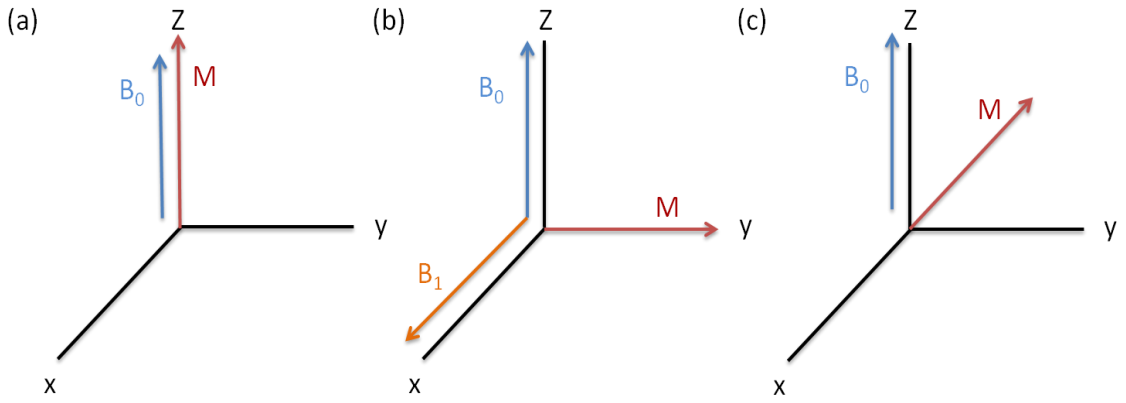


Figure 6.1 Diagrammatic representation of the effect of the application of B_0 and B_1 on the net magnetisation (M) during NMR spectroscopy. Application of B_0 results in net M in the direction of the z axis (a). A 90^o B_1 pulse results in alignment of M with the y axis (b). Over time (M) in the direction of the y axis decreases and subsequently increases in the direction of the x axis as a result of spin-lattice and spin-spin relaxation (c). Diagram adapted from Lambert and Mazzola (2004).

The excess nuclei in the lower energy spin state are excited to the higher energy spin state via the application of pulses of B_1. In an NMR experiment when B_0 is applied there is a net magnetisation (M) in the direction of the z axis, due to the excess of nuclei in the +z spin state (see Figure 6.1). As the spins are randomly distributed about the z axis there is no net magnetisation in the x or y direction. However, when B_1 is applied along the x axis, at a radio frequency equal to the Larmor frequency of the nuclei, some of the excess nuclei are excited to the higher spin state thus a force is exerted on M resulting in it tipping towards the y axis. This can be detected electronically. During a pulsed experiment B_1 is pulsed for a sufficient length of time to align M with the y axis (a 90^o pulse), at which point M can be detected. Magnetisation decays over time via 'spin-lattice' and 'spin-spin' relaxation, resulting in a decrease in y magnetisation with time, referred to as free induction decay (FID). Spin lattice

relaxation is the reappearance of M along the z axis and is characterised by time constant T_1. Spin-spin relaxation decay is the of magnetisation along the xy axis and is characterised by time constant T_2. In order to analyse the FID signal it must be Fourier transformed. Fourier transformation is the numerical conversion of data from the time domain to the frequency domain (see Figure 6.2).

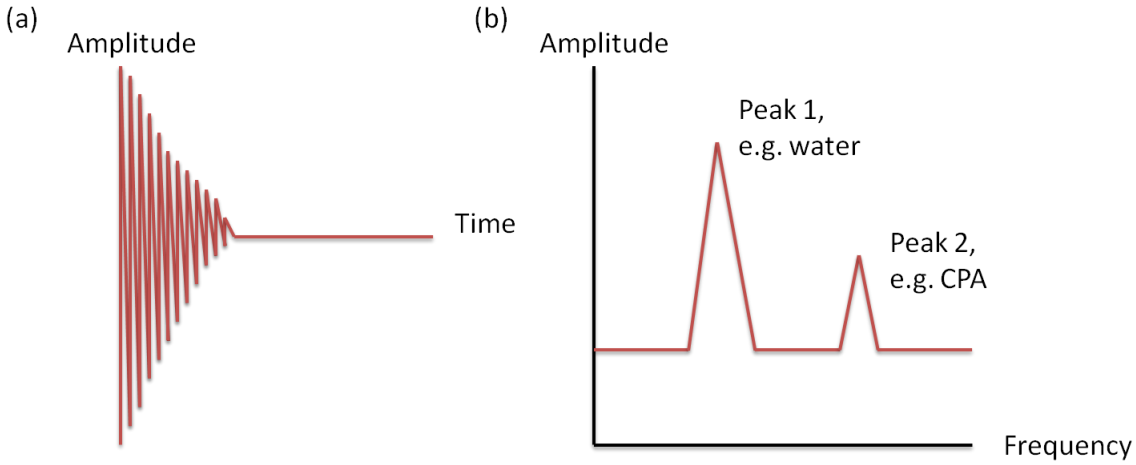


Figure 6.2 Diagrammatic representation of the (a) FID signal collected following an NMR run and (b) data following Fourier transformation. Adapted from Newton (1998).

Following transformation of data in this experimental series 2 peaks should be observed for each sample; the water and the CPA; DMSO. Known concentrations of DMSO were used to calibrate the data to enable quantification of the level of DMSO present. This enabled the determination of the level of CPA permeation following perfusion times of 10, 30 and 60 minutes.

6.1.3 Assessment of the effect of CPA perfusion time on ovarian tissue and follicle survival and developmental potential

As discussed in Chapter 1, the exposure of ovarian tissue, oocytes and follicles to CPAs can be detrimental to their health, as CPAs have cytotoxic effects (Meryman, 1971). There are also risks that if CPAs are not used at the optimal concentration and/or applied/ removed at the correct rate their use can cause cell shrinkage prior to cryopreservation and the swelling of cells once they are thawed and returned to hypotonic solution, possibly leading to cell lysis (Picton et al., 2000). The use of DMSO as a CPA during the cryopreservation of mature murine oocytes has led to zona hardening and disruption of the plasma membrane and cytoskeleton (Trounson and Kirby, 1989). Additionally, histological evaluation of ovine ovarian tissue

revealed that exposure to 1.5M DMSO resulted in significantly fewer normal preantral follicles compared to control tissue, which had not been exposed to any CPA (Santos et al., 2006). Furthermore the percentage of follicles in which oocyte damage was observed doubled, and the percentage with degenerating GCs and oocytes tripled in DMSO-exposed tissues compared to control tissues, as a result of osmotic stress and the chemical toxicity of DMSO. Therefore, in the current study in addition to direct quantification of CPA penetration by NMR spectroscopy, the effects of varying lengths of DMSO exposure on follicle and oocyte health will also be assessed histologically and compared to control tissue perfused with either L-15 medium for an equivalent length of time and to control tissue that has not been perfused with either 1.5 M DMSO or L-15 medium with 10% FCS. Follicle survival and growth in DMSO perfused and control tissues will be assessed using the cortex culture methodologies detailed in Chapters 3 and 4. During *in vitro* culture the level of release of the enzyme- lactate dehydrogenase (LDH), into spent culture media. The enzyme LDH is present in the cell cytoplasm and it will be released into the media upon damage to the cell membrane (Decker and Lohmann-Matthes, 1988, Koukourakis et al., 2003). In this experimental series the level of LDH in the spent culture media will therefore be used as an indicator of the level of ovarian cell membrane damage caused by exposure to cytotoxic DMSO for varying lengths of time. Details of the principles of the assay used to quantify LDH concentrations in spent culture media are provided in Section 6.2.7.

6.1.4 Aims

The first aim of this experimental series was to determine the minimum perfusion time needed for 1.5M DMSO to fully permeate the sheep ovarian cortex and the vascular pedicle in whole ovaries using NMR spectroscopy. The second aim of this experimental series was to assess the effects of tissue perfusion on the level of tissue damage and follicle and cortex health using the LDH assay of cell membrane damage in conjunction with NR staining as acute assays of tissue damage/ health, and 8 day cortex culture and histological analysis to assess the longer term impact of CPA cytotoxicity on follicle and oocyte health and developmental competence.

6.2 Materials and Methods

6.2.1 Preparation of equipment

To reduce the risk of contamination all white paper towel referred to in this method was soaked in 70% alcohol and left to dry prior to use. All re-usable equipment used to perform cannulations, dissections and perfusions was sterilised by soaking in 70% alcohol prior to the tissue arriving in the lab. This includes all catheters, metal guides and three-way taps. Following soaking equipment was flushed through; using a syringe, with 70% alcohol followed by and excess volume of sterile culture water (W3500) to remove any residual alcohol.

6.2.2 Tissue preparation

Ovarian tracts were transported to the laboratory at room temperature where they were examined on clean white paper towel to enable full inspection. Tracts with both ovaries intact and with sufficiently large vasculatures, to enable the cannulation to be performed, were selected. Once selected the uterus and fat were trimmed from the tracts using scissors, with care being taken to not cut off the ovarian pedicle or any of the ovarian or uterine vasculature. The tracts were then washed in pre-warmed (37°C) OWM (see Section 2.2) to remove any dirt and transferred into a sterile beaker containing 100iuml^{-1} (v/v) heparinised Ringer's solution (see Appendix IV). A summary of the protocol used in the current study is provided in Figure 6.3.

6.2.3 Ovarian artery cannulation

Cannulations were performed under laminar flow conditions using a dissecting microscope (Olympus). Individual reproductive tracts were placed on white paper towel to aid visualisation and to help hold the tissue in place. Throughout the cannulation procedure the ovary and artery were kept hydrated using fresh heparinised Ringer's solution. Once the ovarian artery had been identified, a Mersilk 45mm suture needle and prolene/ polypropylene thread (Ethicon, Somerville, US) were used to tie off any arterial branches which led to the uterus and/or oviduct. Using forceps blunt dissections were carried out to remove connective tissue above and surrounding the artery, with care being taken not to cut through the artery. The ovarian vein and artery were then separated, thus creating an exposed loop through which the handle of a scalpel could then be inserted to aid further manipulations by creating tension.

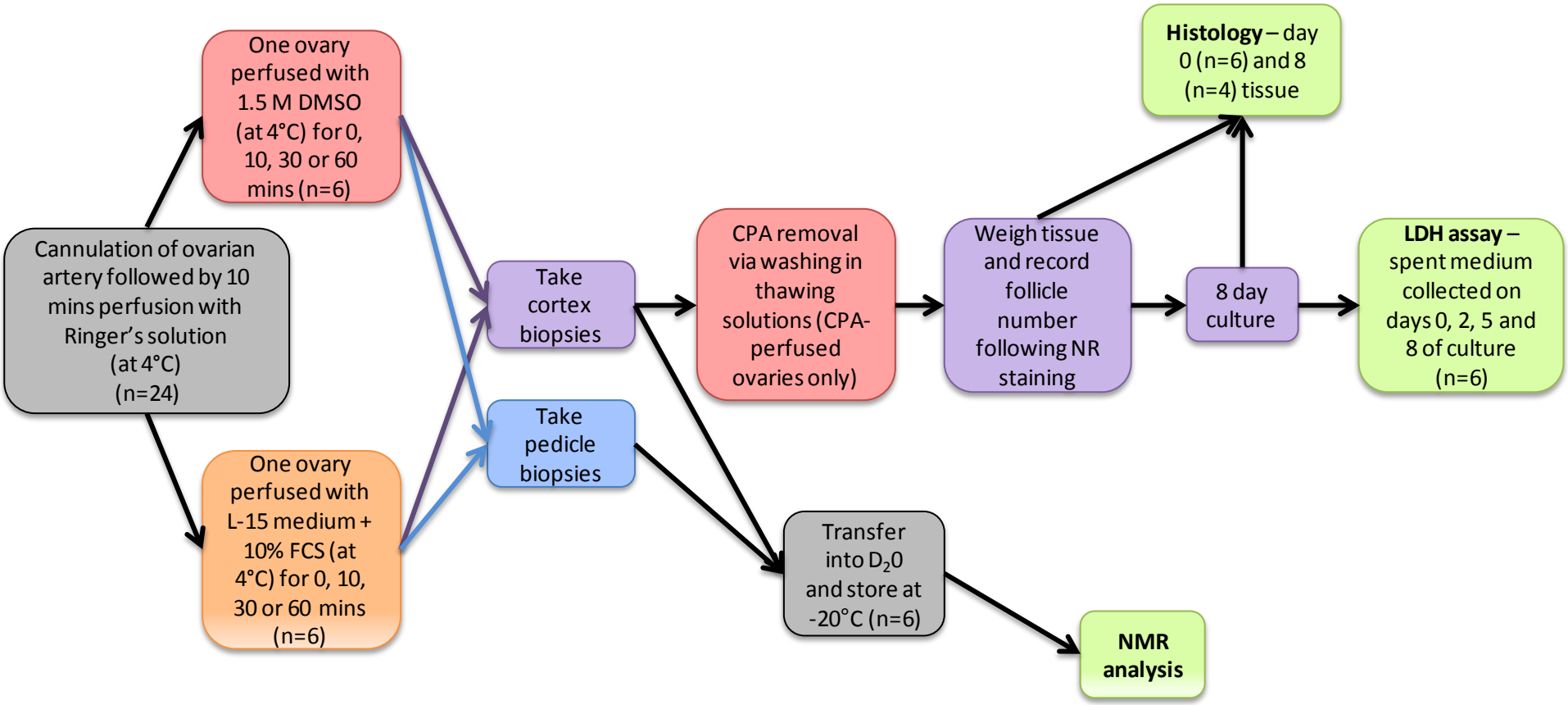


Figure 6.3 Summary of the protocol used in the current study with the number of repeats (n) shown at each stage.

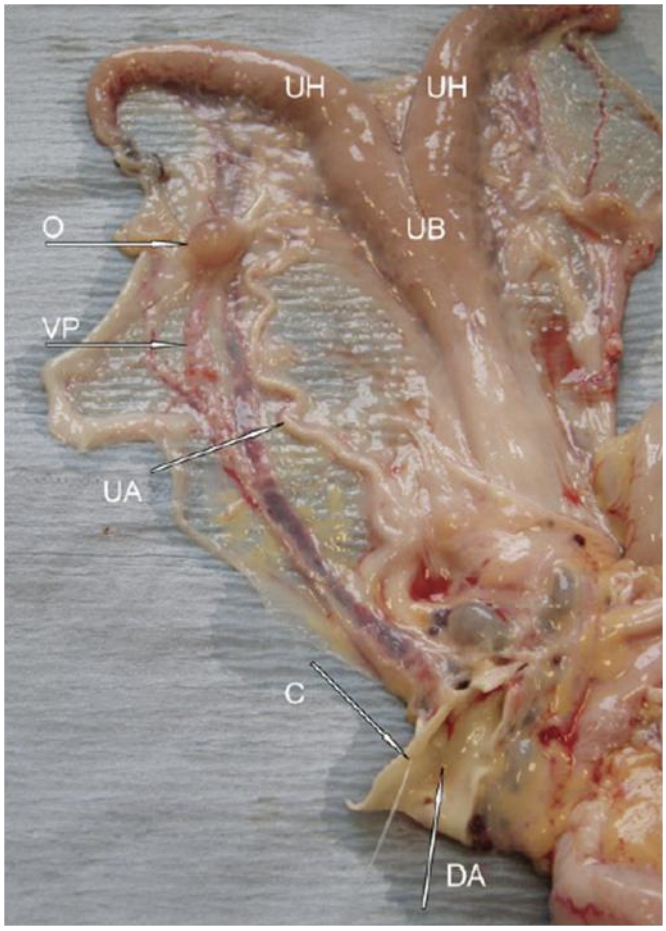


Figure 6.4 Image of an ovine reproductive tract with a cannulated ovarian artery. Image is taken from (Onions et al., 2008). The cannulation (C) of the dorsal aorta (DA) of the ovarian artery is shown with the ovary (O), vascular pedicle (VP), and uterine horns (UH), body (UB) and artery (UA) also labelled.

Using iris scissors a small V-shaped incision of around 10-15 mm was made in the arterial wall. Through this incision a vascular catheter with metal guide (Leaderflex 22G (0.7 mm x 200 mm; Vygon Ltd, Wiltshire, UK) was inserted a distance of approximately 1cm into the artery. Once in place the metal guide was pulled out of the cannula slightly. Two prolene/ polypropylene sutures (Ethicon) were tied around the cannula and the ovarian artery, just above the entry point, in order to secure it. In order to ensure that the cannula had been inserted in the correct direction- which is necessary as the artery is very convoluted, and that there were no leaks through arterial branches that had not been tied off, 2-5mls of Ringer's solution was flushed through the cannula and vasculature using a sterile syringe via a 3-way tap. Once the cannulation was complete the ovary and vasculature were dissected from the uterus and placed into a sterile Nunc 90mm Petri dish on ice (4°C). This procedure was then repeated for the second ovary of the tract.

6.2.4 Ovary perfusions

All perfusions were carried out on ice at 4°C. Two syringe-driven perfusion pumps (Precidor) were used in this investigation. The pumps were supplied by ChemLab Scientific Products (Essex, UK). The pumps were set up as shown in Figure 6.5. Note that although 50ml syringes were initially used these were prone to sticking. It was also found that if the syringes were kept in the fridge for too long before use the rubber seal would lose some of its elasticity and the syringes again would be prone to becoming stuck. After this was discovered 20ml syringes were set-up just prior to use to eliminate this problem. In order to ensure that the pump was working correctly such that the cannulae were not occluded and that media was being pumped through the ovary from the very start of the perfusion time, each time a new syringe was attached to the pump it was tested immediately prior to perfusion using a spare catheter.

All perfusions were carried out on ice at 4°C and all solutions used were kept at 4°C until use to minimise the cytotoxic impact of DMSO. The ovaries were first perfused with Ringer's solution (see Appendix IV) for 10 minutes, at a rate of $1 ml min^{-1}$. During the perfusion the Petri dishes were tilted slightly with the ovary positioned in the lower half of the dish in the used media to help keep it hydrated. Following perfusion with Ringer's solution one ovary from each tract was then perfused with CPA solution containing 1.5M DMSO at a rate of $0.5 ml min^{-1}$ (see Appendix IV) and the other, control ovary was perfused with Leibovitz L-15 medium supplemented with 10% heat-inactivated Fetal Calf Serum (FCS), from here on in referred to as L-15 medium, at a rate of $0.5 ml min^{-1}$. Perfusions were run for 0, 10, 30 and 60 minutes. Once the perfusion was complete the ovary was transferred to the flow hood, the cannulae removed and the vasculature trimmed away. The ovaries were then transferred to sterile 5ml universals before processing. Two punch biopsies were taken from the vasculature, using a 4mm biopsy punch (Glaxosmithkline, Middlesex, UK) and stored individually in 400µl D_20 in 0.5ml eppendorf tubes, sealed immediately using parafilm, at -20°C for later NMR assay analysis (see Section 6.2.8).

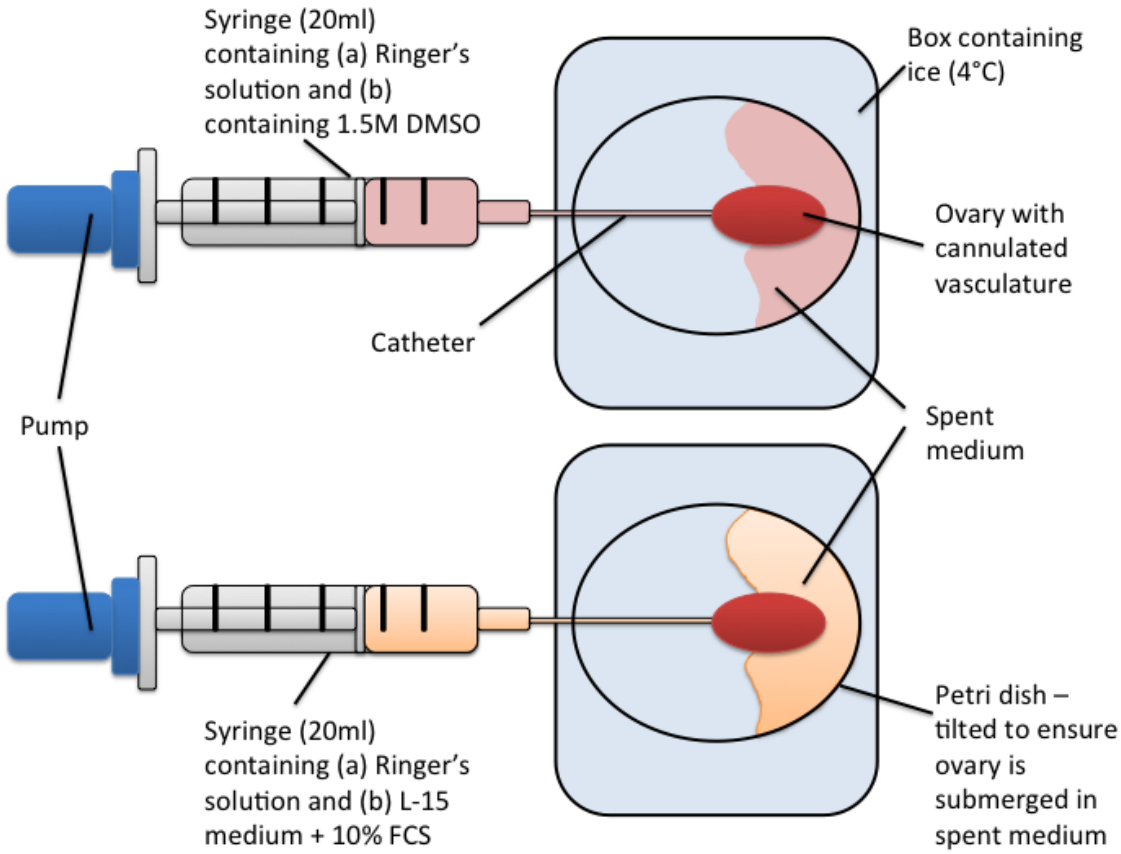


Figure 6.5 Diagram showing how the pumps were set-up for the ovary perfusion. Pairs of ovaries from the same reproductive tract were simultaneously first perfused with (a) Ringer's solution for 10 minutes, then with (b) either 1.5M DMSO (pink) or L-15 medium + 10% FCS (orange) for 0, 10, 30 or 60 minutes.

