Errata

p127, 3rd line: "the results obtained in Study 2 indicate" for "the results obtained Study 2 indicate"

p135 para 3, 10th line: "they received dunnart ovarian xenografts" for "they were received dunnart ovarian xenografts"

Ovarian Xenografting for the Conservation of Endangered Species

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Abbreviations

AI	Artificial insemination	**MII**	Metaphase II
ALS	Antilymphocyte serum	**µg**	Microgram
ANOVA	Analysis of variance	**µL**	Microlitre
ART's	Assisted reproductive technologies	**µm**	Micrometre
		mg	Milligram
BLO	Bilaterally ovariectomised	**ml**	Millilitre
cm	Centimetre	**mm**	Millimetre
COC	Cumulus oocyte complex	**MT6**	Modified T6
°C	Degrees Celsius	**n**	Number
DMSO	Dimethyl sulfoxide	**NOD-SCID**	Non-obese diabetic SCID
EG	Ethylene glycol	**OT**	Ovarian tissue
EMEM	Eagle's modified essential media	**%**	Percentage
		PBS	Phosphate buffered saline
FCS	Fetal calf serum	**PCOS**	Polycystic ovarian syndrome
FIGα	Factor in the germline alpha	**PCR**	Polymerase chain reaction
FSH	Follicle stimulating hormone	**PMSG**	Pregnant mare serum gonadotrophin
g	Gram		
gDNA	Genomic DNA	**PROH**	1,2-propanediol
GV	Germinal vesicle	**RAG**	Recombinase activation gene
h	Hour	**RT**	Room temperature
hCG	Human chorionic gonadotrophin	**SCID**	Severe combined immunodeficiency
ICSI	Intra-cytoplasmic sperm injection	**SPF**	Specific pathogen free
		Subcut	Subcutaneous
IU	International units	**TGF β**	Transforming growth factor beta
IVF	*In vitro* fertilization		
IVM	*In vitro* maturation	**ULO**	Unilaterally ovariectomised
LH	Luteinizing hormone	**w/v**	Weight for volume
LN_2	Liquid nitrogen	**w/w**	Weight for weight

Summary

The principal aim of this thesis was to investigate the feasibility of employing ovarian xenografting as a method for generating mature oocytes for use in assisted reproductive technologies (ART's) for the conservation of endangered species.

In the initial studies, the factors affecting the viability of ovarian tissue collected post-mortem were evaluated. Mouse ovaries were subjected to differing durations (three-48 hours), temperatures (on ice versus room temperature (RT)) and methods (*in situ* versus *in vitro*) of storage and then grafted under the kidney capsule of bilaterally ovariectomised recipient mice. Two weeks after grafting the number of surviving follicles was assessed. Ovaries grafted following a period of storage contained approximately 50% fewer follicles than non-stored fresh grafts. Ovaries, which were removed from the donor at the time of death, then stored *in vitro*, on ice or at RT, for up to 48 hours before being grafted, contained some viable follicles. By contrast ovaries that were left *in situ* (within the donor) until the time of grafting contained no viable follicles after 24 hours storage if the carcass was stored at RT. The results show that ovarian tissue collected post-mortem does contain viable follicles and may be of use in conservation biology.

The second series of experiments aimed to establish whether oocytes matured within ovarian xenografts are normal and capable of yielding live young. Ovaries from young mice were xenografted under the kidney capsule of adult immunocompromised rats (female and male) for three weeks. Oocytes were collected from the xenografts, matured overnight and fertilized. Oocytes from mouse ovarian xenografts to both male and female rat recipients, matured, and cleaved to 2-cell embryos following fertilization. The

subsequent transfer of embryos to foster mothers resulted in the birth of fertile live young; thus demonstrating, for the first time, that oocytes matured within xenografted ovarian tissue are normal and capable of producing live young.

The third experimental chapter examined the value of ovarian xenografting for Australian marsupials. Ovarian tissue from three marsupial species (the tammar wallaby, pademelon wallaby and eastern grey kangaroo) was xenografted to NOD-SCID mice to investigate the effect of the recipient's gonadal status, the graft site and cryopreservation protocol. Follicular development to antral stages was observed in xenografts from all three species, and full sized oocytes could be collected from tammar wallaby xenografts to bilaterally ovariectomised, unilaterally ovariectomised and intact female recipients. For grafted pademelon wallaby ovarian tissue the kidney capsule proved a more superior graft site than under the skin. Studies on eastern grey kangaroo ovarian tissue showed that for both slow and rapid-cool freezing, dimethyl sulfoxide based cryoprotectant solutions were more effective than solutions, which contained ethylene glycol.

The fourth experimental chapter reports the investigation of whether immunologically tolerized or immunosuppressed animals could replace immunocompromised mouse and rat strains as xenograft recipients. To attempt to initiate neonatal tolerance, newborn rat pups were injected subcutaneously with a single whole 12-day old mouse pup ovary. While, some mouse (tolerizing) ovaries could be recovered three weeks after injection, none of the ovaries subsequently grafted to the kidney capsules of these tolerized recipients were recovered. This indicates that the neonatal tolerance was unsuccessful. In contrast, juvenile mice that underwent immunosuppression, through the repeated administration of antilymphocyte serum, successfully supported rat ovarian xenografts. Further investigation into the use of immunosuppressed recipients in ovarian xenografting studies is therefore warranted.

The final experimental chapter employed ovarian grafting as a tool to examine follicle growth in transgenic mice that die at birth. The role of follistatin in ovarian function was investigated. Ovaries were collected from both normal and follistatin knockout fetuses and assessed either immediately or after grafting for three weeks or four weeks under the kidney capsule of RAG mice or after seven days of *in vitro* culture. Follistatin knockout fetal ovaries contained significantly fewer oocytes than the normal ovaries, however the oocytes formed follicles that underwent normal follicular development. The results indicate that follistatin plays a crucial role in fetal ovarian function.

In summary, oocytes matured within ovarian xenografts are normal and, when fertilized, yield healthy live young. Ovarian grafting and more specifically, xenografting, provides a valuable tool for the *in vivo* maturation of oocytes and for studying folliculogenesis. Ovarian xenografting thus remains a promising prospect for use in conservation biology.

Declaration

I hereby declare that, to the best of my knowledge, this thesis contains no material previously published or written by another person, nor material that has been accepted for the award of any other degree or diploma in any university, or other institution, except where due reference is made within the text of this thesis. Preliminary studies investigating the viability of mouse ovarian tissue collected after death reported in Chapter Two were undertaken during the honours research year of my Bachelor of Science degree. Approximately half of the grafts from the treatment groups indicated by an asterisk in Chapter Two (See Table 2.3.) were obtained from the preliminary study.

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Publications

Peer Reviewed Papers

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~ *Chapter One* ~

Review of the Literature

1.1. Introduction

In excess of eleven thousand species of animals and plants worldwide are currently facing the threat of extinction as a result of habitat loss and degradation (Ptak *et al.*, 2002). Australia's marsupial species are no exception, with almost half of the native species currently threatened by extinction (Szabo, 1995). Efforts must therefore be made now to ensure the survival of threatened species.

Habitat protection, the control of introduced plant and predator species and captive breeding programs have been the preferred solutions for species protection. These strategies have offered a solution for some marsupial species. For example, in five years the eastern barred bandicoots' population was raised from 110 to 550 through the combined efforts of captive breeding, radio tracking and computer modelling (Szabo, 1995). Similarly, the establishment of sanctuaries that are enclosed by electric fences, providing habit protection and the exclusion of predators, have helped protect the survival of brush-tailed bettongs, long-nosed potoroos and pademelon wallabies (Anderson, 1993). However, both captive breeding programs and habitat protection as strategies for preserving endangered species have limitations.

Limited space and a lack of knowledge of how to create artificial habitats for captive populations result in many captive animals not breeding to full potential. Of more than 4000 mammalian species, the reproductive physiology and behaviour has only been studied extensively in fewer than 100 species (Lasley *et al.*, 1994). As a consequence the conditions for optimal breeding remain unknown for most species. Furthermore, the physical restraints of captive breeding programs restrict the number of animals contributing to the gene pool, hence resulting in reduced genetic diversity. Similarly, even with the best forethought by curators at zoos, matings designed to expand the genetic pool, are not always successful as, like humans, many animals too have partner preferences (Wildt *et al.*, 1997). Habitat protection is frequently also not feasible as, for many species, insufficient space exists to sustain reproductively viable wild populations (Wildt *et al.*, 1997). In light of these difficulties, recent years have seen increasing interest in the use of assisted reproductive technologies (ART's) for the preservation of endangered wildlife species.

Numerous ART's have been suggested for use in the propagation of threatened species including, superovulation, *in vitro* fertilization (IVF), artificial insemination (AI), intra-cytoplasmic sperm injection (ICSI), embryo transfer, cross fostering, *in vitro* maturation (IVM) of oocytes, *in vivo* maturation of germ cells through the transplantation of reproductive tissues and the cryopreservation of gonadal tissue, embryos, oocytes and sperm (gene banking). Recent years have seen increased use of ART's in conservation biology. AI with frozen/thawed sperm has resulted in the birth of cheetah litters (Wildt *et al.*, 1997). While in the rhesus monkey, ICSI has been used to fertilize eggs which can develop into blastocysts (Hewitson *et al.*, 1998) and more recently live young (Nusser *et al.*, 2001). In the wild European mouflon, a species at risk of extinction, healthy offspring have been produced through the combination of oocyte collection, IVM, IVF, embryo

culture and blastocysts transfer to receptive domestic sheep fosters (Ptak *et al.*, 2002). Although some of the ART's developed for eutherian species can be applied to marsupials, the reproductive biology of most endangered marsupial species is different from eutherians. Successful fertilization has been achieved in the brush tail possum and tammar wallaby following superovulation and intrauterine insemination (Molinia *et al.*, 1998a). However most ART's that require mature oocytes have been hampered by the difficulties in determining the exact time of ovulation and difficulties in the development of successful and reliable superovulation protocols (Hinds *et al.*, 1996; Hickford *et al.*, 2001).

Despite the advances in the transfer of established ART's to non-domestic species, most (e.g. IVF, ICSI and AI) are limited by their requirement for mature oocytes. Mature oocytes are particularly difficult to obtain because they can only be collected from sexually mature females at specific stages of the reproductive cycle. Ovarian tissue can be collected from both live and dead females of all ages, at any stage of the reproductive cycle. Methods, which allow mature oocytes to be generated and collected from ovarian tissue may therefore be useful.

Both the *in vitro* maturation of oocytes (Eppig & O'Brien, 1996; Obata *et al.*, 2002; O'Brien *et al.*, 2003) and the transplantation of ovarian tissue to recipients of the same or a different species (Carroll *et al.*, 1990; Mattiske, 1997; Choo, 1999; Waterhouse *et al.*, 2001) provide means of generating fertilizable oocytes. Follicular development through the xenografting of ovarian tissue has been achieved in a number of species (Gosden *et al.*, 1994b; Candy *et al.*, 1995; Gunasena *et al.*, 1998; Mattiske *et al.*, 2002) and by oocyte exchange in the mouse and rat (Eppig & Wigglesworth, 2000). By comparison full follicular development *in vitro* has only been achieved in the mouse (Eppig & O'Brien, 1996; Obata *et al.*, 2002; O'Brien *et al.*, 2003) and, in the mouse is most successful when used in combination with serial nuclear transfer (Obata *et al.*, 2002). As nuclear transfer

itself requires mature enucleated oocytes, it is not likely to be of value for rare or endangered species unless a universal donor can be identified. Furthermore, the oocytes matured entirely *in vitro* were smaller in size than oocytes grown *in vivo* and successful embryonic development occurred at a low frequency (Eppig & O'Brien, 1996; O'Brien *et al.*, 2003). Consequently, the use of oocytes matured *in vivo* by means of ovarian grafting or oocyte exchange would appear to be the preferred method for generating mature oocytes for use in ART's for the preservation of endangered wildlife until *in vitro* maturation techniques can be perfected.

Ovarian grafting can also provide a valuable additional tool for studying follicular survival and development in both well documented and poorly documented species (Gosden *et al.*, 1994b; Gunasena *et al.*, 1998; Cleary *et al.*, 2001; Mattiske *et al.*, 2002). The aims of this thesis were therefore to investigate the feasibility of using ovarian grafting in the conservation of endangered wildlife and as a tool for studying follicular development.

1.2. Reproduction

1.2.1. Mouse and Rat Reproduction

Mice and rats are widely used models for scientific research. They are both small in size, breed rapidly, easy to handle, relatively cheap, well characterized and available in a wide range of outbred, inbred, mutant, transgenic and knockout lines. These attributes make rodents a convenient model for the study of ovarian function through the development of ovarian grafting techniques, and for use as recipients in xenografting studies.

In mice and rats, reproduction, and specifically follicular development, follows a similar pattern. The mouse is the main focus of this literature review as this is the more frequently utilized species within this thesis.

1.2.1.1. Anatomy of the female reproductive tract

The female reproductive tract consists of paired ovaries, oviducts, a bicornuate uterus, a cervix, vagina, clitoral glands and clitoris (Figure 1.1.). The mouse and rat ovaries are located in the abdominal cavity each suspended slightly below a kidney. They are enclosed within a thin connective tissue capsule, known as the ovarian bursa, and covered by a fat pad.

Ovaries from mature animals can be divided into two zones. The medulla which lies innermost and is the smaller of the two regions consists of loose fibroelastic connective tissue (Leeson & Leeson, 1976). The cortex, the outer and major portion of the ovary, contains most of the follicles (Hummel *et al.*, 1966; Rugh, 1968; Leeson & Leeson, 1976; Fortune *et al.*, 2000).

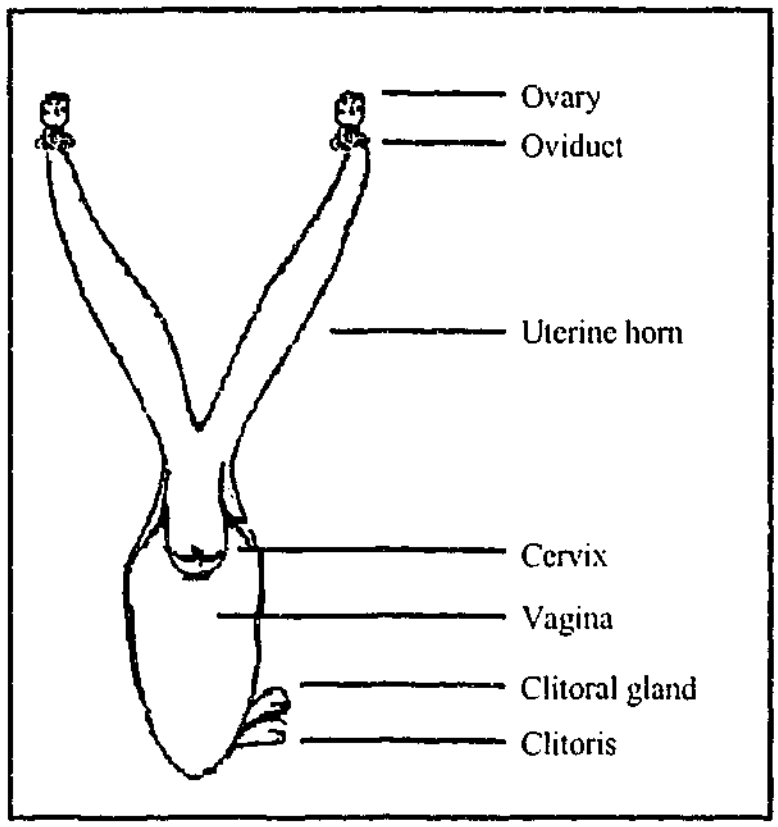


Figure 1.1. Schematic illustration of the anatomy of the female mouse/rat reproductive tract (adapted from (Hummel *et al.*, 1966).

1.2.1.2. The estrous cycle and reproduction

Reproduction in mice and rats is characterized by an estrous cycle of approximately four to five days (Long & Evans, 1922; Bronson *et al.*, 1966; Perry, 1971) and a short period of gestation of 19-21 days (Long & Evans, 1922; Rugh, 1968; Perry, 1971). Both species are polyestrous, having an estrous cycle that can be divided into four stages; proestrus, estrus, metestrus and diestrus. These stages (Table 1.1.), which can be identified by changes in the appearance of the ovary, oviduct, uterus, vagina and vaginal cytology, have been well described (Long & Evans, 1922; Bronson *et al.*, 1966; Perry, 1971; Gude *et al.*, 1982; Evans *et al.*, 1990).

Proestrus and estrus are both anabolic stages during which active growth in many parts of the reproductive tract occurs (Rugh, 1968). While proestrus lasts approximately 24 hours (Rugh, 1968; Perry, 1971; Evans *et al.*, 1990), it is during estrus that ovulation occurs and the female is receptive to mating (approximately one day in the rat (Perry, 1971) and one to three days in the mouse (Rugh, 1968)). In the absence of pregnancy or

pseudopregnancy, estrus is followed by the catabolic phase metestrus, which is characterized by degenerative changes in the reproductive tract (Bronson *et al.*, 1966). Metestrus lasts between one and five days in the mouse (Rugh, 1968). The final stage of the cycle, diestrus, is a quiescent period of slow growth that lasts for over two days in the mouse and rat (Rugh, 1968; Perry, 1971).

Table 1.1. Effect of the stage of the estrous cycle on mouse and rat uterine morphology and vaginal smear cytology (adapted from Bronson *et al.*, 1966; Biggers *et al.*, 1971; Perry, 1971).

Stage of estrous cycle	Appearance of uterus	Vaginal smear cytology
Proestrus	Increased distension and vascular supply.	Nucleated round epithelial cells.
Estrus	Distension reaches maximum before returning to normal.	Epithelial cells lose nucleus and become larger cornified cells.
Metestrus	Normal size.	Numerous cornified cells, with some epithelial cells and leukocytes.
Diestrus	Anaemic and shrunken.	Mainly leukocytes.

Mating can be detected by the presence of a vaginal plug. The vaginal plug, which can be visibly observed extending from the cervix to vagina, is formed by the solidification of the secretions from the vesicular and coagulating glands of the male (Bronson *et al.*, 1966; Perry, 1971). Vaginal plugs are generally highly efficient indicators of mating and pregnancy (Bronson *et al.*, 1966). However, while mice and rats have a postpartum estrus, the duration of pregnancy may be extended (by diapause) by up to two weeks in a lactating female (Rugh, 1968; Perry, 1971; Rowlands & Weir, 1984). Similarly, cervical stimulation or sterile mating usually results in pseudopregnancy. This lasts for approximately 14 days, however the duration of a pseudopregnancy is variable in both the mouse and rat (Bronson *et al.*, 1966; Perry, 1971).

1.2.1.3. Follicular development in the mouse

In 1968 Pedersen and Peters proposed a scheme for the classification of oocytes and follicles within the mouse ovary (Figure 1.2.). The scheme, which is still in wide use and has been adapted for a number of species including the tammar wallaby (Alcorn, 1975), differentiates oocytes on the basis of size, and follicles on the basis of morphology and size as determined by the number of follicle cells surrounding the oocyte.

The first stage of ovarian development in the fetal female mouse involves the appearance of the primordial germ cells in the yolk sac splanchnopleure at eight days gestation and then their migration and arrival at the genital ridge at nine to ten days of gestation (Rugh, 1968). A few days later at fetal day 12, the ovary can be differentiated from the testis (Odor & Blandau, 1969a; Odor & Blandau, 1969b; Hogan *et al.*, 1986), because oogonia, the largest cells are only present in the ovary (Odor & Blandau, 1969a). Between days 12 and 14 the most rapid oogonial mitotic divisions are observed (Odor & Blandau, 1969a). By day 15 after fertilization, most of the oogonia have entered the prophase stage of the first meiotic division, thus forming oocytes (Odor & Blandau, 1969b; Wassarman & Albertini, 1994).

At day 16, oocytes start to reach the pachytene stage (Monk & McLaren, 1981; Wassarman & Albertini, 1994). During this differentiative process, the oocyte proliferation ceases (Jost & Magre, 1993) and instead the programmed atresia of oocytes that continues until after birth, begins. At the time of birth (day 18 to 21), the ovary consists mainly of stomal cells and small oocytes (Peters & Pedersen, 1967). Some oocytes are still in the transitory stages of development (pachytene and early diplotene), while others have reached the resting primordial follicle state (late diplotene and dictyate) in which they remain until the resumption of meiosis just prior to ovulation (Peters & Pedersen, 1967).

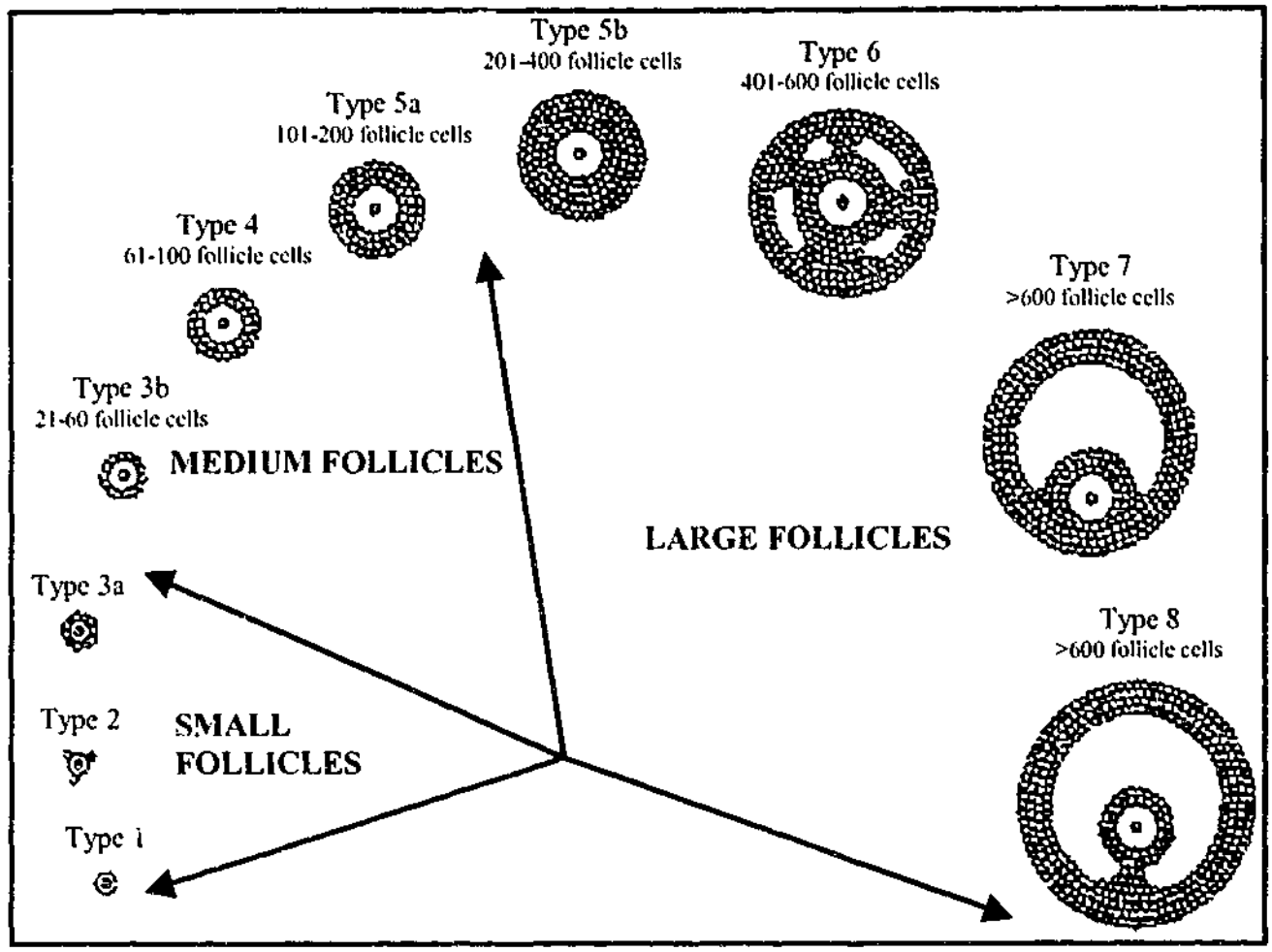


Figure 1.2. Schematic illustration of the scheme used for the classification of mouse oocytes and follicles (adapted from Pedersen & Peters, 1968).

Within a few days of birth, follicle cells begin to attach to the surface of some oocytes (Peters & Pedersen, 1967). By seven days post-partum, the mouse ovary has become larger in size, with the majority of oocytes bordered by one of more follicle cells. Oocyte growth and development has also begun with both small and medium sized follicles observed (Peters & Pedersen, 1967; Peters, 1969). A week later, the follicles contained within the ovaries have reached further stages of development, and additional follicles have initiated growth (Peters *et al.*, 1975). By three weeks of age, the mouse ovary contains small, medium and large sized follicles (Peters *et al.*, 1975). The structure of the ovary is also changed. The ovary, which was once a solid organ, is now an organ with a delicate centre formed by an elaborate vascular system (Peters, 1969). The ovary of a one-month old animal begins to take on the form of an adult ovary, but without corpora lutea. Sexual maturity is reached at around six weeks of age in the female mouse.

This vast knowledge of the reproductive system of the mouse makes it an ideal model for scientific research into ovarian function and ART's.

1.2.2. Marsupial Reproduction

The three sub-classes of mammals, Eutheria, Monotremata and Marsupialia can be differentiated by their modes of reproduction. Marsupial reproduction is characterized by the immature state in which the young are born and, in most species, the presence of a pouch in females. After a relatively short gestation, it is within the pouch that marsupial young undergo most of their development.

The tammar wallaby, *Macropus eugenii* is one of the most extensively studied marsupial species and as a consequence the reproductive anatomy and physiology are well understood. This knowledge, in association with the ability of the tammar wallaby to be bred in captivity and its convenient size and availability, make it an excellent model for the research of ART's including ovarian grafting (Mattiske *et al.*, 2002), superovulation (Molinia *et al.*, 1998a; Molinia *et al.*, 1998b) *in vitro* maturation (Mate & Rodger, 1993) and fertilization (Renfree & Lewis, 1996). While relatively less information is available pertaining to the reproductive physiology of the eastern grey kangaroo and the pademelon wallaby, their availability has also made them suitable models for scientific research (Every, 2000).

1.2.2.1. Anatomy of the female reproductive tract

The reproductive tract of female marsupials differs substantially from that of many species of Eutheria. The tract consists of paired but separate uteri, fallopian tubes, ovaries, the lateral vaginae and the median vagina (Tyndale-Biscoe, 1973; Tyndale-Biscoe, 1984; Figure 1.3.).

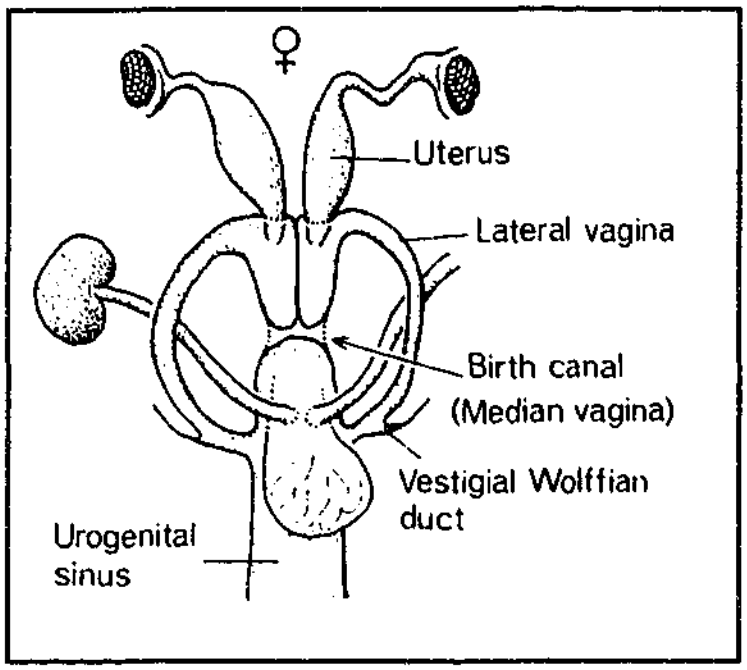


Figure 1.3. Schematic illustration of the anatomy of the female marsupial reproductive tract, from Tyndale-Biscoe, 1973.

The uteri of marsupials are paired, but separate structures (Figure 1.3.), which each possess their own cervices (Tyndale-Biscoe & Renfree, 1987). The two uterine necks, open into the anterior vagina expansion by way of their cervices (Tyndale-Biscoe & Renfree, 1987). The marsupial vagina (Figure 1.3.) consists of two lateral vaginae that connect the uterus to the urogenital sinus and a median vagina (Tyndale-Biscoe & Renfree, 1987). While the lateral vaginae are used exclusively for sperm ascent (Short *et al.*, 1988), the median vagina serves as the birth canal.

1.2.2.2. Marsupial reproduction

Several features are common to the reproductive physiology of most marsupial species. Most are polyestrous, monovular, ovulate spontaneously and, if conception occurs, pregnancy typically occupies a period less than the duration of the estrous cycle (unless the embryo enters diapause; Hinds *et al.*, 1996). In the tammar wallaby both the estrous cycle and gestation are approximately 30 days in length (Tyndale-Biscoe & Renfree, 1987).

Two common breeding patterns are recognised in marsupials (Tyndale-Biscoe & Renfree, 1987; Hinds *et al.*, 1996). The majority of marsupials, including possums and opossums, have a gestation period that is substantially shorter in duration than the estrous cycle

(Tyndale-Biscoe & Renfree, 1987; Hinds *et al.*, 1996). In these species, the luteal phase occupies 60% of the estrous cycle, and following parturition, lactation suppresses estrus and ovulation (Tyndale-Biscoe & Renfree, 1987).

In the majority of kangaroos and wallabies, including the tammar wallaby, postpartum estrus and ovulation are observed (Tyndale-Biscoe & Renfree, 1987; Renfree, 1994). Their gestational period and estrous cycle are similar in length, with the luteal phase occupying more than 90% of the estrous cycle (Hinds *et al.*, 1996).

However, despite these differences observed in the modes of reproduction, the estrous cycle of marsupials is not dissimilar to that observed in many other mammals, with a likeness existing in the cytology of both the ovaries and uterus (Renfree, 1994).

1.2.2.3. Follicular development in marsupials

As in eutherians, the oocyte and follicular growth patterns of marsupials conforms to a uniform biphasic pattern (Lintern-Moore *et al.*, 1976; Lintern-Moore & Moore, 1977). Follicular development within the tammar wallaby (Alcorn, 1975; Alcorn & Robinson, 1983) follows a similar pattern to that proposed for the mouse by Pedersen and Peters (1968; see Section 1.2.1.3.).

At parturition the ovary of the tammar wallaby pouch young is not yet differentiated (Alcorn, 1975), despite the migration of 3200-4000 primordial germ cells to the gonadal ridges before birth (Ullmann *et al.*, 1997).

In tammar wallaby pouch young, follicular development and growth can be divided into four stages; differentiation, proliferation, degeneration and follicular (Alcorn, 1975). The differentiation stage, which lasts from birth until day 22 postpartum, is characterized by steady growth in the ovary (Alcorn, 1975). At the end of this period a clear distinction can be made between the cortex and medulla of the ovary (Tyndale-Biscoe, 1984). Similarly,

it is now that the first oogonia develop from primordial germ cells and germ cell nests are formed (Alcorn, 1975). The proliferative stage (between days 24 and 30) is a period of rapid growth that is characterized by the initiation of the transition of oogonia to oocytes (Alcorn & Robinson, 1983). Between days 50 and 110, the degeneration stage, a rapid decrease in the germ cell population is observed (Alcorn, 1975), however it is also during this time (around day 95) that a cohort of primordial follicles begin to develop into primary follicles (Alcorn & Robinson, 1983). Despite this, it is not until 150 days postpartum, during the follicular stage, that medium to large sized follicles are observed (Alcorn, 1975; Mattiske, 2003). Subsequently, by about day 210, normal pre-follicular germ cell development has ended and the follicle population has been reduced, by means of atresia to 25% of its peak (Alcorn & Robinson, 1983).

1.2.3. Mammalian Ovarian Function

Ovarian function (oogenesis, folliculogenesis, ovulation and atresia) involves the complex interaction between the ovary and hypothalamic and anterior pituitary synthesised factors (Matzuk, 2000). However, while the roles of many extra-ovarian factors in ovarian function have been identified, the roles and/or identity of numerous intra-ovarian factors are yet to be determined.

Within the field of reproductive biology, the term recruitment has been employed to describe two very distinct processes that occur during follicular development. Namely; the initial growth of primordial follicles and the selection of follicles for the final stage of development before ovulation. To distinguish between these processes, McGee and Hsueh (2000) have proposed the use of the term 'initial recruitment' to describe the mechanisms by which follicles are selected from the resting population to undergo growth and 'cyclic recruitment' for follicles when they are rescued from the pre-antral follicle pool, to undergo gonadotrophin dependent maturation as opposed to atresia. To avoid confusion

this terminology will be employed throughout this thesis. In addition the term 'dominant follicle selection' will be used to describe the method by which a follicle is selected for ovulation.

1.2.3.1. Initial recruitment

Most of the research in the field of initial recruitment has been undertaken in the human, rat and mouse. Consequently, these species are the focus of the majority of the references below.

The initial recruitment of quiescent primordial follicles into the growing follicle pool is characterised by the transition of granulosa cells from flat to cuboidal in shape, and an increase in the oocyte size (Baker, 1982; Gougeon, 1996; Fauser & Van Heusden, 1997; Matzuk, 2000; Eppig, 2001; Braw-Tal, 2002). While, the factors involved in this initiation of growth are unknown, the process is not believed to be gonadotrophin dependent (Peters *et al.*, 1973; Baker, 1982; Roche, 1996; Elvin & Matzuk, 1998) and results in follicles that are not recruited remaining dormant (McGee & Hsueh, 2000). Once a follicle enters the growing pool it is committed to growth, with this period of growth either ending with the ovulation of the mature oocyte from the follicle or death of the follicle and oocyte by means of atresia (Peters *et al.*, 1975).

The period of development at which initial recruitment first occurs varies between species. However, once commenced, follicular growth is continuous through life (Richards, 1980). The first follicles begin growth in the first week of extrauterine life in the mouse (Peters *et al.*, 1975; Smitz & Cortvrindt, 2002), while, in larger mammals such as the human, the earliest initial recruitment occurs during fetal life (Picton *et al.*, 1998; McGee & Hsueh, 2000; Smitz & Cortvrindt, 2002). Following the initial recruitment in all species, follicles develop through primary and secondary stages before reaching the gonadotrophin sensitive pre-antral stage of development. If gonadotrophin levels are sufficient to support

development the follicle will form an antral cavity. This growth phase takes only 17 days in the mouse and rat (Hogan *et al.*, 1986; Driancourt *et al.*, 1993) while, it takes more than 200 days to complete in the human (McGee & Hsueh, 2000). However, until puberty is reached and its associated rise in gonadotrophins, atresia is the pre-determined fate of all growing follicles (McGee & Hsueh, 2000).

1.2.3.2. Cyclic recruitment

Cyclic recruitment, the gonadotrophin driven rescue and continued growth of a small number of antral follicles, commences with the onset of puberty (McGee & Hsueh, 2000). It is this follicle stimulating hormone (FSH) driven process that leads to the emergence of pre-ovulatory follicles which, if selected as a dominant follicle, undergo ovulation (Roche, 1996).

Growing follicles gain gonadotrophin sensitivity during the late pre-antral stage of follicular development, following the acquisition and subsequent expression of FSH receptors on the granulosa cells of pre-antral and antral follicles (Carson *et al.*, 1979; Wang & Greenwald, 1993; Oktay *et al.*, 1997). It is not until the antral stage of development that responsiveness to gonadotrophins is crucial, with studies demonstrating that follicular development to the antral stages can be achieved in the absence of FSH (Kumar *et al.*, 1997). In rodents early antral follicles are recruited at 0.2-0.4mm in diameter, while in humans the recruitment occurs when follicles are comparably much larger (2-5mm) and have already acquired an antrum (McGee & Hsueh, 2000). The duration of time from selection for cyclic recruitment and dominant follicle selection for ovulation also differs greatly between rodents and humans. This process only takes two to three days in the rat (McGee & Hsueh, 2000), while in the human it takes two weeks for an antral follicle to become the dominant pre-ovulatory follicle (Driancourt *et al.*, 1993; Fauser & Van Heusden, 1997; McGee & Hsueh, 2000). Luteinizing hormone (LH) appears to play a far

less crucial role than FSH in cyclic recruitment, with LH receptors only found on the granulosa cells of large, pre-ovulatory follicles (Driancourt *et al.*, 1993; Xu *et al.*, 1995), thus possibly indicating a role for LH in the selection of dominant follicles.

1.2.3.3. Dominant follicle selection

Dominant follicle selection is the process by which one follicle (e.g. in the human) or multiple follicles (e.g. in rodents), is/are selected from the cohort of growing follicles for ovulation, while the remaining subordinate follicles enter atresia due to insufficient support by reduced FSH levels (Fauser & Van Heusden, 1997). While the specific mechanisms involved in the selection of dominant follicles remain unknown, FSH, LH, estrogens and inhibin are all believed to be involved (McGee & Hsueh, 2000).

Dominant follicles are characterised by a faster growth rate and the increased production of estrogens and inhibin compared to non-dominant follicles (Zeleznik & Benyo, 1994). This production of estrogens and inhibin thus further ensures dominance by exerting negative feedback on FSH release, thus promoting the atretic demise of gonadotrophin dependent non-dominant follicles. An advanced stage of development has also been proposed as a potential characteristic of follicles selected for dominance (Fauser & Van Heusden, 1997), with the success of dominant follicles provided additional support from the increased production of autocrine and paracrine growth factors that promote increases in both vasculature and FSH responsiveness (McGee & Hsueh, 2000).

1.2.3.4. Follistatin and folliculogenesis

Ovarian grafting provides a valuable tool with which ovarian viability and function can be examined. In this thesis ovarian grafting has been employed to investigate the role of follistatin in ovarian function, and specifically folliculogenesis. A brief review of follistatin is therefore given below.

Follistatin, a glycosylated monomeric protein, and its binding partner activin, are believed to regulate folliculogenesis through both paracrine and autocrine interactions (Woodruff *et al.*, 1990; Findlay, 1993; Gilfillan & Robertson, 1998; Sidis *et al.*, 1998). Activin, a member of the transforming growth factor β (TGF β) superfamily, stimulates FSH secretion (Gilfillan & Robertson, 1998; Knight & Glister, 2001). Follistatin (along with inhibin) has an established role of suppressing pituitary FSH synthesis and secretion, through binding to and neutralizing activin activity (Nakamura *et al.*, 1990; Shimonaka *et al.*, 1991; de Winter *et al.*, 1996; Gilfillan & Robertson, 1998). While the ability of follistatin and activin to regulate FSH levels is well established, their specific role in the initial recruitment of follicles and oocyte maturation remains unclear.

Studies thus far have established a role for activin in oocyte maturation. In an *in vitro* culture model, activin stimulates meiotic maturation within rat (Itoh *et al.*, 1990; Gilfillan & Robertson, 1998), human (Alak *et al.*, 1998), bovine (Silva & Knight, 1998) and zebrafish (Wu *et al.*, 2000) oocytes. Furthermore, consistent with its agonist/antagonist activity, follistatin on its own inhibits oocyte maturation (Gilfillan & Robertson, 1998). In an extension to these studies, it can be postulated that follistatin and activin may have a role in determining the destiny of any given follicle (i.e. towards atresia or ovulation). Additionally, a recent study examining follistatin expression during fetal life, found follistatin to be more abundantly expressed in ovaries than in testes (Menke & Page, 2002). This observation suggests that follistatin may play a role in fetal ovarian function.

Some further insight into the roles that follistatin and activin play in reproduction has been provided through the development of the activin and follistatin, knockout and knockin mouse strains (Matzuk *et al.*, 1995a; Guo *et al.*, 1998; Brown *et al.*, 2000; Cipriano *et al.*, 2000; Lau *et al.*, 2000). The over-expression of follistatin results in infertility (Guo *et al.*, 1998), with histological analysis of ovaries from selected individuals exhibiting both

blocks in folliculogenesis and the absence of corpora lutea. These observations, lend further support to the previously demonstrated ability of activin to promote oocyte maturation (Itoh *et al.*, 1990; Alak *et al.*, 1998; Gilfillan & Robertson, 1998; Silva & Knight, 1998; Wu *et al.*, 2000) and follistatin to inhibit oocyte maturation (Gilfillan & Robertson, 1998). A relative elevation in activin and a decrease in follistatin concentrations therefore appear to be crucial during the later stages of oocyte maturation.

Polycystic ovarian syndrome (PCOS) is a common reproductive disorder that is characterized by hyperandrogenism, anovulation and follicular arrest. Recent clinical studies examining PCOS indicate that elevated follistatin levels (Eldar-Geva *et al.*, 2001; Norman *et al.*, 2001), due to variations at or near the follistatin gene (Urbanek *et al.*, 1999) may contribute to the disorder. A high follistatin to activin ratio therefore appears a likely candidate for blocking late stage oocyte maturation (Eldar-Geva *et al.*, 2001; Norman *et al.*, 2001).

Further insight into the role of follistatin in ovarian function may be provided through the examination of folliculogenesis in an environment totally devoid of follistatin. While, a follistatin knockout strain of mouse has been developed (Matzuk *et al.*, 1995b), examination of folliculogenesis has been made impossible by the death of follistatin knockout pups within a few hours of parturition. Methods that enable the examination of folliculogenesis within follistatin knockout ovaries must now be developed.

1.3. Ovarian Tissue Collection and Storage

1.3.1. Short-term Storage and Post-mortem Collection of Ovarian Tissue

With the formation of gene storage banks, reproductive tissues and cells (including ovarian tissue) are being salvaged from the bodies of precious individuals following death. Consideration must therefore be given to the degeneration that occurs in both fresh and post-mortem obtained ovarian tissue during storage prior to cryopreservation and/or use.

Unlike mature oocytes and embryos, ovarian tissue may be collected from females of all ages, at any stage of the reproductive cycle (i.e. without prior superovulation) and following recent death. These attributes make the employment of ovarian tissue in ART's for the conservation of endangered wildlife a popular option.

The ability of both male and female gametes to remain viable for a period of time (hours to days) following death (Moodie & Graham, 1989; Johnston *et al.*, 1991; Schroeder *et al.*, 1991; Songsasen *et al.*, 1998; An *et al.*, 1999; Browne *et al.*, 2001) enables the post-mortem collection of gametes from precious individuals. Theoretically, salvaged gametes may then be employed in ART's to propagate and ultimately help ensure the survival of rare and endangered species.

The post-mortem viability of sperm has been studied extensively (Songsasen *et al.*, 1998; An *et al.*, 1999; Check *et al.*, 1999; Browne *et al.*, 2001; Check *et al.*, 2002; Yu & Leibo, 2002; Hishinuma *et al.*, 2003). Viable mouse (An *et al.*, 1999), dog (Yu & Leibo, 2002) and deer (Hishinuma *et al.*, 2003) spermatozoa can be recovered seven days following death. The viability of female gametes collected post-mortem is less well established, and results obtained indicate that the period of survival is shorter than for sperm (Johnston *et*

al., 1991; Schroeder *et al.*, 1991; Cleary *et al.*, 2001; Takahashi *et al.*, 2001; Takahashi & Nagasu, 2001). In the mouse, ovaries collected up to two hours after death can restore fertility when grafted to recipient females immediately after being salvaged (Takahashi & Nagasu, 2001) or following cryopreservation (Takahashi *et al.*, 2001). Cleary and colleagues (2001) found that frozen/thawed mouse ovarian tissue grafts contained viable follicles when the tissue was collected up to 12 hours after death of the donor female. However, the fertility of these grafts was not examined. More recently, two pregnancies have been reported in the vulnerable wild European mouflon following the transfer of embryos obtained from the fertilization of oocytes collected post-mortem (Ptak *et al.*, 2002). This achievement clearly demonstrates the potential of gametes collected following death in conservation biology.

