The Reproductive Phenotype of the Male Aromatase Knockout Mouse

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Thesis submitted for Doctor of Philosophy
October 2001

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ABSTRACT

The process of spermatogenesis involves a coordinated series of developmental events to form a mature spermatozoon capable of fertilisation from an immature spermatogonium. These maturation steps occur within the seminiferous tubules of the testis, in close communication with the supportive Sertoli cells and surrounding interstitium, and then in the epididymal ducts. This process has been well characterised with respect to androgens and gonadotrophins. Oestrogens were also recognised to be synthesised in many of these reproductive structures. Although abnormal levels of oestrogens at various stages of development were shown to result in abnormalities in spermatogenesis, suggesting the testis was a possible oestrogenic site of action, the exact role of oestrogen remained unclear. A specific and direct role for oestrogen was not unreasonable given that both oestrogen receptors, predominantly ERβ, and aromatase were localised in the testis. Through gene targeting technology, models have been designed to elucidate oestrogen action, with the significance of this 'female' hormone in the process of male fertility now beginning to be realised. The model described in these studies is the aromatase knockout (ArKO) mouse. The ArKO mouse lacks a functional aromatase cytochrome P450 enzyme, thus is unable to catalyse the conversion of C19 steroids (androgens) to C18 steroids (oestrogens).

Initial investigations found that withdrawing endogenous oestrogens resulted in severe disruptions to spermatogenesis between 18 weeks and 1 year of age. Quantitation of germ cell numbers indicated that spermatogonia and spermatocyte numbers were unchanged, however there were significantly fewer round and elongated spermatids, some animals showing complete spermiogenic arrest. This occurred with no change in Sertoli cell number. In addition, abnormalities were observed in early acrosome development and Leydig cell hypertrophy. This specific lesion in germ cell development, resulting in a significant decline in germ cell number by 1 year of age, was suggested to be due to an increase in germ cell apoptosis. This was characterised by an increase in the expression of

apoptotic genes, particularly the Bcl-2 family at 18 weeks and the FAS pathway at 1 year of age. Given that the role for oestrogen as a cell survival factor, these findings were not surprising.

Due to these abnormalities in developmental spermatogenesis, the mature spermatozoa contained in the epididymis were severely reduced in concentration and presented with abnormalities in their functional motility. As such they were unable to fertilize oocytes *in vitro*, resulting in a marked reduction in the ability to sire litters. As the ArKO mice also have impairments in their sexual behaviour, failing to mount receptive females, the reduced fertility may also be a consequence of behavioural abnormalities. Male mice are considered sexually mature by 6-7 weeks of age, however the onset of the spermatogenic disruptions occurred between 18 weeks and 1 year of age. This was puzzling, with one hypothesis being that the consumption of dietary soy by these mice was providing them with an alternative exogenous oestrogen source able to maintain spermatogenesis. The removal of the dietary soy severely exacerbated the phenotype of the ArKO mice, suggesting that the phytoestrogenic constituents of soy were exerting agonistic effects to prevent the severity of the phenotype arising. As animals on the soy free diet presented with the disruption to spermatogenesis no earlier than 14 weeks, it is possible that the soy partially contributed to the late onset phenotype, however other factors such as androgens and growth factors could appear to be important. The ArKO mice on the diet containing no soy represented a completely oestrogen free model, and as such, all ArKO mice are currently raised on this diet.

In conclusion, disruption of the aromatase *Cyp19* gene leads to a progressive disruption to spermatogenesis. Oestrogen appears to have a direct effect on the development and survival of the germ cells, with male mice have severely reduced fertility in its absence. As both ERβ and aromatase co-exist in the germ cells where the lesion is observed, it is postulated that the actions of oestrogen are a result of paracrine and/or intracrine interactions within the cells of the seminiferous epithelium. Thus, the ArKO mice have brought to our attention the hitherto unsuspected role of oestrogen as a crucial male hormone.

STATEMENT OF DECLARATION

The work embodied in this thesis was conducted at Prince Henry's Institute of Medical Research, through The Department of Biochemistry and Molecular Biology, Monash University, during 1998-2001. It contains no material which has been accepted for the award of any other degree or diploma in any university or other institution, and to the best of my knowledge contains no material previously published or written by another person, except where due reference is made in the text.

Kirsten M Robertson

ACKNOWLEDGEMENTS

I would sincerely like to thank Prof. Evan Simpson who was the instigator behind this work with the ArKO mouse. Evan, I will always be grateful to you for giving me the opportunity to become involved with such an exiting project for my PhD. Thanks for your generosity and foresight which enabled me to attend and participate in numerous national and international conferences. I found this incredibly beneficial not only for my career advancement but also for exposing me to the current developments and technologies being made in endocrinology research. I would also like to thank you for introducing me to Prof. Jan-Ake Gustafsson and recommending that he offer me a position as a post doc in his lab at the Karolinska Institute in Stockholm.

I would also like to thank Dr. Margaret Jones who was my supervisor through my honours year. Margaret, it was your enthusiastic approach and guidance that inspired me to undertake my PhD in the first place. Thank you for believing in my abilities to the extent that you were prepared to recommend me to Evan as a suitable PhD student for his lab. I also have to thank you for nominating me for Young Australian of the Year. Not only was this an incredible honour and the most exciting night of my life but it also lead to unbelievable media exposure for both myself, my project and Prince Henry's Institute.

A big thanks to all of the Simpson Lab for their support, encouragement and advice, I thoroughly enjoyed the three and a half years that I spent in the lab. To Yoko for running the lab so well, Emily for keeping us well supplied with mice bits and Colin for the chats and constant trips to stores for gloves, pens etc etc. I would, however, like to single out Wah Chin Boon for a special mention. You were an inspirational mentor, always willing to stop what ever you were doing to answer all my questions no matter how trivial- your tolerance was greatly appreciated. Also, thankyou for being prepared to give up your valuable time to undertake the mammoth task of reading and re-reading my drafts, and please thank Robert for his patience and understanding.

I would also like to thank everyone at Prince Henry's Institute for their support and expertise, which has been invaluable in completing my PhD. In particular Liza O'Donnell for sharing her knowledge of spermatogenesis and other members of the Robbo group for letting me share your lab in my first year. Sue Panckridge for always giving up her time to answer all of my questions, graphic or not. Sue Elger for taking time to chat and for being fun on our Port Douglas get away. To all the tea room people for making lunch a little more interesting. To Anne, Bex and Kate for your friendship.

Of course I would also like to thank all of the PHIMR students. In particular my fellow Simpsonites Kylie and Marie, also Dora and Kara. Thanks guys for keeping me sane and most importantly for making my PhD fun. I'll miss you all. And I leave you with one word of advice, it's not gossiping it's networking!

Last but not least is of course my family, including Brian and Megan and all adopted members. Thanks for being there. To Mum and Dad who not only kept me for an extra 3 and a half years until the ripe old age of 26, an age when most parents would have thrown their kids out of the nest, but were amazingly supportive. Thankyou for letting me 'own' the study for nearly 6 months of writing, letting me sit at that computer for days on end surrounded by organised chaos, only leaving this place of habit when succumbing to Jamie's pleas for internet access. Thanks to Seonad and Warren for taking me out partying when I was close to breaking point. Iain, now you can play all the games that you want, and Jamie the study is finally yours! A special mention to Toby, whose companionship while typing was calming, also Brett and Leanne for amusing drinking sessions. Finally to Karen who went above and beyond the call of duty as Jamie's girlfriend when she was prepared to undertake the arduous task of proofreading the completed works.

Thanks everyone - its finally done!