6.2.5 Processing of ovaries and culture setup

Ovaries were processed on ice at 4°C, under laminar flow conditions. The ovaries perfused with CPA solution were processed before those perfused with L-15 medium. The ovaries were dried using sterile tissue before processing. In both series of experiments a skin graft knife was used to slice thin sections from the outer cortex of the ovary. Punch biopsies of 4mm diameter were then taken from the harvested cortex. The number of biopsies possible to obtain per ovary varied with ovary size - between 7 and 10 biopsies. The maximum number of biopsies possible was collected. Two biopsies obtained from each ovary were immediately transferred to a labelled 0.5ml eppendorf tube containing 400µl of D_20, which were sealed immediately using parafilm and stored at -20°C for later NMR assay analysis (see Section 6.2.8). The remaining biopsies taken from the CPA perfused ovary were washed in 5ml solutions of progressively decreasing DMSO concentration (1.0M, 0.5M and 0M DMSO plus 0.1M sucrose and 10% FCS in L-15 medium) in a 5ml universal. This was necessary as if the discs were placed directly into culture media this would have caused the cells to swell and

potentially burst due to the changes in osmolality. The discs were placed in each solution and shaken using a mini-orbital shaker (Stuart; VWR) set at 80 revolutions per minute (rpm) for 10minutes each, on ice. All biopsies from the same ovary were incubated in 500µl of 50µgml^{-1} NR dye for 2½ hours, the solution had been placed into the incubator at 37°C and 5% CO_2 to pre-warm and gas for 3 hours prior to use, to enable viable follicle quantification (see Section 2.5-2.6). Note that phenol red-free basal culture media was used in the current series of experiments, with the same additives as detailed in Table 2.1 as the LDH assay has previously been validated for use with sheep cortex and phenol red present in culture media has previously been shown to interfere with the LDH assay (Gunson, 2009). Of the most follicle rich biopsies tissue weight was recorded (see Section 2.6) and 4 biopsies were put into culture (2 biopsies per well), 1 biopsy – was retained as an uncultured control and was transferred to 4% (w/v) PFA (see Appendix II) for histological analysis (see Section 6.2.9).

6.2.6 Ovarian cortical culture

Ovarian cortical culture was set up as described in Section 2.7. The only modification in the present experiments was that the phenol red-free α-MEM basal culture media (Invitrogen) was used in the cortical culture media. Tissue was cultured for 8 days, with media changes being carried out on days 2 and 5 and the spent media (250µl) was stored in eppendorfs at 4°C for up to 4 weeks prior to analysis using the LDH assay (see Section 6.2.7). A 250µl sample of tissue free media was also taken on day 0 and at the end of culture for use as controls in the LDH assay.

At the end of culture the tissue weight was recorded (see Section 2.6). A note of the general appearance of the tissue was also made. The tissue was then transferred to NR solution (see Section 2.5) for an hour at 37°C to enable the assessment of the level of NR staining and the number of viable follicles. Note that at the end of culture it was often not possible to count the follicles due to the more intense level of NR staining that was achieved as the tissue had not initially been kept at 4°C, as at the start of culture. The tissue had also often thickened due to cell proliferation and/or balled up after culture which further hindered follicle counting. The cultured biopsies were transferred to 4% (w/v) PFA (see Appendix II) for future histological analysis following NR staining (see Section 6.2.9).

6.2.7 Lactate dehydrogenase assay

The level of the enzyme LDH in the spent culture media can be used as a measure of cell damage as LDH present in the cell cytoplasm will be released into the media following damage to the cell membrane (Decker and Lohmann-Matthes, 1988, Koukourakis et al., 2003). The amount of LDH was measured using an assay kit (Cytotoxicity Detection Kit, Roche Applied Sciences) on an MRX plate reader (Dynex Laboratories Chantilly, Virginia, USA). The level of LDH in the culture media was determined using a coupled enzymatic reaction (see Figure 6.6), in which LDH catalyses the conversion of lactate to pyruvate resulting in the reduction of NAD+ (nicotinamide adenine dinucleotide) to NADH/H+. The catalyst (diaphorase) present in the kit then transfers the H/H+ from the NADH/H+ to the tetrazolium salt thus forming formazan, resulting in a colour change from pale yellow to red. The level of LDH activity directly correlates to the level of formazan formed. Formazan absorbs light at a wavelength of around 500nm. Therefore the absorption of the sample at a wavelength of 490nm was measured using a MRX plate reader (Dynex Laboratories, Virginia, USA) to determine the level of LDH in the sample. A reference wavelength of 630nm was used to determine and subtract background absorbance.

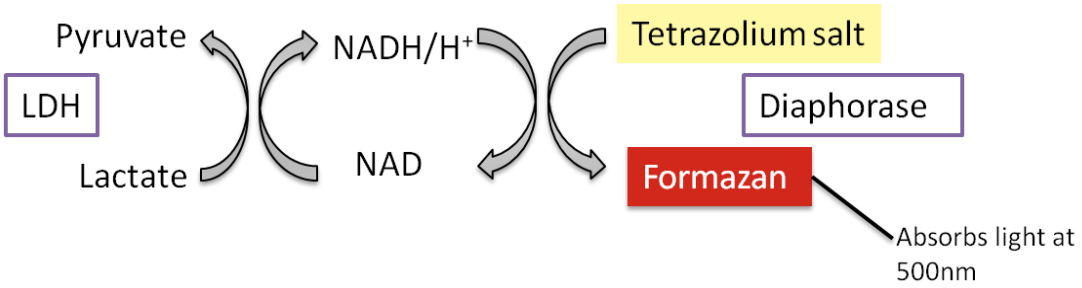


Figure 6.6 Summary of the coupled enzyme reaction used in the LDH assay to determine the level of lactate in the media. Enzymes are highlighted in purple boxes.

Quality controls (QCs) and standards were prepared as detailed in Appendix IV. Prior to use 1ml of distilled water was added to the catalyst then stored for up to 1 month at 4°C. Immediately prior to the assay 11.25ml of the dye solution (iodotetrazolium chloride and sodium lactate) was added to 250µl of catalyst to produce the reaction mix. Standards of LDH, of concentrations: 0.625Uµl^{-1}, 1.25Uµl^{-1}, 2.5Uµl^{-1}, 5Uµl^{-1}, 10Uµl^{-1}, 20Uµl^{-1} and 40Uµl^{-1}, and LDH QCs 1-4 of concentration: 1Uµl^{-1}, 2Uµl^{-1}, 4Uµl^{-1} and 8Uµl^{-1}, respectively, and blanks were measured in triplicate. Test samples were assayed in duplicate in optically clear 96 well microtitre plates (Greiner Bio-one, Frickenhausen, Germany). Firstly, 100µl of standard/ sample was added to each well, then using an eight channel, multichannel pipette (Invitrogen)

150µl of reaction mix was added per well. The plate was then placed in a foil covered box to shield it from light and left for 30 minutes before being transferred to the MRX plate reader. The assay management software; Biolinx 2.21 (Dynex Laboratories) was used to analyse the data. In total 22 assays were conducted and the intra and inter assay coefficient of variance (CV) were calculated for QCs 1-4 using the formula shown in Equation 6.1, values are shown in Table 6.1.

Equation 6.1

$$\% \text{ CV} = \text{standard deviation for QC} \times 100 / \text{mean for QC}$$

Table 6.1 Inter and intra assay coefficients of variation for 22 assays.

QC	QC1	QC2	QC3	QC4
Inter assay % CV	12.5	13.2	10.4	11.1
Intra assay % CV	14.7	13.7	11.0	11.5

6.2.8 Nuclear magnetic resonance assay

As described in Sections 6.2.4 and 6.2.5 punch biopsies were taken from the pedicle and ovary cortex immediately following perfusion and stored in deuterium oxide (D_2O) for NMR analysis. Samples were removed from the freezer the night before analysis and left to defrost at room temperature on a mini-orbital shaker (Stuart) set at 100-120rpm to allow equilibration of the water and CPA (if present) between the tissue and D_2O. Following equilibration 300µl of the sample was transferred to a fresh eppendorf and 300µl of D_2O added. Eppendorfs were inverted to mix, then 350µl of the diluted sample was transferred to a 5mm diameter glass NMR tube (Wilmad Glass Company, New Jersey, USA).

NMR analyses were conducted in the laboratories of Dr. Julie Fisher, School of Chemistry, University of Leeds. Assays were carried out as previously described and validated for sheep cortex by Newton et al. (1998). All the spectra were recorded on a Varian Unity Inova 500 (1H frequency 500MHz). Prior to running the time course experiments, the T1's (longitudinal relaxation times) were measured using an inversion recovery experiment. Spectra were then acquired over 64 transients with spectral width of 3000Hz in 16,384 pairs of data points, resulting in an acquisition time of 5.5 seconds. The relaxation delay between transients was 10 seconds and a 20 degree pulse width (2.5µs duration) was employed. The time period for each transient was approximately 3 times the longest T1 measured. The data were recorded at

20°C, and were processed remotely using the NMR package ACD (ACD Labs, Ontario, Canada). Prior to Fourier transformation the FIDs were multiplied by an exponential line-broadening factor of 1Hz. The area under each peak was measured using the standard procedures within ACD. Calibration data were obtained by recording the spectra of 1.5M DMSO to give a cryoprotectant to water ratio of 0.0339. A tube containing only D_20 was also analysed to obtain a value for the background water signal, which was subtracted from all the water peaks. The uptake of CPA was calculated by dividing the test values by the calibration value. The detection threshold of the assay was a cryoprotectant to water ratio of 0.00001.

6.2.9 Histological analysis

All histological analyses were performed with the assistance of Mrs Ping Jin, as described in Sections 2.9-2.11. The effect of varying the length of perfusion on the integrity of stromal tissue and follicles was assessed histologically, as described in Section 2.11. Representative images of good, adequate and poor quality stroma are shown in Figure 6.7.

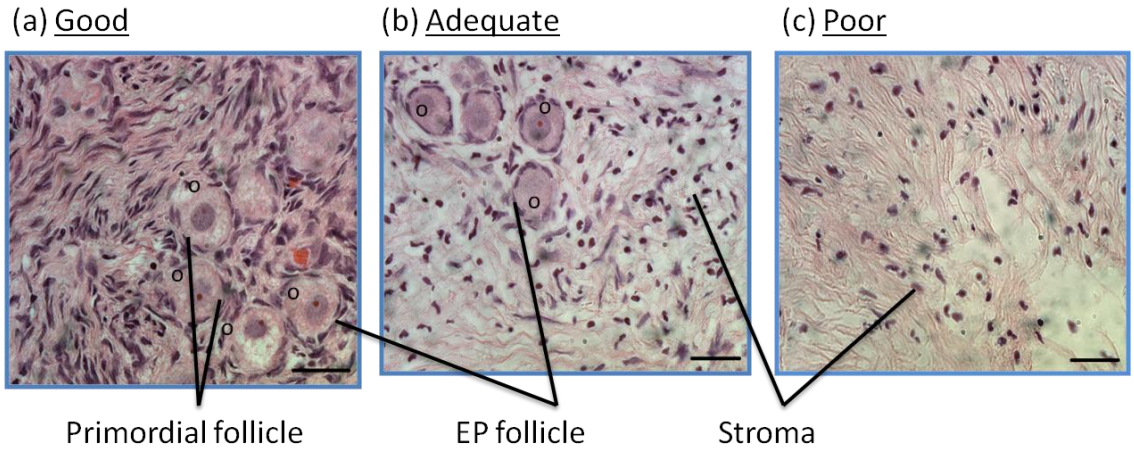


Figure 6.7 Representative images of (a) good, (b) adequate and (c) poor quality stromal tissue. All follicles are at the primordial or EP stage of development. During histological analysis only follicles with a visible nucleolus were counted – the oocyte of these follicles is labelled 'O'. Tissue samples are taken from (a) non-perfused ovary on day 0, (b) an ovary perfused with CPA for 30 minutes after 8 days of culture and (c) an ovary perfused with L-15 medium for 60 minutes after 8 days of culture. Scale bar = 20µm.

6.2.10 Statistical analysis

The distribution of data was analysed for normality using the Anderson Darling test. Data was then analysed across all treatments using one-way ANOVA and between equivalent tissue

samples at the start (day 0) and end (day 8) of culture using the Student's t-test. The LDH assay data presented in Figures 6.9 and 6.1 and the mean number of EP and primary follicles per mg tissue presented in Figure 6.14 was not normally distributed therefore was log-transformed to achieve normality.

6.3 Results

6.3.1 Nuclear magnetic resonance data

In this experimental series ovaries were perfused with CPA or L-15 medium to determine the minimum length of time required for the CPA to fully permeate the different tissue types in the ovary and pedicle whilst maintaining cell viability. The relative level of CPA permeation in tissue perfused with Ringer's solution for 10 minutes followed by either CPA or L-15 medium for 0, 10, 30 or 60 minutes is shown in Figure 6.8. In Figure 6.8 data is separated into pedicle and cortex samples obtained immediately after perfusion, within these categories data is subdivided into non-perfused tissue (i.e. tissue perfused with only Ringer's solution), tissue perfused with L-15 medium for 10, 30 and 60 minutes and tissue perfused with CPA for 10, 30 and 60 minutes. In the pedicle tissue samples there is a clear and significant increase ($p<0.01$) in the level of CPA permeation after 10 minutes. Increasing the perfusion time to 60 minutes did not significantly increase the level of CPA permeation suggesting that the tissue was saturated with CPA after the shortest, 10 minute time period. In contrast in the cortical tissue, a perfusion length of 60 minutes was required to significantly increase ($p<0.01$) the level of CPA permeation over all other CPA infused time points, as well as the time 0 controls and the L-15 treated tissues. After 60 minutes of perfusion the level of CPA permeation in the cortical tissue was not significantly different ($p>0.05$) from that observed in the pedicle tissue suggesting that 60 minutes perfusion was sufficient to allow the CPA to fully saturate the ovary.

Low levels of CPA permeation were observed in some of the cortical tissue samples, which were perfused with L-15 medium only indicating that there must have been some cross-contamination with CPA during tissue processing. However, as indicated in Figure 6.7, it is important to note that the levels of CPA penetration recorded at each perfusion time point were vary variable between samples and none of the levels recorded were significantly different ($p>0.05$) to the non-perfused, time 0 controls. The explanation for this observation is unclear as no contamination was detected in the equivalent pedicle samples after L-15

treatment for any length of perfusion. This suggests that limited cortex contamination with CPA must have occurred when the ovaries were processed immediately following the termination of the perfusion. The CPA contamination could have occurred if the blades used to slice cortical tissue from the ovary were not changed between the CPA- and L-15 medium-perfused tissue as it is possible that some residual CPA may have been transferred between the 2 ovaries as the CPA- perfused ovaries were always processed first. Therefore levels of CPA found in the CPA-perfused cortical tissue would not have been affected as fresh blades were always used for the preparation of each pair of ovaries. Alternatively, residual CPA exposure may have been incurred in the tissue processing dish. The latter explanation is unlikely as although the same Petri dishes were used to process all of the ovaries recovered on the same day, the dishes were always wiped dry with sterile tissue paper between samples so CPA carry over is unlikely to have been sufficient to contaminate the samples.

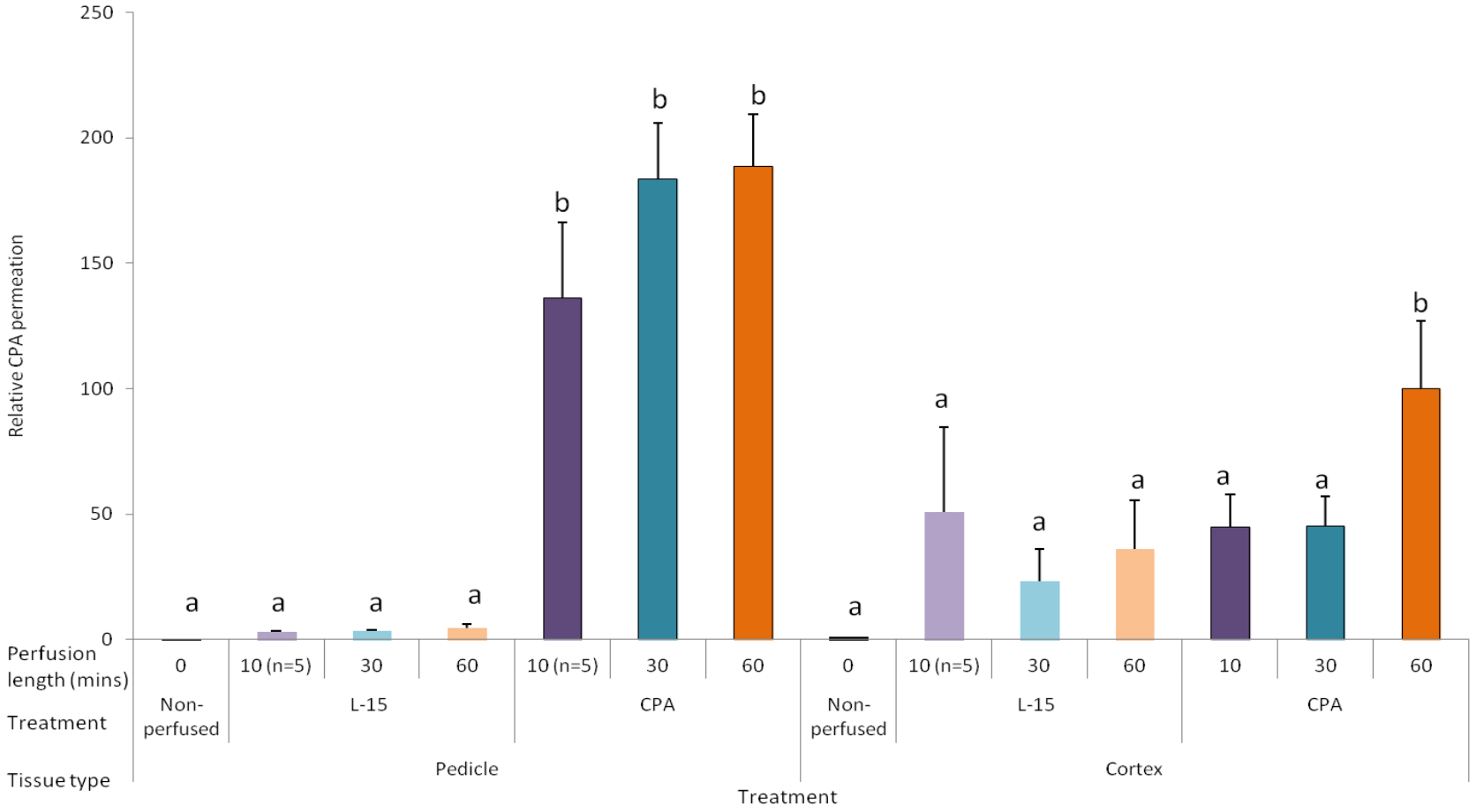


Figure 6.8 Histogram showing the relative level of CPA permeation in tissue perfused with Ringer's solution for 10 minutes followed by either CPA or L-15 medium for 0 (non-perfused control), 10, 30 or 60 minutes. Data shown are mean relative CPA permeation ± SEM for 6 replicate samples, unless otherwise indicated on the graph (n). Statistically significant differences are indicated by different letters across all treatments and tissue types ($p<0.01$).

6.3.2 Lactate dehydrogenase assay data

The concentration of LDH in spent culture media per g of tissue at the start of culture, for media collected on days 2 and 5 and the end of culture for media collected on day 8 , was used as an indicator of the level of damage to the ovarian tissue and follicles following perfusion. Figures 6.9 and 6.1 show that in all treatment groups, independent of perfusion length, the concentration of LDH in spent culture media increased with increasing time in culture, as in all tissue groups the concentration of LDH was significantly higher ($p<0.001$) in spent media collected on day 5 than day 2 and that collected on day 8 compared to days 2 and 5, with the exception of the tissue perfused with CPA for 30 minutes in which there was no statistical difference ($p>0.05$) in the concentration of LDH in media collected on days 5 and 8, although there was a significantly higher ($p<0.05$) concentration than in media collected on day 2. There were no significant differences in the concentration of LDH in spent media, between different treatment groups on days 2 and 5 of culture. However, the concentration of LDH in spent culture media of non-perfused tissue was significantly higher ($p<0.05$) than that of tissue perfused with either CPA or L-15 for 10 or 30 minutes, by day 8 of culture. This high concentration of LDH in the spent culture media of non-perfused tissue after 8 days of culture was unexpected and after this time frame is likely due to subtle differences/stressors in the culture environment rather than differences in tissue treatment at the start of culture as no significant differences were observed between treatments after 2 and 5 days *in vitro*. Furthermore, the LDH concentration in the spent culture media collected on day 8 of culture was not significantly different ($p>0.05$) in tissue perfused for 10 or 30 minutes. However, although the LDH concentration in spent media of tissue perfused for 10 minutes was not significantly different ($p>0.05$) from that in the spent media of tissue perfused for 60 minutes, the LDH concentration in spent media of tissue perfused for 30 minutes was significantly lower ($p<0.05$). It is interesting to note that the patterns of LDH concentration increase with increasing length of culture observed in the CPA perfused ovaries are very similar to those observed in the L-15 perfused ovaries within each time category. As counterpart CPA and L-15 ovaries were taken from the same tract this suggests that the source of the ovary impacts on LDH release. This could also explain the relatively high concentration of LDH observed in the non-perfused tissue and why the lowest concentration of LDH was observed in the tissue perfused for 30 minutes. To aid clarity of interpretation, these results are also presented in Figure 6.10 in line graph form, which demonstrates the strong positive relationship ($p>0.05$) between the duration of culture and the concentration of LDH in spent media.