In comparison, to other organs such as the kidney (Lindell *et al.*, 1991; Lindell *et al.*, 1998), little information exists regarding the optimal conditions for ovarian tissue storage. Numerous solutions have been employed for the short-term storage of ovarian tissue including phosphate buffered saline (PBS; Candy *et al.*, 1995; Cox *et al.*, 1996; Wolvekamp *et al.*, 2001), Leibovitz-L15 culture medium (Gosden *et al.*, 1994b; Newton *et al.*, 1996; Nugent *et al.*, 1998), HEPES-buffered human tubal fluid medium (Gook *et al.*, 1999) and HEPES-buffered M2 medium (Candy *et al.*, 1997; Candy *et al.*, 2000). However most of these media were developed for the handling and culture of embryos and oocytes and despite the variety of media employed, little data exists comparing the efficiency of different media, with the optimal ovarian tissue storage medium still unknown.

One study compared ovarian storage in mouse oocyte and embryo handling media (M2; Hogan *et al.*, 1986) and PBS (Tan, 2002). The PBS gave better support during short-term storage (three hours), but the effect was temperature and time dependent.

Several different temperatures have been used for the short-term storage of ovarian tissue. While, metabolic suppression by means of a low storage temperature is a key factor in maintaining organ viability (Lindell *et al.*, 1998), results obtained by Weissman and colleagues (1999) indicate that low storage temperatures may not be optimal for ovarian tissue. In this study, human ovarian tissue was stored in FSH supplemented PBS, either on ice or at 37°C. Following grafting to female immunocompromised mice, higher follicular survival and development was observed in the ovarian tissue pieces stored at 37°C. However, ovarian tissues from a number of species including the mouse, human, wombat, sheep and cat can resume function following short-term storage on ice (Gosden *et al.*, 1994b; Weissman *et al.*, 1999; Wolvekamp *et al.*, 2001), at room temperature (RT; Cox *et al.*, 1996) and at 37°C (Candy *et al.*, 1997; Gook *et al.*, 1999; Weissman *et al.*, 1999; Candy *et al.*, 2000).

1.3.2. Cryopreservation

Cryopreservation, the low temperature storage of biological tissue, involves the pre-freezing exposure of the tissue to a protective agent known as the cryoprotectant and then the cooling of the tissue to subzero temperatures (-196°C in liquid nitrogen).

Cryopreservation of ovarian tissue has attracted large amounts of interest for its clinical application and for its potential value in the conservation of threatened wildlife. Clinically, the cryopreservation of ovarian tissue is currently being considered for female cancer patients, as chemotherapy and radiation therapy can render patients infertile, with the premature onset of menopause frequently observed. Collection and cryopreservation of ovarian tissue before chemotherapy and/or radiation therapy may provide the only insurance to ensure the protection of these patients fertility (Kim *et al.*, 2001b; Oktay *et al.*, 2001c; Salha *et al.*, 2001; Revel & Laufer, 2002).

Gene banking, by means of cryopreservation of reproductive tissues and cells, has the potential to play a fundamental role in conservation biology (Wildt *et al.*, 1997; Mate *et al.*, 1998; Holt & Pickard, 1999; Ryder *et al.*, 2000). The cryopreservation of ovarian tissue is a well-established technique that, in combination with grafting may enable the preservation of the female germ cell line and the maintenance of genetic diversity of dwindling wildlife populations.

Cryopreservation, has been used for the long-term storage of a number of different reproductive tissues and cells including sperm (Tselutin *et al.*, 1995; Watson, 1995) and testes (Schlatt *et al.*, 2002; Shinohara *et al.*, 2002) in the male, and oocytes (Carroll *et al.*, 1993; Shaw *et al.*, 1993; Agca, 2000), embryos (Trounson, 1990; Nagashima *et al.*, 1995; Fogarty *et al.*, 2000), ovarian tissue (Green *et al.*, 1956; Cox *et al.*, 1996) and ovaries and their associated reproductive tracts (Wang *et al.*, 2002) in females.

1.3.2.1. Background

While, some tissue viability is lost during the cryopreservation cooling process, due to intracellular ice formation and cryoprotectant toxicity, upon reaching the final temperature of –196°C, tissue may be stored indefinitely without any additional loss of viability (Shaw *et al.*, 1993).

The cryopreservation protocols used in reproductive biology can be classified into three main techniques, slow-cooling, rapid-cooling and vitrification, based upon both the rate of freezing and the cryoprotectant concentration. Slow-cooling cryopreservation, which has been employed extensively in the freezing of ovarian tissue from a number of species (Gosden *et al.*, 1994a; Cox *et al.*, 1996; Newton *et al.*, 1996; Sztein *et al.*, 2000; Wolvekamp *et al.*, 2001; Mattiske *et al.*, 2002), involves the controlled slow-cooling (2°C or less per minute) of tissues to low temperatures. Relatively low cryoprotectant concentrations are used in this technique, as the slow rate of cooling helps prevent

destruction by intracellular ice. As with oocytes and embryos (Trounson, 1990; Kasai, 1995; Shaw *et al.*, 1995a; Lee *et al.*, 1998; Vajta, 2000) higher cryoprotectants allow ovarian tissue to be rapidly frozen (e.g. by direct plunging in liquid nitrogen; Shaw *et al.*, 1995a; Lee *et al.*, 1998). Vitrification is a rapid-cooling method that utilizes extremely high cryoprotectant concentrations, to make the solution and its contents vitrify, forming a glass-like solid without any ice crystals to reduce the damage incurred to the cells.

The cryopreservation of ovarian tissue has several advantages over using embryos or oocytes. In addition to each piece of frozen tissue potentially preserving up to thousands of immature follicles (Gosden & Nagano, 2002), the tissue can be collected without prior hormonal stimulation and avoids the requirement of a sperm donor (Wood *et al.*, 1997; Shaw *et al.*, 2000; Kim *et al.*, 2001a; Salha *et al.*, 2001). This is particularly important in the clinical setting, where successful recovery of tissue may be in part determined by the speed by which cancer treatment is initiated. Furthermore, ovarian tissue may be collected from females of all ages and, of particular importance to the conservation of endangered wildlife, following death (see Section 1.3.1.; Shaw *et al.*, 2000).

Cryopreservation of ovarian tissue circumvents the difficulties associated with the freezing of mature oocytes such as their size and susceptibility to spindle damage which can lead to the increased incidence of aneuploidy (Harp *et al.*, 1994; Oktay *et al.*, 1998a; Shaw & Cox, 2003). Cryopreservation of ovarian tissue thus remains the preferred method of storing female gametes clinically, and more importantly to the research reported in this thesis, for the preservation of endangered species.

1.3.2.2. Cryoprotectants

Cryoprotectants, the protective agents in which cells/tissue are frozen, can be classified into two groups; penetrating agents and non-penetrating agents. Glycerol, dimethyl sulfoxide (DMSO), ethylene glycol (EG) and 1,2-propanediol (PROH) are examples of

penetrating cryoprotectants. These compounds, which have molecular weights of less than 100, protect cells after entering through osmosis and minimizing the damaging effects caused by ice formation and the build-up of salts and other solutes (Shaw *et al.*, 1993; Newton *et al.*, 1998). Non-penetrating cryoprotectants such as large sugars (e.g. sucrose, raffinose and ficoll), proteins and lipoproteins remain in the extracellular solution because of their size or polarity (Shaw *et al.*, 1993).

An extensive amount of research has been undertaken to determine the most effective cryoprotectants and cryopreservation protocols for use in female reproductive biology (Pickering *et al.*, 1991; Carroll *et al.*, 1993; Shaw *et al.*, 1995b; Hovatta *et al.*, 1996; Candy *et al.*, 1997; Newton *et al.*, 1998; Saeed *et al.*, 2000). However, despite this extensive investigation, no protocol can be considered to be optimal (Fahy, 1986; Gosden & Nagano, 2002).

Cryoprotectant selection for use in the freezing of ovarian tissue requires a balance to be obtained between the damaging toxicity of the solute, and its ability to prevent ice crystal formation (McGann & Walterson, 1987; Gosden & Nagano, 2002). DMSO, EG, PROH and glycerol are four penetrating cryoprotectants that have been routinely employed in the cryopreservation of ovarian tissue. However, while cryopreserved ovarian tissue has been demonstrated to be capable of restoring fertility (Parrott, 1960; Gosden *et al.*, 1994a; Candy *et al.*, 2000; Shaw *et al.*, 2000), the optimal cryoprotectant and cryopreservation procedures remain unknown. Successful cryopreservation procedures are not only dependent on the cryoprotectant used, but also on the conditions through which the tissue to be frozen is exposed to the cryoprotectant (the equilibration conditions). It is believed that optimal equilibration conditions will depend on both the temperature and duration of the exposure. However, despite this, conflicting reports exists in regards to cryoprotectants and the optimal conditions for their equilibration.

Several different cryoprotectants have been used in the freezing of ovarian tissue, with 1.5M solutions of the penetrating cryoprotectants, EG and DMSO found to perform best in the freezing of human ovarian tissue (Newton *et al.*, 1998) and mouse pup ovaries (Candy *et al.*, 1997). The optimal equilibration conditions have been more variable. However, it is generally accepted that the time of exposure to the cryoprotectant before cooling must be adequate to enable penetration of the tissue to be frozen, but not too long that it incurs toxic damage. Newton and colleagues (1998) indicated that, for human ovarian tissue, the freezing success was optimised by equilibrating the ovarian tissue in the cryoprotectant, EG or DMSO, for 30 minutes at 4°C. By contrast Gook and colleagues (1999) suggested, on the basis of histological analysis, that ovarian tissue equilibrated with the cryoprotectant PROH, for 90 minutes at RT resulted in a higher proportion of intact human primordial and primary follicles following slow-cooling. These results suggest that long equilibration conditions prior to freezing may be superior to shorter equilibration times. However, this observation contradicts those made by Candy and colleagues (1997) in the mouse, where shorter equilibration times gave better follicle survival. These studies clearly demonstrate that the optimal equilibration conditions may vary both with the cryoprotectant used and the species from which the tissue was obtained.

1.3.3. Cryopreservation for the Long-term Storage of Ovarian Tissue

1.3.3.1. Cryopreservation of ovarian tissue to date

Ovarian tissue freezing was attempted long before embryo freezing. Green and colleagues (1956) made one of the earliest attempts at cryopreserving ovarian tissue and live mice were born following grafting of cryopreserved ovarian tissue in 1960 (Parrott, 1960). In more recent years, many have successfully slow-cool cryopreserved the ovarian tissue of a number of species, with the success of protocols used, routinely evaluated by the ability of the frozen/thawed tissue to regain function following transplantation. Cryopreserved

ovarian tissue of mice (Harp *et al.*, 1994), rats (Aubard *et al.*, 1998), sheep (Gosden *et al.*, 1994a; Gunasena *et al.*, 1997a; Baird *et al.*, 1999), elephants (Gunasena *et al.*, 1998) humans (Newton *et al.*, 1996), tammar wallabies (Mattiske *et al.*, 2002), wombats (Wolvekamp *et al.*, 2001), marmosets (Candy *et al.*, 1995), cats (Bosch *et al.*, 2002) and cows (Semple *et al.*, 2000; Herrera *et al.*, 2002) all regain function (i.e. become established and resume follicular development) following freezing and transplantation to an immunocompatible recipient. Furthermore, it has been demonstrated that even with prolonged storage prior to freezing, ovarian tissue can be successfully cryopreserved and resume follicular development following thawing and grafting (Cleary *et al.*, 2001).

In addition, the full restoration of fertility and the birth of live young has been achieved using slow-cool cryopreserved fetal (Cox *et al.*, 1996; Shaw *et al.*, 2000), juvenile (Candy *et al.*, 2000) and adult (Parrott, 1960; Gunasena *et al.*, 1997b) mouse ovarian tissue. Similarly, lambs have been born following the autografting of frozen/thawed cortical slices (Gosden *et al.*, 1994a; Salle *et al.*, 2002). Recent years have seen the clinical extension of these studies, with reports of restored follicular development and menstruation following slow-cool cryopreserved ovarian tissue autografting in female patients (Oktay *et al.*, 2001a; Radford *et al.*, 2001). However, before this technology can become routine practice (i.e. in the preservation of fertility in cancer patients), the potential risk of reinfection with disease must be eliminated.

Rapid cooling has also been used for ovarian tissue cryopreservation. Shaw and colleagues (1995a) obtained comparable, but low fertility for both slow and rapid-cooled and vitrified mouse ovarian tissue. Mouse pup ovaries were found to withstand vitrification (Lee *et al.*, 1998). However, contrasting results have been obtained in the human (Gook *et al.*, 1999). In a report by Gook and colleagues (1999), histological examination found both pre-granulosa cell and oocyte survival to be improved with slow-cool cryopreservation upon

comparison with rapid-cooled tissue. However, despite this result, the convenience of rapid-cooling (speed by which it can be completed, non-requirement for specialized equipment) and its potential application in the field warrant its further investigation.

1.3.3.2. Cryopreservation of marsupial ovarian tissue

Marsupial ovarian tissue, cryopreserved by a standard slow-cool freezing protocol of 1.5M DMSO and a 30-minute equilibration period has permitted follicular development in pademelon wallaby (Every, 2000), wombat (Wolvekamp *et al.*, 2001), and tammar wallaby (Mattiske, 1997; Snow, 1999; Mattiske *et al.*, 2002) frozen/thawed xenografted ovarian tissue. In addition to these studies, some investigation has been undertaken into alternative cryoprotectants and freezing protocols. Mattiske (1997) found that rapid-cooled tammar wallaby ovarian xenografts resumed follicular development and were similar in appearance to xenografts that were slow-cooled, thus supporting the potential employment of rapid-cool cryopreservation in the field.

The protective actions of EG in slow-cooling have been examined in both tammar wallaby (Snow, 1999) and pademelon wallaby (Every, 2000) ovarian xenografts, with tissue from both species exhibiting follicular development to antral stages. Furthermore, preliminary results obtained with tammar wallaby ovarian xenografts frozen in both DMSO and EG have indicated that longer equilibration (two hours as opposed to 30 minutes) may be beneficial to both follicle survival and follicular development (Snow, 1999). However, it is highly feasible that these observations may be species specific and be related to the density of the ovary. Further investigation of marsupial ovarian tissue cryopreservation is therefore required.

1.4. Ovarian Grafting

1.4.1. Background

The grafting of ovarian tissue to a recipient, which will not reject the graft provides a means for the *in vivo* maturation of oocytes contained within the ovarian graft, and a convenient model for the investigation of follicular development. Following grafting, ovarian tissue resumes normal function and can yield live young when returned to the normal anatomical site (Russell & Hurst, 1945; Gosden *et al.*, 1994a; Cox *et al.*, 1996; Petroianu *et al.*, 2002; Salle *et al.*, 2002). Alternatively, mature oocytes can be collected from the ovarian graft and fertilized *in vitro*. Embryos can then be transferred to a recipient to generate live young (Carroll *et al.*, 1990; Liu *et al.*, 2001; Yang, 2002). While fertilizable oocytes have been generated from normal ovaries by the *in vitro* maturation of pre-antral follicles (Eppig & Schroeder, 1989), primordial follicles (Eppig & O'Brien, 1996; Obata *et al.*, 2002; O'Brien *et al.*, 2003) and germ cells (Obata *et al.*, 2002), the proportion that form live young is much lower than that for *in vivo* matured oocytes. Additionally, ovarian tissue is well suited to grafting, it may be collected from females of all ages, at any stage of the reproductive cycle and even following recent death. The *in vivo* maturation of oocytes by means of ovarian grafting is therefore presently the preferred method for generating mature oocytes for use in ART's aimed at the preservation of endangered species.

1.4.1.1. Types of grafts

Ovarian grafts can be classified by both, the graft recipient and graft site. Recipients fall into three main categories autograft, allograft (or isograft) and xenograft recipients. *Autografting* involves the transfer of tissue within one individual while, in contrast,

allografting and *xenografting* involve the transfer of tissue from one individual to another. In *allografting*, the tissue is transferred to another member of the same species, while *xenografting* involves the transplantation of tissue from a member of one species to a member of a different species.

The terms orthotopic and heterotopic are used to refer to the graft site. *Orthotopic* grafts are transplanted to the normal anatomical position, while *heterotopic* grafts are transplanted to a site in the body different to which it had occupied in the donor.

1.4.1.2. History of ovarian grafting

Ovarian tissue transplantation is by no means a new concept, with the earliest reported attempt at ovarian grafting dating back to 1863 (reviewed by Nugent *et al.*, 1997). While these early rabbit allografts failed, presumably as a result of rejection, others reported greater success. In 1895, Morris performed the first human, ovarian allografts and autografts, and reportedly achieved pregnancy in one female (Morris, 1895). While the validity of this early work remains uncertain (Nugent *et al.*, 1997), this research in conjunction with other studies performed around the same period of time, raised significant interest in the area of gonadal tissue transplantation.

Ovarian grafting was attempted by numerous researchers between the 1940's and 1960's. Russell and Hurst (1945), clearly demonstrated the concept, through the development of a protocol that resulted in the birth of pure strain mice to hybrid mothers that had received ovarian grafts from pure strain donors. Jones and Krohn (1960) developed a successful procedure for the orthotopic transplantation of adult mouse ovarian tissue. Excellent results were obtained with 97% of recipients demonstrating estrous activity. Ovarian grafting in combination with cryopreservation as a means of storing ovarian tissue was also examined in the mouse (Green *et al.*, 1956) with the resumption of the estrous cycle observed in some recipients.

Similarly, research was also undertaken in other species. Harris and Eakin (1949), examined the survival of autografts, allografts and xenografts using mouse and rat ovarian tissue. Their results were the first to indicate that the presence of gonads in the recipient plays a role in determining graft success, as growth and regeneration was maximised in grafts to ovariectomised recipients (Harris & Eakin, 1949). This finding, which has been further supported by studies using mouse (Cox *et al.*, 2000) and wombat (Cleary *et al.*, 2002) ovarian tissue, has formed the basis of the majority of grafting studies to date (see Section 1.4.3.1.).

The 1990's saw a resurgence in the interest in ovarian auto and allografting (Gosden *et al.*, 1994a; Cox *et al.*, 1996; Candy *et al.*, 1997; Baird *et al.*, 1999; Salle *et al.*, 1999). In addition the generation of immunocompromised mouse and rat strains enabled the investigation of ovarian xenografting as a tool for both examining ovarian function and generating mature oocytes. Follicular development to the antral stage was observed within ovarian xenografts from a number of species (reviewed by Paris *et al.*, 2003) providing great hope for ovarian xenografting both clinically and in the conservation of endangered species.

1.4.2. Applications of Ovarian Grafting

Most autografting and allografting studies have been undertaken with the human clinical potential of the technique in mind. Xenografting has been examined for its prospects both clinically and as a tool for the generation of mature oocytes for use in conservation biology. In addition ovarian grafting has been employed as a tool to examine ovarian function and tissue viability (e.g. following cryopreservation or prolonged storage). In the mouse ovarian grafting has been employed to assess the viability of cryopreserved ovarian tissue obtained post-mortem (Cleary *et al.*, 2001; Takahashi *et al.*, 2001). While in the

sheep, ovarian autografting was used to investigate the role of FSH in folliculogenesis (Campbell *et al.*, 2000).

1.4.2.1. Clinical applications of ovarian grafting

Ovarian tissue grafting is a promising prospect for cancer patients. Cancer treatments, such as chemotherapy, frequently render patients sterile. The collection of ovarian tissue from female patients prior to treatment, and the subsequent autotransplantation of the tissue back to the female once the treatment has been completed may potentially safeguard the fertility of recovered patients. Research undertaken in this field is optimistic to date. An elegant study by Gosden and colleagues (1994a) first demonstrated the potential of this technology, when both fresh and frozen sheep ovarian tissues resumed function and were capable of producing offspring following autografting back to the donor animals. This has since been reiterated by Salle and colleagues (2002). The long-term function of cryopreserved sheep ovarian autografts was further demonstrated by Baird and colleagues (1999), with cyclical ovarian function observed in all animals for the duration of the study (22 months). In addition to sheep (Gosden *et al.*, 1994a; Baird *et al.*, 1999; Salle *et al.*, 1999; Salle *et al.*, 2002), autografts and/or allografts have been found to restore hormone activity in the mouse (Brem *et al.*, 1990; Harp *et al.*, 1994; Cox *et al.*, 1996; Jenkin *et al.*, 1996; Gunasena *et al.*, 1997a; Gunasena *et al.*, 1997b; Candy *et al.*, 2000; Shaw *et al.*, 2000), rat (Aubard *et al.*, 1998; von Eye Corleta *et al.*, 1998; Callejo *et al.*, 1999), rabbit (Petroianu *et al.*, 2002) and non-human primates (Schnorr *et al.*, 2002), with long-term fertility demonstrated in the mouse (Candy *et al.*, 2000; Shaw *et al.*, 2000), and rabbit (Petroianu *et al.*, 2002).

Recent years have seen the increased interest in the autotransplantation of cryopreserved human ovarian tissue and much debate surrounding the ethics of this technology (Newton, 1998; Gosden, 2000; Robertson, 2000; Kim *et al.*, 2001a; Salha *et al.*, 2001; Gosden &

Nagano, 2002; Hardy *et al.*, 2002; Revel & Laufer, 2002). Both fresh and frozen/thawed tissue has been found to produce steroid hormones following the return of the tissue to the donor (Oktay & Karlikaya, 2000; Callejo *et al.*, 2001; Oktay *et al.*, 2001a; Radford *et al.*, 2001). Furthermore, in an extension to this work, the restoration of ovarian function and oocyte retrieval from subcutaneous grafts has been reported in the human (Oktay *et al.*, 2001b) and primate (Schnorr *et al.*, 2002). However, valid concerns exist regarding the application of this technology to cancer patients. Justification for this was clearly illustrated when the development of cancer in healthy mice following allografting with ovarian tissue from donor mice with lymphoma was reported (Shaw *et al.*, 1996). While, a more recent study involving the xenotransplantation of ovarian tissue from patients with lymphoma to mice has indicated that ovarian tissue collected prior to treatment may be safe for autografting (Kim *et al.*, 2001b), the potential risk to the patient cannot be ignored. Similarly, consideration must be given to the possible transmission of other diseases.

The xenografting of human ovarian tissue to immunocompromised mice or rats has been used for research. During the past decade, numerous studies have been undertaken investigating the xenotransplantation of both fresh and cryopreserved human ovarian tissue (Newton *et al.*, 1996; Oktay *et al.*, 1998b; Weissman *et al.*, 1999; Oktay *et al.*, 2000; Revel, 2000; Gook *et al.*, 2001; Kim *et al.*, 2001b). Furthermore, despite genetic and reproductive differences between the human and mouse, follicular development to gonadotrophin dependent antral stage follicles has been observed following ovarian transplantation both with (Oktay *et al.*, 1998b; Weissman *et al.*, 1999; Gook *et al.*, 2001) and without (Gook *et al.*, 2001) exogenous hormone stimulation. In an extension to this work, Revel and colleagues (Revel, 2000; Revel & Laufer, 2002) have reported the successful retrieval of oocytes from xenografted human ovarian cortex following stimulation with FSH for nine to 12 weeks, and human chorionic gonadotrophin (hCG)

stimulation 36 hours prior to oocyte collection. In addition, following *in vitro* maturation normal metaphase II (MII) oocytes were obtained. It may be speculated that xenografting could be used to protect patients from reinfection, however such a approach could not be pursued in the state of Victoria in Australia, where it is forbidden to grow or maintain human gametes. In countries where such bans are not present careful consideration would have to be given to clinical employment of human ovarian xenografting. Specifically, the possibility of retrovirus transmission, genetic modification and the moral ethics of the technique warrant debate.

1.4.2.2. Ovarian xenografting for the conservation of endangered wildlife

As mentioned previously, ovarian xenografting offers great promise as a tool that may enable the generation of mature oocytes from ovarian tissues that are salvaged from endangered wildlife species or stored cryopreserved. Oocytes harvested from grafts could then be used in IVF or ICSI to produce offspring, hence increasing population numbers. Of great advantage, and in contrast to the situation with respect to mature oocytes and embryos, ovarian tissues can be collected from females irrespective of age, the stage of the reproductive cycle and even following recent death. These factors make the xenografting of ovarian tissue an attractive ART for use in the preservation of endangered wildlife.

Recent years have seen substantial interest in the xenografting of ovarian tissue, with tissue from a number of non-human mammalian species including the sheep (Gosden *et al.*, 1994b; Gunasena *et al.*, 1997a), cow (Weissman *et al.*, 1998; Semple *et al.*, 2000; Herrera *et al.*, 2002), cat (Gosden *et al.*, 1994b; Bosch *et al.*, 2002), elephant (Gunasena *et al.*, 1998), marmoset (Candy *et al.*, 1995), wallaby (Mattiske *et al.*, 2002), wombat (Wolvekamp *et al.*, 2001; Cleary *et al.*, 2002), dog (Metcalfe *et al.*, 2001) and rabbit (Muirhead *et al.*, 2002) successfully xenografted to immunocompromised mouse or rat recipients, with graft establishment and follicular development observed. Regardless of

species differences, follicular development within ovarian xenografts has been observed to proceed normally, with antral follicles observed within ovarian xenografts from a diverse range of species (Gosden *et al.*, 1994b; Gunasena *et al.*, 1998; Mattiske *et al.*, 2002; Muirhead *et al.*, 2002). The results imply that the biological activities of FSH and LH are not exclusively species specific (Gosden *et al.*, 1994b), and hence, that hormones produced by the eutherian graft recipient are capable of supporting follicular development within the graft, whether it be of eutherian or marsupial origin.

As will be discussed further in Section 1.4.3.1., the gonadal status of the recipient is known to have an effect on graft function. While bilaterally ovariectomised females have typically been employed as ovarian xenograft recipients for their elevated gonadotrophin concentrations that are believed to be beneficial to graft establishment and follicular development (Dissen *et al.*, 1994; Gosden *et al.*, 1994b; Candy *et al.*, 1995; Wolvekamp *et al.*, 2001; Mattiske *et al.*, 2002), conflicting reports exist in regards to the optimal gonadal status of the recipient. In addition to bilaterally ovariectomised females, intact females (Mattiske *et al.*, 2001), castrated males (Bosch *et al.*, 2002) and intact males (Waterhouse, 2001) have all successfully supported follicular development within ovarian xenografts. Further investigation is thus required to clarify the exact role of the recipient hormone environment.

Ovarian xenografting has been extended in some species, by the collection of oocytes from ovarian xenografts. Oocytes have successfully been recovered from wallaby (Mattiske *et al.*, 2000; Mattiske *et al.*, 2001), cow (Herrera *et al.*, 2002), mouse (Waterhouse, 2001) and marmoset (Choo, 1999; Waterhouse, 2001) ovarian transplants, with fertilization and embryo cleavage reported in oocytes harvested from both wallaby and marmoset xenografts (Choo, 1999; Mattiske *et al.*, 2000). However, in more recent years no advancement in this field of research has been reported, with the viability (measured by the

ability to produce healthy live young) of oocytes matured within ovarian xenografts still not proven. The challenge now is for live young to be produced from oocytes matured within ovarian xenografts, and the full potential of ovarian xenografting to be realized.

1.4.3. Grafting of Ovarian Tissue

Small ovaries have typically been grafted whole, relying on their small size to ensure rapid revascularization (Gosden, 1992), while larger ovaries have tended to be cut into sections enabling the tissue to be distributed among numerous recipients (Shaw & Cox, 2003) and reducing the duration of time to the complete revascularization of the tissue. Even though revascularization can be complete within 24 hours, transplantation does reduce ovarian tissue follicle numbers by approximately a third in the mouse (Candy *et al.*, 1997) and human (Newton *et al.*, 1996). This loss is even greater if grafting follows cryopreservation (Cleary *et al.*, 2001).

1.4.3.1. Effect of gonadal status of recipient

As mentioned previously, bilaterally ovariectomised female recipients have typically been employed in ovarian grafting studies. Their elevated gonadotrophin levels are believed to be beneficial to both graft establishment and follicular development (Harris & Eakin, 1949; Dissen *et al.*, 1994; Gosden *et al.*, 1994b; Cox *et al.*, 1996). However, recipients with alternative gonadal statuses have been studied.

Cleary and colleagues (2002) found follicular development was accelerated in wombat ovarian tissue xenografts to bilaterally ovariectomised recipients compared to intact recipients. This observation may reflect the capacity of gonadotrophins to increase mRNA levels of some angiogenic factors in ovarian grafts (Dissen *et al.*, 1994). In contrast, a wallaby xenografting study indicated that mature oocytes were more readily collected from xenografts to intact recipients than ovariectomised recipients (Mattiske *et al.*, 2001),

possibly indicating a species-specific effect for the role of the hormonal environment on graft establishment and development.

Unilaterally ovariectomised individuals may provide an alternative recipient for the ovarian tissue of some species. Unilateral ovariectomy is known to cause a temporary increase in circulating FSH (Welschen & Dullaart, 1974; Butcher, 1977; Findlay & Cumming, 1977; Redmer *et al.*, 1984), and a rise in angiogenic factor synthesis (Dissen, 1994). However, little information currently exists regarding the use of unilaterally ovariectomised recipients in ovarian grafting studies. Deansely found that neonatal rat ovaries exhibit retarded development when grafted subcutaneously to unilaterally ovariectomised recipients (Deansely, 1956). Similarly, Cox and colleagues (1996) reported that when fetal mouse ovaries are grafted to unilaterally ovariectomised recipients they either failed to become established, or failed to form antral follicles. It was speculated that the circulating gonadotrophin levels in unilaterally ovariectomised recipients were insufficient to establish graft development (Cox *et al.*, 1996).

The use of male graft recipients has also been examined. Follicular growth and survival is improved in primate oocytes undergoing *in vitro* maturation in the presence of exogenous androgens (Vendola *et al.*, 1998), suggesting that increased androgen concentrations as offered by male ovarian graft recipients, may be beneficial to the graft function. This notion is supported by a study reported by Weissman and colleagues (1999), in which, more developing follicles were observed in subcutaneous human ovarian xenografts to male mice recipients than female mice following exogenous hormone stimulation. Follicular development to antral stages in cat ovarian tissue has also been supported following xenografting to castrated male mice (Bosch *et al.*, 2002), clearly demonstrating that bilaterally ovariectomised recipients are not the only suitable recipients for use in grafting studies.

1.4.3.2. Effect of graft site

While, orthotopic grafts can completely restore normal fertility, including the capacity to conceive naturally (Shaw & Cox, 2003), heterotopic grafts sites have other benefits. Over the years numerous sites in the body have been successfully used for the transplantation of ovarian tissue including the ovarian bursa (Russell & Hurst, 1945; Jones & Krohn, 1960; Parkening *et al.*, 1984; Parkening *et al.*, 1985; Gosden *et al.*, 1994a; Candy *et al.*, 2000; Petroianu *et al.*, 2002), kidney capsule (Rumery & Blandau, 1976; Cox *et al.*, 1996; Newton *et al.*, 1996; Gunasena *et al.*, 1998; Oktay *et al.*, 1998b; Gook *et al.*, 1999; Oktay *et al.*, 2000; Wolvekamp *et al.*, 2001; Mattiske *et al.*, 2002), intra-muscular (Lightman *et al.*, 1998; Revel, 2000), subcutaneous sites (Green *et al.*, 1956; Byskov *et al.*, 1977; Lightman *et al.*, 1998; Weissman *et al.*, 1999; Semple *et al.*, 2000; Oktay *et al.*, 2001b) and the corneal chamber of the eye (Ben-Or, 1965).

Of these heterotopic sites, the kidney capsule became the most popular site. In addition to the convenient pocket in which the graft can be easily secured and recovered from, the highly vascular environment provided by the kidney capsule permits rapid revascularization of grafts (Gosden *et al.*, 1994b). Subcutaneous sites are substantially less vascularized but have the advantage of providing a surgically less intrusive alternative and permit monitoring of graft establishment and follicle development by palpation. These benefits are particularly valuable in the clinical setting. Few studies have directly compared the efficiency of graft sites. However, these studies indicate that the kidney capsule provides a better graft site than under the skin, as it gave faster follicular development in wombat ovarian xenografts (Cleary *et al.*, 2002) and significantly higher rates of oocyte recovery and embryo cleavage in mouse allografts (Yang, 2002).

1.4.4. Immunology of Transplantation

The success of any organ or tissue transplant is determined by the immunocompatibility of the organ/tissue donor and recipient. Where the donor and recipient are immunologically incompatible, the graft recipient must be rendered immunologically tolerant or undergo immune suppression if graft rejection is to be avoided. Alternatively, for the purpose of scientific investigation and ovarian grafting studies, the employment of immunocompromised strains of mice and rat enable the problem of graft rejection to be avoided.

1.4.4.1. Immunocompromised graft recipients

The development of immunocompromised strains of mice and rat such as the severe combined immunodeficiency (SCID) mouse, and nude mouse and rat has enabled the investigation of ovarian xenografting using ovarian tissue collected from a range of species including the wallaby (Mattiske *et al.*, 2002), elephant (Gunasena *et al.*, 1998) and human (Oktay *et al.*, 1998b; Gook *et al.*, 2001). The host environment enables xenografted ovarian tissue to regain function and the *in vivo* maturation of oocytes. The oocytes matured via this process may potentially be capable of generating viable offspring following employment of the oocytes in alternate ART's such as IVF, ICSI and embryo transfer.

The SCID mutation in the mouse was first discovered in C.B-17 inbred mice homozygous for the autosomal recessive mutation, *scid*, 20 years ago (Bosma *et al.*, 1983). The SCID trait, which is the result of the *scid* locus on chromosome 16 (Bosma *et al.*, 1989), is characterised by the absence of functional T and B cells (Bosma *et al.*, 1983; Steinsvik *et al.*, 1995; Renz *et al.*, 1996). Consequently, mice expressing this trait are incapable of initiating a normal immune response to foreign antigens, thus rendering them a suitable model for cancer, HIV and transplantation research. In ovarian xenografting studies, SCID

mice have successfully acted as hosts for ovarian tissue from a number of species including the dunnart (Lamden, 1996), cat (Gosden *et al.*, 1994b), dog (Metcalfe *et al.*, 2001) and human (Oktay *et al.*, 1998b; Oktay *et al.*, 2000). However, both the partial restoration of lymphocyte production (Bosma *et al.*, 1988; Sandhu *et al.*, 1996) and the presence of non-specific immune response circulating natural killer cells and macrophages (Sandhu *et al.*, 1996) result in SCID mice being unsuitable for long-term studies (>5 months). However, the development of the non-obese diabetic (NOD)-SCID (Shultz *et al.*, 1995) has provided a viable alternative model. NOD-SCID mice, which have successfully supported follicular development within human (Weissman *et al.*, 1999) and cat (Bosch *et al.*, 2002) ovarian xenografts, are in addition to being devoid of lymphocytes, largely deficient in macrophages and natural killer cells (Shultz *et al.*, 1995).

Nude mice and rats, that are devoid of T lymphocytes due to the absence of the thymus as a result of the recessive *nude* mutation, have also been shown to support follicular development within human (Lightman *et al.*, 1998; Revel, 2000), elephant (Gunasena *et al.*, 1998), cow (Herrera *et al.*, 2002) and wombat (Wolvekamp *et al.*, 2001) ovarian tissue xenografts. In the study by Lightman and colleagues (1998), the efficiency of nude mice as xenograft recipients was clearly demonstrated with 97% of fresh and 93% of frozen grafts able to be recovered after surgery. Furthermore, the majority of xenografts contained primordial and pre-antral follicles that, when assessed visually, were deemed to be normal in appearance.

More recently recombination activation gene (RAG) mice have been generated. In RAG mice, portions of the RAG-1 or RAG-2 coding regions have been deleted, resulting in the absence of mature T and B lymphocytes (Mombaerts *et al.*, 1992; Shinkai *et al.*, 1992), providing an alternative model for transplantation studies, in which the leakiness associated with SCID mice, has not been observed (Steinsvik *et al.*, 1995). Furthermore,

preliminary results obtained in our laboratory have demonstrated that RAG mice too are suitable recipients for ovarian xenografts (Snow, 1999).

1.4.4.2. Tolerization and immune response suppression

The high cost and specific housing requirements of immunocompromised mouse and rat strains has led to the investigation of alternate recipient models for ovarian xenografts (Lamden, 1996; Cleary, 1999). Two models that have been suggested for use in ovarian transplantation studies are immunologically tolerized and immunosuppressed recipients.

The ability to induce tolerance through the exposure of embryos or young neonates to foreign antigens was first demonstrated by Billingham and colleagues in 1953. While, this concept has since formed the basis of numerous scientific investigations, conflicting views exist regarding both the basis and existence of neonatal tolerance (Gammon *et al.*, 1986; Forsthuber *et al.*, 1996; Stockinger, 1996; Garza *et al.*, 1997). Furthermore, results obtained by Garza and colleagues (1997) propose that immunological tolerance may not be inducible to all antigens. The successful use of this phenomena in ovarian transplant studies may therefore benefit by the relatively low immunogenicity of ovarian tissue in comparison to other tissues such as the skin (Billingham & Parkes, 1955; Krohn, 1965).

Immunosuppression has been achieved in clinical transplantation using a number of different agents including antilymphocyte serum (ALS) and cyclosporine (Starzl, 2000), both of which have subsequently been employed in ovarian transplantation studies (Barnes & Crosier, 1969; Cleary, 1999). Over thirty years ago, ALS treatment was found to extend the longevity of mouse ovarian allografts (Barnes & Crosier, 1969), however its efficiency in preventing ovarian xenograft rejection has not previously been examined. Similarly, immunosuppression by means of treatment with cyclosporine, has been found to suppress wombat ovarian tissue xenograft rejection in rats (Cleary, 1999) and ovarian allograft rejection in rabbits (Petroianu *et al.*, 2002). The use of immunosuppressed or

immunologically tolerized recipients in ovarian xenografting studies therefore appears to provide a potentially viable alternative to the employment of expensive immunocompromised mouse and rat strains for research purposes.

1.5. Summary and Aims

Worldwide thousands of plant and animal species are being faced with the threat of extinction. Australia is by no means an exception. The Australian Terrestrial Biodiversity Assessment 2002 found that 22 native mammalian species have been lost since settlement of the country, while another eight species no longer persist on the mainland and are now isolated to islands. In the same assessment the marsupial leadbeaters possum, long-footed potoroo and spot-tailed quoll were all identified as threatened species. ART's must be developed to ensure the protection of endangered species and the preservation of their germline, or these species may be lost forever. The opportunistic collection and salvaging of ovarian tissue for use in ART's is a favourable option, due to both the ease by which ovarian tissue may be collected, and the potential of each piece of tissue. Subsequent transplantation of collected ovarian tissue provides a valuable means for the *in vivo* maturation of oocytes. Matured oocytes may then potentially be employed in ART's to propagate species and help ensure their survival.

The central aim of this thesis was to investigate the feasibility of employing ovarian xenografting as a strategy for the conservation of endangered species. However, there are many aspects to the use of ovarian transplantation in conservation biology. To maximise the genetic pool of threatened species, ovarian tissue must be scavenged from the carcasses of valuable females following death and be collected opportunistically from healthy surviving females undergoing gonadectomy. Information on the degeneration and loss of viability that occurs within ovarian tissue contained within a carcass post-mortem or during short-term storage during transportation from the site of tissue collection to a laboratory for freezing or tissue utilization is thus required. The aim of the first experimental study

described in this thesis (Chapter Two) was to use a mouse model to investigate the effect of varying storage conditions (duration, temperature and method of storage) on the viability of ovarian tissue collected following death.

While xenografting has proved an efficient means for supporting follicular development within the ovarian tissue of a wide range of species, the viability of oocytes matured within ovarian xenografts has not been proven. The study presented in Chapter Three aimed to use a basic mouse to rat model to demonstrate the full potential of ovarian xenografting and thus determine whether live young can be produced from oocytes matured within ovarian xenografts.

The three studies described in Chapter Four were designed as an extension to Chapter Three. The general aim of this chapter was to investigate ovarian xenografting using ovarian tissue obtained from three marsupial species. The specific aim of the chapter was to obtain mature oocytes from marsupial ovarian xenografts. It is highly likely that the optimal conditions for xenografting may be species dependent. Therefore, additional aims of Chapter Four were to investigate the effect of the gonadal status of the recipient, the graft site and cryopreservation protocols on marsupial ovarian xenograft function.

Immunocompromised mouse and rat strains have typically been employed in ovarian xenografting studies. However, these strains are both more expensive to purchase and difficult to maintain. The purpose of the two preliminary studies described in Chapter Five was to evaluate the feasibility of employing immunologically tolerized and immunosuppressed recipients in ovarian xenografting studies as an alternative to immunocompromised mouse and rat strains.

In addition to providing a valuable means for the *in vivo* maturation of oocytes, ovarian grafting provides a tool for examining ovarian function. The aims of the studies presented

in Chapter Six were to employ ovarian grafting and *in vitro* culture to examine oogenesis and folliculogenesis within ovaries devoid of follistatin, and in doing so gain a greater understanding of the role that follistatin plays in ovarian function.

~ *Chapter Two* ~

Viability of Mouse Ovarian Tissue Collected After Death

2.1. Introduction

Worldwide, conservation groups have begun to establish genome resource banks that consist of a range of materials including reproductive tissues and somatic cells from common and endangered species (Holt & Pickard, 1999; Ryder *et al.*, 2000). These materials are being collected to help protect genetic diversity, and for use in ART's to propagate threatened species. In many instances the materials (tissue, gonads, embryos or gametes) are collected opportunistically (salvaged) from rare or endangered species, following their death or after gonadectomy, and then transported to a suitably equipped laboratory where they are cryopreserved for long-term storage.

However, the banking of the female germline has several constraints. Mature eggs and embryos are scarce and their cryopreservation properties differ widely. The collection and cryobanking of ovarian tissue, rather than eggs and embryos, from rare and endangered species has many advantages. Ovarian tissue can be easily collected from live and recently deceased animals (Cleary *et al.*, 2001; Wolvekamp *et al.*, 2001), it is easy to cryopreserve (Gosden *et al.*, 1994a; Agca, 2000; Shaw *et al.*, 2000) and commonly contains very large numbers of oocytes at differing stages of development.

While the cryopreservation protocols developed to date are effective for ovarian tissue of a number of mammalian species including the mouse (Parrott, 1960), rat (Aubard *et al.*, 1998), elephant (Gunasena *et al.*, 1998), sheep (Gosden *et al.*, 1994a), cow (Semple *et al.*, 2000), marmoset (Candy *et al.*, 1995) and human (Newton *et al.*, 1996; Oktay *et al.*, 2000), the disadvantage of cryobanked ovarian tissue is that most of the oocytes it contains are immature and require further maturation by *in vitro* culture or by grafting before they are mature and ready to be fertilized (by IVF or ICSI). Ovarian tissue grafted back to the donor (autografting) or another animal of the same species (allografting), has been found to successfully resume normal reproductive function in the mouse (Candy *et al.*, 2000; Shaw *et al.*, 2000), sheep (Gosden *et al.*, 1994a; Baird *et al.*, 1999; Salle *et al.*, 2002) and rabbit (Petroianu *et al.*, 2002) and endocrine function in the human (Oktay *et al.*, 2001a; Oktay *et al.*, 2001b). However, the requirement for the donor and recipient to be immunologically compatible restricts the use of autografting and allografting for the ovarian tissue of rare and endangered species. In such a situation, ovarian tissue can be xenografted to immunodeficient recipients in order to study follicular development in a particular species or as a method of maturing oocytes for subsequent use in ART's (Gosden *et al.*, 1994b; Gunasena *et al.*, 1998; Paris *et al.*, 2003).