PUBLICATIONS AND PRESENTATIONS

Publications

1. **Kirsten M. Robertson**, Liza O'Donnell, Margaret E.E. Jones, Sarah J. Meachem, Wah Chin Boon, Carolyn R. Fisher, Kathy H. Graves, Robert I. McLachlan and Evan R. Simpson 1999. Impairment of spermatogenesis in mice lacking a functional aromatase (*Cyp 19*) gene (ArKO). *Proc. Natl. Acad. Sci* (USA) **96**: 7986-7991
2. Evan Simpson, Gary Rubin, Colin Clyne, **Kirsten Robertson**, Liza O'Donnell, Sue Davis and Margaret Jones 1999. Local estrogen biosynthesis in males and females. *Endocrine Related Cancer* **6**: 1-99
3. Evan Simpson, Gary Rubin, Colin Clyne, **Kirsten Robertson**, Liza O'Donnell, Sue Davis and Margaret Jones 2000. The role of local estrogen biosynthesis in males and females. *TEMS* **11** (5) 184-188
4. Margaret E.E. Jones, Kara L. Britt, Kylie N. Hewitt, Anne Thorborne, Nigel G. Wreford, Joe Proietto, Orhan K. Oz, **Kirsten M. Robertson** and Evan R. Simpson. Aromatase Deficient (ArKO) mice have an Obese Phenotype 2000. *Proc. Natl. Acad. Sci.* (USA) **97**: 12735-40
5. **Kirsten M. Robertson**, Orly Lacham-Kaplan, Evan R. Simpson and Margaret E.E Jones. Characterization of the fertility of young aromatase knockout (ArKO) male mice 2001. *Journal of Andrology* **22** (5): 825-830
6. Liza O'Donnell, **Kirsten Robertson**, Margaret Jones and Evan Simpson. Estrogen and spermatogenesis 2001. *Endocrine Reviews* **22** (3): 289-318
7. Margaret E.E. Jones, Kara L. Britt, Kylie N. Hewitt, Anne Thorborne, Nigel G. Wreford, Joe Proietto, Orhan K. Oz, **Kirsten M. Robertson** and Evan R. Simpson. Aromatase Deficient ArKO mice accumulate excess adipose tissue. *Journal of Steroid Biochemistry and Molecular Biology*- in press.

8. **Kirsten Robertson**, Evan Simpson, Liza O'Donnell and Margaret Jones. The ArKO mouse reveals a role for dietary phytoestrogen in male reproductive development. Submitted *Proc. Natl. Acad. Sci.* (USA)
9. **Kirsten Robertson**, Wah Chin Boon, Evan Simpson and Margaret Jones. The aromatase knockout mouse reveals a role for oestrogens in the regulation of testicular apoptotic genes. Submitted *Molecular Endocrinology*
10. Evan Simpson, Colin Clyne, Gary Rubin, Wah Chin Boon, **Kirsten Robertson**, Kara Britt, Caroline Speed, Margaret Jones. Aromatase- A Brief Overview. Submitted *Annual Review of Physiology*

Conference presentations

1. **Kirsten Robertson**, Liza O'Donnell, Margaret Jones and Evan Simpson Disruption of Spermatogenesis in the Aromatase Knockout Mouse 1998. *Endocrine Society of Australia*, Perth - oral presentation
2. **Kirsten Robertson**, Liza O'Donnell, Margaret Jones, Sarah Meachem, Robert McLachlan and Evan Simpson 1999. Impairment of Spermatogenesis in Mice Lacking the Aromatase (*Cyp 19*) Gene (ArKO). *American Endocrine Society*, San Diego- oral presentation
3. **Kirsten Robertson**, Liza O'Donnell, Evan Simpson and Margaret Jones. The Effects of Phytoestrogens on the Phenotype of the Male Aromatase Knockout (ArKO) mouse 1999. *Endocrine Society of Australia*, Melbourne - Junior Scientist Award
4. **Kirsten Robertson**, Liza O'Donnell, Evan Simpson and Margaret Jones. The Late Onset Phenotype of the Male Aromatase Knockout (ArKO) Mouse 2000. *11th European Workshop on Molecular and Cellular Endocrinology of the Testis*, Saint Malo, France – mini poster presentation
5. **Kirsten Robertson**, Wah Chin Boon, Evan Simpson and Margaret Jones. Oestrogen regulated genes in the testis of the aromatase knockout (ArKO) mouse 2000. *Aromatase and the third generation*, Port Douglas, Queensland, Australia- poster presentation

6. **Kirsten Robertson**, Wah Chin Boon, Evan Simpson and Margaret Jones. Oestrogen regulated genes in the testis of the aromatase knockout (ArKO) mouse 2001. *American Endocrine Society*, Denver - oral presentation.

Conference proceedings

1. Evan Simpson, Gary Rubin, Colin Clyne, **Kirsten Robertson**, Liza O'Donnell, Susan Davis and Margaret Jones 1998. Local Estrogen Biosynthesis in Males and Females. *Aromatase and its Inhibitors*, Prague, The Czech Republic
2. Margaret Jones, **Kirsten Robertson**, Liza O'Donnell, Wah Chin Boon and Evan Simpson. The Aromatase Knockout (ArKO) Mouse...re-evaluating a role for estrogen in male physiology 2000. *Proceedings of the first European Congress of Andrology*. March pp 299-324. L'Aquila, Italy.
3. Yoko Murata, **Kirsten Robertson**, Margaret Jones and Evan Simpson. Role of Estrogens in the Male: ArKO mice. *Molecular and Cellular Endocrinology* - in press.

ABBREVIATIONS

3βHSD	3β-hydroxysteroid dehydrogenase
17β-HSD	17-hydroxysteroid dehydrogenase
ABC	Avidin biotinylated conjugate
ABP	Androgen binding protein
AIS	Androgen insensitivity syndrome
AMH	Anti-mullarian hormone
AMV	Avian myeloblastosis virus
AP-1	Activator protein–1
AR	Androgen receptor
ArKO	Aromatase knockout
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CAD	Caspase-activated DNase
ICAD	CAD inhibitory subunit
Cdk	Cyclin dependant kinase
DAB	3,3'-diaminobenzidine tetrahydrochloride
DES	Diethylstilbesterol
DEHP	Di-(2-ethylhexyl) phthalate
DEPC	Diethyl pyrocarbonate
DHT	Dihydrotestosterone
DIABLO	Direct IAP-binding protein with low pI
DIG	Digoxigenin-peroxidase
DMSO	Dimethyl sulfoxide
dNTP	Deoxynucleotide
dpc	Days post coital
DTT	Dithiothreitol

E2	Oestradiol
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
ER	Oestrogen receptor
ERKOα	ER knockout -alpha
ERKOβ	ER knockout -beta
ERE	Oestrogen response element
ERK	Extracellular signal-regulated protein kinase
ES cells	Embryonic stem cells
EtBr	Ethidium bromide
ETOH	Ethanol
FADD	FAS associated death domain
FAF	FAS associated factor
FAP-1	FAS associated phosphatase
FSH	Follicle Stimulating Hormone
G1	Gap 1
gld	Generalized lymphoproliferative disease
hpg	Hypogonadal
Het	Heterozygote
Hrp	Horseradish peroxidase
IGF	Insulin-like growth factor
IL-1β	Interleukin-1β
INK4	Inhibitors of only cdk4 or cdk6
KO	Knockout
LH	Luteinising hormone
lpr	Lymphoproliferation
MAPK	Mitogen activated protein kinase
MOPS	3-(*N*-morpholino)propanesulfonic acid
MPF	M-phase promoting factor
MPOA	Medial preoptic area
NADP	Nicotinamide adenine dinucleotide phosphate

