

# **The role of TrkB in neonatal ovary development**

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## **Declaration**

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

**Tamsin R. M. Lannagan**

**2009**

## Abstract

The signalling cascade induced by the binding of neurotrophins (NGF, BDNF, NT3 and NT4) to their high-affinity tyrosine kinase receptors (TrkA, B and C) is well documented to be important for neuronal cell survival, proliferation and differentiation. Evidence has accumulated demonstrating the importance of these signalling pathways in non-neuronal tissues, including the ovary where all neurotrophins and their receptors are expressed. In the mouse, effects on ovulation have been demonstrated but the role of Trk signalling in neonatal ovary development is less clear. Previous work had found that TrkB expression is upregulated at the time of follicle formation in the mouse and transgenic mice null for the TrkB receptor demonstrate significant loss of oocytes neonatally (TrkB knockouts, KO, die shortly after birth). This thesis examines the phenotype of the TrkB KO using morphological, histological and surgical techniques with the aim being to further investigate the role of TrkB signalling in oocyte survival, and to contribute to our understanding of neonatal ovary development. The main questions addressed are: 1) what developmental defects are occurring on a morphological level that result in the phenotype of the TrkB KO; 2) can these defects be quantified; and 3) what are the long-term survival prospects for TrkB KO oocytes. Morphological assessment revealed that TrkB KO ovaries exhibit poorer follicle health than their Controls and this was confirmed by assessment of basement membrane (BM) composition. TrkB KO brain and kidney were also assessed and found to have similarly affected BM. It is well known that cells require contact with the BM to maintain survival, thus it is postulated that TrkB signalling contributes to oocyte survival through regulation of the BM. Due to the postnatal lethality of the mutation, TrkB KO ovaries were transplanted to ascertain long-term oocyte survival. Unexpectedly it was found that TrkB KO oocytes are able to survive and follicles grow as

well as they do in the Control transplants. Consequently, the *in vivo* effect has to be indirect. It is known that oocytes in the neonatal ovary undergo an increased rate of cell death but it is not known how the cell debris is removed. A novel observation of a neonatal ovarian immune response has been made in this thesis and is postulated to be a physiological mechanism for cell debris clearance. In conclusion, this thesis has demonstrated that signalling through TrkB has an effect on regulating BM in the ovary and other organs, but that surprisingly it has an indirect effect on oocyte survival.

## **Presentations arising, or containing work from this thesis**

### **Oral presentations**

Lannagan T, Spears N

**Heterotopic transplantation of newborn TrkB KO ovary reveals an indirect effect on oocyte survival.**

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Spears N, Lannagan T, Edwards D, Murray AA

**Role of the TrkB neurotrophin receptor in the neonatal mouse ovary.**

9<sup>th</sup> National Ovarian Workshop, Cambridge, 2007

Lannagan T, Spears N

**The role of the neurotrophin receptor, TrkB, in the development of the neonatal mouse ovary.**

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### **Poster presentations**

Lannagan T, Price D, Spears N

**Immune-like response in the developing mouse ovary.**

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Lannagan T, Spears N

**TrkB and basement membrane formation in early ovary development.**

XVI<sup>th</sup> Ovarian Workshop, San Antonio, 2007

## **Assistance given throughout this investigation**

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

Chapter 4: P0 brains were collected and fixed by Dr Alison Murray and Rowena Smith.

Chapter 5: Ovaries were dissected from neonates for transplantation by Rowena Smith.

Chapter 6: Sections were prepared for TEM (and toluidene blue stained) by Stephen Mitchell, EM Suite, Edinburgh University.

*Dazl* ovaries were obtained already embedded in wax from Professor A. McNeilly, MRC Human Reproductive Sciences Unit, Edinburgh. Vivian Allison sectioned and H&E stained the *Dazl* ovaries.

I was also assisted with PCR genotyping by Rowena Smith and Dr Alison Murray.

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## Table of Contents

Declaration .....	3
Abstract .....	4
Presentations arising, or containing work from this thesis.....	6
Assistance given throughout this investigation.....	7
Acknowledgments .....	8
Abbreviations .....	13
<b>Chapter 1 General Introduction.....</b>	<b>16</b>
1.1. Introduction .....	17
1.2. Embryonic ovary .....	18
1.2.1. Primordial germ cells to oogonia .....	18
1.2.2. Oogonia to oocytes .....	22
1.3. Perinatal ovary development .....	25
1.3.1. Primordial follicle formation.....	25
1.3.2. Gonadotrophin independent follicle growth.....	29
1.4. Cell death in the developing mammalian ovary.....	39
1.5. Neurotrophins and Trk receptors .....	43
1.5.1. Neurotrophins: the ligands.....	43
1.5.2. Trks: the receptors .....	44
1.5.3. p75: the receptor .....	49
1.5.4. Detecting <i>TrkB</i> mRNA and protein .....	50
1.6. Roles of Trk receptors in the development of the nervous system .....	52
1.6.1. Cell survival .....	52
1.7. Roles in the development of non-neuronal tissues .....	54
1.8. Neurotrophins, Trks and the developing mammalian ovary .....	55
1.8.1. Ovarian innervation.....	56
1.8.2. TrkA, TrkC and p75.....	57
1.8.3. TrkB .....	59
1.9. Aims of PhD.....	63
<b>Chapter 2 Materials and Methods.....</b>	<b>64</b>
2.1. <i>TrkB<sup>FL</sup></i> KO mice .....	65
2.2. Genotyping of mutant mice .....	65
2.2.1. Collection of tissue .....	65
2.2.2. Extraction of DNA.....	65

2.2.3. Genotyping of DNA by polymerase chain reaction.....	65
2.2.4. Analysis of PCR products on agarose gel.....	66
2.2.5. Visualisation of DNA products.....	67
2.3. Tissue collection .....	67
2.3.1. Gross dissection .....	67
2.4. Wax Histology .....	67
2.4.1. Ovary fixation .....	67
2.4.2. Wax embedding.....	67
2.4.3. Sectioning and mounting wax blocks.....	67
2.4.4. Haematoxylin and eosin staining wax sections.....	68
2.5. Plastic Histology.....	69
2.5.1. Resin embedding .....	69
2.5.2. Sectioning and mounting resin blocks .....	69
2.5.3. Haematoxylin and eosin staining resin sections .....	70
2.6. Oocyte cell counts and follicle classification .....	70
2.6.1. Cell counts.....	70
2.6.2. Follicle classification .....	72
2.7. Immunohistochemistry and immunofluorescence .....	73
2.7.1. Dewaxing and rehydration .....	73
2.7.2. Antigen retrieval.....	73
2.7.3. Immunohistochemistry.....	73
2.7.4. Immunofluorescence .....	75
<b>Chapter 3 <i>TrkB<sup>FL</sup></i> KO mouse ovary: a time-course of neonatal development .....</b>	<b>77</b>
3.1. Introduction .....	78
3.2. Aim .....	80
3.3. Materials and Methods .....	85
3.4. Results.....	88
3.5. Discussion.....	99
<b>Chapter 4 ECM of the <i>TrkB<sup>FL</sup></i> KO mouse: laminin-<math>\alpha</math>1 .....</b>	<b>108</b>
4.1. Introduction .....	109
4.2. Aim .....	117
4.3. Materials and Methods .....	118

4.4. Results.....	122
4.5. Discussion.....	142
<b>Chapter 5 <i>TrkB<sup>FL</sup></i> KO mouse ovary development beyond the first postnatal week: assessment by ovarian transplantation.....</b>	<b>153</b>
5.1. Introduction.....	154
5.2. Aim.....	156
5.3. Materials and Methods.....	157
5.4. Results.....	161
5.5. Discussion.....	177
<b>Chapter 6 Novel description of an immune response in the neonatal mouse ovary.....</b>	<b>188</b>
6.1. Introduction.....	189
6.2. Aim.....	195
6.3. Materials and Methods.....	198
6.4. Results.....	200
6.5. Discussion.....	221
<b>Chapter 7 General Discussion.....</b>	<b>227</b>
7.5. Further research.....	235
7.6. Concluding remarks.....	238
<b>References.....</b>	<b>240</b>
Appendix A. PCR protocols.....	263
Appendix B. TEM solutions.....	265

## Abbreviations

ADAM	A disintegrin and metalloproteinase
ADAMTS-1	ADAM with thrombospondin type 1 motifs-1
AhR	Aryl-hydrocarbon receptor
AOD	Autoimmune ovarian disease
AOI	Area of interest
AMH	Anti-Müllerian hormone
bFGF	Basic fibroblast growth factor
BAX	Bcl2-associated X protein
Bcl2	B-cell lymphoma/leukaemia 2
BDNF	Brain derived neurotrophic factor
BM	Basement membrane
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
Ca <sup>2+</sup>	Calcium
CNS	Central nervous system
CSF	Cerebrospinal fluid
DAB	3,3'-Diaminobenzidine tetrahydrochloride
Dazl	Deleted in azoospermia-like
ddH <sub>2</sub> O	Double distilled water
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglion
ECM	Extracellular matrix
Figla	Factor in the germ line, $\alpha$
FGF	Fibroblast growth factor
FSH	Follicle stimulating hormone
FSHR	Follicle stimulating hormone receptor
GCNA	Germ cell nuclear antigen
GDF-9	Growth differentiation factor-9
GnRH	Gonadotrophin-releasing hormone
GPCR	G-protein coupled receptor

H&E	Haematoxylin and eosin
hpg	Hypogonadal
HRB	Hugh Robson Building
HRP	Horseradish peroxidase
IGF	Insulin-like growth factor
IL	Interleukin
ISH	<i>In situ</i> hybridisation
IVC	Individually ventilated cage
KL	Kit ligand
KO	Knockout
LF	Little France
LIF	Leukaemia inhibitory factor
LH	Luteinising hormone
LHR	Luteinising hormone receptor
Lhx8	LIM-homeobox protein 8
MIS	Müllerian inhibiting substance
MMP	Matrix metalloproteinase
MPI	Mean pixel intensity
mRNA	Messenger RNA
MVH	Mouse vasa homologue
Na <sup>+</sup>	Sodium
NGF	Nerve growth factor
NT3	Neurotrophin 3
NT4	Neurotrophin 4
OSE	Ovarian surface epithelium
PA	Plasminogen activator
PAMS	Periodic acid-methenamine (Jones) silver stain
PBS	Phosphate-buffered solution
PCD	Programmed cell death
PFA	Paraformaldehyde
PGC	Primordial germ cell
PI	Propidium iodide

PI3K	Phosphatidyl inositol trisphosphate
PLL	Poly-L-lysine
PML	Polymorphonuclear leukocyte
PNS	Peripheral nervous system
POF	Premature ovarian failure
RA	Retanoic acid
RNA	Ribonucleic acid
SCG	Superior cervical ganglion
SCP-1	Synaptonemal complex protein-1
SEM	Standard error of the mean
siRNA	small interfering RNA
Sohlh1	Spermatogenesis and oogenesis helix-loop-helix1
STWS	Scott's tap water solution
TEM	Transmission electron microscopy
TF	Transcription factor
TGF	Transforming growth factor
TIMP	Tissue inhibitor of MMP
TNF	Tumour necrosis factor
TNFRS	Tumour necrosis factor receptor superfamily
Trk	Tropomyosin-related tyrosine kinase
TSA	Tyramide signal amplification
TZP	Transzonal projection
ZP	Zona pellucida

# **Chapter 1**

## **General introduction**

## 1.1. Introduction

During the 17<sup>th</sup> century it was postulated that babies grew from a miniature man (or homunculus) found inside the head of sperm. In the 350 years or so that have since passed, our knowledge and understanding of reproduction has increased greatly, yet there is still much to be learnt and dogmas to be challenged. Early forays into speculating on mechanisms of reproduction fixated on sperm as the crucial component. This is probably understandable, given that ovulation is not externally visible, but production of semen is quite apparent. Research has revealed that in fact sperm are not as essential as an oocyte which can support embryo development. This was demonstrated when the first viable parthenogenetic mouse from two maternal genomes was produced (Kono *et al.*, 2004).

Research has also unveiled aspects of female reproductive physiology that our knowledge of is now taken for granted. Some of these include uncovering: the cyclical changes that occur in ovarian tissue; the influence of gonadotrophin hormones upon these cycles; and the follicles containing the oocyte and somatic cells that are the linchpin of this endocrine system. These discoveries and more, have contributed greatly to understanding how a mature, fertilizable oocyte is produced and, where possible, how fertility disorders may be treated. However, the nature of science has taught us that there are usually more questions than answers. Currently, there are groups focusing their research on the early development of the ovary. Of particular interest is how and what factors regulate formation of the finite complement of primordial follicles, how are sufficient numbers preserved to maintain fertility, how are some activated to the growth phase and of these, why are some supported and some die. The focus of this thesis is neonatal mouse ovary development and the role of TrkB signalling in it.

## 1.2. Embryonic ovary

The vertebrate gonad forms as part of the genitourinary tract. This comprises the organs responsible for the production, storage and release of urine, and the internal and external reproductive organs. The genitourinary tract forms from mesoderm and endoderm, although it is known that the internal reproductive organs form from intermediate mesoderm (Little *et al.*, 2007). The tract is sexually undifferentiated until week 8 of pregnancy in the human and 11.5 days post coitum (commonly referred to as embryonic age E11.5) in the mouse. Thus, the tract follows an identical process of formation in both sexes until the sex is phenotypically determined by genetic factors. The developing gonads are attached to the mesonephros (early evolutionary kidney) and consist of the coelomic epithelium, covering the gonad, and an underlying ridge of mesenchyme which will accommodate the primordial germ cells (PGCs). There are also ducts associated with the gonads which will go on to form the reproductive tracts (Wolffian in male, Müllerian in female).

### 1.2.1. Primordial germ cells to oogonia

The male and female gametes (sperm and oocyte respectively) are products of a series of differentiation events that can be charted from E6.25 in mouse development. This is the earliest observable point of segregation of the precursor primordial germ cells (PGCs) from the extraembryonic mesoderm (Ohinata *et al.*, 2005). The precursor cells differentiate to form a population of lineage-restricted PGCs. The PGCs then migrate from the allantois of the embryo towards the developing gonadal ridges, using amoeboid-like locomotion (Blandau *et al.*, 1963) and are guided by responding to paracrine signalling molecules (Hoyer *et al.*, 2005). The PGCs rapidly proliferate as they migrate and continue to do so once the gonad is reached. The gonad is colonized by PGCs at E10.5-E11.5 before they

lose their motile behaviour. Many genes are important in PGC specification, proliferation and migration have been characterised through transgenic manipulation of the mouse (Rodrigues *et al.*, 2008).

Male differentiation begins with the expression of the male-specific *Sry* gene (located on the Y chromosome). *Sry* upregulates expression of anti-Müllerian hormone (AMH; also referred to as Müllerian inhibiting substance, MIS) from the testis, causing regression of the female Müllerian duct. *Sry* also upregulates expression of testosterone, which is then secreted by the testis. Testosterone supports the survival and development of the Wolffian duct into the epididymis and vas deferens. *Sry* is absent in females, thus AMH is not produced. Müllerian ducts are not regressed and can instead form the oviduct and uterus, the lack of testosterone results in regression of the Wolffian duct.

It was assumed that formation of the female tract was a default pathway, and would only form without active expression of male-specific genes. However, the proposal that formation of the female tract is also under positive genetic regulation is gaining more credence. For example, in the mouse, *Wnt4* is a signalling molecule produced by ovarian somatic cells at the time of sex determination (Vainio *et al.*, 1999). Newborn males null for this gene do not exhibit any deformities in reproductive tract development, but newborn females are distinctly masculinised (Vainio *et al.*, 1999). Thus, female-specific gene expression is required to ensure a female ovarian phenotype.

Once the sex of the gonad is determined, PGCs located in the developing ovary are then referred to as oogonia. The ovary is initially a collection of interspersed oogonia, epithelial and mesenchymal cells (Byskov, 1986). The proliferating oogonia eventually form clusters which longitudinally, are revealed to be cords extending from medulla to cortex. These cords are separated

by a basement membrane (BM) from the interleaving stroma (see Figure 1.1).



### 1.2.2. Oogonia to oocytes

The oogonia continue to undergo mitosis until they replicate DNA one last time and then enter the first phase of meiosis, prophase 1. Once an oogonium enters meiosis it is called an oocyte. It is the specific process of an oogonium entering meiosis and becoming an oocyte that I shall refer to as oogenesis (although the term is sometimes applied more loosely by others).

An intercellular connection (or “bridge”) is left connecting the oogonia to each other resulting from incomplete cell division during mitosis (Gondos and Zamboni, 1969). Through these connections organelles, cytoplasm and signalling molecules can be passed (Pepling and Spradling, 1998). The onset of meiosis can be observed to begin in distinct clusters of oogonia in the ovary, while there may still be other clusters continuing to undergo mitosis. Within individual clusters the oocytes are at the same stage of meiosis as each other, thereby suggesting that the “bridges” facilitate synchronised germ cell development (Gondos and Zamboni, 1969; Pepling and Spradling, 1998). They are, though, not required for fertility in female mice as *Tex14* null mice lack “bridges” between oocytes at birth but are fertile (Greenbaum *et al.*, 2009).

To reach the quiescent dictyate stage associated with oocytes in follicles, oocytes must progress through leptotone, zygotene, pachytene and diplotene stages before reaching the dictyate stage of prophase I. In the mouse the majority of oocytes have achieved this by the time of, or shortly after, birth. Entry into meiosis occurs cell-autonomously at ~E13.5 in the mouse (McLaren, 1984; Ohkubo *et al.*, 1996; McLaren and Southee, 1997). In contrast, oogonia also enter meiosis when exposed to the high levels of retinoic acid (RA) produced by the mesonephros and associated tubules in the female gonad (Bowles *et al.*, 2006). This latter finding fits in with the observation that a cortico-medullary boundary (see Figure 1.1) is formed in the ovary at the time oocytes enter meiosis, indicating that

this region and its gene expression potentially play a role in regulating entry to meiosis (Byskov, 1986; Byskov *et al.*, 1997).

Other examples of locally produced factors contributing to the regulation of meiosis exist. For example prior to entering meiosis the tyrosine kinase receptor (KIT) is expressed by mouse and human oocytes and its ligand (Kit ligand; KL, also called stem cell factor) is expressed by supporting somatic cells. Expression of both components is downregulated at meiosis and both are upregulated once the diplotene stage of prophase I has been reached (Driancourt *et al.*, 2000; Hoyer *et al.*, 2005).

The KIT/KL signalling loop is an example of paracrine signalling and evidence of how its expression may be regulated at meiosis is found in the human fetal ovary. Prior to follicle formation, activin (a member of the transforming growth factor- $\beta$  family; TGF- $\beta$ ) is expressed in oocytes but the downstream signaling components (Smad2) are expressed in somatic cells (Coutts *et al.*, 2008). Thus, activin must exert its effects on somatic cells and, indeed, was found to reduce the amount of KL produced by human fetal somatic cells in culture. Coutts *et al.*, (2008) also used immunohistochemistry to reveal that KIT is downregulated in oocytes when activin is elevated. This allowed speculation that activin, secreted by oocytes, reduces KL production by granulosa cells which in turn reduces KIT expression by the oocyte. Oogonia are then able to progress through prophase I of meiosis and form primordial follicles, which do not express activin, therefore allowing the expression of KIT to return (Coutts *et al.*, 2008).

The period of oogenesis in the developing mouse ovary continues until approximately E16.5, by which point nearly, if not all, oogonia have at least entered meiosis (Peters, 1970). Progression to the diplotene stage is required for oocytes to form follicles as demonstrated by interfering with the meiotic machinery involved. Synaptonemal complex protein-1 (SCP-1) is a protein which

maintains the close association of paired chromosomes recombining during the pachytene stage of prophase I. SCP1 expression disappears at entry to the diplotene stage and this disappearance precedes follicle formation. It was demonstrated that prematurely and artificially downregulating SCP-1 by using antisense RNA results in precocious entry to the diplotene stage and early follicle formation (Paredes *et al.*, 2005). Expression of the RNA binding protein deleted in azoospermia-like (Dazl) is also implicated in this process. The ovaries of mice null (knockout; KO) for *Dazl* appear normal at E15 and enter meiosis, but arrest so that by E19 only a few oocytes remain, with the rest dying (Ruggiu *et al.*, 1997). Follicles are completely absent by adulthood although the remaining somatic cells maintain normal steroidogenic activity (McNeilly *et al.*, 2000). This result, along with the expression of Dazl protein in wild-type oocytes throughout oocyte and follicle development (Ruggiu *et al.*, 1997), indicates a direct requirement for *Dazl* expression in oocytes to reach the dictyate stage of meiosis: if oocytes cannot reach this stage, they cannot direct folliculogenesis and die.

Oocytes arrested in dictyate state maintain it throughout the rest of follicle growth and development. If growing follicles are not lost to atresia, which most are, meiosis is resumed upon ovulation, the oocyte undergoes a second arrest and meiosis is only actually completed upon fertilisation.

### **1.3. Perinatal ovary development**

The next defined stage of ovarian development in the rodent is the process of folliculogenesis which occurs around the time of birth. As the term folliculogenesis is also another term used differently by different authors (often to describe the growth of follicles) I shall define folliculogenesis specifically to be the formation of primordial follicles, and not to refer to any later stages of follicle development. Folliculogenesis is reported in the literature to occur within either the last days of embryogenesis immediately prior to birth (mouse) or within the first two to three postnatal days (rat). However, there is enough variation in the observed timing of folliculogenesis in published material to make it difficult to determine whether or not such strict boundaries can be defined.

#### **1.3.1. Primordial follicle formation**

The precise molecular and cellular mechanisms by which formation of primordial follicles occurs are not fully understood. It is known, however, that the purpose of this process is to ensure oocyte survival and this is achieved by the oocyte gaining somatic cell support through formation of a primordial follicle. A primordial follicle is an oocyte surrounded by a single layer of flattened, non-proliferating somatic pregranulosa cells.

Although the formation of primordial follicles is not fully understood for all mammals, there are some basic observations that hold true for this process across species: oocyte number is dramatically reduced; oocyte clusters fragment and form closer associations with pregranulosa cells; follicles first form at the boundary of the ovarian cortex and medulla; and a BM is laid down around the follicle to separate it from the surrounding stroma (see Figure 1.2).



It has been proposed that primordial follicles in the rat ovary do not lay down a BM until the pregranulosa cells are opposed by a layer of mesenchymal prethecal cells, only then are they truly a primordial follicle (Rajah *et al.*, 1992). Similarly, in the fetal sheep ovary it is also postulated that colonisation of the ovary by streams of mesenchymal cells from the mesonephros contribute to follicle formation (McNatty *et al.*, 2000).

The initial mixing of cell-types (epithelial and mesenchymal) in the ovary has complicated which lineages could contribute to the supporting somatic granulosa cells. Extremely detailed histological studies of the ovary at follicle formation in the rat (Rajah *et al.*, 1992) and the sheep (Sawyer *et al.*, 2002) demonstrate that pregranulosa cells are found alongside the oogonia within the cords and these are likely to be the source of supporting granulosa cells in the primordial follicles. As both rat and sheep cords are open and continuous with the ovarian surface epithelium (OSE) but enclosed by BM at the medulla, it is possible to speculate that the source of these cells within the cords could include the OSE.

Sawyer *et al.* (2002) propose that one way primordial follicles could form is by oocyte and pregranulosa cells “budding off” from cords at the medulla. An alternative mechanism whereby oocytes within clusters undergo apoptosis has been suggested (Pepling and Spradling, 2001). This facilitates fragmentation of the cluster by allowing easier access of somatic cells to the surviving oocytes. It has also been postulated that surviving oocytes benefit from the deaths of others by gaining nutrients and there is also the aspect of reduced competition for growth factors.

Evidence demonstrating that apoptosis of oocytes within clusters contributes to follicle formation has been shown by the influence of hormones. Treatment of neonatal female mice with genistein, a synthetic estrogen (Jefferson *et al.*, 2006), results in reduced primordial follicle formation. As does estradiol, progesterone

and genistein treatment of neonatal mouse ovaries in culture (Chen *et al.*, 2007). These studies reveal a reduced rate of apoptosis and increased oocyte survival; consequently there is reduced oocyte cluster breakdown and increased numbers of multi-oocyte follicles in response to hormone treatment. This led to the hypothesis that *in utero* exposure to maternal estrogen prevents precocious follicle formation but at birth the sharp decrease in hormones triggers oocyte cell death in the clusters, allowing follicle formation. Prenatal formation of primordial follicles (as occurs in humans and baboons, for example) takes place in the presence of maternal estrogens, thus, the effects of hormone signalling on this process cannot be the same across species. In fact, reducing estrogen levels during pregnancy in baboons reduces primordial follicle formation in the fetus (Zachos *et al.*, 2002; Pepe *et al.*, 2006). This information, combined with the finding that estrogen receptors are synthesised in human fetal ovaries at the time of follicle formation (Fowler *et al.*, 2009) implies that maternal/fetal estrogens are actually required for primordial follicle formation in these species. In addition, it has been demonstrated that effects of estrogen are mediated through a G-protein coupled receptor (GPCR) in hamster ovary to promote primordial follicle formation (Wang *et al.*, 2008). Interfering with the receptor by siRNA prevented this promotion of follicle formation. It, therefore, may be possible to speculate that mice are unique in forming follicles in response to a decrease in estrogen levels as other species demonstrate a requirement for it.

Whichever way it is achieved, it is of paramount importance to oocytes that they acquire associations with pregranulosa cells; once they do, the support provided is sufficient to maintain the follicle in a quiescent state throughout the female's reproductive life, which can be decades in the human (Maheshwari and Fowler, 2008).

### 1.3.2. Gonadotrophin independent follicle growth

Instead of remaining quiescent some follicles immediately enter the growth phase upon formation. This usually occurs at the medulla of the ovary and across species it is the norm to see a gradient of follicle development, with clusters of quiescent primordial follicles in the cortex and growing follicles in the medulla (Rajah *et al.*, 1992; Sawyer *et al.*, 2002). The factors regulating entry of follicles to the growth phase are not yet completely understood. However, it is clear that there are a panoply of factors at work, on morphological and genetic levels.

In the mouse ovary, regulation of growth initiation can be partially attributed to the boundary between the cortex and the medulla (Byskov *et al.*, 1997; see Figure 1.1). *In vitro* culture of embryonic mouse ovaries revealed that failure to form a cortico-medullary boundary resulted in all follicles commencing growth, however, if the boundary is formed then only a specific number of follicles will activate growth at the boundary (Byskov *et al.*, 1997). Ultimately this was found to be dependant on a sufficient population of oocytes in the ovary, leading Byskov *et al.* (1997) to propose that the growing follicles in the medulla require contact with non-growing follicles in the cortex to regulate the size of the cohort recruited and prevent premature follicle activation.

The physical features of each stage of growth have been described thoroughly; initially one or two of the flattened granulosa cells become cuboidal in shape, indicating differentiation has occurred (a transitional follicle). The change in appearance is used as the hallmark of granulosa cell proliferation and activation of follicle growth. This first phase of follicle growth is considered slow compared to the later gonadotrophin dependent stages. It can take rodents weeks and larger species months for follicles to progress to the primary (one complete layer of cuboidal granulosa cells),

secondary (two layers) and preantral stages (multiple layers; see Figure 1.2).

The follicles prepare themselves for entry to the gonadotrophin dependant stage by becoming semi-responsive to available hormones but there is compelling evidence that follicle growth does not require gonadotrophins up until antrum formation. Follicle stimulating hormone (FSH) and luteinising hormone (LH) are released from the anterior pituitary under the action of the gonadotrophin-releasing hormone (GnRH). Mutation of the gene encoding GnRH in the *hypogonadal (hpg)* mouse prevents release of GnRH from the hypothalamus which in turn, ablates FSH and LH levels (Halpin *et al.*, 1986) resulting in *hpg* mice lacking antral follicles. Prevention of FSH $\beta$  subunit expression (Kumar *et al.*, 1997) or of FSH receptors in the mouse by mutation (Abel *et al.*, 2000) also stops follicles from progressing beyond the preantral stage.

Independence of these systemic hormones indicates that other factors must be involved in initiating and supporting early follicle growth. One such factor is the ability of the cells to communicate with each other. Gap junctions are cell-to-cell points of contact forming channels which allow second messengers, small metabolites and inorganic ions to pass. The junctions are formed by each cell presenting one half on the channel (a connexon). Each connexon is made up of six protein subunits from the connexin family, of which there are fifteen members. Connexin43 (encoded for by the gene *Gja1*) is important in follicle growth although mutation in mice (*Gja1* KO) is neonatal lethal. Heterotopic transplantation of the ovaries for three weeks revealed that the follicles block at the primary follicle stage of growth, or secondary stage depending on strain background (Ackert *et al.*, 2001). Connexin43 protein is expressed between the pregranulosa cells of primordial follicles from P1 (Juneja *et al.*, 1999) allowing Ackert *et al.* (2001) to hypothesise that its expression is required in gap junctions for granulosa cell proliferation in early

follicles. However, it was also demonstrated that the germ cell population of the *Gja1* KO is reduced in the E11.5 and E17.5 gonads (Juneja *et al.*, 1999). Although the evidence is compelling that subsequent growth of surviving follicles is affected in *Gja1* KOs, the possibility that the already depleted population of oocytes transplanted may have altered the intraovarian signalling, and therefore the outcome of the transplants, was not addressed.

The factors considered most likely to be activators of follicle growth are those that stimulate, promote and maintain granulosa cell proliferation.

The TGF- $\beta$  superfamily receives much attention as many of its members facilitate roles in ovarian function (Shimasaki *et al.*, 2004; Drummond, 2005; Knight and Glister, 2006). One such member is growth differentiation factor-9 (GDF-9) which is normally expressed in the oocyte from primary follicle stage onwards. Expression of this growth factor is required for follicle growth past the primary stage as ovaries from mice unable to express the protein are blocked at this point due to granulosa cells unable to proliferate (Dong *et al.*, 1996). Other oocyte expressed TGF- $\beta$  superfamily members shown to be involved are members of the bone morphogenetic protein family (BMP-15 and BMP-7). A natural mutation occurring in the sheep homologue of *Bmp-15* (*FecX*<sup>l</sup>) results in blocked follicle growth at the primary stage (Galloway *et al.*, 2000). In addition, although mice null for *Bmp-15* did not show an effect on fertility (Yan *et al.*, 2001) rat granulosa cells co-cultured with oocytes and BMP-15 did show an increase in mitosis (Otsuka and Shimasaki, 2002). BMP-7 stimulates rat granulosa cell proliferation and promotes the primordial to primary follicle transition *in vivo* (Lee *et al.*, 2001b).

In contrast to the positive regulation by these oocyte-expressed TGF- $\beta$  superfamily members described so far, it has been found that the member, AMH, has a role as a repressor of primordial follicle recruitment. AMH protein is expressed in the granulosa cells of

secondary follicles onwards (Durlinger *et al.*, 2002) and mice null for the factor exhibited precocious follicle activation such that at four months significantly more preantral follicles were found with a concomitant reduction in the number of primordial follicles retained (Durlinger *et al.*, 1999). Thus, it is proposed that AMH functions as a way for already growing follicles to prevent premature activation of quiescent follicles.

The KIT/KL pathway (already known to be important for PGC migration, oogonia survival, and entry to meiosis as discussed above), also seems to be a central mediator of primordial follicle activation through paracrine signalling (Driancourt *et al.*, 2000). The pattern of expression indicates that this would occur between the granulosa cells and the oocyte, and between the granulosa cells and the thecal layer. This is because mRNA and protein for the receptor KIT is found in oocytes from the diplotene stage onwards and mRNA in the thecal layer (Manova *et al.*, 1990). However, mRNA for the ligand, *KL*, is contrastingly expressed in granulosa cells from the primary stage onwards (Manova *et al.*, 1993). Evidence for a role in follicle activation can be found: KL stimulates oocyte growth (Reynaud *et al.*, 2000), added to ovarian culture it promotes follicle activation (Nilsson and Skinner, 2004), it has a mitogenic effects on rat granulosa cells in culture (Otsuka and Shimasaki, 2002); and if the murine *KL* gene is mutated or if signalling is blocked (either by injecting pups with neutralising antibody or by adding it to mouse ovary culture) primordial follicle activation is reduced (Yoshida *et al.*, 1997; Driancourt *et al.*, 2000).

The KIT/KL pathway can also regulate and be responsive to the expression of other factors. *KL* mRNA is increased in the granulosa cells of *Gdf-9* KO mouse ovaries (Elvin *et al.*, 1999), possibly explaining the continuation of oocyte growth observed in the null ovaries even though the granulosa cells do not proliferate.

BMP-15 stimulates *KL* mRNA expression in granulosa cells co-cultured with oocytes (Otsuka and Shimasaki, 2002). However, KL added to the co-culture results in a dose-dependent reduction in *Bmp-15* mRNA oocyte expression. This suggests that there is a negative feedback loop between the two. In addition, although BMP-15 stimulates granulosa cell proliferation *in vitro*, blocking the KIT receptor on the oocyte with an antibody, prevents the mitotic effect of BMP-15 on granulosa cells, leading Otsuka and Shimasaki (2002) to speculate that the effect on mitosis seems to be mediated by the KIT receptor.

As well as members of the TGF- $\beta$  superfamily, the KIT/KL pathway also interacts with other growth factors implicated in the primordial to primary activation. Basic fibroblast growth factor (bFGF) is detected in the oocytes of primordial follicles, the granulosa cells of growing follicles and upregulates primordial follicle activation (Nilsson et al., 2001). The interactions of bFGF and the KIT/KL pathway were tested using a P4 rat ovary culture system. Addition of a bFGF neutralising antibody abrogated the KL induced activation, and addition of a KIT receptor blocking antibody prevented the bFGF induced activation (Nilsson and Skinner, 2004) demonstrating that both pathways contribute to follicle activation. While KL had no effect on *bFGF* mRNA levels in cultured ovaries, bFGF did increase *KL* mRNA.