Although procedures for obtaining mature oocytes from banked ovarian tissue should improve, it is essential that the material that is stored is viable. At the time of commencement of the work contained in this thesis, no real consideration was being given to the quality of the material at the time of collection or the most appropriate means of storing this material before it was cryopreserved.

Both male and female germ cells remain viable for a variable length of time (hours to days) following the death of an animal (An *et al.*, 1999; Browne *et al.*, 2001). The post-mortem viability of sperm has been studied in a number of species including the deer (Hishinuma

et al., 2003), dog (Yu & Leibo, 2002), cane toad (Browne *et al.*, 2001), mouse (Songsasen *et al.*, 1998; An *et al.*, 1999) and human (Check *et al.*, 1999; Check *et al.*, 2002). Live offspring have been produced from sperm collected following death in both a mouse (Songsasen *et al.*, 1998; An *et al.*, 1999) and human model (Check *et al.*, 2002). Considerably less work has been undertaken investigating the viability of female gametes following death. The developmental competence of *in vitro* matured germinal vesicle stage oocytes collected 12 and 48 hours post-mortem has been investigated in the mouse (Schroeder *et al.*, 1991) and cat (Johnston *et al.*, 1991) respectively. Similarly, in the cow (Yang *et al.*, 1990) and sheep (Moodie & Graham, 1989), viable oocytes have been obtained from ovaries stored *in vitro* for periods of less than 12 hours. These studies demonstrated that oocytes collected post death are capable of maturation, and in the mouse, cat and cow, fertilization following *in vitro* culture.

Despite these studies, further practical data showing how rapidly ovarian tissue viability changes within a dead animal's body, or after ovariectomy is needed. Data is also required to determine the optimal conditions to store or transport ovarian tissue to the laboratory. As a result it is currently unknown whether ovarian tissue should be collected opportunistically from deceased animals (e.g. a road kill victim), especially in instances where the animal may have been dead for periods greater than 12 hours, prior to tissue removal, or can be transported to a suitably equipped laboratory, as often occurs in less populated and remote areas (e.g. Australia and Africa). Similarly, little data currently exists regarding the optimal storage temperature for ovarian tissue following collection. While metabolic suppression has always been thought to be fundamental to successful organ preservation (Lindell *et al.*, 1998), a study comparing warm and cold transport media for human ovarian cortex has indicated that cold storage may not be superior (Weissman *et*

al., 1999). A thorough investigation of the conditions that are optimal for the storage of ovarian tissue is therefore required.

2.1.1. Aims of this Chapter

This chapter reports the use of a mouse model to investigate the effects of storage duration (three-48 hours), temperature (on ice versus RT) and storage out of and in the body (*in vitro* versus *in situ* stored ovaries) on the follicle population contained within ovarian tissue collected from deceased mice.

2.2. Materials and Methods

2.2.1. Animals

Four-week old inbred BALB/c mice were used as both donors (n=41) and recipients (n=41). Four-week old animals were used as they contain a high abundance of follicles at all stages of follicular development and they do not contain corpora lutea (Peters, 1969). In the absence of corpora lutea, the volume of the ovary is small, thus ensuring a uniform opportunity for revascularization at the time of grafting. Recipients and donors were used at the same age to enable some mice to be utilised as both donors and recipients.

The mice were obtained from Central Animal Services at Monash University and were housed under a 12 hour light-dark regimen at 21°C. Ethical approval for this study was obtained from Monash University Department of Physiology Animal Ethics Committee and complied with the conditions laid down by the NH&MRC/CSIRO/AAC Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1997).

2.2.2. Ovarian Tissue Collection

Donor mice, not also serving as recipients, were killed by cervical dislocation and then their ovaries subsequently collected. Depending on the treatment group, the ovaries were either collected immediately or at a later stage. To collect the ovaries, the abdominal cavity was opened and the intestines moved to the side, enabling the visualization of the ovaries that were anatomically suspended slightly below each kidney. Using forceps, the ovaries were carefully removed from the body of the donor animal before being dissected free of the bursa and fat in PBS (Gibco BRL – Life Technologies, NY, USA) at RT.

2.2.3. Ovarian Tissue Storage Conditions

2.2.3.1. *In situ*

Ovaries were randomly allocated to one of four experimental storage groups or the zero hours fresh control group (fresh non-stored; Table 2.1.). To simulate the collection of ovarian tissue from deceased animals in the wild (e.g. a road kill) the ovaries in Groups 1 and 2 were left in the bodies of the donor animals for the duration of the storage period (*in situ*). At the end of the storage period, the *in situ* stored ovaries (Groups 1 and 2) were collected from the body of the animal using the same methods as described above (Section 2.2.2.) and cut in half using a scalpel blade prior to grafting.

2.2.3.2. *In vitro*

To simulate the collection and transportation of ovarian tissue in the field or from a zoo to a laboratory for storage (as frequently occurs at the Animal Gene Storage and Resource Centre of Australia), ovaries from Groups 3 and 4 were removed immediately (*in vitro*) from the bodies of the animals, cut in half using a scalpel blade, and then placed in PBS supplemented with Penicillin (62units/ml; Penicillin G; Sigma, MO, USA) and Streptomycin (62µg/ml; Streptomycin sulfate; Sigma, MO, USA). PBS was used, as it is a simple and readily available media that is frequently used for the short-term collection and storage of ovaries under laboratory conditions. PBS is also routinely utilized by zoos and in the field for the collection and transportation of ovarian tissue.

2.2.3.3. Time and temperature

The bodies of the animals (stored in a sealed plastic bag; Groups 1 and 2), or dissected ovaries (in a petri dish containing PBS; Groups 3 and 4), were stored either at RT (21°C; Groups 2 and 4) or placed directly on ice and water (0°C; Groups 1 and 3). The ovarian tissues remained in their respective storage conditions (*in situ* or *in vitro*) for 3, 6, 12, 24 or 48 hours following the death of, or removal from the donor mouse, giving 20 treatment

groups in total (Table 2.1.). The shorter time points were investigated to reflect the degeneration that would occur within ovarian tissue that was collected from an animal at the time of gonadectomy, euthanasia or within a short period following death. The longer time points simulated conditions in the field where an animal may have died and 12-48 hours passed before the tissue was recovered, or alternatively, where the tissue was transported from the field or a zoo to an equipped laboratory for storage or the use of the tissue.

2.2.3.4. Controls

As a control, a group of recipients (n=4) received ovarian tissue that was grafted immediately (0 hours) following collection from the donors (n=4; Table 2.1.).

Table 2.1. Number (n) of grafts grafted for each experimental group following storage *in situ* or *in vitro* for 3, 6, 12, 24 or 48 hours (h) or grafted fresh following no storage (0h).

Experimental group	**Ovarian tissue storage conditions**	**n Grafted following storage**					
		0h	**3h**	**6h**	**12h**	**24h**	**48h**
Group 1	Ovaries stored *in situ* on ice	-	8	8	8	6	4
Group 2	Ovaries stored *in situ* at RT	-	8	8	6	6	4
Group 3	Ovaries stored *in vitro* on ice	-	10	8	6	12	12
Group 4	Ovaries stored *in vitro* at RT	-	6	8	8	9	6
Control	0 hours (non-stored fresh tissue)	13	-	-	-	-	-

2.2.4. Ovarian Tissue Grafting

Each ovarian half was grafted under the kidney capsule of age matched recipients to assess the number and viability of follicles within the ovarian tissues of each of the treatment groups and the control group.

2.2.4.1. Anaesthetic

Recipients were anaesthetised using a filter sterilised 2.5% (w/v) Avertin solution. The Avertin stock contained 1g 2,2,2 Tribromethanol (Aldrich Chemical Company Inc., WIS, USA) and 0.5g tertiary amyl alcohol (Sigma-Aldrich Chemical Company Inc., WI, USA), made to volume with distilled water. The Avertin was administered to graft recipients through an intra-peritoneal injection. The depth of anaesthesia was assessed using the toe pinch test. When no response was elicited, surgery was commenced. The depth of anaesthesia was monitored for the duration of the surgery.

2.2.4.2. Ovarian grafting to the kidney capsule

The back of each recipient was sterilised with 70% alcohol, and a 1cm mid dorsal incision was made through the graft recipient's skin. Using scissors, an incision was made into the peritoneal cavity overlying the left flank to expose one ovary. Using the ovarian fat pad, the ovary was exteriorised. The ovarian fat pad was then carefully pulled to locate and exteriorise the kidney through the peritoneal incision. The kidney capsule was gently lifted from the kidney and carefully torn using fine forceps, to create a pocket between the kidney and its capsule. Two halves of the same ovary from a donor animal, were then carefully placed under the capsule before returning the kidney to its normal anatomical position. The recipient's own ovary was then removed using scissors to cut the entire ovary at the level of the oviduct before returning the reproductive tract to the abdominal cavity. The grafting procedure was then repeated on the right hand side of the graft recipient, such that each female recipient received four grafts (equivalent to two ovaries). The dorsal incision was closed using 9mm wound clips (Becton Dickinson Primary Diagnostics, MD, USA). The grafting took a maximum of 10 minutes per animal grafted.

Generally, the left kidney of recipient animals received grafts from one treatment group and the right kidney grafts from another treatment group. This ensured that ovarian tissue

from the one treatment group went into more than one recipient. At least three mice were used as recipients for each treatment group (excluding the 48 hours *in situ* treatment groups where n=4 half ovaries were grafted and hence only two recipients used).

2.2.5. Assessment of Graft Function

2.2.5.1. Graft recovery

Grafts were recovered from recipients two weeks later as this allows surviving primordial follicles to reach the early antral stage of follicular development in allografted mouse ovarian tissue (Cox *et al.*, 1996).

Recipients were killed by cervical dislocation and an incision made across the width of the abdomen through both the skin and peritoneum to enable access to the kidneys. The kidneys were removed using forceps and a pair of scissors, and the grafts carefully dissected free from the kidney using forceps and a scalpel blade and placed into PBS. A section of kidney was removed around the peri .cter of the graft to ensure the complete removal of the graft. Grafts were then taken for histology to enable assessment of the follicle population (Section 2.2.5.2.).

2.2.5.2. Histology

Grafts were placed in Bouin's fixative (Sigma Diagnostics, MD, USA) for 24 hours in preparation for histological assessment. Tissues were then transferred to a 70% alcohol solution until they were processed and embedded in paraffin wax. During the processing, the tissue was dehydrated, cleared and impregnated with paraffin using a histokinette machine (Thomas Optical and Scientific Co, UK) set on a 24-hour cycle. During this process the tissue was dehydrated and cleared in two changes of 70% alcohol and 90% alcohol, followed by four changes of 100% alcohol and histosol (Interpath Services, West Heidelberg, Australia). Impregnation of the tissue was achieved by three changes of

paraffin wax at 60°C, with two of these changes occurring on the histokinette and a final change occurring in a vacuum-sealed bath for 20 minutes.

The ovarian tissues were embedded in paraffin blocks and serially sectioned at 7µm thickness using a rotating microtome. Sections were floated on a 45°C water bath with gelatine before being collected onto slides. Sections collected onto slides were dried overnight in a 37°C incubator.

Dried sections were stained using the step-by-step procedure described in Appendix 1. The nuclei of the cells were stained with haematoxylin followed by the counterstaining of the cytoplasm with eosin. Once stained, slides were cover-slipped using 24mm x 64mm cover-slips, (Mediglass, Australia) using DPX (BDH, UK). Following cover-slipping, slides were left to dry on the bench for a minimum of 24 hours before being analysed under a light microscope.

2.2.5.3. Classification and counting of follicles

Blind follicle counts were performed to assess the estimated total number of follicles in recovered grafts. Follicles containing oocytes that had a visible nucleolus were counted. To ensure accurate counting of follicles, only every third section was examined. Follicles were classified using the scheme proposed by Pedersen and Peters (1968), which is summarised in Table 2.2 and Figure 1.2. Follicles that appeared morphologically normal were termed normal and follicles with signs of atresia were termed atretic (Table 2.2.).

Table 2.2. Scheme used for the classification of follicles during histological analysis. The scheme is based on the follicle classification scheme for the mouse proposed by Pedersen and Peters (1968).

Follicle type	Description of follicle type
Small	Oocyte surrounded by one or less layers of flattened or cuboidal follicle cells (up to a maximum of 20 follicle cells within the largest cross-section).
Medium	Oocyte surrounded by one to three layers of follicle cells (up to a maximum of 200 follicle cells within in the largest cross-section).
Large	Oocyte surrounded by greater than three layers of follicle cells, with or without the presence of follicle fluid.
Atretic	Oocyte showing signs of degeneration (e.g. loss of nuclear membrane or fragmentation) or the presence of three or more pyknotic nuclei in follicle cells in a single section.

2.2.6. Statistical Analysis

The total number of follicles and types of follicles from the different treatment groups were analysed using one and two-way analysis of variance ($p<0.05$ was considered to be significant) and the least significant difference post hoc test. Two-way analysis of variance established the effect of the storage model, temperature and duration of storage. One-way analysis of variance was used to identify differences between treatment groups. Differences in graft recovery rates were determined using the Chi-Square test. Analysis of variance was made with the aid of the computer statistics package SPSS 9.0.1 for Windows (1999). Results obtained are presented as the mean ± SEM.

2.3. Results

2.3.1. In Vitro *Versus* In Situ *Storage*

Follicles that were normal in appearance were observed in all recovered grafts that had been stored *in vitro* prior to grafting (Groups 3 and 4; Figure 2.1.a.) and in grafts that had been stored *in situ* and kept on ice (Group 1) for up to 48 hours (Figure 2.1.b.; Table 2.3.), following the death of the donor animal prior to grafting. No grafts were recovered from ovarian tissue stored *in situ* at RT (Group 2) for 24 or 48 hours. Therefore, these time-points were not included in the statistical analysis (other than the graft retrieval rates).

Table 2.3. Number of grafts, grafted and recovered, for each of the experimental groups following storage under different conditions for varying lengths of time. See Table 2.1. for description of experimental groups. $p<0.01$ for the graft recovery rate of all treatment groups without a superscript letter. *See declaration (page v).

Experimental group	n Grafts retrieved/n grafted following storage					
	0h	3h	6h	12h	24h	48h
Group 1	-	5/8	6/8	6/8	5/6*	4/4*
Group 2	-	6/8	6/8	5/6	0/6[a]	0/4[a]
Group 3	-	6/10	7/8	5/6	7/12*	6/12*
Group 4	-	4/6	6/8	6/8	4/9*	6/6*
Control	8/13*	-	-	-	-	-

In successfully recovered grafts from all treatment groups, follicles of all sizes were observed. *In vitro* storage (Groups 3 and 4) significantly improved the number of normal small and medium sized follicles and the number of total normal follicles ($p<0.01$) present in recovered grafts compared to *in situ* storage (Groups 1 and 2). In contrast, the number

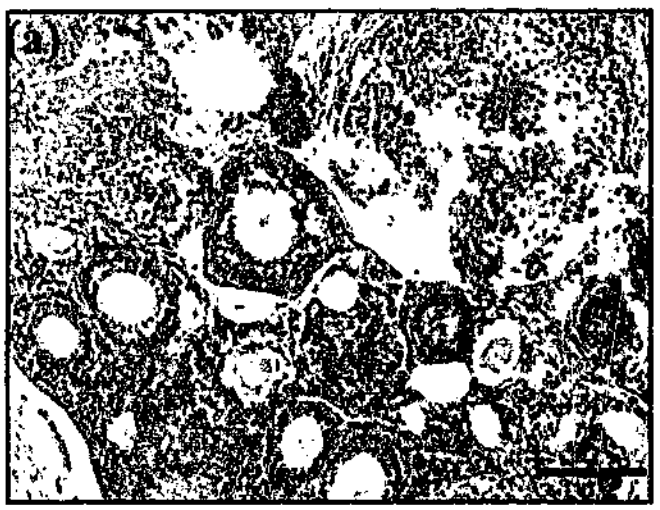
of normal large sized follicles contained within recovered grafts was not altered by *in situ* or *in vitro* storage ($p>0.24$), with a small number of follicles containing antral fluid observed in recovered grafts from all storage treatment groups and the non-stored fresh control group (Figure 2.1.c.). No corpora lutea were observed in grafts belonging to any of the treatment groups or the non-stored fresh control group.

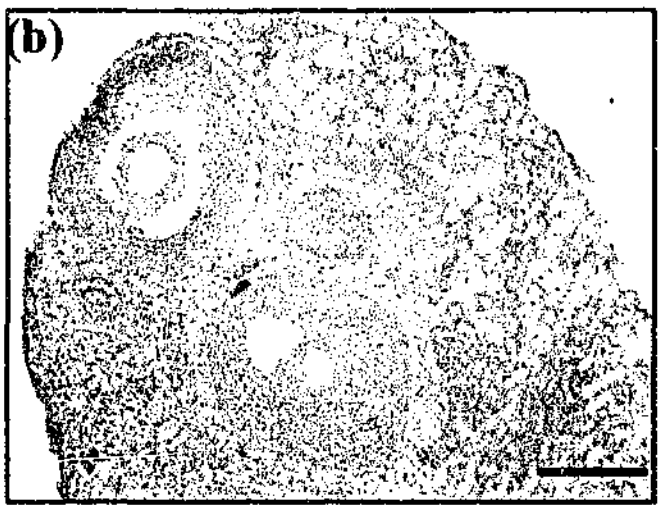
2.3.2. Storage Duration

The duration of storage had a significant effect on the number of normal small, medium and large sized follicles and the total number of normal follicles in recovered ovarian grafts ($p<0.03$; Figure 2.2.). Non-stored fresh control ovarian tissue ($n=8$; Figure 2.1.c.), collected from the donors within a few minutes of death and grafted immediately to a new recipient, contained significantly more normal small and large sized follicles and total normal follicles than grafts belonging to any of the storage treatment groups (Groups 1-4; $p<0.01$). There was a significant decline in the total number of normal follicles after only three hours storage of the ovarian tissue post death in all treatment groups, when compared to non-stored fresh ovarian tissue grafts ($p<0.01$; Figure 2.1.c.).

As the storage duration increased, a decline in the total normal follicle numbers was observed (Figure 2.2.). Following 24 hours storage and beyond, none of the ovarian tissue stored *in situ* at RT (Group 2) became established following grafting (Table 2.3.). However, despite this, when these two treatment groups were disregarded the combined effect of the storage conditions had no effect on the graft recovery rate when comparing the remaining treatment groups and the non-stored fresh control group ($p>0.99$).

Figure 2.1. Ovarian histology of grafts recovered two weeks following transplantation. Viable follicles at all stages of folliculogenesis were observed in ovarian tissue stored both **(a)** *in vitro* on ice (bar=100µm) (and at RT) and **(b)** *in situ* on ice (bar=200µm) for 48 hours prior to grafting. **(c)** Non-stored fresh grafted control ovarian tissue was densely packed with viable small, medium and large sized follicles (bar=200µm). The non-stored fresh control ovarian tissue contained significantly more viable follicles than any of the treatment groups.





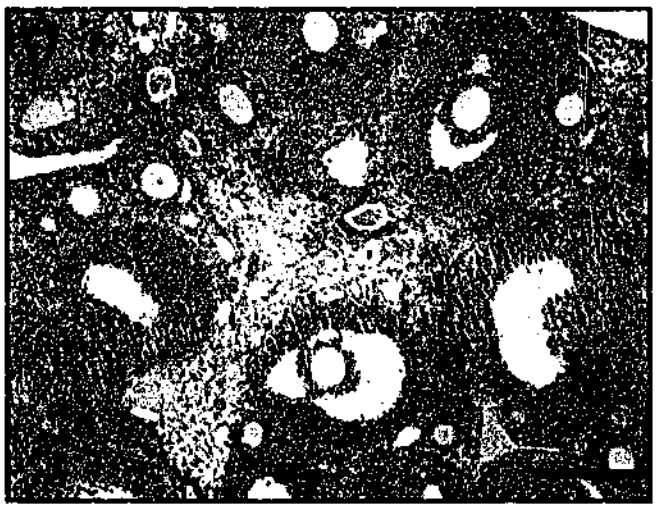
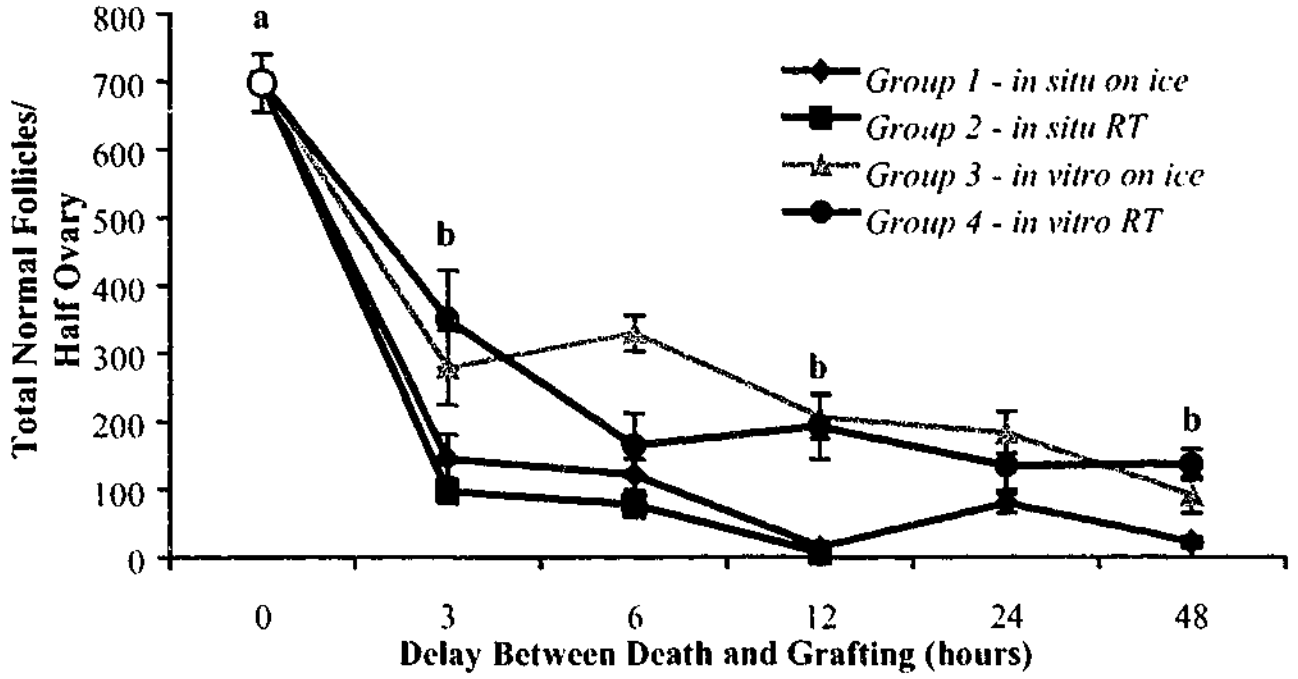


Figure 2.2. Effect of storage condition, temperature and duration on the total number of normal follicles in ovarian tissue grafts recovered two weeks following grafting. Results are presented as the mean ± SEM. **a** $p<0.01$ for fresh grafts (0 hours) compared with Groups 1-4; **b** $p<0.05$ for Groups 3 and 4 (*in vitro*) compared with Groups 1 and 2 (*in situ*).



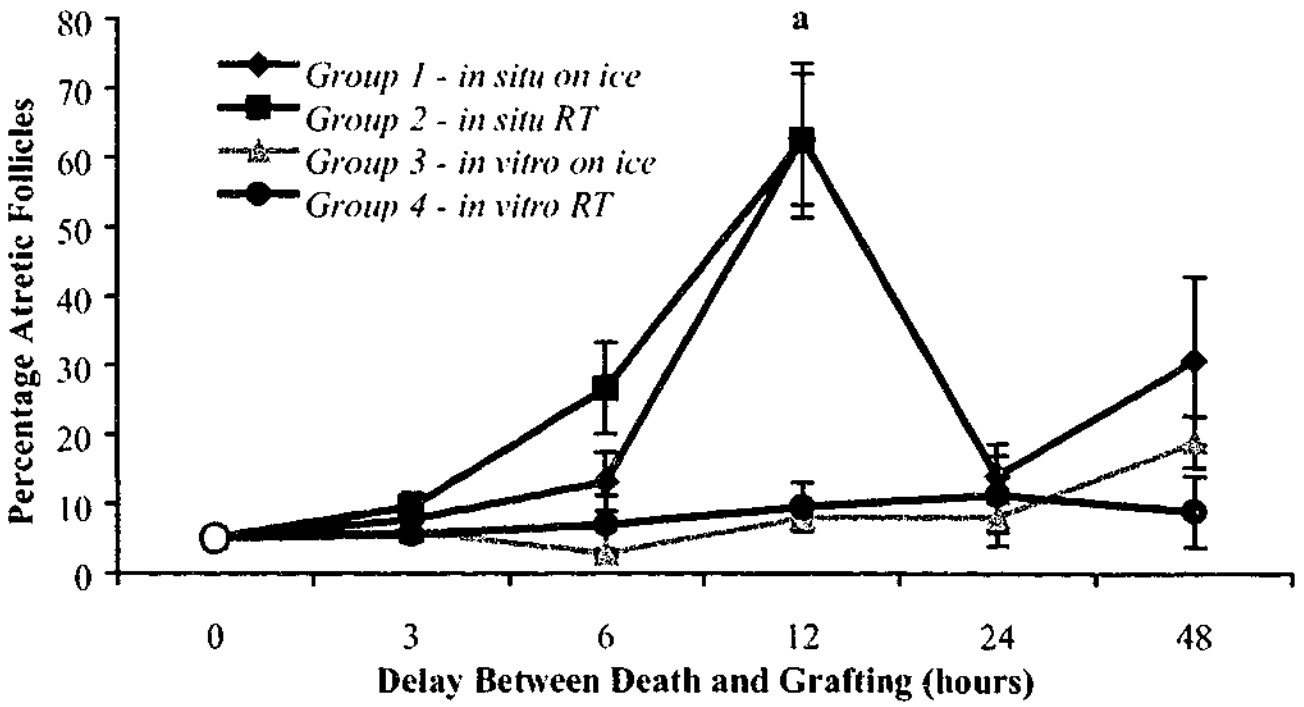
2.3.3. Storage Temperature (RT versus on ice)

The number of normal small, medium and large sized follicles and consequently the total number of normal follicles present in grafts established from the tissue that had been stored on ice or at RT, was not significantly different ($p>0.05$). However, ovarian tissue stored on ice (Groups 1 and 3), numerically but not statistically, contained a greater number of total normal follicles than ovarian tissue stored at RT (Groups 2 and 4; Figure 2.2.). Furthermore, in combination, the storage temperature and the duration of storage had a small significant effect ($p<0.05$) on the number of normal small sized follicles and the total number of normal follicles contained within recovered grafts. As a consequence of this, no grafts were recovered from tissue that had been stored *in situ* at RT (Group 2) for 24 hours or more (Table 2.3.). In contrast, tissue stored *in situ* on ice (Group 1) for up to 48 hours was successfully recovered following grafting (Figure 2.1.b.; Table 2.3.).

2.3.4. Atretic Follicles

Atretic follicles were present in grafts from all storage treatment groups (Groups 1-4) and the non-stored fresh control group (Figure 2.3.). Both the storage conditions and the duration of storage prior to grafting had a highly significant effect on the percentage of atretic follicles present within the grafts, when represented as a proportion of the total follicle numbers ($p<0.01$). In contrast, the storage temperature had no significant effect on the percentage of atretic follicles ($p>0.76$).

Figure 2.3. Effect of storage condition, temperature and duration on the percentage of atretic follicles, as a proportion of total follicles (normal and atretic), in grafts recovered two weeks after grafting. The results are presented as the mean ± SEM. **a** $p<0.01$ for Groups 1 and 2 (*in situ*) compared with Groups 3 and 4 (*in vitro*).



As can be seen in Figure 2.3., the percentage of atretic follicles tended to increase with the duration of storage. This was most apparent in the *in situ* stored ovarian tissues (Groups 1 and 2), with the six hours at RT and 12, 24 and 48 hours (on ice and RT) stored ovarian tissues containing a higher percentage of atretic follicles than the non-stored fresh control ovarian tissue ($p<0.03$). Of the *in vitro* stored ovarian tissue (Groups 3 and 4) only those

stored on ice for 48 hours contained a significantly higher percentage of atretic follicles than the non-stored fresh control tissue ($p<0.01$).

2.4. Discussion

Gene banking of male and female germ cells from rare or endangered species has the potential to help preserve the genetic diversity of many rare species. Gene banks would be most effective if they could represent as much as possible of the species genetic diversity, to reduce problems such as inbreeding in subsequent populations. One strategy to achieve this is for reproductive tissues and germ cells of threatened species to be opportunistically collected and scavenged from individuals belonging to both wild and captive populations. In the study reported in this chapter, the viability of ovarian tissue salvaged from deceased animals and stored under different conditions was investigated using a mouse model. This study demonstrated that some follicles remain morphologically normal for up to 48 hours following the death of the donor animal. However, this only occurred when the ovarian tissue was stored *in vitro* after removal from the animal at the time of death (regardless of the storage temperature), or when the body of the animal was immediately placed on ice after death.

2.4.1. In Vitro *Versus* In Situ *Storage*

During the period of tissue storage following the death of the donor, the *in vitro* and *in situ* stored ovarian tissues would have been subjected to extremely different conditions and hence different damaging effects. The removal of the ovarian tissue from the body was less detrimental than storage in the body. Similarly, it was in the *in situ* stored ovarian tissues that the greatest percentage of atretic follicles were observed. While, it previously has been shown that small sized follicles develop in a relatively hypoxic environment (Gosden & Byatt-Smith, 1986), the higher oxygen concentration offered by the PBS *in vitro* storage, in comparison to the presumably hypoxic *in situ* storage, may explain why

improved survival was observed in *in vitro* stored ovarian tissue. Conversely, the high oxygen concentrations provided by the PBS may also have been of detriment to the *in vitro* stored tissue due to the presence of reactive oxygen species. High oxygen concentrations have previously been implicated as a cause of apoptosis (Stoian *et al.*, 1996). This possibly accounting for the massive follicle loss that was observed in the *in vitro* stored tissues after only three hours storage.

Another consideration is the differing sizes of the *in vitro* (stored as half ovaries) and *in situ* (stored intact) ovarian tissue pieces. As smaller pieces of tissue, the *in vitro* stored half ovaries would have benefited from improved diffusion of oxygen during storage as a result of their relatively greater surface area, in comparison to the to the whole *in situ* stored ovaries. Similarly, the addition of the antibiotic to the *in vitro* storage solution may have offered protection to the ovarian tissue both prior to and post grafting from the damaging effects of bacterial agents. Previous studies have demonstrated that a penicillin-streptomycin combination is effective in protecting tissue from bacterial damage (Cram *et al.*, 1983; Cram & Domayer, 1983). In contrast, *in situ* stored tissues would have been offered no such protection. However, despite the damage incurred to the tissue by *in situ* storage, the results obtained indicate that damage may be minimised by on ice storage and hence prolong the survival of the ovarian follicles to 48 hours.

Further studies are now required to investigate more suitable storage media that may improve ovarian tissue viability. A more complex nutritive media or the inclusion of a free radical scavenger such as vitamin E may prove effective at improving follicular survival within *in vitro* stored ovarian tissue.

2.4.2. Storage Duration

Despite the encouraging result that grafts became established and showed evidence of follicle growth up to 48 hours post death following *in vitro* storage, and *in situ* storage on

ice, at least 50% of the follicles were lost in the first three hours of storage in comparison to freshly grafted ovarian tissue. The fact that the majority of loss had occurred within only three hours of storage, demonstrates the importance of collecting and grafting or freezing ovarian tissue as quickly as possible after the death of the animal or in a clinical situation after tissue removal from a patient.

Since the bioassay of viability used in this study involved ovarian grafting, the results are likely to provide a stringent estimate of follicular viability. It is well established that at least one third of follicles are lost in ovarian tissue grafted immediately after collection (Newton *et al.*, 1996; Candy *et al.*, 1997), due to damage inflicted by ischaemia and re-perfusion injury by free radicals (Nugent *et al.*, 1998; Shaw *et al.*, 2000). Therefore if these damages could be minimized more follicles may survive for up to 48 hours, or longer than this after the death of the animals. Alternatively, the improvement or development of other strategies for recovering oocytes from the tissue and growing them to maturity may also yield a higher number of viable oocytes from salvaged ovarian tissue. However, there are currently no effective alternatives to grafting.

Until recently, there had been only one report in which primordial follicles were successfully grown into mature fertilizable oocytes *in vitro* (Eppig & O'Brien, 1996). While, more recently, this method was improved it has still only been achieved in the mouse (O'Brien *et al.*, 2003). The successful maturation *in vitro* of ovarian germ cells using nuclear transfer technology was also reported. However, mature oocytes were needed for this to be accomplished (Obata *et al.*, 2002), thus making the application of this technique to endangered species a remote possibility at present. Ovarian grafting (auto, allo, xeno) thus remains the technique of choice for the maturation of immature oocytes within ovarian tissue.

Early studies suggested that small sized follicles were the only survivors of ovarian grafting (Jones & Krohn, 1960). More recently, evidence has suggested that larger follicles too can also survive cryopreservation (Sztein *et al.*, 2000) and grafting, possibly due to technical advances in cryopreservation procedures (Candy *et al.*, 1995). In the study undertaken in this chapter, most large sized follicles did not survive the storage and grafting procedure. This is evidenced by the fact that very few large follicles with the presence of antral fluid, and no corpora lutea were observed following two weeks of grafting. Corpora lutea would have been evident within the ovarian grafts had large sized follicles survived grafting. However, when comparing the percentages of different follicle types in the recovered grafts, similar patterns were observed in grafts from all treatment groups. This indicates that small and medium sized follicles were the main survivors in the grafted ovarian tissue, with large sized follicles probably being damaged by the hypoxic storage conditions and/or the ischaemic conditions of grafting. This can be explained by both the high metabolic demand and central location of large sized follicles in the ovary. In comparison, small sized follicles are least affected by ischaemia, due to both their location in the ovary and low metabolic requirements as a result of their quiescence (Gosden, 1992; Kim *et al.*, 2001a).

However, despite the resilience of small sized follicles, the loss of approximately 50% of follicles after only three hours storage clearly demonstrates that the small follicles, which constitute the majority of the ovary (Greenwald & Roy, 1994; Vom Saal *et al.*, 1994), have also died during storage. This loss is most likely the combined result of apoptosis, bacteria infiltration and enzymatic degradation, and potentially, as a consequence of this, augmented ischaemia post grafting. Furthermore, while normal follicles were observed in grafts stored for up to 48 hours prior to grafting, the follicle populations contained within these grafts (in particular *in situ* on ice stored grafts) was extremely low, and consequently

may serve very little purpose due to a shortened graft longevity and as a result a decreased likelihood of being able to collect mature oocytes for use in alternate ART's.

2.4.3. Storage Temperature (RT versus on ice)

It had been anticipated that ovarian tissue survival would greatly benefit from on ice storage due to metabolic suppression caused by the lower storage temperature (Lindell *et al.*, 1998). Degradation of the tissue was also expected to be more pronounced in RT stored tissues, as it has previously been shown that enzyme reaction rates are slowed 10- to 13-fold by storage at 0-4°C (Fuller, 1991). Similarly, in hamster ovary cells, apoptosis, as a result of oxyradical species, has previously been established to be reduced with hypothermia (Slikker *et al.*, 2001). Surprisingly however, in the study reported in this chapter, the storage temperature did not have a significant effect on the survival of the grafted ovarian tissue. Despite this, the storage temperature and duration of storage together played a role in determining the rate of deterioration that occurred in the ovarian tissue. This suggests that as the duration of storage increased, the storage temperature became a more important factor in determining the rate of deterioration occurring in the tissue.

The *in situ* on ice and RT stored grafts best illustrate this. In the grafts belonging to these experimental groups, similar follicle populations were observed at each time point following storage for up to 12 hours prior to grafting. However, following greater periods of storage, grafts stored at RT could not be recovered from recipients. In contrast, grafts stored on ice were recovered and contained normal follicles. Therefore, in practice ovarian tissue that is being stored for short lengths of time (such as between collection and use) may be stored at either RT or on ice. In contrast, ovarian tissue being stored for a greater period, such as during transportation from the field to the lab, should be stored on ice to minimise deterioration incurred within the tissue.

2.4.4. Summary

The results presented in this chapter demonstrate that a greater proportion of ovarian follicles survive when the ovarian tissue is removed from the body at the time of death, compared to when there is a delay between death and tissue collection. The results indicate that optimal conditions require the ovarian tissue to be removed from the body and either grafted or cryopreserved immediately after the death of the animal to maximise ovarian follicle numbers. However, in the field this is not always feasible. Functional ovarian tissue may be salvaged from the bodies of deceased animals, which are exposed to low temperatures such as those experienced during winter months, in regions with cooler climates, or when the body is refrigerated immediately following death. However, if the body has been exposed to warm temperatures, rapid removal of the tissue is preferred. Once removed from the body the time to tissue utilisation, but not storage temperature, appears to be important, thus enabling ovarian tissue to be easily transported from the field to a suitably equipped laboratory for freezing.

~ *Chapter Three* ~

Ovarian Xenografting to Immunocompromised Recipients

3.1. Introduction

Recent years have seen increased interest in the use of ART's to preserve endangered wildlife. However, most ART's require the use of mature oocytes, thus limiting their application as mature oocytes are generally difficult to obtain in the numbers required for fertilization and the subsequent production of offspring. As a result methods are required for the generation of mature oocytes. The culture of primordial follicles, as a method of oocyte maturation, initially had very limited success (Eppig & O'Brien, 1996). Recently, this technique was improved, however its application is still limited to the mouse (O'Brien *et al.*, 2003). Similarly, live young have been produced from ovarian germ cells matured *in vitro*. However, due to defective cytoplasmic maturation the donor oocyte nucleus required serial transfer to normal mature enucleated oocytes in order to achieve complete maturation (Obata *et al.*, 2002). This requirement of mature oocytes is a major limitation to the application of the technique in conservation biology.

Ovarian grafting provides a potential alternative method for the generation of mature oocytes. Oocytes matured within ovarian tissue autografts and allografts to female recipients have been shown to produce live young after fertilization *in vitro* (Liu *et al.*,

2001; Yang, 2002) or through natural conception (Gosden *et al.*, 1994a; Agca, 2000; Candy *et al.*, 2000; Petroianu *et al.*, 2002). Interestingly, male mice can also support follicular development, with viable fetuses produced following the *in vitro* fertilization of recovered oocytes (Waterhouse *et al.*, 2001). Thus raising the issue of the importance of the female hormonal environment of ovarian graft recipients.

Ovarian xenografting provides a tool for the maturation of oocytes of endangered wildlife. Immunodeficient rodent recipients can support follicular development within ovarian tissue xenografts from a number of species including the sheep (Gosden *et al.*, 1994b), marmoset (Candy *et al.*, 1995), elephant (Gunasena *et al.*, 1998), wallaby (Mattiske *et al.*, 2000; Mattiske *et al.*, 2002), cow (Semple *et al.*, 2000; Herrera *et al.*, 2002) and human (Oktay *et al.*, 1998b; Weissman *et al.*, 1999; Gook *et al.*, 2001). Furthermore, when assessed histologically, the oocytes contained within ovarian xenografts appear similar to those of the donor species in both size and appearance (Gosden *et al.*, 1994b; Gook *et al.*, 2001; Mattiske *et al.*, 2002).

Fertilization and early-stage embryo cleavage has also been reported in oocytes recovered from both marmoset (Choo, 1999) and wallaby (Mattiske *et al.*, 2000) xenografts. However, the viability of oocytes matured within ovarian xenografts and resulting embryos has not previously been evaluated or demonstrated, in that it has not been demonstrated that the oocytes are fully functionally competent and if fertilized can produce normal live young.

3.1.1. Aims of this Chapter

This chapter reports the use of a mouse to rat model, to investigate whether ovarian xenografting generates mature oocytes that, when fertilized, would be capable of yielding live young. If successful this would establish the potential application of ovarian xenografting for the conservation of endangered wildlife.

3.2. Materials and Methods

3.2.1. Animals

All animals used in this study were obtained from Central Animal Services, Monash University. Animals were maintained under a 12 hour light-dark regimen at 21°C. Specific pathogen free (SPF) animals were housed under sterile conditions in micro-isolators and were fed food and water *ad libitum* that was sterilized by irradiation. Non-SPF animals were housed under conventional conditions with food and water *ad libitum*.

Ethical approval for this study was obtained from the Monash University Department of Physiology Animal Ethics Committee and complied with the conditions laid down by the NH&MRC/CSIRO/AAC Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1997).

3.2.1.1. Mice

The ovarian tissue was obtained from three-week old SPF inbred FVB mice (n=12). This strain of mouse has a white coat colour. SPF FVB male mice were used as sperm donors for the *in vitro* fertilization procedure (n=3). SPF FVB strain mice were selected to minimise the chance of disease transmission and for their high fertility (Taketo *et al.*, 1991).

Six-week old conventional agouti F1 females (C57BL×CBA; n=6) were used as controls (for the collection of oocytes) for the *in vitro* maturation and *in vitro* fertilization procedures. Conventional agouti F1 males (n=2; eight-weeks old) were used as sperm donors for the *in vitro* fertilization of F1 control oocytes.

Conventional agouti F1 foster females (n=11; six-weeks old) were used as pseudopregnant embryo transfer recipients. Recipients were declared pseudopregnant if a copulatory vaginal plug was observed by visual examination, following mating with vasectomized male mice. Embryo transfer recipients were used on day zero of pseudopregnancy.

3.2.1.2. Rats

Adult female (n=7; six-week old) and male (n=2; six-12 week old) SPF nude rats (CBH-mu/rnu) served as the xenograft recipients for this study. Nude rats are immunologically compromised and do not reject ovarian xenografts (Wolvekamp *et al.*, 2001).

3.2.2. Ovarian Tissue Collection

Donor mice and control females were killed and their ovaries collected as described in section 2.2.2. Following collection from donor animals FVB ovaries were placed in PBS at RT and cut in half using a scalpel blade in preparation for grafting.

3.2.3. Ovarian Tissue Grafting

3.2.3.1. Anaesthetic

All surgical procedures on the nude rats were carried out in a laminar flow cabinet using sterile technique.

Nude rats were anaesthetised with a 2mg xylazine hydrochloride (Troy Laboratories, NSW, Australia) and 10mg ketamine hydrochloride (Parnell Laboratories, NSW, Australia) solution made to 10ml with PBS. The anaesthetic was administered via an intra-peritoneal injection. The depth of anaesthesia was assessed and monitored using toe pinch and blinking reflexes. When no response was elicited to either stimuli surgery was commenced.

3.2.3.2. Ovarian grafting to the kidney capsule

Between two and six half FVB ovaries were xenografted to each nude rat recipient (Table 3.1.). Ovarian tissue was xenografted under the kidney capsules of recipient male and female nude rats using the procedure outlined in section 2.2.4.2. with minor modifications (below). The incision made through the dorsal skin of recipient rats was approximately 1.5cm to enable the rat kidneys to be exteriorised. At the time of xenografting female rat recipients were ovariectomised as outlined in section 2.2.4.2. Male rat recipients were not castrated at the time of transplantation. In the absence of the ovarian fat pad, male recipient's kidneys were exteriorised by gentle palpation. Dorsal skin incisions were closed using 9mm wound clips.