NRS	Normal rabbit serum
OVX	Ovariectomised
P450scc	Cholesterol side chain cleavage
PII	Promoter II
PBS	Phosphate buffered saline
PGC	Primordial germ cells
pRb	Retinoblastoma protein
RIA	Radio-immuno assay
S-	Soy free
S+	Soy containing
SSC	Tri-sodium citrate
SF-1	Steroidogenic factor-1
Smac	Ssecond mitochondria-derived-binding protein with low pI
STAR	Steroidogenic acute regulatory
SRY	Sex-determining region of Y gene
T	Testosterone
TBE	Tris/borate/EDTA
Tfm	Testicular feminised male
TGF-β	Transforming growth factor beta
TNF-α	Tumour necrosis factor alpha
TNFR1	Tumour necrosis factor receptor
TUNEL	Terminal deoxynucleotide transferase-mediated deoxy-UTP nick end labelling
WT	Wildtype

Amino acid abbreviations

Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N

Aspartic acid	Asp	D
Asparagine or aspartic acid	Asx	B
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glutamine or Glutamic acid	Glx	Z
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Chapter One

Literature Review

1.1 INTRODUCTION

For decades oestrogens were defined as 'female' hormones and androgens as 'male' hormones. Each had well classified functions in male and female reproductive physiology, including the maturation of secondary sexual organs and the onset and maintenance of spermatogenesis and folliculogenesis. Although it was recognised almost 70 years ago that the testes displayed oestrogenic activity (Zondek 1934), it has only been in the last few years that oestrogen was considered to have an essential function in male reproduction (Sharpe 1997; Sharpe 1998). Subsequent studies indicated a possible role for oestrogen in the function of male reproductive structures, with the administration of this hormone during crucial developmental periods causing abnormalities such as epididymal cysts, microphallus, cryptorchidism, and testicular hypoplasia in adulthood (McLachlan *et al.* 1975; Gill *et al.* 1979; Stillman 1982; Bullock *et al.* 1988). There was also concern that environmental factors, believed to have oestrogenic action, were contributing to the onset of reproductive abnormalities such as testicular carcinomas, decreases in sperm counts and infertility (Colborn *et al.* 1993; London 1993; Sharpe & Skakkebaek 1993).

Despite these findings, it wasn't until 1993, with the generation of the oestrogen receptor-alpha (ERα) knockout mouse (Lubahn *et al.* 1993), that a specific role for oestrogen in male fertility was elucidated. The classification of oestrogen as only a 'female' acting sex hormone was now finally obliterated. Oestrogen, acting through its ERα, was found to be important for male fertility, although this was an indirect effect via the reabsorption of the luminal fluid required to optimise the testicular environment (Eddy *et al.* 1996; Hess *et al.* 1997a). A direct effect on the developing germ cells was not found. The discovery of two adult men who possessed mutations in their aromatase gene (Morishima *et al.* 1995; Carani *et al.* 1997), thus were unable to synthesise oestrogens, and a single man who had a mutation in his ERα gene (Smith *et al.* 1994), highlighted the importance of oestrogen in bone physiology and cardiovascular maintenance. Nonetheless, a role for oestrogen in fertility remained largely unanswered.

By the late 1990's, a local and specific role for oestrogen in male reproduction had not yet been defined. As aromatase and the ERα and β are localised to the male reproductive structures, in particular the seminiferous epithelium, it was highly probable that oestrogen did have a direct role in spermatogenesis (Hess *et al.* 1995; Carreau *et al.* 1999; Carreau 2000). To investigate this, we generated an aromatase knockout (ArKO) mouse, through targeted disruption of exon IX of the *Cyp19* gene, in which oestrogen synthesis was abolished (Fisher *et al.* 1998). Preliminarily characterisation of the male ArKO mice found that they became progressively infertile, but the cause of this infertility was unknown.

The studies presented in this thesis investigate the effect of abolishing oestrogen on spermatogenesis and other related reproductive functions, providing evidence that oestrogen have a hitherto unknown local action on the seminiferous epithelium specifically in the maturation and survival of germ cells. This review will first provide an overview of the structure and function of aromatase and the oestrogen receptors, then of oestrogen biosynthesis in the testis. It will continue to investigate the cellular structure and associated function of the testis, including the process of spermatogenesis. Following this will be a description of the localisation of both aromatase and the ER in the testis. Then an overview of the importance of cell cycle progression and apoptosis in the testis and finally a description of the oestrogen receptor knockout models and the humans with mutations in both the ER and aromatase.

1.2 OESTROGEN SYNTHESIS AND ACTION

Oestrogen biosynthesis involves the irreversible conversion of androgens into oestrogens by the action of the enzyme aromatase P450. Oestrogens then act in an autocrine, paracine or endocrine mediated fashion to influence the growth, development and homeostasis of specific reproductive process, and other physiological systems such as cardiovascular, bone, brain and liver. Part of the steroid hormone superfamily, oestrogens are lipophilic transcription factors, consequently able to diffuse in and out of cells, however are retained with high affinity in specific target cells that possess an intracellular oestrogen receptor (ER).

1.21 Aromatase

1. 211 Structure and function

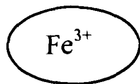
Aromatase is a member of a superfamily of hemoproteins known as cytochrome P450. These enzymes are iron containing electron transfer proteins, located in the smooth endoplasmic reticulum. They play an important role in the metabolism of exogenous substances, such as pollutants and drugs, and endogenous substances, converting them to steroid hormones, cholesterol and fatty acids (for review see Guengerich 1992; Slaughter & Edwards 1995; Chang & Kam 1999). However, further studies have suggested these enzymes are also involved in homeostatic mechanisms such as hypertension (Makita *et al.* 1996). As of 1995, the P450 superfamily consisted of 74 gene families, with a total of 481 gene members (Nelson *et al.* 1996).

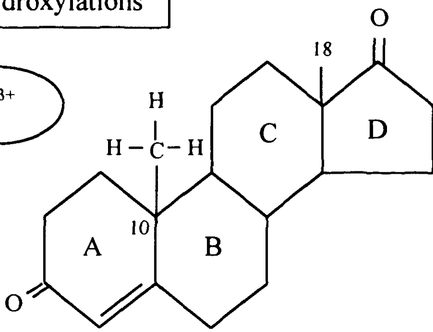
Steroid hormones, including oestrogen, progesterone, testosterone, aldosterone and cortisol, are all derived from the precursor molecule cholesterol. Aromatase cytochrome P450 is a member of the gene family CYP19, designated this as the C19 methyl group of the cholesterol molecule is the site of attack by oxygen (**Figure 1.211**), located in region 15q21.1 on human chromosome 15. Its primary role is to bind to the C19 androgen

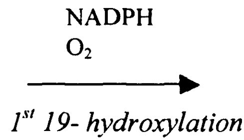
Figure 1.211 The Aromatase Reaction

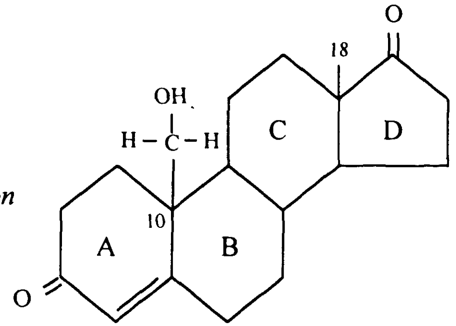
The conversion of C19 androgens into C18 oestrogens by P450aromatase occurs in 2 main reactions. The first involves oxidization of the C19 methyl group by 2 hydroxylation reactions, forming 19-hydroxy androstenedione then 19-oxo androstenedione. The C19-oxo group is then peroxidatively attacked resulting in the release of the C19 group as formic acid, removal of a proton from the C1 position and the aromatisation of the A ring.