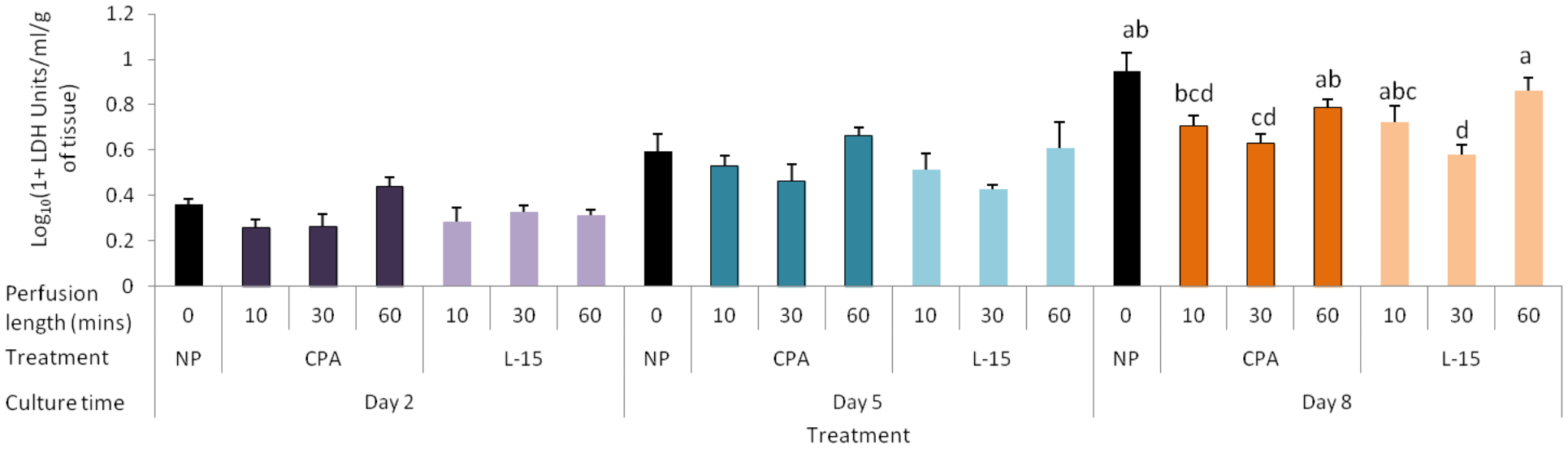


Figure 6.9 Histogram showing the concentration of LDH present in spent culture media/g of tissue on days 2, 5 and 8 of culture following whole ovary perfusion with either CPA or L-15 for 0, 10, 30 and 60 minutes. Data is presented as mean ± SEM for 6 culture repeats. Data was analysed using one-way ANOVA within each time point. Statistical differences are denoted using different letters ($p<0.05$).

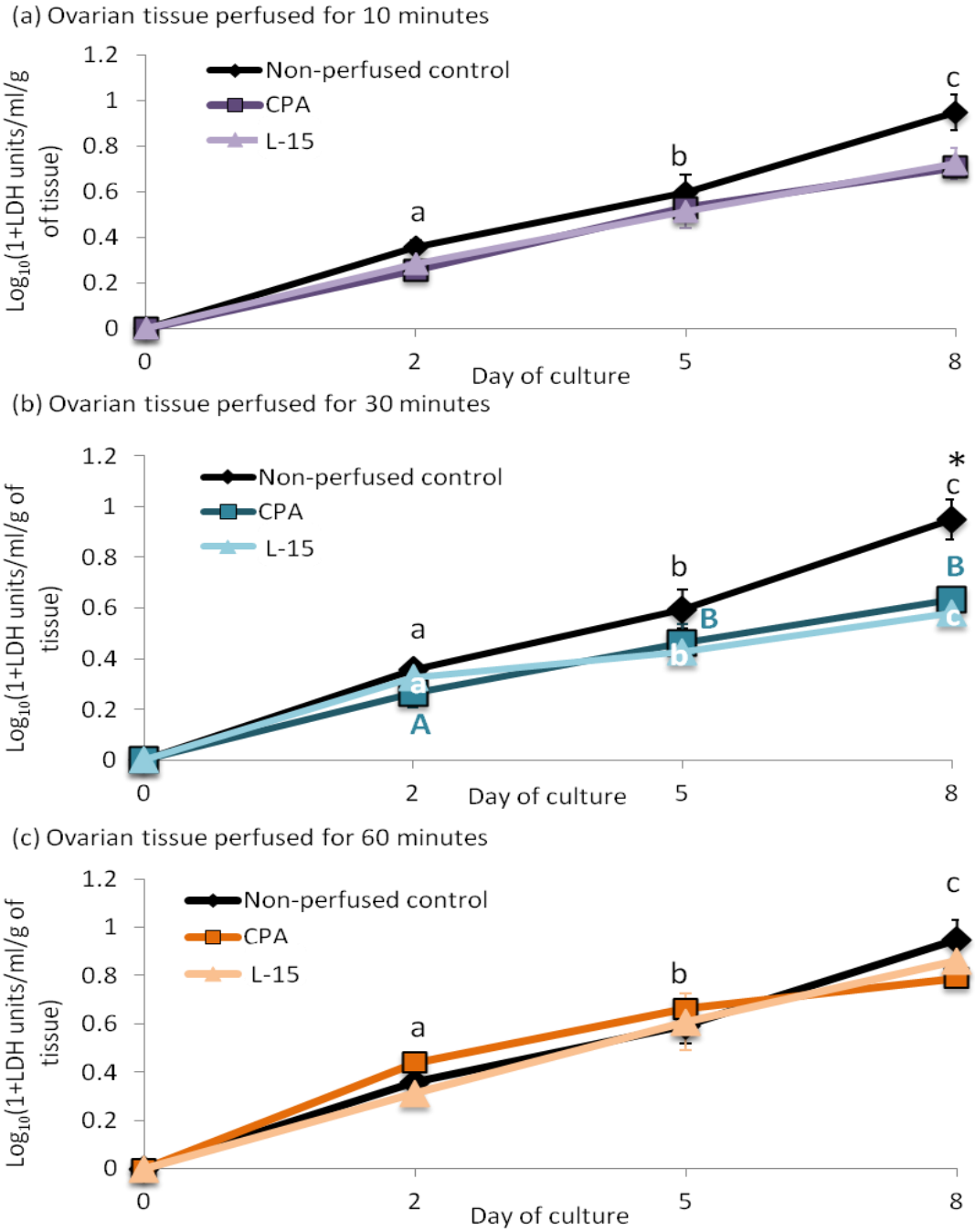


Figure 6.10 Concentration of LDH present in spent culture media of tissue on days 0, 2, 5 and 8 of culture following whole ovary perfusion with either CPA or L-15 for non-perfused control tissue and tissue perfused for (a) 10, (b) 30 and (c) 60 minutes. Data is presented as mean ± SEM for 6 culture repeats. A strong positive correlation ($p<0.05$) between duration of culture and LDH concentration in the spent media was observed in all data sets, analysed using ANCOVA. The data was also analysed using one-way ANOVA. Statistical differences in LDH concentrations between different lengths of culture, within treatment groups are shown using different lower case letters ($p<0.001$) and upper case letters ($p<0.05$). Note that the statistical differences observed are the same in Figures (a) and (c) therefore the black letters are representative of all treatment shown on those graphs, whereas black, teal and white letters were used in Figure (b) to represent non-perfused control, CPA and L-15 perfused data, respectively. Statistical differences within culture lengths and between treatments are denoted using * ($p<0.05$).

6.3.3 Tissue viability following perfusion

Immediately prior to culture tissue biopsies were incubated in NR dye in order to determine the number of viable follicles present (see Figure 6.11). In all treatment groups, with the exception of the 30 minute CPA perfused group, 95-100% of biopsies contained viable follicles. Following 30 minutes perfusion with CPA a lower percentage (86%) of biopsies contained viable follicles, which was due to biopsies obtained from one ovary in which only 37.5% contained viable follicles. Hence, this highlights the importance of the use of NR staining prior to cultures to screen for dead tissue and tissue with depleted follicles counts. Only tissue biopsies containing viable follicles were used for cortical culture. Following 8 days of culture tissue biopsies were incubated in NR dye in order to determine the level of tissue viability (see Figure 6.11). The percentage of the tissue biopsy positively stained with NR was scored and the mean for all biopsies from ovaries treated in the same way was obtained and presented in Table 6.2. The lowest percentage of NR positive staining was observed in the tissue obtained from ovaries perfused for 30 minutes with CPA, however, unlike prior to culture, this was due to low percentage staining observed in a number of samples.

Table 6.2 Mean percentage NR stain of tissue pieces cultured for 8 days following whole ovary perfusion with either CPA or L-15 for 0, 10, 30 and 60 minutes. The SEM is shown for the number of repeat cultures indicated. The data was normally distributed and analysis using one-way ANOVA revealed no statistically significant ($p>0.05$) differences.

Treatment	Perfusion length (minutes)	% NR stain in tissue pieces on day 8 of culture	SEM	Number of repeats
Non-perfused control	0	76.7	6.7	5
CPA	10	78.3	7.3	5
	30	58.3	12.0	6
	60	79.9	6.8	6
L-15	10	77.8	6.3	6
	30	90.3	5.5	6
	60	90.0	3.1	5

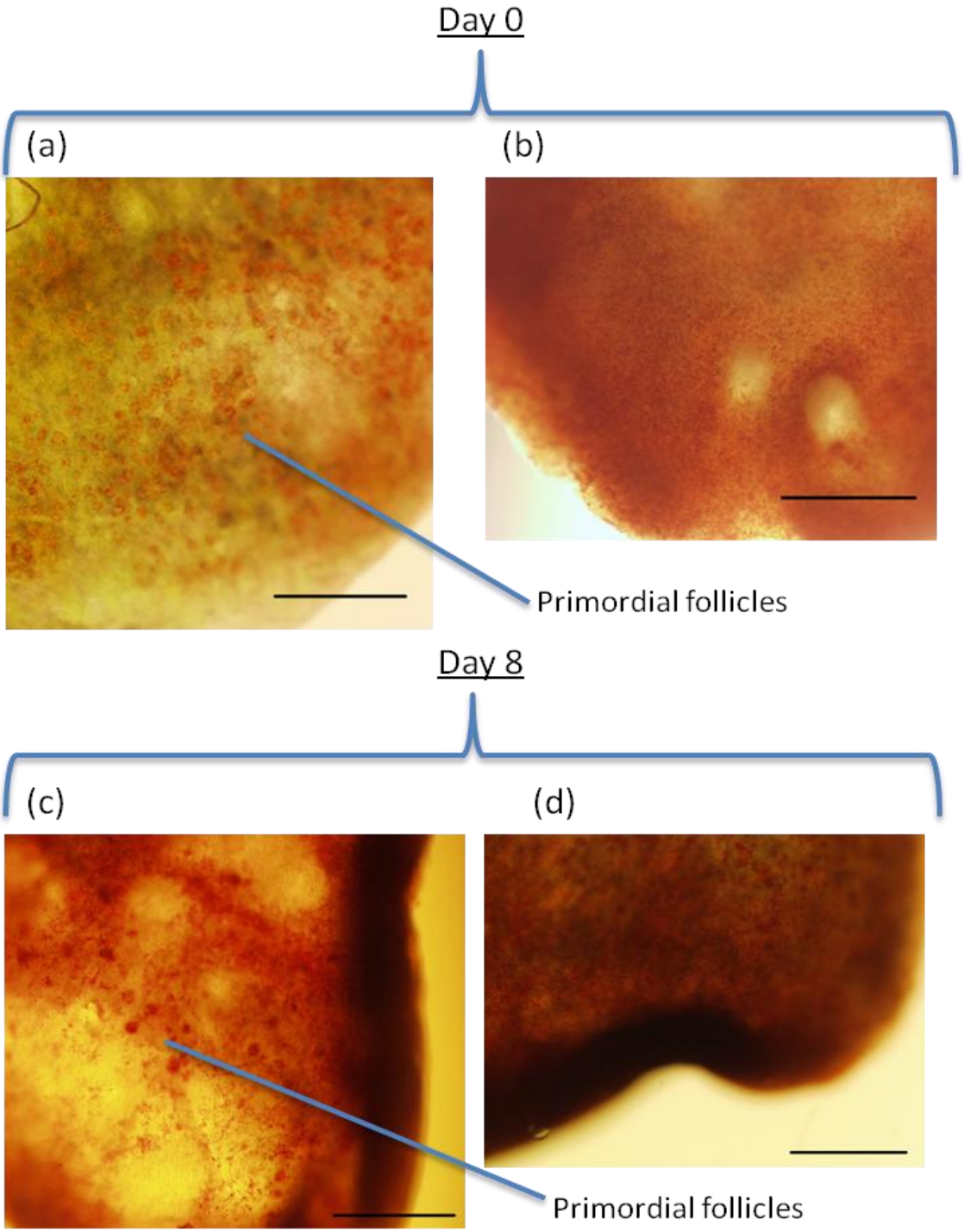


Figure 6.11 Representative images of biopsies after whole ovary perfusion and following NR incubation on days 0 (a and b) and 8 (c and d) of culture. The tissue biopsies in (a) and (c) are primordial follicle rich, whereas the tissue in (b) does not contain any follicles and therefore it was not used for culture. Due to the intensity of staining in (d) it was not possible to identify and/or count the number of follicles present after culture, however, the stromal tissue in both (c) and (d) has a relatively high level of NR staining and was therefore considered as viable. In addition the stromal tissue had proliferated after 8 days culture as the edges of the tissue have curled and thickened. Scale bar = 500µm.

6.3.4 Histological analysis

In this experimental series the effect of perfusing ovaries with either CPA solution or L-15 control medium on the follicle health and population dynamics in cortical tissue, before and after culture, was investigated. Three different perfusion times were compared relative to time 0, namely 10, 30 and 60 minutes. Paired ovaries from the same reproductive tract were first perfused with Ringer's solution for 10 minutes, then with either CPA or control medium, L-15 for the lengths of time stated above. Non-perfused tissue obtained from ovaries that were perfused with Ringer's solution alone was also used as a control for all comparisons. Follicle population dynamics were analysed in tissue samples from all treatment groups described above; obtained on days 0 and 8 of culture. A summary of all the types of samples used for histological analysis is provided in Table 6.3 and the total numbers of follicles of each classification analysed in each treatment group and provided in Table 6.4.

Table 6.3 Summary of all the types of samples used for histological analysis.

Treatment	Non-perfused tissue	Perfused tissue											
Medium	N/A	CPA						L-15					
Perfusion time (minutes)	0	10		30		60		10		30		60	
Day of culture	0	0	8	0	8	0	8	0	8	0	8	0	8

Table 6.4 Total number of each classification of follicle analysed histologically following each treatment.

Treatment	Perfusion length (minutes)	Day	Follicle classification					
			Prd	EP	1°	Trans	2°	EA
Non-perfused control	0	0	3890	1615	170	90	35	5
		8	5715	2115	60	35	5	0
CPA	10	0	1370	1380	315	50	15	0
		8	2660	1000	80	190	40	0
	30	0	680	440	28	36	4	0
		8	1400	725	30	45	25	0
	60	0	805	805	50	90	5	0
		8	670	300	10	5	0	0
L-15	10	0	1870	700	40	45	15	0
		8	2765	621	19	5	0	0
	30	0	1333	898	117	114	29	0
		8	2030	1165	100	240	60	0
	60	0	1355	920	115	85	10	0
		8	1210	480	10	20	5	0

Follicle population dynamics were analysed histologically. The mean number of follicles present in day 0 and day 8 tissue is presented in Figure 6.12. The mean number of follicles in all groups on days 0 and 8 were compared. When all day 0 tissue samples were compared, although the mean number of follicles in all perfused tissue samples was lower than in the non-perfused tissue this was not statistically significant ($p>0.05$). However, the p value showed a trend towards significance ($p=0.059$) therefore if more repeats had been carried out these differences might have reached statistical significance. This suggests that the process of perfusing the ovary with either CPA or L-15 damaged the tissue and caused more follicles to degenerate than in the non-perfused tissue. The mean number of follicles in CPA or L-15 perfused tissue was significantly lower ($p=0.002$) than in the non-perfused tissue on day 8. Therefore the level of follicular degeneration during culture was consistently higher in the ovaries that had been perfused with CPA or L-15, compared to non-perfused control tissues that had been washed with Ringer's solution only. The lower mean follicle number in all perfused tissues on day 0 could partly account for this observation. In order to examine this further the mean number of follicles on days 0 and 8 of culture within each perfusion time group were compared. In all perfusion groups the number of follicles decreased after 8 days of culture via follicle degeneration. However, only in the tissue perfused for 30 and 60 minutes with CPA ($p=0.042$ and $p=0.003$, respectively) and 60 minutes with L-15 ($p<0.001$) was the decrease significant. Therefore, treatment with CPA consistently resulted in higher levels of follicle degeneration and loss than L-15 following a 30 minute perfusion period. Whereas a 60 minute of perfusion, regardless of whether the media contained only L-15 or CPA, resulted in higher levels of follicular degeneration and loss.

Follicle population dynamics were examined in Figure 6.13 with respect the percentage of primordial follicles present in tissue on days 0 and 8 of culture following whole ovary perfusion. The percentage of primordial follicles was higher in tissue fixed on day 8 compared to day 0 in all CPA and L-15 perfused tissue and non-perfused tissue. This suggests that the proportion of primordial follicles relative to growing follicles increased after culture. As the mean number of follicles is lower in day 8 tissue, compared to day 0 tissue (see Figure 6.12) this suggests that the growing follicles are more susceptible to atresia during culture following L-15 or CPA perfusion. The increase in the proportion of primordial follicles on day 8 was not statistically significant in any treatment group except the 60 minutes CPA perfusion group ($p<0.05$). This suggests that 60 minutes of CPA perfusion is the most damaging treatment for growing follicles.

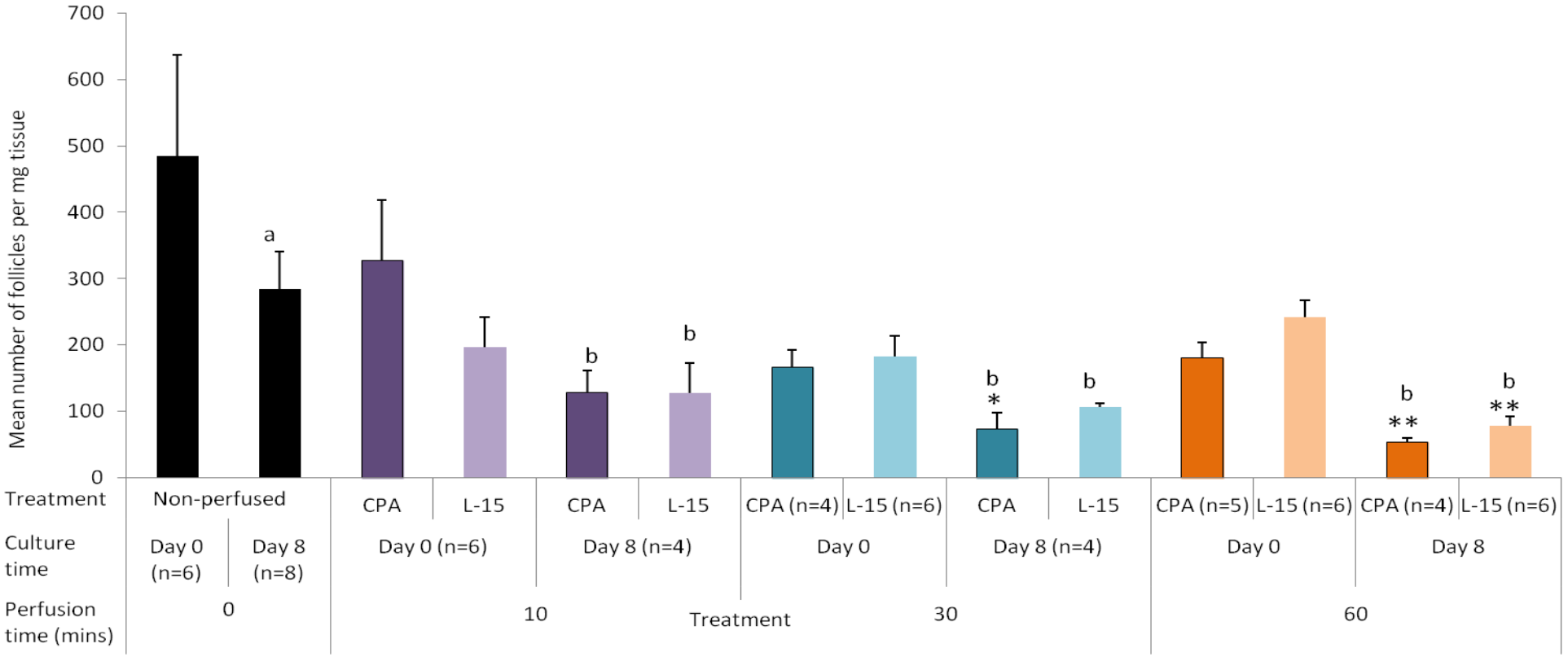


Figure 6.12 Mean number of follicles present in tissue on fixed days 0 and 8 following whole ovary perfusion with either CPA or L-15 for 0, 10, 30 and 60 minutes. Data is presented as mean ± SEM for the number of culture repeats (n) indicated. Statistical differences between follicle counts in day 0 and 8 tissue within each perfusion time were analysed using the Student's t-test and are indicated using * ($p<0.05$) or ** ($p<0.001$). Statistical differences between the day 8 follicle counts between different perfusion times were analysed using one-way ANOVA and are indicated by different letters ($p<0.05$).

The population dynamics of EP and primary follicles present in tissue on days 0 and 8 are presented in Figures 6.14 and 6.15, as the mean number and mean percentage of follicles, respectively. The population dynamics of multi-layered follicles are shown in Table 6.5. Following 10 minutes perfusion there was no difference in the mean number of EP, primary or multi-layered follicles on days 0 and 8 between treatment groups (see Figure 6.14 and Table 6.5). Although the number of follicles decreased in day 8 tissue relative to day 0 tissue this difference was not significant ($p>0.05$). However, when the mean percentage of follicles was considered there was a significant decrease ($p=0.028$) in the tissue perfused for 10 minutes with L-15 relative to both its day 0 counterpart and all other EP follicle counts (see Figure 6.14). It is unclear why perfusion with L-15 would have caused a decrease in EP follicle counts. The SEM for 10 minute CPA EP follicle percentage is quite high therefore it is possible that if more repeats were carried out a decrease would also be seen in this category. There were no significant differences in the mean percentage of primary or multi-layered follicles following 10 minutes perfusion (see Figure 6.14 and Table 6.5).

Following 30 minutes of perfusion there were no statistically significantly changes ($p>0.05$) in the percentage of EP, primary and multi-layered follicles, relative to non-perfused control tissue or on days 0 and 8 of culture (see Figure 6.7 and Table 6.1). It should be noted, however, that all follicle counts decreased on culture day 8 relative to equivalent culture day 0 samples. There was also no significant decrease ($p>0.05$) in the mean number of EP, primary and multi-layered follicles, relative to non-perfused control tissue in CPA and L-15 perfused tissue on days 0 and 8 of culture, apart from the tissue perfused with L-15 for 30 minutes which had, after 8 days *in vitro*, a significantly lower ($p<0.05$) number of EP follicles relative to non-perfused control, 30 minute L-15 perfused day 0 tissue (see Figure 6.14 and Table 6.5). The number of primary follicles present in tissue perfused for 30 minutes with L-15 was not, however, significantly lower ($p>0.05$) than that observed in non-perfused tissue on day 8 or tissue perfused for 30 minutes with CPA and analysed on days 0 or 8 of culture. Furthermore, the number of primary follicles did not decrease after 8 days of culture relative to that observed in the same treatment group on day 0 therefore these findings are unlikely to be indicative of a higher level of damage to the tissue. Again it should be noted that the mean number of follicles in tissue perfused for 30 minutes decreased on day 8 of culture relative to their day 0 counterparts, although not significantly ($p>0.05$).