In a similar manner the role of the cytokine leukaemia inhibitory factor (LIF) on follicle activation was assessed (Nilsson et al., 2002). Normally expressed in the somatic cells of quiescent and primary follicles, LIF promotes the levels of primordial follicle activation in P4 cultured rat ovaries and a LIF neutralising antibody abrogates the effect. LIF can also increase the amount of *KL* mRNA granulosa cells can produce *in vitro*, therefore Nilsson et al. (2002) hypothesise that LIF may promote activation through regulation of *KL* expression.

Thus, hormones and growth factors produced by granulosa cells exert effects on follicle assembly and growth, but the oocyte is considered to be the master organiser of these effects (Eppig, 2001). There is now strong evidence that oocyte-specific transcription factors (TFs) regulate expression of some of the genes just discussed and their mutation results in oocyte loss through improperly regulated primordial follicle formation, follicle activation and oocyte survival (Suh *et al.*, 2002; Skinner, 2005; Pangas and Rajkovic, 2006; Pepling, 2006; Rodrigues *et al.*, 2008).

Null mutation of a gene encoding murine newborn ovary homeobox TF (*Nobox*) results in oocyte loss by P14 (Rajkovic *et al.*, 2004). Wild-type and null mice have comparable numbers of oocytes and primordial follicles on the day of birth, although by P7 it is apparent that complete follicle formation, activation and subsequent growth is affected with more oocyte clusters found in the KO than in the wild-type ovary. Downregulation of the TGF superfamily members, *Gdf-9* and *Bmp-15*, in *Nobox* null mice indicates how the mutation may cause the phenotype.

As well as the BM deposited around the follicle another form of extracellular matrix (ECM) is formed around the oocyte. This is referred to as the zona pellucida (ZP) and it is instrumental in mediating fertilisation and preventing polyspermy from occurring. ZP mRNA is expressed in newborn mouse ovaries (Soyal *et al.*, 2000) and in human ovaries the protein is expressed in primordial follicles onwards (Gook *et al.*, 2008). Rajkovic *et al.* (2004) demonstrated that expression of ZP proteins is not affected in the *Nobox* null mutant although it is when another TF (murine factor in the germ line, alpha; *Figla*) is mutated (Soyal *et al.*, 2000). *Figla*, a helix-loop-helix TF, is specifically expressed in oogonia and oocytes in the neonatal ovary peaking around the time of birth in the mouse. *Figla* mutation results in almost complete loss of oocytes by P1 as oocytes are unable to form primordial follicles (Soyal *et al.*, 2000). It is proposed by

Soyal *et al.* (2000) that this effect on primordial follicle formation is attributable to oocyte inability to recruit supporting somatic cells, resulting in oocyte apoptosis, rather than lack of ZP.

Recent studies have revealed that these TFs are under the control of another oocyte-specific TF important for ovary development (spermatogenesis and oogenesis helix-loop-helix1; *Sohlh1*). Pangas *et al.* (2006) examined the ovaries from *Sohlh1* null mice; in addition to reduced primordial follicle activation and subsequent oocyte loss, mRNA transcripts for *Nobox* and *Figla* are both reduced, as are the genes they regulate (*Gdf-9*, *Bmp-15* and *ZP*). However, NOBOX did not co-immunoprecipitate with *Sohlh1* indicating that it is not a direct target of *Sohlh1*. Transcripts of another TF (LIM-homeobox protein 8; *Lhx8*) were significantly reduced in the *Sohlh1* KO at the time of birth (Pangas *et al.*, 2006). *Lhx8* is expressed in a similar pattern to *Sohlh1* and KO mice have a similar phenotype culminating in oocyte loss (Choi *et al.*, 2008). The newborn *Lhx8* KO is shown to have reduced mRNA for *Figla* (and consequently the *ZP* genes), *Nobox* (and consequently *Gdf-9*) and both *Kit* and *KL* (Choi *et al.*, 2008). Thus, it is demonstrated that *Sohlh1* can exert transcriptional regulation over *Lhx8* but it is *Lhx8* which can regulate the expression of growth factors important in follicle formation and activation through regulating *Figla* and *Nobox* expression and by stimulating *Kit/KL* expression.

These examples demonstrate the myriad of factors within the ovary that are acting in concert to repress and promote follicle activation. Follicles that have commenced growth begin to prepare themselves for entering the gonadotrophin dependent stage, although this will not be possible until puberty. Only then will sufficient levels of gonadotrophins be released from the anterior pituitary (FSH and LH) which support full follicle growth and ovulation. Preparation for gonadotrophin dependence, and entry to the fast phase of growth, commences with FSH receptor (FSHR) synthesis by the granulosa

cells of secondary follicles. In addition, formation of the prethecal layer occurs at this time by cells derived from the mesenchyme orientating themselves against the follicular BM. LH receptor (*Lhr*) mRNA is found in the thecal layers of immature follicles (Camp *et al.*, 1991) but subsequent growth to the preantral follicle stage is considered to be responsive, but not dependant on gonadotrophins, as discussed above. Once the follicles become gonadotrophin dependent, the majority of follicles die as they do not receive sufficient support. The surviving follicles progress through subsequent stages of growth to the ovulatory stage as part of the reproductive cycle. The end of the preantral stage is characterised by formation of a fluid-filled cavity in the granulosa cells (antral follicle; see Figure 1.2). This leads to differentiation of mural granulosa cells (those found in the follicle wall) and the cumulus granulosa cells, found next to the oocyte.

The preovulatory (or Graffian) follicle is associated with final stages of growth. Up until this point, gonadotrophin support is utilised by the follicles in a two-cell model between the granulosa and thecal cells. The thecal layer expresses LHR, and under the effects of LH provides increasing amounts of aromatisable androgens in the form of testosterone to the granulosa cells of growing follicles where it is converted into estrogens. Granulosa cells of antral follicles also express LHR (Camp *et al.*, 1991). The follicles grow due to the proliferative action of FSH on granulosa cells and, thus, produce increasing concentrations of oestrogen and inhibin, the TGF- $\beta$  superfamily member. These hormones negatively act on the hypothalamic-pituitary axis to reduce FSH release thereby suppressing the growth of smaller follicles, but by this stage the larger follicles are able to withstand this reduction in FSH. Eventually in the cycle, oestrogen levels reach a point where it actually causes a surge in the release of gonadotrophins (a small FSH surge precedes a large LH peak mid-cycle). The surge in LH ruptures

the follicle wall and the oocyte is released. The remaining granulosa cells of the ovulated follicle luteinise and form a corpus luteum. This structure secretes progesterone, which supports pregnancy, should it occur. Each cycle a cohort of follicles is taken through the stages of antral and preovulatory development. How the primordial follicles are initially recruited from the pool is unclear, although it is eventually depleted resulting in senescence (see Figure 1.3). The steroidal control and the intraovarian factors regulating this process, and their interactions with each other, have been extensively reviewed (Baker and Spears, 1999; McGee and Hsueh, 2000; Hillier, 2001; Suh *et al.*, 2002; Gilchrist *et al.*, 2004; Barnett *et al.*, 2006; Drummond, 2006).



#### **1.4. Cell death in the developing mammalian ovary**

The factors regulating cell death of oogonia and oocytes in the developing mammalian ovary are not fully understood, although it has long been recognised to occur in rodents and human ovaries on a large scale (Beaumont and Mandl, 1962; Baker, 1963). Across mammals, cell death in the ovary during oogenesis is a key factor in regulating the ovarian reserve and ultimately the reproductive life-span of the female since the remaining germ cells have by then exited mitosis and entered meiosis.

Beaumont and Mandl (1962) characterized the embryonic and neonatal rat ovary in terms of germ cell number and morphology. Their observations suggest that morphologically distinct cell death events take place in waves at different points in ovary development. They also observed that the dying cells are removed within 24 hours, making this a rapid process, which is consequently difficult to observe. Phagocytosis of apoptotic oocytes by somatic cells is one possible way to remove the debris, and this has been observed in immature human ovaries (Baker and Franchi, 1967).

Morphologically, the cells dying bear the hallmarks of classic programmed cell death (PCD) referred to as apoptosis. These include condensed chromatin and cytoplasm, a wrinkled nuclear envelope, cell shrinkage and eventually the membrane of the cell breaks down so that smaller apoptotic bodies (blebbing) are formed but retained within a membrane. The controlled destruction of the cell, in particular the retention of the debris within a membrane, is thought to contribute to the anti-inflammatory nature characteristic of apoptosis. This is in contrast with another form of PCD, necrosis, which results in the loss of the plasma membrane inducing an inflammatory response.

The first wave of cell death occurs in the oogonia prior to meiosis, during meiosis and another as the oocytes reach the

diplotene stage (coincident with the formation of the primordial follicles; Beaumont and Mandl, 1962). These peaks have also been observed in mice (Coucouvanis *et al.*, 1993; Ghafari *et al.*, 2007) and a peak has been observed in human fetal ovary at the time of folliculogenesis (Fulton *et al.*, 2005). Although it has been proposed that there is a basal level of loss of oogonia and oocytes during embryogenesis with a peak during meiosis and at follicle formation (McClellan *et al.*, 2003). The loss of oocytes continues into the neonatal period in rodents, with oocyte number only stabilising from P6/7 (Kerr *et al.*, 2006; Rodrigues *et al.*, 2009).

The occurrence of a peak in apoptosis at entry to meiosis is open to speculation although there is evidence that apoptosis is occurring in PGCs/oogonia prior to entry to meiosis. One mechanism postulated to account for cell death is the failure of germ cells to obtain sufficient amounts of growth factors from supporting somatic cells. This would be a useful mechanism, allowing regulation of surviving cell number within a structure, such as the ovary, and could promote the apoptosis of any cells that have migrated incorrectly to an area without trophic support. For example, when murine PGCs from E12.5 embryos are cultured *in vitro* for 4-5 hours almost half of them exhibited signs of apoptosis. Addition of the oocyte growth promoting cytokine KL, significantly reduced the percentage of PGCs exhibiting an apoptotic marker (Pesce *et al.*, 1993) although this effect was not found over longer culture periods. In addition, mice heterozygous for *Kit* exhibited increased oocyte apoptosis in E15.5 mouse embryos (Driancourt *et al.*, 2000).

The aryl-hydrocarbon receptor (AhR) is a basic helix-loop-helix TF proposed to be a mediator of prenatal germ cell apoptosis. *In vitro* culture of embryonic ovaries without the support of serum normally results in elevated levels of apoptosis. E13.5 *Ahr* KO ovaries, however, exhibit more non-apoptotic germ cells when cultured without support than wild-type ovaries, demonstrating that its

expression would normally contribute to germ cell death under those conditions (Robles *et al.*, 2000). The next peak of apoptosis is proposed to occur during meiosis (at the pachytene stage in particular); it may be that this is a way of removing oocytes with abnormal chromosomes. The Bcl2-associated X protein (BAX) is a component of the pro-apoptotic pathway. The function of BAX has been investigated in relation to apoptosis occurring during meiosis. Consistent with BAX's apoptotic properties, mice null for the *Bax* gene contain approximately twice as many oogonia as wild-type ovaries at E14.5 (Alton and Taketo, 2007). However, upon entering meiosis, and up to its completion, the proportion of oocytes lost in *Bax* KO mice was comparable with the proportion lost in wild-type ovaries, thus oocyte apoptosis occurring during meiosis is not regulated by BAX.

The prevention of prenatal oocyte apoptosis (as demonstrated by the *Ahr* and *Bax* KO mice) results in increased numbers of oocytes which are maintained during folliculogenesis. *Ahr* KO ovaries contain significantly more primordial follicles than wild-types at P4 (Robles *et al.*, 2000) and *Bax* KO ovaries contained significantly more cells stained for an oocyte marker (mouse vasa homologue, MVH) than *Bax* heterozygotes at P5 (Alton and Taketo, 2007).

Likewise disruption of pro-survival genes such as *Bcl2* (B-cell lymphoma/leukemia 2) have an effect on oocyte and follicle number. Null expression of *Bcl2* in the mouse results in a reduced primordial follicle pool, demonstrating that oocytes do require protection from apoptosis, although the mice are still fertile reiterating that apoptosis is not the only mediator of neonatal oocyte loss (Ratts *et al.*, 1995). The opposite could also be induced in mice by overexpressing *Bcl2*, the ovaries contain increased numbers of primordial follicles, indicating that more oocytes than normal are saved from an apoptotic fate (Flaws *et al.*, 2001).

There are studies, though, that are beginning to emerge to suggest that apoptosis is not the only mechanism of oocyte loss, in fact some actively proclaim that it is not associated with the loss of primordial follicles (Tingen *et al.*, 2009). As discussed in Section 1.2.2, loss of oocytes can be associated with failure to reach the diplotene stage of meiotic prophase I. Observations made in wild-type mice have provided additional support for this hypothesis. Oocytes that had not reached the diplotene stage (as demonstrated by continued germ cell nuclear antigen expression; GCNA) were observed to be extruded through the OSE of neonatal mouse ovaries (Kerr *et al.*, 2006). Thus, it is postulated to be a mechanism which actively contributes to reduction in oocyte number in the ovary during the neonatal period (Wordinger *et al.*, 1990).

Other forms of PCD are active during oocyte cell death *in vivo* and *in vitro*, such as caspase-independent pathways (Lobascio *et al.*, 2007) and autophagy (Lobascio *et al.*, 2007; De Felici *et al.*, 2008; Rodrigues *et al.*, 2009).

Thus, it can be concluded from the literature that although apoptosis was considered to be the main mechanism of cell death in the developing ovary, it is not the only way to remove oocytes and its blockade is not the only way to save them. Therefore, there are multiple pathways which can regulate oocyte death in the developing ovary. However, it would seem manifest that fate of the germ cell depends on the stage of differentiation it is in and the stimulus received. Cell death in the developing ovary and subsequent cell death associated with atresia of growing follicles has been extensively reviewed (Tilly, 1996; McGee *et al.*, 1998; Morita and Tilly, 1999; Reynaud and Driancourt, 2000; De Felici *et al.*, 2005; Krysko *et al.*, 2008).

## 1.5. Neurotrophins and Trk receptors

The neurotrophins are a family of polypeptide growth factor molecules which bind to a family of tyrosine kinase receptors (TrkA, B and C). The ligands are named for their characteristic role in the development and nourishment (troph) of the nervous system (neuro; neuron). They are released from neuronal targets in the developing nervous system to promote growth of the innervating neurons. In some instances there is competition for these neurotrophins, and without sufficient levels the neurons competing for them may succumb to apoptosis. Hence they are best known for their roles in neuronal growth and survival. There is a very large field of literature available on neurotrophin and Trk research (the number of available publications through pubmed returning hits for “neurotrophins” is 34,567, for “TrkB” alone it is 2,136 hits). Considering this, I have decided to focus the majority of the introduction on Trk signalling to TrkB and its ligands.

### 1.5.1. Neurotrophins: the ligands

The neurotrophin nerve growth factor (NGF) was first discovered by Stanley Cohen and Rita Levi-Montalcini in the 1950's. They were awarded the 1986 Nobel Prize in Physiology or Medicine, and the discovery of NGF was followed by that of other members of the same family. These include brain derived neurotrophic factor (BDNF) and neurotrophins 3 and 4 (NT3 and NT4). More recently NGF-like neurotrophins 6 and 7 have been discovered but it is not yet apparent if they have significant signalling roles in mammalian development and they are, therefore, not discussed further here (Gotz *et al.*, 1994; Lai *et al.*, 1998).

Neurotrophins are initially synthesised in an immature form called proneurotrophins. Once considered to be inactive, these precursors are actually capable of signalling via the p75 receptor which they bind to with high affinity (Lee *et al.*, 2001a). The mature

neurotrophins are formed by the action of convertases (enzymes responsible for the cleavage of proteins) including members of the furin serine proteinase family (Seidah *et al.*, 1996). Mature ligands form homodimers which then bind to the p75 receptor with low affinity and the Trk receptors with high affinity (Lewin and Barde, 1996; Lu *et al.*, 2005).

### **1.5.2. Trks: the receptors**

Although the first ligand (NGF) was discovered several decades ago, it wasn't until much later that the receptor for this family of growth factors was identified. The *Trk* locus encoding the receptors was first recognised in humans when it transformed into an active oncogene in a colon carcinoma through fusion with tropomyosin (Martin-Zanca *et al.*, 1986). This allowed identification of three tropomyosin-related tyrosine kinase receptors: TrkA, TrkB and TrkC. These receptors are comprised of an extracellular binding domain, a transmembrane domain and an intracellular region containing the tyrosine kinase domain. The extracellular sequences are distinct between the receptors allowing them to bind, with high specificity, their respective ligands. NGF binds to and activates TrkA, BDNF and NT4 to TrkB and NT3 to TrkC (Dechant, 2001). It has been found in some *in vitro* models that NT3 can activate TrkA and TrkB, although with less affinity than for TrkC (Ip *et al.*, 1993; Barbacid, 1994; Davies *et al.*, 1995).

Each Trk receptor exists in a full-length form which expresses the intracellular tyrosine kinase domain responsible for triggering the downstream signalling cascade. Some additionally express truncated receptors, which do have an intracellular domain although it does not contain the tyrosine kinase domain (see Figure 1.4).

Neurotrophin homodimers bind to the Trk receptor, causing it to dimerise (Jing *et al.*, 1992) resulting in phosphorylation of cytoplasmic tyrosine residues and activation of the kinase domain

(Klein *et al.*, 1991). The signalling cascades are initiated by adaptor molecules and/or enzymes attaching to the phosphorylated tyrosine residues (Schlessinger and Ullrich, 1992). Due to the sequence homology of the intracellular sequences across the Trk receptor family, the downstream signalling cascades initiated can be the same, although activated by different ligands, see Figure 1.5 (Patapoutian and Reichardt, 2001; Huang and Reichardt, 2003; Segal, 2003; Reichardt, 2006).





### 1.5.2.1. Truncated Trk receptors

The truncated Trk receptors are able to bind neurotrophins, as they express the extracellular binding domains, however, they lack the intracellular tyrosine kinase activity required to transduce intracellular signals (see Figure 1.4). Thus, truncated receptors were considered to be incapable of downstream tyrosine kinase signalling. At odds with this seeming lack of function considerable upregulation of truncated receptor isoform expression was observed in chicken and mouse neuronal and non-neuronal tissues during embryogenesis (Escandon *et al.*, 1994). The reasons for this remain unclear, although possible functions for these truncated receptors have since been postulated. It was suggested that where ligand is of limited availability the truncated receptors could compete with the full-length receptors for their respective ligands. Unable to transduce a signal effectively makes them negative regulators of the activity of the pathway (Biffo *et al.*, 1995). A more subtle effect was also considered plausible as truncated receptors could theoretically act as “mops” to bind excess signal without over-stimulating the signalling pathway. In addition the ability of full-length TrkB and its truncated isoforms to dimerise together was postulated to result in inhibition of downstream signalling (Eide *et al.*, 1996). However, it has actually been demonstrated that these truncated receptors are capable of some forms of immediate cell signalling.

Rose *et al.* (2003) assessed the action of BDNF on cultured astrocytes and neurons through full-length and truncated TrkB receptors (T1 and T2). Expression pattern analysis revealed the detection of full-length receptors to be high in neurons and low in astrocytes but the reverse was true for truncated (T1) receptors. Application of BDNF results in Ca<sup>2+</sup> release from astrocytes and neurons but when a Trk inhibitor (K252a) is applied it only blocks BDNF-induced Ca<sup>2+</sup> release in neurons, not in astrocytes. K252a inhibits tyrosine kinase activity of Trk receptors by prevention of

autophosphorylation of intracellular tyrosine residues (Ohmichi *et al.*, 1992; Tapley *et al.*, 1992). Thus, BDNF must be inducing  $\text{Ca}^{2+}$  release in the astrocytes by activation of truncated receptors. The evidence for truncated TrkB signalling is strengthened by the finding that astrocytes from mice mutant for the full-length receptor release  $\text{Ca}^{2+}$  in response to BDNF as well as astrocytes from mice expressing the full-length receptor (Rose *et al.*, 2003).

These results indicate two separate signalling pathways activated by BDNF binding. The known signalling pathway in neurons consists of BDNF binding to full-length TrkB receptor, opening  $\text{Na}_v1.9$   $\text{Na}^+$  channels depolarising the cell. This causes voltage-gated  $\text{Ca}^{2+}$  channels to open resulting in  $\text{Ca}^{2+}$  influx (Blum *et al.*, 2002). Blocking  $\text{Na}^+$  channels prevents BDNF-induced  $\text{Ca}^{2+}$  signal in neurons but not astrocytes. The alternative mechanism was shown to involve the  $\text{Ins}(1,4,5)\text{P}_3$  pathway that activation of (through ligand binding) results in  $\text{Ca}^{2+}$  release from intracellular stores. Inhibition of this pathway significantly reduces BDNF-induced  $\text{Ca}^{2+}$  signal in astrocytes. What has not been answered is how truncated receptors initiate the signalling cascade required to activate the  $\text{Ins}(1,4,5)\text{P}_3$  pathway when they lack the tyrosine kinase domain.

### **1.5.3. p75: the receptor**

Widely considered to be a receptor that signals death to the cell p75 is a member of the tumour necrosis factor receptor superfamily (TNFRS). Each mature neurotrophin has a similar affinity for p75 as the recognition domain is highly conserved across neurotrophins (He and Garcia, 2004). The affinity mature forms of neurotrophins have for p75 is low; in contrast, immature forms of neurotrophins (proneurotrophins) bind to and activate p75 receptors with high affinity, see Figure 1.5 (Lee *et al.*, 2001a; Woo *et al.*, 2005). Transduction of proneurotrophin signals are dependant on

interactions of p75 with co-receptor sortilin, a type I transmembrane protein (Bronfman and Fainzilber, 2004; Teng *et al.*, 2005).

In addition to neurotrophin-p75 interactions there are also different receptor-receptor associations which can alter neurotrophin affinity (identified by co-immunoprecipitation techniques). Trk and p75 receptors can interact directly to form a complex able to increase ligand specificity; in particular TrkB was demonstrated to have an increased specificity for BDNF when the TrkB-p75 complex was formed (Bibel *et al.*, 1999). Truncated TrkB receptors are also able to interact with p75 (Hartmann *et al.*, 2004). Structural analysis of crystalline NGF homodimers showed that they have the potential to recruit both p75 and TrkA receptors into trimolecular multiplexes (He and Garcia, 2004). With evidence for the existence of p75 isoforms (Schor, 2005), these findings combined reveal how difficult it may be to predict the outcome of neurotrophin signalling.

Overall it appears that the end effect on the cell is dependent on the type(s) of receptor present, the form of the neurotrophin, and ultimately the cell type receiving the signal. Clearly the dogma that Trk receptors solely mediate cell survival and p75 receptors solely mediate cell death should be considered redundant.

#### **1.5.4. Detecting *TrkB* mRNA and protein**

There is a lack of precise molecular data available relating to the nature of TrkB and its receptor isoforms, with complete nucleotide sequence data for full-length TrkB only available on NCBI Entrez Gene database towards the end of the course of this PhD and TrkB T2 expression is still not confirmed, except in the rat. This impacted considerably on determining reliably which receptor isoforms are expressed in which tissues, when and at what level. It also meant that it is not possible to be entirely sure what is generated by the transgenic mouse null for full-length *TrkB*, *TrkB<sup>FL</sup>* (Klein *et al.*, 1993) either in terms of truncated isoforms or other splice variants

induced by the mutation, although Klein *et al.* (1993) did demonstrate by Western blot that truncated receptor protein was unchanged in the brains of *TrkB<sup>FL</sup>* KO newborn mice.

Protein detection has also been greatly hampered due to the lack of information available, and although many papers have been published on expression patterns of TrkB receptors in many tissues, the supposed expression pattern has differed depending on: the tissue; the antibody; and the protocol used. Considering the size of the field of Trk signalling research it seems amazing that there is a paucity of reliable antibodies for TrkB. This, however, is what we believe to be the case (and others have come to the same conclusion, personal communication with Dr Minichiello, EMBL, Italy). For example, the commercially available antibody for full-length TrkB was purchased from Santa-Cruz and tested with wild-type brain, yet mass spectrometry on bands from 2D gels revealed that full-length TrkB was not detected by the antibody. A separate antibody considered to be specific for full-length TrkB (a gift from Professor Kaplan, Hospital for Sick Children, University of Toronto, Canada) actually reacted with *TrkB<sup>FL</sup>* KO tissue. Given that it is possible for some antibodies to work under only very strict conditions it remains feasible that further refinement of the protocols may need to be carried out to ensure optimisation but they could not be considered robust antibodies.

Thus, bearing in mind the issues described here the discussion of mRNA detection and protein expression patterns should not be considered absolute. Future research in this field may be able to make use of transgenic mice which can express a reporter gene under the control of TrkB promoters to establish expression patterns. For example the *TrkB* allele has been floxed and a *tau-lacZ* gene attached allowing the targeted removal of the gene from neurons (Xu *et al.*, 2000). The transgenic mice were used to assess the role of TrkB in the hippocampus and to visualise where *TrkB* expression is replaced

by lacz (Xu *et al.*, 2000). Use of heterozygotes expressing the *lacz* transgene may provide more reliable detection of TrkB expression in neuronal tissues.

## **1.6. Roles of Trk receptors in the development of the nervous system**

A large amount of the information available on the roles of Trk signalling has been generated from research using transgenic mice null for the ligand or receptor of choice. These mice tend to either show no apparent phenotype due to compensatory roles or are lethal in early postnatal development. It may be possible to assume that mutations arising in humans would follow the same pattern and therefore would not be apparent, however, the activity of Trk signalling contributes to many conditions. For TrkB these include: metastatisation of cancer; epileptogenesis; schizophrenia; and obesity.

As mentioned at the start of this section Trk signalling is best characterised for its roles in neuronal growth and survival, although it is also involved in neuronal differentiation and synaptic function: as cell survival is particularly pertinent to the development of the ovary it will be focused on here.

### **1.6.1. Cell survival**

The roles of Trk signalling in cell survival in the developing peripheral and central nervous systems have been exemplified by studies using mutant mice null for *TrkA*, *TrkB*, *Ngf* and *Bdnf*. It has been well reviewed that across differing neuronal populations all KOs suffer some degree of neuronal cell loss (Barbacid, 1994; Klein, 1994; Snider, 1994; Conover and Yancopoulos, 1997; Tessarollo, 1998) and almost all exhibit neonatal lethality. These effects are found mainly in the sympathetic and sensory neurons of the peripheral nervous

system (PNS) where the ligand is secreted in limited amounts to control the number of innervating neurons according to the size of the target tissue. Generally *TrkA* KOs exhibit loss of sympathetic neurons, in particular the superior cervical ganglia, SCG (Fagan *et al.*, 1996); *TrkB* KOs lose nodose ganglion neurons; and *TrkC* KOs lose parvalbumin (PV)-positive dorsal root ganglion (DRG) neurons (Ernfors *et al.*, 1994). Klein *et al.* (1993) assessed in detail the phenotype of mice null for *TrkB*. Homozygous mutant pups do not survive past the first postnatal week due to an inability to feed. Neuronal counts found significantly less ganglion and facial motor neurons, both involved in feeding.

It seems in the central nervous system (CNS) that there is greater redundancy in Trk signalling, for example in the hippocampus survival roles of growth factors can overlap such as is found for neurotrophins and insulin-like growth factor-1, IGF-1 (Lindholm *et al.*, 1996). However, it has also been demonstrated in the mouse embryo that BDNF (produced by the cerebral cortex) regulates the survival of dorsal thalamic neurons as they innervate the cerebral cortex (Lotto *et al.*, 2001). Artificially increasing cortical levels of BDNF *in vivo* resulted in a reduced level of thalamic neuronal cell death and blocking BDNF increased the levels of cell death.

Another way this has been demonstrated is through pharmacological inhibition of Trk signalling (Chen *et al.*, 2005). Transgenic mice were generated for each Trk receptor by the insertion of alleles into the kinase domain which render the receptor susceptible to specific pharmacological inhibition by the molecule PP1. Effects of PP1 administration during pregnancy on cell populations already known to be reliant on Trk signalling for survival were assessed by cell count at P0.5. *TrkA* inhibited mice exhibited a 50% loss in SCG neurons; *TrkB* inhibited mice a 60% loss in nodose ganglion neurons; *TrkC* inhibited mice a significant decrease in the number of PV-positive DRG neurons. Thus, these pharmacologically

controlled *Trk* KO mice exhibit the same phenotype as the other transgenic KO mice generated when exposed to PP1. The intended use of these mice is ultimately to allow research into the role of Trk receptors in adult neuronal cell survival (which cannot be done in the other KOs generated due to their postnatal lethality).

The potential administrative routes for PP1 (oral and/or intraperitoneal) and the fact that it can cross the placenta to the embryo means that it would allow highly specific points of development to be assessed for their requirement for the kinase activity of each Trk receptor; as such, they could be extremely informative in studies on fertility.

## **1.7. Roles in the development of non-neuronal tissues**

In spite of extensive characterisation of the roles of neurotrophins and their receptors in the nervous system, through the use of transgenic mice, it wasn't until relatively recently that functions were also identified in non-neuronal tissues (for review see Tessarollo, 1998).

Mice null for *Nt3* exhibit heart defects that are attributed to defective survival and/or migration of neural crest cells (Donovan *et al.*, 1996). A similar phenotype is observed when expression of *TrkC* is ablated by mutation (Tessarollo *et al.*, 1997). Thus, it appears that the NT3-TrkC signalling pathway is critical in regulating development of the heart and it has not yet been demonstrated to be under the control of other neurotrophins or their receptors. A variant of the p75 receptor has, however, been implicated in vasculature development. In addition to exhibiting nervous system defects, mice null for the *p75* variant also had dilated blood vessels with thinner walls (von Schack *et al.*, 2001). Blood vessels consequently rupture and blood loss causes death of the pup by late gestation.

The immune system can be considered to be another area of major non-neuronal Trk signalling with expression of the Trk receptors in many immune tissues and subsets of cells (Vega *et al.*, 2003). Lymphocytes in particular are strongly regulated by Trk signalling, as is one of the tissues they develop in, the thymus. NGF can regulate proliferation of lymphocytes *in vitro* (Thorpe and Perez-Polo, 1987; Otten *et al.*, 1989) and mutation of *TrkA* results in abnormal development of the murine thymus (Garcia-Suarez *et al.*, 2000). Similarly *TrkB* KO mice demonstrated significant loss of thymocytes (lymphocyte precursors) in the thymus (Garcia-Suarez *et al.*, 2002) and *Bdnf* null mice exhibit a decrease in circulating and stored (spleen or bone marrow) lymphocytes (Schuhmann *et al.*, 2005). In addition to regulating the physiologically normal immune system, Trk signalling is also implicated in pathological immune conditions such as chronic inflammation in lung disease (Nockher and Renz, 2003). Thus, there is considerable potential for Trk signalling in the development and regulation of the immune system.

## **1.8. Neurotrophins, Trks and the developing mammalian ovary**

The subject of mature ovary development and Trk signalling has been covered much more extensively than roles in neonatal ovary development. To maintain brevity I will limit the breadth of this Chapter to reviewing the available literature on early ovary development and the roles of Trk signalling in relation to this.

The developing and mature ovary is now a recognised site of functional neurotrophin-Trk signalling (Dissen *et al.*, 2002; Dissen *et al.*, 2009). mRNA and protein detection has revealed neurotrophins and receptors to be expressed in the ovary across many species: human (Anderson *et al.*, 2002); primate (Dees *et al.*, 1995); rodent (Dissen *et al.*, 1995; Spears *et al.*, 2003); and hen (Jensen and

Johnson, 2001). The majority of the literature available relates to their effects on follicular growth and ovulation (postnatal events), although there is also a growing body of evidence demonstrating roles for the neurotrophin-Trk signalling pathway in the developing mammalian ovary.

### **1.8.1. Ovarian innervation**

A principal factor one would consider to be important when contemplating the phenotypes of ovaries where neurotrophin signalling has been altered is assessing any effect on neuronal innervation of the ovary.

Ovaries receive innervation from the PNS through sympathetic and sensory neurons and in the rhesus macaque ovary, p75 expressing neuron-like cells extend into the vasculature, the interstitium, and primordial and growing follicles (Dees *et al.*, 1995; Dees *et al.*, 2006). Originally it was thought that the sole site of neurotrophin action in the ovary was on the nerves innervating the gland. Disrupting innervation of the ovary can be achieved by administering antibodies directed against the ligand or receptor. For example administration of an antibody raised against NGF resulted in loss of the sympathetic innervation of neonatal rat ovary (Lara *et al.*, 1990). This in turn caused abnormal ovary function with a reduction in preantral follicle development and abnormal estrous cyclicity. Thus, an assumption is made that neural defects in the ovary can be considered unlikely to be responsible for neonatal ovarian phenotypes as follicle development is supported up to the preantral stage.

## 1.8.2. TrkA, TrkC and p75

The majority of the information available in relation to Trk signalling and neonatal ovary development focuses on TrkB, however, some information is available pertaining to the other neurotrophin receptors which shall be summarised first.

### 1.8.2.1. TrkA

The role of NGF-TrkA signalling in neonatal rodent ovary development has recently been concluded to contribute to the regulation of primordial follicle formation and early follicle growth through stimulation of somatic cell proliferation and FSHR expression (Dissen *et al.*, 2009 review). Assessment of P7 *Ngf* KO mouse ovaries revealed an apparent reduction in mesenchymal cell proliferation (Dissen *et al.*, 2001). This is proposed to cause a reduction in primordial follicle formation in the *Ngf* KO at P2 and P4 through structural reorganisation problems (unpublished findings referred to in Dissen *et al.*, 2009). Subsequent differentiation and proliferation defects result in significantly less primary and secondary follicles at P7, although by that point no difference in primordial follicle number is found (Dissen *et al.*, 2001). TrkA protein is found at high levels in the oocyte and granulosa cell layer of primordial and primary follicles, with a reduction in intensity from the secondary stage (Dissen *et al.*, 2001). The same group only identified low levels of TrkA in the interstitium of P7 mouse ovaries. Thus, it is unclear if it is an autocrine pathway (for example, NGF-TrkA is expressed by and acting only on granulosa cells), paracrine pathway (mesenchymal cells to granulosa cells/oocyte), or both in effect regulating these processes.