Hydroxylations

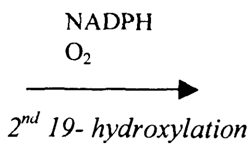


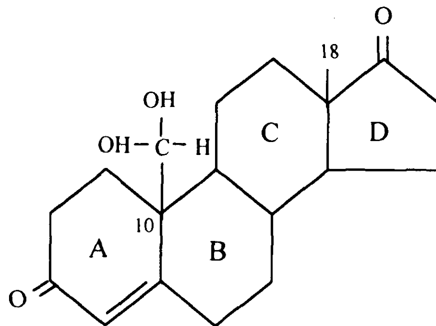


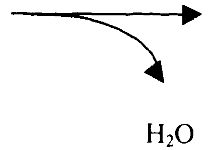




Androgen substrate (C19)

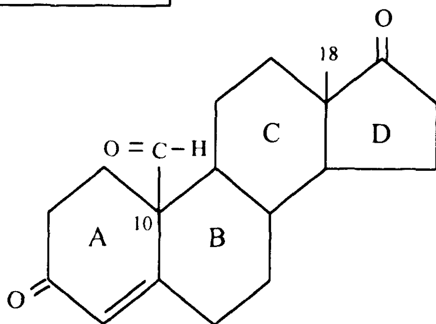




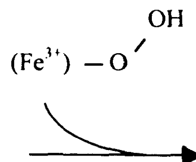


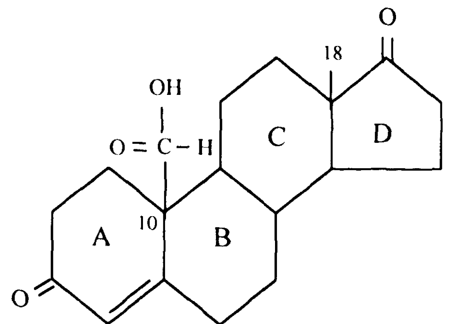
19-hydroxy-androstenedione

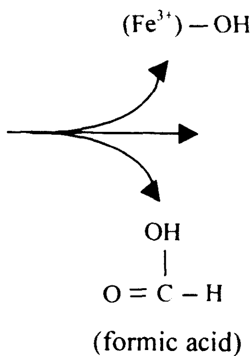
Aromatisation

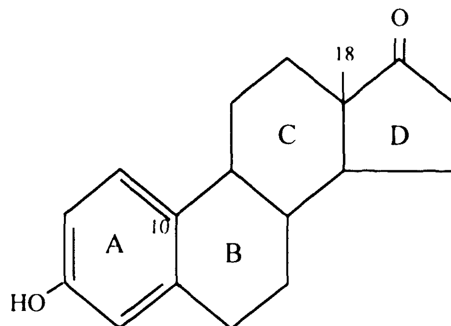


19-oxo androstenedione









Oestrogen (C18)

substrate and catalyze the series of reactions required to irreversibly produce C18 oestrogens (for review see Simpson *et al.* 1997). The proposed mechanism involves 3 steps (**Figure 1.211**). Firstly, the heme group, common to all cytochrome P450 enzymes, interacts with NADPH to incorporate a single oxygen molecule into the androgen substrate. This occurs in two hydroxylation reactions of the C19 methyl group, the first forming 19-hydroxy-androstenedione, which is closely followed by a second reaction forming 19-oxo androstenedione, with water released as a by-product. The second aromatisation reaction occurs quickly, involving a peroxidative attack on the C19 methyl group that then converts it to formic acid and its subsequent removal. At the same time, a proton is removed from the C1 position causing the unique reaction that results in the aromatisation of the A ring to form the structure associated with oestrogens.

The P450aromatase enzyme has been localised to numerous tissues in the human such as adipose tissue, bone, certain areas of the brain, ovary, foetal liver, placenta and testis, whereas in the rodent it is in the brain, adipose tissue and gonads (for review see Simpson *et al.* 1997). The human CYP19 gene spans at least 70kb and is comprised of 10 exons, from I to X (**Figure 1.212**), with the coding region spanning from exon II to X (Means *et al.* 1989; Toda *et al.* 1990; Harada *et al.* 1990). The heme group common to all cytochrome P450 enzymes is located on exon X. To date, exon I consists of 9 distinct first exons; exon I.1, I.2, I.3, I.4, I.5, 1.6, II, 2a and 1f, each driven by their own specific promoter (Simpson *et al.* 1993). This allows for tissue specific expression of aromatase. For instance, aromatase expression in adipose tissue is driven by promoter 1.4 and regulated by glucocorticoids and class one cytokines (Mahendroo *et al.* 1993). Each untranslated exon I is spliced into the aromatase transcript, resulting in the differing sequence and length of the 5' terminus of the transcript. In contrast, as the coding region is always the same, this results in a structurally identical enzyme in different tissues.

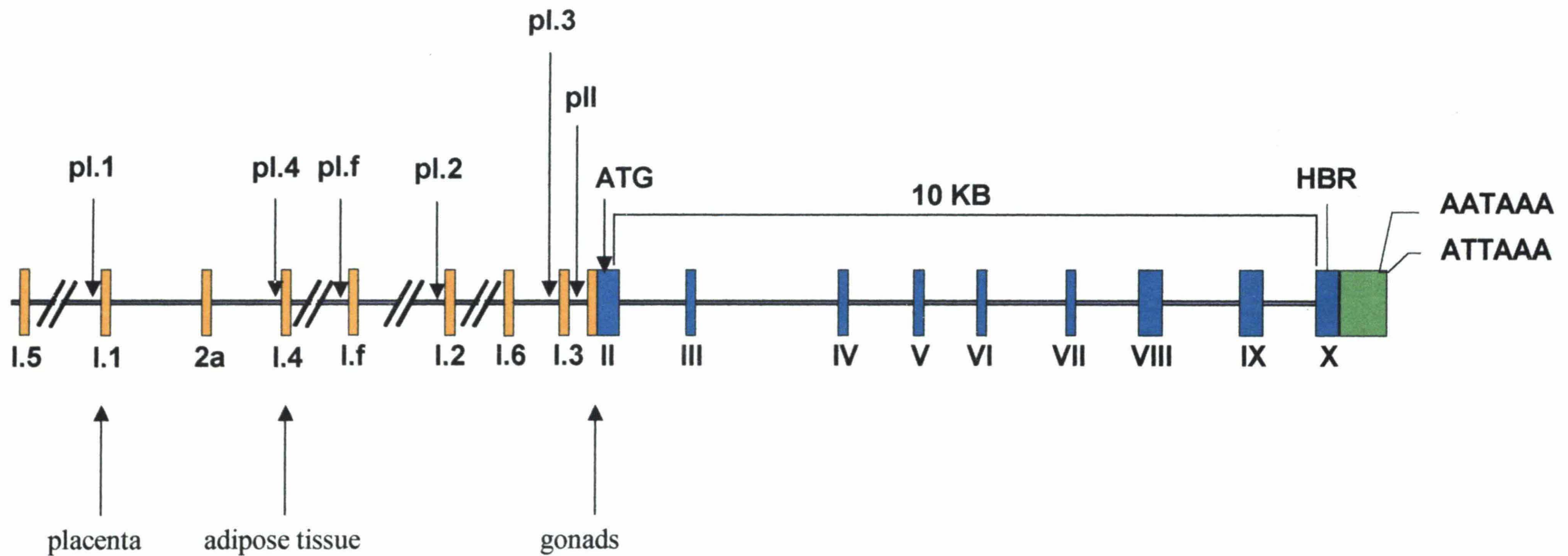


Figure 1.212 The structure of the human CYP19 gene

The aromatase CYP19 gene consists of 10 exons, exon II to X forming the coding region of approximately 35kb in length (blue). The heme binding region (HBR) is located in exon X, as are two alternative polyadenylation signals. There are 9 alternative exon I (I.5, I.1, 2a, I.4, I.f, I.2, I.6, I.3 and II), each containing their own specific promoter to allow for tissue specific expression (not all have been identified). For example, in the placenta, promoter I.1 drives aromatase expression, whereas aromatase expression in adipose tissue is driven by promoter 1.4 and regulated by glucocorticoids and class one cytokines. Whereas in the gonads, expression is regulated by gonadotrophins via cAMP and promoter II.