Following 60 minutes of perfusion there were no statistically significant ($p>0.05$) changes in the percentage of EP, primary and multi-layered follicles, relative to non-perfused control tissue or to perfused tissue fixed on days 0 and 8 of culture (see Table 6.5 and Figure 6.15). However, just as was observed for the 30 minute perfusions, detailed above, it was noted that all counts decreased on culture day 8 relative to culture day 0. There was also no significant decrease ($p>0.05$) in the mean number of EP follicles, relative to non-perfused control tissue or on days 0 and 8 of culture (see Figure 6.14 and Table 6.5). However, there were significantly fewer primary ($p=0.011$) and multi-layered ($p=0.036$) follicles present in tissue cultured for 8 days, which had been perfused with either L-15 or CPA for 60 minutes relative to non-perfused tissue follicle counts on day 0 and 8. Additionally, the number of primary follicles in day 8 tissue had decreased significantly relative to their day 0 culture control counterparts, following 60 minute perfusion with L-15 or CPA. However, although the absolute number of multi-layered follicles in day 8 cultured tissue decreased relative to their day 0 culture control counterparts, following 60 minute perfusion with L-15 or CPA, this difference was not statistically significant ($p>0.05$).

Table 6.5 Histological data regarding the mean multi-layered follicle number per mg of tissue and mean percentage of multi-layered follicles in tissue fixed on days 0 and 8 of culture following treatment with Ringer's solution only (non-perfused control) or whole ovary perfused with Ringer's solution followed by perfusion with either CPA solution or L-15 for 10, 30 or 60 minutes. The values are mean ± SEM for the 6 and 4 culture repeats, for days 0 and 8, respectively. Data were analysed using one-way ANOVA. No statistical differences ($p>0.05$) in number or percentage of multi-layered were found between treatments.

Perfusion time (minutes)	Treatment	Day	Mean per mg tissue	SEM of mean per mg tissue	Mean %	SEM of mean %
0	Non-perfused control	0	9.0	2.4	5.0	3.0
		8	0	0	0	0
10	CPA	0	5.8	3.0	1.5	0.5
		8	8.3	2.8	7.3	3.1
	L-15	0	7.2	3.2	4.3	2.2
		8	1.1	0.7	1.5	1.3
30	CPA	0	3.8	1.8	0.8	0.4
		8	2.9	1.8	2.0	1.0
	L-15	0	10.3	2.1	4.4	2.8
		8	2.9	1.8	1.8	0.9
60	CPA	0	9.7	2.7	3.8	1.1
		8	2.9	1.8	7.7	4.7
	L-15	0	10.3	2.7	2.9	1.6
		8	2.9	1.8	2.0	1.0

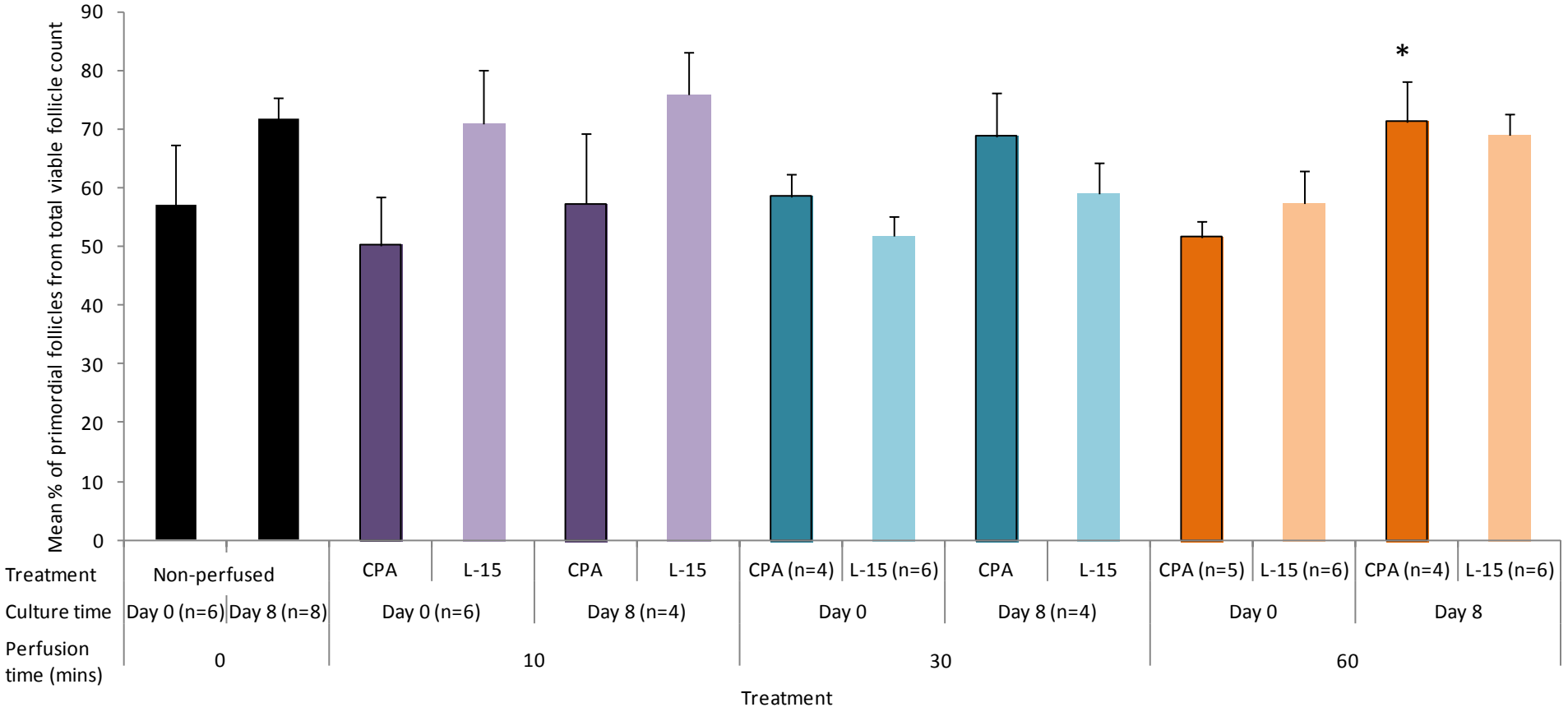


Figure 6.13 Mean percentage of primordial follicles present in tissue fixed on days 0 and 8 of culture following whole ovary perfusion with either CPA or L-15 for 0, 10, 30 and 60 minutes compared to non-perfused tissue exposed to Ringers' solution only. Data is presented as mean ± SEM for the number of culture repeats (n) indicated. Statistical differences between follicle counts in day 0 and 8 tissue within each perfusion time are indicated using * ($p<0.05$), data was analysed using one-way ANOVA.

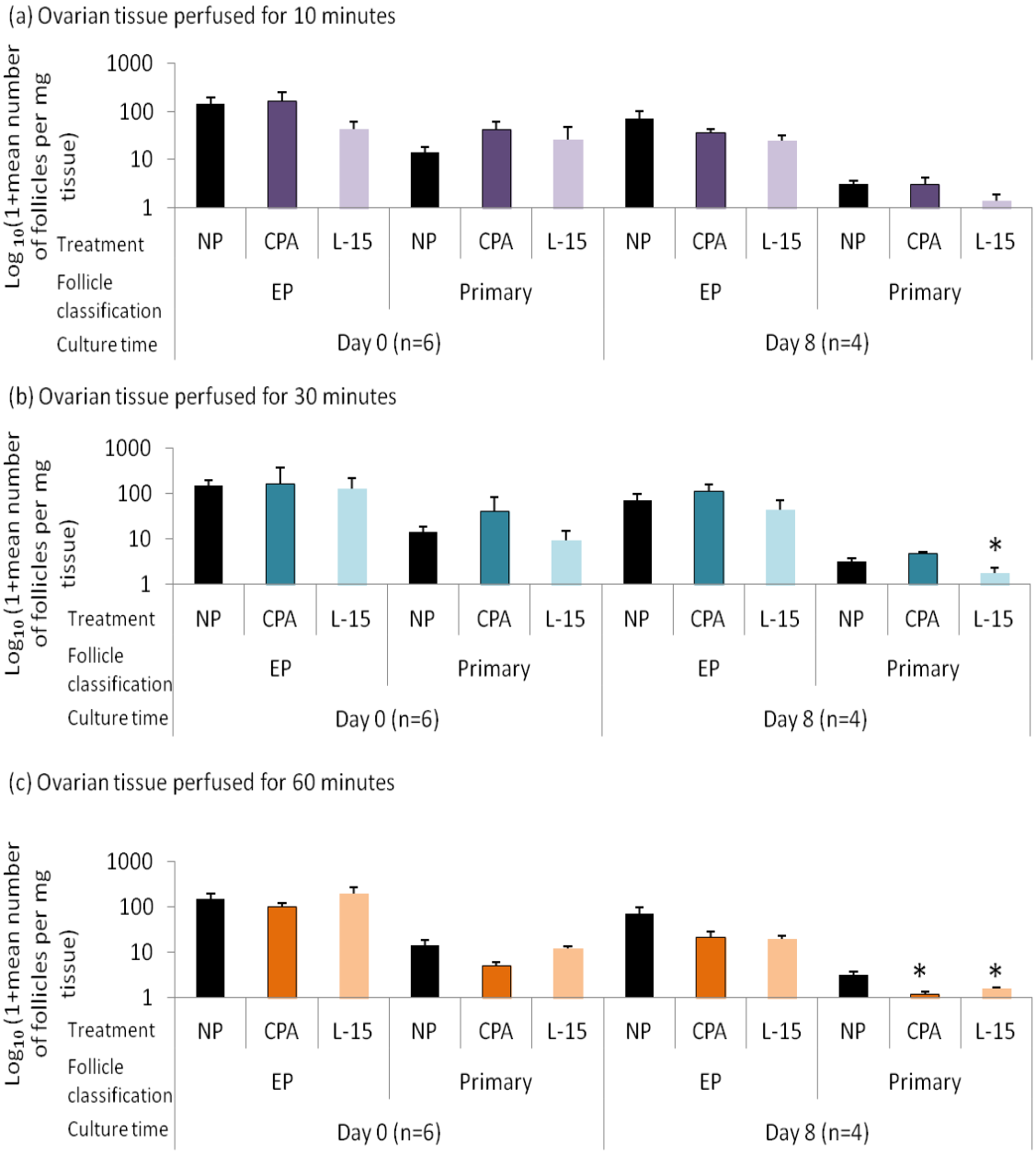


Figure 6.14 Mean number of EP and primary follicles per mg of tissue fixed on days 0 and 8 of culture that has been exposed to Ringer's solution only (Non-perfused - NP) or whole ovary perfusion with CPA solution or L-15 for (a) 10 minutes, (b) 30 minutes and (c) 60 minutes. Data is presented as mean number ± SEM for the number of repeats shown (n). Data was analysed using one-way ANOVA, statistical differences between day 0 and day 8 tissue are indicated using * ($p<0.05$).

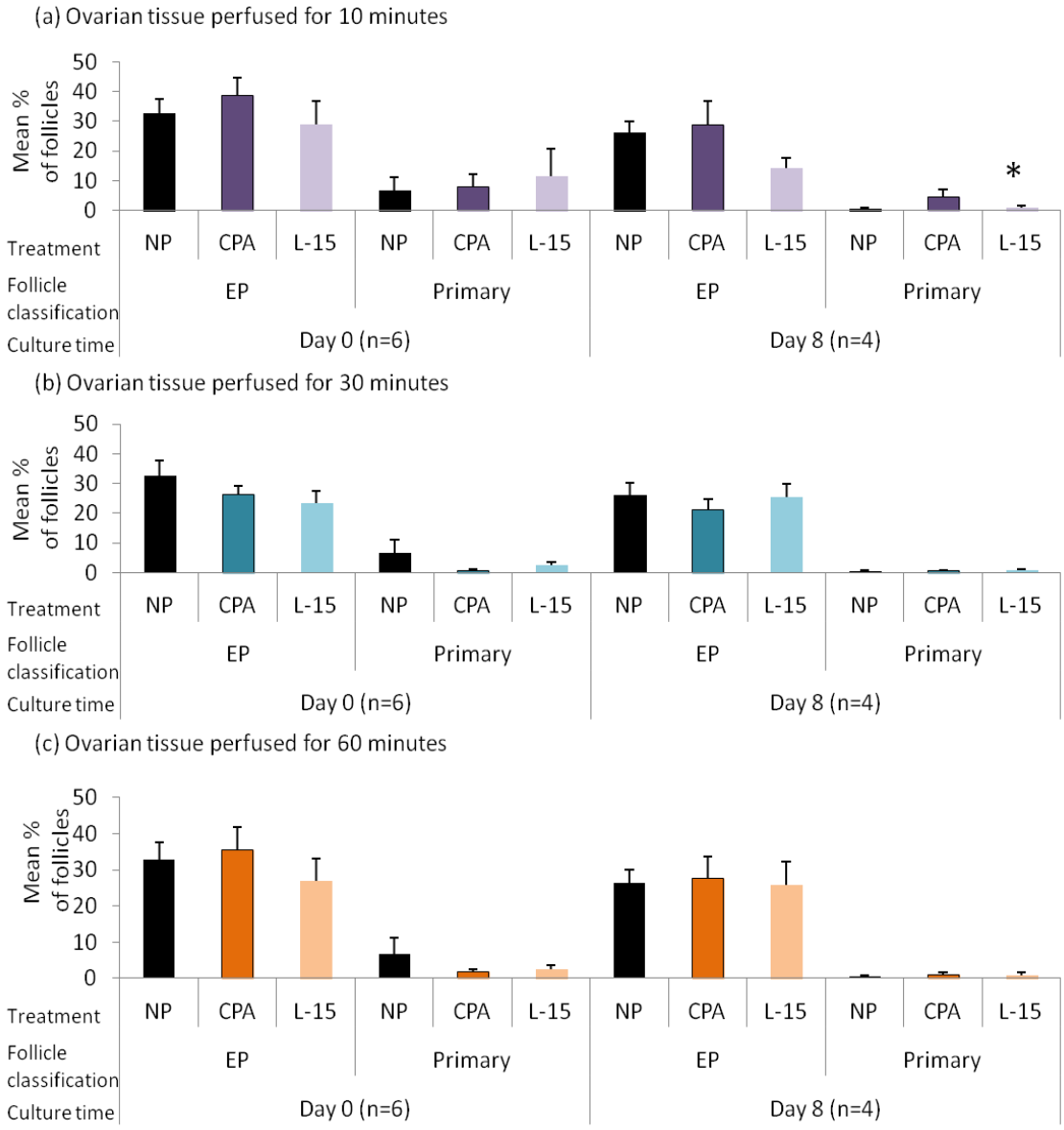


Figure 6.15 Mean % of EP and primary follicles present in tissue fixed on days 0 and 8 of culture that has been exposed to Ringer's solution only (Non-perfused - NP) or whole ovary perfusion with CPA solution or L-15 for (a) 10 minutes, (b) 30 minutes and (c) 60 minutes. Data is presented as mean % ± SEM for the number of repeats shown (n). Data was analysed using one-way ANOVA, statistical differences between day 0 and day 8 tissue are indicated using * ($p<0.05$).

The integrity of follicles and stromal tissue was assessed histologically in samples fixed on days 0 and 8 of culture, as shown in Figure 6.16. The stromal tissue score decreased on day 8, compared to day 0 of culture within each treatment group, however, this was only statistically significant ($p<0.05$) following 10 minutes perfusion with L-15, and 60 minutes perfusion with either L-15 or CPA. The effect of whole ovary perfusion and subsequent cortical culture on tissue weight was examined in Figure 6.17. Neither ovary perfusion nor culture had a significant effect on tissue weight at the start or end of culture ($p>0.05$).

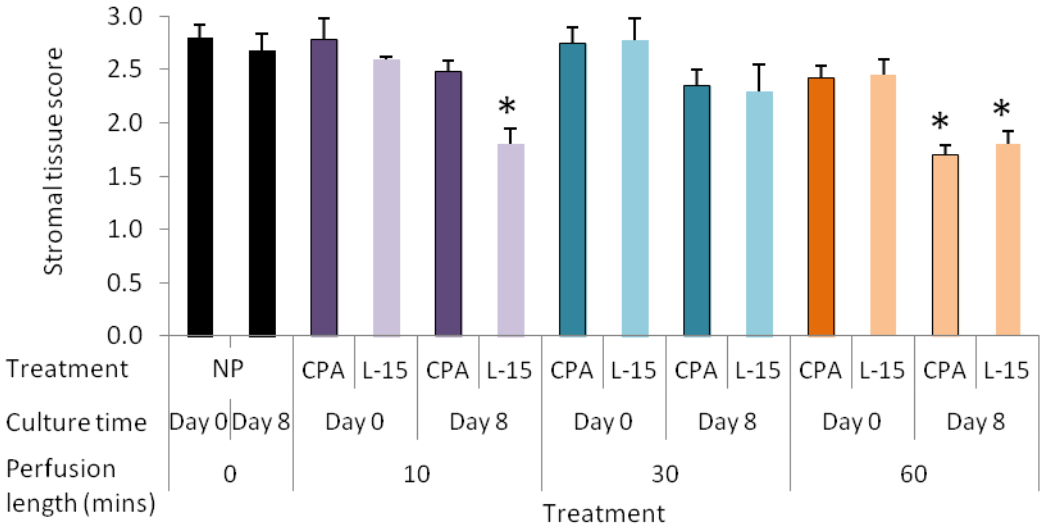


Figure 6.16 Histological analysis of stromal tissue integrity in tissue fixed on days 0 and 8 of culture that has been exposed to Ringer's solution only (Non-perfused - NP) or whole ovary perfusion with CPA solution or L-15 for (a) 10 minutes, (b) 30 minutes and (c) 60 minutes. The overall appearance of the tissue and follicles in the section of tissue was rated as good (3), adequate (2) or poor (1). Values are shown as the mean ± SEM for 4 culture repeats. Stromal tissue score was compared across all treatments using one-way ANOVA and statistically significant differences relative to all other treatments ($p<0.05$) are denoted using *.

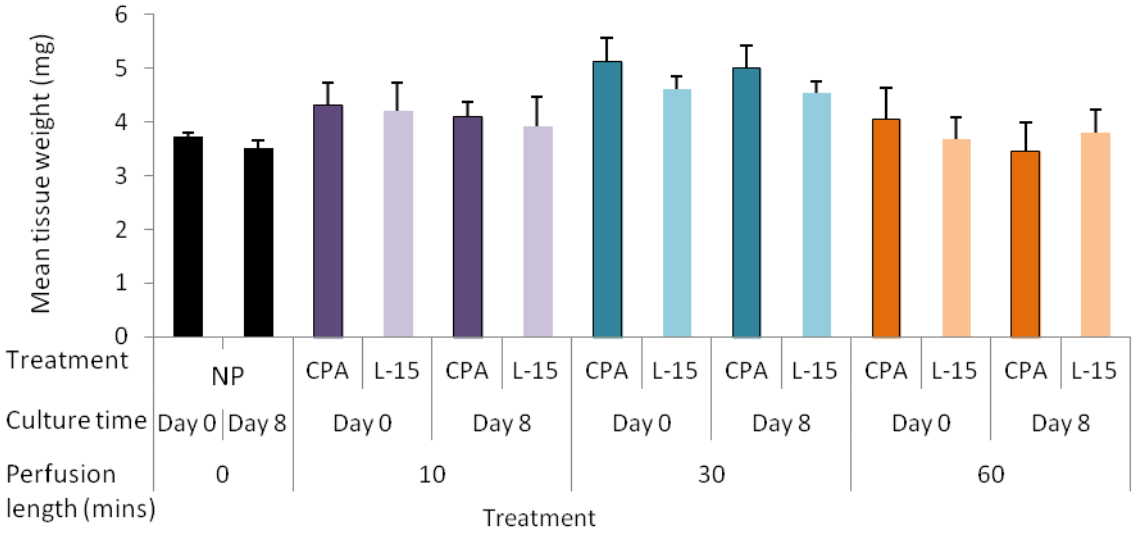


Figure 6.17 Mean tissue weight on days 0 and 8 of culture of tissue that has been exposed to Ringer's solution only (Non-perfused - NP) or whole ovary perfusion with CPA solution or L-15 for 0, 10, 30 and 60 minutes. Data is presented as mean ± SEM for 6 culture repeats. Data was analysed using one-way ANOVA. No statistical differences ($p>0.05$) were found either within treatment groups between day 0 and 8 tissue, or between treatment groups.