It is also proposed by the same group that NGF-TrkA signalling contributes to follicles attaining gonadotrophin support. This is based on the observation that *Fshr* mRNA was decreased in P7 *Ngf* KO ovaries (Romero *et al.*, 2002). In the same study, pre-treatment of

cultured P2 rat ovaries with NGF then treatment with FSH resulted in increased numbers of multi-layered follicles. This result was only induced by FSH following NGF treatment and not by FSH or NGF treatment alone, demonstrating that NGF treatment does induce *Fshr* mRNA transcription which results in functional protein synthesis. However, Romero *et al.* (2002) do not seem to consider that the reduced mRNA of *Fshr* in the P7 *Ngf* KO ovaries may simply be caused by the reduction in secondary follicles (which they also did not count in the ovaries cultured with NGF). Therefore, it seems plausible to me that the reduction of early growing follicles in the P7 *Ngf* KO may actually be a primary effect of reduced granulosa cell proliferation and the reduced *Fshr* mRNA is a secondary effect caused by reduced secondary follicles present. In conclusion, there is strong evidence to suggest that NGF-TrkA signalling is not required for oocyte survival but is for getting the process of follicle formation and early follicle growth underway.

#### **1.8.2.2. TrkC**

Less evidence exists for the role of the NT3-TrkC pathway in neonatal ovary development, as illustrated by its exclusion from a recent review on the roles of neurotrophin signalling (Dissen *et al.*, 2009). Dissen *et al.* (1995) found that mRNA levels of rat *Nt3* and *TrkC* remain unchanged during follicle formation, which may indicate their non-requirement. In addition, as well as examining the ovaries of *TrkB* KO mice, Spears *et al.* (2003) assessed those of a *TrkC* KO strain. TrkC receptors appear not to be required for oocyte survival as no difference in oocyte number was found between the neonatal wild-type and *TrkC* KO ovaries.

#### **1.8.2.3. p75**

Examination of the protein distribution of the p75 receptor has revealed that in the developing rat, mouse and human ovary, it is

strongly localised to the mesenchyme and ovarian stroma (Dissen *et al.*, 1995; Dissen *et al.*, 2001; Anderson *et al.*, 2002). As the expression of neurotrophins and receptors which exert functional roles (NGF-TrkA and BDNF/NT4-TrkB) are expressed in granulosa cells, the oocyte, or both, it raises the possibility that p75 serves no role in follicle formation or oocyte survival. Or at least this would be unlikely to occur through the receptor multiplexes (discussed in Section 1.5.3) as the Trk and p75 receptors are simply not localised in the same areas of the ovary. However, specific and consistent localisation of p75 to the stroma may suggest it serves a function there.

### **1.8.3. TrkB**

Through RT-PCR, *Nt4* and *Bdnf* mRNA have been detected in human and rodent ovaries during follicle formation (Anderson *et al.*, 2002; Spears *et al.*, 2003; Harel *et al.*, 2006). Transcription of *Nt4* mRNA increases at the time of follicle formation in the rat (Dissen *et al.*, 1995). As well as variations in the amount of transcription there are changes in where it and translation occur during folliculogenesis. Using *in situ* hybridisation (ISH), *Nt4* mRNA is detected mainly in the oogonia of human fetal ovaries prior to follicle formation, though once incorporated into a primordial follicle NT4 protein is mainly localised to the flattened granulosa cells, with lower levels in the oocyte (Anderson *et al.*, 2002). There is also a switch in expression of the protein in the mouse, non-growing follicles exhibit NT4 and BDNF protein mainly in the oocytes, but they are found at a greater intensity in the granulosa cells of growing follicles (Paredes *et al.*, 2004).

The expression of full-length and truncated *TrkB* receptor isoform mRNA is confirmed in the ovaries of mice (Spears *et al.*, 2003) and humans (Anderson *et al.*, 2002; Harel *et al.*, 2006) at the time of folliculogenesis. In addition, detection of full-length *TrkB* mRNA

increases in the neonatal rat ovary at the time of follicle formation (Dissen *et al.*, 1995). Immunohistochemistry for full-length TrkB receptor protein reveals consistent localisation in mouse and human oocytes prior to and during follicle formation (Anderson *et al.*, 2002; Spears *et al.*, 2003; Paredes *et al.*, 2004; Harel *et al.*, 2006). It has also been detected in pregranulosa and primordial follicle granulosa cells in human fetal ovaries (Anderson *et al.*, 2002; Harel *et al.*, 2006).

Intriguingly, the full-length receptors capable of signalling are expressed at much lower levels than truncated receptors. This has been demonstrated in mouse ovary at the level of RNA (RT-PCR; Spears *et al.*, 2003) and protein (Western blot; Paredes *et al.*, 2004). Paredes *et al.* (2004) also used antibodies able to distinguish the expression pattern of the T1 truncated TrkB receptor protein from that of the full-length isoform on P7 mouse ovary sections. The intensity of the immunofluorescent signal generated was used to interpret expression levels. Comparatively, the level of full-length TrkB receptor expression in oocytes from primordial to secondary follicles was low against expression of the TrkB T1 isoform. The expression of the truncated receptor in primordial follicle oocytes is low but plentiful and localised to the oocyte cell membrane of growing primary and secondary follicles. Recent unpublished work from this lab (Murray and Spears) found extremely low levels of TrkB T1 in newborn mouse ovaries. This is consistent with the findings of Paredes *et al.* (2004) as newborn mouse ovaries consist mainly of primordial follicles. In contrast with the localisation studies, unpublished findings were recently reported to show that denuded oocytes from infantile mouse ovaries only express *TrkB T1* (RT-PCR; Garcia-Rudaz *et al.* discussed in a recent review on the role of neurotrophins in the ovary; Dissen *et al.*, 2009).

Thus, in the mammalian neonatal ovary, localisation of the different TrkB signalling components indicates strong potential for

different combinations of autocrine and paracrine signalling to occur between the granulosa cells and the oocytes. It seems that these relationships change dynamically by alteration of expression levels depending on the stage of follicle development and the compartment of the follicle examined.

As discussed in Section 1.6.2. mice null for Trk receptors or their ligands have been generated and their phenotypes described with particular emphasis on the effects on neuronal cell populations. Assessment of mice null for the ligand *Nt4* has revealed that as well as not suffering from developmental abnormalities *Nt4* KO mice have normal fertility (Conover *et al.*, 1995) and a normal complement of secondary follicles at P10 (Paredes *et al.*, 2004). In contrast, *Bdnf* KO mice die shortly after birth (Conover *et al.*, 1995) and although their fertility has not been assessed, mice null for both *Bdnf* and *Nt4* exhibit significantly reduced numbers of secondary follicles (Paredes *et al.*, 2004). This compensatory effect by the ligands has also been demonstrated *in vitro*. Addition of either anti-BDNF or anti-NT4 antibodies to P0 wild-type ovary cultures had no effect on healthy oocyte density, but in combination the antibodies resulted in a significant decrease in healthy oocyte density (Spears *et al.*, 2003).

Spears *et al.* (2003) also assessed ovary development of mice null for the full-length TrkB receptor (*TrkB<sup>FL</sup>*; Klein *et al.*, 1993) on a congenic C57BL/6 background. This mutation prevents downstream signalling from the tyrosine kinase domain but does not prevent expression of the truncated receptors. *TrkB<sup>FL</sup>* KO pups die within the first postnatal week and assessment of the ovaries was only possible up to P4/5. At this point the ovaries contained significantly less oocytes than wild-type ovaries with reduced numbers of follicles. Spears *et al.* (2003) replicated this effect *in vitro* by culturing P0 wild-type mouse ovary with the pan-Trk inhibitor (K252a, previously discussed in Section 1.5.2.1). After a week in culture with the strongest dose of K252a tested, P0 mouse ovaries exhibited

significantly reduced follicle numbers and extensive cell death with more than 85% of oocytes lost.

These observations led Spears *et al.* (2003) to hypothesise that limited neurotrophin availability creates competition amongst oocytes forming primordial follicles. Oocytes with insufficient support do not survive and undergo apoptosis, should sufficient tyrosine kinase activity be generated then the oocyte can survive.

The presence of truncated TrkB receptors during folliculogenesis and their apparent upregulation in growing follicles (Paredes *et al.*, 2004) imply a functional role. The nature of the role is not clear although it does not appear to be in oocyte survival. Trk activity inhibition by K252a causes significant oocyte loss (Spears *et al.*, 2003) implying that any activity of non-catalytic Trk isoforms is not sufficient for oocyte survival. Furthermore, a less severe effect on oocyte survival was found when expression of truncated receptors was also removed by mutation of all murine TrkB receptor isoforms (*TrkB<sup>FLTr</sup>*; Paredes *et al.*, 2004). Ovaries from P7 *TrkB<sup>FLTr</sup>* mice contain normal primordial and primary follicle numbers, although secondary follicles are significantly reduced. Survival must eventually become an issue for these *TrkB<sup>FLTr</sup>* KO ovaries as transplantation for two weeks (necessary due to postnatal lethality) revealed that follicles were unable to sustain development and were found to be degenerating or missing altogether (Paredes *et al.* 2004).

The *TrkB<sup>FLTr</sup>* mouse may be puzzling in its less severe response, but it may point towards the possibility that the truncated receptors exert a dominant negative effect in the *TrkB<sup>FL</sup>* KO. The truncated receptor can bind ligand but cannot transduce a signal, thus it binds available ligand preventing it from activating another pathway. In contrast, no isoforms are expressed in the *TrkB<sup>FLTr</sup>* KO leaving ligands free to potentially activate other survival pathways.

## 1.9. Aims of PhD

The formation of the pool of primordial follicles is one of the most important processes that the mammalian ovary undergoes to ensure long-term fertility of the individual, yet it is relatively poorly understood. The regulation of follicle growth and ovulation by the actions of gonadotrophins is a very well studied process and a consequence of this has been that infertility arising from difficulties in these latter (post-pubertal) stages can be treated in a variety of ways. However, integral for this is the availability of primordial follicles. Thus, without a supply of primordial follicles fertility treatments, for the most part, are redundant. It is, therefore, crucial that the factors acting during this period of development to regulate: oocyte survival; successful incorporation into primordial follicles; and their initiation to the growth phase, are understood.

The roles of many growth factors and their signalling pathways in facilitating these crucial, concomitant processes are beginning to be elucidated, however, by no means is there a complete picture. One signalling pathway has been demonstrated to exert a prominent role in neonatal murine oocyte survival, namely that of full-length TrkB signalling, yet the mechanisms of its action are not yet fully understood. My thesis aimed to investigate this.

Specifically my goals were to:

1. Decipher and investigate the chain of events occurring in the *TrkB<sup>FL</sup>* KO mouse that leads to the phenotype of severe oocyte loss seen shortly after birth.
2. Extrapolate to determine how TrkB signalling contributes to the normal loss of oocytes that occurs at the time of ovarian follicle formation.

# **Chapter 2**

## **Materials and Methods**

## 2.1. *TrkB<sup>FL</sup>* KO mice

Expression of murine full-length *TrkB* was disrupted by targeted gene mutation of the tyrosine kinase domain (Klein *et al.*, 1993) and subsequently placed on a C57BL/6 congenic background (Spears *et al.*, 2003). *TrkB<sup>FL</sup>* null offspring were generated by mating *TrkB<sup>FL</sup>* heterozygous breeding pairs. Wild-type and *TrkB<sup>FL</sup>* heterozygous littermates acted as Controls for the *TrkB<sup>FL</sup>* KOs. Mice were kept in accordance with UK legal requirements: housed under a temperature-controlled, 12 hour light: 12 hour dark regime with food and water *ad libitum*.

## 2.2. Genotyping of mutant mice

### 2.2.1. Collection of material

*TrkB<sup>FL</sup>* KO mice do not survive beyond the first postnatal week and as such, do not reach weaning age when ear clips would normally be obtained for genotyping. Therefore, pups collected had a piece of tail tip taken after tissue collection for genotyping. Tail tips and ear clips (to genotype breeding stock) were placed in 1.5ml eppendorf tubes and stored at -20°C until extraction.

### 2.2.2. Extraction of DNA

DNA was extracted from the ear clip/tail tip using the hotSHOT method, see Appendix A (Truett *et al.*, 2000). Briefly, 75µl of alkaline lysis reagent was added to samples in 1.5ml eppendorfs followed by heating to 95°C on a thermal cycler (MJ Research) for 30 minutes. The eppendorfs were cooled to 4°C before 75µl of neutralising reagent was added. The extracted DNA was stored at -20°C until genotyping.

### 2.2.3. Genotyping of DNA by polymerase chain reaction

Prior to genotyping by polymerase chain reaction (PCR) the DNA sample was further diluted: embryonic/neonatal DNA was diluted 1:5 in MilliQ autoclaved ddH<sub>2</sub>O and adult DNA was diluted 1:10. This initial dilution allowed 5µl of extracted DNA (from either adult or non-adult tissue) to be used with the same genotyping PCR bulk mix consisting of PCR buffer, primers and polymerase enzyme necessary to drive the reaction (see Appendix A). Thin walled PCR tubes (0.5ml) were used for the reaction on a thermal cycler (MJ Research). The PCR programme was specific to genotyping *TrkB* (see Appendix A). In each reaction appropriate controls were used (DNA samples previously shown to be wild-type, heterozygous or null for *TrkB*) and in addition a water blank tested for contamination.

#### **2.2.4. Analysis of PCR products on agarose gel**

Agarose gels were prepared by adding agarose powder to TBE (see Appendix A) and heating in a microwave to dissolve the powder. Once the gel had cooled slightly SYBRsafe (Invitrogen) was added to allow eventual visualisation of the bands (see Appendix A). The mixture was then poured into the chamber of a tank with a well comb inserted before being left to cool for approximately 30 minutes. The well comb was then removed before sufficient TBE buffer was added to the tank to cover the gel.

After the PCR reaction the tubes were centrifuged before 4µl of loading buffer was added to each sample. This resulted in a final volume of 24µl per sample, 8-10µl of which was then pipetted into a well in the gel. The tank was then connected to a power pack and run at 60 millivolts for 30 minutes (mini-gel) or at 90 millivolts for 45 minutes (midi-gel).

## **2.2.5. Visualisation of DNA products**

The gel was visualised using a UV transilluminator linked to software (Syngene). The PCR products were compared with molecular weight markers to confirm the genotype of the sample.

## **2.3. Tissue collection**

### **2.3.1. Gross dissection**

Neonates were killed by decapitation and tail tips were collected for subsequent genotyping as described in Section 2.1. Pups were pinned to a corkboard and a ventral incision was made to allow access to the gonads which were collected under magnification. Tissue was immediately immersed in fixative.

## **2.4. Wax Histology**

### **2.4.1. Ovary fixation**

The ovaries were fixed in Bouins or 4% paraformaldehyde (PFA) for a minimum of an hour but not more than 24 hours. Ovaries were washed in 70% ethanol to remove traces of fixative before storage in fresh 70% ethanol until processing.

### **2.4.2. Wax embedding**

Processing consisted of ovaries being passed through a series of alcohol solutions increasing in strength (70%, 90%, 100% x 2) before immersion in a paraffin wax/xylene solution which allows penetration of the tissue by the molten paraffin wax. Ovaries were then placed into a plastic mould before it was filled with wax and allowed to cool.

### **2.4.3. Sectioning and mounting wax blocks**

Wax blocks were removed from plastic moulds and a heated chuck was fused with the base of each block. Blocks were then carefully trimmed around the tissue and orientated on a microtome (Reichert-Jung) before sectioning at 5µm. The wax ribbons were floated onto a waterbath (50°C), placed onto poly-L-lysine (PLL) coated slides and allowed to dry overnight in a 37°C oven.

#### **2.4.4. Haematoxylin and eosin staining wax sections**

Slides were dewaxed in xylene (10 minutes x2) before rehydration through decreasing ethanol concentrations (100%, 90%, 70%, 5 minutes each) to tap water. Freshly filtered haematoxylin (30 seconds) was used to stain nuclei before the slides were returned to tap water. The intensity of the stain was determined under a microscope without letting the sections dry out. If the stain was too strong then the sections were dipped in acid alcohol before microscopically checking again. Once the level of desired stain was achieved the slides were “blued up” in Scott’s tap water solution (STWS; 3 minutes) before a 3 minute wash in tap water. To stain the cytoplasm of the cells the slides were immersed in eosin stain for 30 seconds, briefly washed in tap water before placing in potassium alum to fix the stain (30 seconds). The slides were given a final rinse in tap water before quick dehydration through the ethanol solutions (70%, 90%, 95%, 100% x2). The sections were then cleared in xylene (6 minutes x2) before a xylene-based mountant (DPX) was used to apply coverslips. Once slides were dry the sections were examined and photographed under brightfield microscopy (DMLB microscope, DFC480 camera and IM50 software all supplied by Leica).

## **2.5. Plastic Histology**

### **2.5.1. Resin embedding**

In addition to wax-embedding ovaries two different types of resin were also used (Technovit and LR White purchased from TAAB).

#### **2.5.1.1. Technovit**

Ovaries stored in 70% ethanol were dehydrated by moving through increasing concentrations of ethanol (96% and 100%) before being pre-infiltrated with a 1:1 mixture of the polymer and ethanol (2 hours in each solution). The ovaries were then equilibrated overnight in the polymer alone before embedding in plastic moulds. The embedding solution consists of polymer, hardener and softener. It polymerises at room temperature in approximately 2-3 hours. After polymerisation has taken place the chuck is adhered to the block using polymer plus hardener. The blocks were then left to dry for at least 24 hours before removing from the mould and cutting.

#### **2.5.1.2. LR White**

Ovaries stored in 70% ethanol were dehydrated through increasing concentrations of ethanol (90%, 95%, 100% x2, 30 minutes each). The ovaries were equilibrated in LR White polymer overnight before embedding in fresh polymer in a gelatine capsule (LR White degrades plastic). The reaction for LR White requires reduced exposure to air (the capsule has a lid), increased temperature (60°C oven) and takes longer to polymerise (overnight). The gelatine capsule is removed by running in hot tap water for 1-2 hours. The block is left to dry before supergluing to a wooden chuck.

### **2.5.2. Sectioning and mounting resin blocks**

Blocks were cut on a Reichart-Jung microtome using glass blades. After orientation of the block, 5µm sections were cut and collected. As a ribbon does not form in the same way as for wax blocks, each section was collected under magnification and floated on room temperature tap water. Sections were then placed on PLL slides and air dried.

### **2.5.3. Haematoxylin and eosin staining resin sections**

Dry slides were immersed in freshly filtered haematoxylin (2 minutes) to stain nuclei before being washed in tap water. The intensity of the stain was microscopically determined and if it was too strong acid alcohol was used to reduce its intensity before microscopically checking the slides again. Once the level of desired stain was reached the slides were “blued up” in STWS (2 minutes) before washing for 2 minutes in tap water. One minute in eosin was sufficient to stain the cytoplasm of the cells. The slides were then given another brief wash in tap water before immersion in potassium alum for one minute. A final wash of tap water then the slides were left to air dry completely before coverslips were mounted using DPX. Once slides were dry the sections were examined and photographed under brightfield microscopy (as described in Section 2.4.4).

## **2.6. Oocyte cell counts and follicle classification**

### **2.6.1. Cell counts**

The beginning and end sections of each ovary were identified and every “n”th section in between was photographed under brightfield microscopy (as described in Section 2.4.4). To obtain the P0 counts in Chapter 3 every 9<sup>th</sup> section was counted, to obtain the P0-P7 counts, every 18<sup>th</sup> section was counted, and in Chapter 5,

every 10<sup>th</sup> section was counted. Each section was then reconstructed in Powerpoint before being merged and compressed into a GIF file (graphics interchange format) which reduces the file size but doesn't reduce visual quality. Cell counter tool in ImageJ allowed several cell types of interest to be counted simultaneously in each section. The analysis carried out for this experiment (and all others) was done so blind to genotype.

#### **2.6.1.1. Cell types counted**

Oocytes were counted when the nucleus was visible in the section. Apoptotic oocytes were recognised from their appearance of a shrunken cytoplasm and heavy eosin stain. Apoptotic cells were identified by their lack of cytoplasm and their very heavy punctate haematoxylin stain.

#### **2.6.1.2. Count correction**

An estimated total number of cells can be obtained for each ovary by multiplying the total number of cells counted by "n". Where size of the object being counted exceeds section thickness the chance of the object being double counted is increased and the final number will be an overestimate. To address this problem a methodology was devised whereby a correction factor is applied to the estimated total (Abercrombie, 1946). It requires calculation of the mean diameter of the object being counted. This can then be taken into account in relation to the thickness of the section and the estimated total of cells. Mean diameter for each cell type was obtained by measuring and averaging the two widest perpendicular cross-sections of the object (in ImageJ). Where possible, a minimum ten examples of each cell type were measured to obtain the mean. The corrected estimated total for each cell type counted in each ovary was calculated by using the Abercrombie equation shown below.

Abercrombie equation:

$$\text{estimated total } x \left\{ \frac{\text{section thickness}}{\text{section thickness} + \text{mean diameter}} \right\}$$

## **2.6.2. Follicle classification**

To assess follicle development, oocytes counted were also categorised in terms of their somatic cell associations. Oocytes yet to form primordial follicles were found in clusters with other oocytes and were referred to as pre-primordial oocytes. Primordial follicles were considered to consist of an oocyte clearly associating with one or more flattened pre-granulosa cells. The oocytes in follicles transitioning to the growth phase (primordial-primary follicles) were surrounded by only one layer of somatic cells but some had differentiated into cuboidal granulosa cells. One complete layer of cuboidal granulosa cells identified the primary follicles and the beginnings of a second layer identified the primary-secondary follicles. Two complete layers were used to identify secondary follicles. Preantral follicles were considered to have three layers of granulosa cells and appearance of spaces in the granulosa compartment indicated development of an antrum (hence they were called early antral follicles).

### **2.6.2.1. Follicle measurements**

As described in Section 2.6.1.2 oocyte nucleus was measured to allow correction of estimated total oocyte counts. In addition to the nucleus, oocyte and follicle (up to the follicular BM) measurements were also obtained using ImageJ.

## **2.7. Immunohistochemistry and immunofluorescence**

### **2.7.1. Dewaxing and rehydration**

Slides were dewaxed in xylene (5 minutes x2) before rehydration through decreasing ethanol concentrations (100% x2, 95%, 90%, 70%, 5 minutes each) to MilliQ ddH<sub>2</sub>O (minimum 5 minutes).

### **2.7.2. Antigen retrieval**

Fixation of tissue can prevent access of antibodies to epitopes, these sites can be re-exposed by high-temperature antigen retrieval (achieved by pressure cooking). To 1600ml of MilliQ ddH<sub>2</sub>O, 15ml of Vector Antigen Unmasking Solution (citrate buffer; Vector H-3300) was added and the solution heated to boiling. At that point the slides were added and the cooker closed, once steam was apparent the slides were left for 5 minutes. The slides were then left to cool in the solution for 20 minutes before tap water was added to cool the slides further, a 5 minute wash in phosphate-buffered saline (PBS) on a shaker followed. To ensure that the sections were always covered with solution during the subsequent stages a hydrophobic pen was then used (ImmEdge) to encircle the sections. This also facilitated using lower volumes of more expensive reagents.

### **2.7.3. Immunohistochemistry**

Sections were prevented from drying out by carrying out blocks and antibody exposure in a hydrated tray. In addition, the sections were covered with parafilm during overnight primary antibody exposure.

#### **2.7.3.1. Blocking**

Prior to antigen retrieval endogenous peroxidases were blocked to prevent non-specific reactions occurring by immersing slides in

0.5% H<sub>2</sub>O<sub>2</sub> in methanol at room temperature for 10 minutes. To further reduce non-specific antibody binding and background staining, non-immunised animal serum solution (2ml serum, 8ml PBS and 0.5g BSA) with avidin (1ml of serum solution:4 drops avidin) was applied to the sections for one hour at room temperature. The slides were washed in PBS/0.01% tween for 5 minutes with agitation. A biotin block (1ml serum solution:4 drops biotin) was applied overnight with the primary antibody. Avidin-biotin block kit from Vector laboratories.

### **2.7.3.2. Primary antibody**

Primary antibody for immunohistochemistry was diluted in the serum/biotin solution (see Section 2.7.3.1) and applied at 50µl per slide overnight at 4°C. Control slides with primary antibody omitted were exposed to the serum/biotin solution only.

### **2.7.3.3. Secondary antibody**

Parafilm was removed and the slides were washed (2 x 5 minutes) in PBS/0.01% tween. The secondary antibody (biotin conjugated) was diluted in the serum solution made on day one of the protocol and was applied at 100µl per slide for one hour at room temperature. The slides were then washed (2 x 5 minutes) in PBS/0.01% tween.

### **2.7.3.4. Antigen detection**

Detection of the biotinylated secondary was facilitated by using the Vectastain ABC Elite kit (Vector laboratories). The strong affinity and multiple binding sites that avidin has for biotin means that it can form a complex with a biotinylated peroxidase enzyme (ABC) and still bind the biotinylated secondary antibody. The ABC kit was prepared 30 minutes in advance and agitated until use to facilitate complex formation: 2 drops of solution A and 2 drops of solution B in 5ml of

PBS. Three to four drops of ABC were applied per slide for 30 minutes at room temperature. The slides were then washed (2 x 5 minutes) in PBS/0.01% tween.

Detection of the ABC complex is achieved by application of 3,3'-diaminobenzidine (DAB) which acts as the substrate for the peroxidase enzyme, depositing a dark-brown stain. To 5ml of MilliQ ddH<sub>2</sub>O two drops of buffer stock are added, followed by four drops of DAB stock, two drops of H<sub>2</sub>O<sub>2</sub> were only added immediately prior to use. It was tested on excess ABC to ensure the reaction worked before the slides were exposed to DAB. Reaction visualisation took between 30 seconds and 5 minutes depending on the antibody and tissue. The reaction was ended by rinsing the slides with MilliQ ddH<sub>2</sub>O. Slides were counterstained in haematoxylin before dehydration, mounting and image acquisition as described in Section 2.4.4.

#### **2.7.4. Immunofluorescence**

Sections were dewaxed, rehydrated and processed through antigen retrieval as described in Sections 2.6.1 and 2.6.2.

##### **2.7.4.1. Blocking**

Prior to antigen retrieval endogenous peroxidases were blocked to prevent non-specific reactions occurring using 3% H<sub>2</sub>O<sub>2</sub> in methanol at room temperature for 30 minutes. To reduce background staining non-immunised animal serum solution (2ml serum, 8ml PBS and 0.5g bovine serum albumin, BSA) was applied for one hour at room temperature before 2x 5 minute washes in PBS with agitation.

##### **2.7.4.2. Primary antibody**

Primary antibody for immunofluorescence was diluted in the serum solution (see Section 2.7.4.1) and applied at 50µl per slide

overnight at 4°C. Control slides were exposed to the serum solution only.

#### **2.7.4.3. Secondary antibody**

Parafilm was removed and the slides were washed (2 x 5 minutes) in PBS. The secondary antibody (horseradish peroxidase, HRP conjugated) was diluted in the serum solution made on day one of the protocol and was applied at 100µl per slide for 30 minutes at room temperature. The slides were then washed (2 x 5 minutes) in PBS. The subsequent steps were carried out with minimal light exposure to the fluorescent reagents or slides (once they had been exposed to fluorescent reagents).

#### **2.7.4.4. Fluorescence visualisation**

Detection of the secondary antibody was amplified using the tyramide signal amplification kit (TSA™ Plus Fluorescein; PerkinElmer). TSA uses the HRP-conjugated secondary antibody to catalyse deposition of the fluorophore labelled tyramide onto tissue sections. A 1:50 dilution of the tyramide was prepared in the kit diluent before 50µl per slide was applied for 10 minutes at room temperature. The slides were then washed (2 x 5 minutes) in PBS. To counterstain the nuclei, 100µl of propidium iodide (PI; 1mg/ml in PBS) was applied to each slide for 15 minutes at room temperature. The slides were then washed (2 x 5 minutes) in PBS before mounting with Vectashield (Vector) and sealing coverslip with nailvarnish.

Slides were stored in the dark at 4°C until they were visualised by confocal microscopy (Zeiss LSM510 attached to an upright Axioskop research microscope). The green TSA was visualised by an argon/krypton laser at 488nm, the red PI counterstain was visualised by a helium/neon laser at 543nm.

## Chapter 3

### ***TrkB<sup>FL</sup>* KO mouse ovary: a time-course of neonatal development**

### 3.1. Introduction

Over many decades, morphological studies, and more recently genetic studies, have revealed the complex topology of the embryonic and neonatal ovary. After initially forming, the ovarian anlagen is comprised of a heterogeneous population of cells seemingly without organisation, but within a short space of time (taking only days in the mouse) the fundamental units of the ovary (primordial follicles) are formed and commence growing (see Figure 1.2).

As the follicles progress through each stage of development, the oocyte and supporting somatic cells respond to the effects of growth factors which can be endocrine, paracrine or autocrine, as discussed in Chapter 1. It is not until the individual reaches sexual maturity that the effects of this orchestra of cell-to-cell communication on fecundity are usually revealed. In extreme cases it may be possible to assess the effect of a mutation on fertility, even if it results in the death of the neonatal pup, as oocyte survival at the time of birth will have direct consequences on the fertility of the individual at maturity.

Spears *et al.* (2003) established that the ovaries of mice lacking the full-length TrkB receptor (*TrkB<sup>FL</sup>* KO) on a congenic C57BL/6 background contained significantly less oocytes than their wild-type Controls at P4/5. Similarly, culturing wild-type newborn ovaries with pan-Trk receptor blocker K252a significantly reduced the number of oocytes surviving, as did adding antibodies directed against the specific TrkB ligands, BDNF and NT4. K252a is a protein kinase inhibitor which prevents the phosphorylation of all Trk receptors, inhibiting downstream signalling (Tapley *et al.*, 1992). Human fetal ovary cultured with K252a for two days also exhibited a significant reduction in density of healthy germ cells (Spears *et al.*, 2003).

In an attempt to decipher the role of Trk signalling in the mouse ovary prior to folliculogenesis, Spears *et al.* (2003) cultured E16.5 wild-type mouse ovaries in the presence of K252a.

Unexpectedly, this showed that K252a does not affect oocyte survival or the growth of oocytes at this stage. Instead, the experiments revealed that in contrast to the culture of newborn ovaries (which supports formation of primordial and early preantral follicles) culturing E16.5 ovaries bypasses the normal formation of primordial follicles, with follicles forming directly at the primary stage. This observation critically provided the backbone for the interpretation that Trk signalling is essential specifically for primordial follicle survival in the mouse, as an effect was only found when primordial follicles were present.

The role of the truncated TrkB receptors in neonatal ovary development was partially addressed by examination of the ovaries from mice null for all TrkB receptor isoforms (*TrkB<sup>FLTr</sup>*; Paredes *et al.*, 2004). P7 *TrkB<sup>FLTr</sup>* KO ovaries exhibit a significant reduction in the number of secondary follicles but no difference in primordial or primary follicle numbers. The block in follicle growth is attributed to a significant reduction in granulosa cell proliferation, thereby preventing the formation of secondary follicles. This subsequently causes a significant reduction in *Fshr* mRNA synthesis, preventing follicle gonadotrophin dependence and possibly further growth (Paredes *et al.*, 2004).

Prior to commencement of this project, the role of the truncated TrkB receptors (T1 and T2) was difficult to determine as no KO was available for analysis. This was due to the truncated receptors having an almost identical sequence to the full-length receptor apart from short intracellular tails that terminate before the tyrosine kinase domain. Thus, it was easier to disrupt expression of all isoforms through mutation of the signal peptide motif, as was done to generate the *TrkB<sup>FLTr</sup>* KO (Rohrer *et al.*, 1999). A *TrkB T1* KO has since been generated and in collaboration with Dr Lino Tessarollo's research group (National Cancer Institute, Frederick, Maryland, USA) its phenotype has been assessed by the Spears group. The mice appear

normal and fertile and examination of their neonatal ovaries does not reveal any abnormalities (Spears and Murray; unpublished findings, 2008).

Thus, at the start of this project it could be intimated that prevention of full-length TrkB signalling has a significant role in the survival of oocytes during folliculogenesis (Spears *et al.*, 2003), whereas prevention of both full-length and truncated TrkB signalling prevents early follicle growth (Paredes *et al.*, 2004).

## **3.2. Aim**

The aim of this Chapter was to assess the neonatal development of the *TrkB<sup>FL</sup>* KO ovary.

### **3.2.1. Limitations**

During the course of any PhD many problems, small and large, are overcome; unfortunately during this project there were significant issues which need to be addressed before presentation of the results. These issues can be subdivided into three topics: *TrkB<sup>FL</sup>* KO survival; *TrkB* genotyping; and histology.

#### **3.2.1.1. *TrkB<sup>FL</sup>* KO survival**

Immediately prior to the start of this PhD, the on-site animal house where the TrkB colony had been maintained was closed down and all colonies were re-derived into a new facility for almost the entire duration of the project. The TrkB colony was originally housed in the Hugh Robson Building (HRB; a conventional breeding unit with no barrier to pathogens); these mice were used for the research published by Spears *et al.* (2003). The new unit at Little France (LF) was a much cleaner facility with individually ventilated cages, IVCs, and a pathogen-free status; these mice were used for this investigation.

As discussed in Chapter 1, the *TrkB<sup>FL</sup>* KO pups do not survive for more than a few days after birth, thus, it was expected that collection of postnatal material beyond P7 may be difficult. However, it became apparent that even fewer pups than anticipated were surviving with many *TrkB<sup>FL</sup>* KOs now dying on the day of birth. Pup weight was recorded at the point of collection and a significant reduction in the mean birth weight of the entire LF colony was found compared to the original HRB colony (see Figure 3.1). This indicates that the colony as a whole was disadvantaged at birth in the LF unit when compared to the HRB unit. Figure 3.1 reveals significant weight differences between Control and *TrkB<sup>FL</sup>* KO pups at P4 and P6, but at P0 there is no difference between the genotypes irrespective of which animal unit they were born in. Thus, *in utero* the *TrkB<sup>FL</sup>* KOs do not suffer significant growth retardation compared to their littermates, it is only once the pups are dependent on suckling that the retarded growth of the *TrkB<sup>FL</sup>* KO pups becomes apparent. There was, though, an effect taking place *in utero* which reduced the weight of all LF pups.