Reproduced from Simpson *et al* (1997), Carreau *et al* (1999)

1.212 Expression of aromatase in the male gonads

Aromatase expression is driven by the proximal promoter II (PII) in all major cell types in the male gonad, and is regulated by gonadotrophins and cAMP (**Figure 1.213**) (Lanzino *et al.* 2001; Jenkins *et al.* 1993; Bulun *et al.* 1994). Gonadotrophins are well recognized to regulate oestrogen expression in the testes. In foetal and immature rats, it is the Sertoli cells that possess high levels of aromatase expression under the stimulation of FSH (Levallet & Carreau 1997). Whereas in adult testes, the Leydig cells take over this role and aromatase expression is stimulated by LH (**Section 1.4**) (Valladares & Payne 1979b; Papadopoulos *et al.* 1986; Genissel *et al.* 2001). Many studies have been conducted in the ovary and the testis to characterise the role of gonadotrophins in stimulating aromatase expression. Following the binding of FSH to its seven transmembrane receptor, protein kinase A is activated via the elevation of intracellular cAMP (**Figure 1.213**). This in turn activates transcription factors such as cAMP response element binding protein (CREB) and steroidogenic factor-1 (SF-1), which stimulate aromatase gene expression though binding to their response elements located in the proximal promoter region of exon II (Bulun *et al.* 1994; Michael *et al.* 1995; Michael *et al.* 1997; Young & McPhaul 1998). The activation of these response elements has been found to be crucial for aromatase activity in the testes (Young & McPhaul 1997).

The testes have also been reported to express abundant levels of a newly discovered first exon, exon I.6 (Shozu *et al.* 1998). It has been hypothesized that promoter I.6 is regulated by an Activator Protein–1 (AP-1) binding site, suggesting that other important regulatory factors may be influencing testicular aromatase transcription in a manner independent of gonadotrophin induced cAMP (Papadopoulos *et al.* 1987; Carreau *et al.* 1988; Rigaudiere *et al.* 1989).

In summary, aromatase expression in the male gonads appears to be positively regulated by gonadotrophins, androgens and oestrogens via PII. However, it is hypothesised that the CYP19 gene may also contain motifs for negative regulation through growth factors and

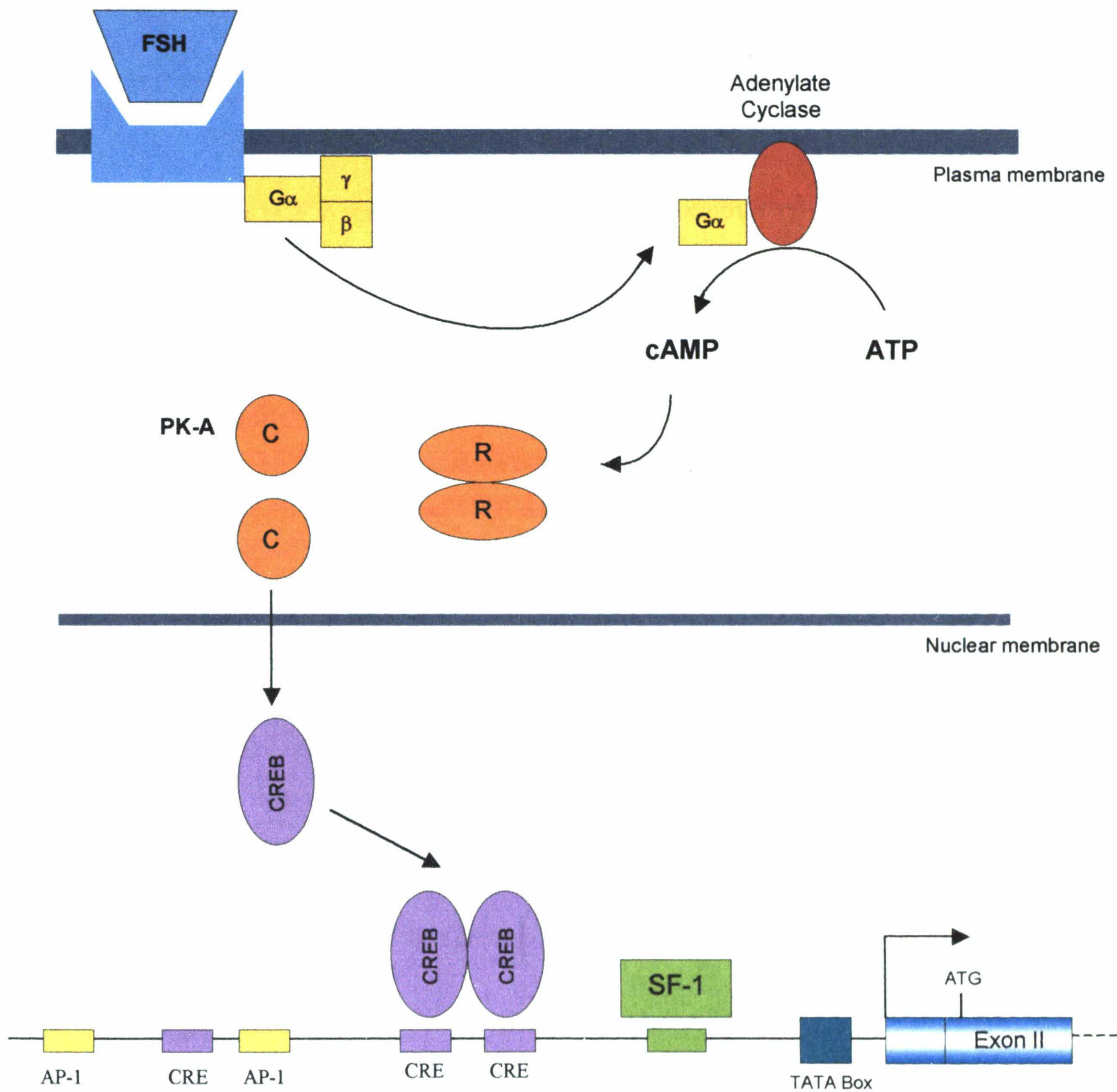


Figure 1.213 Schematic diagram depicting the regulation of aromatase expression in the testes

FSH binds to its membrane receptor and via G protein activation increases cAMP. This activates protein kinase A (PK-A), by the binding of the regulatory (R) elements with the catalytic elements (C), which then promotes the binding of CREB (cAMP response element binding protein) to the cAMP response element in promoter II of the aromatase gene. SF-1 may also play role.

Adapted from Simpson *et al* (1997)

cytokines, such as TGF-β and TNF-α, released from the seminiferous epithelium (Genissel *et al.* 2001).

1.22 Oestrogen receptors

Oestrogen receptors belong to a nuclear receptor superfamily of ligand-inducible transcription factors, consisting of over 150 members (Evans 1988; Mangelsdorf *et al.* 1995). The existence of a receptor that bound 17β-oestradiol was first reported in the 1960's (Jensen & Jacobson 1962), then characterised in the early 1970's (Jensen & DeSombre 1973) and cloned in 1986 (Green *et al.* 1986; Greene *et al.* 1986). For many years following, it was believed that all of the biological actions of oestrogens occurred through this single ER, until a second ER was cloned from rat (Kuiper *et al.* 1996) and human (Mosselman *et al.* 1996) in 1996, and mouse in 1997 (Tremblay *et al.* 1997). The original ER was then redefined as ERα and the new ER as ERβ. Each subtype does not arise through differential splicing, but from two separate genes located on chromosome 6 and 14 (Enmark *et al.* 1997). The discovery of this new ER indicated that the oestrogen signalling is far more complex than predicted.