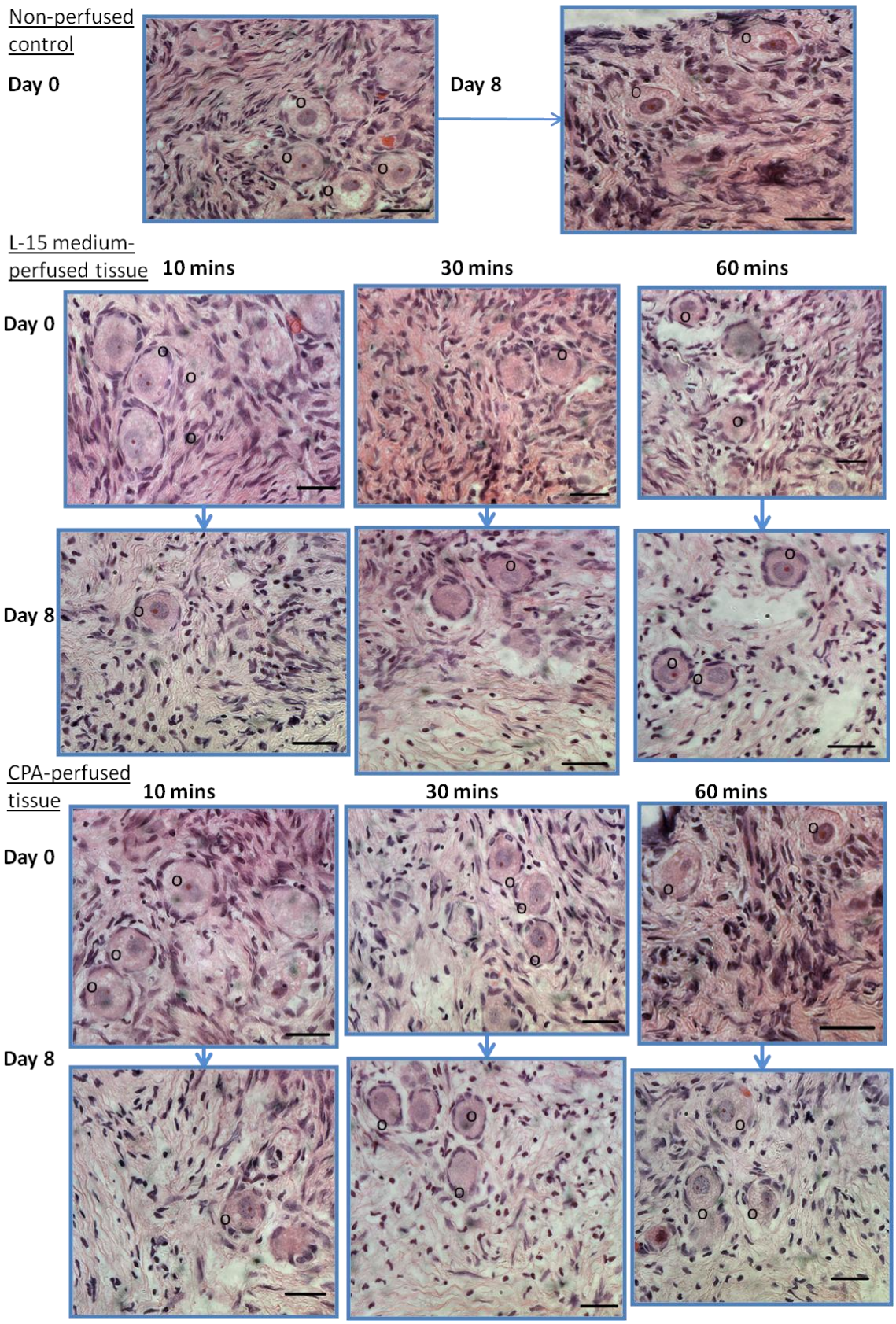


Figure 6.18 Representative images of tissue fixed following perfusion with either L-15 medium or CPA for 0, 10, 30 and 60 minutes either on day 0 or day 8 of culture compared to non-perfused control tissues. All follicles are at the primordial or EP stage. During histological analysis only follicles with a visible nucleolus were counted – the oocyte of these follicles is labelled 'O'. Scale bar = 20µm.

Table 6.6 Mean diameter of primordial and EP follicles fixed on days 0 and 8 of culture following whole ovary perfusion with either CPA or L-15 for 0, 10, 30 and 60 minutes compared to non-perfused control tissues. The mean and SEM for the number of culture repeats indicated (n) are shown. Data was analysed using one-way ANOVA. No statistical differences in follicle diameter were found between all treatments within all day 0 and all day 8 data in either primordial or EP follicles.

Perfusion time (minutes)	Treatment	Day	Primordial diameter (μm)	Primordial diameter SEM	EP diameter (μm)	EP diameter SEM
0	Non-perfused control	0 (n=6)	24.3	0.9	31.4	0.7
		8 (n=4)	24.2	0.1	30.7	0.4
10	CPA	0 (n=6)	24.8	0.4	29.2	2.8
	L-15		23.3	0.3	32.4	0.4
	CPA	8 (n=4)	24.9	0.6	32.6	0.8
	L-15		24.2	0.3	30.9	0.7
30	CPA (n=4)	0	24	0.4	31.7	0.6
	L-15 (n=6)		24.9	0.2	33	0.5
	CPA	8 (n=5)	24.5	0.4	32.5	0.1
	L-15		24.8	0.2	32.8	0.4
60	CPA	0 (n=6)	24.5	0.4	31.6	0.5
	L-15		24.4	0.4	32.8	0.5
	CPA	8 (n=4)	24.6	0.2	32	0.3
	L-15		24.2	0.1	31.7	0.2

In order to examine whether perfusing the ovary with CPA had any dehydrating effects that would cause cell shrinkage, the diameter of oocytes (see Figure 6.19) and follicles (Table 6.6) at the primordial and EP stages was analysed histologically. Data regarding diameters of follicles more developed than the EP stage is not shown because the vast majority of follicles present in the ovary are the earlier preantral stages. Follicles at primordial and EP stages are more resistant to cryopreservation procedures and therefore more likely to survive, thus dehydration effects upon follicles of these stages are more relevant. In addition, as follicle development progresses there is more variation in size within each category and it becomes more difficult to determine whether differences in size were due to osmotic effects or normal variability, especially as there were fewer primary, transitional, secondary and EA follicles present. Analysis revealed that perfusion of the whole ovary, regardless of the presence or absence of CPA or of the perfusion duration, did not affect the diameter of primordial or EP oocytes or follicles. Therefore, the rehydration steps following CPA perfusion prior to culture were sufficient to ensure the cells did not sustain permanent damage due to dehydration as a result of the perfusion technique and/or CPA exposure.

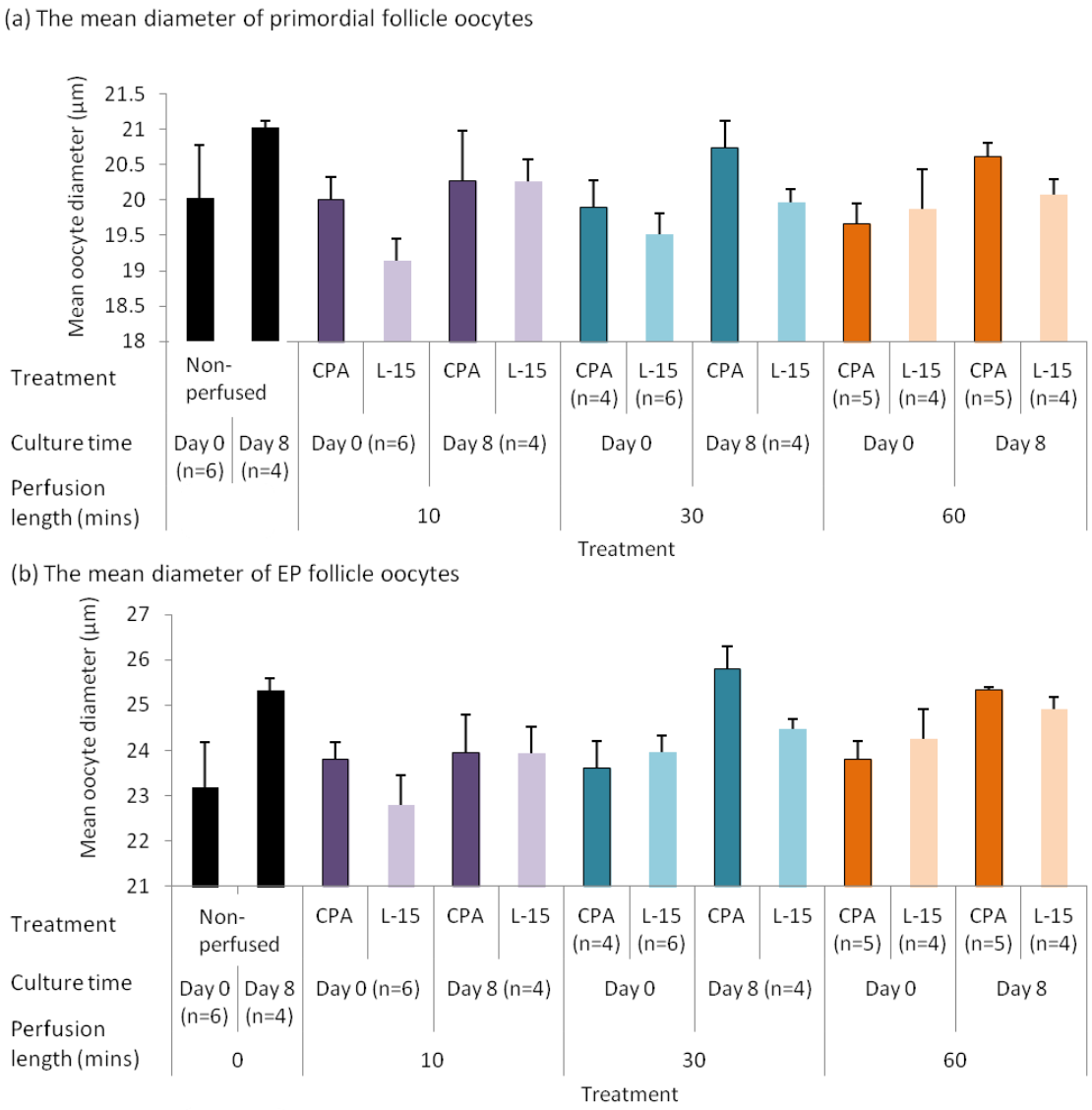


Figure 6.19 Mean oocyte diameter of (a) primordial and (b) EP follicles present in tissue fixed on days 0 and 8 of culture following whole ovary perfusion with either CPA or L-15 for 0, 10, 30 and 60 minutes or following exposure to Ringer's solution only (non-perfused controls). Data is presented as mean ± SEM for 6 culture repeats. Data was analysed using one-way ANOVA. No statistical differences in oocyte diameter were found between any of the treatments within all day 0 and all day 8 data in primordial and EP follicles.

6.4 Discussion

The data obtained via NMR analysis indicates that the level of DMSO detected in the pedicle tissue increased significantly after a much shorter perfusion time than the cortical tissue, 10 and 60 minutes respectively. This is to be expected as the pedicle tissue is much softer and more permeable than the more fibrous cortical tissue (Bedaiwy et al., 2003). The increased length of exposure to the CPA, when the pedicle already has a high level of DMSO permeation,

is therefore likely to contribute towards to the micro-vascular damage that has previously been observed and thought to result in the high levels of follicle depletion following re-transplantation of frozen-thawed whole ovaries (Onions et al., 2009). Note that the process of cannulating the ovary may also have mechanically damaged the pedicle vasculature, but this parameter was not analysed. In a recent study the effect on the permeability of blood vessels in ovine ovaries was assessed using fluorescent microspheres 7 days after autotransplantation (Onions et al., 2013). The ovaries were either fresh (i.e. they had not been perfused), perfused with 1.5M DMSO but not cryopreserved, or perfused with 1.5M DMSO and cryopreserved. Subsequent analysis of ovarian sections showed that the perfusion process itself damaged the ovarian vasculature as in 50% of ovarian medullae from perfused but not cryopreserved ovaries, unlike in non-perfused ovaries; the 100nm fluorescent microspheres were able to penetrate the smooth muscular wall of the blood vessel. Ovaries that were both perfused and frozen-thawed prior to autotransplantation exhibited even greater signs of microvascular damage as the 100nm fluorescent microspheres were detected in the smooth muscular wall of the blood vessel in 80-90% of ovarian medullae from these ovaries. It would be interesting to use this technique to assess the level of damage to the pedicle vasculature following the different perfusion times used in the present studies.

Surprisingly, successful cryopreservation of whole ovine ovaries has been reported following only 3 minutes of perfusion with 1.3M DMSO (Arav et al., 2010). The fact that the ovaries remained functional for 6 years suggests that this was a sufficient length of time to fully permeate the ovarian tissue. However, the details of the perfusion rate are not given and CPA penetration was not reported and therefore it is difficult to directly compare the two studies. Furthermore a novel multithermal gradient freezing methodology was used in this study, whereas the protocol being developed and tested in the current study is being optimised for whole ovary, slow-freezing methods. In addition, the animals used in the published study were all ~9 months of age, whereas older animals were used in the current study, therefore the ovaries perfused in the current study were larger, more fibrous and less permeable to DMSO, thus it was expected a longer perfusion time would be required. In addition the ovaries of lambs have a larger primordial follicle reserve than the ovaries of older animals.

The length of time and the rate at which ovaries are perfused with DMSO varies between studies, furthermore some studies utilised pumps (Onions et al., 2008, Onions et al., 2009) whereas others performed perfusion manually (Imhof et al., 2006). There are a number of

studies in which ovaries were perfused with 1.5M DMSO at the same rate as used in our study (Onions et al., 2008, Onions et al., 2009, Onions et al., 2013). In these investigations ovaries were perfused with DMSO for 60 minutes and the data should be directly comparable to that obtained in the current investigation.

The highest level of DMSO permeation was reported after 60 minutes perfusion in the current study. Histological examination of the tissue indicated that the length of perfusion *per se* had a detrimental effect on subsequent follicle survival after 8 days of culture in both the CPA and L-15 perfused ovaries (see Figure 6.12). Indeed, CPA perfusion for only 30 minutes significantly reduced follicle numbers on day 8 of culture. This is unsurprising as DMSO is known to have cytotoxic effects via its chemical toxicity and the osmotic stress experienced by exposed cells, as detailed in Section 6.1.3 (Meryman, 1971). As the follicle counts on day 0 did not significantly differ between the non-perfused tissue and the perfused tissue in any treatment group this shows that the effects of CPA exposure on follicle survival are not immediate, rather the damage is sub-lethal. This is similar to observations in previous studies in which primordial follicle density, assessed histologically, was not significantly different in perfused and non-perfused ovine ovaries (Courbiere et al., 2005). The data regarding oocyte and follicular diameter shows that there are no obvious short-term or long-term dehydration effects of perfusion or CPA exposure resulting in cell shrinkage at the start or end of culture (see Figure 6.19 and Table 6.6). Therefore the method of rehydration utilised was adequate and unlikely to be the cause of the increased levels of follicle degeneration in CPA perfused tissue.

Information from small rodent models regarding follicle population dynamics is scarce as many articles focus on the presence or absence of follicles at different stages, rather than the actual follicle counts (Harp et al., 1994, Candy et al., 2000). However, Yin et al. (2003) reported a significant decrease in number of both primordial and growing follicles following the orthotopic transplantation of cryopreserved whole ovaries, compared to fresh ovaries in rats. Similarly, a reduction in the number of primordial and growing follicles was reported following the WOCP and orthotopic transplantation of mouse ovaries, with primordial follicles exhibiting a higher survival rate than growing follicles (Candy et al., 1997).

Previous studies have shown a decrease in follicle number following CPA exposure and cryopreservation of ovine ovarian tissue (Newton et al., 1996, Amorim et al., 2003). Although, Hovatta et al. (1996) reported no significant difference in the level of follicular atresia in

human tissue following 1.5M DMSO exposure and cryopreservation compared to fresh tissue. The use of DMSO as a CPA ameliorates these effects on follicle survival to a greater extent than other CPAs in ovine ovarian tissue (Amorim et al., 2003, Santos et al., 2006). Less research has been conducted regarding CPA exposure alone, however, exposure to 1.5M DMSO has been shown to significantly reduce the proportion of viable isolated ovine primordial follicles and primordial follicles *in situ* in cortex, both prior to and after 5 days culture (Santos et al., 2007).

The increase in the percentage of primordial follicles after 8 days of culture compared to day 0, shown in Figure 6.13, is most pronounced in the 60 minute CPA perfused tissue and reached statistical significance only in this group ($p<0.05$). As cortical tissue culture is not optimal for follicles that have progressed past the transitional stage of development (see Chapter 4) the more developed follicles present on day 0 were more prone to degeneration than primordial and EP follicles (Hovatta et al., 1999). Furthermore, follicles that activated prior to culture may have started to degenerate *in vivo* but were not yet displaying any morphological signs of atresia (Telfer et al., 2008). The data presented here support the notion that primordial follicles are more tolerant to the perfusion process and cytotoxic insults than other classifications of follicles. This finding is advantageous in terms of fertility preservation strategies for a number of reasons. Firstly primordial follicles are the most abundant follicles present in the ovaries (Gosden and Telfer, 1987b) and secondly they display the highest level of tolerance to the cryopreservation procedure (Newton et al., 1996). Furthermore, previous studies utilising ovine ovarian tissue slices have shown that more developed preantral follicles can survive the cryopreservation procedure (Newton et al., 1999b) but that these follicles degenerate soon after grafting due to ischaemia (Gosden et al., 1994, Baird et al., 1999).

Follicle losses following orthotopic transplantation of whole ovaries have been attributed partly to the cannulation and perfusion procedure, as these processes can result in poor vascular patency so affecting follicle survival following grafting (Bedaiwy et al., 2003). However, more recent research has shown that full patency of the vasculature can be achieved following whole ovary perfusion and cryopreservation (Onions et al., 2009). Despite this, significant primordial follicle loss was still observed following grafting. It should be noted that there was some evidence of damage to the microvasculature of the pedicle (Onions et al., 2013) that may have been the cause of this effect and that this damage can be overcome by use of anti-thrombotic agents (BK Campbell and HM Picton, unpublished data). In a more recent study whole ovine ovaries were perfused with Indian ink following 1.5M DMSO

perfusion, cryopreservation and thawing (Maffei et al., 2013). This revealed that the vasculature in the cortex and medulla appeared to remain intact. The perfusion duration used, however, was only 5 minutes therefore, in light of the present findings it would be essential to repeat this investigation to assess the impact of increasing the perfusion duration to 60 minutes to match the optimal time for penetration of the whole ovary by DMSO.

Collectively, the results presented in the current study, along with previous investigations which utilise a similar perfusion protocol suggest that it may not be damage to the vasculature that results in the large loss of follicles following grafting, but some other aspect of the perfusion process itself (Onions et al., 2009, Onions et al., 2013). In this investigation increasing the perfusion duration rather than the cannulation process resulted in increasing levels of follicle loss (see Figure 6.12). Indeed, mechanical damage to the pedicle vasculature and capillaries cannot have caused the follicular loss observed in the current culture experiments as increasing the length of the perfusion increased the level of follicle loss, in both the CPA and L-15 medium. It would be interesting to explore the use of other markers of cell stress and damage to assess the effects of perfusion pressure and cryopreservation on follicle number and health and cortex integrity.

While the focus of many of the earlier studies into the effects of ovarian tissue cryopreservation has been the survival, health and developmental competence of follicles and oocytes, a novelty of the current work is the investigation of the impact of CPA exposure and perfusion on stromal integrity. The importance of maintaining the health of the stroma is increasingly being recognised. Maintenance of stromal tissue integrity and morphology is of great importance to both the maintenance of the primordial reserve and to primordial activation and continued development as it provides the scaffold for the follicles and oocytes, without which oocyte-somatic cell interactions may be compromised. Receptors and growth factors expressed by ovarian stromal cells are required for primordial follicle activation, such a c-kit, and development, and BMP4 and BMP7 (Knight and Glister, 2006). Therefore, more recent studies of ovarian tissue health and whole ovary cryopreservation have used methods to assess stromal viability. In the current study the health of the stromal tissue was assessed both in fresh tissue and histologically. As described earlier the process of whole ovary perfusion with either CPA or L-15 medium for 0-60 minutes did not have an immediately apparent detrimental effect on follicle viability. Nor did it appear to affect long-term stromal tissue viability following 8 days culture (see Table 6.2). Similarly, when stromal tissue and

follicle integrity were assessed histologically, there was no apparent immediate effect on stromal health (see Figure 6.16). Tissue fixed after 8 days culture, however, exhibited loss of integrity, particularly following 60 minutes perfusion with either CPA or L-15 medium ($p<0.05$). A significant decrease in integrity was also observed in day 8 tissue that had been perfused with L-15 medium for 30 minutes. Although it is unclear why the follicle and tissue integrity was this poor in this group as the variation in the data is low, in support of the ideas presented here, stromal tissue damage has been proposed to be a major contributing factor in follicular burn out and premature ovarian failure following exposure to chemotherapeutic agents (Gavish et al., 2014). Furthermore, a decrease in ovine ovarian stromal tissue integrity following exposure to cryoprotectants has been observed in a number of previous studies. Faustino et al. (2010) used light microscopy to determine the mean number of stromal cells per 100µm in ovine ovarian tissue following exposure of fragments to 1.0M or 1.5M EG followed or not by cryopreservation. A significant reduction in density was observed following CPA exposure alone for 5-20 minutes and a further reduction was observed following cryopreservation. The effect of CPA exposure and cryopreservation was also assessed by Oskam et al. (2010) using a slightly more rigorous methodology. Ovine ovarian tissue was assessed histologically. Acid Fuchsin Orange G was used to stain the tissue which was then examined using transmission election microscopy and scored according to the percentage of pyknotic stromal cells and the level of oedema. Tissue fragments were exposed to either 1.5M EG or 1.5M PROH for 30 minutes. Although an increase in the percentage of pyknotic cells and the level of oedema was observed this was not significantly higher than that observed in control tissue. Exposure of ovarian tissue to PROH, however, resulted in a significant increase in the aforementioned parameters. More recently the same group extended this study by using similar techniques to assess the quality of ovine stromal tissue following 1.5M PROH exposure and subsequent cryopreservation followed by either *in vitro* culture or xenotransplantation into SCID mice, which revealed an irreversible level of damage to the stromal tissue (Oskam et al., 2011). The effect of WOCP on the health of stromal tissue has also been previously investigated (Maffei et al., 2013). Here whole ovine ovaries were perfused with 1.5M DMSO for 5 minutes then cryopreserved using conventional freezing. Ovaries were then thawed and analysed histologically. Stromal density was assessed by counting nuclei, revealing a significant reduction in the cryopreserved compared to the control tissue. The level of proliferation was also analysed using a polyclonal antibody for Ki67, with no significant effect observed on day 0 but a significant reduction in expression observed following 7 days *in vitro* culture, compared to control tissue. On this basis it would be of value

to conduct a more detailed evaluation of stromal cell number in the tissue for the different CPA and perfusion treatments used in the current series of experiments.

As discussed in detail in Chapter 4 the identification of markers of stromal normality and health would be highly useful in determining the effect of damage to stromal tissue on follicle loss as a result of ovarian perfusion, CPA exposure and freeze-thaw protocols. In addition determination of the exact mechanisms of follicle loss induction may enable the development of methods to reduce/ counteract said mechanisms (Morgan et al., 2012, Morgan et al., 2013).

In a recent study conducted by Onions et al. (2013) the expression levels of genes and/or proteins associated with apoptosis – *Bax, Bcl2* and *Casp6*; shear stress – *ET-1*; wound repair-thrombospondin 1 (*THBS1*); and hypoxia - *THBS1*, Ki67, *ET-1* and *ET-2*, were quantified in response to the whole ovary perfusion and cryopreservation. Up-regulation of *ET-2* expression by the cells in the pedicle accompanied by the down-regulation of *THBS1* expression in both the pedicle and medulla suggests that these cells experienced hypoxia as a result of the perfusion process. In addition increased levels of Ki67 were observed in the ovarian stromal cells. Up-regulation of Ki67 is observed in cells in hypoxic conditions. Up-regulation of Ki67, however, is also a sign of cell proliferation, as mentioned above, suggesting that the stromal cells were healthy and able to proliferate following whole ovary perfusion and cryopreservation. Although expression of *Bax* was up-regulated and *Bcl2* down-regulated in response to perfusion and cryopreservation in both the pedicle and medulla, thus promoting apoptosis, the level of *Casp6* expression was down-regulated in the medulla suggesting that in this tissue type apoptosis was suppressed. As mentioned above *THBS1* expression was down-regulated in both the pedicle and medulla in response to ovary perfusion suggesting that this process results in injury and induces wound repair. It would be interesting to combine the methods utilised in the current study and that conducted by Onions et al. (2013) in order to determine the effects of varying perfusion duration on the expression levels of genes associated with the stress response of cells at the start and end of cortical culture. Finally, the process of transferring the ovarian tissue, which was kept at 4°C throughout the perfusion process, to media, pre-warmed to 37°C immediately prior to auto-transplantation of the tissue may result in heat shock. In order to further evaluate this the expression levels of heat shock proteins could be analysed (Samali and Orrenius, 1998).