The small number of *TrkB<sup>FL</sup>* KOs generated by the LF colony meant that there were not enough samples collected of each age (for each experiment) to allow a thorough statistical analysis to be carried out. To maximise the scope of the project in line with the available material, achieving objectives took longer than anticipated.



### 3.2.1.2. TrkB colony genotyping

During the initial 10 months of the PhD, genotyping of the TrkB colony proved unreliable, often giving incorrect results. This problem contributed to the reduction in *TrkB<sup>FL</sup>* KOs produced by the colony, as wild-type breeders were misidentified as heterozygous, resulting in breeding pairs unable to generate *TrkB<sup>FL</sup>* KOs. A forward primer common to both genotypes and two separate reverse primers (one to detect the wild-type allele and the other to detect the mutant allele) were used to genotype the colony (Schimmang *et al.*, 1995). The variable results were due to the *TrkB<sup>FL</sup>* KO reverse primer occasionally being detected by wild-type samples to give a heterozygous result. This would not impact on the correct identification of *TrkB<sup>FL</sup>* KOs (such as those used for the research published in Spears *et al.*, 2003) as only wild-type or heterozygotes would be misidentified. A *TrkB<sup>FL</sup>* KO mouse would only give a genotyping result if the reaction worked (*TrkB<sup>FL</sup>* KO band) or a blank if it did not, in which case the sample would be repeated. A significant portion of time was spent analysing the only sequence data available at that time: the putative *TrkB* sequence of a cDNA clone which lacks the intron/exon details (Klein *et al.*, 1989). As discussed in Chapter 2, expression of the full-length TrkB protein was disrupted by insertion of a neo cassette into exon K2 of the catalytic domain (Klein *et al.*, 1993). Once the location of exon K2 was identified by basepair number in the cDNA clone sequence, primers were designed from within this coding sequence to genotype the TrkB colony reliably, as confirmed by BLAST.

### 3.2.1.3. Histology

During this project the low rate of sample collection was compounded by histological problems which resulted in a number of samples unsuitable for particular analyses. It was identified that the in-house made Bouins fixative caused poor preservation of the

ovarian ultrastructure. Consequently, poor morphology only allowed simple counts to be carried out and prevented further assessment of the ovaries. Freshly purchased Bouins fixative and resin embedding the ovaries greatly enhanced morphological preservation and assessment, although, once embedded in resin the tissue could not be utilised for other protocols such as immunohistochemistry which require wax embedded or frozen tissue. Thus, it was decided to fix one ovary in Bouins for resin embedding and the other in paraformaldehyde (PFA) for wax embedding. Ideally, all suboptimally fixed material would have been discarded, but as *TrkB<sup>FL</sup>* KO tissue was so limited, it was used for some analysis. It is essential to stress, though, that in all comparisons made between Control and *TrkB<sup>FL</sup>* KOs, like was only ever compared with like.

#### **3.2.1.4. Outcome**

Ultimately, the end result of these problems outlined above was extremely few *TrkB<sup>FL</sup>* KOs to analyse. Therefore, taking into account the limitations of this project, the aim was completed by carrying out a detailed developmental analysis of a sample-limited time-course of neonatal C57BL/6 congenic Control and *TrkB<sup>FL</sup>* KO ovaries.

### 3.3. Materials and Methods

#### 3.3.1. Animals

Transgenic animals were generated and genotyped as described in Chapter 2. Control (wild-type and *TrkB<sup>FL</sup>* heterozygote) and *TrkB<sup>FL</sup>* KO pups were collected from P0 through to P7. Pups were weighed at collection. The phenotype had previously been apparent at P4/5 but collecting *TrkB<sup>FL</sup>* KO ovaries closer to that age was rare due to neonatal *TrkB<sup>FL</sup>* KO lethality occurring earlier in the LF colony (see Section 3.2.1.1). Therefore, it became protocol that if a pup seemed weak it was collected before it expired regardless of age, but where possible the pups were left as long as was feasible before being collected. Hence there were some ovaries older than P4/5 collected, providing an almost complete P0-P7 time-course by the end of the study. Imminent demise can be considered an unlikely contributing factor to abnormal ovarian phenotypes. This assumption is based on the observation by Spears *et al.* (2003) that although *TrkC* KO mice exhibit the same postnatal lethality as *TrkB* KO mice (thus, would also have been collected close to death) they do not exhibit an ovarian phenotype such as the one observed in the *TrkB* KO mice.

#### 3.3.2. Histological assessment of mouse ovaries

Initial P0 counts were carried out on ovaries with the bursa removed. Ovaries were fixed, embedded in Technovit resin, sectioned at 5µm and stained for haematoxylin and eosin (H&E) as described in Chapter 2.5.3. Upon embedding in the resin, the “cleaned up” ovaries moved to the base of each mould, resulting in loss of the start of each ovary when sectioning the block. As each of these ovaries had been treated the same way, it is assumed that a similar portion of each ovary has been lost and, therefore, the counts are at least comparable with each other (although not with ovaries collected subsequently). Ovaries subsequently collected were left intact within

the bursa providing a “buffer” region of tissue that could be safely discarded at the start of histological sectioning. One pair of P0 ovaries were treated in this way, hence they are included in the P0-P7 counts. Oocyte counts and follicle classifications were carried out as detailed in Chapter 2.6.

#### **3.3.4. Follicle health assessment**

Follicle health was assessed on a rating basis of 1 = poor health, 2 = medium health, 3 = good health, see Figure 3.2. The poor follicle health rating of 1 was applied where the follicle lacked an obvious basement membrane (BM), exhibited poor somatic cell-to-oocyte arrangement and contained an oocyte of poor quality. Medium health rating of 2 was used where follicles exhibited a discontinuous BM and/or poor somatic cell-to-oocyte arrangement. Good health rating of 3 was given where a continuous visible BM could be seen, the oocyte had somatic cell support and a healthy oocyte appearance. Each P0-P7 Control and *TrkB<sup>FL</sup>* KO ovary was assessed for each category of follicle (primordial, primordial-primary and primary). The number of poor, medium and good follicles counted in each ovary was then calculated as a percentage of the total follicles in each category.

To assess how the *TrkB<sup>FL</sup>* KOs compared to their Controls in terms of follicle health from primordial through to primary stages, the mean percentage of poor follicles was calculated for the P0-P7 Control ovaries and compared with the mean percentage of poor follicles in the P0-P7 *TrkB<sup>FL</sup>* KOs. This was repeated for the mean percentages of good follicles.

#### **3.3.5. Statistical analysis**

As discussed previously, low sample sizes mostly prohibited statistical analysis, although in some instances it was feasible and appropriate to compare aspects of the sibling Control and *TrkB<sup>FL</sup>* KOs using Student’s t-test.



### 3.4. Results

#### 3.4.1. The P0 *TrkB<sup>FL</sup>* KO ovary does not exhibit a reduction in health

Total counts for healthy oocytes, apoptotic oocytes and apoptotic cells were obtained for Control (n=2) and sibling *TrkB<sup>FL</sup>* KO (n=6) P0 ovaries. As expected the counts obtained for healthy oocyte number varied between Control ovaries (Rodrigues *et al.*, 2009) and no clear difference was observed between those cells or any others assessed in the *TrkB<sup>FL</sup>* KO ovaries (see Figure 3.3).

#### 3.4.2. The P0-P7 *TrkB<sup>FL</sup>* KO ovary does not exhibit a reduction in health

Ovaries were collected from P0-P7, where possible. The following ages were not available: P1, P2 and P5. One or two ovaries of each remaining age was assessed for healthy oocyte, apoptotic oocyte and apoptotic cell number (see Figure 3.4). As expected the healthy oocyte counts revealed a sharp decline in number which plateaus by P7 in the Controls (Kerr *et al.*, 2006; Rodrigues *et al.*, 2009) but no effect of *TrkB<sup>FL</sup>* KO was found. The sharp decrease in oocyte number is reflected in the initially high numbers of apoptotic oocytes counted. Again, no difference was apparent between Control and *TrkB<sup>FL</sup>* KO ovaries in counts obtained for apoptotic oocytes or other apoptotic cells.

During the analysis it became apparent that some of the ovaries were infiltrated with an unknown cell-type similar in appearance to apoptotic cells. For this reason these ovaries, and their corresponding Control or *TrkB<sup>FL</sup>* KO ovary, were omitted from the apoptotic cell results. The infiltrate of unknown cell-type is the subject of Chapter 6.





### **3.4.3. Follicle development in the P0-P7 *TrkB<sup>FL</sup>* KO ovaries**

The results show that up to P7, *TrkB<sup>FL</sup>* KO ovaries are capable of supporting oocyte survival. Thus, the logical progression was to determine how the *TrkB<sup>FL</sup>* KO ovaries compared to their Controls in terms of follicle development. This has been broken down into three stages: the incorporation of oocytes into primordial follicles; follicle growth initiation; and follicle growth progression.

#### **3.4.3.1. The P0-P7 *TrkB<sup>FL</sup>* KO ovary supports folliculogenesis**

Oocytes yet to form primordial follicles (pre-primordial oocytes) and those that had formed primordial follicles were calculated as a percentage of the total oocytes in these two categories. These oocytes are the dormant, non-growing population of oocytes. There is no obvious difference between Control and *TrkB<sup>FL</sup>* KO ovaries, although the results (Figures 3.5 A and B) demonstrate three interesting points: firstly, at P0 follicle formation is well underway in both groups; secondly, the percentage of primordial follicles formed in both genotypes gradually increases with age; so that thirdly, regardless of genotype >80% of dormant oocytes were found in primordial follicles by P7 with some pre-primordial oocytes remaining.

#### **3.4.3.2. The P0-P7 *TrkB<sup>FL</sup>* KO follicles initiate growth**

To determine how the *TrkB<sup>FL</sup>* KO ovaries compare to Control ovaries in terms of follicle growth initiation, the growing and non-growing oocytes were calculated as a percentage of the total oocytes. Figures 3.6 A and B demonstrate that from P0 to P7, irrespective of genotype, the majority (>80%) of oocytes are found in a dormant state. The remaining oocytes are found in growing follicles, revealing that at P0 there are already follicles which have activated growth and that this proportion increases with age in both genotypes.





### 3.4.3.3. Early follicle growth is delayed in the *TrkB<sup>FL</sup>* KO ovary

It has been demonstrated in the previous sections that the *TrkB<sup>FL</sup>* KO ovaries exhibit morphologically normal neonatal ovary development: primordial follicles are formed; a dormant pool of oocytes is maintained; and follicles initiate growth. The analyses so far, however, do not determine to what stage these growing follicles progress in the *TrkB<sup>FL</sup>* KO ovaries. Therefore, the follicles were characterised as stated in Chapter 2.6.2 and calculated as a percentage of the total growing follicles.

Figures 3.7 A and B demonstrate that regardless of genotype the most common type of growing follicle observed in the neonatal ovary is the primordial-primary follicle with both genotypes containing ~80% primordial-primary follicles, even at P7.

Figure 3.7 A reveals that primary follicles are first observed in Control ovaries at P4, with primary-secondary follicles appearing at P6. In stark contrast the *TrkB<sup>FL</sup>* KO ovaries do not produce primary follicles until P6 and no primary-secondary follicles were observed even at P7 (Figure 3.7 B). This clearly demonstrates that the *TrkB<sup>FL</sup>* KOs exhibit a delay in progression through the early stages of growth initiation (from primordial-primary to primary follicle stage). Statistical analysis using a two-tailed, paired Student's t-test rejected the null hypothesis, thus from P4-P7 there is a significant reduction in the mean number of primary follicles produced by *TrkB<sup>FL</sup>* KO ovaries compared to their sibling Controls ( $P < 0.05$ ; see Figure 3.8).





#### **3.4.4. *TrkB<sup>FL</sup>* KO ovaries exhibit poorer follicular health than their Controls**

Some of the P2/3 *TrkB<sup>FL</sup>* KO follicles demonstrated poor follicular arrangement. This was judged from abnormal granulosa cell-to-oocyte orientation and the absence of an intact follicular BM (see Figure 3.2). It was decided that due to its excellent maintenance of morphology, in particular that of the BM, the resin embedded tissue would be ideal for quantifying this observation from P0-P7, as described in Section 3.3.4. *TrkB<sup>FL</sup>* KOs exhibit a larger percentage of unhealthy follicles than Controls at each stage (see Figure 3.9 A). A one-tailed, paired Student's t-test was used to determine if this trend was significant. The difference is significant at the primordial and primordial-primary follicle stages ( $P < 0.05$ ). The same analysis was used to determine if Controls had a higher percentage of follicles in good health than the *TrkB<sup>FL</sup>* KO ovaries. This was found to be true with Controls exhibiting significantly more healthy follicles at all stages (see Figure 3.9 B).



### 3.5. Discussion

It was previously demonstrated that *TrkB<sup>FL</sup>* KO ovaries exhibit mass apoptosis and significant loss of oocytes and follicles by P4/5 (Spears *et al.*, 2003). The main results of this Chapter in relation to the *TrkB<sup>FL</sup>* KO have revealed that the phenotype is not apparent in the re-derived colony, instead the *TrkB<sup>FL</sup>* KO ovaries assessed for this PhD present a phenotype of reduced early follicle growth and a measurable reduction in follicle health compared to their Controls.

#### 3.5.1. Early follicle growth

The aim of this project was to describe the *TrkB<sup>FL</sup>* KO ovary. As previous assessment of the colony indicated a severe effect by P4/5 (Spears *et al.*, 2003) ovaries were examined here at an earlier stage of development. P0 *TrkB<sup>FL</sup>* KO ovaries were examined from the re-derived colony and it was found that these ovaries demonstrate no effect on oocyte number, the presence of dying oocytes or general apoptosis, as compared to their Controls, demonstrating that full-length *TrkB* is not required for oocyte survival at the time of follicle formation.

A time-course of P0-P7 ovaries from the re-derived colony was then subjected to a detailed analysis. Again, genotype had no effect on oocyte number, oocyte death, or apoptosis in general. From the *in vivo* material collected, the single *TrkB<sup>FL</sup>* KO P7 ovary assessed contained approximately half the number of oocytes of the Control ovary. This is not out-with the realms of innate variability in terms of oocyte number, although the P0-6 *TrkB<sup>FL</sup>* KOs had exhibited similar numbers to the Controls. It is possible that if further P7 *TrkB<sup>FL</sup>* KO ovaries were collected (which would simply take too long to be feasible) then an *in vivo* phenotype on oocyte survival may become apparent, although with a sample size of only one no conclusion can be drawn.

Thus, P0-P7 *TrkB<sup>FL</sup>* KOs for the most part appear normal, they do not display any defect in their ability to incorporate oocytes into primordial follicles, and they are able to maintain a proportion in a quiescent state as well as the Controls. Growth initiation from the primordial to the primordial-primary stage of follicle development occurs normally, however, it is clear that completing this transition and reaching the primary stage is affected, as *TrkB<sup>FL</sup>* KOs exhibit significantly less primary follicles than their Controls.

The maintenance of a quiescent pool of primordial follicles in the *TrkB<sup>FL</sup>* KO ovary potentially indicates that the pathways responsible for repression of follicle activation are intact. One such pathway, discussed in Chapter 1.3.2, is regulated by anti-Müllerian hormone (AMH). A known repressor of primordial follicle activation, it is expressed in murine granulosa cells from the primary follicle stage onwards (Durlinger *et al.*, 2002). Strongest expression is found in small and large preantral follicles, although it is also possible to observe staining in the granulosa cells of follicles that have activated growth (primordial-primary). Thus, it is possible to propose that in spite of the reduction in primary follicle number in *TrkB<sup>FL</sup>* KO ovaries, the transitory follicles present may express sufficient AMH to suppress primordial follicle activation.

Paredes *et al.* (2004) examined P7 ovaries from mice lacking all TrkB receptor isoforms (*TrkB<sup>FLTr</sup>* KO) and found a significant reduction in the formation of secondary follicles but none in the formation of primordial or primary follicles. Therefore, the *TrkB<sup>FL</sup>* KO appears to exhibit a slightly different phenotype by demonstrating a block in follicles reaching the primary stage.

It must be pointed out that the *TrkB<sup>FL</sup>* KO and the *TrkB<sup>FLTr</sup>* KO are mutations placed on different backgrounds. The *TrkB<sup>FL</sup>* KO generated by Klein *et al.* (1993) was placed on the C57BL/6 background, whereas the *TrkB<sup>FLTr</sup>* KO generated by Rohrer *et al.* (1999) was placed on an Institute for Cancer Research (ICR) strain.

The reasoning behind using the ICR strain was that both litter size and KO survival were improved on this background compared to C57BL/6. It is feasible that strain differences may contribute to differences in observed follicle development but in spite of this, I propose that the phenotypes of the *TrkB<sup>FL</sup>* KO and the *TrkB<sup>FLTr</sup>* KO are actually more similar than they may first appear and in fact it is possible that the *TrkB<sup>FL</sup>* KO phenotype is a precursor of the *TrkB<sup>FLTr</sup>* KO phenotype. The reasoning behind this is that Paredes *et al.* (2004) actually address that they would have expected there to be higher numbers of primary follicles found in their *TrkB<sup>FLTr</sup>* KOs if the block was solely in reaching secondary stage. This increase would be created by follicles activating growth, reaching primary stage, but not progressing to secondary stage. However, this is not the case, as *TrkB<sup>FLTr</sup>* KOs actually exhibit a slight (albeit insignificant) reduction in primary follicle number (Paredes *et al.*, 2004). I propose that the significant reduction in secondary follicle number in the *TrkB<sup>FLTr</sup>* KO is a consequence of follicles taking longer to reach the primary stage of growth as I have shown here occurs in *TrkB<sup>FL</sup>* KO mice. Examination of *TrkB<sup>FLTr</sup>* KO ovaries at an earlier age would be required to confirm if this is the case. There is supporting evidence for my hypothesis, as mice null for the neurotrophin, *Ngf*, exhibit significantly less primary and secondary follicles but no difference in primordial follicle number at P7 (Dissen *et al.*, 2001). Subsequent examination of the *Ngf* null ovaries at P2 and P4 revealed a reduction in primordial follicle number which they propose to account for the reduction in primary and secondary follicles observed at P7 (unpublished findings referred to in Dissen *et al.*, 2009). Thus, a reduction in formation of early follicle stages may subsequently cause a reduction in follicle growth; though by that point, the effect on the population of early follicles may no longer be so marked.

Contrary to the *Ngf* KO study, *TrkB<sup>FL</sup>* KO ovaries do not exhibit a reduction in the ability to form primordial follicles, thus it is

proposed that both the *TrkB<sup>FL</sup>* and *TrkB<sup>FLTr</sup>* KO ovaries exhibit slower growth from the primordial-primary follicle stage onwards, reducing the number of primary follicles in the *TrkB<sup>FL</sup>* KO, and the number of secondary follicles in the *TrkB<sup>FLTr</sup>* KO.

As discussed in Chapter 1, growth factors which exert mitogenic effects on granulosa cells are potential activators of follicle growth. To determine if *TrkB* signalling exerts its effects on follicle growth by regulating expression of growth factors or their receptors, Paredes *et al.* (2004) examined two such paracrine signalling systems in the Control and *TrkB<sup>FLTr</sup>* KO ovaries at P7. The first is oocyte-expressed growth differentiation factor-9 (GDF-9) and its putative receptor, bone morphogenetic protein type II receptor (BMPRII), found on granulosa cells (Shimisasi *et al.*, 2004). The second is the oocyte-expressed KIT receptor and the granulosa cell expressed ligand, KL. No difference was found between the genotypes in their transcription of *Kit*, *KL* or *Gdf-9*. *BmprII* mRNA was significantly higher in the *TrkB<sup>FLTr</sup>* KO, although this is not thought to be of major importance as the *BmprII* and *KL* mRNA levels were corrected in the *TrkB<sup>FLTr</sup>* KO to account for the reduced numbers of granulosa cells in the *TrkB<sup>FLTr</sup>* KO. Thus, in the P7 *TrkB<sup>FLTr</sup>* KO ovary, these growth factor signalling pathways are normal. There is, though, a significant reduction in the expression of proliferation markers (bromodeoxyuridine, BrdU, and proliferating cell nuclear antigen, PCNA) in granulosa cells explaining the reduction in secondary follicles (Paredes *et al.*, 2004). It was also found in newborn *Ngf* KO ovary that somatic cell proliferation is reduced, contributing to the significant reduction in primary and secondary follicles (Dissen *et al.*, 2001).

The endocrine status of these mice was also examined and it was found that serum from *TrkB<sup>FLTr</sup>* heterozygous and KO pups contained significantly less FSH, compared to serum from Control mice. *TrkB<sup>FLTr</sup>* heterozygotes, though, did not display an effect on follicle development, indicating that this is not the cause of the KO

phenotype (Paredes *et al.*, 2004). The lack of secondary follicles (which are characterised by granulosa cell *Fshr* expression) was confirmed by a significant reduction in *TrkB<sup>FLTr</sup>* KO *Fshr* mRNA.

It is not possible to address here the capacity of *TrkB<sup>FL</sup>* KO primary follicles to continue growing *in vivo* in the KO. Primary-secondary stage follicles were not observed (although apparent in P6/7 Control ovaries) but, since follicle growth is delayed in *TrkB<sup>FL</sup>* KO mice, ovaries would need to be collected from mice older than P7 to determine if these follicles could develop further. It is unlikely that *TrkB<sup>FL</sup>* KO mice would survive for the required number of days to allow this to be undertaken.

It is interesting to note that the rate of small preantral follicle growth that occurs normally in the C57BL/6 mouse ovary is known to be reduced significantly when compared to other strains at P4 (Canning *et al.*, 2003). It may be possible that the opportunity to study the reduced rate of follicle development observed in the *in vivo* *TrkB<sup>FL</sup>* KO was impinged upon by this trait of the C57BL/6 mouse.

I propose that in addition to the *TrkB<sup>FLTr</sup>* and *Ngf* KOs, a granulosa cell proliferation defect is present in the phenotype of the *TrkB<sup>FL</sup>* KO. Thus, neurotrophin signalling in the ovary contributes to the proliferative capacity of the somatic cells through unknown pathways parallel to and not associated with the GDF-9 or KL pathways. The role of the truncated TrkB receptor isoforms in follicle growth initiation and its support is not clear, although as I have proposed that the phenotypes between the *TrkB<sup>FLTr</sup>* and the *TrkB<sup>FL</sup>* KO are actually quite similar, the conclusion could be drawn that additionally removing expression of the truncated receptors does not worsen the phenotype. This is supported by the fact that the *TrkB T1* KO does not present an ovarian phenotype as discussed in the introduction.

### 3.5.2. Follicle health

The arrangement of the follicle is very precise, with an oocyte at the centre, enclosed by layers of granulosa cells, and a BM separating it from the steroidogenic thecal cells. Observations had previously been made that sections from *TrkB<sup>FL</sup>* KO ovaries often appeared fuzzy (Spears *et al.*, 2003). In addition, poor follicular organisation is apparent. This abnormality is characterised by poor granulosa cell orientation around the oocyte and BM support of the follicle. Clear disruption of follicles was first observed in P2/3 *TrkB<sup>FL</sup>* KOs and this effect was confirmed by blind assessment of the P0-P7 Control and *TrkB<sup>FL</sup>* KO ovaries.

The health of primordial, primordial-primary and primary follicles was assessed, and one of the parameters used was the integrity of the follicular BM. In the majority of the follicle categories examined, *TrkB<sup>FL</sup>* KO ovaries exhibit significantly higher percentages of follicles with poor health and significantly lower percentages of follicles with good health, compared to Controls. The fact that any “good” follicles are observed suggests that in the *TrkB<sup>FL</sup>* KO some follicles are maintained appropriately. This is reinforced by Spears *et al.* (2003) who demonstrated that culturing P0 wild-type ovaries with K252a results in ~85% loss of oocytes, but those that survived had normal oocyte diameters, demonstrating normal growth.

It has also been observed in the *TrkB<sup>FLTr</sup>* KO that there is disruption of the BM (Paredes *et al.*, 2004) and from the sections shown of the *Ngf* KO (Dissen *et al.*, 2001) it is possible to see poor follicular BM. Therefore, it is proposed that the poor follicular health exhibited by the *TrkB<sup>FL</sup>* KOs, in particular disruption of the BM, is of physiological significance and may contribute to the reduction in follicle growth.

The significance of reduced follicle health has been implicated through studies investigating the importance of the BM. The BM can influence cell shape, cell behaviour, cell death and cell anchorage. It

also provides support for the tissue and presents signalling molecules to granulosa cells, and can ultimately affect oocyte quality (Irving-Rodgers and Rodgers, 2005). If the structure of the follicle is disrupted then it is possible to hypothesise that so will the connections between the oocyte and its supporting somatic cells. An important connection between the oocyte and granulosa cell is the transzonal projection (TZP). Many of these granulosa cellular extensions cross the zona pellucida (ZP) to the oocyte (Albertini *et al.*, 2001). Gap junctions are found at the ends of TZPs and as discussed in Chapter 1.3.2 gap junctions are composed of connexins. The connexin found between oocyte and somatic cells is connexin37 and mice null for the gene (*Gja4* KO) exhibit a block in follicle growth at the preantral stage (Carabatsos *et al.*, 2000). The segregated expression pattern of the connexins in ovarian follicles (connexin37 between oocyte and somatic cell and connexin43 between somatic cells) implied separate functions, yet fertility could be restored in the *Gja4* KOs through ectopic oocyte expression of *Gja1* (Li *et al.*, 2007).

In summary, even though the severe phenotype is no longer apparent in the re-derived *TrkB<sup>FL</sup>* KO ovaries, there is a subtle phenotype exerted by the mutation on the health of the follicles, crucially indicated by the BM. Thus, it is proposed that the abnormal follicular structure of the *TrkB<sup>FL</sup>* KO ovaries contributes to the proliferative defect in *TrkB<sup>FL</sup>* KOs. Accordingly, the next Chapter will examine the BM of the *TrkB<sup>FL</sup>* KOs in detail.

### 3.5.3. Re-derivation

There are two issues to be addressed in relation to the re-derivation of the *TrkB<sup>FL</sup>* colony. The first is the lower birth weight resulting in fewer *TrkB<sup>FL</sup>* KOs collected and the second is the change in phenotype of the *TrkB<sup>FL</sup>* KOs.

Comparing the mean birth weight of the LF and HRB colonies revealed that the LF colony was significantly smaller in weight on the

day of birth compared to the previous colony. It has been demonstrated that housing in IVCs can affect pup survival and weanling weight, depending on air changes per hour and cage change intervals (Reeb-Whitaker *et al.*, 2001). Thus, re-derivation of the colony into IVCs is a credible cause of the reduction in TrkB colony weight at P0. Given that *TrkB<sup>FL</sup>* KOs perish neonatally due to their inability to feed, it seems likely that postnatal survival may be influenced by birth weight (what reserves of body fat they manage to accumulate *in utero* may help them to survive longer postnatally). A lower birth weight means a reduced fitness of newborn pups, resulting in increased loss of *TrkB<sup>FL</sup>* KOs.

There is also evidence to indicate that animal housing may influence the variation in survival of mice with the same mutation on the same background. The *TrkB<sup>FL</sup>* KO was generated by Klein *et al.* (1993) on a C57BL/6 background and the majority of mutants perished within the first 48 hours of birth. The mice assessed by Spears *et al.* (2003) were descendants of the Klein *TrkB<sup>FL</sup>* KO maintained on the same background and it was found that these KO pups could survive to P4/5 in conventional caging. However, upon re-derivation most *TrkB<sup>FL</sup>* KO pups did not survive that long, although a few did very occasionally. Intriguingly, Dr Vega's group at the University of Oviedo in Spain routinely published papers describing the same Klein *TrkB<sup>FL</sup>* KO on the same C57BL/6 background surviving to P15 (Garcia-Suarez *et al.*, 2006; Perez-Pinera *et al.*, 2008). Through personal correspondence with Dr Silos-Santiago (who provided these mice from Vertex Pharmaceuticals, San Diego, USA) it was established that these *TrkB<sup>FL</sup>* KOs were able to survive for up to a month and the colony was housed in a conventional breeding unit. These observations strongly indicate that there is a potential role for unit sterility to act as a factor in the robustness of the *TrkB<sup>FL</sup>* KOs.

How the re-derivation could have affected the phenotype of the colony is unclear. It has been proposed, in evolutionary theoretical scenarios, that phenotypes can alter depending on the environment, this is referred to as phenotypic plasticity (Pigliucci *et al.*, 2006). It has also been found through studying respiratory responses in mice, that a pathogen-free environment can result in loss of phenotype (Pinto *et al.*, 2008). This led the group to conclude that pathogens and environment can affect presentation of mutant allele phenotypes. It may be that the *TrkB<sup>FL</sup>* KOs were able to exhibit a robust phenotype when housed in the conventional caging of the HRB unit but re-derivation into the sterile LF unit was a sufficient enough change in environment to cause a shift in the phenotype.

## Chapter 4

### ECM of the *TrkB<sup>FL</sup>* KO mouse: laminin- $\alpha$ 1

## 4.1. Introduction

The extracellular matrix (ECM), although once considered of limited purpose, actually has incredibly diverse functions in normal and pathological conditions, from growth and wound healing to tumour formation. The multicellular organism simply could not exist without it due to the structural support it provides in the form of connective tissue. The ECM can generally be described as proteins and proteoglycans secreted by epithelial, stromal and fibroblast cells. The proteins self-assemble into macromolecular structures located in the extracellular spaces of the tissue (Bosman and Stamenkovic, 2003; Gelse *et al.*, 2003).

The ECM has many functions including: the provision of support to cells and strength and elasticity to tissues; creation of microenvironments within tissue by separating functional regions from supportive structures (parenchyma and stroma respectively); a substrate for cells to move on, thereby facilitating cell migration; binding biologically active components which regulates availability of growth factors, nutrients and hormones. Subsequently the latter can influence cell differentiation, proliferation, migration, survival and death. The ECM orientates and polarises the cells into basal and apical regions, and can regulate tissue turgidity by controlling the movement of fluid (Tanzer, 2006).

Part of the ECM is a thickened layer, referred to as the basement membrane (BM), which is found immediately adjacent to the epithelial cells it supports (see Figure 4.1). The BM is in contact with and supported by ECM components found in the stroma (also referred to as the interstitial matrix). The composition of both the BM and the interstitial matrix varies according to tissue and cell type and can be remodelled by the actions of proteases, allowing the ECM to be regulated in a dynamic, temporal and organ-specific fashion (Bosman and Stamenkovic, 2003; Tanzer, 2006).



The ECM component found in greatest quantity in most animals is the glycoprotein collagen. It forms a scaffold (which provides structural integrity) along with another glycoprotein, laminin (which provides biological activity). This mesh of macromolecules is interconnected by accessory proteins, generally proteoglycans and nidogen (Hallmann *et al.*, 2005; Tanzer, 2006). Members of the collagen family are made up of three polypeptide chains ( $\alpha$ -helices) which assemble into a triple helix. They can be heterotrimeric (comprising two or more different chains) or homotrimeric (three identical chains). With more than 20 collagens produced from distinct genes the possible ECM compositions are extensive, although mainly fibrillar collagens are synthesised including types I, II, III, V and XI (Bosman and Stamenkovic, 2003; Gelse *et al.*, 2003). Fibrillar collagens include those found in skin, bone, tendons and cartilage as they confer strength due to the rigidity of their triple helix. They contribute mainly to stromal ECM but there is also evidence of collagen type V fibrils projecting from the stroma into the scaffold of the BM, increasing epithelial cell anchorage (Adachi and Hayashi, 1994).

The BM (also referred to as the lamina densa) is considered to be a specialised layer of ECM. The proximity of its location, adjacent to the epithelial cells, allows biologically active components of the BM to regulate cell behaviour. The BM is similar to the stromal ECM in that it is comprised of a network of collagen and laminin linked by accessory proteins, except that the collagen is the more flexible, non-fibrillar collagen type IV (Bosman and Stamenkovic, 2003; Gelse *et al.*, 2003; Hallmann *et al.*, 2005). Laminin is composed of three chains ( $\alpha$ ,  $\beta$ ,  $\gamma$ ) and each type of chain has a subset of isoforms, facilitating tissue-specific expression of up to 15 different laminins (Colognato and Yurchenco, 2000; LeBleu *et al.*, 2007; Miner, 2008). Laminin was first recognised as a non-collagenous component of ECM when it was isolated from large quantities of ECM produced by

a tumour cell line. Characterisation subsequently revealed its presence in normal tissues (Timpl *et al.*, 1979). Laminin 1 (composition  $\alpha 1\beta 1\gamma 1$ ) is the major laminin expressed embryonically in several tissues, with laminin- $\alpha 1$  the chain best characterised (Colognato and Yurchenco, 2000).

As well as deposition of ECM, maintenance of tissue form and function is regulated by the turnover of ECM which is achieved partly by the actions of proteases and their inhibitors. Proteases include: the matrix-metalloproteinases (MMPs); plasminogen activators (PAs); the a disintegrin and metalloproteinase (ADAM) family and cathepsins. Protease activity is subject to the tissue inhibitors of MMPs, TIMPs (Curry and Osteen, 2003; Mazaud *et al.*, 2005).