Table 3.1. Number of graft recipients, control ovary donors, grafts grafted, control ovaries and hormone treatment administered for xenograft and control groups.

Xenograft groups	**n Graft recipients**	**n Grafts**	**Hormone treatment**
Female –PMSG	4	19	Nil
Female +PMSG	3	18	10 IU PMSG
Male +PMSG	2	11	10 IU PMSG
Control Groups	**n Donor mice**	**n Ovaries**	**Hormone treatment**
F1 –PMSG	2	4	Nil
F1 +PMSG	2	4	10 IU PMSG
F1 PMSG + hCG (ovulated)	2	4	10 IU PMSG + 10 IU hCG

3.2.4. Graft Recovery and Oocyte Collection

3.2.4.1. Hormone treatment

Nineteen days after xenografting, three of the female and both the male rats were given a single hormone treatment of 10 IU PMSG (+PMSG; Intervet Pty. Ltd., Bendigo East,

Australia) to stimulate follicle growth (Table 3.1.). As outlined in Table 3.1., the remaining rats received no hormone treatment (-PMSG).

Four control animals also received hormone treatment (Table 3.1.). Two F1 females were given a single treatment of 10 IU PMSG (F1 +PMSG control) to serve as a control for the +PMSG xenograft groups in order to test the *in vivo* maturation conditions. Two additional females were superovulated by standard treatment with 10 IU PMSG followed, 48 hours later, with 10 IU hCG (Intervet Pty. Ltd., Bendigo East, Australia; Hogan *et al.*, 1986). Fourteen hours later, the ovulated oocytes were collected. The remaining two F1 control females (F1 -PMSG control) received no hormone treatment (Table 3.1.). These controls were used to test the *in vitro* maturation and fertilization conditions.

3.2.4.2. Graft recovery

Twenty-one days after grafting (48 hours after hormone treatment) ovarian xenograft recipients were killed by CO_2 inhalation. Ovarian xenografts were then collected from nude rat recipients using the procedure described in section 2.2.5.1.

3.2.4.3. Oocyte collection

Upon collection, mouse ovarian xenografts and control ovaries were placed directly in $KSOM^{AA}$ handling media (Appendix 2) that was pre-warmed on a heating stage to 37°C. $KSOM^{AA}$ handling media is a HEPES buffered version of KSOM and $KSOM^{AA}$ that has previously been shown to be highly successful for mouse IVF (Lawitts & Biggers, 1993; Summers *et al.*, 1995; Summers *et al.*, 2000). It is prepared from stock solutions as shown in Appendix 2. Oocytes were then released from follicles within the grafts and control ovaries using 26G needles (Terumo Medical Corporation, MD, USA).

3.2.5. In Vitro *Culture and Embryo Transfer*

3.2.5.1. *In vitro* maturation

Germinal vesicle (GV) stage oocytes and expanding cumulus oocyte complexes (COC's) obtained from all xenograft treatment groups and the F1 +PMSG and F1 –PMSG control groups were collected from the handling media by mouth pipette and transferred to 30µL drops (a maximum of 10 oocytes per drop) of gas and temperature equilibrated maturation media covered with mineral oil (Sigma, USA). The maturation media consisted of 2.02g/L Eagles Minimum Essential Media (EMEM; M0894; Sigma, MD, USA) supplemented with 10% FCS (Gibco-BRL, MD, USA), 0.044g/L $NaHCO_3$ (BDH AnalaR, Australia), 1 IU/ml PMSG and 1 IU/ml hCG made to volume with sterile deionised water (CSL, Australia). Oocytes were then incubated at 37°C with 5% CO_2 for 18 hours to enable maturation and extrusion of the first polar body.

F1 PMSG + hCG ovulated control oocytes did not undergo *in vitro* maturation.

3.2.5.2. *In vitro* fertilization

In order to obtain sperm for the fertilization of mature oocytes, the males were killed by cervical dislocation and the abdomen sterilized with 70% alcohol. An incision across the width of the lower abdomen just above the scrotum allowed the fat pads of the testes to be accessed. The fat pads were then gently pulled to exteriorise both testes and caudae epididymides. The caudae epididymides were removed and then each cut a few times before being placed separately in 1ml of equilibrated modified T6 (MT6) fertilization media (Fraser, 1984; Appendix 3) which was pre-equilibrated at 37°C, 5% CO_2 for 12 hours prior to use. The caudae remained in the fertilization media in the incubator for one hour to enable capacitation and sperm swim up to occur.

Following maturation and sperm incubation, xenograft matured oocytes were inseminated with sperm from adult FVB males. All of the control F1 oocytes were inseminated with

sperm from adult F1 males. 30µL drops of the sperm fertilization media were plated out on 35mm culture dishes (Falcon, Becton Dickinson Pty. Ltd., Scoresby, Australia) and covered with pre-equilibrated mineral oil. A maximum of ten MII xenograft matured, or control oocytes were then transferred to each sperm drop and the dishes returned to the incubator for two hours in order for fertilization to occur.

3.2.5.3. Embryo culture

Fertilized oocytes were collected and transferred by mouth pipette to equilibrated KSOM^{AA} embryo culture media (Summers *et al.*, 2000); Appendix 2), which was pre-equilibrated at 37°C, 5% CO_2 for two hours prior to use. The embryo culture media was then returned to the incubator for 24 hours at which time the number of 2-cell embryos was scored.

3.2.5.4. Embryo transfer

Embryo transfer recipients were anaesthetized using a 1mg xylazine hydrochloride and 5mg ketamine hydrochloride solution made to 10ml with PBS. The anaesthetic was administered and monitored as described in Section 3.2.3.1.

Two-cell embryos were transferred to the oviducts of foster mice using a standard technique (Shaw & Kasai, 2001). This was performed by Dr Jillian Shaw. Briefly, once the mice were anaesthetised the oviducts of foster females were located and exteriorised. Embryos were then inserted into the opening of the oviducts using a thin glass capillary. In each foster mouse the oviduct on one side received control embryos (maximum of six) while the contralateral oviduct received embryos from one of the xenograft groups (maximum of eight embryos). This was to minimise individual variation between foster mice. Once the embryo transfer was completed, the oviducts were returned to the abdominal cavity and the skin closed with wound clips.

3.2.6. Statistical Analysis

Statistical analysis on the graft retrieval, 2-cell cleavage, implantation and pups born rate was performed using the Chi-Square Test. Statistical significance was determined as $p<0.05$.

3.3. Results

3.3.1. Graft and Oocyte Recovery Rate

Ovarian xenografts were successfully recovered from both female and male nude rat recipients. At the time of graft collection, grafts were healthy in appearance and well vascularized. Antral sized follicles were evident on the surface of most grafts.

Graft recovery rates were comparable for the Female –PMSG (78.95%) and Female +PMSG (83.33%) xenograft groups, with 15 grafts recovered for both groups (Table 3.2.). A significantly lower ($p<0.05$) graft recovery rate was obtained for the mouse ovarian xenografts placed in the male recipients, with only one of 11 grafts visible three weeks after grafting (9.09%; Table 3.2.).

Despite, the poor graft recovery rate obtained for the male rat recipients, a total of ten oocytes were collected from the single graft retrieved (Table 3.2.). Female xenografts gave fewer oocytes (average of 5.67 and 3.80 oocytes collected per graft for the Female –PMSG and Female +PMSG groups respectively).

Seventy-five and 53 oocytes were collected from the F1 – PMSG and F1 +PMSG control groups respectively. A total of 53 oocytes were collected from the superovulated F1 PMSG + hCG control ovaries. Upon comparison to the xenograft groups, the number of oocytes collected per half control ovary was slightly higher in most of the control groups, with an average of 9.38, 6.63 and 6.63 oocytes collected from the F1 –PMSG, F1 +PMSG and F1 PMSG + hCG control groups respectively.

Table 3.2. Effect of hormone treatment on oocyte number and cleavage rates for xenograft and control groups of mice. Same superscript letters within columns indicate groups that are statistically the same ($p>0.05$).

Xenograft groups	**n Grafts retrieved/n grafted (%)**	**n Oocytes**	**n 2-cells**	**% Oocytes to 2-cell**
Female –PMSG	15/19 (78.95)[a]	85	34	40.00[a]
Female +PMSG	15/18 (83.33)[a]	57	33	57.89[b]
Male +PMSG	1/11 (9.09)[b]	10	2	20.00[a]
Control groups	**n Ovaries**	**n Oocytes**	**n 2-cells**	**% Oocytes to 2-cell**
F1 –PMSG	4	75	49	65.33[b]
F1 +PMSG	4	53	37	69.81[b]
F1 PMSG + hCG (ovulated)	4	53	50	94.33[c]

3.3.2. Embryo Cleavage Rate

Of the 85 and 57 oocytes collected from the Female -PMSG and Female +PMSG xenografts, 34 (40.00%) and 33 (57.89%) formed 2-cell embryos following insemination (Table 3.2.). Only two (20.00%) of the 10 oocytes collected from the Male +PMSG xenograft formed 2-cell embryos. The rate of cleavage to 2-cell embryos was comparable for the Male +PMSG and Female –PMSG xenograft groups ($p>0.05$), while a significantly higher rate of cleavage was obtained for the oocytes collected from the Female +PMSG xenografts ($p<0.05$; Table 3.2.)

The rate of 2-cell formation in the F1 –PMSG (65.33%) and F1 +PMSG (69.81%) control groups (Table 3.2.) was comparable to that obtained for the Female +PMSG xenograft group (57.89%; $p>0.05$). However, the rate of cleavage was significantly higher than for the Female –PMSG (40.00%) and Male +PMSG (20.00%) xenograft groups ($p<0.05$). A significantly greater proportion of oocytes formed 2-cell embryos in the superovulated F1

PMSG + hCG (94.33%) control group than in any of the xenograft groups or in the other control groups ($p<0.05$).

3.3.3. Generation of offspring

Following embryo transfer live young were produced from the embryos of all of the xenograft and control groups except the Male –PMSG xenograft group. Three FVB pups were born to females receiving embryos derived from the xenografts from non-hormone treated rats (Female –PMSG). Two pups resulted from xenografts from female rats that received hormone treatment (Female +PMSG; Table 3.3.). All five FVB pups (4 male, 1 female) were healthy in appearance at birth, developed normally and were successfully weaned at 3 weeks of age (Figure 3.1.).

Table 3.3. Effect of hormone treatment on the pregnancy rate of xenograft and control groups. Same superscript letters within columns indicate groups that are statistically the same ($p>0.05$). *Expressed as n and as a % of 2-cells transferred. †One pup in each of these groups died within 24 hours of birth.

Xenograft groups	**n 2-cells transferred (n fosters)**	**Total implantation scars***	**Pups weaned***
Female –PMSG	32 (5)	12 (37.50)[a]	3 (9.38)[a]
Female +PMSG	33 (5)	8 (24.24)[a]	2 (6.06)[a]
Male +PMSG	2 (1)	0 (0.00)[a,b]	0 (0.00)[a,b]
Control groups	**n 2-cells transferred (n fosters)**	**Total implantation scars***	**Pups weaned***
F1 –PMSG	12 (2)	7 (58.33)[a,b]	1 (8.33)[a]
F1 +PMSG	18 (3)	14 (77.77)[b]	6† (33.33)[b]
F1 PMSG + hCG (ovulated)	36 (6)	26 (72.22)[b]	15† (41.67)[b]

In the F1 –PMSG control group a single pup was born. The pup was developmentally normal and was weaned at 3 weeks of age. In comparison, seven pups were born from the F1 +PMSG control group. Of the seven pups that were born, six reached weaning age with one pup dying within 24 hours of birth (Table 3.3.). Similarly, in the superovulated control F1 PMSG + hCG group, one pup was found dead shortly after birth. The remaining 15 pups from this control group were healthy and were successfully weaned at three weeks of age (Table 3.3.).

Figure 3.1. Offspring born and weaned following the transfer of 2-cell embryos derived from xenografted (white coat colour) ovarian tissue.

3.3.4. Implantation Scars

Following the weaning of offspring, the foster mothers were killed and their uterine walls examined for implantation scars. The number of implantation scars on the uterine walls of foster mothers exceeded the number of pups born for all xenograft and control group, thus indicating that some fetal loss had occurred (Table 3.3.). However, when the number of implantation scars to pups born was compared for all groups, no difference in fetal loss was found to exist between the xenograft and control groups ($p>0.05$).

The foster mother that received both 2-cell embryos from the Male +PMSG xenograft in addition to six F1 PMSG + hCG control embryos, failed to become pregnant and did not have any implantation scars.

3.3.5. Fertility of Offspring

Upon reaching eight weeks of age FVB offspring generated from the oocytes grown within the xenografted ovarian tissue were mated with agouti F1 animals to assess their fertility. All FVB individuals were found to be highly fertile, with large healthy litters produced within 4 weeks of pairing. The four male FVB mice sired a total of 32 pups (14 female and 18 male). The female delivered nine pups of which four were female and five were male. All pups produced were normal in appearance and were successfully weaned at three weeks of age.

3.4. Discussion

Numerous ART's have been suggested for use in the conservation of endangered wildlife. However, the application of most ART's is limited by the requirement for mature oocytes. For most endangered species the collection of mature oocytes is made difficult by insufficient knowledge of the reproductive physiology of the species. In contrast ovarian tissue may be readily collected, however, once collected, methods that enable the maturation of follicles are required. To date, culture conditions that enable complete follicular development *in vitro* do not exist for any species other than the mouse (Eppig & O'Brien, 1996; Obata *et al.*, 2002; O'Brien *et al.*, 2003). Alternative methods for generating mature oocytes are therefore required.

This is the first study that proves that xenografting can be used for generating mature oocytes for use in the propagation of another species. During the past 10 years, ovarian xenografting has been the focus of many studies (reviewed by Paris *et al.*, 2003). Ovarian tissue from a diverse range of species including the elephant (Gunasena *et al.*, 1998), wallaby (Mattiske *et al.*, 2002), cat (Gosden *et al.*, 1994b) wombat (Wolvekamp *et al.*, 2001), sheep (Gosden *et al.*, 1994b), dog (Metcalfe *et al.*, 2001) and human (Newton *et al.*, 1996; Oktay *et al.*, 1998b; Weissman *et al.*, 1999; Gook *et al.*, 2001) develop large fluid filled follicles following xenografting to immunocompromised rodent recipients. Development occurs despite the genetic and reproductive differences between the ovarian tissue donor and the xenograft recipient, thus clearly demonstrating that the biological activities of FSH and LH are not exclusively species specific (Gosden *et al.*, 1994b).

While the priming of the xenograft recipients with PMSG prior to graft retrieval was expected to increase oocyte numbers and quality (as indicated by embryo yield; Hogan *et al.*, 1986), there was no effect on either the number of oocytes collected from the xenografts or the viability of the resulting embryos. While a slightly higher rate of cleavage was observed in the oocytes collected from female xenograft recipients that received hormone treatment (Female +PMSG) compared to xenograft recipients that received no hormonal stimulus (Female –PMSG), no differences were found to exist in the implantation rate or the number of resulting pups born. This is consistent with results by others in our laboratory (Waterhouse, 2001; Yang, 2002) and has led to the speculation that exogenous hormones when given, may not elicit a response in ovarian grafts under the kidney capsule. The specific reason for this remains unclear, however it is possibly attributable to the clearance of the hormone from the blood supply upon entering the kidney (i.e. prior to reaching the ovarian graft) or the metabolism of the hormones (e.g. by kidney or liver) before reaching the graft.

In contrast to the xenograft groups, control groups appeared to benefit slightly from stimulation with PMSG prior to graft collection. While not statistically significant, oocytes collected from the ovaries of females treated with PMSG appeared to have a higher rate of cleavage and implantation than oocytes collected from females that received no prior hormone stimulus. Furthermore, significantly more pups were born for 2-cell embryos transferred in the hormone treatment controls than the no hormone control group. These findings further support the theory that ovarian tissue grafts do not respond to exogenously administered hormones. However, further studies looking at factors such as hormone receptor expression and hormone binding need to be undertaken to confirm this.

While the fertilization and pregnancy rates obtained for the female xenograft groups were consistently lower that those obtained for control groups, 2-cell embryos from the Female

–PMSG and Female +PMSG xenograft groups, formed at rates comparable ($p>0.05$), to those reported in other studies for *in vitro* matured and fertilized mouse oocytes collected from grafted follicles (Carroll *et al.*, 1990; Liu *et al.*, 2001). Furthermore, the proportion that developed to live young following transfer in the xenograft groups was similar ($p>0.90$) to that previously reported in allografting combined with *in vitro* culture studies (Liu *et al.*, 2001), indicating that xenografts behave in a similar manner to both autografts and allografts.

Offspring produced from within the xenograft groups were normal and fertile. This is of particular importance for the conservation of endangered wildlife. Litters resulting from the matings of the xenograft produced mice with normal F1 mice were phenotypically normal and consisted of healthy young that followed a normal developmental growth pattern.

While, in most ovarian xenografting studies female recipients have been utilized, male recipients have also successfully been employed (Weissman *et al.*, 1999; Bosch *et al.*, 2002). In a study utilizing human ovarian tissue, more developing follicles were observed in subcutaneous grafts to male xenograft recipients than grafts to female recipients (Weissman *et al.*, 1999). Castrated male mice can also successfully support follicular development to the antral stages within xenografted cat ovarian tissue (Bosch *et al.*, 2002). In the present study follicular development occurred within the mouse ovarian tissue xenografted to a male recipient. While, the graft recovery rate was significantly lower than in the female recipients, indicating a lower efficiency rate, the single graft recovered contained a large number of healthy oocytes. When fertilized some cleaved to 2-cell embryos. The successful oocyte maturation within this xenograft is possibly attributable to the increased androgen levels offered by a male recipient. Conflicting reports exist regarding the effect of androgens on follicular development and oocyte maturation.

Anderiesz and Trounson (1995) found that testosterone exposure to immature mouse oocytes undergoing *in vitro* maturation was detrimental to the oocytes capacity to mature and undergo normal embryonic development. In contrast, exogenous androgen administration has been found to enhance follicular growth and survival in the primate (Vendola *et al.*, 1998) and to be of no detriment to pig oocytes during *in vitro* maturation (Herrick & Pope, 2002).

No offspring were produced from oocytes matured within xenografts to male recipients in the present study, and it remains unclear whether this is due to the gonadal status (i.e. androgen levels) of the xenograft recipient or the unsuitability of the foster. The foster that received both these 2-cell embryos alongside six 2-cell embryos from the PMSG + hCG superovulation control group (control group that had the highest rate of cleavage and implantation), had no implantation scars on either side of the uterine horn, suggesting that the female was not receptive at the time of embryo transfer or alternatively suffered from an infertility problem. Offspring should be able to be produced from oocytes matured within male xenograft recipients as in a mouse allografting model, viable fetuses were produced from oocytes matured within grafts to male recipients (Waterhouse *et al.*, 2001). With increased embryo numbers and receptive fosters, offspring may have been produced from oocytes matured within mouse ovarian xenografts to male recipients. However, this is yet to be proven.

Despite the success enjoyed in this study several obstacles still need to be overcome before xenografting is employed in the conservation of endangered wildlife. Particularly, for most threatened species optimal *in vitro* fertilization and embryo culture conditions are unknown, thus limiting the application of ovarian xenografting at present. With this in mind, ovarian xenografting provides a tool for generating mature oocytes, which could subsequently be used in the investigation and development of *in vitro* culture conditions.

On a more cautious note, careful consideration must be given before ovarian xenografting is employed in the conservation of endangered wildlife and equally importantly in a clinical setting. Specifically, little information is currently available on the possible transmission of retroviruses and diseases during xenografting. Additionally, the possibility of epigenetic modification within the oocyte requires investigation.

However, with these issues in mind, the results reported in this chapter remain extremely exciting. The fact, that the rat supports complete follicular development within mouse ovarian tissue despite an estimated 11 million years of evolutional divergence (Catzeflis *et al.*, 1993) is highly encouraging and warrants the extension of this research to more divergent species.

3.4.1. Summary

In summary, this study demonstrates for the first time, the full potential of ovarian xenografting as a strategy for the conservation of endangered wildlife. As reported in this chapter, the rat has successfully supported follicular development within mouse ovarian tissue, generating viable mature oocytes that when fertilized produce fertile offspring. Ovarian xenografting hence remains a promising prospect for conservation biology.

~ *Chapter Four* ~

Xenografting of Marsupial Ovarian Tissue

4.1. Introduction

With almost half of Australia's native species, many of which are marsupials, currently being threatened with extinction (Szabo, 1995), it has become increasingly apparent that, to ensure the survival of our native wildlife, ART's will need to be employed. However, little is known about the reproductive physiology of many threatened species making it difficult to collect oocytes, sperm and embryos, and therefore employ standard ART's such as IVF. Thus, the collection of ovarian tissue is possibly the only practical way of obtaining female gametes from endangered species. The tissue can be collected from live or, more importantly, recently deceased animals and can be used in combination with cryopreservation to preserve, indefinitely, the genetic identity of individuals within a small population.

To be of potential use in the conservation of endangered wildlife and in particular marsupial species, methods are not only required for the short-term storage of ovarian tissue (as investigated in Chapter Two) but also for the long-term storage of tissue prior to ovarian xenografting. Studies have demonstrated that both tammar wallaby (Mattiske, 1997) and wombat (Wolvekamp *et al.*, 2001) ovarian tissue can be successfully frozen in DMSO using the standard slow-cooling procedures developed for mammalian ovarian tissue. Similarly, marmoset (Candy *et al.*, 1995), elephant (Gunasena *et al.*, 1998) and cat

(Bosch *et al.*, 2002) ovarian tissue has been successfully cryopreserved using standard slow-cool procedures. In light of the results obtained in Chapter Two, that demonstrate that a massive follicle loss occurs within ovarian tissue after only three hours storage (*in vitro* or *in situ*), it is clear that methods for the collection and freezing of tissue in the field are required. Despite this, little information is available on the use of more field applicable rapid-cooling procedures. Similarly, no investigation into the use of alternate cryoprotectants has been reported to date.

Ovarian xenografting provides a valuable tool for the *in vivo* maturation of oocytes. Follicular development to the antral stage has been reported within ovarian xenografts from a wide range of eutherian species including the elephant (Gunasena *et al.*, 1998), cat (Gosden *et al.*, 1994b; Bosch *et al.*, 2002), cow (Semple *et al.*, 2000; Herrera *et al.*, 2002) marmoset (Candy *et al.*, 1995) and human (Oktay *et al.*, 1998b; Weissman *et al.*, 1999; Gook *et al.*, 2001). Furthermore, as demonstrated for the first time in this study (Chapter Three) oocytes matured within ovarian xenografts are normal and when inseminated yield fertile live young.

Ovarian xenografting has been applied to marsupial species, with reports of successful ovarian xenografts using tammar wallaby (Mattiske *et al.*, 2000; Mattiske *et al.*, 2002), fat tailed dunnart (Lamden, 1996), wombat (Wolvekamp *et al.*, 2001; Cleary *et al.*, 2002) and pademelon wallaby ovarian tissue (Every, 2000). However, despite the research conducted in this field, the optimal conditions for xenografting of marsupial ovarian tissue remain unknown.

Bilaterally ovariectomised females have typically been used as ovarian xenograft recipients (Gosden *et al.*, 1994b; Candy *et al.*, 1995; Wolvekamp *et al.*, 2001; Mattiske *et al.*, 2002), since they have elevated circulating gonadotrophin levels that are believed to be beneficial to both follicular development and graft establishment (Dissen *et al.*, 1994). However,

recipients with other gonadal statuses can also be employed, with intact females (Cleary *et al.*, 2002), intact males (Weissman *et al.*, 1999) and castrated males (Bosch *et al.*, 2002) successfully supporting follicular development within wombat, human and cat ovarian xenografts, respectively. Additionally, as demonstrated in Chapter 3 of this thesis, intact male rats support follicular development of mouse ovarian xenografts. However, a study by Cox and colleagues (2000) has indicated that both the age of the donor and the gonadal status of the recipient influence follicular development within grafted mouse ovarian tissue. In that study, follicular development was inhibited in both fetal and neonatal ovaries grafted to intact recipients. However, full follicular development was observed when the recipient's ovaries were removed. In contrast, full follicular development was observed in immature ovaries (day 10) after transplantation, regardless of the gonadal status of the recipient (Cox *et al.*, 2000).

While the kidney capsule has generally been the graft site of choice for the rapid vascularization it provides (Kim *et al.*, 2001a), alternate grafts sites have also been considered in ovarian autografting and xenografting studies. Follicular development and oocyte retrieval has been achieved in human ovarian tissue intra-muscular xenografts (Revel, 2000). For the ease of graft monitoring and to enable less invasive surgery (Weissman *et al.*, 1999), the feasibility of both subcutaneous ovarian xenografts (Weissman *et al.*, 1999; Kim *et al.*, 2002) and autografts (Oktay *et al.*, 2001b) have been investigated with antral follicle development being achieved in both situations. However, to date there is little information available on the use of alternate grafts sites in marsupial ovarian xenografting studies. In the study conducted by Cleary and colleagues (2002) both the graft recovery rate and follicular development were improved in wombat ovarian xenografts to the kidney capsule compared to under the skin.

4.1.1. Aims of this Chapter

Ovarian tissue from three marsupial species; the tammar wallaby (Study 1), pademelon wallaby (Study 2) and eastern grey kangaroo (Study 3) was xenografted to generate mature oocytes for *in vitro* fertilization and embryo production. The effect of the gonadal status of the recipient, the graft site and different cryopreservation protocols were evaluated, with the aim of determining the xenografting conditions that are optimal for maximum follicle growth within marsupial ovarian tissue.

4.2. Materials and Methods

4.2.1. Animals

Ethical approval for the three studies reported in this Chapter was obtained from the Monash University Department of Physiology Animal Ethics Committee and complied with the conditions laid down by the NH&MRC/CSIRO/AAC Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1997).

4.2.1.1. Donors

Ovarian tissue was collected from three different marsupial species. In Study 1, ovarian tissue was collected from eight d75–d127 tammar wallaby pouch young. Pouch young were obtained from a colony bred and maintained by the Department of Zoology at the University of Melbourne (courtesy of Professor Marilyn Renfree and Associate Professor Geoff Shaw). The pouch young were removed from the pouch on the day of tissue collection and xenografting.

Pademelon wallaby (*Thylogale billardierii*; Study 2) ovarian tissue was salvaged from the carcasses of two female wallabies that were approximately 12 months in age. The pademelon females had been shot dead less than one hour prior to the collection of ovarian tissue. Wallaby carcasses were obtained from Mr Graham Farrington, Department of Biological Sciences, Monash University. The wallabies were culled legally during routine population control.

Eastern Grey kangaroo (*Macropus giganteus*) ovarian tissue used in Study 3, was obtained from a single adult female eastern grey kangaroo legally culled in the field. The culling of

the kangaroo and collection of tissue from the field was undertaken with the approval of the Department of Natural Resources and Environment (Wildlife permit #10001005).

4.2.1.2. Recipients

Adult female and male NOD-SCID mice were used as the xenograft recipients in the three studies described in this chapter. The mice were housed under SPF conditions as described in Section 3.2.1. NOD-SCID mice are immunocompromised and do not reject ovarian xenografts (Weissman *et al.*, 1999; Semple *et al.*, 2000).

4.2.2. Ovarian Tissue Collection

4.2.2.1. Adult ovarian tissue collection

Pademelon wallaby and eastern grey kangaroo ovaries were collected following the death of the donor. Deceased animals were placed on their backs. The ovaries were accessed and removed through a lateral incision across the width of the abdomen. Extracted ovaries were stored in PBS on ice until xenografted.

4.2.2.2. Pouch young ovarian tissue collection

Pouch young were killed by decapitation using a large scalpel blade. The abdomens of the pouch young were swabbed with 70% alcohol and then opened using scissors. Ovaries were located and removed using scissors and forceps. Collected ovaries were stored on ice in PBS supplemented with 62units/ml Penicillin G and 62μg/ml Streptomycin sulfate, until grafted.

4.2.3. Slow-cool Cryopreservation of Eastern Grey Kangaroo Ovarian Tissue

4.2.3.1. Solutions

The solutions used in the slow-cooling of ovarian tissue consisted of either 1.5M DMSO (Sigma, MO, USA) or EG (Sigma, MO, USA) with 0.1M sucrose (Sigma, MO, USA),

made to volume with PBS. Both solutions were filter sterilized before use with 0.22μm Millex syringe driven filter units (Millipore Corporation, USA).

Ovarian tissue to be cryopreserved was placed in 2ml cryovials (5 pieces of ovarian tissue per vial; Greiner, Australia) containing 1ml of cryopreservation solution. Cryovials containing tissue were stored on ice (0°C) for 30 minutes to enable penetration of the tissue by the cryoprotectant.

4.2.3.2. Slow-cooling procedure

Tubes were slow-cooled in the field applicable cooling device "Mr Frosty" (Sigma, USA) that had previously been tested by myself and Michelle Cleary using mouse ovarian tissue (Cleary *et al.*, 2001). Temperature equilibrated cryovials were placed into a pre-cooled (0°C) "Mr Frosty". The device was then placed in a polystyrene esky containing dry ice. After 2 hours in the dry ice, the cryovials were plunged into liquid nitrogen (-196°C) for storage.

4.2.3.3. Thawing and washing procedure

Cryovials containing slow-cooled ovarian tissue were removed from liquid nitrogen and held in liquid nitrogen vapour for approximately 30 seconds to initiate thawing and avoid cracking of the sample. Vials were then immersed in a 37°C water bath until the samples were completely thawed.

Following thawing, cryopreserved tissues were removed from cryovials and serially washed to dilute out the cryosolution. The first wash solution consisted of 0.75M cryoprotectant (either DMSO or EG depending on the cryosolution used in the freezing of the tissue) supplemented with 0.25M sucrose made to volume with PBS. The second solution consisted of 0.25M sucrose in PBS. The final wash solution was PBS supplemented with Penicillin G (62units/ml) and Streptomycin sulfate (62μg/ml). The

cryopreserved tissue remained in both the first and second wash solution for 10 minutes at RT. Tissue was placed in the final solution at RT until grafted.

4.2.4. Rapid-cool Cryopreservation of Eastern Grey Kangaroo Ovarian Tissue

4.2.4.1. Cryosolutions

Two cryosolutions were used in the rapid-cooling of ovarian tissue. In both protocols tissues were placed in the first cryosolution for five minutes at RT. Tissues were then transferred to the second cryosolution for one minute before being blotted dry on tissue and being placed on small pieces of foil ready for freezing. All cryosolutions were filter sterilized before use.

The first tested cryoprotectant was developed by Professor Gabor Vajta (Vajta *et al.*, 1998). The first solution consisted of 10% DMSO and 10% EG made to volume in PBS. The second solution contained 20% DMSO, 20% EG and 20% sucrose made to volume with PBS.

The second cryoprotectant was developed by Kasai and colleagues (Kasai *et al.*, 1990). The first solution consisted of PBS supplemented with 10% EG. The second solution consisted of 40% EG supplemented with 1.8g Ficoll 70,000 (Sigma, MO, USA) and 1.02g sucrose per 10ml made to volume with PBS.

4.2.4.2. Rapid-cooling procedure

Ovarian tissues were placed on pieces of household grade aluminium foil, and the foil then transferred into 1.8ml cryovials containing liquid nitrogen (five pieces of tissue per vial). The cryovials were then submerged in liquid nitrogen for storage. The rapid-cooling protocol took less than 10 minutes in total.

4.2.4.3. Thawing and washing procedure

Cryovials containing rapid-cooled ovarian tissue were thawed using the protocol described in Section 4.2.3.3.

Upon thawing, cryopreserved tissues were washed serially in three thawing solutions. Solution 1 consisted of 0.5M sucrose made to volume with PBS (five minutes at RT). Tissues were then transferred to Solution 2 (0.25M sucrose made to volume in PBS) for a further five minutes at RT. Finally, tissues were then transferred to PBS supplemented with Penicillin G (62units/ml) and Streptomycin sulfate (62µg/ml), at RT until grafted.

4.2.5. Ovarian Tissue Grafting

4.2.5.1. Anaesthetic

Xenograft recipients were anaesthetised using a 1mg xylazine hydrochloride and 5mg ketamine hydrochloride solution made to volume with PBS. The anaesthetic was administered and the animals monitored as described in section 3.2.3.1.

4.2.5.2. Ovarian grafting to the kidney capsule procedure

Ovarian tissue pieces were xenografted to the kidney capsule of NOD SCID recipients using the procedures outlined in Section 2.2.4.2.

4.2.5.3. Subcutaneous grafting of ovarian tissue procedure

Subcutaneous grafts were inserted at the same time as kidney capsule grafts. Once the kidney capsule xenografts were secured, the kidney was returned to its normal anatomical position. Subcutaneous grafts were then placed between the abdominal wall and skin on each flank. A piece of non-absorbable suture was placed with each piece of ovarian tissue to indicate the location of the graft at the time of retrieval. The recipient's skin was then brought together, aligned and closed with wound clips.

4.2.6. Assessment of Graft Function

4.2.6.1. Blood collection and graft recovery

To enable the collection of blood, xenograft recipients were anaesthetised as described in Section 4.2.5.1. Once anaesthetised, a blood sample from each recipient mouse was taken by cardiac puncture, using a 30G needle (Terumo Medical Corporation, MD, USA). Blood samples were centrifuged at 4°C at 3000g for 10 minutes to ensure separation of the plasma. Plasma samples were then stored in the freezer (–20°C) for later analysis of FSH levels.

FSH hormone analysis was kindly organised by Anne O'Connor (Monash Institute of Reproduction and Development, Monash University, Melbourne, Australia) and undertaken by Sue Hayward (Monash Institute of Reproduction and Development, Monash University, Melbourne, Australia) using a standard procedure (Wreford *et al.*, 1994). All reagents used in the assay were obtained through, NHPP, NIDDK and Dr. A.F. Parlow (Bethesda, MD, USA). The antiserum used was rabbit anti-rat antiserum (NIDDK rabbit anti-rFSH S-11). The second antibody used was goat-anti rabbit serum #12. The standard and tracer used for the study were, 0.31-40.40ng/ml of NIDKK rFSH-RP-2 and iodinated NIDDK rFSH-I-9 respectively. Three quality controls were used consisting of plasma from a normal mouse and both a male and female rat. All samples were analysed in a single assay using 20µL duplicates. The intra-assay co-efficient of variation was 6.3% and the limit of detection was 2.13ng/ml.

Following the collection of blood, recipients were killed by cervical dislocation. Xenografts to the kidney capsule were recovered as described in Section 2.2.5.1. Subcutaneous pademelon wallaby xenografts were recovered using forceps and scissors. Once collected, pademelon wallaby and kangaroo xenografts were placed in Bouin's fixative (Sigma Diagnostics, MD, USA) for histological analysis. Tammar xenografts

were placed in handling media for oocyte collection. Following oocyte collection, some grafts (see Section 4.2.7.1.) were placed in fixative to enable histological analysis.

4.2.6.2. Vaginal smear cytology

Following xenograft collection, vaginal smears were taken from all graft recipients to establish the stage of the estrous cycle, as a means of assessing graft function. A drop of PBS was placed into each mouse's vagina using a glass pipette. The PBS was aspirated twice and then transferred to a slide and allowed to dry. Once dry, a drop of methylene blue was placed on each slide to stain the smear. Slides were then cover-slipped. Stained vaginal smears were examined under the microscope using the criteria described in Section 1.2.1.2. and Table 1.1., to establish the stage of the estrous cycle.

4.2.6.3. Uterine weight and morphology

As an additional means of assessing graft function, the uteri of graft recipients were collected and weighed. Uteri were dissected out of the abdominal cavity of recipients using scissors and placed in PBS until the time of weighing.

4.2.6.4. Histology

Recovered ovarian xenografts were processed, sectioned and stained as described in Section 2.2.5.2.

4.2.6.5. Classification and counting of follicles

Follicle numbers and types contained within the marsupial ovarian xenografts were determined using the methods described in Section 2.2.5.3., with some slight modifications described below.

The nucleolus of kangaroo and wallaby oocytes is larger than that of mouse oocytes (Lintern-Moore *et al.*, 1976). Therefore, every fifth section of eastern grey kangaroo, tammar wallaby and pademelon wallaby xenografts were counted to avoid the double

counting of follicles. The types of follicles present in each graft were classified using a modification of the Pedersen and Peters (1968) classification scheme that has previously been used for the classification of tammar wallaby follicles (Alcorn, 1975). This scheme is summarised in Table 4.1.

Table 4.1. Marsupial ovarian follicle classification scheme. The scheme is based on the follicle classification scheme for the mouse proposed by Pedersen and Peters (1968) with modifications made for the tammar wallaby (Alcorn, 1975).

Follicle type	**Description of follicle type**
Small	Oocytes surrounded by up to a complete ring of follicle cells.
Medium	Oocytes surrounded by at least two layers of follicle cells. No antral fluid.
Large	Oocytes surrounded by several layers of follicle cells and the presence of antral fluid.
Atretic	Oocytes showing signs of degeneration (e.g. loss of nuclear membrane or fragmentation) or the presence of three or more pyknotic nuclei in follicle cells in a single section.

4.2.7. Experimental Protocol

4.2.7.1. Study 1 – Xenografting of tammar wallaby pouch young ovarian tissue

This study was done in collaboration with Deidre Mattiske (Department of Zoology, University of Melbourne). Both the oocyte collection and *in vitro* culture of oocytes were undertaken within the Department of Zoology at the University of Melbourne.

Tammar wallaby pouch young ovaries (n=16) were collected as described in Section 4.2.2.2. Ovaries were cut into quarters and xenografted, one piece of ovarian tissue per kidney, under the kidney capsule of female NOD-SCID recipients (n=25), using the procedures outlined in Sections 4.2.5.1. and 4.2.5.2. At the time of surgery, recipients

were either left intact (n=12), bilaterally ovariectomised (BLO; n=9), or unilaterally ovariectomised (ULO; n=4; Table 4.2.).

Table 4.2. Description of tammar wallaby pouch young ovarian xenograft treatment groups (gonadal status of recipient, number of pieces of ovarian tissue xenografted, number of recipients and the *in vitro* culture protocol). OT=ovarian tissue.

Treatment group	Xenograft recipient	n Pieces of OT grafted	n Graft recipients	Handling/maturation media
T1	BLO	10	5	Protocol 1
T2	Intact	14	7	Protocol 1
T3	BLO	8	4	Protocol 2
T4	Intact	10	5	Protocol 2
T5	ULO	8	4	Protocol 2

Three months after graft placement, xenografts were recovered from recipients using the methods described in Section 4.2.6.1. One of two protocols was then used for the collection and maturation of oocytes (Table 4.2.). In treatment groups T1 and T2, Protocol 1 was used. The ovarian xenografts were mashed using 30G needles to puncture the antral follicles and release all of the oocytes contained within the grafts into warmed (37°C) handling medium. The HEPES buffered EMEM based handling medium was developed by Dr David Pushett (Monash Institute of Reproduction and Development, Australia) originally for the collection of cat oocytes (Appendix 4).

Following the unsuccessful maturation of oocytes using protocol 1, oocytes in treatment groups T3-T5 were collected using Protocol 2. Using a 30G needle, large antral follicles visible on the surface of grafts were ruptured to release mature oocytes. The oocytes were released into pre-warmed (37°C) handling media that was specifically designed for marsupial oocytes by Nadine Richings (Department of Zoology, University of Melbourne;

Appendix 5.). Following the collection of oocytes, xenografts were placed in Bouin's fixative for histological processing (Section 4.2.6.4.) and analysis (Section 4.2.6.5.).

Tammar wallaby oocytes collected using both protocols were recovered from the relevant handling media and transferred by mouth pipette to the matched gas (5% CO_2) and temperature (37°C) equilibrated maturation media (treatment groups T1 and T2, Protocol 1, Appendix 4; treatment groups T3-T5, Protocol 2, Appendix 5). Oocytes remained in the maturation media for up to 120 hours post collection. Oocytes were assessed daily for maturation (extrusion of the first polar body) and signs of degeneration (darkening of cytoplasm, changes in shape and size, fragmentation).

Blood samples (Section 4.2.6.1.), vaginal smears (Section 4.2.6.2.) and uterine weights (Section 4.2.6.3.) were collected from xenograft recipients as an additional means of evaluating graft function.

4.2.7.2. Study 2 – Xenografting of pademelon wallaby ovarian tissue

Pademelon wallaby ovaries (n=4) were collected as described in Section 4.2.2.1. Once extracted, ovaries were cut into small pieces (approximately 1-2mm^3; n=73), yielding between 17 and 19 pieces of ovarian tissue from each ovary. Ovarian tissue pieces were then xenografted fresh to NOD-SCID female mice recipients (n=8), using the procedures outlined in Sections 4.2.5.2. and 4.2.5.3. Each NOD-SCID recipient received a total of eight pademelon wallaby ovarian tissue xenografts. To each recipient, four pieces of ovarian tissue were grafted subcutaneously and a further four pieces under the kidney capsule. At the time of grafting, recipients were either bilaterally ovariectomised (BLO; n=4), unilaterally ovariectomised (ULO; n=2) or left intact (n=2; Table 4.3.). Four pieces of fresh ungrafted ovarian tissue were placed directly into Bouin's fixative as a control.

Table 4.3. Description of pademelon wallaby xenograft treatment groups. Subcut=subcutaneous.

Gonadal status of recipient	Graft Site	n Pieces of OT grafted	n Graft recipients
BLO	Kidney	16	4
BLO	Subcut	16	4
Intact	Kidney	8	2
Intact	Subcut	8	2
ULO	Kidney	8	2
ULO	Subcut	8	2

Pademelon wallaby ovarian xenografts remained in recipients for two months. Xenografts and blood samples were then collected using the procedures outlined in Section 4.2.6.1. Recovered ovarian xenografts were processed and sectioned as described in Section 4.2.6.4. To assess graft function, follicle counts were performed recording both the number and types of follicles present (Section 4.2.6.5.).

4.2.7.3. Study 3 – Xenografting of eastern grey kangaroo ovarian tissue

Kangaroo ovaries (n=2) were collected from a single adult eastern grey kangaroo as described in Section 4.2.2.1. One whole ovary was placed directly in Bouin's fixative for histology. The remaining ovary was cut into 50 pieces (approximately 1-2mm^3 in size) that were allocated to one of ten cryopreservation treatment groups as shown in Table 4.4.

Ovarian tissue to be cryopreserved by slow-cooling was equilibrated in the relevant cryosolution as described in Section 4.2.3.1. and then slow-cooled using the procedure outlined in Section 4.2.3.2. Kangaroo ovarian tissue to be cryopreserved by rapid-cooling was placed in the appropriate cryosolutions according to the treatment group (Table 4.4.; Section 4.2.4.1.) and then rapid-cooled as outlined in Section 4.2.4.2.

Table 4.4. Description of cryopreservation treatment groups for eastern grey kangaroo ovarian xenografts.

Treatment group	Xenograft recipient	Cryoprotectant	Freezing Protocol	n Pieces of OT
K1	Intact female	DMSO	Slow-cool	5
K2	Intact female	EG	Slow-cool	5
K3	BLO	DMSO	Slow-cool	5
K4	BLO	EG	Slow-cool	5
K5	Male	DMSO	Slow-cool	5
K6	Male	EG	Slow-cool	5
K7	BLO	20% v/v DMSO, 20% v/v EG 20% w/v sucrose (Vajta's)	Rapid-cool	5
K8	BLO	40% v/v EG, 18% w/v Ficoll, 10% w/v sucrose (Kasai's)	Rapid-cool	5
K9	Male	20% v/v DMSO, 20% v/v EG 20% w/v sucrose (Vajta's)	Rapid-cool	5
K10	Male	40% v/v EG, 18% w/v Ficoll, 10% w/v sucrose (Kasai's)	Rapid-cool	5

Slow-cooled and rapid-cooled cryopreserved kangaroo ovarian tissue pieces were thawed and washed as described in Sections 4.2.3.3. and 4.2.4.3., respectively and then xenografted to the kidney capsule of recipient NOD-SCID mice (Section 4.2.5.).