1.221 Structure

The two ERs have a similar overall structure (**Figure 1.221**), with almost identical DNA binding domains, showing 97% amino acid homology. Therefore, as expected both ERα and β interact with the same DNA response elements (Kuiper *et al.* 1997). However, they are only moderately conserved in their ligand-binding domain with 55% similarity (Tremblay *et al.* 1997). The ERβ product not only has only 17% homology with the ERα A/B domain, but is shorter than the ERα by 114 amino acids (599 vs 485) (Tremblay *et al.* 1997). Due to this, the ERβ does not contain a strong AF-1 domain (Hall & McDonnell 1999).

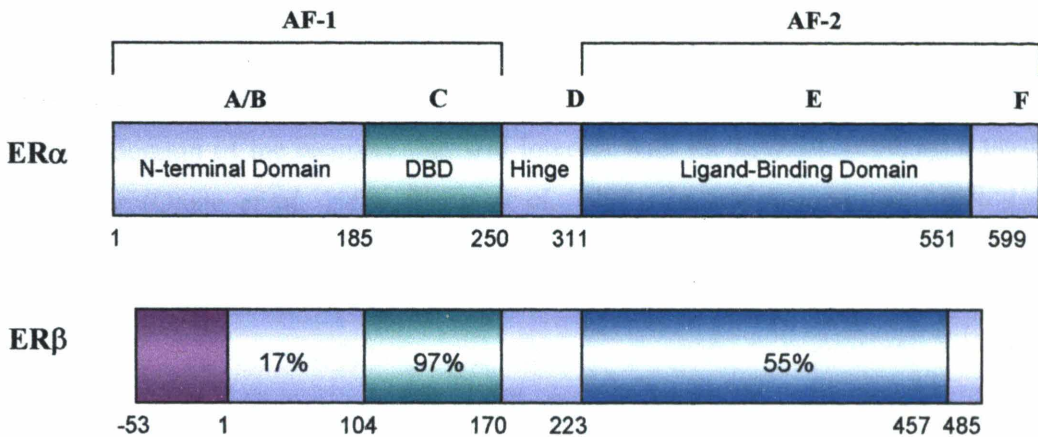


Figure 1.221 ***Comparison of the structure and percentage amino acid identity between human ERα and ERβ***

The expression of both ERα and ERβ appears to be distinct to particular tissues (for review see Couse & Korach 1999)). ERα appears to be predominantly expressed in the kidney, liver, heart, skeletal muscle, mammary gland and the reproductive tissues- vagina, uterus, and testis (**Section 1.4**). In addition to kidney and testis, ERβ is found also in the spleen, small intestine, colon, bladder, prostate, ovary, bone and lung (Couse *et al.* 1997). Differential expression is also found in the brain, with ERα localised to the pituitary, whereas ERβ is found in the hypothalamus, limbic system, cerebral cortex and cerebellum (Couse *et al.* 1997; Shughrue & Merchenthaler 2000). Therefore ERβ may function as a sole ER, or interact with ERα to alter its function within specific cells.

1.222 Activation of the ER

i Ligand dependant activation

Following binding of a specific ligand to the receptor, inhibitory complexes are removed (Pratt & Toft 1997) and the receptor undergoes a ligand induced conformational change enabling it to interact with varying co-activators and co-repressors (for review see Shibata

et al. 1997; McKenna *et al.* 1999; Katzenellenbogen *et al.* 2000). For instance, if an agonist binds (such as E2, DES), the ERs highly conserved helix 12 is repositioned over the ligand-binding pocket forming a lid (Brzozowski *et al.* 1997), exposing the AF-2 activation domain of the receptor that will interact with the LXXLL motif of a particular co-activator (Onate *et al.* 1995; Shiau *et al.* 1998). In comparison, the binding of an antagonist (such as raloxifene, 4-OH-tamoxifen), prevents the movement of helix 12 over the ligand-binding pocket (Brzozowski *et al.* 1997), thus the AF-2 domain remains inactive and co-activator recruitment fails to occur (Shiau *et al.* 1998). This particular conformation also inhibits receptor activity through the binding of specific co-repressors (for review see McKenna *et al.* 1999).

This ability for specific factors to inhibit either AF-1 or AF-2 leads to the introduction of the term SERMs (selective ER modulators) (for review see McDonnell 1999; Dutertre & Smith 2000). As both ERα and β differ in their conformational change following the binding of an agonist or antagonist, particularly with respect to helix 12, their ability to recruit specific co-activators is varied (Kraichely *et al.* 2000).

Once the ER complex has altered its conformation it is activated through one of its five phosphorylation sites (Smith 1998). It is then capable of binding to its specific oestrogen response element (ERE) in the regulatory region of target genes to transcriptionally activate the downstream gene by activating the general transcriptional apparatus (Orti *et al.* 1992). Not only can the ER bind to their consensus ERE on target genes, but there is also evidence that they can interact with other DNA bound transcription factors, such as AP-1 (Sukovich *et al.* 1994; Webb *et al.* 1995).

ERα and ERβ are not only able to form homodimers, but there is evidence suggesting that they preferentially form heterodimers (Cowley *et al.* 1997). Irrespective of what ligand is binding, both ERα and ERβ can have opposite actions in a cell, suggesting possible opposing effects in a particular cell type. It is also proposed that if both subtypes are

expressed in a cell, it is the ratio of ERα and ERβ that will determine oestrogen responsiveness (Ogawa *et al.* 1998a; Hanstein *et al.* 1999).

When the structural differences between ERα and β were analysed, it was specifically the LBD that differed substantially in its degree of homology. However, the area of the LBD specifically involved in ligand binding was quite similar, with the low homology in other areas of this domain leading to the different relatively affinities for various synthetic or naturally occurring ligands. For instance, the phytoestrogens (genistein and coumesterol) and the xenoestrogens (methoxychlor and bisphenol) bind to the ERβ with higher affinity (Kuiper *et al.* 1998). However, once bound, ERβ has significantly lower transcriptional activity when compared to ERα (Barkhem *et al.* 1998) (for review see Pettersson & Gustafsson 2001). The affinity for the natural ligand oestrogen did not differ between receptor isoforms, however ERβ required a higher concentration for maximal activity.

***ii** Alternative ligands*

It had been realised for almost 50 years that androgens can have estrogen-like effects in female reproductive organs (see Kuiper *et al.* 1997). Those originally observed to successfully compete with oestradiol for the ER included 5α-androstane-3β,17β-diol and 5-androstene-3β,17β-diol (Garcia & Rochefort 1979). Then in 1997, Kuiper *et al* studied the binding affinity of various compounds for ERα and β, confirming these original results with both 5α-androstane-3β,17β-diol and 5-androstene-3β,17β-diol binding with relative high affinity to both ERs but particularly ERβ (**Figure 1.222**). Other studies have also shown this (Boccuzzi *et al.* 1992; Le Bail *et al.* 1998; Maggiolini *et al.* 1999). In contrast, testosterone and DHT are poor activators of either ER. This brings to our attention the complexity of receptor activation (**Figure 1.222**).