Laminin not only contributes to tissue structure and integrity but also confers biological activity upon the cells interacting with the BM by binding to cell surface receptors (integrins, see Figure 4.1). There are also adhesive connections that form laterally between the cells sitting on the BM (see Figure 4.1). Cell-cell connections include tight junctions, adherens (the cadherins), desmosomes and gap junctions. Other cell-ECM connections include dystroglycan and other proteoglycans. These adhesive associations discussed form complexes with intracellular components including  $\beta$ -catenin, which in turn associates with actin attached to the microtubules of the cytoskeleton. This regulates the behaviour of the cell including its shape. The complexes can also regulate the activation of cellular signalling cascades, which can control gene expression and cell function (Hagios *et al.*, 1998; Bosman and Stamenkovic, 2003; Zahir and Weaver, 2004).

#### **4.1.1. Ovarian ECM**

Proliferating PGCs migrate from the allantois to the undifferentiated gonad of the developing embryo. There they are incorporated along with somatic cells (epithelial cells derived from the

mesonephros and ovarian surface epithelium, OSE) into the sex cords which are segregated from the mesenchymal and endothelial cells of the underlying stroma by a BM (Byskov *et al.*, 1986; Rajah *et al.*, 1992; Luck, 1994; Rodgers *et al.*, 2000; Rodgers *et al.*, 2003). It is during the perinatal period of rodent ovary development that the primordial follicles are formed. Many oocytes undergo cell death at this time but those that survive are incorporated into follicles and are surrounded by a BM which separates them from the surrounding stroma. The ovarian stroma has been implicated in primordial follicle formation due to the expression of growth factors and/or receptors expressed there, which may facilitate the co-ordinated breakdown of the nests to individual follicles. It has also been proposed by Rajah *et al.* (1992) that primordial follicles are not truly formed in the rodent ovary until the epithelial pregranulosa cells are opposed by mesenchymal (stromal) cells to induce deposition of a BM.

The advent of the electron microscope has greatly facilitated studies of the ovarian ECM (Zamboni, 1974). It was observed that the quiescent primordial follicles in the ovary of the rhesus monkey were separated from the surrounding tissue by a thin basal lamina (the follicular BM) and collagen fibrils. The BM is essential in maintaining the avascular nature of the oocyte and granulosa cell compartment of the follicle as well as supporting its structure and facilitating growth. It is also important in conferring signalling molecules to follicular cells which act on follicle development and ultimately are important in ensuring the goal of the ovary: to produce a mature fertilisable oocyte at ovulation, capable of supporting embryo development.

Immunohistochemical techniques have begun to establish the spatiotemporal expression of ECM components in the ovary. This is integral to understanding the requirements of healthy follicles at each stage of growth, and how an atretic follicle may be identified. The research group of Professor Ray Rodgers (Research Centre for

Reproductive Health, Adelaide, Australia) is dedicated to understanding the role of ECM in mammalian ovarian development and function. However, their model organism of choice is the bovine ovary (Rodgers *et al.*, 2003).

The component identified to be first laid down around the mouse primordial follicle by Berkholtz *et al.*, (2006) is laminin. This component is also present in the BM from primordial follicles onwards in rabbit and pig ovaries and was also found in the interstitium between oocyte cords prior to follicle formation (Lee *et al.*, 1996). However, another study detected collagen type IV in the BM of the mouse primordial follicle (Nakano *et al.*, 2007). It has also been found in the primordial follicles of rat (Mazaud *et al.*, 2005) and cow (Rodgers *et al.*, 2000), thus not all studies are in agreement.

Berkholtz *et al.* (2006) also described the BM components expressed in growing follicles of the mouse ovary. Laminin and collagen type IV are found in the BM of primary follicles, aggregates of fibronectin (another cell adhesion glycoprotein) and diffuse fibrillar collagen type I is found in the granulosa cell layer. In the secondary and larger multi-layered follicles laminin and collagen type IV are additionally observed in the thecal layer. Fibronectin in these larger growing follicles is still found in aggregates in the granulosa cell layer as is diffuse collagen type I, both are also found in the thecal layer. Antral follicles maintain the same BM composition but also exhibit deposits of fibronectin and collagen type I in the follicular fluid (Berkholtz *et al.*, 2006). Collagen (types I and IV), laminin and fibronectin were all identified in the ovarian stroma, and all except for laminin were detected in the OSE.

The aggregates of ECM that form in the granulosa layer of preantral and antral follicles were originally observed by two Austrian scientists (Call and Exner) in rabbit ovulatory follicles in the 19<sup>th</sup> century. They described these aggregates as clumps of granulosa cells arranged around a cavity and named them Call-Exner bodies. It

has since been shown that the cavity of these structures contains ECM components similar to those of the follicular BM (namely collagen type IV and laminin) and are actually located in the granulosa layer of preantral follicles onwards in mammals (van Wezel *et al.*, 1999). Thus, it is apparent that the regulation of the composition of the follicular BM, the thecal layer, and the ECM within the follicle itself varies in a species and follicle stage-dependant fashion.

Once puberty is reached, the ovary continually undergoes cycles of follicle growth, atresia, and ovulation. This requires a great degree of ECM remodelling which must be carried out in a controlled fashion: too much degradation and not enough synthesis results in the follicle not being maintained, too little degradation and follicle growth is impaired. As previously mentioned, it is the role of proteases to degrade the different components of the ECM, and this has been well documented for ovulation (Curry and Osteen, 2003).

Prior to ovulation the proteases also play a role in ovary development. The first stage in folliculogenesis is the degradation of the ECM which segregates the ovigerous cords from the surrounding stroma. This allows the oocytes to forge somatic associations with the pregranulosa cells, ultimately resulting in the formation of primordial follicles. Mazaud *et al.* (2005) revealed that in the rat this process is assisted by the actions of the MMP and PA families of proteases. The importance of controlling BM remodelling has also been demonstrated in follicle growth in the mouse. The protease, a disintegrin and metalloproteinase with thrombospondin type 1 motifs-1 (ADAMTS-1) protein is found in the granulosa cells of follicles from the secondary stage onwards (Brown *et al.*, 2006). The mouse null for this gene (assessed by the same group) suffers from a defect in ovulation, again demonstrating that the ultimate role is in releasing the oocyte. However, the *Adamts-1* KO mouse also exhibited reduced numbers of antral follicles alongside increased

numbers of dysgenic follicles. These defects in follicular ECM components were tracked back to earlier stages of follicle growth (Brown *et al.*, 2006), demonstrating how defective protease regulation can affect normal fertility.

#### 4.1.1.2 Neurotrophin signalling and the ovarian ECM

A role for neurotrophin signalling in relation to the ECM has been implicated in different *in vitro* and in *in vivo* disease systems. It has been demonstrated *in vitro* that downstream signalling components of tyrosine kinase receptors (Akt/PKB) are implicated in the production of ECM components such as laminin and collagen type IV by embryoid bodies (Li *et al.*, 2001). Activation of the Akt/PKB pathway through TrkB is sufficient to prevent anoikis in a rat intestinal epithelial cell line (Douma *et al.*, 2004): anoikis is when a cell loses contact with ECM and undergoes apoptosis. In tumours, it is this ability of cells to suppress anoikis that allows the tumours to grow unchecked and eventually metastasise. Human ovarian epithelial cancers overexpress *Bdnf* and *TrkB* mRNA, with particular overexpression of the full-length receptor in high-grade carcinomas revealing an *in vivo* role (Yu *et al.*, 2008). In conjunction with assessing samples of cancerous tissue, Trk signalling in cancer cell lines was assessed. Cells which had survived anoikis (spheroid anchorage-independent cells) had increased levels of full-length *TrkB* mRNA and increased levels of phosphorylated phosphatidylinositol 3'kinase (PI3K), demonstrating the role of TrkB signalling in cell survival pathways (Yu *et al.*, 2008). It was also found that silencing TrkB signalling by siRNA reduced the amount of phosphorylated Akt and increased the amount of anoikis induced in the cells (Yu *et al.*, 2008). These observations demonstrate that *TrkB* overexpression protects cells from a fate of cell death (anoikis) by activating the PKB/Akt survival pathway, apparently through full-length TrkB.

Other evidence of involvement of Trk signalling in the ovary regulating follicular BM deposition includes observations of BM breaches in preantral follicles of the *TrkB<sup>FLTr</sup>* KO (Paredes *et al.*, 2004). In addition, Dissen *et al.* (2001) examined the ovaries of P7 mice null for *Ngf*; their figures reveal that the follicular BM of primary and secondary follicles appears abnormal (although they did not comment on this). Thus there is evidence in the literature to support the hypothesis that Trk signalling may regulate ovarian ECM deposition, and signalling survival in response to a loss of contact with ECM.

## 4.2. Aim

Chapter 3 showed that *TrkB<sup>FL</sup>* KO ovaries contain follicles with a hazy BM. This was assessed morphologically in Chapter 3 and a reduction in follicle health from the primordial through to the primary stage was shown, when compared to Control ovaries. The aim of this Chapter was to investigate the role of TrkB signalling on BM formation by histologically comparing the follicular basement membrane of Control and *TrkB<sup>FL</sup>* KOs.

## **4.3. Materials and Methods**

### **4.3.1. Animals**

Transgenic animals were generated and genotyped as described in Chapter 2.1-2.2. Control (wild-type and *TrkB<sup>FL</sup>* heterozygote) and *TrkB<sup>FL</sup>* KO pups were collected from P0 through to P7.

### **4.3.2. Tissue collection**

Ovary, testis, kidney and brain were collected from P0 (ovary, testis, brain), P3 (ovary and kidney), P4 (ovary), P6 (ovary, kidney, brain) and P7 (ovary) Control and *TrkB<sup>FL</sup>* KOs as described in Chapter 2.3. In addition, non-experimental wild-type ovaries (P2) and kidney (P0) were collected to test stains. The high content of BM in the tubules of kidney acts as an excellent positive control for ECM stains.

### **4.3.3. Tissue preparation**

Ovaries were fixed in Bouins or PFA as described in Chapter 2.4.1. Testes were fixed in 4% PFA for approximately one hour, kidneys and brains were fixed in 4% PFA overnight.

Post-fixation, all tissue types were treated the same way as detailed in Chapter 2.4. Briefly, the tissue was embedded in wax (kidneys and brains were embedded coronally) before being sectioned at 5µm on a microtome.

### **4.3.4. Histological assessment by general BM stains**

Prior to specific assessment of the BM, adjacent sections from the ovaries, testes and kidneys were stained with H&E (as detailed in Chapter 2.4.4) to ensure morphology was adequate for assessment.

#### **4.3.4.1. Van Gieson stain**

Van Gieson stain turns collagen fibres red and is used to stain connective tissue. The protocol was tested on wild-type P0 kidney

and P2 Bouins and PFA fixed ovary to see if it would be appropriate for use on experimental material.

The van Gieson stain was prepared a day in advance: 15ml of 0.5% aqueous acid fuchsin, 75ml of saturated picric acid, 50ml of MilliQ ddH<sub>2</sub>O and 0.25ml of HCl. Picric acid precipitate formed and was avoided when decanting the stain. Slides were dewaxed and rehydrated to tap water as described in Chapter 2.4.4 before immersion in van Gieson stain for 2-10 minutes. The slides were then washed in tap H<sub>2</sub>O before the level of stain was checked microscopically. To minimise loss of stain, the dehydration steps from 70%-95% were omitted and instead, slides were immersed in 100% ethanol (x2). The slides were then cleared in xylene, mounted and images were obtained as described in Chapter 2.4.4. Only 10 minutes of exposure to van Gieson stain revealed a detectable staining of fibres.

#### **4.3.4.2. Periodic acid-methenamine (Jones) silver stain**

Periodic acid-methenamine (Jones) silver stain (PAMS) oxidises polysaccharides in BM to aldehyde groups. Aldehyde groups reduce silver ion to a black-coloured silver precipitate on the BM revealing its location in the tissue. As for the van Gieson stain, this protocol was first tested on wild-type P0 kidney and P2 Bouins and PFA fixed ovary.

Prior to each experiment all glass and plastic-ware to be used was washed in specialised cleaning detergent (Sigma) to prevent precipitation of the silver ions elsewhere than on the tissue. The methenamine silver nitrate solution was prepared in advance (5ml of 5% silver nitrate plus 100ml of 3% methenamine), if stored in an amber bottle in the fridge it would last for up to one month. Slides were dewaxed and rehydrated to MilliQ ddH<sub>2</sub>O (see Chapter 2.4.4). The sections were oxidised in freshly made 0.5% periodic acid at room temperature for 20 minutes. During this time the working

silver solution was prepared and heated to 62°C in a waterbath: 25ml of methenamine silver nitrate solution, 2 ml of 5% borax and 23 ml of MilliQ ddH<sub>2</sub>O. The slides were removed from the periodic acid, washed in running tap H<sub>2</sub>O before being rinsed in MilliQ ddH<sub>2</sub>O. The slides were then immersed in the 62°C working silver nitrate solution. The speed of the reaction varied greatly (10-40 minutes), thus slides were regularly checked for desired staining levels microscopically and were agitated to promote the reaction. Once staining was complete, the slides were washed in MilliQ ddH<sub>2</sub>O (x4) before being toned (to emphasise stain) in 0.2% gold chloride for 30 seconds. The slides were washed twice more in MilliQ ddH<sub>2</sub>O before being dehydrated, cleared in xylene, mounted and photographed as described in Chapter 2.4.4.

#### **4.3.5. Histological assessment by antibody detection of laminin- $\alpha$ 1**

To improve the detection of ovarian BM, immunohistochemistry was carried out for a specific isoform of laminin ( $\alpha$ 1) known to be detectable in the neonatal ovary (Uda *et al.*, 2004; Berkholtz *et al.*, 2006).

##### **4.3.5.1. Immunohistochemistry**

Laminin- $\alpha$ 1 was detected in ovary, testis, kidney and brain by DAB as described in Chapter 2.7.1-2.7.3. The primary antibody used was a rabbit anti-rat polyclonal (Abcam) at a 1:100 dilution. The secondary used was a biotinylated goat anti-rabbit antibody (Dako) at a 1:200 dilution. Non-immunised goat serum was used to make antibody solutions.

##### **4.3.5.2. Immunofluorescence**

Laminin- $\alpha$ 1 was detected in ovary, testis, kidney and brain by immunofluorescence as described in Chapter 2.7.1, 2.7.2 and 2.7.4. The same primary antibody was used as for DAB detection except at

a dilution of 1:300. The secondary used was a HRP-conjugated chicken anti-rabbit antibody (Santa Cruz) at a 1:200 dilution. Non-immunised chicken serum was used to make antibody solutions.

## 4.4. Results

### 4.4.1. Basement membrane – general stains

Initially, I planned to use a general ECM stain which would allow assessment of Control and *TrkB<sup>FL</sup>* KO ovary ECM, particularly that of the follicular BM. However, it became clear for different reasons that the protocols would not be suitable.

#### 4.4.1.1. Van Gieson stain

The van Gieson stain was tested on P0 kidney and P2 non-experimental ovary, both of which demonstrated normal morphology as shown by H&E in Figure 4.2 A and B. In the kidney, collagen fibrils between tubules were distinctly picked up by the stain, and more lightly, the BM of the glomeruli and renal tubules (Figure 4.2 D). In the ovary the stain appeared to work better in the collagen fibrils of the bursa than in the ovary itself (Figure 4.2 C). Crucially, in the ovary proper, it was not feasible to distinguish between stain at cell membranes and stain around follicles, thus, the protocol was not used on *TrkB* material.

#### 4.4.1.2. PAMS stain

Sections from the same kidney and ovary were used to refine the PAMS protocol, although after numerous attempts this method also failed to stain the ovarian BM in the desired manner. Figure 4.2 E-H demonstrate examples that were either too light to clearly visualise BM or too heavy. It was possible to achieve a level of stain between these extremes, but it was not possible to do so consistently. It was, therefore, not used on *TrkB* tissue.



#### 4.4.2. Immunohistochemical analysis of BM

Due to the varying success of the general stains it was decided that immunohistochemistry (including immunofluorescence) could be more reliably carried out on the limited tissue available. It would also allow the examination of a specific ECM component whereas the general stains could not do that.

##### 4.4.2.1. Laminin- $\alpha$ 1 protein expression in the ovary

Laminin- $\alpha$ 1 was selected as it is located in the BM of the murine follicles from the primordial stage onwards (Berkholtz *et al.*, 2006). Initial trials with the antibody using DAB detection had proved problematic, thus, it was tested with fluorescent antibody detection as well as DAB (see Figure 4.3). Ultimately both protocols revealed intense staining located in: the BM of follicles; ECM of stroma; vasculature (ovary proper and bursa); and ECM immediately below the OSE. Low levels were also apparent in the granulosa layer of follicles (see Figure 4.4 C). Primary antibody titration with the TSA fluorescent detection system demonstrated that it could be used at a greatly reduced dilution. A lower concentration of primary antibody reduces problems associated with background signal, thus it became the protocol of choice to analyse the Control and *TrkB<sup>FL</sup>* KO ovaries.

An initial trial of laminin- $\alpha$ 1 protein detection on Control and *TrkB<sup>FL</sup>* KO ovary indicated two aspects of laminin- $\alpha$ 1 deposition that appeared to be affected in the *TrkB<sup>FL</sup>* KO. Notably, there was an overall reduction in signal present and secondly, the laminin- $\alpha$ 1 in the BM surrounding the follicles was not continuous (see Figure 4.4). It was decided to focus the study on the overall reduction of laminin- $\alpha$ 1 signal, as breaks in continuity of signal around a follicle do not necessarily represent areas of abnormal BM (personal correspondence with Professor Ray Rodgers, Adelaide, Australia). This was confirmed by observations of discontinuous laminin- $\alpha$ 1 signal in Control ovaries.





#### 4.4.2.2. Semi-quantification of laminin- $\alpha$ 1 protein

Limited availability of tissue ruled out quantifying the reduction in laminin- $\alpha$ 1 by established means such as Western blot. Thus, it was decided to use the immunofluorescent protocol to semi-quantify the signal. This is based on the principle that the amount of fluorescent signal generated reflects the amount of available antigen for primary antibody to bind to, indicating protein levels. This would allow an attempt at addressing the question of whether or not laminin- $\alpha$ 1 is reduced in the *TrkB<sup>FL</sup>* KO ovary. Although not ideal, this principle has been used in several other studies to quantify a protein of interest (Costes *et al.*, 2000; Seino *et al.*, 2002; Szeszel *et al.*, 2005; Wang *et al.*, 2005). Given that no exact protocol existed, and that this is not the standard way of quantifying protein in tissue, how the images were obtained and the method of analysis had to be carefully considered to ensure that it would give meaningful results. Accordingly, suitability of differing protocols was investigated but the one considered best practice is described herein.

To limit variability, paired Control and *TrkB<sup>FL</sup>* KO tissue were always included in the same run of laminin- $\alpha$ 1 immunohistochemical detection. The pair was also always imaged on the confocal in the same session. The Control section was imaged first to set the gain which best demonstrated signal in follicular BM (gain is the level of excitation used by the laser to generate fluorescence). The *TrkB<sup>FL</sup>* KO sibling was then imaged at the same gain, thus fluorescence detected would be comparable to that of the Control. The confocal laser point scans across the section in one plane of focus, it then moves a set distance to another plane in the section before repeating the scan. The resulting image is called a “z” stack as it is made up of several layers. Importantly, point scanning means that each layer of the stack is in focus, no blurred light from out of focus layers is collected which would affect true signal.

Each image is made up of pixels which record the intensity on a scale from 0 (no signal) to 255 (maximum signal, it is possible to elicit signal greater than this value but it will only record it as 255). Initially, the mean pixel intensity (MPI) of confocal stacks was measured using the software, ImageJ. MPI indicates the average level of fluorescence in all of the pixels in the image. The basic interpretation of this is: a reduction in MPI represents a reduction in available antigen.

The MPI value was obtained by the following steps in ImageJ. Initially a variance filter was applied to the stack of confocal slices (laminin- $\alpha$ 1 signal only), this allows analysis of the follicular BM as it detects areas of high contrast (such as the edge of a follicle). The mask is then applied to the original stack so that the MPI of the image can be obtained with reference to these areas of high contrast only. However, this could only be done to the entire image which introduced two problems. Firstly, some of the images obtained included bursa, which, as shown in Figure 4.3 B and C, exhibits very strong laminin- $\alpha$ 1 signal. So it follows that signal from the bursa could affect the MPI, thus preventing a true measure of the signal from the ovary proper only. Secondly, for some other ovaries the area of the section was smaller than its sibling. This results in a greater number of pixels at 0, reducing the MPI of the ovary proper. This second problem was partially overcome by thresholding both Control and *TrkB<sup>FL</sup>* KO images. Thresholding allows selection of intensity values you wish to measure (0-255) and by applying the threshold to just above 0, the pixels without signal are not included in the MPI value. The resulting MPI is of pixels with signal only. However, the area issue could not be rectified, which meant that non-ovarian signal could not be excluded, thereby preventing a comparison of signal from the ovary proper only.

Image-Pro Analyzer software (COIL facility, Edinburgh University) allows selection of areas for analysis and so it was used to

compare the Control and *TrkB<sup>FL</sup>* KO ovaries. With Image-Pro Analyzer, instead of obtaining an MPI for the image, the area of signal was measured. In the same way that the MPI reflects amount of antigen binding, the area of signal present can also be considered to represent the amount of laminin- $\alpha$ 1 deposition. With Image-Pro Analyzer, an area of interest (AOI) is selected and assessed. The AOI is then thresholded so that low level signal from stroma is excluded and the strongest signal from the BM is measured. The area of the objects in the AOI that exceed the threshold are calculated and totalled. This process is carried out on the Control ovary before the same AOI and threshold is applied to the sibling *TrkB<sup>FL</sup>* KO. The area of signal from the Control is considered to be the basal level of signal and is normalised to 1. The area of signal obtained from the sibling *TrkB<sup>FL</sup>* KO is divided by the Control area to represent the value as a fold change. The results for each pair can then be compared to other pairs.

There were some *TrkB<sup>FL</sup>* KO tissues which elicited no signal at the threshold set by the Control; however that is not to say there was no signal at all. In these instances, the 0 value obtained for the *TrkB<sup>FL</sup>* KO area of laminin- $\alpha$ 1 signal would prevent representation of the area of signal in the *TrkB<sup>FL</sup>* KO as a fold change of the Control. Thus, for these samples (marked as \* in the figures) it was considered an acceptable alternative to set the threshold according to the signal in the *TrkB<sup>FL</sup>* KO, this threshold was then applied to the Control and the fold change calculated as before.

#### 4.4.3. Semi-quantitative laminin- $\alpha$ 1 expression in the P0-P7 sibling pairs of Control and *TrkB<sup>FL</sup>* KO ovary

Ovaries were available for analysis at P0, P3, P4, P6 and P7. At P0, *TrkB<sup>FL</sup>* KO ovaries (n=3) exhibited a reduced area of thresholded fluorescent signal when presented as a fold change relative to the Controls (see Figure 4.5 A).

From P3 through to P7 the trend of laminin- $\alpha$ 1 expression is less clear. Figure 4.5 B shows that at P3 one *TrkB<sup>FL</sup>* KO had virtually no signal compared to its Control while the other had the same. Similarly, both P4 and P6 ovaries exhibited a *TrkB<sup>FL</sup>* KO with virtually no signal compared to its Control, but the other showed an increase in signal (Figures 4.5 C and D), while the P7 *TrkB<sup>FL</sup>* KO had almost four times as much thresholded signal as its Control (Figure 4.5 E).

In spite of the apparent variability, it can be concluded that P0-P7 Control ovaries always produced measurable laminin- $\alpha$ 1 signal in the BM of follicles. On the other hand, six out of ten *TrkB<sup>FL</sup>* KOs had reduced signal with five of those giving almost no signal at all.

As discussed in the introduction to Chapter 3, newborn ovaries were obtained from the *TrkB T1* KO mouse courtesy of Dr Tessarollo. These ovaries did not appear morphologically abnormal and laminin- $\alpha$ 1 detection did not reveal a reduction compared to the Control (not shown).

Due to the low sample sizes for most of the ages assessed, statistics were not feasible. The P0 *TrkB<sup>FL</sup>* KOs did have a sample size of three; however, the same gain was not used for each pair which would have allowed the area of signal generated to be averaged for each genotype.



#### **4.4.4. Semi-quantitative laminin- $\alpha$ 1 expression in non-ovarian tissue of *TrkB<sup>FL</sup>* KO mice**

The indication that loss of TrkB signalling disrupts laminin- $\alpha$ 1 deposition in the ovary prompted the consideration that this could be a general mechanism of ECM synthesis in response to TrkB activation. If this were true, then laminin- $\alpha$ 1 deposition in the *TrkB<sup>FL</sup>* KO may also be disrupted in other tissues in which TrkB has a known function. It was possible to test this hypothesis on Control and *TrkB<sup>FL</sup>* KO testis, kidney and brain by using the same protocol used to analyse the ovaries.

##### **4.4.4.1. Laminin- $\alpha$ 1 in the *TrkB<sup>FL</sup>* KO testis**

P0 Control and *TrkB<sup>FL</sup>* KO testis were stained for H&E. This did not reveal any obvious morphological defect in the *TrkB<sup>FL</sup>* KO testis (see Figure 4.6 A and B). Laminin- $\alpha$ 1 signal was detected in the BM of seminiferous tubules and blood vessels in the testis. At the same level of excitation (gain) the fluorescent signal was virtually absent in the *TrkB<sup>FL</sup>* KO (Figure 4.6 C and D). This was confirmed by the reduced area of thresholded fluorescent signal exhibited by the *TrkB<sup>FL</sup>* KO as a fold change relative to the Control testis (Figure 4.6 E). As only one *TrkB<sup>FL</sup>* KO testis was collected statistics were not possible.



#### 4.4.4.2. Laminin- $\alpha$ 1 in the *TrkB<sup>FL</sup>* KO kidney

P3 and P6 Control and *TrkB<sup>FL</sup>* KO kidneys were obtained and processed for wax histology. H&E stained sections did not reveal any gross morphological defect in the *TrkB<sup>FL</sup>* KO kidneys (see insets Figure 4.7 A-D). Laminin- $\alpha$ 1 signal was clearly detected in the BM of the proximal renal tubules located in the cortex of the kidney, and appeared reduced in the *TrkB<sup>FL</sup>* KOs (see Figure 4.7 A-D). Calculating the area of thresholded signal in the *TrkB<sup>FL</sup>* KOs and presenting it as a fold change relative to the Control kidneys confirmed that the signal was reduced (see Figure 4.7 E and F). As only one pair of kidneys at P3 and one pair at P6 were obtained for analysis statistics were not possible.



#### 4.4.4.3. Laminin- $\alpha$ 1 in the *TrkB<sup>FL</sup>* KO brain

P0 Control (n=4) and *TrkB<sup>FL</sup>* KO (n=2) sibling brains were processed for immunohistochemistry as were one pair of sibling P6 Control and *TrkB<sup>FL</sup>* KO brains.

DAB detection of laminin- $\alpha$ 1 in P0 brain confirmed deposition in the blood vessels, including those of the choroid plexus in the lateral ventricles, and in the developing meninges, including the pia mater and the arachnoid membrane (see Figures 4.8 A and B). Difficulties were encountered in trying to ensure that equivalent coronal sections were compared between genotypes at P0. To reveal if a difference was present, it was decided to compare the signal in a region of the brain that did not significantly change in morphology throughout the tissue, thus ensuring the signal was measured reliably. The area of signal was, therefore, calculated for the meninges (see Figures 4.8 C and D) as described for the ovaries, but as the same gain was used for each sample the area of signal obtained could be averaged and then compared between genotypes (see Figure 4.8 E; as only two *TrkB<sup>FL</sup>* KOs were collected it was not possible to compare statistically). It was found, though, that the level of signal was reduced in the meninges of the P0 *TrkB<sup>FL</sup>* KO brains.

Comparable coronal sections were obtained for the P6 brains allowing assessment of the different laminin- $\alpha$ 1 expressing structures as confirmed initially by DAB (see Figures 4.9 A and B). Signal was measured in choroid plexus of the third ventricle, meninges and lateral ventricles (Figures 4.9 C-H). The area of signal was then calculated for the three different regions of P6 brain and compared between genotypes (see Figure 4.10). Across the three areas of P6 brain, the *TrkB<sup>FL</sup>* KO presented a reduced area of thresholded signal compared to its Control, although statistics were prohibited due to the sample size.







#### 4.4.4.4. Summary of laminin- $\alpha$ 1 expression in *TrkB<sup>FL</sup>* KO tissue

It can be summarised that although only limited samples were able to be collected from P0-P7, laminin- $\alpha$ 1 signal was measured in 13 different *TrkB<sup>FL</sup>* KO pups (see Figure 4.11). Out of these 13 assessed it was found that nine exhibited a reduction in laminin- $\alpha$ 1 signal (in the ovary, testis, kidney or brain). Two *TrkB<sup>FL</sup>* KOs did not exhibit a consistent trend across tissues: one did not exhibit a difference in the ovary but showed a reduction in the kidney, another exhibited an increase in laminin- $\alpha$ 1 in the ovary but a decrease in the kidney and brain. The remaining two *TrkB<sup>FL</sup>* KOs examined exhibited an increase in the ovary but no other tissue was assessed from these animals.



## 4.5. Discussion

Chapter 3 outlined that H&E sections of *TrkB<sup>FL</sup>* KO ovaries often exhibited a hazy ultrastructure, and demonstrated that *TrkB<sup>FL</sup>* KO ovaries measurably contain follicles of poorer health than their Controls. To assess this phenotype further, laminin- $\alpha$ 1 protein expression was assessed as its known deposition in the BM of primordial follicles onwards would allow assessment of the follicular BM in the P0-P7 ovaries collected.

Analysis of immunofluorescent histochemistry demonstrated that the *TrkB<sup>FL</sup>* KO pup exhibits a reduction in laminin- $\alpha$ 1 in the ovary, the testis, the kidney and the brain as compared to Control siblings. General conclusions that can be drawn from this experiment are considered before the significance of these findings in relation to the phenotype of the *TrkB<sup>FL</sup>* KO ovary shall be discussed.

### 4.5.4. Detection and analysis of laminin- $\alpha$ 1 expression in the ovary

The pattern of ovarian laminin found in the ovary here, matches that in the literature for the most part (Frojdman *et al.*, 1995; Pan and Auersperg, 1998; Gomes *et al.*, 1999; Berkholtz *et al.*, 2006). It was, though, additionally found in the granulosa layer of follicles from the primordial stage onwards. For the purposes of this investigation, the detection of laminin- $\alpha$ 1 in follicular BM was considered reliable and accurate. In Chapter 3 it was shown at P0 that for both *TrkB<sup>FL</sup>* KO and Control ovaries, the majority of oocytes are either found in clusters or enclosed in non-growing primordial follicles. *TrkB<sup>FL</sup>* KO ovaries, however, exhibit a significantly higher percentage of poor primordial follicles than Control ovaries. From these observations, and in conjunction with the finding that P0 *TrkB<sup>FL</sup>* KO ovaries exhibit a reduction in laminin- $\alpha$ 1, I propose that the poor health observed in the *TrkB<sup>FL</sup>* KOs is partially evidenced by the reduction in expression of laminin- $\alpha$ 1. The physiological relevance of this in terms of oocyte survival or follicle formation in the

*TrkB<sup>FL</sup>* KO is not clear as no difference was detected in the distribution of follicle development or follicle number at P0 (see Chapter 3).

As shown in Chapter 3, the phenotype of the P4-P7 *TrkB<sup>FL</sup>* KO ovary is one of retarded follicle growth, with significantly less primary follicles and no primary-secondary follicles observed when compared to Control ovaries. The hypothesis put forward here is that a reduction in deposition of laminin- $\alpha$ 1 from the time of primordial follicle formation contributes towards a reduction in follicle growth. However, this cannot be categorically stated, as from P3-P6 ovaries displayed variable levels in laminin- $\alpha$ 1 (three exhibited a reduction, one no difference, and three an increase). There are, though, possible explanations for this discrepancy. Firstly, it may be that another source of laminin- $\alpha$ 1 signal in the P3-P7 ovaries is responsible for the increase. The threshold was set by signal observed in the follicular BM but it was not possible to exclude non follicular BM laminin- $\alpha$ 1 signal if it was above the threshold, such as the signal from ovarian vasculature. This scenario is depicted in Figure 4.12.



Secondly, it has been shown that the absence of primary follicles is apparent from P4 in the *TrkB<sup>FL</sup>* KO (see Chapter 3). Therefore, it seems likely that, although the ovaries do contain comparable proportions of total growing follicles, the distribution of follicles (primary/primary-secondary) is different, making it difficult to obtain an area absolutely comparable between pairs in terms of the follicle stages found in that section. This may prevent a fair comparison of laminin- $\alpha$ 1. This suggestion is reinforced by the finding that two of the pups examined, which did not exhibit a reduction in ovarian laminin- $\alpha$ 1 did exhibit a reduction in other organs assessed (kidney and/or brain) which show less variability in their architecture.

Assessment of only the follicular BM would need to be carried out to prove that there really is a reduction in laminin- $\alpha$ 1 in the follicles of the older ovaries. One potential way of doing this would be to assess the BM of individual follicles using a variation of the analysis used here to assess the whole ovary. From each Control and *TrkB<sup>FL</sup>* KO ovary, several sections throughout the ovary would be used for immunofluorescent laminin- $\alpha$ 1 detection. By using multiple sections from the same ovary, the sample size of each follicle type (primordial, primordial-primary and primary) may be large enough for statistical analysis. Following thresholding of the images it is possible, using the software described in Section 4.4.2.2, to draw a line across a follicle (bisecting it) which can then display the intensity of the signal along that line as a histogram. The histogram of the follicle intensity has two peaks representing where the line crosses the BM on each side of the follicle. The intensity of the peaks represent the strength of signal (and effectively are a measure of the amount of laminin- $\alpha$ 1) in the BM. If this were carried out for several follicles in each category from several ovaries it seems likely that if a difference does exist in the BM between the genotypes then it could be revealed in this way. This assessment would require a continuous

ring of laminin- $\alpha$ 1 staining around the follicles. This has been found by some groups (Brown *et al.*, 2006; Gomes *et al.*, 1999) and its disruption in continuity is claimed to be indicative of disrupted BM integrity. However, as discussed in Section 4.4.2.1 it is not considered by all to indicate this. Another possibility would be to trial other antibodies for laminin to establish if there is one that specifically localises only to the follicular BM. The same analysis used in this study could then be repeated without other sources of laminin skewing the results.