Briefly, xenograft recipients were anaesthetised as described in Section 4.2.5.1. One piece of ovarian tissue was grafted to each kidney capsule using the methods described in Section 4.2.5.2. Each recipient received a graft from one treatment group to the left kidney and a graft from another treatment group to the right kidney. Ovarian xenografts remained in recipients for one month.

At the time of graft recovery, blood samples and kidney xenografts were recovered from recipients using the procedures outlined in Section 4.2.6.1. Recovered xenografts were placed directly in Bouin's fixative for histological analysis (Sections 4.2.6.4 and 4.2.6.5.). As an additional means of assessing graft function, vaginal smears were taken, (Section 4.2.6.2.) and the uteri of recipients collected and weighed (Section 4.2.6.3.).

Ovarian xenografts were assessed histologically with the number and types of follicles contained within xenografts recorded (Sections 4.2.6.4. and 4.2.6.5.).

4.2.8. Statistical Analysis

The follicle counts from the different recipient gonadal statuses, graft sites and cryopreservation protocols were analysed using one and two-way analysis of variance ($p<0.05$ was considered to be significant) and the least significant difference post hoc test. One-way analysis of variance was used to identify differences between treatment groups, while two-way analysis of variance determined if there were any combined effects of the cryopreservation protocol and gonadal status of the xenograft recipient. The uterine weights and oocyte diameters were compared using one-way analysis of variance. Differences in the graft recovery rates were assessed using the Chi-Square test. Analysis of variance was made with the aid of the computer statistics package SPSS 9.0.1 for Windows (1999). Results obtained are presented as the mean ± SEM.

4.3. Results

4.3.1. Study 1 – Xenografting of Tammar Wallaby Pouch Young Ovarian Tissue

4.3.1.1. Graft recovery

Tammar wallaby pouch young ovarian xenografts were recovered from 21 NOD-SCID recipients (n=2 recipients died while recovering from the anaesthetic, n=1 recipient died prior to graft collection, probably as a of post-surgical infection, n=1 recipient was killed two months after surgery because of ill health) three months after graft placement (Table 4.5.). One or two ovarian xenografts were recovered from each of the surviving recipients (n=21), except for one recipient, from which no ovarian xenografts were recovered. Statistically, the gonadal status of the recipient had no effect on the graft recovery rate ($p>0.48$).

4.3.1.2. Uterine weight, vaginal smear and hormone analysis

The gonadal status of the recipient had no effect on the uterine weight of graft recipients ($p>0.14$; Table 4.5.). Uteri collected from females belonging to all of the treatment groups were highly vascularized and of normal size. The vaginal smear cytology at the time of xenograft collection was similar for females from each of the treatment groups, with all but two females in estrus or metestrus.

Similarly, the circulating FSH levels were unaffected by the gonadal status of the recipients ($p>0.73$; Table 4.5.).

Table 4.5. Effect of gonadal status on tammar wallaby pouch young ovarian xenograft, retrieval rates and function (uterine weights and FSH levels). *Results only given for xenograft recipients that survived the duration of the study. †Results presented as the mean ± SEM. Same superscript letter within columns indicate groups that are statistically the same ($p>0.05$). See table 4.2. for description of treatment groups.

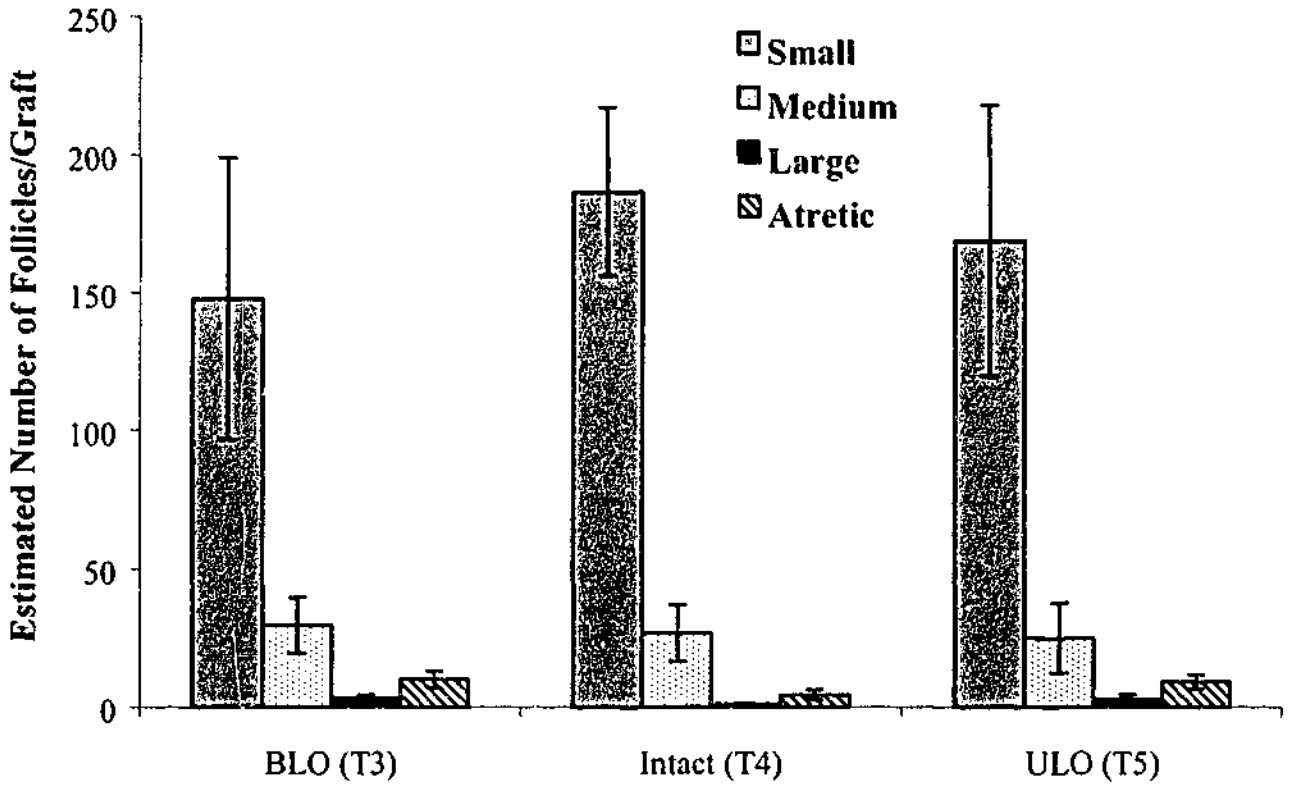
Gonadal status of recipient	n Grafts retrieved/n grafted (%)*	Uterine weight† (g) (n)	Circulating FSH level† (ng/ml) (n)
BLO (T1,T3)	13/16 (81.25)[a]	0.20 ± 0.03[a] (8)	7.16 ± 3.14[a] (5)
Intact (T2,T4)	17/18 (94.44)[a]	0.28 ± 0.03[a] (9)	5.79 ± 2.52[a] (5)
ULO (T5)	7/8 (87.50)[a]	0.20 ± 0.01[a] (4)	3.84 ± 1.23[a] (2)

4.3.1.3. Follicular development

Ovarian xenografts were large in size (approximately double their original size), and well vascularized at the time of graft collection. Antral sized follicles were visible on the surface of numerous grafts, enabling the collection of full sized oocytes (see Section 4.3.1.4.). Upon histological analysis, grafts recovered from recipients belonging to treatment groups T3-T5 (treatment group T3, BLO, n=7; treatment group T4, intact, n=5; treatment group T5, ULO, n=7) were generally highly populated with follicles. However as demonstrated in Figure 4.1., the follicle counts obtained for the viable small, medium and large sized follicles and total atretic follicles were highly variable.

On analysis of the viable follicle population numbers contained within grafts recovered from treatment groups T3-T5, small sized (type 3a and type 3b) follicles were the most abundant follicle type (Figures 4.1. and 4.2.a.). Medium sized follicles were present (Figure 4.2.b.), but at a lower frequency than the small sized follicles. Even after the collection of oocytes from the largest antral follicles, viable type 6 and 7 (antral) follicles were observed in grafts collected from ovarian tissue transplanted to BLO and ULO recipients, and type 6 follicles in grafts recovered from intact recipients (Figure 4.2.c.).

Figure 4.1. Effect of the gonadal status of the recipient on the number of small sized, medium sized, large sized and atretic follicles contained within tammar wallaby pouch young ovarian xenografts. Results are presented as the mean ± SEM. $p>0.05$ for all treatment groups for each of the criteria assessed.

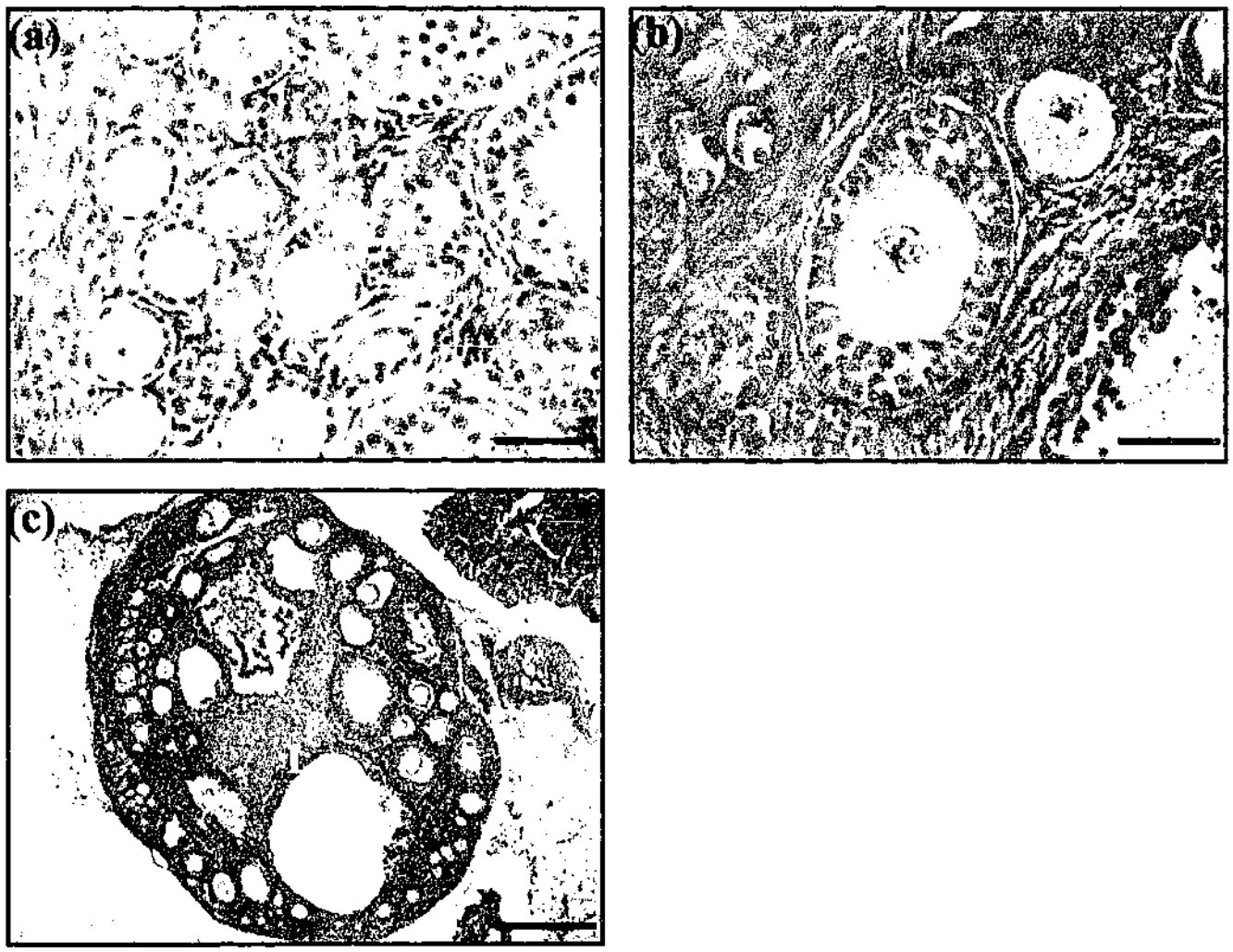


The gonadal status of the xenograft recipient had no effect ($p>0.54$) on either the number of viable small, medium or large sized follicles contained within recovered xenografts. Similarly, the gonadal status had no effect on the number of atretic follicles, with few atretic follicles being found in xenografts recovered from recipients with each of the three gonadal statuses (treatment groups T3-T5).

4.3.1.4. Oocyte collection

Expanded oocytes were successfully collected from antral follicles visible on the surface of tammar wallaby ovarian xenografts recovered from BLO, intact and ULO NOD-SCID graft recipients (Table 4.6.). The gonadal status of the recipient influenced the number of recovered oocytes, but this difference was non-significant ($p>0.49$; Table 4.6.).

Figure 4.2. Histological appearance of tammar wallaby pouch young ovarian tissue following xenografting to NOD-SCID mice. Three months after grafting tammar wallaby ovarian tissue xenografts were densely packed with healthy **(a)** small (S) sized and **(b)** medium (M) sized follicles (bars=50µm). **(c)** Despite the prior collection of oocytes from antral sized follicles visible on the surface of the xenografts, follicular development to antral (L) stages was observed in tammar wallaby pouch young ovarian tissue xenografts (bar=500µm).



Of the oocytes released from the xenografts and collected into maturation media, great variability was observed with regard to both the maturity and health of the oocytes. No pattern could be established between the appearance of the oocytes collected and the gonadal status of the xenograft recipient.

Table 4.6. Effect of gonadal status on the number of tammar wallaby pouch young ovarian xenografts yielding oocytes (as a proportion of recovered xenografts) and the mean diameter of the oocytes collected. See table 4.2. for description of treatment groups. Same superscript letter within columns indicates groups that are statistically the same ($p>0.05$).

Gonadal status of the recipient (treatment groups)	**n Grafts yielding oocytes/n grafts recovered (%)**	**n Oocytes collected**	**Mean ± SEM oocyte diameter (µm)**
BLO (T1,T3)	7/13 (53.85)[a]	15	152.50 ± 5.75[a]
Intact (T2,T4)	7/17 (41.18)[a]	8	143.00 ± 10.00[a]
ULO (T5)	2/7 (28.57)[a]	2	181.25 ± 12.25[a]

Although, the majority of oocytes collected were released from fluid filled blisters that were visible on the surface of the xenografts (presumably antral sized follicles), no oocytes with extruded polar bodies were observed. Instead, only less mature oocytes that had not undergone complete nuclear maturation were obtained. Some of the collected oocytes had no cumulus oophorus (Figure 4.3.a.), while others were surrounded completely or partially by cumulus cells (Figures 4.3.b. and 4.3.c.). However, no pattern could be established between the presence or absence of cumulus cells, the degree of expansion and the gonadal status of the xenograft recipient.

The diameters of the oocytes were measured at the time of oocyte collection to determine the mean diameter for oocytes collected from recipients with each of the gonadal statuses. The gonadal status of the recipient had no effect on the oocyte diameters ($p>0.05$; Table 4.6.).

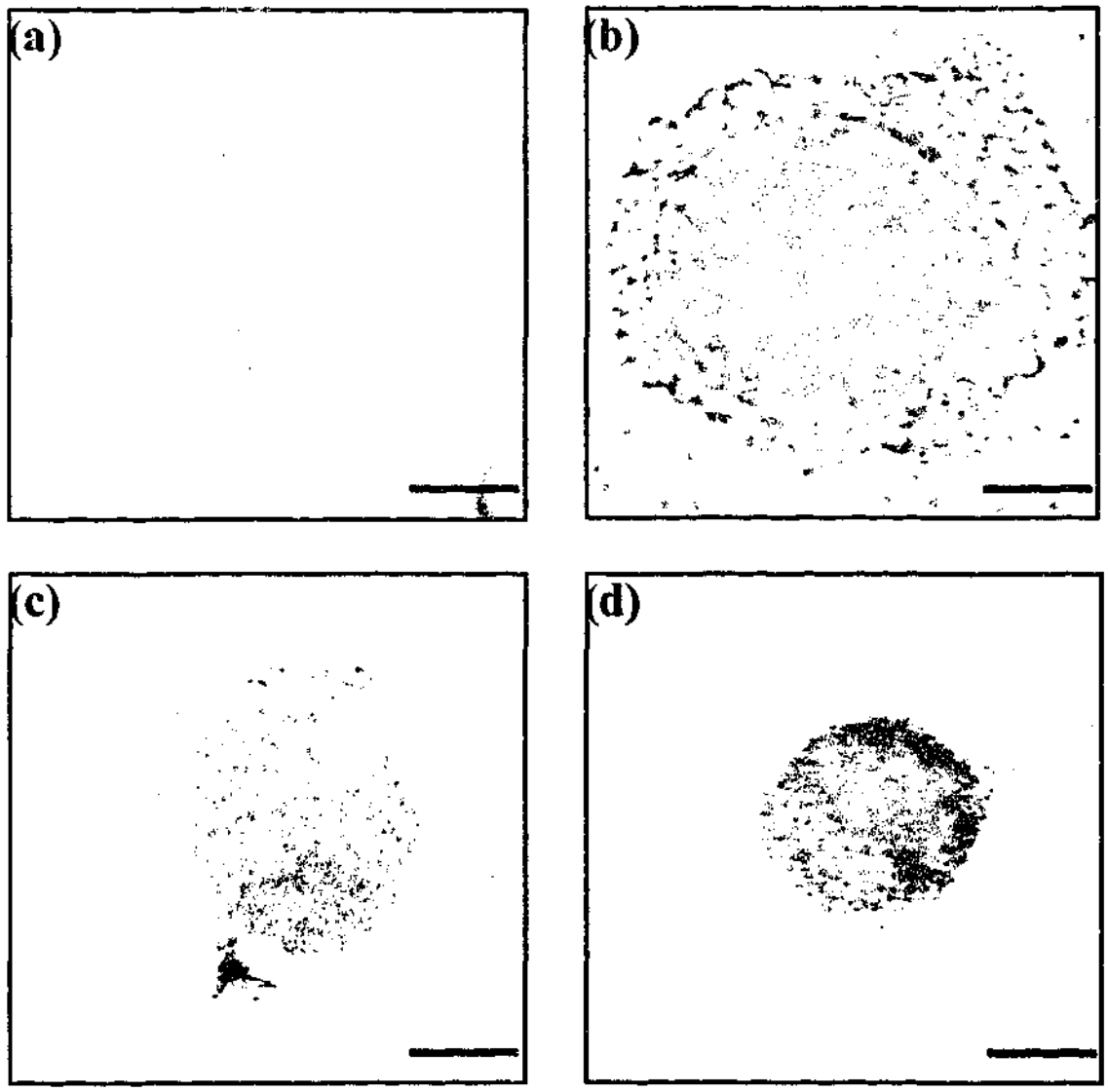
The cytoplasm of three oocytes collected from xenografts to intact recipients and one oocyte collected from a xenograft to a BLO recipient were dark in colour and granular in appearance, possibly indicating degeneration and/or fragmentation (Figure 4.3.d.). A further four oocytes collected from xenografts to BLO recipients were irregular in shape, and/or shrunken in size at the time of oocyte collection, thus also suggesting poor viability.

Oocytes cultured in maturation media were examined every 24 hours and signs of maturation or degeneration observed for. Regardless of the maturation media used none of the oocytes extruded the first polar body.

For the first 48 hours of culture, oocytes generally retained their shape and viability as determined by visual examination. However, after 48 in culture, the majority of oocytes had become irregular in shape, with the oocytes cytoplasm frequently shrinking away from the zona and becoming dark and granular. After 96 hours in maturation media all cultured oocytes showed signs of degeneration. Between 96 and 120 hours post oocyte collection, the oocytes were removed from culture and discarded.

In addition to the oocytes that were collected and placed in maturation media, numerous highly immature oocytes (tight cumulus) were also released from the grafts belonging to treatment groups 1 (BLO) and 2 (intact) at the time of oocyte collection. These oocytes were discarded due to their immaturity. The justification for this was provided by previous experience in our laboratory that has found that highly immature oocytes collected from ovarian xenografts do not mature in culture.

Figure 4.3. Oocytes released from tammar wallaby pouch ovarian xenografts. Ovarian xenografts were grafted under the kidney capsule of intact, BLO and ULO female NOD-SCID mice for three months. At the time of xenograft collection, healthy oocytes were released from xenografts in BLO, intact and ULO recipients. **(a)** A nude tammar wallaby oocyte collected from a xenograft in a ULO recipient. **(b)** A cumulus enclosed oocyte collected from a xenograft in a BLO recipient. **(c)** An oocyte with a few cumulus cells attached collected from a tammar wallaby ovarian xenograft in an intact NOD-SCID mouse recipient. In addition to healthy oocytes, oocytes showing signs of degeneration and fragmentation were observed at the time of oocyte collection. **(d)** A dark and shrunken (unhealthy) tammar wallaby oocyte collected from an ovarian xenograft to an intact recipient. (bars=50μm).



4.3.2. Study 2 – Xenografting of Pademelon Ovarian Tissue

4.3.2.1. Graft recovery

Of the eight NOD-SCID females to receive pademelon wallaby ovarian xenografts in this study, only three females survived the duration (2 months) of the study. Two mice were found dead between weeks four and five post surgery. No cause of death could be established. An additional three females were culled five to eight weeks after the surgery while displaying signs of ill health, namely weight loss and lethargy. Interestingly, despite the ill health of these five animals, post-mortem analysis revealed that some of the xenografts were still present and appeared to be well vascularized.

All four ovarian xenografts under the kidney capsule were recovered from each of the xenograft recipients (n=3; Table 4.7.). Not all subcutaneous xenografts could be recovered. However, when assessed statistically, no difference existed between the kidney capsule and subcutaneous graft recovery rates upon comparison of the six treatment groups ($p>0.19$).

Table 4.7. Effect of the graft site and gonadal status of the recipient on pademelon wallaby ovarian tissue xenograft retrieval rates. *Results given for xenograft recipients that survived the duration of the study. Same superscript letters indicate groups that are statistically the same ($p>0.05$).

Treatment group/gonadal status of recipient	Graft site	n Grafts retrieved/n grafted (%)*
BLO	Kidney	4/4 (100.00)[a]
BLO	Subcut	3/4 (75.00)[a]
Intact	Kidney	4/4 (100.00)[a]
Intact	Subcut	2/4 (50.00)[a]
ULO	Kidney	4/4 (100.00)[a]
ULO	Subcut	2/4 (50.00)[a]

4.3.2.2. Hormone analysis

Circulating FSH levels varied according to the gonadal status of the xenograft recipient. The highest circulating FSH level was found in the BLO recipient (12.63ng/ml). The ULO recipient (<2.13ng/ml) had a lower circulating FSH level than the intact recipient (5.26ng/ml).

4.3.2.3. Follicular development

Small and medium sized follicles (viable and/or atretic) were observed in ovarian xenografts from each of the six treatment groups (Figures 4.4. and 4.5.). Small sized follicles were the most abundant follicle type (Figure 4.6.a.). Grafts to BLO recipients generally contained the greatest follicle population, followed by grafts to ULO recipients, however, this difference was non-significant ($p>0.05$). Grafts to intact recipients contained significantly fewer small sized follicles than xenografts to BLO recipients ($p<0.03$; Table 4.8.). Kidney capsule grafts overall contained a greater follicle population than subcutaneous grafts (Figure 4.4.).

Few medium and large sized follicles were observed in recovered kidney capsule and subcutaneous ovarian xenografts (Table 4.8.), and antral sized follicles were only observed in kidney xenografts to BLO (up to type 7) and ULO (up to type 6) recipients (Figure 4.6.b.). Corpora lutea were not observed in any ovarian xenografts. There was no statistical difference in the number of medium and large sized follicles present in xenografts when comparing all xenograft treatment groups ($p>0.08$; Table 4.8.).

Figure 4.4. Effect of graft site and gonadal status of the recipient on the total number of viable follicles contained within pademelon wallaby ovarian tissue xenografts compared to fresh ungrafted control tissue. Results are presented as the mean ± SEM. Same superscript letters indicate groups that are statistically the same ($p>0.05$).

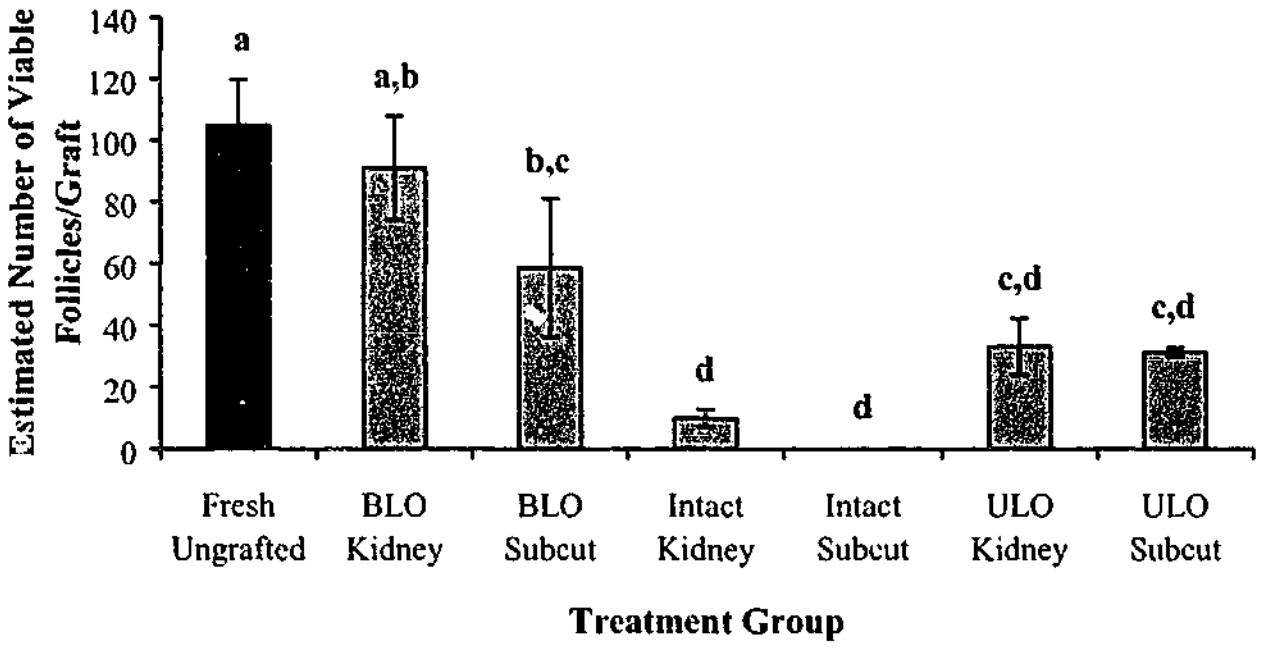
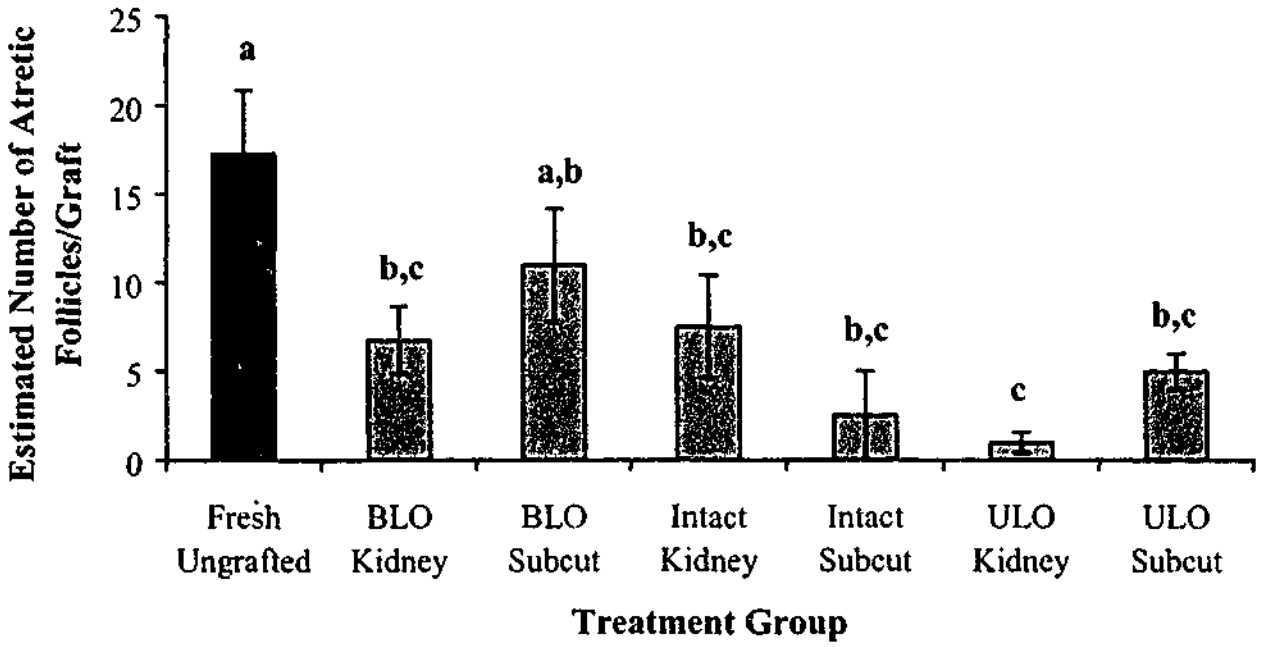


Figure 4.5. Effect of graft site and gonadal status of the recipient on the total number of atretic follicles contained within pademelon wallaby ovarian tissue xenografts compared to fresh ungrafted control tissue. Results are presented as the mean ± SEM. Same superscript letters indicate groups that are statistically the same ($p>0.05$).

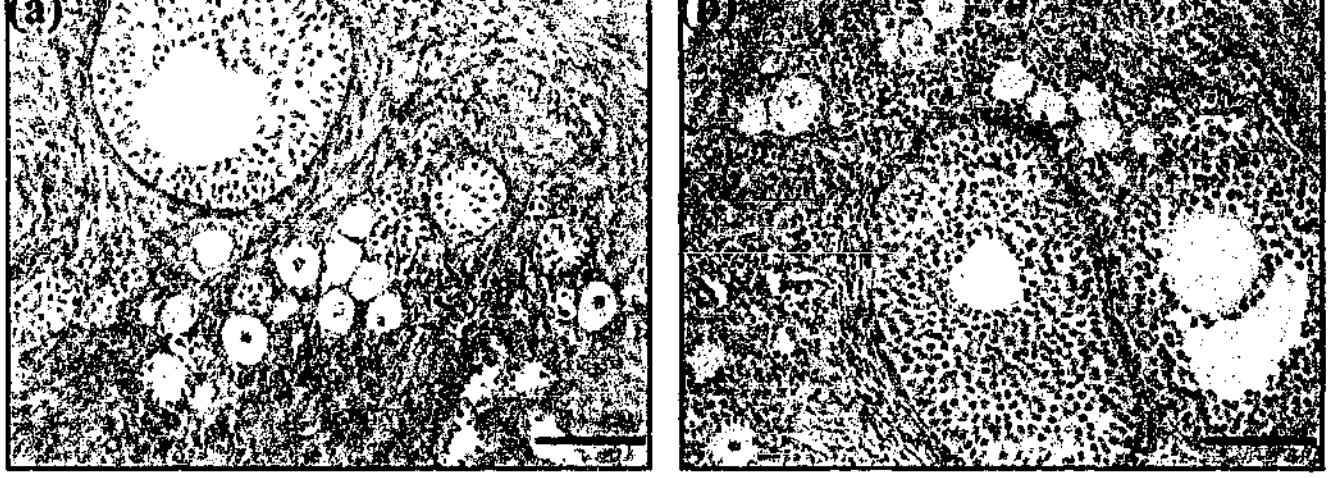


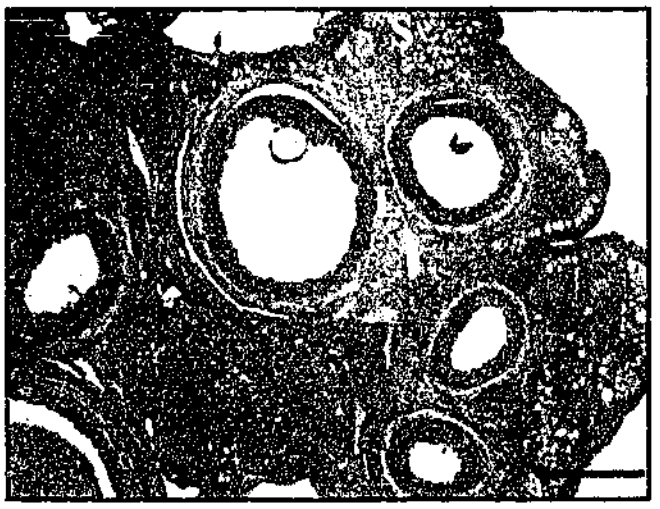
As in the ovarian xenografts, in the fresh ungrafted control ovarian tissue, small sized follicles were the most abundant follicle type (Figure 4.6.c.). However fresh ungrafted ovarian tissue contained significantly more small sized follicles ($p<0.04$; Table 4.8.), and as a result total viable follicles ($p<0.03$; Figure 4.4.), than in xenografts from all of the treatment groups other than the BLO kidney capsule xenografts ($p>0.44$; Table 4.8.; Figure 4.4.). The numbers of medium and large sized follicles were equivalent in all treatment groups and the ungrafted fresh control tissue ($p>0.19$; Table 4.8.).

Table 4.8. Effect of the gonadal status of the recipient and the graft site on the number of viable small, medium and large sized follicles and atretic follicles within pademelon wallaby ovarian tissue xenografts compared to fresh ungrafted ovarian tissue. Same superscript letters within columns indicate groups that are statistically the same ($p>0.05$). *Results are the mean ± SEM number of viable follicles. †Results presented as the percentage of atretic follicles as a proportion of total follicle numbers.

Treatment group	Small sized follicles*	Medium and large sized follicles*	% Atretic follicles†
Fresh Ungrafted	102.30 ± 14.82^{a}	2.50 ± 1.19^{a}	14.14
BLO Kidney	$88.50 \pm 16.47^{a,b}$	2.50 ± 1.32^{a}	6.91
BLO Subcut	$58.33 \pm 22.26^{b,c}$	0.33 ± 0.33^{a}	15.78
Intact Kidney	9.25 ± 2.56^{d}	0.75 ± 0.48^{a}	42.86
Intact Subcut	0.00 ± 0.00^{d}	0.00 ± 0.00^{a}	100
ULO Kidney	$30.55 \pm 8.91^{c,d}$	2.75 ± 0.25^{a}	2.92
ULO Subcut	$27.50 \pm 2.50^{c,d}$	4.00 ± 4.00^{a}	13.70

Figure 4.6. Histological appearance of pademelon wallaby ovarian tissue. **(a)** Small (S) sized follicles were the most abundant follicle type observed in pademelon wallaby ovarian tissue xenografts (subcutaneous graft to a BLO recipient) at two months after grafting (bar=100µm). **(b)** In addition to small and medium sized follicles pademelon wallaby ovarian tissue xenografts (kidney capsule graft to a BLO recipient) contained antral (L) sized follicles at two months after grafting (bar=200µm). **(c)** Fresh ungrafted pademelon wallaby ovarian tissue was more densely packed with follicles than the recovered ovarian tissue xenografts (bar=500µm).





Atretic follicles were present in all xenografted groups and in fresh ungrafted control ovarian tissue (Table 4.8.; Figure 4.5.). Significantly more ($p<0.02$) atretic follicles were observed in the fresh ungrafted control tissue than all xenografted groups other than the BLO subcutaneous xenografts. The remaining xenograft treatment contained the same number of atretic follicles ($p>0.11$), despite significant differences in the total number of viable follicles. When analysed as a percentage of total follicle numbers, the proportion of atretic follicles (Table 4.8.) was comparable and less than 16% in all grafted and fresh groups, except the intact kidney and intact subcutaneous xenograft groups, in which the lowest follicle populations were observed.

The gonadal status ($p<0.01$), but not the graft site ($p>0.22$) had a significant effect on the total number of viable follicles, further indicating that the bilaterally ovariectomised recipient is optimal. The combined effect of the gonadal status and graft site was non-significant ($p>0.52$). Neither the gonadal status or graft site affected the number of atretic follicles ($p>0.07$).

4.3.3. Study 3 – Xenografting of Kangaroo Ovarian Tissue

4.3.3.1. Graft recovery

Kangaroo ovarian xenografts were recovered from 23 NOD-SCID recipients one month after graft placement (n=2 recipients were found dead three to four weeks post surgery, for unidentified reasons). Between three and five ovarian xenografts from each treatment group were recovered from graft recipients (Table 4.9.). There was no difference in the graft recovery rate between treatment groups ($p>0.26$).

Table 4.9. Effect of both the gonadal status of the recipient and the cryopreservation protocol on eastern grey kangaroo ovarian xenograft retrieval rates. See Table 4.4. for full description of treatment groups. *Results only given for xenograft recipients that survived the duration of the study. Same superscript letters indicate groups that are statistically the same ($p>0.05$).

Treatment group	Description of cryopreservation group	n Grafts retrieved/n grafted (%)*
K1	Intact DMSO	5/5 (100.00)[a]
K2	Intact EG	4/5 (80.00)[a]
K3	BLO DMSO	5/5 (100.00)[a]
K4	BLO EG	3/5 (60.00)[a]
K5	Male DMSO	3/5 (60.00)[a]
K6	Male EG	5/5 (100.00)[a]
K7	BLO Vajta's	3/4 (100.00)[a]
K8	BLO Kasai's	4/4 (100.00)[a]
K9	Male Vajta's	4/4 (75.00)[a]
K10	Male Kasai's	4/4 (100.00)[a]

4.3.3.2. Uterine weight, vaginal smear and hormone analysis

The uteri of xenograft recipients from the different treatment groups were visibly different in appearance at the time of graft collection. Uteri collected from intact females (treatment groups K1 and K2) were large in size and highly vascularized. Uteri collected from BLO xenograft recipients (treatment groups K3, K4, K7 and K8) were shrunken and pale in colour. These observations are supported by the recorded uterine weights (Table 4.10.), with uteri collected from intact female recipients (treatment groups K1 and K2) found to be significantly heavier than those collected from BLO recipients (treatment groups K3, K4, K7 and K8; $p<0.02$).

Table 4.10. Effect of both the gonadal status of the recipient and the cryopreservation protocol on the uterine weight and circulating FSH levels of graft recipients at the time of graft retrieval. See Table 4.4. for full description of treatment groups. *Results given as mean ± SEM. Same superscript letter within columns indicates groups that are statistically the same ($p>0.05$).

Gonadal status of the recipient (freezing protocol)	Treatment groups	Uterine weight (g)*	Circulating FSH concentration (ng/ml)*
Intact (Slow)	K1,K2	0.19 ± 0.02^{a}	3.70 ± 0.77^{a}
BLO (Slow)	K3,K4	0.09 ± 0.03^{b}	17.09 ± 0.58^{c}
Male (Slow)	K5,K6	NA	13.69 ± 0.24^{b}
BLO (Rapid)	K7,K8	0.08 ± 0.01^{b}	14.81 ± 0.75^{b}
Male (Rapid)	K9,K10	NA	13.81 ± 0.18^{b}

In addition to differences in uterine weights, differences were also observed in the vaginal smear cytology. Intact recipients (treatment groups K1 and K2) were all in either estrus or metestrus. The same observation was made for BLO recipients that received slow-cool cryopreserved ovarian tissue (treatment groups K3 and K4). In contrast BLO recipients that received rapidly cooled ovarian tissue (treatment groups K7 and K8) were all in the diestrus stage of the cycle with the exception of a single recipient in the estrus stage. For each individual the uterine weight and vaginal smear cytology were correlated, with the heaviest uteri obtained from females that were in the estrus stage of the cycle and lightest uteri from females in the diestrus stage of the cycle at the time of xenograft collection.

The circulating FSH levels were as expected, with the lowest mean levels obtained for the intact recipients that received the slow-cool cryopreserved ovarian tissue (treatment groups K1 and K2). Significantly higher levels ($p<0.01$) were obtained for all other treatment groups (treatment groups K3-K10). These results generally correlated with the uterine weights obtained from female xenograft recipients (treatment groups K1-K4, K7 and K8),

with the highest mean uterine weights obtained from females with intact ovaries (Table 4.10.).

4.3.3.3. Follicular development

Viable follicles were observed in xenografts from all treatment groups other than those that were cryopreserved by slow-cooling in EG and then xenografted to intact female recipients (n=4; treatment group K2; Figures 4.7., 4.8., 4.9.). There was no statistically significant difference in the number of small sized follicles in xenografts from all treatment groups ($p>0.16$; Figure 4.7.). There was high variability in the results for all xenografted groups.

The cryopreservation protocol ($p<0.02$) but not the gonadal status ($p>0.80$) had an effect on the number of small sized follicles contained within the xenografts. In comparison to slow-cooling in DMSO and rapid-cooling using Vajta's protocol, slow-cooling in EG resulted in statistically significantly ($p<0.04$) fewer small sized follicles within the kangaroo ovarian xenografts. The combination of the gonadal status and freezing protocol had a non-significant effect ($p>0.80$).

While very few medium (Figure 4.8.) and large (Figure 4.9.) sized follicles were observed within the kangaroo xenografts, follicular development to the antral stage was observed in xenografts recovered from intact recipients (treatment group K1; Figure 4.10.a.), BLO recipients (treatment group K3) and male recipients (treatment groups K5, K6 and K10; Figure 4.10.b.). However, due to the low number of both medium and large sized follicles contained within xenografts from each of the treatment groups, no statistically significant differences were found to exist when comparing all of the xenograft treatment groups ($p>0.25$). Additionally, neither the cryopreservation protocol used in the freezing of the tissue or the gonadal status of the recipient, were found to affect the number of medium or large sized follicles contained within recovered xenografts ($p>0.21$).

Statistically significant differences existed in the number of atretic follicles contained within xenografts (Figure 4.11.). Ovarian tissue xenografts rapidly frozen using Vajta's protocol and xenografted into a BLO recipient (treatment group K7) contained significantly more atretic follicles than in all of the other treatment groups, with the exception of the intact DMSO, BLO DMSO and BLO Kasai's treatment groups (treatment groups K1, K3 and K8). A direct correlation between the number of viable follicles and the number of atretic follicles was generally seen (Figures 4.7., 4.8., 4.9. and 4.11.). However, xenografts from the intact EG, BLO EG, Male EG and BLO Kasai's treatment groups (treatment groups K2, K4, K6 and K8) contained a greater number of atretic follicles than viable follicles.

Figure 4.7. Effect of treatment group (cryoprotectant, freezing protocol and gonadal status of the graft recipient) on the total number of small sized follicles contained within kangaroo ovarian tissue xenografts. See Table 4.4. for the description of treatment groups. Results are presented as the mean ± SEM. $p>0.05$ for the number of small sized follicles contained within xenografts belonging to all treatment groups.