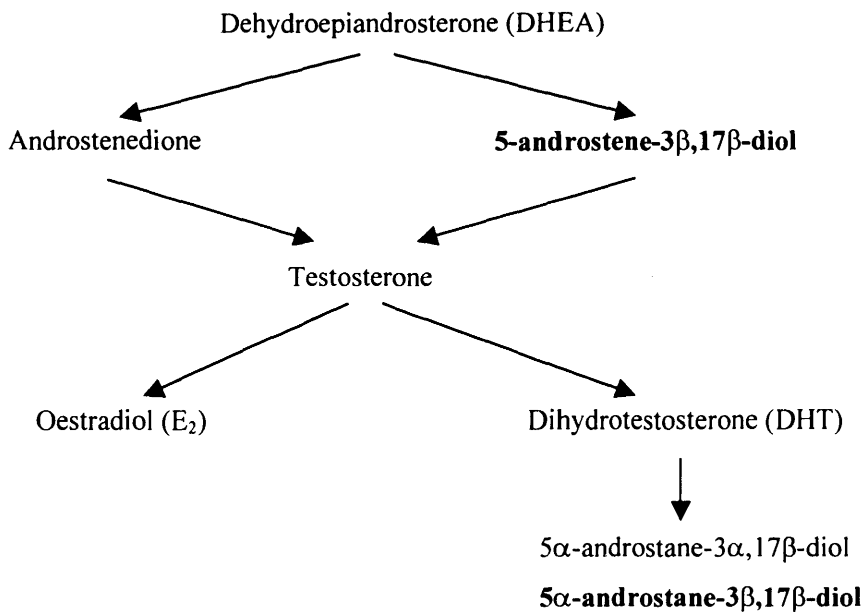


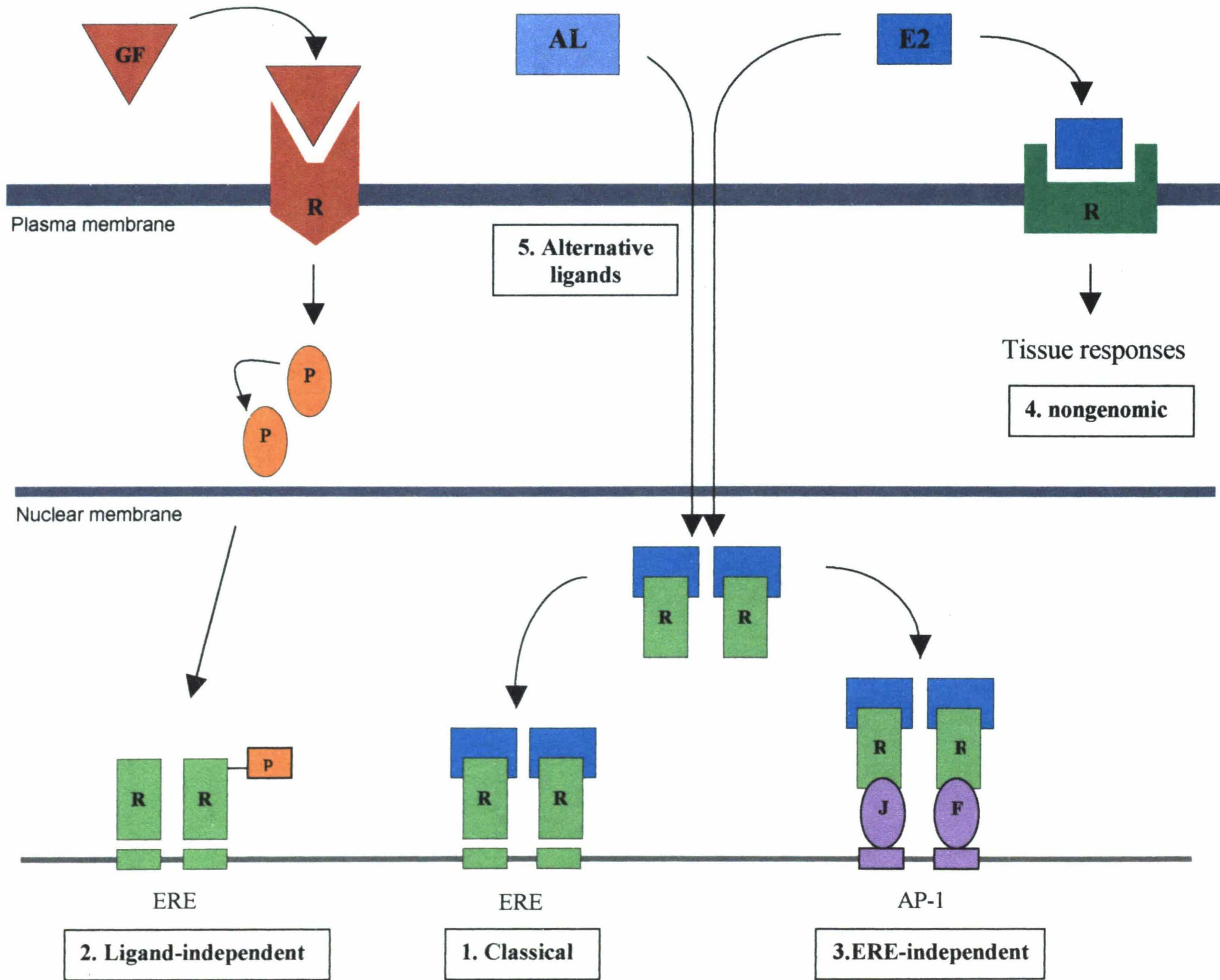
Figure 1.222 ***A simplified pathway highlighting (bold) the androgens capable of ER activation.***

***iii** Ligand independent activation*

Alternative ligand pathways also exist, whereby the receptor is activated in a ligand independent means (**Figure 1.223**) (for review see O'Malley *et al.* 1995). Growth factors, such as insulin-like growth factor (IGF) (Aronica & Katzenellenbogen 1993) and epidermal growth factor (EGF) (Ignar-Trowbridge *et al.* 1992), are able to activate a signalling cascade that leads to the downstream activation of MAPK (intracellular mitogen activated protein kinase) able to then phosphorylate and thus activate both ERs (Kato *et al.* 1998; Tremblay *et al.* 1999; Kato 2001). Other factors can also activate the ER independent of oestrogen, including forskolin (Cho & Katzenellenbogen 1993), cell cycle proteins (Neuman *et al.* 1997; Zwijsen *et al.* 1997; Trowbridge *et al.* 1997) and neurotransmitters (Power *et al.* 1991).

Figure 1.223 Illustration of the complexity of oestrogen action and ER activation within a cell.

The effects of oestradiol (E2) can be mediated in the following ways. (**1**) Firstly, by the classical ligand-dependent manner, whereby oestradiol bind to its intra-cytoplasmic receptor (ER) to activate target genes via its oestrogen response element (ERE). (**2**) Secondly, growth factors (GF) via the activation of an intracellular phosphorylation (P) signaling pathway, can phosphorylate the ER, activating it in a ligand-independent manner. (**3**) Thirdly, the ER-oestradiol complex can bind to other DNA bound transcription factors, such as AP-1, via jun (J) and fos (F), to activate target genes. (**4**) Oestradiol can also bind to its cell membrane oestrogen receptor (R), and in a non-genomic manner activate signal transduction pathways to result in specific tissue responses (ie. cAMP elevations, Ca^{2+} influxes). (**5**) And finally, alternative ligands (AL), such as androgens, can bind to and activate the ER. Adapted from Hall *et al* (2001).


GF
R
Plasma membrane
P
P
Nuclear membrane
AL
E2
5. Alternative ligands
R
R
R
Tissue responses
4. nongenomic
R
R
R
R
P
ERE
2. Ligand-independent
ERE
1. Classical
R
R
J
F
AP-1
3.ERE-independent

These alternative phosphorylation pathways initiated from the plasma membrane may have synergistic actions when oestrogen levels are low, sensitise the receptor to allow it to respond optimally to these low concentrations, or even take the role of oestrogen in the absence of hormones.