In addition to assessing gene expression profiles to determine whether the tissue encountered hypoxic conditions the levels of metabolites in spent culture media collected following perfusion could be measured. An enzyme-linked ultra-microfluorescence assay has previously been used to assess the consumption and/ or production of glucose, pyruvate and lactate by murine oocytes and embryos (Leese and Barton, 1984, Harris et al., 2005, Harris et al., 2009) and it has been used as a means of assessing glucose metabolism as an index of tissue stress during ovine cortex culture (Gunson, 2009) This method could be developed and adapted for the current experimental series to quantify the levels of metabolites in spent media following 0, 10, 30 and 60 minutes perfusion such that levels of pyruvate consumption relative to glucose consumption could be used to determine whether the duration of perfusion affects which energy production pathway is favoured- namely glycolytic conversion of glucose to lactate or the Krebs cycle (Berg et al., 2002, Harris et al., 2009). Decreasing levels of pyruvate: glucose consumption would be indicative of increasingly hypoxic conditions. The ratio of glucose consumption to lactate production could also be used as an indicator of the level of anaerobic glycolysis, with increasing levels of lactate production relative to glucose consumption being observed in increasingly hypoxic conditions (Berg et al., 2002, Harris et al., 2009).

One of the causes of increased follicular loss observed in the current experimental series may have been the increased length of time the tissue was kept in either CPA or L-15 medium plus 10% FCS, both of which are utilise L-15 as the basal medium. The composition of L-15 medium, Ringer's solution and partial composition of the ovine oviduct plasma (Black et al., 1970) is shown in Appendix IV. L-15 medium is not of the exact same composition as blood and therefore is not optimal for maintenance of the health of the ovarian somatic cells and follicles. The metabolic needs of the oocytes may not have been met during this period although this is unlikely as L-15 medium contains 9mgml^{-1} galactose, which can be converted into glucose and used as a metabolite (Berg et al., 2002). The final concentration of galactose in both perfusion media used is ~8mgml^{-1} which is therefore much higher than the concentration of glucose (0.000285mgml^{-1}) in blood in the ovine oviduct (Brackett and Mastroianni Jr, 1974). The concentrations of ions in the medium are different to that in the blood (Black et al., 1970). For example, the concentration of chloride ions in the oviduct blood is 3.94gL^{-1}, which differs slightly from that of the culture media (4.40gL^{-1}) and the both perfusion media (5.4gL^{-1}). Therefore differences in the composition of the blood and perfusion media used, with respect to ions, metabolites and amino acids, as well as differences in the viscosity and flow rate *in vivo* compared to *in vitro*, may have a detrimental effect on the

stromal tissue and/or the follicles and oocytes (Onions et al., 2013). Note that maintaining the health of the stromal tissue is of great importance for the continued development of the follicles (Onions et al., 2013). In a recent study ovine ovaries were perfused with blood, however, the ovaries were not then grafted back into the animal (Onions et al., 2013). It would be interesting to investigate the effects of perfusing the ovaries with ovine blood and also of adjusting the perfusion flow rates and hence pressure and then either grafting the ovaries or culturing the cortical tissue and assessing follicle dynamics in order to determine whether this improves the subsequent follicle survival rate.

The increased length of time for which the ovaries were kept at 4°C may have had a detrimental effect on the subsequent health of the follicles *in vitro*. However, previous research suggests this may not be the case. In a similar investigation, conducted by Onions et al. (2008), in one group cannulations were performed immediately after oophorectomy whereas in another group cannulations were delayed whilst ovaries were transported from the abattoir to the laboratory. If the follicles in ovaries in the former group exhibited higher rates of survival than in the latter, following orthotopic transplantation this may suggest that the lack of metabolites during this short period of time does have a detrimental effect on follicle survival. However, this was not the case and similar levels of follicle survival were exhibited in both groups.

It is possible that the ovaries started to 'dry-out' during the perfusion process. Even though care was taken to pipette media over the ovaries throughout the perfusion period this may not have been sufficient. Therefore in order to optimise the protocol it may be better to completely submerge the ovary in the perfusion media throughout the entire perfusion period. As one of the aims of this investigation was to determine the level of DMSO permeation into the cortical tissue from the ovarian artery this was not appropriate in the current experimental series. It is also possible that the perfusion process did not allow the media to effectively penetrate all areas of the ovary. Never-the-less the perfusion method utilised here has been successfully used to cryopreserve ovaries therefore it is unlikely that the permeation was incomplete (Onions et al., 2008, Onions et al., 2009, Onions et al., 2013). This protocol could, however, be improved via better optimisation of flow rate and pressure in combination with perfusion time.

6.5 Conclusion

This experimental series has confirmed that the ovarian pedicle and cortex have different optima for CPA penetration following perfusion. To maximise follicle health and stromal integrity following WOCP it is therefore necessary to consider the needs of the different key tissues in complex organs such as the ovary as well as the supporting vasculature of the ovarian pedicle. We have successfully used cortex culture as a means to evaluate ovary health and follicle developmental competence after perfusion with CPA. The results of this investigation suggest that a minimum length of 60 minutes perfusion is required to significantly increase the level of DMSO permeation in the cortical tissue. However, perfusing the ovaries for 60 minutes with CPA is detrimental the subsequent health of the follicles *in vitro* such that perfusion time and CPA exposure time must be balanced against perfusion flow rate and temperature to maximise penetration but minimise cytotoxic damage. These results also suggest that it is some aspect of the perfusion process, other than damage to the vasculature *per se*, that results in the increased follicle loss observed *in vitro*. In order to determine the cause of the increases in follicle loss it would be interesting to repeat the investigation with a number of variations. Perfusion of the ovary with blood, rather than L-15 control medium would help elucidate whether the composition of the perfusion medium has a detrimental effect on follicle survival. Additionally submerging the ovaries in media to ensure the tissue does not dry-out whilst facilitating CPA penetration from the outside into the tissue, in conjunction with CPA permeation from inside the medulla outwards towards the outer cortex may also help to improve follicle survival and development rates. More detailed measurements of cell and molecular markers of cortex integrity and/or stress and measurement of molecular and functional markers of follicle, oocyte apoptosis and hypoxia would be of value.

7 General Discussion

7.1 Introduction

Oogenesis and folliculogenesis are complex and protracted processes. Increasing survival rates of cancer patients, who have been exposed to aggressive treatments that can cause infertility, as well as the increased incidence of POF as a result of trauma, disease or the use of ablative therapies to treat genetic conditions mean that the development of fertility restoration techniques for young girls and women will become increasingly valuable in the future. Current methods of fertility preservation, such as oocyte or embryo cryopreservation are not suitable for prepubertal girls, nor are they ideal for women of reproductive age who do not have a life partner. Recent advances in cryobiology have meant that it is now possible to freeze-store intact ovarian tissue for girls and young women requiring fertility preservation. The development of methods that facilitate the complete *in vitro* growth of oocytes from the primordial to pre-ovulatory stages, from frozen-thawed tissue for fertility restoration would overcome many of the problems posed by the alternative fertility restoration methods. It is essential that prior to the therapeutic use of these methods in humans, parameters for determining the normality and competence of *in vitro*-derived oocytes are established and that following fertilisation the health of the resultant offspring can be guaranteed. In addition to this an IVG system that is able to support the normal growth and development of oocytes would be an invaluable tool for the advancement and testing of novel freezing methods and as a means to quantify the ovotoxicity of new chemotherapy agents and combination therapies.

Therefore the overall aim of this thesis was to develop and validate an IVG culture system that could be used to validate and develop new and existing therapies, such as WOCP. The culture system should be able to support the *in vitro* activation, growth and development of ovine oocytes from the primordial follicle stage to produce EA oocytes that are normal and healthy, within a physiologically-relevant timeframe. In order to achieve this goal the first aim of this thesis was to optimise the separate steps required to support a multistep IVG culture system and then link the steps together, which had not yet been achieved using ovine oocytes. The second aim of this thesis was to compare the use a physiologically-relevant IVG system with an accelerated IVG system, with respect to the effects on follicle and oocyte activation, growth and normality as well as stromal tissue health and normality. In addition the third aim of this thesis was to identify cellular, histological and molecular markers of follicle and oocyte development which could be used to characterise the health and assess the normality of the resultant *in vitro*-derived oocytes. In order to achieve this it was necessary to establish the

normal *in vivo* expression patterns of oocyte- and somatic cell-specific genes which have been identified as key to the process of oogenesis and folliculogenesis, as well as to evaluate imprinted gene expression and genes encoding enzymes that regulate the imprinting process, the aberrant expression of which has previously been linked to epigenetic and growth disorders. Once the normal expression patterns of candidate oocyte, somatic and epigenetic markers were established *in vivo* these could then be compared to those exhibited by *in vitro*-derived oocytes and follicles.

Current methods of ovarian tissue cryopreservation have been optimised for the storage of slices of ovarian tissue. Cryopreserving the whole ovary and its vasculature would greatly increase the number of follicles that are preserved and potentially transplanted back into the patient. The process of optimising WOCP is challenging particularly in terms of establishing the length of time for which the ovary should be perfused with the CPA. The perfusion time must be sufficient for complete permeation of the ovary to occur however as CPAs have cytotoxic effects exposure must be kept to a minimum. Therefore the final aim of this thesis was to use the follicle culture system developed in earlier chapters as a tool to evaluate WOCP for fertility preservation and to help define the optimal perfusion time for penetration of whole ovaries with CPA by using follicle development *in vitro* to assess the level of damage to the ovarian tissue following CPA perfusion.

7.2 Development of a multi-phase culture system

Previous research suggests that the IVG of oocytes from the primordial follicle stage will best be achieved using a multi-step, serum-free culture system in order to support the changing needs of the oocytes and follicles as growth and development progresses (Wandji et al., 1997, Wright et al., 1999, Newton et al., 1999b, Hreinsson et al., 2002, Picton et al., 2008). The conditions in the first phase of the culture system must support the activation, growth and development of primordial follicles to the preantral stage. As primordial follicles are not able to survive in isolated culture some form of support matrix is required, such as a gel matrix, ECM or - as utilised in this thesis - the ovarian cortex itself (Hovatta et al., 1997, Hovatta et al., 1999). An *in situ* ovarian cortical slice culture system was selected in this investigation as in previous studies it has been shown to support the growth and development of oocytes and provides an environment similar to that encountered by the oocytes *in vivo* (Eppig and O'Brien, 1996, Wandji et al., 1996b, Wandji et al., 1997, Wright et al., 1999, Scott et al., 2004a, Telfer et al., 2008, McLaughlin and Telfer, 2010, Chambers et al., 2010, Chambers, 2002, Peng et al.,

2010, Magalhaes-Padilha et al., 2012, Fujihara et al., 2012, Liebenthron et al., 2013, Parte et al., 2013). Chapters 3 and 4 of this thesis confirmed and considerably extended previous studies which used an ovarian cortical slice culture system to support the activation, growth and development of ovine primordial follicles and oocytes over extended periods *in vitro*.

The rates of primordial follicle activation and development observed *in vitro* are often greater than those observed in the adult ovary *in vivo* (Wandji et al., 1996b, Wandji et al., 1997, McLaughlin and Telfer, 2010, Telfer et al., 2008). In some culture systems the increased rates observed are similar to those observed in ovarian tissue in which few or no developing follicles are present, such as the fetal ovary or in frozen-thawed ovarian tissue which has subsequently been grafted back into an ovariectomised animal (Chambers, 2002, Kerr et al., 2006, Sawyer et al., 2002, Konishi et al., 1986, Oktay et al., 1998, Campbell et al., 2000). In other culture systems a much more accelerated rate of development is observed. The optimal rate of follicle development *in vitro* is a point of discussion amongst research groups working in this field. Some research groups propose that the use of accelerated culture systems reduce both the length of time for which the oocytes are exposed to suboptimal conditions and the proportion of atretic follicles at the end of culture (Telfer et al., 2008, McLaughlin et al., 2010), thus resulting in a greater yield of competent oocytes capable of developing further *in vitro*. Advocates of the use of a physiologically-relevant timescale to support the IVG of oocytes suggest that increasing the rate of development to an extent greater than that observed *in vivo* may be damaging to the health and developmental competence of *in vitro*-derived oocytes, as insufficient time would be provided for the necessary accumulation of RNAs and proteins required for oocyte maturation to occur (Picton et al., 1998). In addition there are concerns that the epigenetic modifications that occur during oogenesis will not be correctly established if there is insufficient time during the period of accelerated growth and development, which could be highly detrimental to the subsequent health and developmental competence of oocytes and embryos (Kono et al., 1996, Obata et al., 1998, Bao et al., 2000, Obata and Kono, 2002).

The aim of Chapter 3 was therefore to determine whether ovine primordial follicles which have activated and developed to the preantral stage at a physiological rate *in situ* (Chambers, 2002, Chambers et al., 2010) were competent to develop to the EA stage when isolated and put into individual culture (Newton et al., 1999b). The results presented here clearly demonstrate that the first step of this multi-phase culture system was able to support the

growth and development of ovine primordial follicles up to the multi-layered preantral stages. Follicles, EP to secondary- staged, derived following a culture period of 16-23 days were isolated and cultured individually. Only follicles at the secondary stage at the point of isolation were competent to develop to the EA stage in individual culture, in agreement with what has previously been reported with *in vivo*-derived follicles (Hovatta et al., 1999, Newton et al., 1999b). This is the first report of a 2-step culture system able to support the growth and development of ovine primordial follicles to the EA stage at a physiological rate. Similar survival, growth and development rates were exhibited by *in vivo*- and *in vitro*-derived secondary follicles up to day 19 of preantral culture. It may be interesting to use transmission electron microscopy to assess the effects of both length of isolated preantral follicle culture and follicle derivation (i.e. *in vivo* or *in vitro*) on the maintenance of oocyte-GC interactions during culture, as has been done previously in rats (Zhao et al., 2001).

The duration of the first phase of *in situ* culture was evaluated in Chapter 3 to test how the length of cortex culture would impact on the yield and health of the preantral follicles so derived. To address one of the outstanding questions highlighted above regarding the optimal developmental time frame *in vitro* (i.e. rapid/ accelerated growth rates vs. slow/ physiological growth rates) cultures were conducted over a 6 day period in order to determine whether follicle activation, growth and development to the secondary stage could be supported in defined, serum-free conditions over this short period as has previously been advocated by Telfer et al. (2008). Indeed, previous studies utilising bovine and human ovarian tissue have reported the development of primordial follicles to the transitional stage following cortical culture for 6 days, using an accelerated culture system developed in Edinburgh (Telfer et al., 2008, McLaughlin and Telfer, 2010). Therefore in order to determine whether this was possible using ovine tissue and to assess the effects on oocyte, follicle and stromal health using an accelerated culture, the system developed by the Edinburgh group was adapted and compared to the physiologically-relevant, slow growth rate culture system developed in Leeds, in Chapter 4.

One parameter by which the physiological and accelerated culture systems were compared was the follicle population dynamics at the end of culture. Collectively, the data reported in this thesis (Figure 7.1) clearly show that the overall mean number of primary, transitional and secondary follicles present at the end of short-term accelerated culture is significantly reduced compared to the numbers generated following long-term physiological rate culture, after

correction for tissue weight *in vitro*. Use of the long-term physiological rate culture system resulted in significantly more transitional ($p<0.01$) and secondary ($p<0.05$) follicles at the end of the culture period than the short-term accelerated system. The number of secondary follicles derived using the accelerated 6 day system was simply too small to progress to step 2 and conduct meaningful isolated follicle cultures, therefore it was not possible to compare the developmental competence of *in vitro*-derived secondary follicles, grown using the accelerated- or the physiological culture systems.

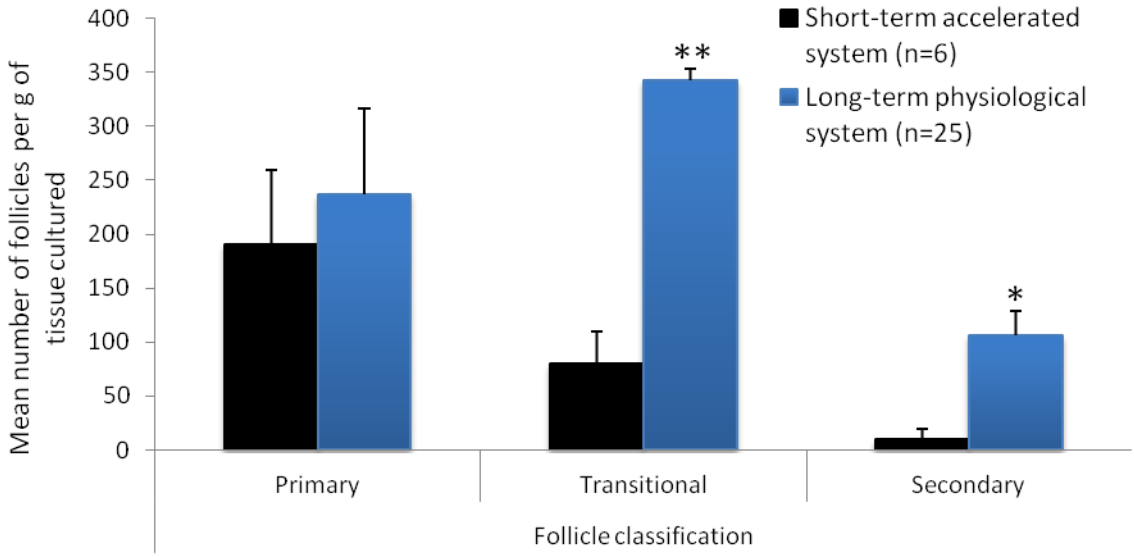


Figure 7.1 Histogram comparing the effect of the use of a short-term accelerated system to a long-term physiological system, in which tissue was cultured for 6 or 16-23 days, respectively, on follicle population dynamics. The mean number of primary, transitional and secondary follicles per g of tissue present at the end of the culture period ± SEM for the number of culture repeats (n) indicated, is shown * and ** indicate statistical significance at $p<0.05$ and $p<0.01$, respectively.

As previously mentioned, advocates of the use of an accelerated culture system suggest that a lower proportion of follicles would be degenerating at the end of short-term culture, compared to long-term culture. Collation of the results from Chapters 3 and 4 of this thesis with regard to the mean number of degenerating follicles per mg tissue at the end of culture (Figure 7.2), shows that this is not necessarily the case. While it must be acknowledged that increasing the length of cortical culture from 6 to 16-23 days resulted in a significant increase ($p<0.05$) in the mean number of degenerating follicles when using step 1 of the physiological culture system, the short-term 6 day accelerated culture system resulted in a higher mean number of degenerating follicles both before and after culture, than any of the culture durations tested in the physiological culture system. The large amount of variability in the data

mean that these differences are not statistically significant (p>0.05). Therefore it is likely that the combination of the method of tissue preparation and/or suboptimal culture conditions have resulted in the increased levels of follicle degeneration in the accelerated culture system. As described in Chapter 4 the method of tissue preparation used in the studies published by the Edinburgh group for bovine and human tissues differs substantially from the method used by the Leeds group in that the majority of medulla is removed and the tissue is teased apart prior to culture, in order to reduce the concentration of inhibitory factors present. This strategy appeared to be very damaging to the ovine cortical tissue and could be one of the causes of the increased rates of follicle degeneration observed when the accelerated culture approach was applied to this species. It is possible that the bovine and human tissue used in previous published studies was tougher and therefore less susceptible to mechanical damage. The results presented suggest that it would be of value to conduct further studies to investigate the impact of methods of reducing damage and/or reducing or conversely increasing the amount of medullary or stromal tissue present at the start of culture on follicle and oocyte health *per se* and the developmental potential of primordial follicles and hence yield of *in vitro*- derived secondary follicles.

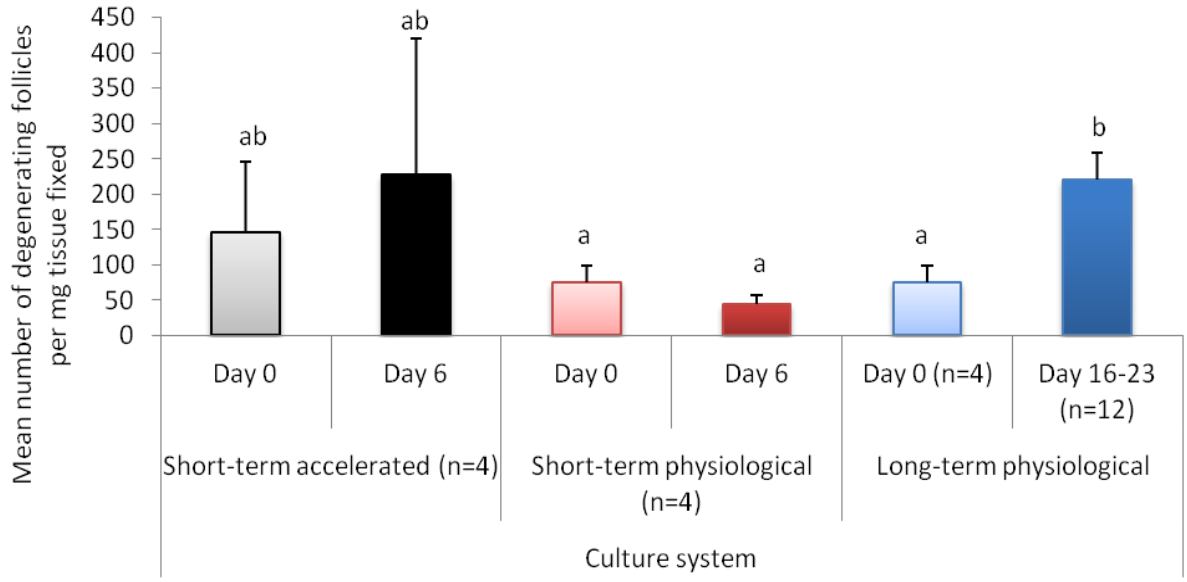


Figure 7.2 Histogram showing the effect of culture system conditions on levels of follicle degeneration assessed histologically. The mean number of degenerating follicles per mg fixed tissue at the start and end of culture ± SEM for the number of culture repeats (n) indicated. Data was analysed using one-way ANOVA and statistical differences are denoted with the use of different letters (p<0.05).