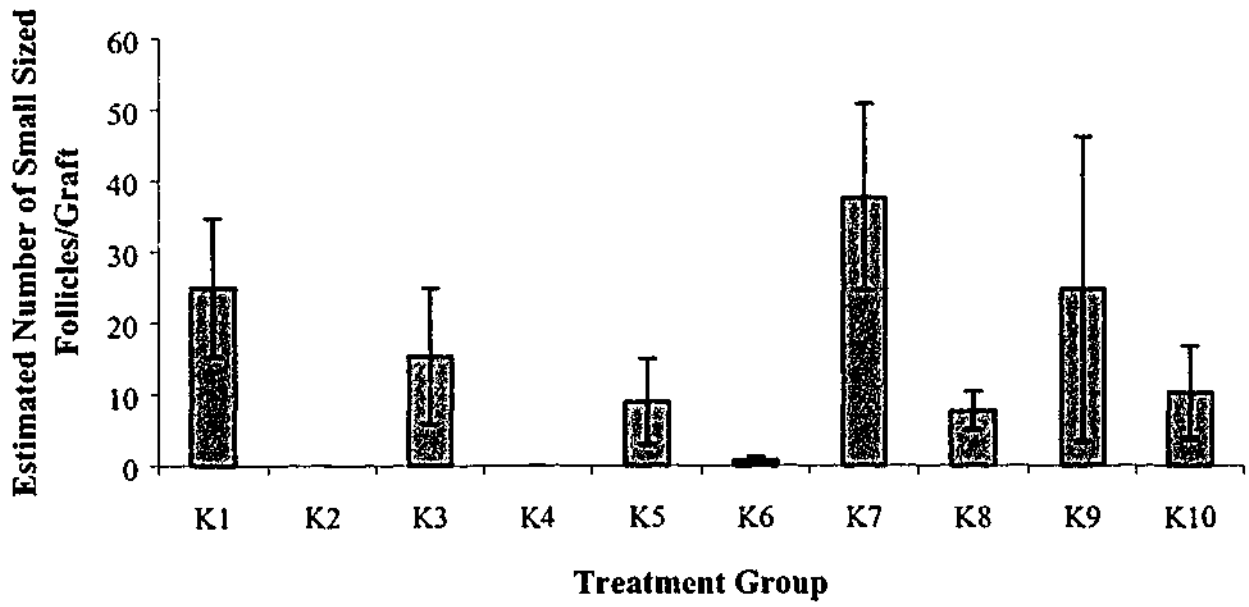


Figure 4.8. Effect of treatment group (cryoprotectant, freezing protocol and gonadal status of the graft recipient) on the total number of medium sized follicles contained within kangaroo ovarian tissue xenografts. See Table 4.4. for the description of treatment groups. Results are presented as the mean ± SEM. $p>0.05$ for the number of medium sized follicles contained within xenografts belonging to all treatment groups.

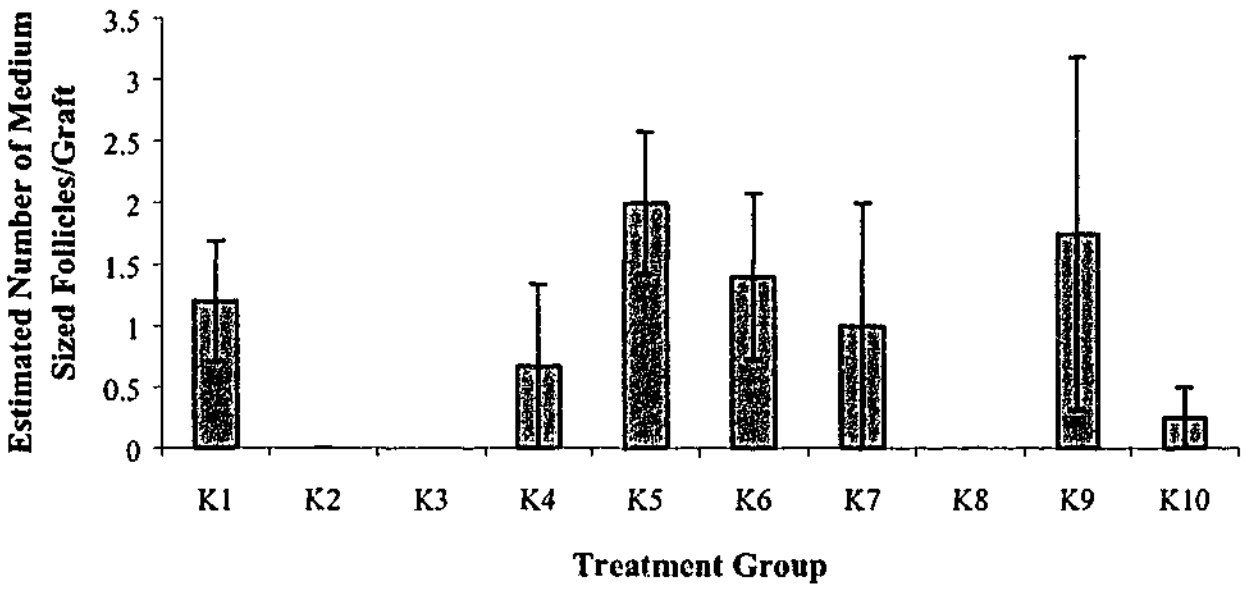


Figure 4.9. Effect of treatment group (cryoprotectant, freezing protocol and gonadal status of the graft recipient) on the total number of large sized follicles contained within kangaroo ovarian tissue xenografts. See Table 4.4. for description of the treatment groups. Results are presented as the mean ± SEM. $p>0.05$ for the number of large sized follicles contained within xenografts belonging to all treatment groups.

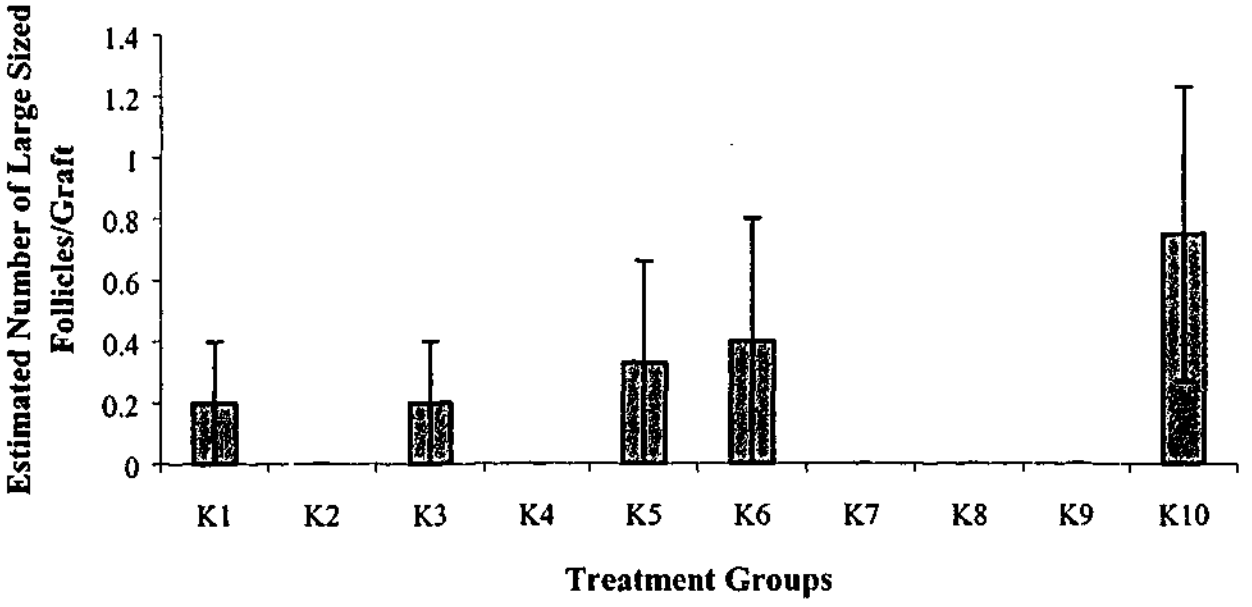
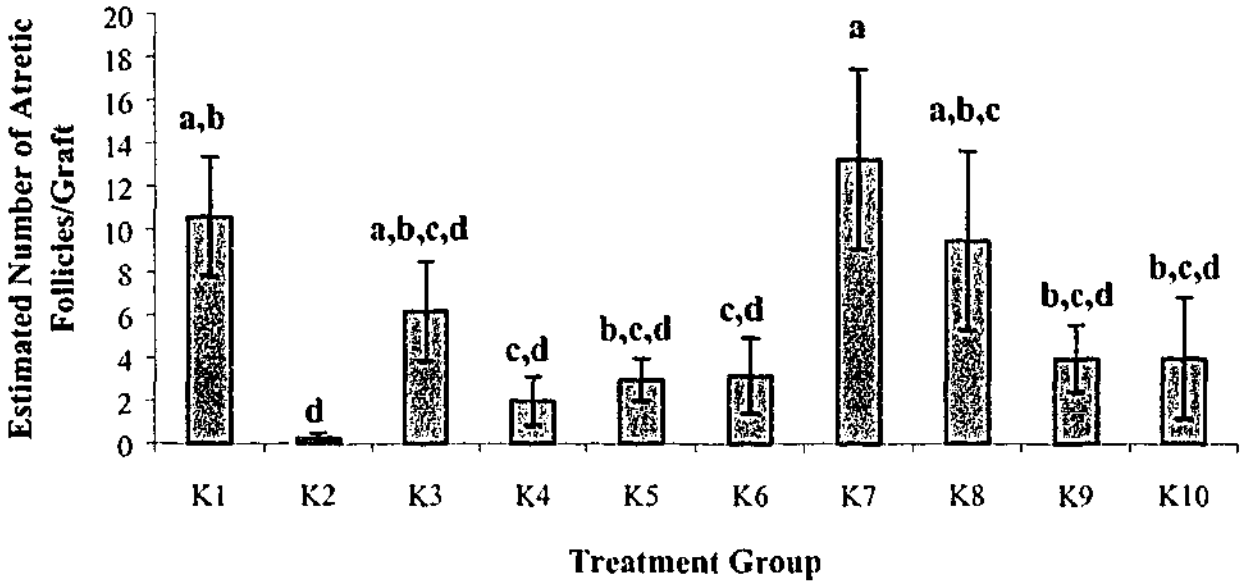


Figure 4.10. Histological appearance of eastern grey kangaroo ovarian tissue xenografts one month after transplantation under the kidney capsule of adult NOD-SCID mice. Follicles of all stages of folliculogenesis were observed within the kangaroo ovarian tissue xenografts, including antral (L) sized follicles within xenografts recovered from **(a)** intact (treatment group K1) (bar=200μm), BLO, and **(b)** male recipients (treatment group K6) (bar=500μm).

Figure 4.11. Effect of the treatment group (cryoprotectant, freezing protocol and gonadal status of the graft recipient) on the total number of atretic follicles contained within kangaroo ovarian tissue xenografts. See Table 4.4. for description of the treatment groups. Results are presented as the mean ± SEM. Same superscript letters indicate groups that are statistically the same ($p>0.05$).



4.4. Discussion

Xenografting of ovarian tissue has the potential to help ensure the survival of threatened and endangered wildlife species. However, to be of the greatest benefit conditions that enable optimal follicular development within ovarian tissue xenografts, thus facilitating the collection of mature oocytes, must be established. While this study was unsuccessful in facilitating the collection of mature fertilizable oocytes a number of interesting points pertaining to marsupial ovarian tissue xenografting were found.

4.4.1. Effect of Gonadal Status of Recipient

Bilaterally ovariectomised females have typically been utilised as ovarian tissue allograft and xenograft recipients (Russell & Hurst, 1945; Gosden *et al.*, 1994b; Cox *et al.*, 1996; Candy *et al.*, 2000; Mattiske *et al.*, 2002). Recent years have seen the more frequent use of recipients with alternate gonadal statuses in xenografting studies (Weissman *et al.*, 1999; Besch *et al.*, 2002; Cleary *et al.*, 2002). However, little practical data is available that compares the efficiency of recipients with differing gonadal statuses. Using human ovarian tissue, the suitability of intact female and intact male NOD-SCID mice recipients was evaluated, by comparing follicle survival and follicular development within subcutaneous ovarian xenografts (Weissman *et al.*, 1999). Interestingly, while no significant difference existed in the number of medium or small sized follicles contained within the human xenografts, significantly more pre-antral and antral sized follicles were observed in the xenografts to the male recipients than the female recipients following hormone stimulation. This result indicating that male recipients may provide a better model for generating mature oocytes for use in other ART's.

It is feasible, that the accelerated follicular development observed in xenografts to male recipients, may have been the result of high circulating androgen levels. Exogenous androgen administration enhances follicular growth and survival in the primate (Vendola *et al.*, 1998). In contrast, testosterone is detrimental to mouse oocytes undergoing *in vitro* maturation (Anderiesz & Trounson, 1995). The specific role and benefits of androgen exposure in oocyte maturation thus remain unknown.

Intact and bilaterally ovariectomised female recipients both support follicular development of xenografted wombat ovarian tissue (Cleary *et al.*, 2002). However, follicular development was faster in xenografts of bilaterally ovariectomised recipients than in intact recipients, supporting the theory that the elevated circulating gonadotrophin levels after ovariectomy are beneficial to graft establishment (Dissen *et al.*, 1994) and follicular development, as FSH levels were higher in these recipients.

In Study 1, investigating ovarian xenografting using tammar wallaby pouch young ovarian tissue, the results indicated that ovariectomy, and hence (initial) gonadotrophin concentrations were not crucial in determining either graft retrieval rates, follicle numbers, follicles types or the maturity and potential of the oocytes collected. However, this may in part be due to the FSH levels. Regardless of the gonadal status, the circulating FSH levels were lower than that which has previously been obtained for bilaterally ovariectomised mice (15.00 ± 0.70ng/ml; Cox *et al.*, 2000), thus indicating that the production of estrogen by follicles contained within the xenografts had a negative feedback effect on the pituitary gonadotrophin release. This is supported by the presence of antral sized follicles within the ovarian xenografts.

While full oocyte maturation was not achieved in any group regardless of the handling and maturation media used, the oocytes collected were comparable in size to what has previously been reported for ovulated tammar wallaby oocytes (126 ± 13μm; Tyndale-

Biscoe & Renfree; 1987). Since full sized oocytes could be collected ovarian xenografting may be useful as a means of generating mature marsupial oocytes. With the success of the work reported in Chapter Three in mind, improved marsupial *in vitro* culture and fertilization conditions should permit further extension of this work with the production of both embryos and live young.

In Study 2, using pademelon wallaby ovarian tissue xenografts, the graft retrieval rates and hence graft establishment rates were unaffected by the gonadal status of the recipient. However, due to the low sample numbers in Study 2, it remains unclear whether this is a genuine finding or if, with increased numbers a different result would be obtained. The latter appears to be most probable if the follicular development observed within the pademelon wallaby xenografts is an accurate indicator, with grafts to the kidney capsule of bilaterally ovariectomised recipients containing a significantly higher number of follicles than those to either unilaterally ovariectomised or intact recipients. Further support to an effect is offered by the high circulating FSH level found in the bilaterally ovariectomised recipient, as graft establishment has been previously demonstrated to be enhanced with elevated gonadotrophin levels (Dissen *et al.*, 1994).

While a significant difference was not observed in the number of follicles contained within xenografts to unilaterally ovariectomised recipients and intact recipients this may have been the result of low sample numbers. This appears likely, as the xenografts to unilaterally ovariectomised recipients, while not statistically significant, did generally contain a greater number of follicles than grafts to intact recipients. Unilateral ovariectomy does result in a temporary increase in circulating FSH (Welschen & Dullaart, 1974; Butcher, 1977; Findlay & Cumming, 1977; Redmer *et al.*, 1984). Increased gonadotrophin levels may result in increased synthesis of angiogenic factors (Dissen *et al.*, 1994). The short-lived increase in gonadotrophin may account for the increased follicular

survival and development in unilaterally ovariectomised recipients as opposed to intact recipients. Thus, in contrast to the results obtained using tammar wallaby pouch young ovarian tissue, the results obtained Study 2 indicate that the gonadal status of the recipient does play a role in graft success.

In Study 3, the gonadal status of mice (intact female versus bilaterally ovariectomised female versus male) receiving kangaroo ovarian tissue xenografts determined graft function but had no effect on graft recovery rates. As in the study by Weissman and colleagues (1999), more medium and large sized follicles were generally present within xenografts to male recipients than in xenografts recovered from female recipients. This indicates that follicular development within kangaroo ovarian tissue may be accelerated through exposure to high androgen levels. This is in contrast to both Study 1, where no pattern could be established between the recipient's gonadal status and graft success, and Study 2 in which the BLO female appeared to be optimal. In addition the male recipients had high circulating FSH levels that were comparable to those previously reported for BLO female recipients (Cox *et al.*, 2000), possibly indicating that a combination of high gonadotrophin and androgen levels are beneficial. However, concrete conclusions cannot be drawn from these studies due to both the low number of follicles contained within all of the xenografts examined and the high variability of follicle numbers within xenografts belonging to each of the gonadal statuses, but the results suggest possible species differences and potentially an effect of the donor animal's age as has been previously demonstrated in the mouse (Cox *et al.*, 2000).

4.4.2. Effect of Graft Site

The kidney capsule provides an exceptionally effective site for the transplantation of tissue. It is highly vascularized, enabling the rapid revascularization of grafted tissue and the capsule itself provides a convenient pocket in which grafts can be secured and from

which they can be easily recovered (Gosden *et al.*, 1994b). For these reasons, the kidney capsule has been the most popular graft site in ovarian transplantation studies (Rumery & Blandau, 1976; Cox *et al.*, 1996; Oktay *et al.*, 1998b; Mattiske *et al.*, 2000; Wolvekamp *et al.*, 2001). However, while the kidney capsule has been extensively used, alternate graft sites have also been investigated including the thigh muscle (Lightman *et al.*, 1998), the corneal chamber (Shaffer & Hulka, 1969), the ovarian bursa (Jones & Krohn, 1960) and the tunica albuginea in males (Bamber *et al.*, 1980).

Recent years have seen the increased interest in the placement of grafts under the skin (Lightman *et al.*, 1998; Weissman *et al.*, 1999; Revel, 2000; Oktay *et al.*, 2001b). It is speculated that the grafting of tissue subcutaneously may enable the easy monitoring of grafts by palpation as well as providing a less invasive alternative that may prove particularly useful in the clinical setting (Weissman *et al.*, 1999). Additionally, subcutaneous grafts may have the added advantage of providing additional space that is essential for complete oocyte maturation and ovulation.

In Study 2 the graft site (kidney capsule and subcutaneous) had no effect on either the graft recovery rate, or the number and type of viable or atretic follicles in fresh pademelon wallaby ovarian xenografts. This finding contrasts with results for cryopreserved wombat ovarian tissue xenografts in which the kidney capsule was found to be a superior site (Cleary *et al.*, 2002). This disparity may be attributable to one or more factors. Grafting alone results in the loss of a minimum of a third of follicles (Newton *et al.*, 1996; Candy *et al.*, 1997) and this loss is marginally greater if it is preceded by cryopreservation (Cleary *et al.*, 2001). Grafting under the kidney capsule may rescue follicles compromised by freezing, while the slow revascularization at the subcutaneous site, may result in the loss of the previously compromised follicles. The difference in the results obtained and those previously reported may be the effect of genuine species difference. However, it is

feasible that the small sample size has caused this result. While, no statistically significant difference was found to exist between the two graft sites, the kidney capsule grafts did generally perform better than subcutaneous grafts with regard to both graft retrieval rates and follicle numbers. This indicates that, had the group sizes been increased the results may have supported those reported by Cleary and colleagues (2002).

4.4.3. Effect of the Cryopreservation Protocol

While Study 1 demonstrated that oocytes can be collected from fresh marsupial ovarian xenografts, xenografting of fresh ovarian tissue is not always possible. Reliable cryopreservation methods for marsupial ovarian tissue are thus required.

Successful cryopreservation procedures are not only dependent on the cryoprotectant used, but also both the conditions through which the tissue to be frozen is exposed to the cryoprotectant (the equilibration conditions) and the method of freezing used. DMSO and EG are effective cryoprotectants for the freezing of mouse (Candy *et al.*, 1997) and human (Newton *et al.*, 1998) ovarian tissue. Both cryoprotectants can successfully protect marsupial ovarian tissue during slow-cool freezing (Snow, 1999; Wolvekamp *et al.*, 2001; Mattiske *et al.*, 2002). To date however, there has only been one report of the employment of rapid-cool cryopreservation for marsupial ovarian tissue (Mattiske, 1997). In the study by Mattiske rapid-cooled tammar wallaby ovarian tissue functioned in a similar manner to slow-cool cryopreserved tissue following xenografting to mice. However, asides from this single study, no further comparison of the two freezing techniques has been made.

The cryopreservation procedure had no effect on the graft recovery rate in any treatment group. In contrast, the cryoprotectant did affect follicle survival as higher survival was seen after slow-cooling in DMSO than after slow-cooling in EG. In support of this finding, DMSO has previously been found to be a more effective cryoprotectant than EG for immature mouse ovaries, with the performance of EG found to be dependent on the

equilibration conditions between the tissue to be frozen and the cryoprotectant (Candy *et al.*, 1997). In the study by Candy and colleagues (1997) prolonged exposure to EG (60 minutes), prior to freezing was detrimental to the tissue. It is therefore feasible to suggest that eastern grey kangaroo ovarian tissue may be highly sensitive to EG and that the 30 minute equilibration period used in the slow-cooling process was too long and resulted in EG driven toxic damage being incurred on the tissue.

Similarly, follicle survival was higher in kangaroo xenografts which had been rapidly cooled using the solutions developed by Vajta and colleagues (Vajta *et al.*, 1998), than in those slow-cooled in EG or rapid-cool frozen after equilibration in Kasai's solutions (Kasai *et al.*, 1990). This result may be attributable to EG, as Kasai's cryosolution consists largely of EG (40%), indicating that high EG concentrations are toxic to kangaroo ovarian tissue. It therefore appears probable that EG as a cryoprotectant is not suitable for the cryopreservation of kangaroo ovarian tissue, using any of the freezing methods examined in this study. The current study does have limitations. Firstly, because of the difficulties associated with obtaining the tissue, all of the ovarian tissue from the eastern grey kangaroo used in the study was obtained in the field from a single donor female. Consequently, the age of the donor was unknown, however had more than one donor been used differences in the donor ovarian tissue may have been observed with regard to the age and health of the donor. Secondly, both the follicle population and sample numbers were low.

4.4.4. Summary

The results presented in this chapter demonstrate that marsupial ovarian tissue can be grown within a mouse. Follicular development to the antral stages is supported with full sized oocytes able to be collected from ovarian xenografts. In addition the results have demonstrated that the gonadal status of the graft recipient, the graft site and the

cryopreservation protocol can all affect the graft function and follicle population contained within marsupial ovarian xenografts. Conflicting results were gained regarding whether the gonadal status of the recipient does or does not play a role in determining graft success and function. While this may be a species specific effect and possibly an age effect, the results do broadly agree with the variable results obtained by studies in other species (Weissman *et al.*, 1999; Cox *et al.*, 2000; Cleary *et al.*, 2002).

As anticipated and in support of results previously gained using wombat ovarian tissue (Cleary *et al.*, 2002), the results obtained in this chapter indicate that the kidney capsule is a more superior graft site than under the skin. This difference is most likely attributable to the highly vascularized environment that is offered by the kidney capsule as opposed to the far less vascularly rich subcutaneous environment. Furthermore, the ability to collect mature tammar wallaby oocytes from xenografts clearly demonstrates that the kidney capsule provides both adequate space and support for follicular development to the late antral stages.

While, both DMSO and EG have been used extensively in the slow and rapid-cool freezing of ovarian tissue from a wide range of species (Gosden *et al.*, 1994a; Candy *et al.*, 1995; Shaw *et al.*, 1995a; Candy *et al.*, 1997; Gunasena *et al.*, 1998; Newton *et al.*, 1998), the results reported in this chapter are indicative of EG being a poor cryoprotectant for the freezing of kangaroo ovarian tissue. While it is difficult to draw absolute conclusion due to low follicle numbers, it is clear that DMSO was better a cryoprotectant, regardless of whether it was used in a slow or rapid-cool freezing protocol.

In summary, the cryopreservation and xenografting of marsupial ovarian tissue offer great promise for the conservation of marsupial species. While the oocytes collected failed to mature in either of the media investigated, the results obtained in Chapter Three indicate that with improved culture conditions full maturation should be possible. Due to the

limited availability of tissue and consequently low sample numbers, many of the studies reported in this chapter showed trends rather than significant results. However with the results obtained in this chapter and those previously reported in the literature, it is becoming increasingly apparent that the optimal conditions for both ovarian cryopreservation (cryoprotectant and method of freezing) and xenografting (gonadal status of the recipient and graft site) may be species specific.

~ Chapter Five ~

Preliminary Evaluation of Tolerized and Immune Suppressed Animals as Recipients of Ovarian Tissue Xenografts

5.1. Introduction

The success of tissue transplantation is dependent on the tissue not being rejected by the recipient. In ovarian grafting studies, this has typically been overcome through the use of autografting (Harris & Eakin, 1949; Green *et al.*, 1956; Gosden *et al.*, 1994a; Baird *et al.*, 1999; Oktay & Karlikaya, 2000; Petroianu *et al.*, 2002) or allografting, using highly inbred strains of mice and rats (Russell & Hurst, 1945; Harris & Eakin, 1949; Cox *et al.*, 1996; Candy *et al.*, 2000). However, in xenografting studies, where the donor and recipient are not immunologically compatible, appropriate steps must be taken to prevent graft rejection. In these instances, unless the recipient is made tolerant to the ovarian graft (i.e. tolerized) or the immunogenicity of the graft is reduced, compromising the recipient's immune system is essential (Gosden, 1992).

The development of immunocompromised strains of mouse and rat over the last two decades has permitted the investigation of ovarian xenografting in a broad range of species (reviewed by Paris *et al.*, 2003). The SCID mouse mutation, which results in the lack of

functional T and B cells (Bosma *et al.*, 1983; Steinsvik *et al.*, 1995; Renz *et al.*, 1996), has been employed extensively in ovarian xenografting studies, with tissue survival and follicular development observed within ovarian grafts from a wide range of species including the cat (Gosden *et al.*, 1994b), dog (Metcalfe *et al.*, 2001), sheep (Gosden *et al.*, 1994b), human (Oktay *et al.*, 1998b; Oktay *et al.*, 2000) and wallaby (Mattiske *et al.*, 2000). However, despite the success enjoyed through the use of SCID mice, their application in long-term studies is largely limited by a genetic defect that causes many of them to become 'leaky', resulting in the spontaneous restoration of lymphocytes and hence, immune responses (Bosma *et al.*, 1988; Sandhu *et al.*, 1996). Additionally, SCID mice have circulating natural killer cells and macrophages, which play a role in non-specific immune responses (Sandhu *et al.*, 1996) and potentially may have an effect on graft function.

These limitations are overcome in part through the use of the non-obese diabetic (NOD)-SCID mouse strain. The NOD-SCID mouse developed by Shultz and colleagues (1995), are not only deficient in T and B cells but also have substantially reduced concentrations of macrophages and natural killer cells, enabling the strain to be successfully employed in ovarian grafting studies (Weissman *et al.*, 1999; Bosch *et al.*, 2002). However, similar to normal SCID mice the long-term use of NOD-SCID mice is limited due to a high incidence of thymic lymphomas (Shultz *et al.*, 1995).

Mice and rats with the recessive *nude* mutation that results in the absence of T cells (Janeway & Travers, 1997), have also been employed as recipients in ovarian xenografting studies. (Gunasena *et al.*, 1998; Lightman *et al.*, 1998; Revel, 2000; Wolvekamp *et al.*, 2001). However, similarly to SCID mice, nude mice and rats have both circulating macrophages and natural killer cells.

RAG deficient mice are another immunodeficient strain that, as demonstrated in Chapter Six of this thesis, can be employed in ovarian grafting studies. To date no reports have been published regarding the use of RAG mice in ovarian xenografting studies. However, in unpublished data recorded in our laboratory, RAG mice have supported follicular development within wallaby ovarian xenografts (Snow, 1999).

While, immunocompromised mice and rats are suitable recipients for ovarian xenografts, these strains are both expensive to purchase and difficult to maintain, due to the requirement for housing in SPF conditions. This has led to the investigation of alternative recipient models for ovarian tissue xenografting studies, in particular the use of tolerized and immunosuppressed recipients (Lamden, 1996; Cleary, 1999).

The basis of immunological tolerance was established in 1953 by Billingham and colleagues (Billingham *et al.*, 1953). In the landmark study that defined immunological concepts thereafter (Forsthuber *et al.*, 1996), it was demonstrated that when animals are exposed to foreign tissue during embryonic development or early neonatal life (i.e. prior to immunological competence) they become tolerant to the foreign tissue (tolerized, thus treating the foreign tissue as self), and therefore will subsequently not launch an immune response (Billingham *et al.*, 1953). Based upon this theory, Lamden investigated the feasibility of tolerizing two to three day old mice to fat tailed dunnart ovarian tissue (Lamden, 1996). In the study, mouse pups were tolerized through an ovarian cell suspension subcutaneous injection, four weeks after which, they were received dunnart ovarian xenografts. While, all marsupial ovarian xenografts recovered showed signs of rejection (Lamden, 1996) it remains unclear whether the effectiveness of tolerization would have been greater, had tolerization began on the day of birth when the murine immune system has yet to be fully operational (Billingham *et al.*, 1953).

The use of immunosuppression in transplantation studies has also been investigated. Antilymphocyte serum (ALS) is effective in prolonging the survival of both skin allografts (Levey & Medawar, 1966) and xenografts (Lance *et al.*, 1969) in mice. Furthermore, in 1969, Barnes and Crosier found ALS treatment to promote the survival of mouse ovarian allografts. These studies thus warrant further investigation into the potential role of immune suppressed and tolerized recipients in ovarian xenografting studies.

5.1.1. Aims of this Chapter

The aims of the preliminary experiments described in this chapter were to investigate the feasibility of using immunologically tolerized rats (Study 1) and immunosuppressed mice (Study 2) as ovarian tissue xenograft recipients.

5.2. Materials and Methods

5.2.1. Animals

All animals used in the studies reported within this chapter were obtained from Central Animal Services, Monash University. Animals were maintained under a 12 hour light-dark regimen at 21°C with food and water *ad libitum*. Ethical approval for the studies reported in this chapter, was obtained from the Monash University Department of Physiology and Monash Medical Centre Animal Ethics Committees. The studies complied with the conditions laid down by the NH&MRC/CSIRO/AAC Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1997).

5.2.1.1. Mice

Ovarian tissue used in the tolerization investigation (Study 1) was obtained from day 12 agouti F1 (C57BL×CBA) female mouse pups (n=48).

Three-week old BALB/c female mice (n=4) served as graft recipients in the immunosuppression study (Study 2).

5.2.1.2. Rats

Newborn female (n=26) and male (n=23) DA rat pups were used as the tolerization subjects in Study 1.

In Study 2, investigating immunosuppression, female day 12 Wistar rat pups (n=4) served as the ovarian tissue donors. Pups were removed from dams, and the ovarian tissue collected and utilized on the same day.

5.2.2. Ovarian Tissue Collection

Day 12 F1 mouse pups and Wistar rat pups were killed by cervical dislocation to enable collection of their ovaries. Ovaries were collected using the methods described in Section 2.2.2. Following collection, ovaries were stored in PBS at RT for up to 15 minutes prior to cryopreservation, tolerization or grafting.

5.2.3. Cryopreservation and Thawing of Ovaries

Ovaries to be cryopreserved were first equilibrated for 30 minutes at 0°C in a 1.5M DMSO cryosolution (Section 4.2.3.1.). They were then aspirated whole into 0.25ml cryopreservation straws (one ovary/straw) that were then sealed with Polyvinyl Alcohol (Sigma, St Lewis, USA) and placed into a programmable Cryochamber, Freeze Control CL3000 (Cryologic Pty. Ltd., Melbourne, Australia). The slow-cooling program was then initiated. From a temperature of 0°C, the cryochamber was cooled at 2°C/minute to –6°C. The temperature was then held at –6°C for five minutes to enable the straws to be seeded (initiation of ice formation). Following seeding, the temperature was lowered at 0.3°C/minute to –40°C. Upon reaching –40°C the straws were removed from the cryochamber and plunged directly into LN_2 where they were stored until they were thawed for use in the tolerization or grafting studies. Cryopreserved ovaries were thawed by firstly removing the straws from LN_2 and placing them in the LN_2 vapour (-120°C) for two minutes. The straws were held in the air for five seconds before being plunged into a 37°C water bath until the contents were completely thawed. The ends of the straws were then cut to enable the contents of each straw to be expelled into the first thawing solution (Section 4.2.3.3). Subsequently the ovaries were serially washed as described in Section 4.2.3.3.

5.2.4. Tolerization and Immune suppression

5.2.4.1. Tolerization

To induce immunological tolerance, newborn rat pups (within three hours of birth; day 1) were injected with a single day 12 F1 mouse pup ovary. The ovary, suspended in PBS, was delivered through a subcutaneous injection to the lower rump using a 19G needle (Terumo Medical Corporation, MD, USA) and a 1ml complete dispensing syringe (Henke Sass, Wolf, Tuttlingen, Germany). Pups were returned to their dam immediately following the tolerization injection.

5.2.4.2. Immune suppression with antilymphocyte serum

Immune suppression was induced using ALS. The antibody was raised in New Zealand white rabbits following immunisation of the rabbits with spleen and thymus lymphocytes from BALB/c mice to generate anti-BALB/c lymphocyte antibody. The ALS was a gift from Dr Mauro S. Sandrin (Molecular Immunogenetics Laboratory Austin Research Institute, Austin and Repatriation Medical Centre, Heidelberg, Victoria) for the purpose of this experiment. The ALS was stored frozen (-20°C) until use.

ALS (250µL neat serum per treatment) was administered through an intra-peritoneal injection to BALB/c graft recipients on day 1 (day of grafting) day 3, day 6 and day 11. The ALS treatment regimen was recommended by Dr Mauro S. Sandrin.

5.2.5. Ovarian Tissue Grafting

5.2.5.1. Anaesthetic

DA rat graft recipients were anaesthetised using the methods described in Section 3.2.3.1.

BALB/c mice graft recipients were anaesthetised as described in Section 4.2.5.1.

5.2.5.2. Ovarian grafting to the kidney capsule procedure

Day 12 F1 mouse ovaries and Wistar pup ovaries were xenografted, one whole ovary per kidney, to DA rat and BALB/c mouse recipients respectively, using the procedures described Section 2.2.4.2. At the time of grafting the recipient's own ovaries were removed as described in Section 2.2.4.2.

5.2.5.3. Ovarian grafting to the ovarian bursa procedure

The back of each recipient mouse was swabbed with 70% alcohol. A 1cm mid dorsal incision was made through the graft recipient's skin. Each of the mouse's own ovaries were then carefully removed via a small incision through the flank. A small tear was made in the bursal membrane through which the ovary was gently eased out. Particular care was taken to ensure that the entire ovary was removed, through a pinching action with the forceps. A day 12 Wistar rat pup ovary suspended in PBS was then cut in half using a sterile scalpel blade. One half ovary was placed in the empty bursal cavity. To secure the graft, the bursal membrane was then sutured to the ovarian fat pad using 10.0 Dermalon (Davis and Geck, NJ, USA) non-absorbable suture. The ovariectomy and grafting procedure was then repeated on the other side of the recipient.

Once the grafting was completed, the recipient's skin was brought back together and the incision closed using 9mm wound clips.

5.2.6. Assessment of Tolerizing Ovary/Graft Function

DA rat recipients were killed through CO_2 inhalation and BALB/c mice through cervical dislocation.

5.2.6.1. Recovery of tolerization ovary

Mouse pup ovaries used in the initial tolerization of newborn rat pups were recovered immediately following the death of the recipient. To enable recovery of the ovary, a large

incision was made through the skin across the recipient's shoulder blades. The skin was pulled down to the lower back of the tolerized rat. The underside of the skin, and revealed back, were viewed under a light microscope to locate the injected ovary. Once located, the ovary was removed using forceps and placed in Bouin's fixative for histological analysis.

5.2.6.2. Recovery of kidney capsule grafts

Grafts to the kidney capsule of mouse and rat recipients were recovered using the procedures described in Section 2.2.5.1. and placed in maturation media (Section 5.2.6.5.) for the collection of oocytes.

5.2.6.3. Recovery of ovarian bursa grafts

An incision was made across the width of the abdomen through both the skin and peritoneum to enable access to the ovarian bursa grafts. Once located, the grafts were removed using forceps and scissors. Recovered grafts were placed in maturation media for the collection of oocytes (Section 5.2.6.5.).

5.2.6.4. Histology

Tolerizing ovaries were processed, cut and stained as described in Section 2.2.5.2. Histological sections were examined under a light microscope and the presence or absence of follicles observed. Follicle types were classified using the scheme described in Table 2.2.

5.2.6.5. Oocyte collection and *in vitro* culture

Oocyte collection from ovarian bursa and kidney capsule grafts was undertaken in supplemented EMEM based maturation media equilibrated at 37°C in 5% CO_2. The maturation media was prepared as described in Section 3.2.5.1. Oocytes were released from follicles contained within the grafts using 26G needles. Following collection, oocytes

were transferred to 30μL drops of pre-equilibrated maturation media covered with mineral oil and incubated at 37°C with 5% CO_2.

After 18 hours incubation, the health and maturity of *in vitro* cultured oocytes was evaluated. Signs of degeneration (e.g. loss of shape, cytoplasmic darkening and fragmentation) and maturation (extrusion of the first polar body) were observed for.

5.2.7. Experimental Protocol

5.2.7.1. Study 1 – Tolerization

Day 12 F1 ovaries were collected as described in Section 5.2.2. and either cryopreserved by slow-cooling in 1.5M DMSO (A-C; Section 5.2.3.) or placed directly into PBS (D) until used (Figure 5.1.). In preliminary experiments (A-C), ovaries were cryopreserved (Section 5.2.3.) to eliminate the difficulty of rapidly obtaining day 12 mouse pup ovaries at the time of rat parturition.

Within three hours of birth, newborn female and male DA rat pups were tolerized with either a frozen/thawed (n=40) or fresh (D; n=9) day 12 F1 mouse pup ovary (Section 5.2.4.1.; Figure 5.1.). Both female and male rat pups were used in this study following the success of xenografting ovarian tissue to both sexes described in Chapter Three of this thesis. Rat pups that received a frozen/thawed ovary were divided into three groups (A-C). To determine whether rapid rejection was occurring, seven days after tolerization, rat pups belonging to group A (n=4) were culled by $C0_2$ inhalation and the tolerizing ovary collected (Section 5.2.6.1.). Once it was determined that the tolerizing ovaries were able to survive the first seven days, the tolerizing ovary was recovered from rat pups belonging to treatment group B using the same procedure (Section 5.2.6.1.). In treatment group B, 19 days after tolerization the recipients were stimulated with 10 IU PMSG in order to help locate the tolerizing ovary (Figure 5.1.). The tolerizing ovary was recovered 48 hours later.

Following the successful collection of tolerizing ovaries both, seven and 21 days after injection, rats belonging to treatment groups C (n=14) and D (n=9) were grafted with a frozen/thawed or fresh day 12 F1 mouse pup ovary to each kidney, respectively (Section 5.2.5.2.; Figure 5.1.) 21 days after tolerization. Nineteen days later, recipient rats were hormonally stimulated (10 IU PMSG) and 48 hours later the ovary grafts were recovered (Section 5.2.6.2.; Figure 5.1.).

Recovered tolerizing ovaries were placed in Bouin's fixative and processed, cut and stained for histological analysis (Section 5.2.6.4.). Ovarian kidney capsule grafts recovered from rat recipients were taken for oocyte collection (Section 5.2.6.5.).

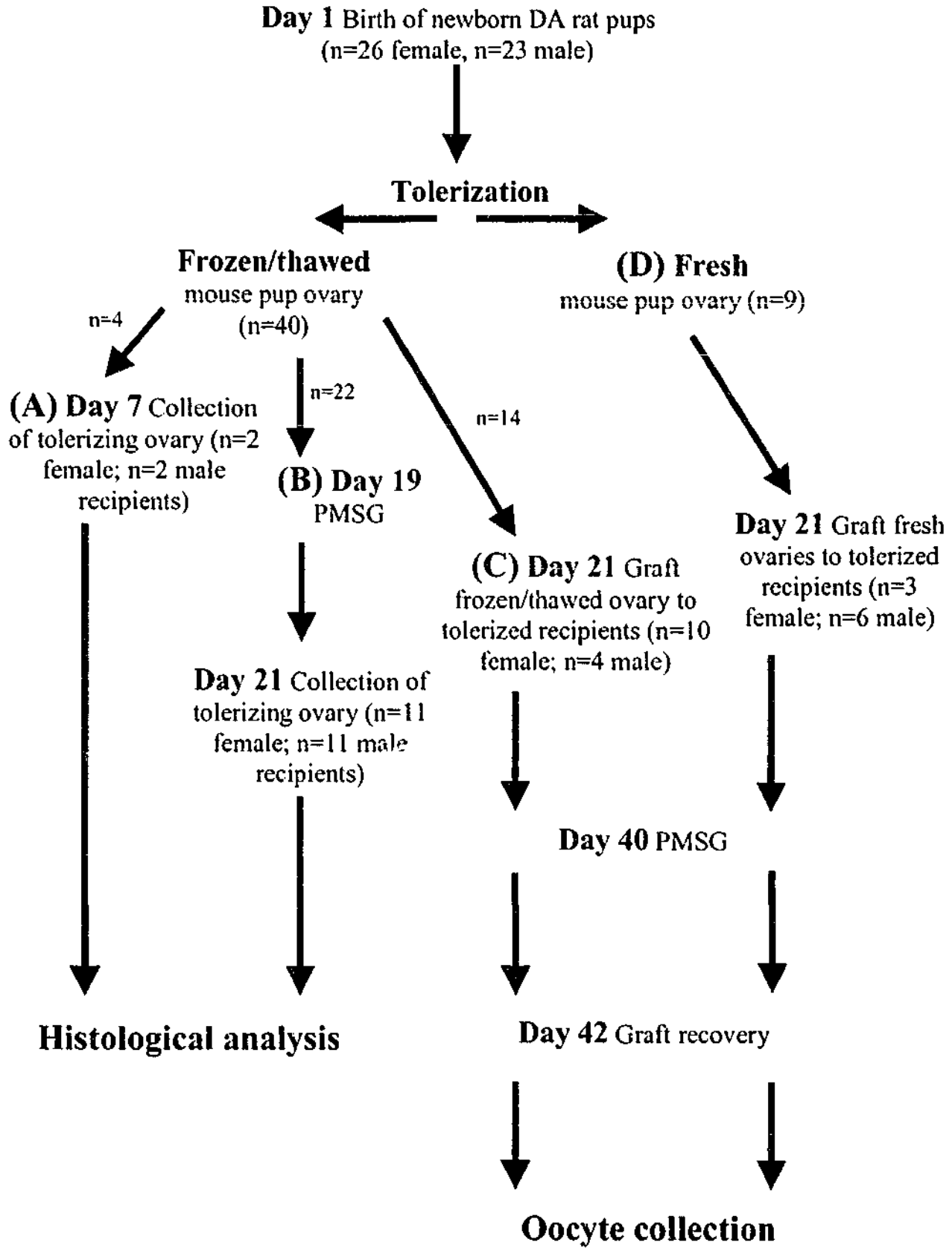


Figure 5.1. Schematic representation of experimental protocol for Study 1 (Tolerization).

5.2.7.2. Study 2 – Immune suppression

Day 12 Wistar rat ovaries (n=6) were collected as described in Section 5.2.2. (Figure 5.2.). Ovaries were grafted whole (one ovary/kidney; n=2 recipients) or as half ovaries (one half ovary/bursa; n=2 recipients) to the kidney capsule (Section 5.2.5.2.) or ovarian bursa (Section 5.2.5.3.) of anaesthetised (Section 5.2.5.1.) three-week old bilaterally ovariectomised BALB/c female mice (n=4). At the time of grafting (day 1) each recipient was treated with ALS (Section 5.2.4.2.). Graft recipients were subsequently treated with ALS on days three, six and 11, post grafting as described in Section 5.2.4.2 (Figure 5.2.).