A final caveat from the experiments carried out here is that it is not possible to distinguish if the reduced detection of laminin- $\alpha$ 1 is from a reduction in synthesis or an increase in degradation. Collection of Control and *TrkB<sup>FL</sup>* KO ovaries for analysis of mRNA and/or protein levels of laminin- $\alpha$ 1 or MMP could to be used to reveal if it is increased synthesis or increased degradation that is responsible. However, using this technique with the antibody used in this study here, would not have allowed discrimination between the sources of laminin- $\alpha$ 1 (follicular BM or stromal/vasculature). Thus, considering the resources available (tissue and antibody) the protocol used here to assess laminin- $\alpha$ 1 in the Control and *TrkB<sup>FL</sup>* KO ovaries was the most appropriate.

#### **4.5.5. Laminin- $\alpha$ 1 expression in non-ovarian tissue**

Given that laminin 1 ( $\alpha$ 1 $\beta$ 1 $\gamma$ 1) is produced by many cell types including epithelial, muscle (smooth, skeletal and cardiac) and endothelial (Bosman and Stamenkovic, 2003 review; Tanzer review, 2006) it would be expected that if laminin- $\alpha$ 1 were affected in the *TrkB<sup>FL</sup>* KO that it would not just be limited to the ovary or indeed just the female *TrkB<sup>FL</sup>* KO.

Laminin- $\alpha$ 1 is located in the basement membranes of seminiferous tubules in mouse testis (Sasaki *et al.*, 2002), thus, the pattern of expression shown for the testis is accurate. It was

demonstrated in this Chapter that laminin- $\alpha$ 1 is reduced in the *TrkB<sup>FL</sup>* KO. The morphology of the neonatal *TrkB<sup>FL</sup>* KO testis does not appear to be different from that of its Control, thus, as with the P0 *TrkB<sup>FL</sup>* KO ovary, the reduction in laminin- $\alpha$ 1 does not appear to have deleterious effects on the tissue. However, unlike the P0 ovary, the P0 testis is undergoing a period of relative quiescence, thus it may be the case that the effect of reduced laminin- $\alpha$ 1 does not have any effect during this period. It may be, that upon resumption of testis development at puberty there is an effect, but due to the neonatal lethality of the *TrkB<sup>FL</sup>* KO this can only be conjectured.

The kidney filters blood of waste products; this process occurs by blood flowing through a tuft of capillaries found at the end of the renal tubule of the nephron. This tuft of capillaries is the glomerulus located inside the Bowman's capsule; held in place by a membrane of ECM called the mesangium. Plasma and waste products cross the glomerular BM and enter the renal tubules where fluid can be reabsorbed into the blood stream or end up along with the waste products as urine. The renal glomerular BM is derived from endothelial and epithelial cells, but laminin- $\alpha$ 1 is found only in the epithelial layers including the proximal tubules and the mesangium of the glomerulus, but not in the BM of the glomerulus itself (Aumailley and Smyth, 1998). Thus, despite the P15 *TrkB<sup>FL</sup>* KO exhibiting a reduced glomerular area (Garcia-Suarez *et al.*, 2006) it is unlikely that this is causing the reduction in signal as laminin- $\alpha$ 1 is simply not expressed in the areas affected in the *TrkB<sup>FL</sup>* KO. The physiological relevance of a reduced expression of laminin- $\alpha$ 1 is unclear.

Thus, laminin- $\alpha$ 1 is reduced in the epithelium of the genitourinary tract. Given that these structures originate from the same tissue primordia, it may not be that surprising that they are affected in the same way. Demonstration of reduced laminin- $\alpha$ 1 in *TrkB<sup>FL</sup>* KO brain is, however, strong evidence that the role of TrkB

signalling is a general mechanism of BM deposition. Laminin- $\alpha$ 1 was detected in the meninges, the choroid plexus and the vasculature of the mouse brain. This is in agreement with the literature for mouse (Letourneau *et al.*, 1988; Sasaki *et al.*, 2002) and rat (Krum *et al.*, 1991). The choroid plexus is a highly vascular structure, which extends frond-like processes into the ventricles of the brain. A network of capillaries is surrounded by a layer of epithelium that is continuous with a layer of specialised ependymal cells, which line the ventricles. Fluid leaks out of the capillaries and enters the ventricles as cerebrospinal fluid (CSF). Tight junctions in the epithelium of the choroid plexus act as a CSF-blood barrier and intraventricular pressure is regulated by CSF secretion and/or absorption. The CSF flows throughout the ventricles of the brain and into the subarachnoid space, bathing the brain. Thus, a reduction in laminin- $\alpha$ 1 in the structures lining the ventricles may contribute to abnormal CSF secretion and/or absorption, although this would require further investigation.

Thus, the tissues assessed in *TrkB<sup>FL</sup>* KO mice (ovary, testis, kidney and brain) all exhibit a reduction in laminin- $\alpha$ 1. However, it is clear that ablation of full-length TrkB signalling is not sufficient to prevent deposition entirely. If that were the case, it would most likely cause embryo lethality as found in laminin 1 deficient mice (Alpy *et al.*, 2005). The effect in the ovary is difficult to interpret as the reduction was not found to be consistent in the ovaries of *TrkB<sup>FL</sup>* KOs that were older than P0. The possible reasons for this, however, have been addressed. I believe that the evidence of reduced laminin- $\alpha$ 1 in other organs is convincing of a general mechanism for full-length TrkB signalling regulating BM deposition.

#### 4.5.6. What has the *TrkB<sup>FL</sup>* KO revealed about laminin- $\alpha$ 1 in the ovary?

I proposed previously that the *TrkB<sup>FL</sup>* KO ovary exhibits reduced laminin- $\alpha$ 1 deposition and that this may contribute to the phenotype of reduced follicle growth observed in Chapter 3. In this section I will discuss the evidence that supports this hypothesis.

There are stages in the initiation of follicle growth that can be identified by morphological changes and by molecular markers of differentiation, as discussed in Chapter 1.3.2. The classic morphological marker of growth activation is differentiation of flattened pregranulosa cells in primordial follicles into cuboidal cells. This requires remodelling and polarising of actin filaments in the cells (Zhang *et al.*, 2005). The cue(s) inducing this differentiation in the ovarian follicle are unknown but growth factors which can stimulate proliferation of epithelial cells and/or ECM deposition are implicated. The mammarys of ovariectomised prepubertal heifers exhibit: reduced expression of insulin-like growth factor-1 (IGF-1); its receptor; epithelial cell proliferation; and laminin (Akers *et al.*, 2005). In addition, the cytokines, interleukin-1 $\beta$  and tumour necrosis factor- $\alpha$  (IL-1 $\beta$  and TNF- $\alpha$ , respectively) have also been implicated in regulating ECM deposition as they induce transforming growth factor- $\beta$  (TGF- $\beta$ ) signalling in rat alveolar epithelial cells (Furuyama *et al.*, 2008). TGF- $\beta$  is so called because of its role in wound healing; it regulates the deposition of almost all components of ECM, and in rat alveolar epithelial cells it increases laminin, nidogen and collagen IV deposition (Furuyama *et al.*, 2008).

The potential regulation of follicle activation by ECM components is not completely clear. The eggs of *Drosophila* ovaries are supported by epithelial nurse cells which, amongst other roles, regulate polarisation of different RNAs in the oocyte. Certain polarisation events are executed by remodelling of actin filaments in the posterior follicle cells (Frydman and Spradling, 2001). Mutation of a receptor-like transmembrane tyrosine phosphatase (*Lar*) results

in failure to polarise the cells and revealed that its ligand (laminin) may regulate normal polarisation through transient expression in the ECM (Frydman and Spradling, 2001). The expression of laminin in the mammalian ovary (pig and rabbit; Lee *et al.*, 1996) prior to follicle formation suggests that it is not responsible for epithelial differentiation, although it may still be possible that once follicles activate growth they then require contact with laminin to support continued growth.

Integrins are cell surface receptors signalling contact between ECM and the cell, inducing intracellular signalling in response to ECM exposure. Laminin 1 ( $\alpha 1\beta 1\gamma 1$ ) binds to integrins and in particular, to integrin  $\alpha 6\beta 1$ , which is considered to be integral for cell anchorage (Colognato and Yurchenco, 2000). *Integrin a6* mRNA is highly expressed in oocytes and granulosa cells of primordial to secondary follicles in the mouse, integrin  $\beta 1$  is expressed in the oocyte from the primordial follicle stage onwards (Burns *et al.*, 2002). Thus, the integrin ( $\alpha 6\beta 1$ ) found to be highly expressed in the early stages of follicle growth is commonly associated with laminin- $\alpha 1$ . It is, therefore, feasible that reduction of laminin- $\alpha 1$  in the *TrkB<sup>FL</sup>* KO ovary may contribute to reduced integrin-ECM binding, which as well as disrupting follicle structure by reducing cell anchorage, may also reduce the intracellular signalling which would otherwise occur in response to ECM exposure. However, Burns *et al.* (2002) also assessed the levels of integrins in the *Gdf-9* null mouse and found that *integrin a6* mRNA was increased three-fold in the mutant ovaries. This is in keeping with the expression pattern of *integrin a6* and the phenotype of *Gdf-9* null mouse ovaries (follicles block at the primary stage of growth; Dong *et al.*, 1996). Thus, integrin expression is not sufficient to rescue the follicles from blocked growth, implying that BM-cell interactions are complementary to the actions of growth factors but not compensatory.

The evidence supporting a molecular pathway for TrkB regulating BM deposition actually stems from work carried out with another tyrosine kinase signalling growth factor (fibroblast growth factor; FGF). Prevention of FGF signalling in embryoid bodies results in the downregulation of laminin and collagen synthesis. When this research was extended to disrupting the signalling pathway components (Akt/PKB), the same outcome was achieved (Douma *et al.*, 2004; Yu *et al.*, 2008). In addition, constitutively activating Akt/PKB in embryoid bodies specifically upregulated *laminin- $\alpha$ 1* mRNA and protein levels 57 and 55 fold, respectively, compared to controls (Li *et al.*, 2001). The Akt/PKB pathway is also activated by downstream signalling induced by activation of the TrkB receptor, thus, it is proposed that full-length TrkB signalling may contribute to BM deposition through the Akt/PKB pathway.

The follicles in *TrkB<sup>FL</sup>* KOs exhibiting poor follicle health (as revealed in Chapter 3) also present disorganisation of granulosa cell arrangement in relation to the oocyte. Whether this contributes to the reduction in laminin- $\alpha$ 1 deposition or is secondary to it (caused by a lack of support from the follicular BM) is unclear. It is apparent, however, that in addition to the BM-cell connections, cell-cell contacts are also required for formation of a normal epithelium, thus it may be possible to conjecture that the disruption of the granulosa cell compartment of the follicle contributes to the reduced follicle growth observed in the *TrkB<sup>FL</sup>* KOs.

Within the follicle, structural integrity and cell adhesiveness is also important to maintain the follicle. As discussed in Chapters 1.3.2 and 3.5.2, gap junctions act to couple the oocyte to somatic cells and between the somatic cells themselves. Mutation of the genes encoding the subunits that comprise the gap junctions (connexins) results in blockade of follicle growth (Carabatsos *et al.*, 2000; Ackert *et al.*, 2001). If follicle structure is abnormal, as in the

*TrkB<sup>FL</sup>* KO, it could be that gap junctions do not form normally, thus contributing to the ovarian phenotype of reduced follicle growth.

Another family of junctions responsible for cell-cell connections are the cadherins. These transmembrane molecules consist of E- (epithelial), N- (neural) and P- (placental) cadherins. Protein expression levels of N- and E-cadherins peaks at P7 in the rat ovary with expression found in both oocytes and granulosa cells, although E-cadherin is not expressed from the primary stage onwards indicating a possible role in regulating entry to follicle growth (Machell *et al.*, 2000). In particular, they may be responsible for propagating the cuboidalisation of adjacent epithelial cells seen in the transition to the growth phase.

It appears that the truncated TrkB receptor does not play a role in ECM deposition. This conclusion has been drawn from the following. The *TrkB T1* KO did not demonstrate abnormal or reduced laminin- $\alpha$ 1 detection. The *TrkB<sup>FL</sup>* KO assessed here demonstrates BM defects as does the *TrkB<sup>FLTr</sup>* KO (Paredes *et al.*, 2004), thus, losing potential signalling from truncated receptors does not seem to increase the defect but losing full-length signalling is sufficient to induce it. In addition, it is full-length TrkB receptor isoforms that are upregulated in cancer cells which have survived anoikis (Yu *et al.*, 2008). This again, suggests that it is the full-length receptor which is important, not only in deposition but in the survival signalling pathways that are normally conferred by contact with ECM.

Thus, it may be possible that the processes of follicle activation and growth depend on factors concomitantly inducing cellular differentiation, proliferation and BM deposition. With the evidence presented here, and the known signalling pathways that can be activated through full-length Trk signalling (see Figure 1.5), TrkB could contribute to the regulation of each of these processes required to support normal follicle development.

## Chapter 5

***TrkB<sup>FL</sup>* KO mouse ovary development  
beyond the first postnatal week:  
assessment by ovarian transplantation**

## 5.1. Introduction

As demonstrated in Chapter 3, *in vivo*  $TrkB^{FL}$  KO ovaries are capable of generating primary follicles. Subsequent follicle growth and oocyte survival *in vivo* cannot be assessed, as the  $TrkB^{FL}$  KOs do not survive past their first postnatal week. Thus, to ascertain further postnatal development of the  $TrkB^{FL}$  KO ovary, transplantation was undertaken.

Transplantation of tissues that cannot be regenerated after being lost to disease or injury may be an ancient procedure (skin grafts actually date back several centuries) but it is still an essential tool for surgeons and basic science researchers alike. Over the last four decades the technique has been pioneered in major feats of surgery with successful transplants in humans including heart, kidney, limbs and even partial face transplants.

Like all techniques it has continually been refined through follow-up research and this has contributed to recognising that rejection of grafts can be minimised by matching the immune type of the donor and recipient in the first instance and later through suppression of the recipient's immune response. Tissue storage and graft site are also important. Initially, survival of the grafted tissue is dependant on its ability to use the blood supply of the recipient, either through the ability of the grafted tissue to create a new vasculature (neoangiogenesis) and/or by surgical attachment to the recipient's blood supply (reanastomosis).

In a clinical setting, transplantation of reproductive tissues is certainly not novel and has recently been reviewed (Gosden, 2008). Women who suffer from loss of fertility early in their reproductive lives, for example through premature ovarian failure or some cancer treatments, may be able to recover their fecundity through auto-transplantation of ovarian tissue collected prior to treatment (Donnez *et al.*, 2004). The major drawback is the possibility that potentially

cancerous cells could be transplanted back into a patient, thus, it may not be the first choice for some women.

Transplantation in basic science research is a standard technique carried out mainly using rodents as recipients. This is due, not only to the less complicated nature of small animal surgery, but also the available sites where grafts can be carried (subcutaneously or under the kidney capsule for example), and the graft success rate. This is especially high when carrying out intra-strain transplants or using immunodeficient nude or SCID mice as recipients (Pelleitier and Montplaisir, 1975; Bosma *et al.*, 1983).

Another benefit of using immunodeficient mice is that tissues from different species can be xenografted without rejection. This has been demonstrated particularly well with human ovarian tissue, allowing investigations into the effects of tissue preservation (or storage prior to transplantation) on the development of the graft (Weissman *et al.*, 1999). This research benefits clinical ovarian transplantation techniques by refining the procedure to maximise survival of the oocytes and follicles in the tissue transplanted.

Ovarian grafts have also been utilised when genetic manipulation results in fetal or neonatal death of experimental mice, as described in Chapter 1.3.2 (the mouse null for connexin43, *Gjal* KO; Ackert *et al.*, 2001). In fact, this technique has been used in this field of research: Paredes *et al.* (2004) assessed ovarian follicular development in the mouse null for full-length and truncated *TrkB* (*TrkB<sup>FLTr</sup>* KO; which displays neonatal lethality) using this technique. P4/5 ovaries were transplanted under the kidney capsule of ovariectomised mice and retrieved two weeks later. In the Controls, follicles had reached the antral stage of growth while the KO grafts contained almost no follicles. The follicles that were present in the KO contained degenerating oocytes and the BM was not intact, although it was visible that the follicles had progressed beyond the secondary stage of growth. They therefore concluded that, full-length

and truncated TrkB signalling is required for the long term survival of oocytes.

## 5.2. Aim

As demonstrated in Chapter 3, *TrkB<sup>FL</sup>* KO ovaries are capable of generating primary follicles *in vivo*, however further follicle growth cannot be assessed due to the lethality of the *TrkB<sup>FL</sup>* KO. Thus, the aim was to determine the long-term survival of *TrkB<sup>FL</sup>* KO oocytes and any potential follicle growth. This was achieved by transplanting ovaries under the kidney capsules of recipient mice before any morphological phenotype became apparent in the *TrkB<sup>FL</sup>* KO ovary. It was important to carry out the transplants at this early stage to ensure that should any deviations from normal development be observed following transplantation, it can be interpreted to have resulted as a consequence of a direct requirement for TrkB signalling in the ovary. If ovaries were transplanted with a phenotype already in effect, it cannot then be determined if subsequent development was caused by the mutation or the phenotype already in place.

## **5.3. Materials and Methods**

### **5.3.1. Animals**

Heterozygous matings were set up to generate neonatal homozygous *TrkB<sup>FL</sup>* null mutants. *TrkB<sup>FL</sup>* heterozygous and wild-type siblings were used as Controls. Over a set period, females were checked for seminal plugs to confirm mating had taken place and allow accurate prediction of when potential offspring would be at the correct stage for transplantation. Due to technical constraints (surgical procedure room was not always available), surgery was carried out using E17.5, E18.5 and P0 ovaries (which were actually 18.5 days post coitum).

### **5.3.2. Genotyping**

Tail tips were collected from neonates at the point of ovary collection prior to transplantation. Genotyping was carried out after surgery, as described in Chapter 2.2.

### **5.3.3. Transplantation protocol**

In addition to utilising aseptic technique it was also protocol to collect the ovaries from the neonates in one room, while the recipients were prepared and the surgery carried out in a separate procedure room. CO<sub>2</sub> incubators in both rooms ensured that the ovaries were kept at 37°C.

#### **5.3.3.1. Media**

The dissecting medium consisted of Liebovitz L-15 supplemented with BSA at 3mg/ml. It was filter-sterilised using a 10ml syringe and a 0.22µm cellulose acetate filter into a sterile flask, and kept at 4°C. Prior to use it was pre-heated to 37°C. The holding medium comprised of α-Minimum Essential Medium (α-MEM) supplemented with 1% penicillin/streptomycin antibiotics to reduce

possible infection. It was filter-sterilised, stored and pre-heated as for the dissection medium.

### **5.3.3.2. Preparation of ovaries for transplantation**

As stated in Section 5.2.1, ovaries were collected from E17.5, E18.5 embryos and P0 pups. To collect embryos, pregnant females were killed by cervical dislocation and a ventral incision allowed access to the uterus. Embryos were dissected free from the uterus and placenta in a laminar airflow hood in a sterile glass petri dish with dissection medium, before decapitation. Embryos were then pinned to a cork board before a ventral incision was made to allow access to the gonads. If female, ovaries were collected and placed in a watch-glass of fresh dissection medium. If P0 pups were available, female offspring were removed from the litter, decapitated and ovaries collected in the same way as for the embryos. Ovaries were freed from fat or oviduct using insulin needles. This ensured minimal non-ovarian tissue would be transplanted. Depending on the number of females collected on the day either one ovary per animal was transplanted and the other fixed in Bouins or frozen, or both ovaries were transplanted. Ovaries to be transplanted were moved from the dissecting medium to the holding medium in six-well trays, one ovary per well. The holding medium was pre-heated in a CO<sub>2</sub> incubator.

### **5.3.3.3. Recipients**

6-8 week old, 18-25 gram C57BL/6 females were used as recipients; mice were purchased from an external supplier (Harlan) and allowed to acclimatise in the animal unit for one week before surgery. Mice of the same background as the TrkB colony were used to prevent graft rejection.

#### **5.3.3.4. Anaesthetic**

The anaesthetic Avertin was selected as it is suitable for short surgical procedures. It is batch tested on site and quantities were purchased as required from the animal unit. To anaesthetise an 18 gram mouse, 0.27ml of Avertin was injected intraperitoneally (0.1-0.2ml of additional Avertin was used for each one gram increase in weight). The mice were then placed in a clean cage until unconscious, confirmed by absence of response to foot pad compression.

#### **5.3.3.5. Surgical technique**

Hair was removed from the surgical site by clippers and the unconscious animal was placed on a heated pad (37°C) for the duration of the procedure. A midline incision allowed visualisation of the fat pad attached to the recipient's ovary through the body wall (used as a guide to locate the kidney). The body wall was then opened and pulling on the fat pad allowed the kidney to be accessed and exteriorised. Under magnification, the kidney capsule surrounding the kidney parenchyma was nicked and slightly widened at one end using a pair of fine watchmaker's forceps. A sterile glass Pasteur pipette was then used to place a single ovary under the kidney capsule. The body wall was sutured shut before 9mm stainless steel autoclips were used to close the skin. The recipient was then returned to a cage on its own or with other recipients at a similar stage of recovery. The animals had access to Vetergesic, analgesic purchased from the animal unit, and were kept in a recovery chamber overnight before being returned to their normal caging.

#### **5.3.4. Histological assessment of transplanted ovaries**

The transplanted ovaries were collected either 2 weeks (Control n=8, *TrkB<sup>FL</sup>* KO n=6) or 3 weeks (Control n=5, *TrkB<sup>FL</sup>* KO n=3) after

surgery. Recipients were culled by cervical dislocation and the transplanted ovaries were recovered by opening the abdomen of the recipient and retrieving the kidney that carried the transplant. The kidney was placed in PBS and the transplanted tissue was removed from under the kidney capsule using forceps and irridectomy scissors. 2-week grafts were either: fixed, embedded in resin, sectioned and stained with H&E as described in Chapter 2.5; or were fixed, processed for wax embedding and sectioned as described in Chapter 2.4.2-2.4.3 (as were all of the 3 week grafts). The latter 2 week and all 3 week grafts embedded in wax could be used for either morphometric analysis (H&E stained) or for immunohistochemistry. The morphometric analysis carried out included oocyte counts, follicle classifications and measurements as detailed in Chapter 2.6.

### **5.3.5. Laminin- $\alpha$ 1 assessment by immunofluorescence**

The immunohistochemistry carried out on 2 and 3 week grafts was assessment of laminin- $\alpha$ 1 deposition as described in Chapter 4.4.2.2. Also described therein is the analysis carried out using Imagepro software. This was slightly amended, as a section from each transplant was able to be processed for immunofluorescence at the same time. They were also all exposed to the same level of excitation on the confocal, meaning that all were comparable in the amount of fluorescence detected. Subsequently an average Control value was obtained and compared to the average *TrkB<sup>FL</sup>* KO value.

### **5.3.6. Statistical analysis**

Where appropriate (and not limited by sample size) multiple comparisons of mean oocyte and follicle measurements were made between *in vivo* and transplanted ovaries. The data sets were subject to ANOVA, and where appropriate, followed by Bonferroni's post-hoc test. Student's t-test was also used to compare oocyte and follicle measurements of transplanted Control and *TrkB<sup>FL</sup>* KO ovaries.

## 5.4. Results

### 5.4.1. *TrkB<sup>FL</sup>* KO follicles progress to the secondary stage after 2-weeks transplantation

Paredes *et al.* (2004) found virtually no oocytes in the grafts of P4/5 *TrkB<sup>FLTr</sup>* KO ovaries after two weeks. Thus, as a starting point it was decided to transplant the *TrkB<sup>FL</sup>* KO ovaries for two weeks. Ovarian tissue was recovered for both Control and *TrkB<sup>FL</sup>* KO transplants. Unexpectedly (given the result of the *TrkB<sup>FLTr</sup>* KO transplants) *TrkB<sup>FL</sup>* KOs were just as capable as Control ovaries of producing secondary follicles (see Figure 5.1). Regrettably, numerical analysis of follicle counts was not possible as not all of the transplants worked consistently (see Figure 5.2). Some transplants were considered not to have worked due to the absence of ovarian tissue and/or the presence of cystic structures (Control n=1, *TrkB<sup>FL</sup>* KO n=2). Those deemed to have partially worked contained ovarian tissue with some cysts or a relatively small amount of ovarian tissue (Control n=1, *TrkB<sup>FL</sup>* KO n=2). Those that had worked well were entirely composed of ovary (Control n=6, *TrkB<sup>FL</sup>* KO n=2). All poor transplants originated from the first run of surgery carried out, thus it may be that improved surgical technique from the first experimental run to the second resolved the unknown cause(s) of variability.

Control ovaries transplanted by Paredes *et al.* (2004) reached the antral stage of follicle growth after two weeks. In contrast, the Control ovaries transplanted in this experiment reached only the secondary stage. This may have been due to the fact that the *TrkB<sup>FLTr</sup>* ovaries were transplanted at P4/5 (almost a week older than the *TrkB<sup>FL</sup>* ovary transplants), thus it seemed possible that subsequent follicle growth could be affected in the *TrkB<sup>FL</sup>* KO ovaries. To determine this it was decided to repeat the transplants and leave for longer than two weeks.





#### **5.4.2. *TrkB<sup>FL</sup>* KO ovaries do not exhibit a reduction in healthy oocyte number after 3-weeks transplantation**

Three weeks after transplantation, ovaries were recovered and it was determined morphologically that the grafts had been successful (Control n=5, *TrkB<sup>FL</sup>* KO n=3). Oocyte counts were obtained as described in Chapter 2.6. Control ovaries exhibited oocyte counts ranging from 180-714, and the *TrkB<sup>FL</sup>* KO ovaries exhibited a comparable range of 132-520 oocytes (see Figure 5.3).



### **5.4.3. Transplanted *TrkB<sup>FL</sup>* KO ovaries maintain a dormant pool of oocytes and initiate follicle growth**

During counting, follicle type was categorised as described in Chapter 2.6.2. To ascertain the ability of Control and *TrkB<sup>FL</sup>* KO transplanted ovaries to maintain a dormant pool of oocytes, primordial follicles were categorised as dormant and all other follicles as growing. The two groups were then calculated as a percentage of the total follicles present in each transplant. It can be seen in Figure 5.4 that *TrkB<sup>FL</sup>* KO ovary transplants are able to maintain dormant follicles as well as their Controls.



#### **5.4.4. *TrkB<sup>FL</sup>* KO follicles progress to the early antral stage after 3-weeks transplantation**

To assess growth of activated follicles the categories (as described in section 2.6.2) were simplified, by combination, into: primary (primordial-primary and primary); secondary (primary-secondary and secondary); preantral; and early antral follicles. Each category was then calculated as a percentage of the total follicles counted in each transplant. Figure 5.5 reveals that the proportions of growing follicles found in the *TrkB<sup>FL</sup>* KO ovaries were within the ranges of the Control ovaries, showing no effect of lack of *TrkB<sup>FL</sup>*.



#### 5.4.5. Control and *TrkB<sup>FL</sup>* KO follicles appear morphologically similar after 3-weeks transplantation

Morphological assessment of the follicles in terms of appearance of oocyte, zona pellucida, granulosa cell morphology and their arrangement in relation to oocyte, and presence of thecal layer did not indicate a difference between Control and *TrkB<sup>FL</sup>* KO ovary transplants (see Figure 5.6 A and B). In addition, in comparison with equivalent-age *in vivo* ovary, the growth of the follicles appeared normal, although the oocytes contained in early growth stage follicles (in the transplants) appeared noticeably larger than those *in vivo* (see Figure 5.6 C).

As measurements were available for primordial through to primary-secondary oocytes and follicles (from the Control P0-P7 time-course assessed in Chapter 3) it was decided to compare these with measurements from the transplants. Oocytes from primary follicles in transplanted ovaries (of either genotype) are significantly larger than oocytes from *in vivo* primary follicles (see Figure 5.7 A). However, only oocytes from *TrkB<sup>FL</sup>* KO transplants are found to be significantly larger than *in vivo* oocytes at the primary-secondary follicle stage. In contrast, it is *in vivo* oocytes which are significantly larger than oocytes from transplanted ovaries at the primordial-primary follicle stage. Analysis of follicle measurements between *in vivo* follicles and follicles from transplanted ovaries revealed no difference at the primordial or primary-secondary stages (see Figure 5.7 B). However, at the primordial-primary follicle stage, *in vivo* follicles were found to be significantly larger than those from transplants. This was again reversed at the primary follicle stage with larger follicles in the transplants than found *in vivo*, Control transplant follicles significantly so.





Comparison of the mean oocyte and follicle sizes obtained for Control and *TrkB<sup>FL</sup>* transplants from primordial through to the early antral follicle stage was carried out (see Figure 5.8). Two-tailed Student's t-tests revealed no difference between the Control and *TrkB<sup>FL</sup>* KOs for either oocyte or follicle from primordial to primary stages. *TrkB<sup>FL</sup>* KO mean oocyte size was, however, found to be significantly larger than the mean Control oocyte from the primary-secondary to early antral follicle stages. The mean follicle sizes for the *TrkB<sup>FL</sup>* KO secondary and early antral follicles were also significantly larger than Control follicles. Thus, *TrkB<sup>FL</sup>* KO ovaries appear to grow (only slightly, but significantly) larger oocytes and follicles than Control ovaries when transplanted.



#### **5.4.6. Semi-quantitative laminin- $\alpha$ 1 expression in the 2- and 3-week Control and *TrkB<sup>FL</sup>* KO ovary transplants**

As demonstrated in Chapter 4, P0 *TrkB<sup>FL</sup>* KO ovaries exhibit a reduced area of laminin- $\alpha$ 1 fluorescence. After 2 weeks transplantation, *TrkB<sup>FL</sup>* KO ovaries (n=2) exhibit a reduced area of thresholded fluorescent signal when presented as a fold change relative to the Controls (n=3, see Figure 5.9 A). This is also true for the 3 weeks transplants, *TrkB<sup>FL</sup>* KO ovaries (n=3) Controls (n=5, see Figure 5.9 B). Small sample sizes and large data spreads were prohibitive of statistical analysis.



## 5.5. Discussion

Previous findings had strongly implicated the role of TrkB signalling in regulating oocyte survival at the time of follicle formation. Not only are the receptor and its ligands expressed in the ovary, but *TrkB* expression is actually upregulated at birth in the rodent, and mice with the null mutation (*TrkB<sup>FL</sup>* KO) exhibited significant neonatal oocyte loss (Spears *et al.*, 2003). On the basis of these findings, the obvious hypothesis was that there is a direct requirement for full-length TrkB signalling in the ovary, to maintain oocyte survival and support follicle formation. Following on from this hypothesis, the assumption was made that transplantation of *TrkB<sup>FL</sup>* KO ovaries would not be able to rescue the phenotype. The results presented in this chapter were therefore, unexpected, nevertheless transplants of late fetal/early neonatal *TrkB<sup>FL</sup>* KO ovaries are able to maintain a dormant pool of oocytes and support follicle growth up to the early antral stage of development just as well as Control transplants. The primary interpretation that must be drawn from this is that *TrkB<sup>FL</sup>* signalling is not directly required within the mouse ovarian follicle for survival. It would then follow that the *in vivo* loss of follicles observed in the *TrkB<sup>FL</sup>* KO (Spears *et al.*, 2003) may actually be due to an indirect effect of loss of full-length TrkB signalling. However, the stark contrast between the findings of this Chapter and those of Paredes *et al.* (2004) demand explanation. I propose that the differences between the two experiments conducted raise sufficient queries that would need to be addressed before it could be categorically stated that full-length TrkB signalling is not directly required by the ovary to support oocyte survival. These differences are outlined in the first part of this discussion.

### 5.5.1. Comparison of *TrkB<sup>FL</sup>* and *TrkB<sup>FLTr</sup>* KO ovary transplants

*TrkB<sup>FL</sup>* KO ovaries were transplanted at late stages of gestation (E17.5/18.5) or on the day of birth under the kidney capsule of intact

recipients. *TrkB<sup>FLTr</sup>* KO ovaries were transplanted at P4/5 also under the kidney capsule, although the recipients were ovariectomised (Paredes *et al.*, 2004). Thus, the contrast in findings between those presented in this thesis and those of Paredes *et al.* (2004) provide an intriguing situation whereby several different factors could potentially contribute to the difference in results. The main possibilities are: 1) the difference in *TrkB* mutation; 2) the difference in recipient and/or; 3) the difference in age of the transplanted tissue.

#### 5.5.1.1. The difference in *TrkB* mutation

The first possibility I shall discuss is that the difference in *TrkB* mutation between the two studies is responsible for the differing outcomes. The KO used by Paredes *et al.* (2004) lacks all forms of the TrkB receptor (*TrkB<sup>FLTr</sup>* KO), whereas, the KO studied in this project is null only for the full-length receptor (Klein *et al.*, 1993). The different results could indicate that there is in fact a direct requirement for truncated TrkB receptor signalling to maintain oocyte and follicle survival, evidenced by the loss of oocytes and follicles in the *TrkB<sup>FLTr</sup>* KO transplants. As the *TrkB<sup>FL</sup>* KO transplants were unaffected it could, therefore, be interpreted to demonstrate that removal from the environment of the *TrkB<sup>FL</sup>* KO rescues oocyte survival (indicating that the requirement for full-length TrkB signalling in the ovary is indirect). However, I will outline why I believe that an indirect effect is unlikely to have caused the ovarian phenotype. The *TrkC* KO assessed by Spears *et al.* (2003) also exhibited retarded growth and postnatal lethality but no effect on oocyte survival. Mutations in both *TrkB* and *TrkC* result in loss of sensory neurones (see reviews by Conover and Yancopoulos, 1997; Tessarollo, 1998). Double *TrkB/C* null mutation results in even greater loss of sensory neurones demonstrating the compensatory effects they exert for each other *in vivo* (Silos-Santiago *et al.*, 1997). Thus, it seems unlikely that the *TrkB* and *TrkC* KOs exhibit significantly different enough neurological

effects to explain why one has a consequence for oocyte survival (TrkB) but not the other (TrkC).