Overall the results presented in Chapters 3 and 4 indicate that in the ovine model a physiologically-relevant 2-step system is able to support the activation and development of primordial follicles to the secondary stage within a period of 16-23 days, and that these follicles can then be isolated and put into individual culture and that ~19% are competent to develop to the EA stage. In order to further assess the developmental competence and normality of the *in vitro*-derived follicles and oocytes, in Chapter 5, expression patterns of a panel of candidate genes which are central to oogenesis and folliculogenesis were mapped across development *in vivo* and compared to that observed *in vitro*.

7.3 Assessment of the effect of culture conditions on the health of stromal tissue

The maintenance of the health and integrity of ovarian stromal tissue during cortical culture is of great importance to the health of the follicles and oocytes and their continued growth and development (Knight and Glister, 2006). Therefore a secondary aim of Chapters 3 and 4 was to evaluate the effect of different culture environments on the health of the ovarian stromal by comparing tissue weight at the start and end of culture. The ovarian stroma provides a biological scaffold essential for the maintenance of oocyte-GC interactions within primordial and early preantral follicles, and the stroma is thought to contribute regulatory factor(s) with various functions, including the inhibition of primordial follicle assembly (AMH (Nilsson et al., 2011)), the maintenance of primordial follicle arrest (Telfer and McLaughlin, 2012) and the activation primordial follicle growth *in vivo*, such as KL, KGF, BMP4 and BMP7 (Knight and Glister, 2006, McLaughlin and McIver, 2009, Monget et al., 2012). In addition ovarian stromal cells are essential for folliculogenesis as they are the precursors of theca cells (Orisaka et al., 2006) and produce steroid hormones, A4 and testosterone which promote continued follicle growth and development (Qiu et al., 2013) The survival and growth of the ovarian stroma during *in situ* cortex cultures is therefore of great importance.

The mean tissue weights at the start and end of culture were used an index of stromal viability *in vitro* following cortex exposure to the accelerated 6 day culture system, or short-term culture in the slow growth, physiological system for 6 days or long-term culture in the slow growth, physiological system for 16-23 days (Figure 7.3). In none of the culture systems or timeframes was the change in cortex tissue weight at the end of culture statistically significant ($p>0.05$) when compared to tissue weights at time 0. It is however worth noting, that an increase in tissue weight indicative of stromal proliferation and/or follicle and oocyte growth was recorded at the end of culture for both the short- and long-term time points in the

physiological, slow-growth culture system, in contrast the accelerated culture environment resulted in a small decrease in weight. These preliminary findings suggest that the culture environment provided by the physiological culture system is better able to support the survival and growth of the stromal tissue. The increase in tissue weight observed using the physiological culture system was greater following 16-23 days, than 6 days, which further supports the idea that stromal tissue survival and growth continues throughout the culture period.

In order to further investigate the effects of tissue preparation and culture conditions on stromal tissue proliferation additional markers of stromal health and development are required. In Chapters 4 and 6 the level of stromal cell division was crudely assessed using histological techniques. These methods could be developed in order to determine stromal cell density, for example by counting stromal cell nuclei (Maffei et al., 2013). In addition to this stromal cell proliferation could be assessed using polyclonal antibodies for Ki67 or by assessing levels of thymidine incorporation (Maffei et al., 2013). As discussed in Chapter 4, the use of stromal cell specific markers, such as vimentin, and the identification of other potential markers would aid investigations (Gunson, 2009).

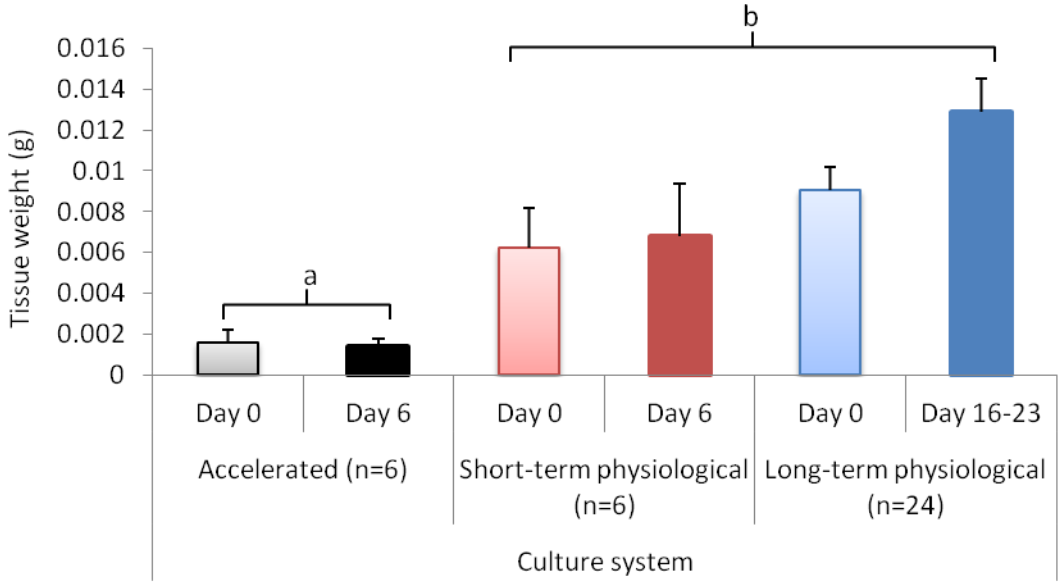


Figure 7.3 Effect of culture system on tissue weight at the start and end of the cortical culture period. The effect of using either a system designed to support the accelerated growth of follicles over 6 days or the physiological growth of follicles over 6 (short-term) or 16-23 days (long-term) on mean tissue weight per well ± SEM for the number of culture repeats (n) indicated, is shown. Data were analysed using one-way ANOVA and statistical differences are denoted with the use of different letters ($p<0.05$).

As discussed in Chapters 4 and 6 the health of the stromal tissue was also assessed in fresh tissue, via assessment of NR staining. As discussed above the method of trimming the tissue into pyramid shapes, rather than squares and use of needles to tease the tissue apart was shown to have a significantly detrimental effect on tissue health in Chapter 4. Follicle viability has been linked to stromal tissue proliferation in feline ovarian tissue *in vitro* (Fujihara et al., 2014). Addition of EGF to the culture media of feline ovarian tissue indirectly resulted in an improvement in primordial follicle viability via the activation of the MAPK and PI3K signalling pathways which promoted stromal cell proliferation. Levels of primordial follicle activation, however, did not increase. Thus these results highlight the importance of stromal tissue health and proliferation in maintaining follicle viability and supporting physiologically-relevant rates of growth and development. Additionally the increased rates of follicle activation observed using the accelerated system compared to the physiological system (See Chapter 4) are likely to have resulted from the method of tissue preparation as reducing the thickness of cortical tissue has been shown to result in an increase in levels of follicle burnout in both fresh and frozen-thawed bovine ovarian tissue, possibly as a result of a reduction in the concentration of stromal-derived inhibitory factors (Gavish et al., 2014). This theory could be further tested by manipulating the physiological culture system developed in Chapters 3 and 4 to determine the effects of cortical slice size, thickness and shape on levels of follicle burnout in ovine ovarian tissue.

The methods of stromal health assessment used in Chapters 4 and 6 were not used on tissue cultured for 16-23 days therefore in the future it would be interesting to assess the tissue in this manner to determine whether the culture conditions are capable of supporting the maintenance of stromal integrity for a period of up to 23 days. It is possible that the increasing rates of follicle degeneration observed following long-term culture compared to short-term culture are due to decreasing stromal integrity. As the primordial and small preantral follicles require more support and are less able to survive and grow in isolated culture compared to multi-layered follicles, as shown in Chapter 3 (Hovatta et al., 1999) it is likely that these follicles were degenerating towards the end of the culture period, whereas the multi-layered follicles were still provided with sufficient support and therefore were able to survive and grow. As the aim of the cortical culture was to produce competent, healthy multi-layered follicles a culture system that supports the integrity of the stromal health for a sufficient length of time for primordial follicles to activate and grow to a stage at which less support is required may be adequate. Indeed a slight reduction in stromal integrity may be preferable to ensure follicle growth is not restricted. It would be interesting to investigate these hypotheses in the future.

The effects of culture conditions could also be assessed using PCNA/ TUNEL staining (Chambers, 2002).

A couple of recent studies have focused on the interaction between caprine GCs and stromal cells during co-culture *in vitro* (Qiu et al., 2013, Qiu et al., 2014). Regulation between the GCs and stromal cells is bidirectional. Co-culture of stromal cells with GCs resulted in increased levels of A4 and testosterone production, *STAR* and *BCL-2* (anti-apoptotic) expression and LH responsiveness as well as a decrease in *BAX* and *BAD* (pro-apoptotic) expression in stromal cells (Qiu et al., 2013). Similarly the effects of stromal cell co-culture on GCs included increased E2 production, *STAR* expression, cell proliferation, *BCL-2* expression as well as a decrease in *BAX* and *CASPASE 3* expression (Qiu et al., 2014). It would be interesting to extend these methods using these markers of stromal health (i.e. A4 and testosterone production, LH responsiveness, expression of steroidogenic enzymes and pro-/anti-apoptotic genes) to ascertain the effects of culture conditions, tissue preparation, exposure to chemotherapeutic agents and novel freezing methods on stromal tissue health.

7.4 Analysis of gene expression patterns

The level of expression of key genes in *in vivo*- and *in vitro*-derived follicles was also used as a measure of normality. Analysis of gene expression patterns suggest that the *in situ* cortical physiological culture system utilised in Chapter 3 was able to provide an environment similar to that encountered by the follicular somatic cell compartment *in vivo* as the expression patterns of key somatic cell genes in primary, transitional and secondary follicles were similar *in vivo* and *in vitro*. The only statistically significant ($p<0.05$) difference observed was the decrease in *IGF1R* expression in *in vitro*- compared to *in vivo*-derived transitional follicles. In contrast the conditions encountered by the follicles during individual preantral culture appear to have resulted in the aberrant expression of *AMH*, *IGF1*, *INHα*, *INHβA* and *FST* as statistically significant increases ($p<0.05$), with the exception of *IGF1* expression, which significantly decreased ($p<0.05$), in levels were detected in *in vitro*-derived compared to *in vivo*-derived EA follicles. The fact that the expression patterns of genes were altered to a greater degree following exposure to isolated culture, compared to *in situ* culture is unsurprising as the conditions were more different in the former environment to those the follicles are exposed to *in vivo*. It is also possible that some/all of these changes may be due in part to the impact of the removal of the theca cell layer during enzyme isolation and the subsequent growth of 'theca-free' follicles, as discussed in Chapter 5. It is interesting to note that secondary follicles

that were isolated from fresh ovarian tissue and individually cultured until they stopped growing and were deemed degenerating also exhibited a significant ($p<0.01$) increase in *INHα*, *INHβA* and *FST* gene expression. Therefore some of the effects of preantral culture conditions on the levels of gene expression in *in vitro*-derived EA follicles can also be observed in secondary follicles. Although these secondary follicles were degenerating, the fact that *INHα*, *INHβA* and *FST* gene expression was higher in follicles that were able to progress to the EA stage *in vitro* show that these aberrations do not necessarily prevent continued growth and development. It would be interesting to investigate the effects of isolated culture on EP, primary and transitional follicles in the future.

The expression patterns of oocyte-specific genes, however, were affected by *in situ* culture conditions to a greater extent than somatic-cell specific genes. Significant decreases ($p<0.05$) in *ZP2, GTSF1, BMP6* and *BMP15* expression were observed in *in vitro*-derived secondary follicles and in *ZP2* and *GTSF1* expression in *in vitro*-derived primary follicles compared to their *in vivo*-derived counterparts. Of the imprinted genes only the expression of *MEST* was significantly affected by the *in situ* cortical culture conditions, as lower levels of expression were observed in *in vitro*-derived primary ($p<0.01$) and transitional ($p<0.05$) follicles compared to *in vivo*-derived counterparts. In order to extend this investigation the levels of DNA methylation of key imprinted genes could be assessed in *in vivo*- and *in vitro*-derived counterpart samples.

As discussed in Chapter 5, based on the expression patterns observed *in vivo* and *in vitro* the genes selected as molecular markers of normality are *AMH, IGF1R, INHα, INHβA, ZP2, GDF9, GTSF1, BMP6, BMP15, MEST, H19, DNMT1o* and *DNMT1s*. These are the genes that would therefore be used to screen all IVG-derived follicles and oocytes in the future.

In this thesis the molecular techniques developed were utilised to compare the expression of genes in *in vivo*- and *in vitro*-derived follicles. In order to extend this work it would also be interesting to compare levels of primordial follicle activation in accelerated and physiological rate culture systems by analysing the expression patterns of genes associated with follicle activation, such as *GDF9*, *LHX8*, *AMH, mTOR* and *PTEN* (McLaughlin et al., 2011, Parte et al., 2013). Additionally, if sufficient numbers of preantral follicles could be derived using an accelerated cortical culture system, it would be interesting to compare levels of gene expression between both *in vivo*-derived follicles and follicles derived *in vitro* using the long-

term physiological rate system in equivalently staged follicles. It would also be interesting to compare the gene expression patterns exhibited by follicles grown *in vitro* following mechanical isolation (i.e. theca intact) vs. enzymatic recovery (theca free), and the effects of using different types of enzymes. In support of this idea, altering ovarian cortical culture conditions has previously been shown to affect the expression levels of genes involved in early folliculogenesis in human tissue (Liebenthron et al., 2013). In this study a conventional cortical culture system and a fluidic dynamic cortical culture system were compared. The expression levels of *KL*, *GDF9* and *INHB* were analysed using RT-PCR. Use of the dynamic fluidic system resulted in a higher number of healthy follicles after 6 days culture as well as increased levels of *KL*, *GDF9* and *INHB*. Authors suggested that the increased expression of these genes indicated increased levels of follicle activation. Therefore it would be interesting to explore methods of optimising particularly the second step of the culture system, in which preantral follicles are cultured whilst using a combination of morphological markers, growth and survival rates, antral cavity formation rates, steroid hormone assays and gene expression analysis to assess the effects of any changes.

7.5 Use of follicle culture as a tool to quantify the impact of cryoprotectant perfusion for whole ovary cryopreservation

The physiological, cortex culture system developed and validated in Chapters 3 and 4 was utilised to assess the effects of whole ovary perfusion on stromal tissue health and follicle population dynamics in Chapter 6. It is well established that primordial follicles display the highest levels of tolerance to the cryopreservation procedure and therefore their health and developmental competence following the perfusion process which precedes WOCP is of great importance. As previously discussed the culture system developed in Chapter 3 supports the activation, growth and development of primordial follicles at a physiological rate as well as maintaining the health of the stromal tissue, it is therefore a useful tool to quantify the effects of CPA exposure and whole ovary perfusion on the immediate health of the stromal tissue and its ability to support the growth and development of the early-staged follicle population.

The main aim of Chapter 6 was to determine the minimum perfusion time required for 1.5M DMSO to fully permeate the ovine ovarian cortex and vascular pedicle in whole ovaries using NMR spectroscopy. As exposure to DMSO is cytotoxic to ovarian tissue (Meryman, 1971, Trounson and Kirby, 1989, Picton et al., 2000, Santos et al., 2006) the second aim of Chapter 6 was to assess the level of damage to the stromal tissue and follicles using the LDH assay of cell

membrane damage in conjunction with NR staining as acute assays of tissue damage/ health, in conjunction with 8 day cortex culture and histological analysis as a means to assess the longer term impact of CPA cytotoxicity on follicle and oocyte health and developmental competence. In order to significantly increase the level of DMSO permeation in the cortical tissue a perfusion period of 60 minutes was required. It was found that it was the perfusion timeframe and/or the perfusion pressure used, rather than the CPA exposure *per se* that was detrimental to the subsequent health of the follicles and stromal tissue *in vitro*.

In order to extend the findings of this experiment it would be necessary to further analyse the integrity or conversely damage to the pedicle tissue by assessing its permeability with fluorospheres (Onions et al., 2013). It would also be of value to use the molecular markers of normality developed in Chapter 5 to assess the normality of gene expression, in addition to assessing the expression of genes and proteins associated with apoptosis, such as *BAX, BCL2* and *CASP6* and hypoxia, such as *THBS1*, Ki67, and *ET-2* (Onions et al., 2013). As discussed in Chapter 6 changes in the expression patterns of *THBS1* and *ET-2* have been detected following whole ovine ovary perfusion and cryopreservation in a manner that suggests the cells of the ovarian medulla and pedicle experienced hypoxia. In addition to this the use of these procedures resulted in the up-regulation of pro-apoptotic gene, *BAX* and down-regulation of anti-apoptotic gene, *BCL2*, in both the pedicle and medulla, however, pro-apoptotic gene, *CASP6* was down-regulated in the medulla, suggesting that in this tissue type apoptosis was suppressed. Down-regulation of *THBS1*, which is also associated with wound repair, also suggests that this process was induced in the pedicle and medulla. Further experiments must be conducted to balance the perfusion time against perfusion pressure and CPA penetration in order to better optimise WOCP. The next step in optimising this protocol for sheep and/or human ovaries is to use the multi-phase follicle culture techniques developed in Chapter 3 to test the integrity, health and developmental competence of follicles and oocytes after WOCP.

7.6 Final conclusions

The research presented in this thesis supports the hypothesis that the complete *in vitro* growth of oocytes is suitable as a tool to both assess the patency of fertility preservation systems and as a means to restore female fertility.

The first aim of this thesis was to develop and validate a culture system that supports the activation, growth and normal development of ovine primordial follicles to the EA stage of development within a physiologically relevant timescale. This was partly achieved in Chapter 3 as the 2-step system was able to support the activation and development of primordial follicles to the secondary stage *in situ*, within slices of ovarian cortex, then following follicle isolation the *in vitro*-derived secondary follicles were competent to develop to the EA stage. However, although the second step of the system was able to support the growth and development of *in vivo*- and *in vitro*-derived secondary follicles, the rates of survival and antral cavity formation were low. The second aim of this thesis was to compare the physiologically-relevant IVG system developed in Chapter 3 with an accelerated IVG system using various markers of normality and in terms of the effects on the rates of follicle activation, growth, development and survival. From the evidence presented in Chapter 4 it can be concluded that the activation, growth and development of ovine oocytes is better supported using a physiologically-relevant IVG system.

The third aim of the thesis was to identify and establish the normal expression patterns of 20 important oocyte- and follicular somatic genes across oogenesis and folliculogenesis *in vivo* and *to* compare these to gene expression patterns in stage-matched oocytes and follicles grown *in vitro*. The comparison of gene expression patterns in stage-matched follicles developed *in vivo* and *in vitro* gave an indication of why rates of development and antral cavity formation in isolated preantral culture were low as the analysis of gene expression profiles revealed aberrant expression of numerous somatic cell genes. In the future gene expression analysis could be used to optimise the preantral follicle culture conditions as culture conditions that support normal patterns of gene expression may also result in increased rates of development and survival. If sufficient EA follicles could be derived *in vitro* following the implementation of improvements to the conditions in the first 2 steps the system could be extended and ultimately the *in vitro* growth and development of primordial follicles up to the MII mature oocyte stage achieved. Continued use of markers of normality could be used to develop this technique in order to optimise it for the use in humans. The fourth aim of this thesis was to utilise the ovarian cortex culture system at a tool to validate WOCP as a fertility preservation strategy, using sheep as a model for humans. This enabled the investigation of the effects of whole ovary perfusion on the growth and survival rates of follicles within the perfused ovarian tissue *in vitro*. In addition the impact of ovarian tissue preparation and culture conditions on stromal tissue integrity *in vitro* was evaluated. The results presented in Chapters 4 and 6 suggest that the aforementioned factors have a significant effect on stromal

tissue integrity. In the future it would be interesting to extend these findings to determine the importance of stromal tissue integrity. Hopefully eventually the combination of whole ovary perfusion and cryopreservation and *in vitro* growth, maturation and fertilisation of oocytes could be used as a safe and reliable method of fertility preservation in large mammals and humans.

The development of the technology to support the IVG and development of oocytes is integral to improving existing methods of fertility reservation and restoration, but it is also vital to assisting in quantifying the likely risk of ovarian tissue and follicle/ oocyte damage or loss following cytotoxic insult - *in vivo* or *in vitro* - and in developing new and improved methods of ovarian preservation, see Figure 7.4.