On day 19 after grafting, n=2 recipients (Figure 5.2.) were hormonally stimulated with PMSG (7.5 IU), followed 48 hours later with hCG (7.5 IU). Twelve hours later (day 21; Figure 5.2.) ovarian grafts were recovered (Sections 5.2.6.2. and 5.2.6.3.) and placed in maturation media for oocyte collection and *in vitro* maturation (Section 5.2.6.5.). The health and maturity of oocytes was evaluated following overnight incubation (Section 5.2.6.5.).

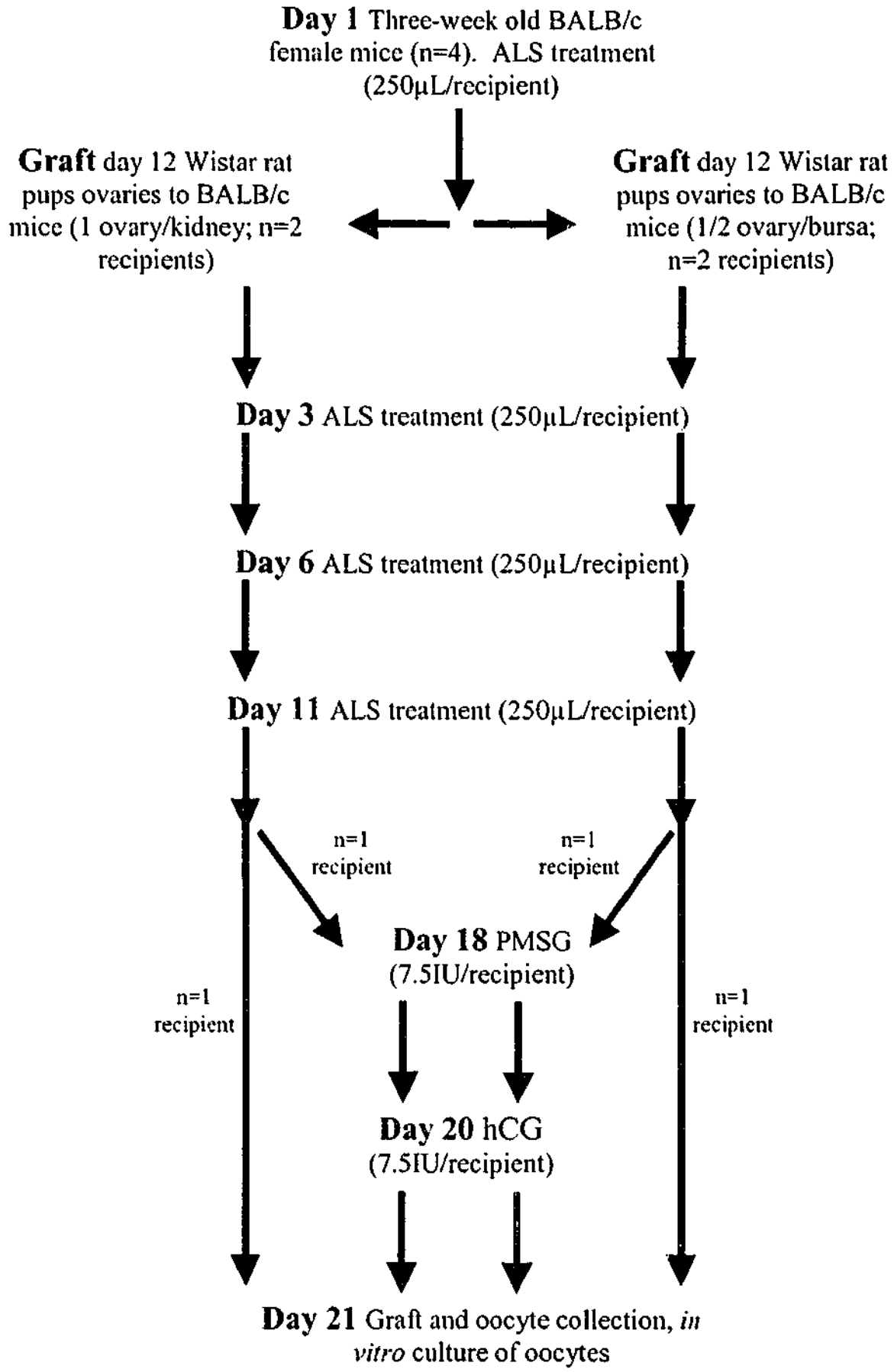


Figure 5.2. Schematic representation of experimental protocol for Study 2 (Immune suppression).

5.3. Results

5.3.1. Study 1 – Tolerization

The tolerizing ovary and graft recovery rates were low for all of the treatment groups (A-D; Table 5.1.). One week after injection the tolerizing (day 12 F1) mouse pup ovaries were successfully located and recovered from three of four recipient rats (treatment group A; Table 5.1.). Three weeks after injection the tolerizing F1 mouse pup ovaries were recovered from 14 of 22 recipient rats. The interval between tolerization and ovary recovery had a statistically non-significant effect ($p>0.66$) on the recovery rate. The histological appearance (number, type and health of follicles present) of the tolerizing ovaries was similar in both female (Figure 5.3.a.) and male (Figure 5.3.b.) recipients. Likewise, no differences were visible between the tolerizing ovaries collected seven days (treatment group A) and twenty-one days (treatment group B) post tolerization.

As evident in Figures 5.3.a.-b., tolerizing ovaries remained relatively intact and were easily identified as ovarian tissue when examined microscopically. However, despite the unchanged gross structure, the follicle population was almost completely depleted in all recovered tolerizing ovaries, with only a few viable small sized follicles observed and signs of cellular infiltration evident (Figures 5.3.a.-b.). While, developing follicles were contained within some of the recovered tolerizing ovaries, they were all deemed to be atretic as signs of oocyte degeneration were evident (Figures 5.3.a.-b.).

Figure 5.3. Histological appearance of frozen thawed tolerizing ovaries recovered from **(a)** female and **(b)** male recipients 21 days after tolerization (bars=100μm). While the gross structure of the recovered tolerizing ovaries remained the same, the ovaries were found to be sparsely populated with follicles and all of the developing follicles were deemed to be atretic (A). Cellular infiltration was observed in the recovered ovaries.

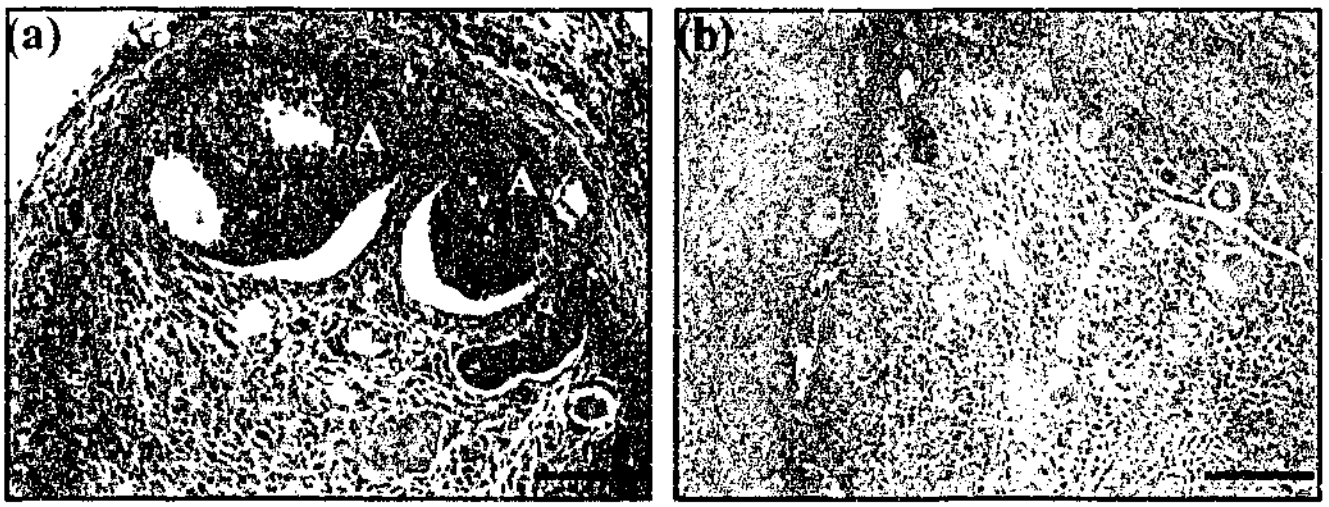


Table 5.1. Effect of treatment group, sex of graft recipient and state of ovarian tissue on tolerizing ovary and ovarian graft recovery rates. Same superscript letters within columns indicate groups that are statistically the same ($p>0.05$). See Figure 5.1. for description of treatment groups.

Treatment group	Sex of recipient (n recipients)	Fresh/ Frozen	n Tolerizing ovaries recovered/n placed	n Grafts recovered/ n grafted
A	Female (2)	Frozen	$1/2^a$	-
	Male (2)	Frozen	$2/2^a$	-
B	Female (11)	Frozen	$7/11^a$	-
	Male (11)	Frozen	$7/11^a$	-
C	Female (10)	Frozen	-	$4/20^a$
	Male (4)	Frozen	-	$2/8^a$
D	Female (3)	Fresh	-	$0/6^a$
	Male (6)	Fresh	-	$0/12^a$

Twenty-one days after grafting to tolerized recipients, frozen/thawed (treatment group C, female and male recipients) ovarian xenografts could be visualised under the kidney capsule of recipient rats. Macroscopically the grafts were poorly vascularized, appearing as white scar tissue on the surface of the kidney, indicating graft resorption, possibly mediated by rejection. Consequently, no oocytes were collected from the grafts. The tolerizing ovaries injected three weeks prior to grafting were not recovered (i.e. no attempt was made).

Interestingly, while some of the frozen/thawed ovarian xenografts could be visualized, no fresh ovarian xenografts were visible at the time of graft retrieval. However, there were no statistically significant differences ($p>0.20$) between the graft retrieval rates for the four graft groups (treatment groups C and D, female and male recipients).

5.3.2. Immune Suppression with Antilymphocyte Serum

All kidney capsule and half of the ovarian bursa rat ovarian xenografts were successfully recovered from immunosuppressed mouse recipients (Table 5.2.). At the time of graft recovery, the grafts appeared reasonably well vascularized and were approximately the same size as at the time of transplantation.

Table 5.2. Effect of graft site and hormone stimulus on graft and oocyte recovery rates following treatment with antilymphocyte serum.

Recipient number	Graft site	PMSG/ hCG	n Grafts recovered/ n grafted	n Oocytes collected	Description of oocytes
1	Kidney	No	2/2	2	compact COC's
2	Kidney	Yes	1/2	3	compact COC's
3	Bursa	Yes	1/2	3	COC's (2 compact, 1 partial)
4	Bursa	No	2/2	2	compact COC's

Oocytes were successfully collected from rat grafts to each of the mouse recipients (Table 5.2.). However, while oocytes were collected, regardless of the graft site and whether the recipient was or was not hormonally primed, the oocytes were immature (small in size and tightly enclosed in cumulus). Additionally, several of the oocytes showed signs of degeneration. This was further evidenced following overnight incubation in maturation media, as all of the oocytes failed to mature, and showed signs of degeneration and poor viability. In light of this, the experiment was subsequently terminated.

5.4. Discussion

Ovarian tissue xenografting provides a valuable tool for the generation of mature oocytes (see Chapter Three of this thesis) and a model in which follicle viability and growth can be examined in a wide range of species (Gosden *et al.*, 1994b; Gunasena *et al.*, 1998; Oktay *et al.*, 1998b; Metcalfe *et al.*, 2001; Wolvekamp *et al.*, 2001; Bosch *et al.*, 2002; Mattiske *et al.*, 2002). However, immunocompromised strains of mice and rat, such as those typically employed in ovarian xenografting studies are both expensive to purchase and maintain. Consequently, alternative ovarian xenograft recipient models have been investigated (Lamden, 1996; Cleary, 1999).

Both induced immunological tolerance and suppression have been employed extensively in tissue and organ transplantation (Levey & Medawar, 1966; Bouwman *et al.*, 1993; Starzl, 2000). However, as reported in this chapter, in the present study neither neonatal tolerization nor immunosuppression using ALS, were entirely effective in preventing ovarian xenograft rejection. Although, some of the tolerizing ovaries and frozen/thawed grafts to tolerized recipients were recovered from recipients in Study 1, the cellular infiltration observed and the fact that no fresh xenografts could be recovered are both indicative of graft rejection.

While the phenomena of neonatal tolerance is widely accepted (Billingham *et al.*, 1953; Gammon *et al.*, 1986; Forsthuber *et al.*, 1996) controversy exists regarding the exact mechanisms of action (Gammon *et al.*, 1986; Forsthuber *et al.*, 1996; Garza *et al.*, 1997). Additionally, there is evidence to suggest that neonatal tolerance is not inducible to all antigenic peptides (Garza *et al.*, 1997). In a study reported by Garza and colleagues

(1997), female neonatal mice were found to develop autoimmune ovarian disease, following injection with the zona pellucida glycoprotein ZP3 in an attempt to induce tolerization. Interestingly, while the tolerization was unsuccessful in female neonates, male neonates that underwent the same treatment became tolerant. These observations led Garza and colleagues (1997) to speculate that neonatal tolerance may only be inducible to foreign antigens or self antigens that are largely sequestered, and that the model may not be applicable to antigens and peptides that are readily accessible to the immune system. Consequently, these observations may provide an explanation for the graft rejection that occurred in the tolerization study reported in this chapter. Further support to this is offered by the failed attempt by Lamden (1996), to initiate tolerance to marsupial ovarian tissue in two to three-day old mice.

Similarly to neonatal tolerization, immunosuppression by means of ALS treatment has had varied success in its ability to prolong graft survival (Levey & Medawar, 1966; Barnes & Crosier, 1969; Lance *et al.*, 1969). While grafts from ALS treated recipients in this study, could be recovered and oocytes were able to be collected from the grafts, the inability of the oocytes to mature and their poor viability (as determined by visual analysis) suggests graft rejection. These results, compared to those reported in Chapter Three, in which healthy mature oocytes and embryos were obtained from mouse ovarian xenografts to immunocompromised rat recipients, provide further evidence of graft rejection. This contrasts previously obtained results in which the longevity of mouse ovarian allografts was extended with ALS treatment (Barnes & Crosier, 1969). However, the discrepancy between the two studies may conceivably be the result of genuine species differences. Evolutionary divergence between the mouse and rat occurred an estimated 11 million years ago (Catzeflis *et al.*, 1993), in contrast immunological differences between two different strains of mice would be presumably less distinct. Furthermore, evidence suggests that

with continuous ALS treatment xenograft rejection may be avoided (Monaco *et al.*, 1966; Lance & Medawar, 1968). Thus, had ALS treatment been maintained for the duration of the study the oocyte collection and viability may have proven more successful.

5.4.1. Summary

The aim of the two studies described in this chapter was to explore the feasibility of employing immunologically tolerized and immunosuppressed animals as alternative recipients in ovarian xenografting studies. While inducing neonatal tolerance was unsuccessful in preventing graft rejection, as evident by histological analysis, immunosuppression through the use of ALS provided a more optimistic outcome in the preliminary experiments described in this chapter. However, while oocytes were collected from xenografts recovered from ALS treated recipients, both the immaturity and visual appearance of the oocytes compared to those previously obtained from ovarian xenografts, were indicative of graft rejection. More extensive studies are now required to determine whether graft rejection can be avoided with the ongoing treatment of graft recipients with ALS. Similarly, qualitative investigation of the viability of oocytes recovered from xenografts to ALS treated recipients must be assessed, to eliminate any detrimental effects of ALS on oocyte and embryo viability and normality.

~ *Chapter Six* ~

The Role of Follistatin in Ovarian Function

6.1. Introduction

Folliculogenesis is a highly controlled process that is regulated by both intra and extra-ovarian factors. Numerous factors including FSH, LH, progesterone, inhibin, activin and follistatin all play a role in follicular growth and development. However, despite the extensive research undertaken in this field, little information is available regarding the exact mechanisms responsible for the initial recruitment of follicles or the precise role that these factors play in oocyte maturation. Specifically, the relationship between follistatin, activin and oocyte maturation is largely unknown.

Activin and follistatin are proteins that are believed to regulate folliculogenesis through paracrine-autocrine interactions (Woodruff *et al.*, 1990; Findlay, 1993; Sidis *et al.*, 1998). Activins are members of the TGF β superfamily of growth and differentiation factors (Sidis *et al.*, 1998) that have many actions including the stimulation of FSH secretion (Gilfillan & Robertson, 1998; Knight & Glister, 2001). Follistatin, a glycosylated monomeric protein, suppresses pituitary FSH synthesis and secretion through binding to activin and as a consequence, neutralizing its activity (Nakamura *et al.*, 1990; Shimonaka *et al.*, 1991; de Winter *et al.*, 1996; Gilfillan & Robertson, 1998).

Activins and follistatin are involved in the regulation of follicle cell proliferation, steroidogenesis, oocyte maturation and corpus luteum function (Knight & Glister, 2001). *In vitro* studies have demonstrated activin's ability to promote oocyte maturation in a wide range of species including the rat (Itoh *et al.*, 1990), bovine (Silva & Knight, 1998; Silva *et al.*, 2003), human (Alak *et al.*, 1998) and zebra-fish (Wu *et al.*, 2000; Wang & Ge, 2003). Follistatin has been shown to inhibit oocyte maturation (Gilfillan & Robertson, 1998) but the exact role of follistatin in oocyte maturation remains unknown. It appears highly probable that follistatin fine-tunes the effects of activin and as a consequence, that the two factors together are responsible for determining the fate of growing follicles, either towards atresia or ovulation. In addition, the recent finding that follistatin is more highly expressed in the fetal ovary than in the testis is suggestive that follistatin may play a role in fetal ovarian function (Menke & Page, 2002).

While, there is a substantial amount of evidence that indicates that activins and follistatin are involved in the regulation of follicle cell proliferation, steroidogenesis and oocyte maturation, the majority of the information to date has been obtained from *in vitro* studies (Knight & Glister, 2001). Appropriate *in vivo* models need to be developed to verify their physiological role (Knight & Glister, 2001). The development and use of activin knockout animals has enabled a greater understanding of the role that activin plays in reproduction (Matzuk *et al.*, 1995a). The over-expression of follistatin in the mouse results in reproductive defects, including blocks in folliculogenesis (prior to antral follicle formation) and the absence of corpora lutea (Guo *et al.*, 1998) giving some insight into the role of follistatin on folliculogenesis. While a follistatin knockout mouse strain has been developed (Matzuk *et al.*, 1995b), thorough investigation of the roles of follistatin in the regulation of FSH secretion and folliculogenesis has been limited due to the post-natal mortality of follistatin-deficient mice. Grafting and *in vitro* culture of ovaries collected

from follistatin knockout fetuses are two tools that may provide a means to overcome this problem.

Ovarian grafting to the kidney capsule is a well-established technique that has been used extensively in the investigation of ovarian function and viability for a wide range of species including the mouse (Rumery & Blandau, 1976; Cox *et al.*, 1996; Candy *et al.*, 1997; Cleary *et al.*, 2001), human (Newton *et al.*, 1996; Oktay *et al.*, 1998b; Gook *et al.*, 2001), elephant (Gunasena *et al.*, 1998), wallaby (Mattiske *et al.*, 2002) and cat (Gosden *et al.*, 1994b; Oktay *et al.*, 1998b). Similarly, the *in vitro* culture of mouse ovarian tissue and oocytes has been studied at length (Odor & Blandau, 1971; Eppig & Schroeder, 1989; Eppig & O'Brien, 1996; Hartshorne, 1997; Liu *et al.*, 2000; O'Brien *et al.*, 2003). In combination the two techniques provide a means of studying folliculogenesis within follistatin knockout ovaries, in an environment with circulating follistatin and one that is totally devoid of follistatin.

6.1.1. Aims of this Chapter

The studies reported in this chapter aim to determine the role of follistatin in oogenesis and folliculogenesis by comparing the *in vivo* and *in vitro* development of ovaries collected from follistatin knockout fetal mice and normal fetal mice. To obtain a comprehensive understanding of the function of follistatin and the pathways through which it acts, oogenesis and folliculogenesis were examined in untreated ovaries (Study 1) and in an *in vivo* environment in which follistatin would be present to assess the role of follistatin in folliculogenesis (Study 2) and an *in vitro* environment totally devoid of follistatin to assess the role of follistatin in the early stages of follicle development (Study 3).

6.2. Materials and Methods

6.2.1. Animals

All mice used in the studies reported in this chapter were obtained from Central Animal Services, Monash University. SPF mice were housed under sterile conditions in micro-isolators and were provided with irradiation sterilized food and water *ad libitum*. Non-SPF animals were housed under conventional conditions with food and water *ad libitum*. All animals were maintained under a 12 hour light-dark regimen at 21°C.

Ethical approval for this study was obtained from the Monash Medical Centre Animal Ethics Committee and complied with the conditions laid down by the NH&MRC/CSIRO/AAC Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1997).

6.2.1.1. Donors

Female day 18 fetuses were used as ovarian tissue donors in the three studies reported in this chapter. Normal fetuses (+/+) and those heterozygous (fs^{ml}/+) and knockout (fs^{ml}/fs^{ml}) for the follistatin gene were produced by mating male and female adults that were heterozygous (fs^{ml}/+) for the follistatin knockout gene. The adults used for the generation of fetuses were obtained from an established colony housed at Central Animal Services, Monash University. The progenitors of the colony were obtained from the Martin Matzuk laboratory (Departments of Molecular and Human Genetics, Pathology and Cell Biology, Baylor College of Medicine, Houston, Texas, USA), where the follistatin knockout strain of mouse was created by a targeted deletion (fs^{ml}) of the 6-exon follistatin gene using

embryonic stem cell technology (Matzuk *et al.*, 1995b). The background strain was C57BL/6J.

6.2.1.2. Recipients

Adult female SPF RAG mice (n=73) were used as graft recipients in this chapter. Ovarian grafting to RAG recipients was undertaken in a laminar flow using sterile technique.

6.2.2. Ovarian Tissue Collection

On day 18 of gestation (day 0 = day plug found), pregnant females heterozygous for the follistatin knockout gene were killed by cervical dislocation. Fetuses were then removed from the dam by caesarean section and killed by decapitation. At this stage, the appearance and weight of each fetus was noted to enable correlation between the phenotype observed and the genotype as later determined by polymerase chain reaction (PCR) analysis. It has been previously noted that follistatin knockout fetuses tend to be both smaller in size and have shinier skin than heterozygous or wildtype fetuses (Matzuk *et al.*, 1995b).

Under a light microscope fetuses were removed from the uterine horns. Using scissors and forceps the abdomen of each fetus was opened and the intestines moved forward to above the kidneys enabling access to the gonads. Fetuses were then sexed as male or female, depending on the size and location of the gonads. At this stage of development fetuses can be identified as being male by the presence of the testes on either side of the bladder. The testes were given to another research group for study. The much smaller fetal ovaries that were located higher in the abdomen suspended below each kidney were removed using fine forceps.

At the time of ovary collection, fetal ovaries were either grafted, *in vitro* cultured or taken immediately for histology (untreated controls).

Following the removal of the ovaries, a sample of tissue was collected from each fetus to confirm their genotype. The samples were snap frozen on dry ice and then stored in a -80°C freezer.

6.2.3. Ovarian Tissue Grafting

To investigate folliculogenesis in follistatin knockout ovaries under *in vivo* conditions containing follistatin, ovaries were grafted to normal adult mice.

6.2.3.1. Anaesthetic

RAG mouse recipients were anaesthetised using a xylazine hydrochloride and ketamine hydrochloride solution as described in Section 4.2.5.1. The anaesthetic was administered as described in Section 3.2.3.1.

6.2.3.2. Ovarian grafting to the kidney capsule procedure

Following collection from the fetus, fetal ovaries were placed in PBS on ice until ready to be grafted. Prior to grafting the ovaries were carefully dissected free of any bursa or uterine tissue that remained attached. Fetal ovaries were then grafted to the kidney capsule of RAG mice recipients, one fetal ovary per kidney. The ovarian grafting followed the procedures outlined in Section 2.2.4.2.

6.2.4. In Vitro *Culture of Fetal Ovarian Tissue*

To investigate the effects of the absence of follistatin on early folliculogenesis in an *in vitro* environment totally devoid of follistatin, ovaries were cultured *in vitro*.

Fetal ovaries to be *in vitro* cultured were placed in a 35mm culture dish containing pre-equilibrated culture media. The *in vitro* culture medium was based on that used by Eppig and O'Brien for the culture of newborn mouse pup ovaries (1996). The medium consisted of Waymouth Medium (MB 752/1; Sigma, USA) supplemented with 0.23mM pyruvic acid (Sigma, USA), 50mg/L Streptomycin sulfate, 75mg/L Penicillin G, 3mg/ml BSA (Fraction

V, lot 527; Bayer, USA) and 2.24g/L $NaHCO_3$ (BDH, AnalaR, Australia). Once in the culture media ovaries were excised from the ovarian bursa.

As described by Eppig and O'Brien (1996), fetal ovaries were then transferred with a drop of medium using a sterile glass Pasteur pipette onto the top of a Millipore, isopore membrane filter (3.0μm pore size, 24mm diameter TSTP Filters; Millipore, Ireland) that rested on 1.3 ml of pre-equilibrated culture media within the centre well of an organ tissue culture dish (Falcon #3037, Becton Dickinson Pty. Ltd., Scoresby, Australia). The outer lying well was filled with pre-equilibrated saline (Baxter Healthcare Pty. Ltd., NSW, Australia). Two ovaries from a single donor fetus were cultured on each membrane for seven days in a 37°C, 5% CO_2 incubator. The culture was fed every second day by adding approximately 1.8ml of pre-equilibrated fresh media and then removing the same volume.

6.2.5. Genotyping of Fetal Ovarian Tissue Donors

At the time of ovary collection for control histology, ovarian grafting or the *in vitro* culture of fetal ovaries, the genotype of the donor remained unknown. While some fetuses were predicted as being follistatin knockouts because they were growth retarded and had shiny skin (Matzuk *et al.*, 1995b), using the phenotype of the fetus alone was not an accurate means of determining the genotype of fetuses. PCR was employed to accurately determine the fetus genotypes.

6.2.5.1. Preparation of gDNA

Genomic DNA (gDNA) was prepared for the PCR as follows. A small sample of tissue (approximately $1mm^3$) from each donor fetus was placed in a well of a 48 or 96 well PCR plate. 100μL of lysis buffer was added to each sample. The lysis buffer consisted of 99μL of lysis stock (10mM Tris-HCl at pH 8.3, 50mM NaCl and 0.2% (v/v) Tween 20 made to volume with dH_2O) with 1μL of proteinase K (10mg/ml; Roche/Boehringer Mannheim, USA). To achieve DNA lysis the samples were incubated in the PCR machine at 55°C for

60 minutes followed by eight minutes at 95°C. The temperature was held at 15°C until the plate was removed from the machine. The gDNA digest was stored on ice until ready for the PCR screening (approximately 15 minutes).

6.2.5.2. PCR Screening

The PCR reaction was performed using 48 or 96 well PCR plates in a PCR machine. Each well contained 5μL of gDNA from the lysis reaction, 3μL of 10x TAQ Polymerase Reaction Buffer (BIOTECH International Limited, Australia), 3μL of 2mM dNTP's, 0.6μL of 50pmol/μL of forward and reverse primers (HPRT 3F, HPRT 3R, foldel.F, foldel.R; Sigma Genosys Australia Pty. Ltd., Australia), 1.8μL of 25mM $MgCl_2$ (BIOTECH International Limited, Australia) and 0.2μL of 5.5Units/μL TAQ DNA Polymerase (BIOTECH International Limited, Australia) all made to a final volume of 30μL with sterile water.

The PCR was run under the following conditions. Three pre-PCR cycles consisting of five minutes at 94°C, 30 seconds at 58°C and 30 seconds at 72°C were run first. This was followed by 40 PCR cycles that consisted of three phases each; the melting phase (30 seconds at 94°C), the primer annealing phase (30 seconds at 58°C) and the extension phase (30 seconds at 72°C). The completed PCR reaction was stored on ice, until it was run on a 2% Agarose gel with a 100 base pair DNA ladder to check for the presence of PCR product. After approximately 75 minutes, the genotypes of donor fetuses were determined by the presence or absence of bands for the two primers. Wildtype fetuses had a band that detected for the mouse follistatin gene (foldel; approximately 157 base pairs) but not a HPRT-3 band, which detected the cassette that replaced the mouse follistatin (approximately 190 base pairs). The knockout fetuses exhibited the opposite bands, while the heterozygous fetuses had a band for both primers.

6.2.6. Assessment of Ovarian Function

6.2.6.1. Graft recovery

Fetal ovaries grafted to RAG mice recipients were recovered as described in Section 2.2.5.1. Recovered grafts were placed in Bouin's fixative for histological analysis.

6.2.6.2. Recovery of *in vitro* cultured ovaries

Fetal ovaries were recovered after seven days of *in vitro* culture. Using forceps, ovaries were carefully removed from the surface of the filter and placed in Bouin's fixative for histological analysis.

6.2.6.3. Histological processing of control and *in vitro* cultured ovaries

After three-24 hours in Bouin's fixative, fetal ovaries were placed in 70% alcohol until ready for processing. To ensure that fetal ovaries were not lost during the dehydration process they were processed manually. The fetal ovaries were serially dehydrated using alcohol and butanol. Ovaries were removed from the 70% alcohol and placed in the second dehydration solution (50% alcohol, 50% butanol) for ten minutes, before being transferred to the final dehydration solution (butanol) for a further ten minutes. To enable impregnation of the tissue, the ovaries were then placed in paraffin wax at 60°C for five to ten minutes before being embedded in paraffin blocks.

Embedded fetal ovaries were serially sectioned and stained as described in Section 2.2.5.2.

6.2.6.4. Histological processing of ovarian grafts

Ovarian grafts from fetuses identified to be wildtype or follistatin knockout by PCR were processed, embedded and stained as described in Section 2.2.5.2.

6.2.6.5. Classification and counting of follicles and oocytes

The number of follicles and follicle types in the wildtype and knockout fetal ovary grafts, and wildtype, heterozygous and knockout *in vitro* cultured ovaries were determined using

the methods described in Section 2.2.5.3. and Table 2.2. The number and types of oocytes contained within the untreated fetal ovaries was determined using the methods described in Section 2.2.5.3. and Table 2.2. in conjunction with the descriptions provided by Odor and Blandau (1969a; 1969b). Confirmation of the oocyte types was provided by Professor David de Kretser (2003).

6.2.7. Statistical Analysis

To determine the effect of the genotype of the fetal ovary donor, in all studies reported in this chapter, follicle counts were compared using one-way analysis of variance ($p<0.05$ was considered to be significant). Analysis of variance was made with the aid of the computer statistics package SPSS 9.0.1 for Windows (1999). Differences in graft recovery rates were determined using the Chi-Square test.

6.2.8. Experimental Protocol

6.2.8.1. Study 1 – Oocyte numbers in untreated follistatin knockout fetal ovaries

Fetal day 18 ovaries were collected (Section 6.2.2.) and placed directly in Bouin's fixative. Wildtype, heterozygous and knockout fetal ovaries (as determined by PCR; Section 6.2.5.) were processed, cut and stained (Section 6.2.6.3.) to enable histological analysis. The total number of oocytes contained within each fetal ovary was assessed and recorded to determine whether the absence of follistatin has any effect on oocyte numbers prior to the formation of follicles.

6.2.8.2. Study 2 – Grafting of follistatin knockout ovaries

Fetal ovaries were collected from day 18 fetuses (Section 6.2.2.) and one ovary was grafted per kidney (Section 6.2.3.) to adult female RAG mice recipients, ensuring that each recipient received ovaries from only one donor fetus. Initial grafts remained in recipients for three weeks. The graft duration was extended to four weeks to permit full follicular development. Grafts were recovered as described in Section 6.2.6.1. Recovered grafts that

were determined as being from wildtype or knockout fetuses by PCR (Section 6.2.5.) were processed histologically (Section 6.2.6.4.), subsequent to which follicle counts were performed (Section 6.2.6.5.), to determine the effect that the absence of follistatin within the ovary has on folliculogenesis.

6.2.8.3. Study 3 – *In vitro* culture of follistatin knockout ovaries

Ovaries were collected from d18 fetuses as described in Section 6.2.2. Fetal ovaries were then *in vitro* cultured (Section 6.2.4.) for seven days to enable follicle formation and the earliest stages of folliculogenesis. After seven days ovaries were recovered (Section 6.2.6.2.) and processed histologically (Section 6.2.6.3.). Follicle counts were performed on wildtype, heterozygous and follistatin knockout *in vitro* cultured ovaries to determine the effect that an environment completely devoid of follistatin has on folliculogenesis (Section 6.2.6.5.).

6.3. Results

6.3.1. Study 1 – Oocyte Numbers in Untreated Follistatin Knockout Ovaries

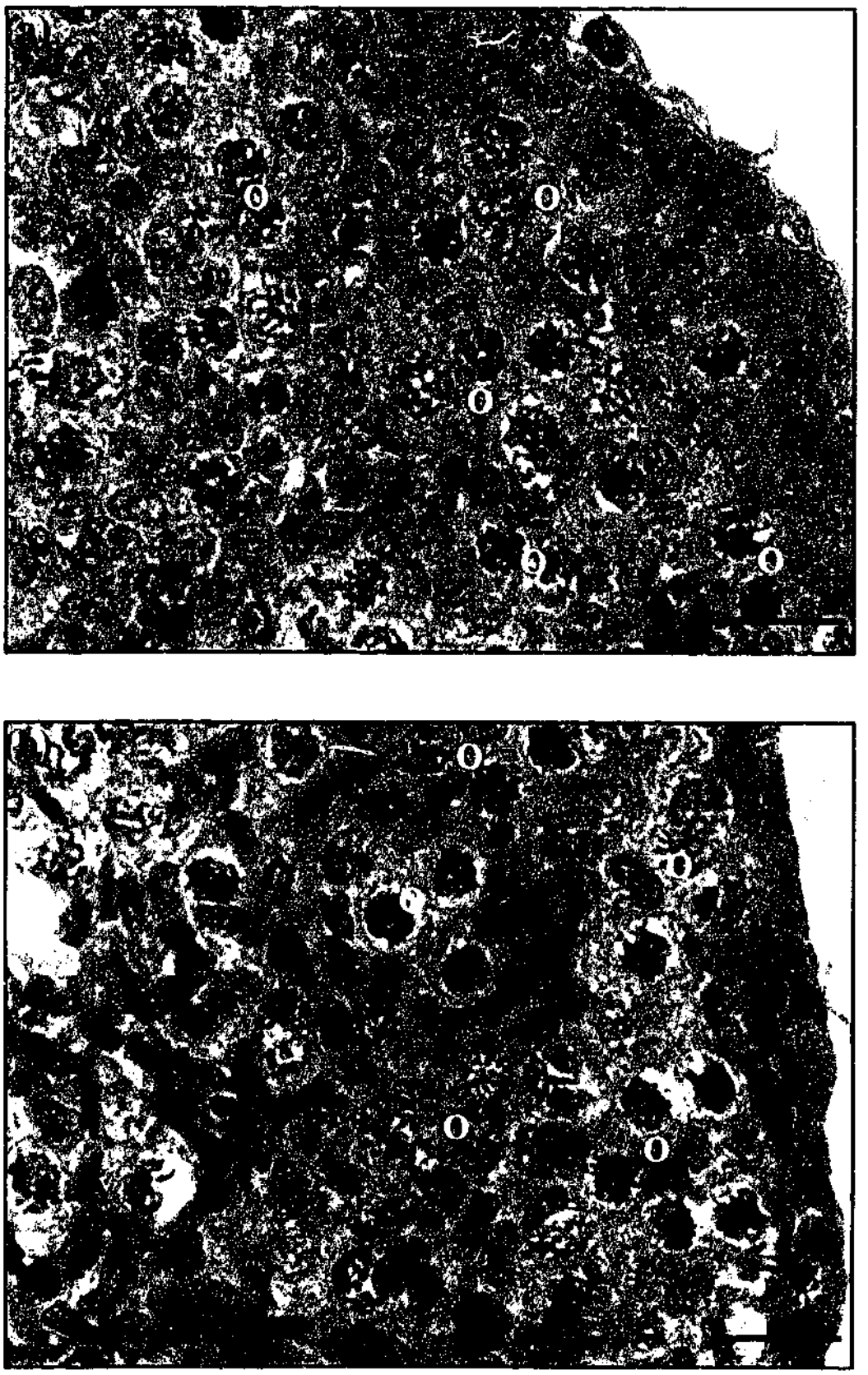
Untreated ovaries were collected from 12, day 18 fetuses (n=6 wildtype; n=3 heterozygous; n=3 knockout). However, due to the extremely small size of the ovaries some ovaries from fetuses with each genotype were lost during collection and histological processing.

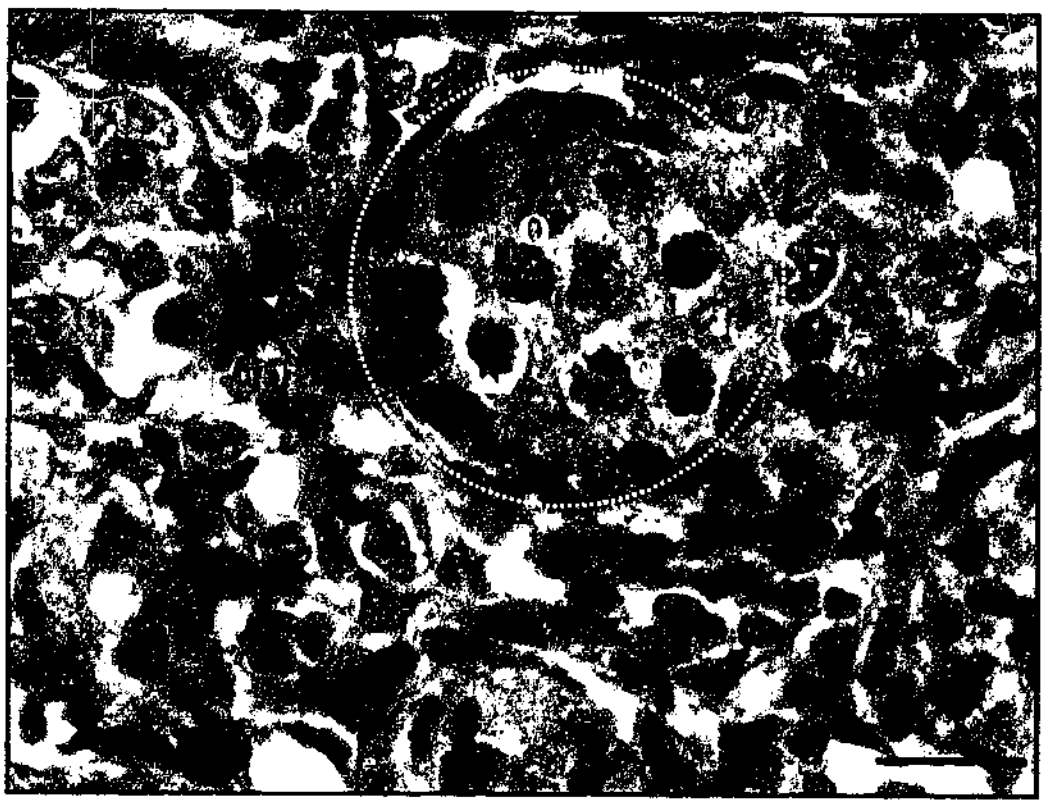
Oocytes were present in wildtype (Figure 6.1.a.), heterozygous (Figure 6.1.b.) and follistatin knockout (Figure 6.1.c.) ovaries (Table 6.1.). The oocytes, which were similar in appearance to what has been previously described for oocytes undergoing meiosis were counted (Odor & Blandau, 1969a; Odor & Blandau, 1969b). Regardless of the donor genotype the oocytes were determined to be between the leptotene and pachytene stages of meiosis (de Kretser, 2003).

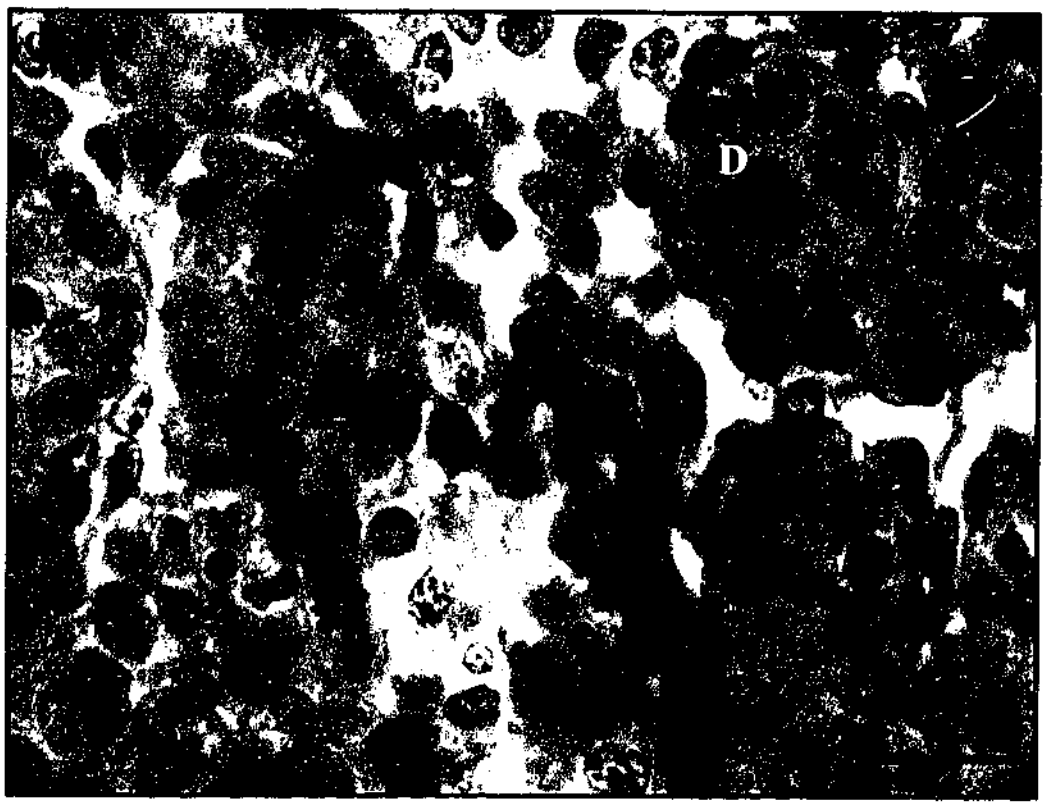
Table 6.1. Effect of donor genotype on the number of oocytes contained within untreated day 18 fetal ovaries. Same superscript letters indicate groups that are statistically the same ($p>0.05$).

Donor genotype (n ovaries assessed histologically)	Mean ± SEM number of oocytes
Wildtype (5)	1396.80 ± 201.40^{a}
Heterozygous (4)	1608.75 ± 190.65^{a}
Knockout (3)	227.33 ± 48.17^{b}

Figure 6.1. Histological appearance of untreated fetal day 18 mouse ovaries. **(a)** Wildtype and **(b)** heterozygous ovaries were densely populated by oocytes in the leptotene to pachytene stages of meiosis (O). **(c)** Follistatin knockout ovaries contained significantly fewer oocytes than the wildtype and heterozygous ovaries with most of the oocytes contained within sex cord like structures (indicated by dotted circle). **(d)** Degenerating (D) oocytes were observed in the follistatin knockout ovaries. (bars=20μm).