There is further evidence demonstrating that indirect effects of *TrkB* mutation are unlikely to be solely responsible for the ovarian phenotype. Spears *et al.* (2003) found that depending on the background the mutation was placed on (mixed or congenic) the phenotype of the *TrkB<sup>FL</sup>* KO would exhibit varying effects on oocyte survival, although all exhibited retarded growth and postnatal lethality. To uphold the indirect theory it would require that the *TrkB<sup>FL</sup>* KOs, which exhibited an ovarian phenotype, exhibited different effects on their general development from those that did not demonstrate an ovarian phenotype, yet all succumbed to the same eventual outcome of retarded growth and death. It seems more likely that other oocyte survival factors were able to compensate to varying degrees for the loss of signalling in the ovary depending on the background. Put simply, in some backgrounds, sufficient tyrosine kinase signalling may be achieved through other receptors (in addition to or instead of TrkB) and so loss of *TrkB* has no great impact. Other backgrounds may express *TrkB* more highly, over other tyrosine kinase receptors, and so mutation has an effect.

The possibility of a direct requirement for truncated TrkB signalling in the ovary cannot be totally ruled out. It is currently thought that the *TrkB* locus gives rise to three proteins, one full length and two truncated receptors, T1 and T2, which lack the catalytic tyrosine kinase domain. As discussed in Chapter 1.5.2.1 lacking the catalytic domain was considered to imply a lack of downstream signalling capabilities, although, in some cell types truncated TrkB T1 receptors have been shown to be capable of inducing intracellular calcium release in response to BDNF (Rose *et al.*, 2003). It, therefore, seems plausible that the truncated receptors are actually capable of generating a signalling cascade in response to the binding of neurotrophins. However, I consider this reason

unlikely to account for the discrepancy discussed here, at least for the TrkB T1 receptor, as mice null for *TrkB T1* have normal ovaries and are fertile as adults (mice supplied by Dr Lino Tessarollo, unpublished findings by Spears lab 2008). This clearly does not exclude a possible role for truncated receptor TrkB T2, but it has yet to be demonstrated to be translated into a functional protein in the mouse (Kumanogoh *et al.*, 2008).

#### **5.5.1.2. The difference in recipient**

The second possibility is that the difference between recipients is responsible. Paredes *et al.* (2004) ovariectomised their recipients, in contrast, for this thesis the females were left intact as it was deemed to be an unnecessary added risk in terms of recipient recovery from the surgery. However, in light of the contrast in results it is reasonable to propose that the intact recipient ovaries may have influenced the survival of oocytes and follicles in the *TrkB<sup>FL</sup>* KO ovaries transplanted.

##### **5.5.1.2.1. Survival factor**

It may be possible that an unidentified survival factor is produced by the intact recipient's ovaries and is carried, systemically, to the transplanted ovaries where it is sufficient to rescue the *TrkB<sup>FL</sup>* KO oocytes. To address this would require repeating *TrkB<sup>FL</sup>* KO ovary transplants using both ovariectomised and intact recipients. Should the oocytes and follicles only be lost in the ovariectomised group it would be convincing evidence that the *in situ* recipient's ovaries do confer protection via some kind of mechanism. It may even be possible that it is due to an intact full-length TrkB signalling pathway in the recipient's ovaries. It could then be hypothesised that although *TrkB<sup>FL</sup>* is not required autonomously in the follicle for its survival it may still be possible that *TrkB<sup>FL</sup>* signalling is required at the level of the intact ovary to support follicle survival. In addition (or

alternatively) it may be that another intraovarian signalling pathway is responsible for the generation of a signal capable of rescuing the transplanted ovaries. A rescue effect has previously been demonstrated *in vitro*; Spears *et al.* (2003) used the tyrosine kinase blocker (K252a) to prevent Trk signalling in newborn mouse ovary cultures (which resulted in significant oocyte loss). Addition of bFGF, a growth factor which signals through a non-Trk tyrosine kinase receptor (Wert and Palfrey, 2000), negated the effect of K252a on oocyte survival.

In the same vein, it may be possible that steroid hormones produced by the intact recipient's ovaries (including estrogen) may have influenced the different outcomes. This is stated as hormone levels (estrogen and progesterone) are high in the neonatal rodent pup but fall over the first few days of life (Kezele and Skinner, 2003). These first few days have been shown to be a period during which ovary development is susceptible to the actions of steroids, as discussed in Chapter 1.3. Kezele and Skinner (2003) were able to repress follicle growth initiation when newborn rat ovaries were cultured in the presence of estrogen and progesterone. Interestingly, they were unable to alter the follicle distribution when culturing rat ovaries from P4 in the presence of estrogen or progesterone.

Therefore, although not an absolute requirement for follicle development, there is a neonatal period where these factors can modulate it. It may be possible that steroids from the recipient's ovaries protected the follicles from loss of *TrkB<sup>FL</sup>* by reducing follicle activation, and repressing the growth of those that had activated such that compensation could be achieved through other survival pathways. Comparatively, even if the *TrkB<sup>FLTr</sup>* KO ovaries had been transplanted during the period when ovaries could be influenced by these hormones (before P4/5) the ovariectomised recipients used for the *TrkB<sup>FLTr</sup>* KO ovary transplants (Paredes *et al.*, 2004) meant that the ovaries would only have been exposed to low levels of circulating

estrogen. Thus, it seems a strong possibility that compensation may have occurred in the *TrkB<sup>FL</sup>* KO transplants.

#### 5.5.1.2.2. Gonadotrophins

Another possibility introduced by the difference in recipient is that the endocrine status of the recipient may have affected the development of the transplanted ovaries. Small growing follicles do not require gonadotrophins for growth, as demonstrated by lack of effect of hypophysectomy on the growth of small follicles. However, as with estrogen there is also evidence for a period during the early postnatal period where effects of gonadotrophins on immature rodent follicle development can be observed. The timing of when these effects can take place are debatable and seem most likely dependant on the species assessed, as mice begin to form follicles prior to birth but rats commence follicle formation on the day of birth. Thus, variations in when ovaries will begin to “respond” to treatments will no doubt depend on the development of the follicles within the ovaries being assessed.

At birth in the rodent, levels of FSH are high but decrease within the first few days of life, which led to speculation upon possible developmental roles in primordial follicle formation and the activation of the first growing follicles (Halpin *et al.*, 1986). The hypogonadal (*hpg*) mouse has undetectable levels of circulating FSH and reduced early follicle growth (Halpin *et al.*, 1986). Conversely, PMSG (which has mainly FSH activity) increased the population of growing follicles in mouse ovaries if treated between P0 and P5 (Lintern-Moore, 1977b). It was confirmed that mouse ovaries do express various transcripts of gonadotrophin receptor isoforms from birth (O'Shaughnessy *et al.*, 1994). It is not, though, until later stages (P5-7) that ovaries in culture are actually gonadotrophin responsive as demonstrated by production of steroids (progesterone and androstenedione; Mannan and O'Shaughnessy, 1991). These

responses by ovaries in culture (LH from P7 and FSH from P15) are attributed to the appearance of primary and secondary follicles and subsequent acquisition of a thecal layer (Mannan and O'Shaughnessy, 1991).

Paredes *et al.* (2004) assessed FSH serum levels and found that they are reduced in the *TrkB<sup>FLTr</sup>* KO, although the time point at which the samples were collected is not stated. It is possible that the P4/5 transplanted *TrkB<sup>FLTr</sup>* KO ovaries may have exhibited reduced early follicle growth prior to transplantation as they did at P7 (Paredes *et al.*, 2004). However, reduced growth was not thought to be the result of reduced serum levels of FSH as the *TrkB<sup>FLTr</sup>* heterozygous females also exhibited reduced FSH but no effect on follicle development.

The removal of *in situ* ovaries from the recipient results in the ablation of the negative feedback loop controlling the release of the gonadotrophins (FSH and LH) from the anterior pituitary. In the absence of *in situ* ovaries, serum FSH levels increase significantly compared to the levels found circulating in intact mice (Cox *et al.*, 2000). The transplanted P4/5 Control and *TrkB<sup>FLTr</sup>* KO ovaries were effectively returned to an environment much more akin to that of the newborn mouse. As Control ovary development was normal the elevated levels do not disrupt normal follicle growth. The *TrkB<sup>FLTr</sup>* KO ovaries, on the other hand, contained only a few atretic follicles, if any (Paredes *et al.*, 2004). The follicles that were present were multi-laminar and had clearly progressed beyond the secondary stage of growth. This led Paredes *et al.* (2004) to hypothesise that, in keeping with their result of reduced *Fshr* mRNA expression in the P7 *TrkB<sup>FLTr</sup>* KO ovary, follicles ultimately fail to become gonadotrophin dependent (as discussed in Chapter 3.1). They conjecture, though, that the fact the follicles grew at all was due to the elevated gonadotrophin levels. It is, therefore, possible to speculate that the elevated gonadotrophin levels were able to override, to some degree, the block in growth that

the *TrkB<sup>FLTr</sup>* KOs exhibit *in vivo*. The fact that the follicles died at the preantral stage meant that, unlike the Control ovaries, the *TrkB<sup>FLTr</sup>* KOs were unable to produce follicles capable of suppressing the activation of dormant primordial follicles (as discussed in Chapter 1.3 and depicted in Figure 1.3). Therefore, it may be possible that the elevated gonadotrophin levels enhanced the phenotype of the *TrkB<sup>FLTr</sup>* KO ovary by accelerating the rate of follicle growth, their demise and the resulting depletion of the pool of primordial follicles. It is not clear how the gonadotrophin levels of the intact recipients used in this investigation may have affected the *TrkB<sup>FL</sup>* KO ovaries transplanted. It does seem reasonable, though, to speculate that they may have influenced the result given that elevated levels may have influenced the outcome of the *TrkB<sup>FLTr</sup>* KO transplants. Thus, it is possible that the endocrine status of the recipients may have affected the outcome of the transplants.

#### **5.5.1.3. The difference in stage of transplantation relative to appearance of phenotype**

A third possibility (and the one I consider to be most likely) is that the age of the ovary transplanted influenced the results. It was demonstrated in Chapter 3 that *in vivo TrkB<sup>FL</sup>* KO ovaries exhibit follicles with poorer health than Control ovaries. The *TrkB<sup>FL</sup>* KO ovary transplants were accordingly carried out prior to the presence of a morphologically obvious phenotype which may influence subsequent development of the ovaries. Another factor known to affect the outcome of a transplant is distribution of follicles in the ovary at the time of transplantation. Growing follicles are lost after transplantation but primordial follicles less so (see review by Gosden, 2008). By transplanting late fetal and early neonatal *TrkB<sup>FL</sup>* KO ovaries it is known that they were unaffected in terms of follicle distribution and oocyte number (see Chapter 3). If a difference in the number of oocytes in the recovered transplants had been found, it

would have been possible to exclude that it was caused by an affect on the follicle population prior to transplantation, thus, making it possible to attribute any effect found to the mutation and not transplantation.

In complete contrast, Paredes *et al.* (2004) did not demonstrate that the *TrkB<sup>FLTr</sup>* KO ovaries they transplanted at P4/5 were unaffected either in terms of follicle health, numbers of oocytes or the distribution of follicle growth. Indeed, we know that they were affected at P7. Paredes *et al.* (2004) observed that the follicles present in the *TrkB<sup>FLTr</sup>* KO ovaries, after transplantation, exhibit a disrupted BM. Thus, it is possible that the *TrkB<sup>FLTr</sup>* KO ovaries exhibited reduced follicular health at the time of transplantation. In addition, it also seems likely that by the time they were transplanted the ovaries were affected in terms of follicle development. Assessment of P7 *in vivo* ovaries of the *TrkB<sup>FLTr</sup>* KO revealed a reduction in secondary follicle formation attributed to a reduction in granulosa cell proliferation (Paredes *et al.*, 2004). It may be that at the point of transplantation the *TrkB<sup>FLTr</sup>* KO ovaries were already irreversibly affected by the mutation, at least in terms of follicle development if not health, whereas, transplantation prior to any obvious defect allows the survival and growth of the follicles in the *TrkB<sup>FL</sup>* KO.

### **5.5.2. Comparison of Control and *TrkB<sup>FL</sup>* KO ovary transplants**

The main finding of interest from the Control and *TrkB<sup>FL</sup>* KO ovary transplants was the demonstration that loss of full-length TrkB signalling has no effect on long-term oocyte survival. There were differences observed between the Control and *TrkB<sup>FL</sup>* KO ovary transplants which may be directly attributable to the mutation and these shall be discussed in this section.

### 5.5.2.1. Oocyte growth

It was observed that oocytes from early growth stage follicles in transplanted Control and *TrkB<sup>FL</sup>* KO ovaries exhibited an intriguing increase in oocyte size. It was confirmed that oocytes in transplanted Control and *TrkB<sup>FL</sup>* KO ovaries were significantly larger at the primary stage than *in vivo* primary follicle oocytes. As this was found for both genotypes it appears that this is attributable to the actual process of transplantation rather than genotype itself. This observation could be interpreted as precocious responsiveness to the new endocrine environment of the recipient. By the primary-secondary follicle stage of growth it became apparent that this was not just a difference between *in vivo* and transplanted ovaries but between Control and *TrkB<sup>FL</sup>* KO oocytes in the transplants. *TrkB<sup>FL</sup>* KO oocytes were significantly larger at this stage of growth than either *in vivo* oocytes or Control transplanted oocytes of the same stage. Comparing the Control and *TrkB<sup>FL</sup>* KO oocyte and follicle measurements from later stages of follicle growth revealed that the *TrkB<sup>FL</sup>* KO has significantly larger sized oocytes from the primary-secondary stage through to the early antral stage. This was also found for the secondary and early antral stage follicles. Thus, it appears that loss of full-length TrkB signalling has an effect on oocyte growth at these stages. It may be possible that this is attributable to a factor produced by the intact ovaries of the recipient, promoting oocyte growth before granulosa cell proliferation initiates. It may also be due to intraovarian signalling pathways affected in the *TrkB<sup>FL</sup>* KO. A similar effect was found in the ovaries of *Gdf-9* null mice. The growth of follicles is blocked at the primary stage as granulosa cells do not proliferate, though the oocyte continues to grow (Dong *et al.*, 1996). This effect was attributed to continued production of the cytokine kit ligand (KL) by granulosa cells acting on the oocyte expressed receptor (KIT) to promote its growth (Elvin *et al.*, 1999). The fact that this increase in size was also observed in the secondary and early antral follicles of

the *TrkB<sup>FL</sup>* KO indicates that this increase in growth is not just limited to the oocyte but is also evidenced by increased growth in the granulosa cell compartment of *TrkB<sup>FL</sup>* KO transplants.

#### **5.5.2.2. Laminin- $\alpha$ 1 deposition**

Assessment of the *TrkB<sup>FL</sup>* KO transplants indicated that the decrease in deposition of laminin- $\alpha$ 1 is maintained, thus the role for regulating BM deposition through TrkB signalling appears to be a direct one. However, given that the follicles were able to survive and grow normally it also appears that the reduced BM is not on its own sufficient to cause loss of oocytes. It may, though, be possible that the endogenous hormones from the intact recipient ovaries rescue the effect of the disrupted BM to some extent either by overcoming the defect or by partially repairing it. This can be postulated as estradiol and FSH have both been found to increase expression of N-cadherin and gap junction components by cultured granulosa cells (Amsterdam *et al.*, 1999). Thus, it could be that upregulated expression of other components involved in regulating follicle structure, induced by endogenous hormones from the intact recipient ovaries, may be able to compensate for the reduction in laminin- $\alpha$ 1.

#### **5.5.3. Summary**

In spite of the caveats discussed in the previous sections it cannot be disputed that the results of the *TrkB<sup>FL</sup>* KO transplants reveal the lack of direct requirement within the follicle for full-length TrkB signalling. This entirely unexpected result requires a complete shift in our understanding of the role of TrkB signalling in ovarian follicle survival and growth.

## **Chapter 6**

### **Novel description of an immune response in the neonatal mouse ovary**

## 6.1. Introduction

The immune system is a form of self-defence evolved to protect against disease-transmitting pathogens such as viruses, bacteria, parasites and fungi. It is a fine-tuned system which acts on two distinct but co-operative levels: the innate immune response (primary reaction to eliminate pathogens mainly by phagocytosis) and the adaptive immune response (secondary reaction to infection which results in life-long pathogen-specific antibody production). In addition to this powerful defence mechanism, cells of the immune system are also functionally involved in normal tissue development and homeostasis. Incorrect regulation of the immune system can result in different pathologies including cancer and autoimmune disease.

The cells responsible for the actions of the innate and adaptive immune systems are the white blood cells (or leukocytes; see Figure 6.1). These cells descend from haematopoietic stem cells (found in the bone marrow) along with red blood cells (erythrocytes) and platelets. Haematopoietic stem cells initially differentiate into two types of specialised progenitor cell which are subsequently responsible for the generation of other immune cells: the myeloid progenitor (innate) and the lymphoid progenitor (adaptive). Myeloid progenitor cells can differentiate into cells including granulocytes, monocytes, macrophages, erythrocytes, megakaryocytes and mast cells. Granulocytes (identified by granules in the cytoplasm) include the polymorphonuclear leukocytes (PMLs), eosinophils and basophils. PMLs are so-called because of their distinctive multilobulated nuclei, but in literature they are also often referred to as neutrophils. Lymphoid progenitor cells are responsible for production of T- and B-lymphocytes, and natural killer cells. In contrast, lymphocytes, monocytes and macrophages are agranular (Bloom and Fawcett, 1962).



In response to inflammation or infection, the innate immune response is quickly triggered and can be exerting its effects within a matter of minutes. Using the vasculature, and by responding to chemokines and cytokines, leukocytes travel to sites in the body where they are required. The most common leukocyte to do this is the PML. Near-mature PMLs are stored in bone marrow ready for quick release, after which they are replenished by increased bone marrow production. PMLs are also found in blood vessel walls ready to migrate, in the circulation and in tissue. Infection-causing agents are ingested by PMLs before they self-destruct by undergoing an apoptotic event. The apoptotic debris is then subsequently cleared by macrophages. Macrophages are present as monocytes in the bloodstream, once recruited they enter the tissue and differentiate into phagocytic macrophages responsible for the removal of pathogens and debris (Janeway *et al.*, 2001).

The acquired immune system is much more specific but slower in its response. Lymphocytes are produced by progenitor cells in the bone marrow and other lymphoid organs such as the spleen and thymus gland. It is in these organs that they importantly acquire the ability to recognize self from non-self. This ensures that only foreign antigens are recognized and removed accordingly, protecting the organism from developing autoimmune diseases. This occurs when lymphocytes recognise self-antigens, effectively becoming auto-antibodies. B-lymphocytes are bone marrow-derived cells which deactivate any antigen they encounter by engulfment. The cells then present the antigen which is recognized by specific thymus-derived T-lymphocytes. This results in production of antibodies by derivatives of B-cells. The binding of T-cells to the antigen-presenting cells leads to their subsequent destruction (Janeway *et al.*, 2001).

Although the primary function of leukocytes has been simplified here to that of phagocytosis and degradation of foreign bodies, leukocytes are also responsible for the propagation and

maintenance or dissolution of an immune response. This is mainly achieved through the actions of cytokines and chemokines. Cytokines are proteins secreted by activated immune cells which act on other immediate immune cells or the tissue they are in. These include members of the interleukin (IL) family 1, 6, 10 and 12, granulocyte macrophage-colony stimulating factor (GM-CSF) and TNF- $\alpha$ . Chemokines (chemotactic cytokines which recruit other immune cells in the vicinity) include IL-8 and monocyte chemoattractant proteins (MCP) 1 and 3. Leukocytes can also release growth factors such as TGF- $\alpha$  and - $\beta$ , IGF, epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF). These growth factors influence the tissue the leukocytes have infiltrated and can regulate normal tissue function, or function in response to immune stimulus.

The role of the immune system in ovarian function has been well documented for later stages of ovary development, namely follicle atresia, ovulation and regression of corpus luteum. The role of macrophages in particular is well studied and is reviewed by Wu *et al.* (2004). Ovulation of a species-specific number of oocytes relies on the appropriate loss of a proportion of growing follicles. This occurs through atresia, the mechanism of which is considered to be apoptosis. In human ovary, more than double the density of PMLs is found in the thecal layer of atretic follicles compared to healthy follicles (although it should be noted that even healthy follicles have four times the density of PMLs in the thecal layer compared to the interstitium; Chang *et al.*, 1998). Thus, it is normal to observe immune cells in apparently healthy follicles, but their increased presence is associated with follicle atresia.

Atresia of preantral and antral follicles in prepubertal mouse ovaries can be induced by  $\gamma$ -radiation (Lee and Yoon, 2005). PMLs and macrophages were observed within atretic follicles (identified by granulosa cell and oocyte apoptosis). In addition, there were

phagocytic granulosa cells and macrophages consuming apoptotic debris and PMLs within the follicles. Thus, it is conjectured that after certain insults or during unnatural levels of apoptosis it is possible for apoptosis to induce an ovarian immune response (Lee and Yoon, 2005).

The role of the immune system in ovulation is the destruction and remodelling of ovarian tissue required to enable ovulation to occur. Macrophages assist in this process by secreting degradative matrix metalloproteinases (MMPs) which can break down collagen surrounding the follicle (Wu *et al.*, 2004). Macrophages are strongly localised to thecal layers of preantral and antral mouse follicles (Wu *et al.*, 2004). It was also found that the presence of macrophages and PMLs actually enhances ovulation rates, whereas depletion of these cell types results in reduced ovulation rates (Russell and Robker, 2007).

Regarding early ovary development, the role of the immune system has been less studied. As discussed in Chapter 1.4, a common feature of neonatal rodent ovary development is loss of oocytes. This occurs through different mechanisms, although large proportions are considered to be lost by apoptosis. Given that apoptosis is a regulated mechanism for the intentional removal of specific cells, it would not be to the benefit of the organism if it induced an inflammatory response each time it occurred. Thus, it has been considered dogma that apoptosis is anti-inflammatory and necrosis (where a cell dies through some kind of trauma) is pro-inflammatory. However, as reviewed by Gregory and Devitt (2004), predicting a response is not straight forward as it is possible to have neutral, pro-, or anti-inflammatory responses depending on the cell types interacting and the cytokines released in the tissue.

In keeping with the notion that apoptosis is anti-inflammatory, existing data indicates that the immune system does not exhibit an apparent role during neonatal ovary development, in particular in

relation to loss of oocytes at follicle formation and growth activation. This is partly based on human ovary studies which indicate that PMLs simply do not associate with primordial or early growth stage follicles (Chang *et al.*, 1998). In addition, it was observed 12 hours after  $\gamma$ -radiation treatment that prepubertal mouse ovaries lost the majority of primordial and primary follicles by apoptosis of granulosa cells and oocytes, however, no immune response was described (Lee *et al.*, 2000).

During the neonatal period the acquired immune response is established. If development is disrupted during this time then it can result in autoimmune diseases. Autoimmune ovarian disease (AOD) is a well-documented incidence of autoimmune disease whereby failure of correct lymphocyte regulation during the neonatal period results in adult onset of oophoritis (inflammation of the ovary). Ovarian failure subsequently occurs, thus linking the disease to premature ovarian failure (POF). As a model of autoimmune disease in the mouse it is very useful to study as it can be induced either through immunisation against a peptide of the zona pellucida protein (ZP3; O'Leary *et al.*, 2008), or by removal of the thymus (Lintern-Moore, 1977a; Taguchi *et al.*, 1980) at P3 (Tung *et al.*, 2001).

The potential for Trk signalling in the development and regulation of the immune system is strongly implicated through the expression of all Trk receptors (including p75) in bone marrow, lymphoid tissue (thymus, spleen and lymph nodes) and subsets of immune cells located within these tissues (see review by Vega *et al.*, 2003). TrkA is expressed in human spleen and lymph nodes and NGF has proliferative effects on lymphocytes *in vitro* (Thorpe and Perez-Polo, 1987; Otten *et al.*, 1989). TrkB, in particular, has been localised to haematopoietic cells, PMLs and erythroblasts of bone marrow as well as macrophages in spleen and thymus (Vega *et al.*, 2003). It is also expressed on isolated lymphocytes (Garcia-Suarez *et al.*, 2002) and eosinophils (Noga *et al.*, 2002). In contrast, isolated B-

lymphocytes from murine thymus express truncated *TrkB* mRNA but not full-length receptor mRNA (Schuhmann *et al.*, 2005). Neurotrophin protein detection in immune tissues has been less successful, although mRNA for all has been detected (Vega *et al.*, 2003). Therefore, exact establishment of expression of receptors, their isoforms and the ligands is not yet complete. However, use of transgenic mice has allowed further analysis of the potential role of Trk signalling. *TrkB<sup>FL</sup>* KO mice (Klein *et al.*, 1993) assessed by Garcia-Suarez *et al.* (2002) exhibited significant loss of lymphocyte precursors (thymocytes) at P15 in the thymus, demonstrating a survival role for TrkB signalling in this cell type, although the exact nature (auto- versus paracrine) is not yet established.

## 6.2. Aim

As discussed in Chapter 3.3.2. some *TrkB<sup>FL</sup>* KO ovaries exhibited a cellular infiltrate of unknown origin (see Figure 6.2). Some of these cells were clearly PMLs. The other type of unknown cell could not be easily identified but was not considered to be apoptotic (personal communication with mouse pathologist Dr David Brownstein, University of Edinburgh), thus they may well also be part of the immune response. The questions addressed in this chapter are:

- 1) what is the nature of this immune response;
- 2) is there an association between the response and lack of full-length *TrkB*; and
- 3) what potential role could this response be fulfilling in the neonatal ovary?

Points two and three are based upon the previously described phenotype of the *TrkB<sup>FL</sup>* KO being one of increased oocyte loss (Spears *et al.*, 2003). Although oocyte attrition is not an unusual process in the neonatal ovary, could it be that the extreme phenotype of the

mutation has overwhelmed the normal process by which the ovary clears apoptotic debris? This novel observation may provide some insight into the mechanism by which the ovary removes this debris.



## **6.3. Materials and Methods**

### **6.3.1. Animals**

Transgenic animals were generated and genotyped as described in Chapter 2.1-2.2. Control (wild-type and *TrkB<sup>FL</sup>* heterozygote) and *TrkB<sup>FL</sup>* KO pups were collected from P0 through to P7.

### **6.3.2. Tissue collection**

Control and *TrkB<sup>FL</sup>* KO ovaries were collected as described in Chapter 2.3. Spleen was collected from Control pups at P2.

### **6.3.3. Tissue preparation**

Ovaries were fixed in Bouins or PFA before embedding in wax and cutting at 5µm, as described in Chapter 2.4, or they were embedded in resin and cut at 5µm (see Chapter 2.5). Spleen was fixed in PFA for 1 hour, wax embedded and sectioned as described in Chapter 2.4, with the exception that sections were cut at 3µm.

### **6.3.4. Morphological assessment of immune response**

Only ovaries that had been left attached to the bursa were used for assessment of the immune response in the ovary proper, hilar region and bursa. One middling section per ovary (where the three areas were visible) was stained with H&E (as described in Chapter 2.4.4 for wax sections and Chapter 2.5.3 for resin sections). Each area of the section assessed was scored for the presence of immune cells, either: no response (0 cells counted); weak (1-9 cells counted); medium (10-29 cells counted); or strong (30+ cells counted).

### **6.3.4. Transmission Electron Microscopy.**

A pair of P5 sibling Control and *TrkB<sup>FL</sup>* KO ovaries were collected and processed for transmission electron microscopy (TEM). See Appendix B for TEM solutions. Ovaries were fixed using 3%

glutaraldehyde in a working buffer of 0.1M sodium cacodylate buffer (NaCo) for 2 hours at room temperature with agitation. Ovaries were then washed in 0.1M NaCo (3 x 15 minutes) before post-fixation in 1% osmium tetroxide in 0.1M NaCo for 45 minutes at room temperature with agitation. Ovaries were washed again in 0.1M NaCo (3 x 15 minutes) before dehydration and embedding in resin as described in Chapter 2.5.1.2. Sections were prepared for TEM by Stephen Mitchell (EM suite, Edinburgh University) and viewed on a Phillips CM120 Transmission electron microscope (FEI UK Ltd). Images were taken using a Gatan Orius CCD camera (Gatan UK).

### **6.3.5. Immunohistochemistry**

Using DAB, as described in Chapter 2.7.1-2.7.3, lymphocytes, PMLs and monocytes/macrophages were identified by the pan leukocyte marker, CD45, also referred to as the common leukocyte antigen (O'Leary *et al.*, 2008). The primary antibody used was a rat anti-mouse polyclonal (BD Biosciences) at a 1:50 dilution. The secondary used was a biotinylated goat anti-rat antibody (Zymax) at a 1:200 dilution. Non-immunised goat serum was used to make antibody solutions. As for assessment of the H&E stained sections, CD45 detection was carried out on a middle section from the available P0-P7, intact, wax embedded Control and *TrkB<sup>FL</sup>* KO ovaries. Spleen was used as the positive control and a section with primary antibody omitted was included as the negative control. Haematoxylin counterstain was used to allow clear identification of the ovarian structures assessed.

### **6.3.6. Assessment of neonatal ovaries from mice null for *Dazl***

Control and *Dazl* KO sibling mouse ovaries (fixed, processed and embedded in wax) were obtained from Professor A. McNeilly (MRC Human Reproductive Sciences Unit, Edinburgh). They were sectioned and stained for H&E as described in Chapter 2.4.4.

## 6.4. Results

To address the first aim of characterising the immune response it was necessary to identify the cells present by morphological and histological methods.

### 6.4.1. The nature of the immune response

Once it had been recognised that these cells were part of an immune response, and due to their distinctive morphologies, it was relatively easy to identify the cells even if the infiltrate was not strong. It was quickly resolved through assessing the Control and *TrkB<sup>FL</sup>* KO ovary P0-P7 time-course that the immune response is not limited to the *TrkB<sup>FL</sup>* KO ovary. Therefore, the ensuing description will be based on the responses observed in both Control and *TrkB<sup>FL</sup>* KO ovaries.

#### 6.4.1.1. The unidentified cells

These cells were originally assumed to be apoptotic due to their small, round, very intense haematoxylin stain and lack of cytoplasm. It was observed that these cells were often adjacent to oocytes within follicles (see Figure 6.3), strongly suggesting that they may be apoptotic granulosa cells. However, a very strong response in a P4 Control ovary (see Figure 6.4) allowed the identification of true apoptotic cells alongside these unidentified cells. Apoptotic cells often exhibit a halo around the remaining cell body and this is not apparent around the unidentified cells. These cells are in fact, quiescent cells, demonstrated by the pattern of chromatin and high ratio of nucleus to cytoplasm. It was actually found that the Control P5 ovary, used for TEM, exhibited the same strong infiltrate of cells, allowing high power examination of these cells (see Figures 6.5 and 6.6). Comparisons of the cell morphology against histological reference (Berman, 2003) revealed that they have very similar morphology to lymphocytes.









In terms of the distribution of these cells in the ovarian tissue assessed, it was apparent that they are only ever found in the ovary proper. They were observed either in discrete pockets or throughout the ovary proper, sometimes extending to the hilar region, but never in the bursa. The pockets appear to be surrounded by a BM separating them from the stroma, but the cells are also able to access the granulosa layer of the follicles (see Figure 6.5). Interestingly, they were never observed without the presence of PMLs and they tended to be present when PML presence was very strong.

Immunohistochemical detection of these cells was initially demonstrated with the pan leukocyte marker CD45 (see Figure 6.7). However, this was deemed to be unreliable as it did not consistently identify these cells in all samples (see Figure 6.8).





#### **6.4.1.2. Identified leukocytes**

During the initial morphological analysis of the neonatal *TrkB<sup>FL</sup>* KO in Chapter 3, several cell types were counted including apoptotic cells. In some *TrkB<sup>FL</sup>* KO ovaries it was observed that there were other cells present at the hilar region which were not apoptotic and not recognisable as belonging to the ovary, these cells are PMLs. Easily identified in H&E sections by their characteristic multi-lobed nuclei, PMLs are most commonly and strongly observed in the bursa and hilar regions (see Figures 6.4 and 6.9). Occasional infiltrations into the ovary proper of varying intensity can also be seen (see Figures 6.2 and 6.8). Large aggregates of PMLs were also sometimes observed in the extraovarian space between the ovarian surface epithelium (OSE) and the bursa, particularly when the response was strong in the ovarian tissue. Immunohistochemical detection of these cells using CD45 was reliable (see Figure 6.8).



It is unlikely that the response was induced by tissue damage incurred at the time of ovary collection as the scale of the response was so great in some samples that it must have been occurring for some time prior to collection. In addition, immature forms of PMLs were observed in the tissue, indicating that leukocytes are actively formed there (myelopoiesis) rather than simply recruited to the tissue. Different stages of formation of leukocytes can be identified in H&E sections by their distinctively shaped nuclei (see Figure 6.9). For example, metamyelocytes (which go on to form eosinophils, basophils or PMLs) have a nucleus larger and more kidney-shaped than mature leukocytes. The subsequent stage of differentiation is the band cell stage where the nucleus becomes more curved but is not yet lobular. Mature eosinophils are clearly distinguished by a halo of pink stain (hence their name), although it was harder to distinguish between mature cells if the nucleus wasn't completely visible (or if stain wasn't picked up heavily enough). Thus, it is possible that in addition to PMLs and eosinophils, mature basophils may also be present.

Macrophages can be identified in H&E sections where there is a cluster of apoptotic debris contained within a halo lacking stain (indicating that debris is contained within one body). This was observed in the ovary proper of some H&E sections. However, the ovary proper is very dense with cells and this prohibited absolute confirmation of their presence. TEM sections obtained revealed the presence of macrophages and their active involvement in phagocytosis of apoptotic debris and cytoplasmic fluid (see Figure 6.10). It also appears that oocytes in ovaries undergoing the immune response are also capable of phagocytosis (see Figure 6.11).