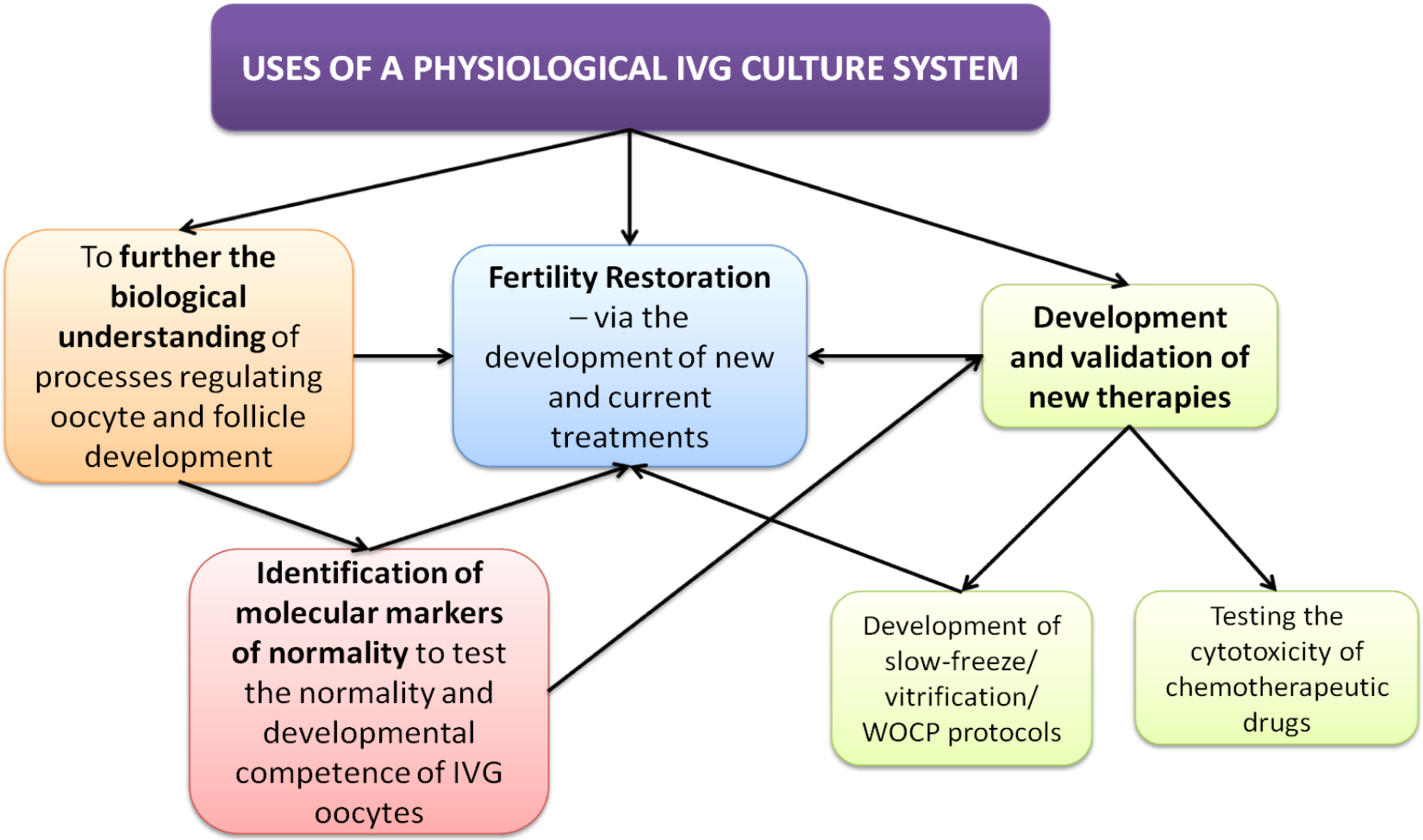


Figure 7.4 Summary of the uses of a physiological IVG culture system.

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Appendix I. *Solutions for Culture Media*

Basal Culture Media

To make a final volume of 50ml 0.5ml Penilcillin G/ streptomycin solution was added to 49.5ml α-MEM with bicarbonate, to give a final concentration of 1%(v/v). BSA Fraction V (0.05g), fatty acid free (A6003);cell culture tested was then added to give a final concentration of 1mgml^{-1}. The solution was filter sterilised and stored for up to 2 weeks.

Human Transferrin (5mgml^{-1})

Human Transferrin (100mg) was dissolved in 20ml α-MEM containing 0.1% BSA then filtered and aliquoted in to 25µl volumes and stored at -20^{o}C.

Sodium Pyruvate (0.47mM)

Sodium pyruvate (51.7mg) was dissolved in 10ml of tissue culture grade water, filtered and stored at 4^{o}C until use, for up to one week.

Sodium Selenite (50µgml^{-1})

Sodium selenite (1mg) was dissolved in 20ml α-MEM containing 0.1% BSA then filtered and aliquoted in to 10µl volumes and stored at -20^{o}C.

L-Glutamine (200mM)

L-Glutamine (200mM) was placed in a sonicator bath to ensure full dissolution then aliquoted into 500µl volumes and stored at -20^{o}C. This solution was not filtered.

Bovine Insulin (10µgml^{-1})

Bovine insulin (100mg) was dissolved in 10ml 3% acetic acid in tissue culture grade water (pH 2-3) then filtered and aliquoted in to 10µl volumes and stored at -20^{o}C, this is stock 1. Immediately prior to use add 1µl of stock 1 to 999µl α-MEM containing 0.1% BSA (10µgml^{-1}).

Long R3 IGF-1 (10ngml^{-1})

Long R3 IGF-1 (1mg) was added to 10mM Hydrochloric acid (1ml). Secondly α-MEM (9ml) containing 0.1% BSA was added. The solution was filtered and aliquoted in to 10µl volumes and stored at -20^{o}C.

Ovine FSH for IVG (0.1Uml^{-1})

Ovine FSH (50U per vial) was added to α-MEM (500ml) containing 0.1% BSA then filtered and aliquoted in to 10µl volumes and stored at -20^{o}C.

Ovine FSH for IVM (200µgml^{-1})

Ovine FSH (9mg) was first dissolved in 4.5ml 10% BSA, then added to 40.5ml M199 (M2154 bicarbonate-buffered) then filtered and aliquoted in to 250µl volumes and stored at -20^{o}C.

Ovine LH (0.0023Uml^{-1})

Ovine LH (25U per vial) was added to α-MEM (10.87ml) containing 0.1% BSA, this is stock 1. Stock 1 (50µl) was added to α-MEM (49.5ml) containing 0.1% BSA then filtered and aliquoted in to 10µl volumes and stored at -20^{o}C.

Ovine LH (200µgml^{-1})

Ovine LH (9mg) was first dissolved in 4.5ml 10% BSA, then added to 40.5ml M199 (M2154 bicarbonate-buffered) then filtered and aliquoted in to 250µl volumes and stored at -20^{o}C.

Solutions for Enzymatic Isolation of Follicles

Hyaluronidase Stock (80IUml^{-1})

Bovine hyaluronidase (12.1mg) was added to 50ml Hepes-MEM then filtered and aliquoted in to 1ml volumes and stored at -20^{o}C.

Collagenase (478.7Uml^{-1})

Collagenase (100mg of 478.7Umg^{-1} solid) was added to Leibovitz L-15 medium containing 10% fetal calf serum, then filtered and aliquoted in to 20ml volumes and stored at -20^{o}C.

Bovine Deoxyribonuclease (DNase; 0.5mgml^{-1})

To one vial of DNase (0.5mg/ ~2,000 Kunitz units per vial) sterile PBS (1ml) was added. The solution was then aliquoted in to 42µl volumes and stored at -20^{o}C.

Bovine Serum Albumin (BSA) Stock (2% (w/v)

Bovine Serum Albumin (A6003; 5mg) was added to Hepes-MEM (250ml), then filtered and aliquoted into 26ml volumes and stored at -20^{o}C.

10x Buffer Stock

The 10x buffer stock was made up in a 500ml volumetric flask. Sodium Bicarbonate (1.6802g), Hepes (free acid; 12.51g) and Hepes (sodium salt; 13.665g) were added to the volumetric flask. Culture tested water was then added to make up a final volume of 500ml. The flask was inverted 3-5 times to mix, then the solution was filtered and kept at 4^{o}C for up to 3 months.

Follicle Isolation Medium (FIM)

Follicle isolation medium was made up in a 500ml volumetric flask as detailed in the table below. Note that the culture tested water was added last to make the solution up to a final volume of 500ml. The flask was inverted 3-5 times to mix, then the solution was filtered and stored at 4^{o}C for up to 2 weeks.

Additive	Volume (ml)	Stock Concentration	Final Concentration
MEM 10x Stock	50	10x	1x
Buffer 10x Stock	50	10x	1x
Penicillin G/ Streptomycin	5	100%	1%
Sodium Pyruvate	0.5	47mM	0.047mM
BSA Stock	25	2% (w/v)	1mgml^{-1}
Heparin	0.2	5000Uml^{-1}	20Uml^{-1}
Culture tested water	Make up to 500ml		

Composition of basal culture media

	α - MEM Media (M4526)	McCoy's 5a Media (M8403)
	[1x]	[1x]
COMPONENT		
Inorganic Salts	g/L	g/L
$CaCl_2$•$2H_2O$	0.2	0.132432
$MgSO_4$ (anhydrous)	0.09767	0.097688
KCl	0.4	0.4
NaHCO3	2.2	2.2
NaCl	6.8	6.46
Na2HPO4 (anhydrous)	0.122	0.504
Amino Acids		
L-Alanine	0.025	0.01336
L-Arginine • HCl	0.126	0.04214
L-Asparagine • H_2O	0.05	0.04503
L-Aspartic Acid	0.03	0.01997
L-Cysteine	-	0.02424
L-Cysteine • HCl •H_2O	0.1	-
L-Cystine • 2HCl	0.0313	-
L-Glutamic Acid	0.075	-
L-Glutamine	-	-
Glycine	0.05	0.00751
L-Histidine • HCl • H_2O	0.042	0.02096
Hydroxy-L-proline	-	0.01967
L-Isoleucine	0.052	0.03936
L-Leucine	0.052	0.03936
L-Lysine • HCl	0.0725	0.03654
L-Methionine	0.015	0.01492
L-Phenylalanine	0.032	0.01652
L-Proline	0.04	0.1787
L-Serine	0.025	0.02628
L-Threonine	0.048	0.01787
L-Tryptophan	0.01	0.00306
L-Tyrosine • 2Na • $2H_2O$	0.0519	0.0261
L-Valine	0.046	0.01757
Vitamins		
Ascorbic Acid	-	0.000563

L-Ascorbic Acid • Na	0.05	-
D-Biotin	0.0001	0.001
Choline Chloride	0.001	0.0002
Folic Acid	0.001	0.005
myo-Inositol	0.002	0.01
Lipoic Acid	0.0002	-
Niacinamide	0.001	0.036
Nicotinic Acid	-	0.0005
D-Panthothenic Acid • 1/2Ca	0.001	0.0002
Pyridoxal • HCl	0.001	0.0005
Pyridoxine • HCl	-	0.0005
Riboflavin	0.0001	0.0002
Thiamine • HCl	0.001	0.0002
Vitamin B_{12}	0.00136	0.002
Other		
D-Glucose	-	3
Glucose	1	-
Glutathione (reduced)	-	0.0005
Peptone	-	0.6
Phenol Red • Na	0.011	0.011
Pyruvic Acid	0.11	-

Appendix II. *Solutions for Histology*

4% Paraformaldehyde (PFA) solution

Paraformaldehyde (4g) was dissolved in phosphate buffer solution (100ml) in a glass bottle inside a flow hood, whilst wearing a mask. The solution was heated to 60^{o}C with agitation to ensure full dissolution. After allowing the solution to cool to room temperature it was transferred to 10ml glass eppendorfs (SLS).

Appendix III. *Solutions for Molecular Analysis*

Lysis buffer mix

Reagent	Volume (µl)
DynalR Lysis Buffer (Invitrogen)	910
RNA later (Invitrogen)	50
20% Sodium dodecyl sulphate	20
Igepal	20

The lysis buffer mix was prepared as detailed in the above table. Note that the DynalR Lysis Buffer was stored at 4^{o}C and needed time to warm up to room temperature before use to prevent precipitation of the RNA later. The solution was vortexed then stored at -20^{o}C.

1x Tris Borate EDTA buffer (1xTBE)

Tris (108g), Boric Acid (55g) and EDTA (7.4g) were added to 10 litres of distilled water and mixed on a magnetic stirrer, then stored at room temperature.

1% agarose gel

Agarose (1g; Bioline Ltd) was added to 100ml 1xTBE and heated in the microwave for ~2minutes, or until the agarose had completely dissolved. The solution was cooled by rinsing under a cold tap prior to the addition of 2µl ethidium bromide (10mgml^{-1}). The mixture was gently swirled to mix before being poured into a gel tray clamp (Bio-rad Laboratories Ltd, Hertfordshire, UK) which contained a comb to form wells. The gel was left for ~20minutes to set, prior to addition of the samples.

Gel loading buffer

Gel loading buffer was prepared by adding Bromophenol Blue (0.125g), Xylene Cyanol FF (0.125g) to Glycerol (15ml) and HPLC grade water (35ml).

1000bp ladder

The 1000bp ladder was prepared by mixing 100bp marker (12µl; Invitrogen Ltd), loading buffer (20µl), 5M NaCl (0.4µl) and HPLC grade water (67.6µl).

Primer sequences of excluded genes. Primers successfully and specifically amplified cDNA however, when used for RT PCR analysis of the melting curve along with high levels of expression in negative samples lead to these genes being excluded from the study, in Chapter 5.

Gene	**Primer sequence**	**Size (base pairs)**	**Reference/ Species**
GREMLIN	F: 5’ GAAGCGAGACTGGTGCAAAAC 3’	93	AY942576 (ovine)
	R: 5’ CCGTAGCAGAAGCGGTTGA 3’		
AREG	F: 5’ AAAAGGGAGGCAAAAATGGA 3’	170	
	R: 5’ CTTTTCCCCACATCGTTCAC 3’		
EREG	F: 5’ AGTCCACAGCTGGCTAGGAA 3’	140	XM2688367 (bovine)
	R: 5’ CGGGTTTTGTGGAAGACAAT 3’		
LHR	F: 5'CACGGTACCAGGAAATGCTT3'	145	L36329 (ovine)
	R: 5'CAGGTGTGCATTCTCCTTCA3'		
FIGLA	F: 5'GCTCCTGGACGACGTGCT3'	114	XM_002691232.1 (bovine)
	R: 5'CTCCAGCACCAACTGCAC3'		

Appendix IV. *Solutions for Ovary Perfusion*

Ringer's Solution

Ringer's solution was prepared as detailed in the table below. The pH was adjusted to 7.2-7.4 using either 1M HCl or 1M NaOH, as required. The solution was filter sterilised and stored at 4 ^{o}C for up to 2 weeks, prior to use. Immediately prior to use 600µl of heparin (25,000 iuml^{-1}) was added per 150ml Ringer's solution, to give a final concentration of 100iuml^{-1}.

Reagent	Quantity	Extra Details
NaCl	8.3g	
KCl	0.3g	
$NaHCO_3$	0.17g	
$MgCl_2$	5g	
$CaCl_2$ (2M)	1ml	Prepared by adding 2.22g to 10ml distilled water
Distilled water	800ml	

Cryoprotective Agent (CPA) solution

A solution of CPA solution was made using the components listed in the table below, then adding Leibovitz solution to give a final volume of 100ml. The pH was adjusted to 7.2-7.4 using either 1M HCl or 1M NaOH, as required. The solution was filter sterilised and stored at 4 ^{o}C for up to 1 weeks, prior to use.

Reagent	Quantity	Final Concentration
Dimethylsulphoxide (DMSO; Fisher)	11.72g	1.5M
Sucrose	3.42g	0.1M
Fetal calf serum (FCS)	10ml	10% (v/v)

Phenol red-free basal culture medium

To make a final volume of 50ml 0.5ml PenilcillinG/ streptomycin solution was added to 49.5ml phenol red-free α-MEM (Invitrogen), to give a final concentration of 1%(v/v). BSA Fraction V (0.05g), fatty acid free (A6003);cell culture tested was then added to give a final concentration of 1mgml^{-1}. The solution was filter sterilised and stored for up to 2 weeks. Note that phenol red-free α-MEM that was close to its expiry was used as this was optimal for the LDH assay.

Composition of L-15 medium (L5520)

Component	gL^{-1}
Inorganic Salts	
Calcium Chloride	0.1396
Magnesium Chloride	0.0937
Magnesium Sulfate (anhydrous)	0.09767
Potassium Chloride	0.4
Potassium Phosphate Monobasic (anhydrous)	0.06
Sodium Chloride	8
Sodium Phosphate Dibasic (anhydrous)	0.19
Amino Acids	
L-Alanine	0.225
L-Arginine (free base)	0.5
L-Asparagine (anhydrous)	0.25
L-Cysteine (free base)	0.12
Glycine	0.2
L-Histidine	0.25
L-Isoleucine	0.125
L-Leucine	0.125
L-Lysine	0.0937
L-Methionine	0.075
L-Phenylalanine	0.125
L-Serine	0.2
L-Threonine	0.3
L-Tryptophan	0.02
L-Tyrosine (free base)	0.3
L-Valine	0.1
Vitamins	
Choline Chloride	0.001
Flavin Mononucleotide • Na	0.0001
Folic Acid	0.001
myo-Inositol	0.002
Niacinamide	0.001
DL-Pantothenic Acid (hemicalcium)	0.001
Pyridoxine • HCl	0.001
Thiamine Monophosphate • HCl	0.001
Other	
D-Galactose	0.9
Phenol Red • Na	0.011
Pyruvic Acid • Na	0.55
Additives	
L-Glutamine	0.3

Concentration of certain components of ovine oviduct plasma. Values presented are means according to that presented by .

Component	Units	Oestrus	Metestrus	Diestrus
Sodium	$mEqL^{-1}$	136-138	123-128	136-146
Chloride	$mEqL^{-1}$	119-126	112-139	127-133
Potassium	$mEqL^{-1}$	8.24-9.90	7.49-8.38	7.84-8.99
Bicarbonate	$mEqL^{-1}$	21.8-22.6	15.6-17.0	17.6-21.8
Calcium	$mEqL^{-1}$	2.72-3.09	2.74-3.81	3.04-3.32
Magnesium	$mEqL^{-1}$	0.58-0.71	0.67-0.74	0.88-1.04
Phosphate	$mEqL^{-1}$	0.98-1.02	0.75-1.38	1.38-1.59
Total phosphorus	$mg100ml^{-1}$	2.28-4.05	1.50-21.3	3.04-4.63
Acid-insoluble phosphorus	$mg100ml^{-1}$	0.77-0.87	0.46-0.80	0.84-2.07
Total carbohydrate	$mg100ml^{-1}$	61.2-69.4	43.7-55.0	67.0-69.1
Protein	$g100ml^{-1}$	0.93-1.45	0.48-2.80	1.59-3.20
Non-protein nitrogen	$mg100ml^{-1}$	109.1	-	-
Lactate	$\mu molesml^{-1}$	2.15-3.91	1.54-3.68	3.44-5.17
Glucose	$mg100ml^{-1}$	28.5	-	-
Citrate	$mg100ml^{-1}$	0.8	-	-
Lipid choline	$mg100ml^{-1}$	1	-	-
Lipid aldehyde	$\mu g100ml^{-1}$	1.1	-	-

LDH assay – quality controls and standard preparations

Quality controls (QCs) and Standards (S) were prepared as detailed in the table below, using LDH from the Cytotoxicity Detection Kit (Roche).

Solution	Reagent	Volume of reagent (µl)	Volume of phenol red-free α-MEM (µl)	Final LDH concentration (Uml^{-1})
Solution 0	LDH ($5500Uml^{-1}$)	20	110	1000
Solution 1	Solution 0	10	90	100
Solution 2	Solution 1	10	990	1
QC4	Solution 2	10	2490	0.004
QC3	QC4	1000	1000	0.002
QC2	QC3	1000	1000	0.001
QC1	QC2	1000	1000	0.0005
S7	Solution 2	20	1980	0.01
S6	S7	1000	1000	0.005
S5	S6	1000	1000	0.0025
S4	S5	1000	1000	0.00125
S3	S4	1000	1000	0.000625
S2	S3	1000	1000	0.000313
S1	S2	1000	1000	0.000156

Appendix V. *Supplier Addresses*

Applied Biosystems, 3 Fountain Drive, Inchinnan Business Park, Paisley, UK, PA4 9RF

Alpha Laboratories Ltd 40 Parham Dr, Eastleigh, Hampshire SO50 4NU, UK

Baird and Tatlock, P.O. Box 1, Romford, RM1 1 HA**.**

Chemlab, Astra House, Christy Close, Southfields Business Park, Laindon, Essex, SS15 6TQ, UK

Cook Ireland Ltd., O'Halloran Road, National Technological Park, Limerick, Ireland

Dynex Technologies Headquarters, 14340 Sullyfield Circle, Chantilly, VA 20151-1621, USA

GE Healthcare, Pollards Wood, Global Headquarters, Diagnostic Imaging Services,

Greiner, Maybachstraße 2, 72636 Frickenhausen, Germany

Invitrogen, LifeTechnologies Ltd**,** 3 FountainDrive, Inchinnan BusinessPark, Paisley PA4 9RF, UK

J Penny and Sons,40 Leeds Road Rawdon, Leeds, LS19 6NU, UK

Life Science, Bio-Rad Laboratories Ltd., Bio-Rad House, Maxted Road, Hemel Hempstead, Hertfordshire HP2 7DX

Linkam Scientific Instruments Ltd., Unit 8 Epsom Downs Metro Centre, Tadworth, Surrey KT20 5LR, UK

New England Bio Lab Ltd. 75-77 Knowl Piece, Hitchin SG4 0TY, UK

Nikon Inc., 1300 Walt Whitman Road, Melville, NY 11747-3064, USA

Nightingales Lane, Chalfont St Giles, Buckinghamshire, HP8 4SP, UK

Nunclon Kamstrupvej 90, Post Box 280 Roskilde, Roskilde-DK4 000, Denmark

National Diagnostics, Unit 4 Fleet Business Park, Itlings Lane, Hessle, East Riding of Yorkshire, HU139LX

Olympus Industrial Stock Road, Southend-on-Sea, Essex SS2 5QH United Kingdom

Richardson Healthcare, Borehamwood, WD6 1QQ, UK

Roche Diagnostics Roche Diagnostics Limited, Applied Science, Charles Avenue, Burgess Hill, West Sussex, RH15 9RY, United Kingdom

Sakura Finetek Europe B.V., Flemingweg 10A, 2408 AV Alphen aan den Rijn, P.O. Box 362, 2400 AJ Alphen aan den Rijn, The Netherlands

Sartorius Stedim UK Ltd. Longmead Business Centre Blenheim Road, Epsom KT19 9QQ Surrey, United Kingdom

Scientific laboratory supplies (SLS, Head Office, Orchard House, The Square, Hessel, East Riding of Yorkshire, HU13 0AE, UK

Sigma-Aldrich Company Limited, The Old Brickyard, New Rd, Gillingham, Dorset, SP8 4XT (UK)

Simport, 2588 Bernard-Pilon, Beloeil, QC J3G 4S5, Canada

Situatedat*:* Freshwater Road, Chadwell Heath, Essex, UK

STARLAB(UK), Ltd, Unit 4 Tanners Drive, Blakelands, Milton Keynes, MK14 5NA, UK

Sterilin Ltd, Parkway, Pen-y-Fan Industrial Estate, Newport, United Kingdom, NP11 3EF

Swann-Morton Limited, Owlerton Green, Sheffield, S6 2BJ

Terumo Europe N.V. 3001 Leuven, Belgium

Thermo Fisher Scientific, Stafford House, 1 Boundary Park, Boundary Way, Hemel Hempstead, Hertfordshire, HP2 7GE, United Kingdom

VWR International,Unit 15, The Birches, Willard Way, Imberhorne Industrial Estate, East Grinstead,

Vygon, The Pierre Simonet Building, V Park, Gateway North, Latham Rd , Wiltshire, SN25 4DL, UK

West Sussex, RH19 1XZ, UK

Wilco Beheer B.V. Wg-Plein 275, 1054 SE Amsterdam, Netherlands

Wilmad, 1172 North West Boulevard, Vineland, NJ 08360