D

While a similar number of oocytes were observed in the wildtype and heterozygous ovaries ($p>0.42$), significantly fewer oocytes were observed in the follistatin knockout ovaries ($p<0.01$; Table 6.1.). Despite the knockout ovaries being largely devoid of oocytes, the majority of the oocytes observed were located in structures that were determined to be sex cords by Professor David de Kretser (2003; Figure 6.1.c.). Oocytes undergoing degeneration were also evident in the knockout ovaries (Figure 6.1.d.). This observation indicated that apoptosis may be the cause of the massive oocyte loss observed in the knockout ovaries.

6.3.2. Study 2 – Grafting of Follistatin Knockout Ovaries

6.3.2.1. Graft recovery

A total of 73 RAG mice were used as fetal ovarian graft recipients. However, because the grafting was done without knowledge of the fetus genotype, 31 RAG recipients received grafts from heterozygous donors. These grafts were not analysed, as only the wildtype and knockout grafts were assessed. Sixteen RAG mice received knockout fetal ovaries (n=16 donors) for a period of three or four weeks, while 26 RAG mice received grafts from wildtype donors (n=26).

A significant proportion of the RAG recipients used in this study either died or had to be culled due to ill health (n=23). It was later established that the poor health of the RAG mice was due to parvovirus infection that was contracted prior to the animals being supplied for use in the experiment. Consequently, the sample numbers per genotype treatment group were substantially reduced (Table 6.2.).

Table 6.2. Effect of the donor genotype and graft duration on ovarian graft retrieval rates. *Results only given for healthy surviving RAG recipients. Same superscript letter indicates groups that are statistically the same ($p>0.05$).

Donor genotype	Graft duration	n Graft recipients*	n Grafts retrieved/n grafted (%)*	n Grafts assessed histologically
Wildtype	3 weeks	12	22/24 (91.67)[a]	14
	4 weeks	9	11/17 (64.71)[a]	10
Knockout	3 weeks	5	10/10 (100.00)[a]	8
	4 weeks	7	11/13 (84.62)[a]	9

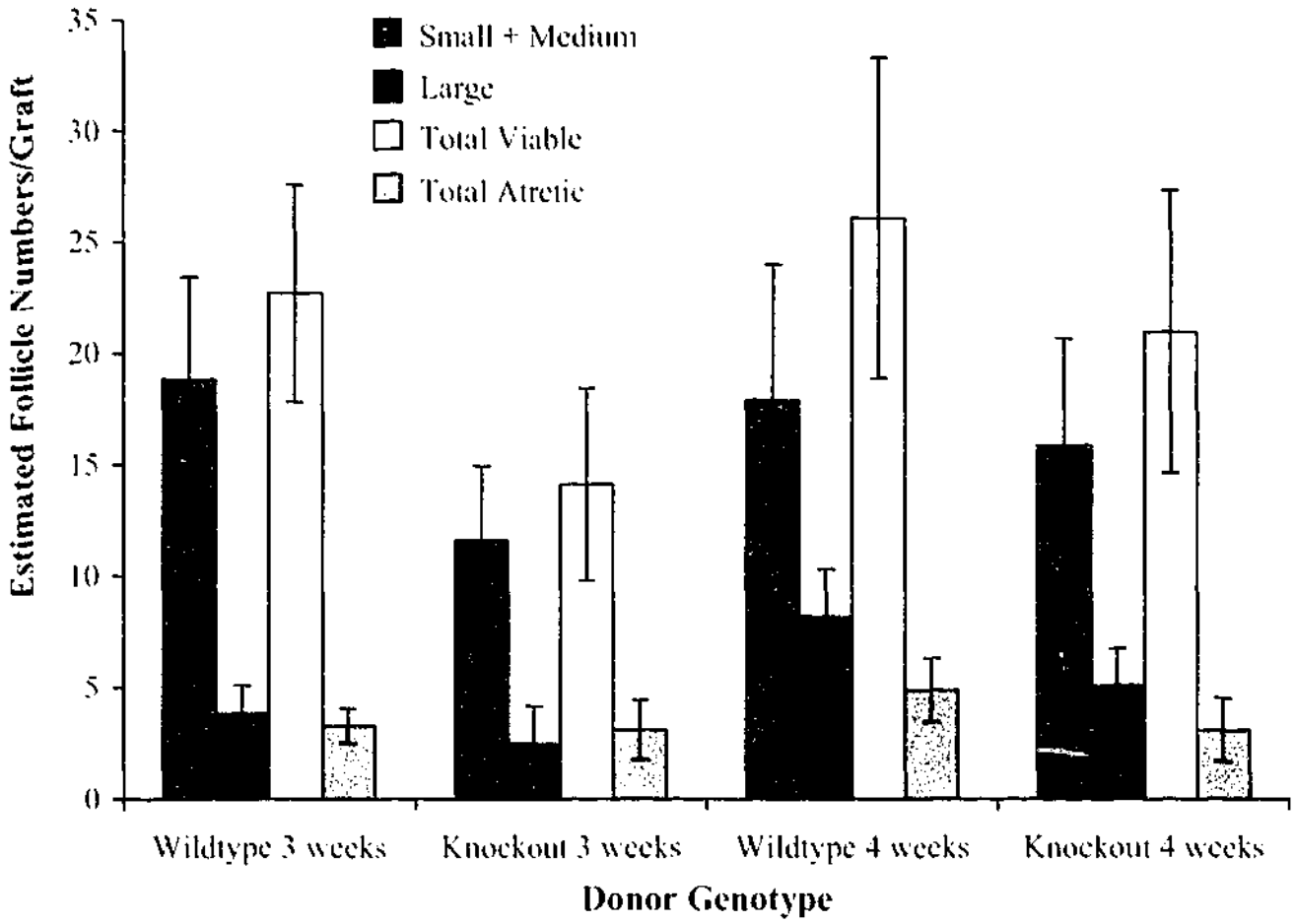
6.3.2.2. Follicular development

Follicles (viable and/or atretic) were observed in wildtype and follistatin knockout fetal grafts both three and four weeks after grafting (Figures 6.2. and 6.3.a.-d.). Very few small sized follicles were observed in any of the grafts, with numerous grafts completely devoid of small sized follicles. Consequently, follicle counts have been presented as viable small and medium sized follicles, viable large sized follicles, total viable follicles and total atretic follicles (Figure 6.2.). In addition to the sparsity of small sized follicles, the total follicle population contained within both the wildtype and knockout grafts was very low, with fewer than 35 follicles in total per graft in all of the treatment groups (Figure 6.2.).

Normal follicle growth (with up to antral sized follicles) was observed in grafts from both wildtype and follistatin knockout fetuses. However slight differences were observed between the three and four-week grafts. With the exception of one graft (that contained three type 7 follicles), type 6 follicles were the most advanced follicle type observed in three-week grafts from both wildtype and knockout fetuses. Furthermore these early antral follicles were sighted in low numbers (Figures 6.3.a.-b.). In four week grafts more advanced follicles were observed, with numerous type 6 and type 7 follicles in both wildtype and knockout grafts (Figures 6.3.c.-d.). Despite the presence of type 7 follicles,

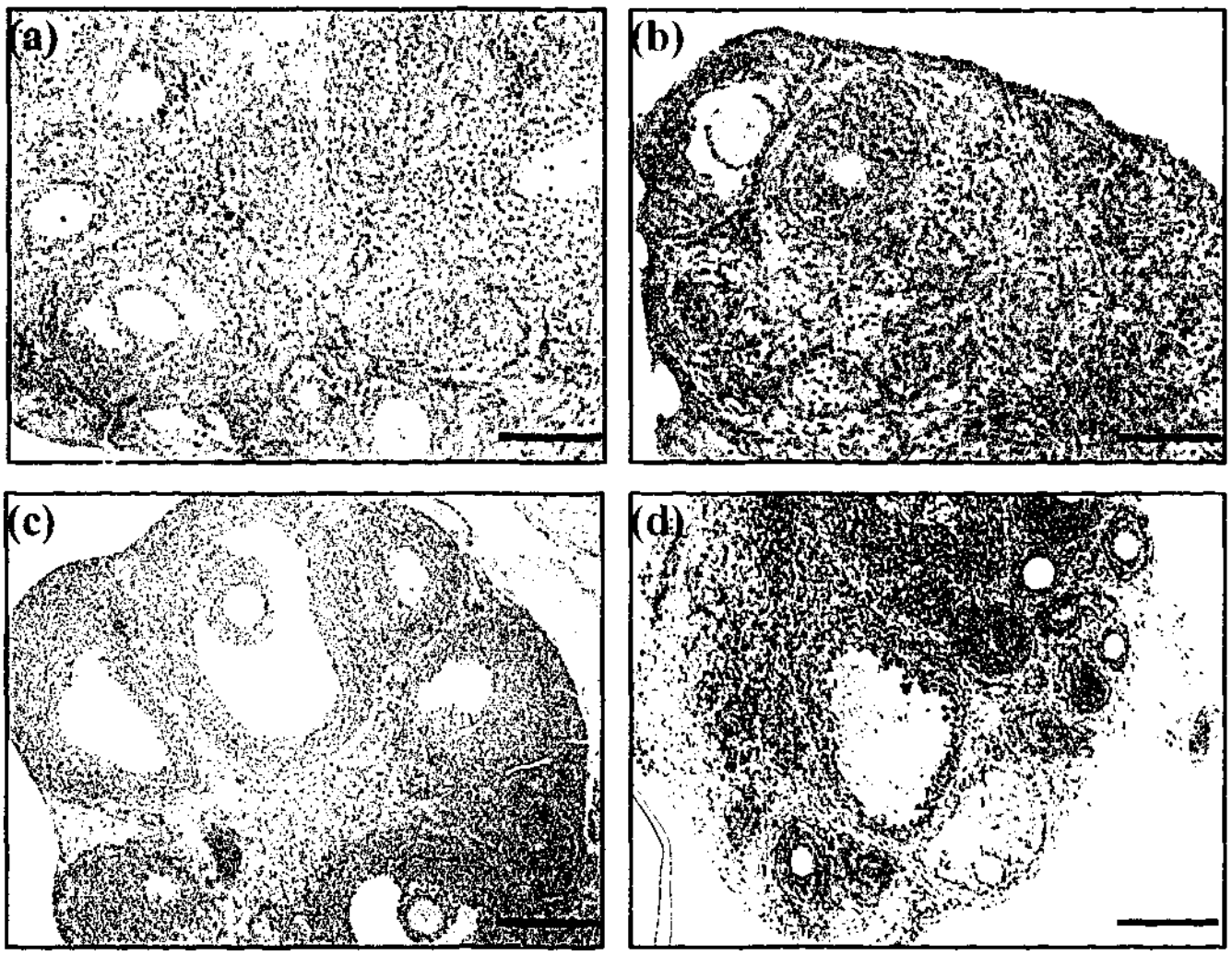
no corpora lutea were observed in any wildtype or knockout grafts indicating that full follicular development and ovulation had not occurred.

Figure 6.2. Effect of the donor genotype on the number of viable small and medium sized, viable large sized, total viable and total atretic follicles contained within fetal ovary grafts three and four weeks post grafting to RAG mouse recipients. Results are presented as the mean ± SEM. $p>0.10$ when comparing the numbers of each follicle type for all donor genotypes.



Upon statistical analysis, no difference was found between the number of viable small and medium sized, viable large sized, total viable and total atretic follicles when comparing grafts from wildtype and knockout fetuses three or four weeks after grafting ($p>0.25$; Figure 6.2.). The duration of grafting had no effect on either the number of viable small and medium sized, large sized, total viable or total atretic follicles contained within either the knockout ($p>0.28$) or wildtype ($p>0.07$) grafted ovaries.

Figure 6.3. Histological appearance of wildtype and follistatin knockout ovaries after grafting to RAG mice for three or four weeks. Three weeks after grafting **(a)** wildtype and **(b)** follistatin knockout ovaries were sparsely populated by follicles (bars=100μm). While very few small sized follicles were contained within the grafts, early antral follicles (up to Type 6) were observed. Four weeks after grafting antral sized follicles (Type 7) were observed in both **(c)** wildtype and **(d)** follistatin knockout ovaries (bars=200μm).



6.3.3. Study 3 – In Vitro *Culture of Follistatin Knockout Ovaries*

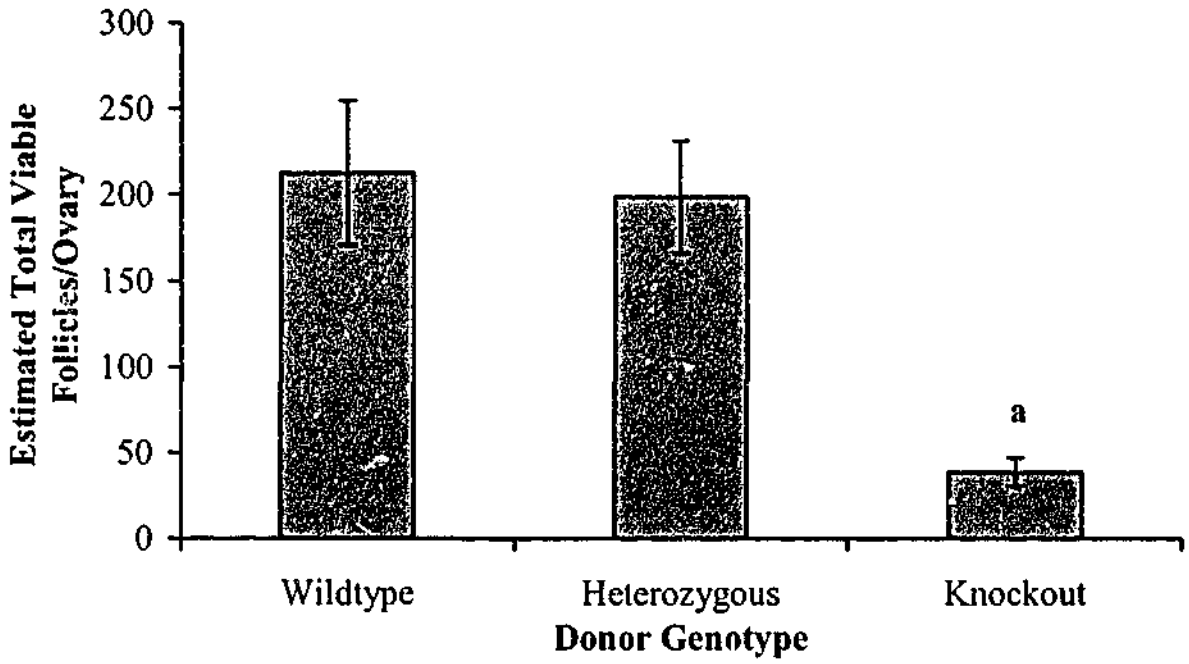
The *in vitro* culture of fetal ovaries was successful, with the majority of cultured ovaries able to be retrieved from the membrane filters after seven days of culture. However, great care needed to be taken while recovering *in vitro* cultured ovaries to ensure that the ovary was removed from the filter intact. Some fetal ovaries ruptured during the retrieval process. These ovaries were not assessed histologically. Due to the small size of the fetal ovaries great care was needed during histological processing. However, some additional fetal ovaries were lost during both the dehydrating and embedding steps of the histological processing (Table 6.3.).

Table 6.3. Number of fetal ovary donors, fetal ovaries *in vitro* cultured and assessed histologically for different donor genotype treatment groups.

Donor genotype	n Fetal ovary donors	n Fetal ovaries *in vitro* cultured	n Fetal ovaries assessed histologically
Wildtype	12	24	11
Heterozygous	12	24	14
Knockout	5	10	10

Both follicle survival and follicular development were evident in the *in vitro* cultured ovaries from follistatin knockout, heterozygous and wildtype fetuses (Figure 6.4.). Regardless of the donor genotype, small sized follicles were the only follicle type observed after seven days of *in vitro* culture. The majority of these follicles were Type 3a follicles, however up to Type 3b follicles were observed (Figures 6.5.a.-c.). While, follicles of the same developmental stages were present in all of the *in vitro* cultured ovaries (i.e. wildtype, heterozygous and knockout), differences existed within the follicle numbers (Figure 6.4.).

Figure 6.4. Effect of the donor genotype on the total number of viable follicles contained within day 18 fetal ovaries after seven days of *in vitro* culture. Results are presented as the mean ± SEM. Superscript letter indicates group that is statistically different ($p<0.01$).



After seven days of *in vitro* culture, wildtype and heterozygous ovaries were both densely packed with viable small sized follicles ($p>0.70$; Figures 6.4. and 6.5.a.-b.). Follistatin knockout ovaries had significantly fewer follicles ($p<0.01$), with a mean of less than 40 follicles in each knockout fetal ovary (Figures 6.4. and 6.5.c.). Ovaries from wildtype or heterozygous donors contained an average of approximately 200 viable follicles. This was significantly more than the follistatin knockout fetuses but substantially fewer than what is typically contained within ovaries of a similar age (Peters, 1969; Canning *et al.*, 2003).

Despite the low number of viable follicles in the ovaries, few atretic follicles were observed, and ranged from eight to 14 percent of the total number of follicles. Furthermore, when compared statistically, the donor genotype was found to have no effect on the number of atretic follicles contained within the *in vitro* cultured ovaries ($p>0.20$; Figure 6.6.).

Figure 6.5. Histological appearance of wildtype, heterozygous and follistatin knockout fetal day 18 ovaries following 7 days of *in vitro* culture in an environment totally devoid of follistatin. **(a)** Wildtype and **(b)** heterozygous ovaries were densely populated with follicles. The majority of follicles were Type 3a follicles (3a) however up to Type 3b follicles (3b) were observed. **(c)** Follistatin knockout ovaries contained significantly fewer follicles than the wildtype and heterozygous ovaries. (bars=50μm).

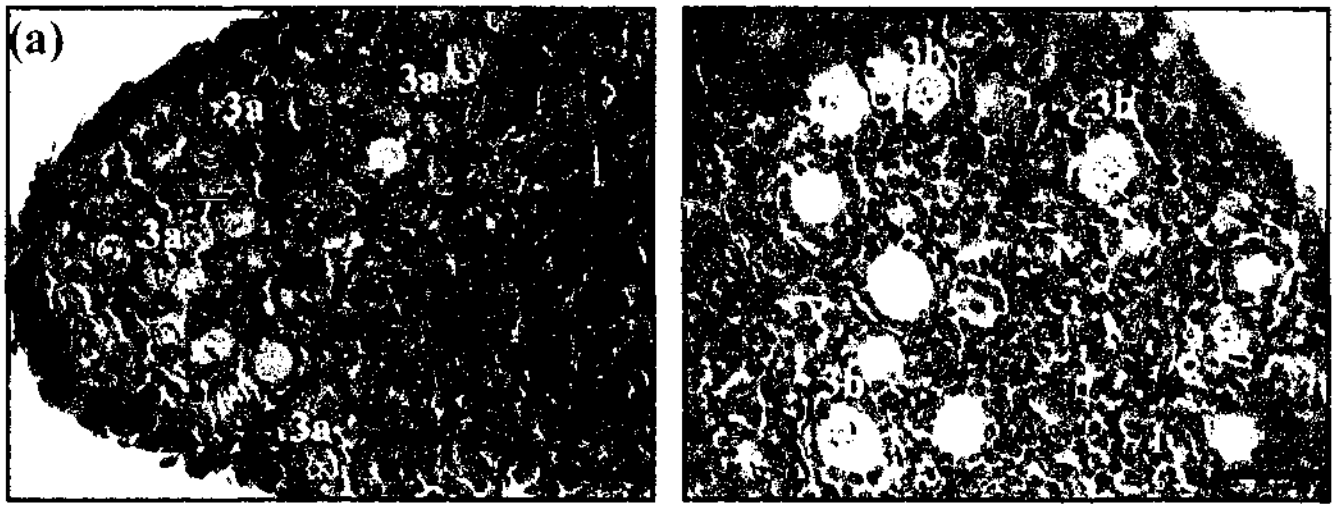
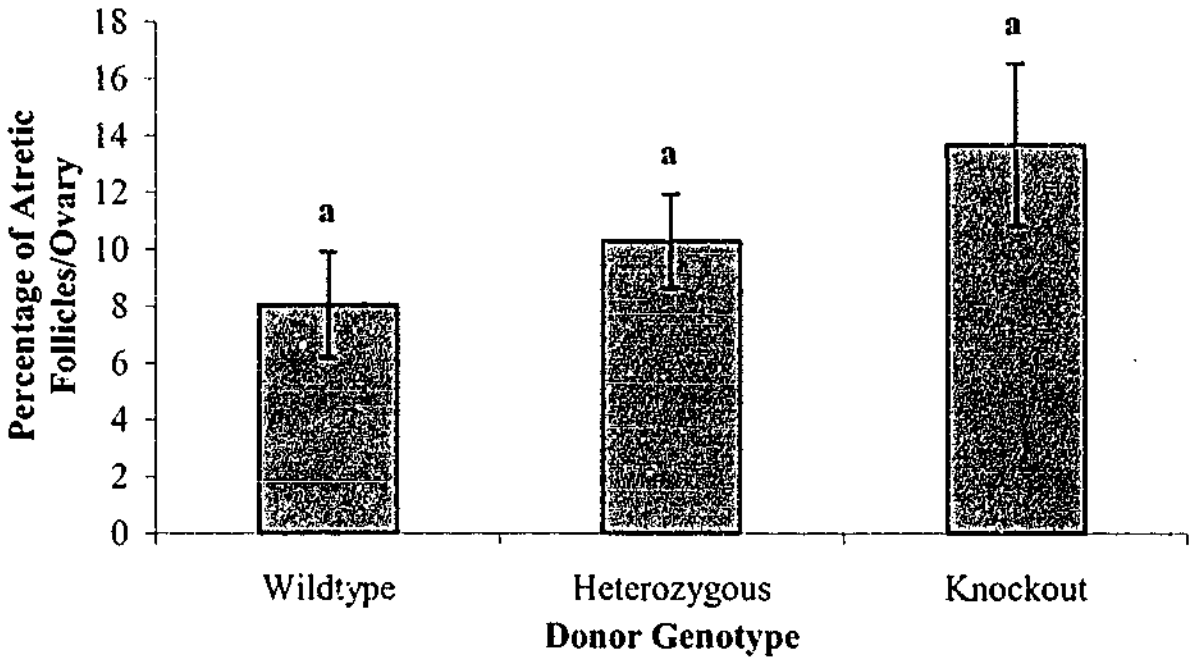


Figure 6.6. Effect of the donor genotype on the number of atretic follicles, as a percentage of total follicle numbers, in day 18 fetal ovaries after seven days of *in vitro* culture. Results are presented as the mean ± SEM. Same superscript letter indicates groups that are statistically the same ($p>0.05$).



6.4. Discussion

Mammalian folliculogenesis involves the developmental progression of small sized follicles, containing a single layer of follicle cells surrounding an oocyte, to large antral sized follicles consisting of multiple layers of follicle cells enclosing a cumulus-oocyte complex (Sidis *et al.*, 1998). This process is governed by numerous factors, including activin and its binding protein follistatin. Together follistatin and activin have established roles in the regulation of FSH secretion (Woodruff, 1998; Knight & Glister, 2001) and as a consequence it is thought that these two factors exert local autocrine-paracrine actions to modulate follicle growth, gonadotrophin responsiveness, steroidogenesis, oocyte maturation, ovulation and corpus luteum function (Knight & Glister, 2001). In addition, a recent study by Menke and Page (2002) identified follistatin to be more highly expressed at embryonic day 12.5 to 15.5 in the ovary than in the testis, leading the authors to speculate that follistatin may play a role in ovarian function during fetal life.

The over-expression of follistatin has previously been shown to result in reproductive defects, with folliculogenesis in some female mice halted between the primary and secondary follicle stages and in most ovaries no corpora lutea were observed (Guo *et al.*, 1998). However, the investigation of ovarian function in the absence of follistatin has previously been made impossible by the post-partum mortality of follistatin knockout mice. The challenge has therefore been to devise methods for investigating folliculogenesis in ovaries devoid of follistatin.

In this study the follistatin knockout mouse (Matzuk *et al.*, 1995b) was employed to investigate the role of follistatin in oogenesis and folliculogenesis. The collection and

grafting of follistatin knockout fetal ovaries provided a novel *in vivo* model by which folliculogenesis could be examined. Similarly, the *in vitro* culture of follistatin knockout ovaries enabled the earliest stages of follicular development to be examined in an environment completely devoid of follistatin.

At day 18 of fetal life, the follistatin knockout mice had a reduced oocyte population compared to ovaries from wildtype and heterozygous fetuses. This interesting finding indicates that follistatin may play a crucial role in germ cell migration, germ cell proliferation and/or the regulation of apoptosis during oocyte meiosis. However, despite the massive oocyte loss observed at day 18 of fetal life, when cultured *in vitro* or grafted, some of the oocytes formed follicles that subsequently proceeded through normal folliculogenesis.

While it is beyond the scope of this study to determine with certainty the function of follistatin in germ cell differentiation, oogenesis or folliculogenesis, some insight into the potential roles for follistatin is provided by previous experience by others with transgenic mouse models.

Through the use of transgenic mice it has been clearly demonstrated that primordial germ cell migration, development and proliferation are controlled by the interplay of numerous factors (Elvin & Matzuk, 1998). For example, in the absence of c-kit, germ cell migration does not occur (reviewed by Elvin & Matzuk, 1998; Driancourt *et al.*, 2000). Similarly, both the transgene insertion at the germ cell deficient locus, and a spontaneous mutation at the atrichosis have been shown to significantly reduce primordial germ cell numbers in the developing mouse gonad (reviewed by Elvin & Matzuk, 1998). Female mice devoid of the zinc-finger protein *Zfx* have poor fertility as a result of substantially reduced primordial germ cell numbers during embryonic life (Luoh *et al.*, 1997). Likewise, follistatin may be partly responsible for the promotion of germ cell migration, development and/or

proliferation as such an effect may explain the sparsity of oocytes contained within the follistatin knockout ovaries at day 18 of fetal life.

Alternatively, or in addition, follistatin may play a role in either, the promotion of the rapid oogonial mitotic divisions that occur between days 12 and 14 of fetal life (Odor & Blandau, 1969a) or the prevention of oogonia loss by apoptosis. The recent discovery that follistatin is more highly expressed in ovaries than in testes at embryonic days 12.5, 13.5 14.5 and 15.5 (Menke & Page, 2002) offers support for these theories. In addition, the presence of structures that were believed to be degenerating oocytes in the follistatin knockout ovaries is indicative of a role for follistatin in the prevention of atresia during oocyte meiosis. However to confirm either primordial germ cell, oogonia or oocyte loss by means of apoptosism, TUNEL staining would need to be employed. In any case, the presence of pachytene stage oocytes in the untreated wildtype, heterozygous and follistatin knockout ovaries clearly demonstrated that follistatin is not the sole factor responsible for enuring that the oocyte meiotic progression occurs.

In the absence of factor in the germline alpha (FIGα), oocytes do not progress through the prophase of the first meiotic division and therefore become arrested and hence do not form primordial follicles (Soyal *et al.*, 2000). They subsequently disappear from the ovary in a matter of days. In contrast to FIGα, the ability of the oocytes contained in the follistatin knockout ovaries to form follicles and commence normal follicular growth clearly demonstrates that follistatin is not the sole factor essential for the early stages of follicular development. However the reduced follicle numbers (compared to oocyte numbers at day 18 of fetal life) in wildtype, heterozygous and follistatin knockout ovaries following *in vitro* culture, is suggestive of a role for circulating follistatin and/or activin in the regulation of follicle development. Alternatively, this massive decrease in follicle numbers may simply be the result of the basic media used for the *in vitro* culture.

Numerous factors including growth differentiation factor-9 and the FSH beta subunit have been established as being critical for stages of folliculogenesis (reviewed by Elvin & Matzuk, 1998). In contrast, follicular development to antral stages within both the follistatin knockout and wildtype ovary grafts indicates that follistatin may not be crucial in the control of either follicle growth or oocyte maturation. However, due to the numerous limitations associated with this study it is impossible to draw absolute conclusions.

Primarily, the low follicle population contained within all grafts is likely to mask any effects that the presence or absence of follistatin may have had on the follicle population. While it is unclear why the follicle survival rate and consequently the follicle population numbers were consistently low for all treatment groups, it is possible that the ovaries were particularly susceptible to ischaemia. This appears likely as attempts at freezing ovaries from both wildtype and knockout fetuses, using standard procedures developed for the mouse, were unsuccessful (personal observation). In addition, other body tissues of C57BL/6 mice (the background strain), are renowned for being susceptible to ischaemia (Yang *et al.*, 1997; Wellons *et al.*, 2000). Alternatively, the developmental stage of the oocytes at day 18 of fetal life (still undergoing meiosis) may render them particularly vulnerable, as in both the mouse (Taketo *et al.*, 1993) and tammar wallaby (Mattiske, 2003) the transplantation of ovarian tissue containing only primordial germ cells has been found to result in complete germ cell loss. However, fetal day 16 mouse ovaries containing meiotic oocytes have previously been shown to survive ovarian grafting with normal fertility observed in recipient animals (Cox *et al.*, 1996).

Consideration must also be given to the circulating follistatin offered by the graft recipient. It is reasonable to suggest that the circulating follistatin may be the driving force for the follicular development and oocyte maturation observed in the follistatin knockout ovaries

(beyond the small sized follicles observed following *in vitro* culture). Consequently, the exact role of follistatin in follicular development and oocyte maturation remains unclear. Similarly, the means through which follistatin acts are yet to be established. However from the present study it is apparent that follistatin is important in the early stages of oogenesis, with the majority of germ cells and/or oocytes lost in follistatin knockout mice. Despite this massive loss, the oocytes present appear able to progress through folliculogenesis in a normal fashion. However, to confirm their viability it must be determined whether the oocytes can be fertilized, and if they form offspring.

It has previously been speculated that together, follistatin and activin exert local autocrine-paracrine actions to regulate corpus luteum function (Knight & Glister, 2001). Three to four weeks after grafting corpora lutea are usually evident in fetal mouse ovarian grafts (Cox *et al.*, 1996). The absence of corpora lutea in both wildtype and knockout ovary grafts was curious and as such this study has failed to investigate the relationship between follistatin and dominant follicle selection, ovulation or corpora lutea formation. However, given the ability of follistatin to regulate oocyte maturation by way of inhibition (Gilfillan & Robertson, 1998), a link between follistatin and, at a minimum, dominant follicle selection appears likely.

The challenge now is to devise further means of investigating the role of follistatin in germ cell differentiation, oogenesis and folliculogenesis. Specifically, these studies have highlighted the need to obtain more information on the role of follistatin in the fetal ovary. A time course study examining the histological appearance of follistatin knockout ovaries from day 12 to 18 of fetal life using stains that detect the factors expressed by primordial germ cells and meiotic oocytes (e.g. 4C9, VASA and alkaline phosphatase) may prove beneficial. In addition, TUNEL staining may help identify the cause of the massive oocyte loss. Once the timing of primordial germ cell, oogonia or oocyte loss has been established,

the *in vitro* culture of follistatin knockout ovaries in the presence follistatin should be of assistance in confirming the role that follistatin plays in fetal ovarian function.

6.4.1. Summary

These studies have demonstrated the value of both ovarian grafting and *in vitro* culture as tools for investigating ovarian function. In the absence of follistatin, oocyte numbers were substantially reduced indicating that follistatin plays a crucial role in germ cell development and/or the early stages of oogenesis. However, the oocytes that were present formed follicles and initiated normal follicular development following *in vitro* culture or ovarian grafting, thus indicating that follistatin is not essential for folliculogenesis.

~ *Chapter Seven* ~

Concluding Remarks

During last 200 years, 22 of Australia's native mammalian species have been lost to extinction. It has become increasingly apparent that to assist in minimizing further losses ART's will need to be employed. However, the application of most ART's is limited by the requirement for mature fertilizable oocytes. Methods that facilitate oocyte maturation are thus required.

Ovarian xenografting provides a valuable means for the *in vivo* maturation of oocytes. In the future oocytes matured within ovarian xenografts could be employed in ART's to assist in the conservation of endangered wildlife species. Meanwhile, ovarian grafting currently provides an effective tool by which ovarian function, and particularly the regulation of folliculogenesis, can be examined. The aims of this thesis were to investigate the feasibility of employing ovarian grafting in the conservation of endangered wildlife and as a tool for investigating ovarian function.

For ovarian xenografting to be of use in conservation biology, the ability to retrieve viable ovarian tissue that would normally be lost or wasted is a necessity. Therefore defining optimal collection and storage conditions for ovarian tissue during transportation from the field to the laboratory is of paramount importance. Information is also required on how

rapidly ovarian tissue degenerates both within the body of a deceased animal and following collection during periods of short-term storage (i.e. during transportation). This study has demonstrated that ovarian tissue can remain functional for up to 48 hours following the death of the animal. This finding has important implications for conservation biology and justifies the opportunistic collection of ovarian tissue from precious individuals following death. Similarly, ovarian tissue can now be collected in the field and transported to the laboratory while remaining confident of its viability if certain basic procedures are followed.

Once viable ovarian tissue has been collected, it can be cryopreserved for long-term storage in a genome resource bank to help protect the genetic diversity of diminishing wildlife populations. With more than a third of Australia's marsupial species currently facing the threat of extinction, the Animal Gene Storage and Resource Centre of Australia has been established to opportunistically collect and store the reproductive tissues and cells of native wildlife. However to be most effective, optimal cryopreservation procedures for native wildlife and particularly marsupial ovarian tissue must be developed. Experiments reported within this thesis have demonstrated that marsupial ovarian tissue can be both slow and rapid-cool cryopreserved in DMSO based cryoprotectant solutions using field applicable techniques developed for eutherian species in order to maintain viability. This finding provides support for the proposal that the opportunistic collection and storage of marsupial ovarian tissue should be continued and encouraged.

Previous studies had demonstrated that both fresh and frozen/thawed ovarian tissue from a range of species resume function and produce large antral sized follicles following xenografting to a suitable host (reviewed by Paris *et al.*, 2003)). Although it had been suggested that this could be a means of maturing oocytes for use in ART's by several authors, the viability of xenograft matured oocytes had not previously been proven. In

addition to demonstrating that oocytes can be recovered from marsupial and eutherian ovarian xenografts, this study has shown for the first time that oocytes matured within ovarian xenografts are viable, and when fertilized, are capable of producing healthy live young. This exciting proof of principal study has demonstrated the full potential of ovarian xenografting as a tool for the *in vivo* maturation of viable oocytes within ovarian tissue that has been obtained opportunistically.

For ovarian xenografting to be of greatest benefit in the conservation of endangered wildlife, methods that facilitate optimal follicular development and hence maximise the mature fertilizable oocyte yield are required. Whether ovarian tissue is xenografted fresh or following storage (short or long-term), both the immunological and gonadal status of the recipient, and the graft site, can influence graft function and thereby have an effect on the oocyte yield. While by no means the cheapest or most convenient recipient model, the NOD-SCID mouse remains the most effective recipient for the *in vivo* maturation of oocytes contained within ovarian xenografts. However, alternatives such as immunosuppression might be preferable, and the successful collection of oocytes from xenografts to immune suppressed recipients provides the impetus for further investigation into the use of immunosuppression in ovarian xenografting studies.

These studies have demonstrated that follicular development is supported to the late antral stages within ovarian xenografts of marsupial (and eutherian) origin in intact male and female recipients and females with one or both ovaries removed. Comparison of the number and types of follicles contained within the ovarian xenografts of different gonadal statues has indicated that the optimal recipient gonadal status is determined by both the species and age of the ovarian tissue donor. Collectively however, the results have demonstrated that a bilaterally ovariectomised female recipient effectively sustains follicular development within marsupial ovarian tissue. Therefore, this recipient model

should be employed in marsupial ovarian xenografting studies where the optimal gonadal status of the recipient is yet to be identified.

While, it is well established that the pocket between the kidney and its capsule provides both a convenient and effective graft site for ovarian transplantation studies, it had been unclear whether the site is suitable for complete follicular development. These studies have shown that the kidney capsule is a superior graft site to under the skin. Furthermore, the proven ability to collect oocytes from large antral sized follicles visible on the surface of grafts to the kidney clearly demonstrates that the kidney capsule is adequate for full follicular development. The kidney capsule should therefore be the graft site of choice for ovarian tissue xenografting studies.

These studies have also shown the value of ovarian grafting as a tool with which ovarian function may be investigated. In addition to ovarian grafting being employed to assess ovarian tissue viability, in conjunction with *in vitro* culture ovarian grafting has established that follistatin plays a crucial role in fetal ovarian function. This information provides an important insight into the intricate process of germ cell differentiation and the role of follistatin in oogenesis and folliculogenesis.

Specifically, the studies contained within this thesis have examined ovarian grafting with regard to its application in conservation biology and to its applicability to the study of ovarian function. The results contained within this thesis justify the employment of ovarian xenografting in conservation biology as a means of producing mature oocytes for use in ART's.

This procedure although possible, is technically demanding, wasteful of recipient animals and somewhat controversial. Realistically, it can therefore only be considered as an interim measure. The challenge now is to use these techniques to enable us to learn more

about the mechanisms involved in follicle and oocyte maturation to assist us to develop *in vitro* culture conditions that permit the maturation, fertilization (and subsequent embryo culture) of oocytes from threatened marsupial species.

In summary, the studies in this thesis have demonstrated that salvaged ovarian tissue may be grafted, fresh or following short or long-term storage, under the kidney capsule of bilaterally ovariectomised female NOD-SCID recipients. Following xenografting, ovarian tissue resumes ovarian function with full follicular development supported, thus enabling the collection of mature oocytes from ovarian xenografts. The subsequent fertilization of oocytes and transfer of embryos to foster mothers results in the birth of healthy live young. The technique has been shown to be a valuable tool for propagating a species and thus holds considerable promise for aiding in the conservation of endangered species. In addition, ovarian grafting is also an important tool for investigating ovarian function, particularly the control of follicular development.

~ Appendices ~

Appendix 1

Haematoxylin and eosin staining procedure used in the preparation of histological slides.

Stain solution	**Time in solution**
Xylene 1	5 minutes
Xylene 2	20 seconds
Xylene 3	20 seconds
Absolute alcohol	20 seconds
Absolute alcohol	20 seconds
Absolute alcohol	20 seconds
Tap water	1 minute
Harris' haematoxylin	7 minutes
Tap water	1 minute
Acid alcohol	2 seconds
Tap water	20 seconds
Scott's tap water	30 seconds – 1 minute
Tap water	10 seconds
Aqueous eosin	3 minutes
Absolute alcohol	20 seconds
Absolute alcohol	20 seconds
Absolute alcohol	20 seconds
Xylene	20 seconds
Xylene	20 seconds
Xylene	until cover-slipped

Appendix 2

Preparation of concentrated stock solutions for KSOM^{AA} handling and embryo culture media. All products were obtained from Sigma (MO, USA) unless indicated otherwise. Stocks were made to volume with deionized cell culture water (CSL, Australia). (Adapted from Lawitts & Biggers, 1993; Summers *et al.*, 1995; Summers *et al.*, 2000).

Stock A	**g/100ml**
NaCl	5.5520
KCl (BDH, AnalaR, Australia)	0.1860
KH_2PO_4 (BDH, AnalaR, Australia)	0.0476
$MgSO_4.7H_2O$	0.0793
Sodium lactate	1.8680
D-Glucose	0.9911
Stock B	**g/100ml**
$NaHCO_3$ (BDH, AnalaR, Australia)	2.100
Stock C	**g/10ml**
Sodium pyruvate	0.0220
Stock D	**g/10ml**
$CaCl_2H_2O$ (BDH, AnalaR, Australia)	0.2500
Stock ED	**g/10ml**
EDTA	0.0035
Stock E	**g/100ml**
HEPES	5 958
Phenol red	0.0100
Stock G	**g/10ml**
Glutamine	0.1460

Preparation of KSOM^{AA} handling and embryo culture media from concentrated stock solutions. EAA=Eagle's essential amino acid (x50 Stock). NEAA=Eagle's non-essential amino acid.

Stock solution	**Handling medium (ml/10ml)**	**Embryo culture medium (ml/10ml**
Stock A	1.00	1.00
Stock B	1.00	0.16
Stock C	0.10	0.10
Stock D	0.10	0.10
Stock ED	0.10	0.10
Stock E		0.84
Stock G	0.10	0.10
EAA (ICN Biomedical Inc., Ohio, USA)	0.10	0.10
NEAA (ICN Biomedical Inc., Ohio, USA)	0.05	0.05
Deionised culture H_2O (CSL, Australia)	7.45	7.45
Bovine Serum Albumin (Gibco BRL – Life Technologies, NY, USA)	1mg/ml	

Appendix 3

Preparation of concentrated stock solutions for MT6 medium. All products were obtained from Sigma (USA) unless indicated otherwise.

Stock A	**g/50ml**
NaCl	3.640
KCl (BDH, AnalaR, Australia)	0.100
$MgCl.6H_2O$	0.050
$NaH2PO_4.H2O$ (BDH, AnalaR, Australia)	0.028
D-Glucose	0.050
Stock B	**g/50ml**
$NaHCO_3$ (BDH, AnalaR, Australia)	1.053
Phenol red	0.010
Stock C	**g/50ml**
$CaCl_2.H2O$ (BDH, AnalaR, Australia)	0.252

Preparation of MT6 from concentrated stock solutions.

Stock solution	**Amount per 10ml**
Stock A	1.0ml
Stock B	1.0ml
Stock C	0.1ml
Deionized culture H_2O (CSL, Australia)	7.9ml
BSA (Fraction V, Lot 527; Bayer, USA)	40.0mg

Appendix 4

Preparation of handling media used in Protocol 1 for tammar wallaby oocytes (developed by Dr David Pushett, Monash Institute of Reproduction and Development for the handling of cat oocytes). All products were obtained from Sigma (USA) unless indicated otherwise.

Component	**Amount/50ml of media**
$NaHCO_3$ (BDH, AnalaR, Australia)	0.0220g
Penicillin (Penicillin G)	0.0038g
Streptomycin (Streptomycin sulfate)	0.0025g
Sodium pyruvate	0.0013g
HEPES	0.0890g
Made to final volume with EMEM (M 4655, Sigma, USA)	

Preparation of concentrated stock for maturation media used in Protocol 1 for tammar wallaby oocytes (developed by Dr David Pushett, Monash Institute of Reproduction and Development for the *in vitro* maturation of cat oocytes).

Stock	**g/50ml**
$NaHCO_3$ (BDH, AnalaR, Australia)	0.1100g
Sodium pyruvate	0.0013g
Penicillin	0.0038g
Streptomycin	0.0025g
Made to final volume with EMEM (M 4655, Sigma, USA)	

Preparation of tammar wallaby maturation medium (Protocol 1) from concentrated stock solution.

Component	**Amount/10ml**
Stock	9.875ml
Fetal Calf Serum (Gibco BRL – Life Technologies, USA)	0.100ml
BSA (A 7906, Sigma, USA)	0.030g
FSH (rhFSH; Organon, Lane Cove, NSW)	0.150 IU

Appendix 5

Preparation of handling and *in vitro* maturation media used in Protocol 2 for tammar wallaby oocytes (developed by Nadine Richings, Department of Zoology, University of Melbourne). All products were obtained from Sigma (USA) unless indicated otherwise.

Component	**Handling medium (amount/50ml)**	**Maturation medium (amount/10ml)**
DMEM	48.5ml	9.5ml
BSA (A 7906, Sigma, USA)	150mg	50.0mg
L-Glutamine	0.5ml	0.2ml
Pen-Strep	1.0ml	0.2ml
FSH (rhFSH; Organon, Lane Cove, NSW)	-	150µL (1.5 IU/ml)

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