Immunohistochemical detection of macrophages and other mature leukocytes is likely with the pan leukocyte marker, CD45. However, detection of immature leukocytes is not guaranteed as not all stages may necessarily express CD45.





#### **6.4.2. Is the immune response influenced by loss of full-length *TrkB*?**

The second aim of the chapter was to investigate the possibility that the novel immune response observed is associated with the mutation in full-length *TrkB*. *TrkB<sup>FL</sup>* KO ovaries were previously found to exhibit substantial levels of cell death and significant oocyte loss during the neonatal period (Spears *et al.*, 2003). Thus, it was proposed that the immune response may be induced by incidences of mass cell death to remove apoptotic debris. The immune response was observed in both Control and *TrkB<sup>FL</sup>* KO ovaries, thus it could not be solely due to the mutation. This, however, does not exclude the possibility that the immune response is a physiological response induced by normal levels of cell death in the ovary. If this were true, Control ovaries would accordingly exhibit a basal level that *TrkB<sup>FL</sup>* KO ovaries may exceed. It was, thus, decided to score the intensity of immune responses in both Control and *TrkB<sup>FL</sup>* KO ovaries to determine if there is a difference between genotypes. As it was apparent that the CD45 antibody would not label all immune cells reliably it was decided to score available H&E sections for intensity of immune response.

##### **6.4.2.1. Immune responses can vary between ovaries from the same animal**

Where available, both ovaries from the same animal were assessed to maximise the number of ovaries graded for the immune response. This revealed that, while a similar response tended to be produced in both ovaries (not shown); it was entirely possible to observe a strong response in one ovary and a weak response in the other (see Figure 6.12).



#### **6.4.2.2. Immune responses vary in intensity irrespective of tissue location or genotype**

Out of 20 *TrkB<sup>FL</sup>* KO ovaries examined, all exhibited leukocytes (PMLs, eosinophils, lymphocytes) in at least one or more of the following areas: the bursa; the hilar region; or the ovary proper. Of 20 Control ovaries examined, all but one exhibited immune cells in at least one or more of the regions mentioned above. Therefore, the response was observed to occur equally in both Control and *TrkB<sup>FL</sup>* KO ovaries. The intensity of the response, though, varied in its location and composition. Figure 6.13 depicts examples of Control ovaries exhibiting weak or strong responses in the different areas assessed and the same is shown for *TrkB<sup>FL</sup>* KO ovaries in Figure 6.14. Thus, as both Control and *TrkB<sup>FL</sup>* KO ovaries exhibit varying degrees of intensity of immune response, no correlation can be drawn between genotype and the intensity of the response.





#### **6.4.2.3. Immune responses vary in intensity from P0-P7.**

Although the immune response varied in intensity irrespective of genotype there appeared to be a trend of intensity across the P0-P7 time-course for both genotypes. Figure 6.15 demonstrates that in terms of the spread of intensity of the response it can be generalised that it is weakest at P0 and at P7, with both Control and *TrkB<sup>FL</sup>* KO ovaries exhibiting the strongest responses in the ages between (P2-P6). Again, no evidence for an effect of lack of full-length *TrkB* was observed.



### 6.4.3. Assessment of *Dazl* KO ovaries

As this immune response is proposed to be involved in the active clearance of apoptotic debris produced by overwhelming oocyte apoptosis, it is postulated that other models of large-scale neonatal oocyte death would possibly exhibit the same immune response. As discussed in Chapter 1.2.2 *Dazl* KO ovaries lose all oocytes perinatally (Ruggiu *et al.*, 1997). It was, therefore, decided that ovaries of the *Dazl* KO mouse would be an ideal model to assess for the presence of an immune response. H&E sections from ovaries were assessed at E17.5 and at P1 in comparison with sibling Control ovaries. At E17.5, all ovaries contained many oocytes with no apparent signs of oocyte loss in the KO. At P1, oocytes were already lost from the KO ovaries assessed and no overt signs of an immune response were visible. Thus, the window of opportunity to observe the active loss of oocytes was missed. Attempts to obtain ovaries of an age between E17.5 and P1 were unsuccessful, therefore, it was not possible to determine if the response is induced in the *Dazl* KO or not.

## 6.5. Discussion

It has been demonstrated here that during establishment of the murine primordial follicle reserve and the initial stages of follicle activation it is typical to observe immune cells in the perinatal ovary. Through morphological and histological assessment, the nature of this response has been identified to consist of cells akin to both the innate and acquired immune responses (eosinophils, PMLs and lymphocytes). The fact that this response was originally observed in neonatal *TrkB<sup>FL</sup>* KO ovary, and its severity in that instance, led to the original hypothesis that the immune response is observed due to increased levels of cell death in the *TrkB<sup>FL</sup>* KO overwhelming the normal method of apoptotic debris clearance. However, this genetic link was not proven as it was confirmed that the severe response is also apparent in Control ovaries.

Thus, as it has now been shown that immune cells are almost always present in neonatal ovaries (at least at low levels in the bursa) I propose that this response may be a normal physiological process that has not been previously described.

Normal ovary development entails a significant loss of oocytes neonatally, therefore, it may be that immune cells are constantly contributing to the clearance of cell debris. Abnormally elevated levels of cell death invoke a larger reaction and additional types of immune cells (such as lymphocytes) may be recruited to the ovary. Evidence for this has been found in the mouse lung where high levels of PML recruitment and subsequent apoptosis actually increase PML recruitment to this organ. The PMLs phagocytose debris until other phagocytes, such as macrophages, are able to control the apoptosis occurring (Rydell-Tormanen *et al.*, 2006). The majority of neonatal oocyte cell death occurs by P2 in the mouse (Rodrigues *et al.*, 2009). Thus, as the strongest reactions were observed in the P2-P6 ovaries assessed, but not at P0, instead of assisting with clearance of debris

at the time of oocyte death, perhaps it is a response that occurs after the peak has occurred. This could then explain the reduction in response by P7. However, again this may also be too simplistic as the responses were so variable not only between animals at the same age but even between ovaries of the same animal.

Alternatively, and as equally novel, is the possibility that the immune response observed may be a case of spontaneous autoimmune ovarian disease (AOD) arising due to lack of immune system stimulation caused by the sterile housing conditions that the TrkB colony is kept in. There is a window in neonatal development (P0-P7 in the mouse) during which the acquired immune system establishes what is self from non-self. Should this not be established appropriately, then the immune system can cause autoimmune disease. AOD can be induced in the mouse by thymectomy at P3 and a strong infiltration of lymphocytes is observed in the ovary some weeks later (Taguchi *et al.*, 1980). Subsequent loss of oocytes links AOD to POF, although normal progression of murine AOD takes 5-6 weeks before it is apparent in the ovary. Thus, it seems that the response observed here would be precocious in its timing. However, AOD can be induced in newborn mice within a few days, when splenic T-lymphocytes from mice exhibiting AOD are transferred (Sakaguchi *et al.*, 1982). It is, therefore, not impossible that a more rapid onset of oophoritis has been observed here.

If this novel observation was to reveal that a form of AOD can be spontaneously induced by limited immune stimulation during the neonatal window then this is a situation that should be considered by all those working with animals in ultra-sterile units. There is already anecdotal evidence of affected animal fertility in very sterile units demonstrated by a decline in breeding (personal communication with Dr N. Spears, Edinburgh University; Mr D. Thomson of Professor R. Ribchester's group, Edinburgh University; and Dr S. Kimber, Manchester University). Thus, it is even not too large a leap to make

the hypothesis that POF in women, which is linked to AOD (Chernyshov *et al.*, 2001), could be related to a lack of immune stimulation during this window, for example premature or newborn babies kept in incubators.

A third option may be that it is a combination of the two. Gregory and Devitt (2004) reviewed that it is sometimes (but not always) possible for defective clearance of apoptotic debris to result in the production of autoantibodies. Thus, it may be possible that overwhelming cell death occurring physiologically in the ovary may trigger AOD and subsequent POF.

Whichever of the above possibilities it could be, the pattern of the response has been observed and described in this Chapter. It was found that PMLs are the cell type most commonly seen in the ovarian tissue. They also appear to be the cell type to reach the ovary first, given that the lymphocytes were never observed without PMLs present, but that the reverse was common.

The role of PMLs is unclear in this model as they are more prevalent in the bursa and hilar region rather than in the ovary proper where oocyte cell death would be occurring. Therefore, although PMLs are capable of cell debris phagocytosis, it seems unlikely that they would be solely responsible for this in the ovary proper. It may be that they have been recruited to the ovary through release of a potent PML recruiter, such as chemokine IL-8, but are not able to penetrate the ovary sufficiently unlike lymphocytes or macrophages. The mode of transport of PMLs into tissue is by the vasculature (they adhere to endothelial cells lining vasculature and migrate between them to enter the tissue). It is, therefore, not surprising that penetration of PMLs into the ovary proper is reduced in comparison to the bursa or hilar region as the vasculature is relatively reduced in the ovary proper (the nests of oocytes and follicles are avascular but there will be vasculature in the interstitial regions).

Macrophages are present and active in the mouse ovary during the process of ovulation (Hume *et al.*, 1984). They have also been detected in the immature (28 day old) rat ovary, along with eosinophils, mast cells, PMLs and T-lymphocytes (Brannstrom *et al.*, 1993). In this study, macrophages have also been shown to be active in the neonatal ovary, ingesting cell debris and cytoplasmic fluid. In addition to this classic phagocyte, granulosa cells of atretic follicles in prepubertal mice exhibit phagocytic properties by ingesting dying granulosa cells and oocytes (Inoue *et al.*, 2000). Material assessed in this chapter also suggests that oocytes are capable of contributing to phagocytosis of cell debris. Characteristically non-phagocytic cells are capable of phagocytosis of apoptotic cells even before they exhibit characteristic signs of apoptosis (Gregory and Devitt, 2004). Thus, it may be that ovarian somatic cells and oocytes are able to phagocytose apoptotic cells before they appear apoptotic histologically, perhaps explaining why the massive cell death known to occur in the neonatal ovary is rarely observed. The ability of oocytes to exhibit phagocytic (or autophagic) properties has also been recently demonstrated by Rodrigues *et al.* (2009).

As it is not possible to distinguish between T- and B-lymphocytes using CD45, the exact type of lymphocyte present in the ovary is unknown. However, from the quiescent morphology of the lymphocytes present it appears that they are functionally silent. The reason for their presence is, therefore, unclear.

The observation in this chapter of lymphocytes and PMLs adjacent to oocytes within follicles indicates loss of BM integrity. It is possible to conjecture that this is a secondary effect, caused by the release of proteolytic enzymes from the granulocytes, breaking down the BM as done by macrophages during ovulation. Alternatively, it may be a consequence of normal follicle loss. During normal follicle atresia the BM of the follicle is compromised, and when atresia is artificially induced immune cells can enter the follicle, possibly due to

a disrupted BM (Lee and Yoon, 2005). Therefore, the ability of the leukocytes to access intrafollicular compartments in the ovaries here could be due to either possibility or a combination of both.

The immune response observed in the ovaries is characteristic of a response to injury or infection, and given that it had not been described in the literature, it was considered that it may have been caused by infection in the colony. This, however, has effectively been ruled out as I have since found that the ovarian immune response has been noted by others (personal communication with Dr J. Visser, Utrecht University; and Professor A. Byskov, University of Copenhagen). In addition, I have observed it myself in ovaries from a separate mouse colony in a separate unit.

There is, however, another possible explanation for the ovarian immune response observed. Professor A. Byskov presented a hypothesis (at the Society for Reproductive Fertility annual conference, 2008, Edinburgh) that stem cells may get trapped in the ovary during development. Thus, it may be that ovaries with a strong immune response may have had haematopoietic stem cells lodged in the ovary during development which have begun to proliferate and differentiate in an uncontrolled manner due to being in the incorrect location. This theory would be supported by the observations of immature forms of the PMLs observed in the ovary and could also explain the presence of the seemingly inactive lymphocytes (which do not comfortably fit in with the presence of innate immune cells). Another observation that may tie in with this notion of trapped stem cells is the occasional collection of a bloody ovary. Could it be that formation of blood cells is occurring in these ovaries and instead of lymphocytes, I have actually observed nucleated erythrocytes, and the apoptotic debris is expelled nuclei? I propose this as one option as it was interesting to note that not all lymphocytes, as identified by H&E, were labelled by the CD45 pan leukocyte marker. It may be that another subset of immune cells, which do not express CD45,

also infiltrate the ovary at the same time. Alternatively, it could be that the cells are at a stage of differentiation where they do not express CD45.

In conclusion, the novel observation of immune cells in the neonatal ovary has been characterised and the possible functions of these cells have been discussed.

# **Chapter 7**

## **General Discussion**

## 7.1. Aim of thesis

The aim of this thesis was to investigate the role of TrkB signalling in the development of the neonatal mouse ovary, in particular to determine its role in oocyte survival at the time of follicle formation. The main tool used to address this was the *TrkB<sup>FL</sup>* KO mouse and the assessment of its ovarian phenotype. The merit of this investigation lies in its contribution to our general understanding of the factors that regulate neonatal ovary development. This period of development in the mouse ovary is a model of the period of development important across many species, including humans, as it is the point at which the female mammal's lifetime supply of follicles is formed. Factors correctly regulating this ultimately determine the reproductive lifespan of the individual and ensure the production of the next generation. Incorrect regulation (including failure to form a pool of follicles) will result in compromised fertility, or even infertility.

## 7.2. Effects of TrkB on the neonatal ovary

The project commenced with deciphering the developmental processes occurring in the *TrkB<sup>FL</sup>* KO mouse which result in the phenotype of severe oocyte loss by P4/5 (Spears *et al.*, 2003). However, as discussed in Chapter 3, the colony had been rederived to a new animal unit prior to beginning the project, consequences of which severely impacted upon my ability to address the aim set out. Namely, the problems encountered were 1) a reduced survival rate of pups in the colony, which resulted in an even lower collection rate of *TrkB<sup>FL</sup>* KO pups than anticipated (due to their postnatal lethal phenotype) and 2) an apparent alteration in the ovarian phenotype from that previously observed (Spears *et al.*, 2003). It was simply unfortunate that point 2 was not determined until some time into the project, due to point 1.

With the benefit of hindsight (and the incredible complexity of Trk signalling, as discussed in Chapter 1.5) it perhaps should not have been so unexpected that the role of TrkB signalling in the ovary could be so difficult to unravel. However, in spite of the difficulties and the reduced available sample size, I have demonstrated that instead of the severe phenotype previously observed there is a more subtle phenotype. The subtle phenotype might, though, have helped reveal how *TrkB* affects ovary development as once the ovary is very affected; it is difficult to determine what has occurred. The phenotype found is one of reduced follicle health in terms of granulosa cell to oocyte arrangement, BM integrity and follicle development. Disruption of follicular BM was assessed in the *TrkB<sup>FL</sup>* KO ovary through analysis of laminin- $\alpha$ 1 deposition (see Chapter 4) and is postulated to be part of the sequence of events which contribute to the resulting ovarian phenotype. The laminin- $\alpha$ 1 defect was not restricted to the ovary, but demonstrable in the *TrkB<sup>FL</sup>* KO testis, kidney and brain. There is evidence that the Akt/PKB pro-survival pathway, activated downstream of tyrosine kinase signalling, can regulate BM deposition *in vitro*, in particular isoform laminin- $\alpha$ 1 (Li *et al.*, 2001). I believe the logical interpretation that can be drawn from these findings is that tyrosine kinase signalling through the full-length TrkB receptor contributes to *in vivo* regulation of laminin- $\alpha$ 1 deposition in the mouse. This reduction is detectable before the reduced rate of primary follicle formation is apparent. This finding, alongside the requirement for epithelial cell-BM contact to direct normal morphogenesis, allows one to conjecture that this effect may have direct consequences on the pregranulosa cells' ability to differentiate into cuboidal granulosa cells before commencing the proliferation required for follicle growth. However, it must also be considered that this sequence of events can also be influenced by other signalling pathways including other Trk receptors. It was discussed in Chapter 1.5.2 that the downstream signalling targets of

the Trk receptors can overlap as the specificity for these receptors lies in the ligand binding domain. It is also possible that the many factors discussed in Chapter 1.3.2 which have been shown to be important in the activation of primordial follicles and their initial stages of growth may compensate for the loss of signalling in the *TrkB<sup>FL</sup>* KO.

Since I showed that *TrkB<sup>FL</sup>* KO oocytes can survive, form follicles and initiate growth *in vivo*, I decided to ascertain their long-term survival prospects. This was done by transplanting the ovaries, as *in vivo* assessment is not possible due to the postnatal lethal phenotype. Considering the previous phenotype of *in vivo* oocyte loss (Spears *et al.*, 2003) and the finding that transplantation of *TrkB<sup>FLTr</sup>* KO ovaries results in mass oocyte loss (Paredes *et al.*, 2004), it was anticipated that oocytes/follicles would also be lost in the *TrkB<sup>FL</sup>* KO transplants. Instead it was found that *TrkB<sup>FL</sup>* KO oocytes and follicles can survive and grow as well as in transplanted Control ovaries (see Chapter 5). This has intriguingly revealed that expression of full-length *TrkB* cannot be autonomously required within ovarian follicles for survival (by granulosa cells or oocytes), otherwise they would have degenerated. How these follicles survive in the first place is unknown, though it is possible to speculate that it could be a survival factor generated by the intact ovaries of the recipient. These ovaries will be producing multiple factors required by the ovary as well as having an intact full-length TrkB receptor signalling pathway. However these follicles in the transplants survive, their subsequent growth may then produce additional survival factors sufficient to rescue the remaining cohort of follicles. The fact that transplanted *TrkB<sup>FL</sup>* KO ovaries continue to exhibit reduced laminin- $\alpha$ 1 compared to Control ovaries, as they did *in vivo*, indicates that this effect is a direct result of loss of full-length TrkB signalling but it is not sufficient to prevent follicle growth or cause atresia. This, however,

does not rule out that laminin- $\alpha$ 1 deposition may influence the very initial stages of follicle growth in the ovary.

The previously proposed mechanism of Trk action within the ovary was that it acted at the level of the follicle: ligand produced by somatic cells and/or oocyte binds to receptors present on the somatic cells and/or oocyte. If it could be proved to be the case that the *TrkB<sup>FL</sup>* KO ovaries have been rescued by an aspect of the transplantation procedure then it may still be possible to speculate that full-length TrkB signalling is required for normal *in situ* ovary development, albeit not directly within the follicle. This would demonstrate an unanticipated mode of *TrkB* action within the ovary.

### **7.3. Variability in inbred mouse strains**

The ability to quantitatively measure change in a phenotypic trait in response to a genetic or environmental factor requires that a baseline be determined for the innate variability of that trait within a population. Determining this innate variability then allows a measure of importance to be placed on factors which, when altered, result in a substantial change to the trait in question. As discussed in a commentary by the late Professor Anne McLaren (1999) there is evidence that in attempting to obtain a normal baseline, by using genetically similar organisms such as inbred strains of mice, it may actually only produce greater variability in response to stressors (changes in genetics/environment). This effectively renders the objective of using inbred strains null and void; the basis for this argument is described herein.

McLaren and Michie (1956) observed that first generation mice (F1) resulting from crossing two inbred strains present a more uniform response to a drug treatment than the original inbred strains do. F1 mice are considered to be more genetically variable than the original inbred strains and this increased heterozygosity seems to

provide them with greater fitness. Fitness here is considered to be the organism's ability (when faced with a stressor) to maintain optimal consistency in development and homeostasis (Waddington, 1942). The problem faced at this time was explaining how adaptive traits (developed in the face of a stressor) become heritable. This was clearly a topic of interest several decades ago but it was only recently that a molecular mechanism was proposed to account for this.

The molecular evidence for the ability to buffer against slight changes in genotype or environment came in the form of the signal transduction chaperone, heat shock protein (Hsp90). Prevention of its normal action in *Drosophila* (either by mutation or pharmacological means) result in presentation of morphological variants which can become heritable when selected for (Rutherford and Lindquist, 1998). This led to the hypothesis that signal transduction chaperones may act as buffers to changes in genetics or environment (stressors) to allow the normal development of the organism. If alteration (genetic or otherwise) affects a chaperone it may prevent its ability to buffer against many other traits which then become apparent, thus instead of affecting only one character many could be affected. The result is a reduction in uniformity within the population. In normal populations, faced with natural selection, the process of stressors inducing small variations in phenotype allows the heritable acquisition of traits that are beneficial. However, for the research scientist this is not so good, as inbreeding (a form of stress as McLaren (1999) puts forth), causes the mouse strains to lose heterozygosity, increasing variability in responses to stress within the population. This effectively means that inbred strains could be unreliable for measuring the significance of some genetic or environmental factors (McLaren, 1999).

The use of inbred strains to generate transgenic mice is, therefore, questionable although commonplace. Many have attempted to circumnavigate the problem of variation between

individuals by increasing the homozygosity of the colony through moving mutations onto inbred congenic backgrounds. Congenic mice differ by only a small genetic sequence and are generated by crossing two strains of inbred mice (one of which expresses a gene, or transgene, of interest). The gene is selected for in each generation and the animal is backcrossed with the other strain for 5-10 generations. This results in the mutation being the only difference between siblings. However, the degree to which a phenotype will be presented can still be variable between congenic strains. This issue has begun to be addressed (Rivera and Tessarollo, 2008) but in general, when this type of problem has arisen in the scientific community it has often been ignored, as the use of congenic strains of mice is considered to be the best practice when assessing the effect of a transgenic mutation.

In terms of mouse ovary development it may not, therefore, be surprising to learn that different inbred strains can exhibit significantly different numbers of follicles shortly after birth (Canning *et al.*, 2003). There is also large variation in oocyte number between individual mice within an inbred strain (Rodrigues *et al.*, 2009). Consequently, when animal availability is limited, as for the *TrkB<sup>FL</sup>* KOs, the innate variability of the ovary magnifies an already existing problem.

#### **7.4. Use of animals in research**

Aside from the *TrkB* colony, it is possible to take the point one step further to the controversial debate that has dogged science for several decades and quite probably always will: the use of animals in research. Rivera and Tessarollo (2008) recently discussed in a commentary that the benefit of using mice as a research tool is their closeness to humans, allowing normal and pathological development to be studied, with or without manipulation. However, disparities in

results, caused by the variation discussed in the previous section, cast a shadow over this logic and question the ability to draw meaningful conclusions from mouse studies. It was proposed that “to minimize inter-animal variations.....experiments might need to be done in a pathogen-free purebred genetic background, in animals of the same age, and only in males; reliable results might even be obtained only when phenotypes are recorded at the same time of day” (Lathe, 1996). Having controlled for these aspects in this study (except, obviously, for only using males) and finding that variability is still apparent in the *TrkB<sup>FL</sup>* KO phenotype it seems that the challenge faced (if we are to continue using transgenic animals in research in a meaningful way) is to develop a reliable robust *in vivo* model. A possible compromise to this has been proposed by Matthaei (2007). The ideal method proposed would be to have a transgene that is under total control of the researcher, in terms of where it is expressed and when it is expressed. The ability to reverse the effects would also allow each animal to act as its own Control (Matthaei, 2007). It has also been proposed by Rivera and Tessarollo (2008) that meaningful interpretations from using transgenic mice can still be obtained if careful consideration is made in relation to what is being investigated and the known idiosyncrasies of individual backgrounds. Additionally, several backgrounds could be studied for effects of the same mutation to achieve a broader spectrum of the potential phenotypes. This would potentially allow differentiation of a true effect from a background effect (Rivera and Tessarollo, 2008). That is not to say that when an effect is observed it is not real but in a situation whereby an effect may appear variable in its presentation it may be useful to build a bigger picture of the nature of the phenotype across several backgrounds. This may be ideal, although it would be very expensive due to the number of animals that would be required.

In spite of the problems that were faced in the project regarding animals, it demonstrated that the whole animal model is yet to be

superseded by new technologies. *In vitro* methods are constantly being refined and their success places them in a position of usefulness that on paper would seem to surpass the mouse. Cell and tissue *in vitro* culture protocols allow almost total manipulation and control of the experiment, however, they cannot truly replicate the complexity of the whole animal model, or reveal possibly wider reaching consequences. This project demonstrated how true this can be with the novel observation of the neonatal immune response. It was ascertained in Chapter 6 that the neonatal ovary is capable of exhibiting severe infiltrations of different types of immune cell including the innate (PMLs, eosinophils and macrophages) and acquired immune responses (lymphocytes). The granulocytes, at least, could be observed in most ovaries from P0-P7 at low levels leading to speculation of potential physiological roles for their presence in the neonatal ovary. In addition the severity of the response was characterised to occur during the developmental period when neonates are susceptible to aberrant immune system formation. This, I believe is an example of the far wider reaching consequences that may come about from mutation or manipulation of the environment. They can occasionally allow scientists to speculate on subjects seemingly at a tangent from their original aim or interest, but it is clear that it would never have been observed had it been cells in a culture dish getting analysed.

## **7.5. Further research.**

During the course of this thesis, although working with limited material, I have been successful in investigating how full-length *TrkB* affects ovary development. However, I do not believe that further work researching the role of full-length *TrkB* signalling only in relation to the ovary would be beneficial to the field due to the lack of molecular tools available and (from this study) the lack of

autonomous requirement for TrkB signalling within the follicle. It did, however, become apparent that a very useful attribute for a researcher to have is an open mind when considering unexpected results. I would say that out of all the results obtained during the course of this PhD it is in fact the unexpected results that have ended up being the most thought-provoking and possibly revealing. Two aspects that I consider would be interesting to take further are discussed here.

Firstly, it was not anticipated that the results of the *TrkB<sup>FL</sup>* KO ovary transplants (carried out in Chapter 5) would give such staggeringly different results from those of Paredes *et al.* (2004). As covered in the discussion of that Chapter, the results raised several possible factors that could account for the different outcomes, although it is apparent that the previously proposed mechanism of TrkB action (autonomously within the follicle) is now redundant as *TrkB<sup>FL</sup>* KO mice examined for this project are able to survive without it. In spite of the lack of requirement for *TrkB* expression within the follicle, it would still be particularly interesting to determine if the variables between the two protocols described influence the outcome of the transplants. This would contribute to our understanding of the optimal way in which this protocol can be used in the future, whether that is to support optimal survival of the transplanted ovaries or it is to decipher autonomous from non-autonomous effects as this experiment was originally intended to do.

To determine the effect of the intact recipient's ovaries on the transplanted ovary would require transplantation of *TrkB<sup>FL</sup>* KO ovaries into ovariectomised recipients. It would also be proper to transplant the *TrkB<sup>FL</sup>* KO ovaries at an older age to see if the mutation has a "tipping point" after which it cannot be rescued. The reverse experiments would require *TrkB<sup>FLTr</sup>* KO ovaries to be transplanted into intact recipients as well as transplanting them at P0, to see if they can be rescued. These steps would be best practice

to try to rule out variables causing the difference in transplant results, however, it would require a sample size of *TrkB<sup>FL</sup>* KO ovaries large enough to statistically compare each combination. This fact, as well as needing access to *TrkB<sup>FLTr</sup>* KO ovaries, unfortunately makes it an unrealistic option.

The second aspect of this project that I believe would merit further investigation is the observation of the neonatal immune response described in Chapter 6. It was originally hypothesised that the response was due to elevated rates of oocyte cell death occurring in the *TrkB<sup>FL</sup>* KO ovary. The observation of the same response in the Control ovaries negated this possibility. However, it was not proven that the response is not associated with cell death occurring in ovaries; therefore, it would still be worth obtaining *Dazl* KO ovaries at the point of their dramatic loss of oocytes to see if the immune cells are present.

I personally consider that the hypothesis of a link between the immune response observed in the colony and the cleanliness of the unit is one worthy of further investigation. The induction, duration and intensity of the immune response may exert effects on the fitness of the animals, in particular, their fertility. To address this would be a major undertaking but if it were possible to access the ovaries from animals on the same background in different units with different degrees of sterility it may be feasible. It would, though, require that improvements be made to the techniques utilised in Chapter 6. The material used to characterise the response in Chapter 6 was a reasonable compromise of some tissue that was good for morphology and some that was good for immunohistochemistry. The pan leukocyte marker, CD45, at least confirmed the immune nature of the cells present. However, as the majority of the antibodies investigated for this chapter are known to work best on frozen sections it would be best to obtain frozen sections and attempt to differentiate between the immune cell types with specific antibodies. It may also be useful

to characterise the chemokine signals present in the neonatal ovary, however this would not necessarily give a physiological explanation.

## 7.6. Concluding remarks

As set out in Chapter 1, the specific aims that I intended to address during the course of this PhD were to:

1. Decipher and investigate the chain of events occurring in the *TrkB<sup>FL</sup>* KO mouse that leads to the phenotype of severe oocyte loss seen shortly after birth.

2. Extrapolate to determine how TrkB signalling contributes to the normal loss of oocytes that occurs at the time of ovarian follicle formation.

In examining the phenotype of the *TrkB<sup>FL</sup>* KO ovary during this PhD it was established that the severe phenotype was no longer apparent. Instead a subtle effect on follicle health, early follicle growth and deposition of laminin was found. Each of these factors contributes to normal development of the ovary but intriguingly the long-term survival prospects of the oocytes in the *TrkB<sup>FL</sup>* KO ovaries is not compromised when they are transplanted. It must be concluded, therefore, that full-length TrkB signalling does not directly regulate oocyte survival. It was possible, though, to speculate that the reduction in follicle health and deposition of laminin may contribute to the reduced follicle growth observed in the *in vivo TrkB<sup>FL</sup>* KO. Thus TrkB signalling, in the context of normal ovary development, may contribute to follicle growth by regulating the extracellular environment of the follicle. This thesis has also highlighted the existence of immune type cells in the neonatal mouse ovary, further investigation of which would be interesting. The difficulties faced during this PhD in addressing these aims allowed

the scope of this thesis to expand to discuss the importance of aspects such as the use of transgenic studies and how animal colonies are maintained. Therefore, although the aims were not able to be fully addressed I believe this thesis has contributed to our understanding of neonatal ovary development and, in the bigger picture, has highlighted areas of working in research that require further attention before similar studies should be undertaken.

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## Appendix A. PCR protocols

### DNA extraction by HotSHOT

Combination of the lysis and neutralising reagents produces a 20mM Tris-HCl, pH 8.1, 0.1M EDTA buffer which DNA can be stored in.

<b>Alkaline Lysis Reagent – pH 12</b>	<b>Neutralising Reagent – pH 5</b>
25mM NaOH	40mM Tris-HCl
0.2mM EDTA	
Dissolve in H <sub>2</sub> O – no pH adjustment	Dissolve in H <sub>2</sub> O – no pH adjustment

### PCR screening for wild-type and mutant *TrkB* genes

Successful PCR genotyping results were obtained mainly through running the reaction for the wild-type gene separately from the mutant gene, although it was also possible to obtain results using primers in combination.

#### **Combined reaction**

<b>PCR Reaction Mixture</b>	<b>Volume per sample (µl)</b>
ddH <sub>2</sub> O (MilliQ-autoclaved)	8.8
10x PCR Buffer	2.0
25mM MgCl <sub>2</sub>	0.6
10mM dNTPs (1:1:1:1)	0.4
Taq	0.2
10mM Primers (1:1:1)	3.0
DNA	5.0
	<b>Final volume = 20µl</b>

#### **Separate reactions for wild-type or mutant gene**

<b>PCR Reaction Mixture</b>	<b>Volume per sample (µl)</b>
ddH <sub>2</sub> O (MilliQ-autoclaved)	9.8
10x PCR Buffer	2.0
25mM MgCl <sub>2</sub>	0.6
10mM dNTPs (1:1:1:1)	0.4
Taq	0.2
10mM Forward Primer	1.0
10mM Reverse Primer	1.0
DNA	5.0
	<b>Final volume = 20µl</b>

All reagents (excluding water) were stored at -20°C, prior to use they were defrosted thoroughly and rotamixed.

## Primer sequences

5' AAGGACGCCAGCGACAAT      Common wild-type forward  
5' GTCCCCGTGCTTCATGTACTC      Wild-type reverse  
5' GATGTGGAATGTGTGCGAGGCC      Mutant reverse

## PCR Parameters

1. Preheat @ 96oC      2 minutes
2. Melt @ 96oC      30 seconds
3. Anneal @ 60oC      30 seconds
4. Extend @ 72oC      30 seconds
5. Steps 2-4 for 34 cycles
6. Extend @ 72oC      5 minutes
7. End

## 100bp ladder

5µl ladder  
45µl ddH<sub>2</sub>O  
10µl loading buffer

## Agarose gel

TBE Buffer pH 8.3	
Tris	10.9g
Boric acid	5.5g
EDTA	0.93g
ddH <sub>2</sub> O	1000ml

The PCR products were run on a 1% agarose gel, either in a standard gel tank or, to allow better separation when using the combined primer PCR reaction, in a midi-gel tank.

### **Mini-gel tank**

50ml TBE  
1g agarose  
1µl Sybresafe

### **Midi-gel tank**

70ml TBE  
1.23g agarose  
1ul SYBRsafe

## **Appendix B. TEM solutions**

### Stock buffer: 0.2M NaCo.

Sodium cacodylate buffer (NaCo) was made as a 0.2M stock in MilliQ ddH<sub>2</sub>O which could be used over 2-3 weeks if stored at 4°C.

### Working buffer: 0.1M NaCo.

Prior to a 1:1 dilution in MilliQ ddH<sub>2</sub>O, the pH of 0.2M NaCo was adjusted to 7.3 by addition of 0.2M HCl.

### 3% glutaraldehyde.

25% glutaraldehyde in 0.1M NaCo (TAAB) was diluted to a 3% solution using the working buffer.

### 1% Osmium tetroxide.

4% osmium tetroxide in H<sub>2</sub>O (TAAB) was diluted to a 1% solution using the working buffer.