***iv** Non-genomic actions of steroids*

Almost 50 years ago steroid hormones were found to have cellular effects that could not be explained by the classical ligand-receptor interaction; including rapid effects (milliseconds to a few minutes), responses in cells incapable of gene transcription and stimulation by steroids incapable of targeting the nucleus. This mechanism of action is suggested to be non-genomic (**Figure 1.223**) (for review see Moss *et al.* 1997; Wehling 1997; Revelli *et al.* 1998; Watson & Gametchu 1999).

Oestrogen has been shown to bind to a cell membrane ERα and β with the same affinity as the nuclear receptor (Razandi *et al.* 1999). It is suggested that this membrane ER is not just similar in structure to the classic nuclear ER (Hardy & Valverde 1994; Pappas *et al.* 1995; Karthikeyan & Thampan 1996), but actually derived from the same transcript (Razandi *et al.* 1999). However, functionally disrupting the nuclear ER does not appear to affect the functioning of the membrane receptor (Gu *et al.* 1999). Once oestrogen has bound, the receptor complex activates signal transduction pathways (Razandi *et al.* 1999) to stimulate the cell via calcium influxes (Morley *et al.* 1992) and cAMP elevations (Minami *et al.* 1990). These intracellular responses have been found to be particularly important in neuronal cells involved in membrane depolarisations and neurotransmitter release, and also in non neural cells leading to calcium influx in uterine, endometrial, myometrial cells and spermatozoa cells, smooth muscle relaxation, pituitary activity and cell proliferation (Moss *et al.* 1997; Wehling 1997; Razandi *et al.* 1999). In this respect, oestradiol can activate both receptor types, the cell surface and the classical receptor, with crosstalk between this pathway and the genomic pathway believed to exist.

Nongenomic actions of oestrogens are important as they allow oestrogen to exert a physiological affect in the absence of a functional nuclear oestrogen receptor. This may be important when investigating the physiological consequences of removing functional ERs.

1.3 THE TESTIS

The testes are responsible for both the production and storage of viable spermatozoa and the manufacture and secretion of steroid hormones. The process of sperm development, or spermatogenesis, involves a coordinated series of developmental events to form a mature spermatozoon capable of fertilization from an immature spermatogonium. These maturation steps occur within the seminiferous tubules of the testis, in close communication with the supportive Sertoli cells and surrounding interstitium. Following maturation, the sperm make their transition through the rete testis and the efferent ductules, to finally reside in the epididymis until ejaculation. This section of the review will explore this process of spermatogenesis and its hormonal regulation by androgens and gonadotrophins. Oestrogen will be discussed in **Section 1.4**. (For further reviews see Weinbauer & Nieschlag 1993; Spiteri-Grech & Nieschlag 1993; Setchell *et al.* 1994a; McLachlan *et al.* 2001).

1.31 Foetal testicular development

During early foetal development, the urogenital ridge is composed of three sections; the pronephros that will form the adrenal, the metanephros that will form the kidney and the mesonephros, also known as the genital ridge, which will differentiate into the male ductal system, or Wolfiaan duct as it is later known (**Figure 1.311**) (for review see Setchell *et al.* 1994a; Capel 2000). In the mouse, the Müllerian ducts also form from an invagination of the mesenephros epithelium between day 11.5 and 12.5dpc, and if allowed to develop will form the female reproductive tract. The primordial germ cells (PGC's), originate in the

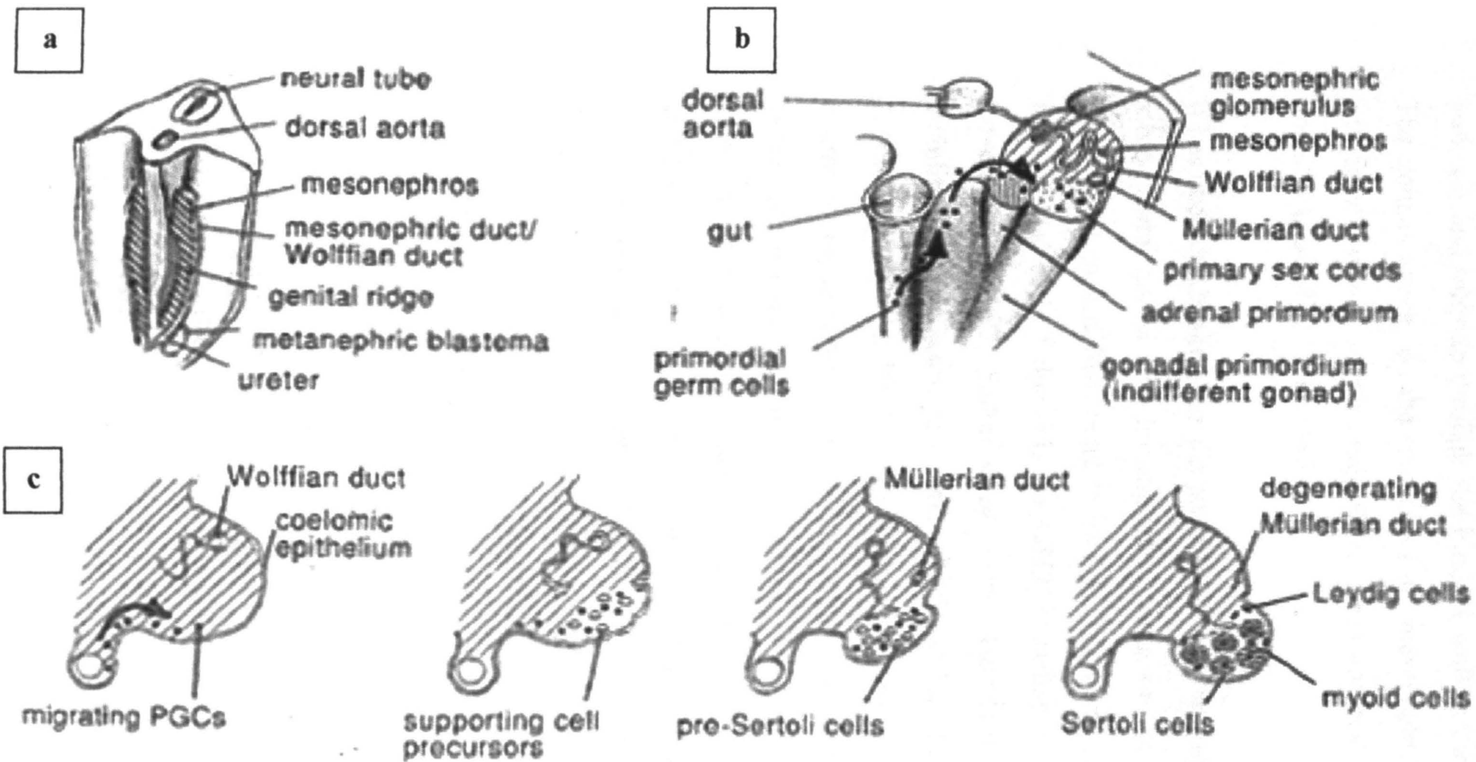


Figure 1.311 Gonadal development

(**a**) The structures that surround the urogenital ridge in the developing foetus. (**b**) The structures of the bipotential gonad, indicating the migration of the primordial germ cells from the hindgut. (**c**) The structure of the developing testis as sex determination and differentiation occur. The Mullarian duct forms by an invagination of the coelomic epithelium in the mesenephros. The supporting cells then organize into testicular cords, which contain the Sertoli cells and gonocytes. The interstitium that surrounds these cords contains the Leydig cells.

Reproduced from Parker *et al* (1999)

yolk sac and migrate through the hindgut wall to the genital ridge (Gardner *et al.* 1985). The gonads appear on the surface of the mesenephros epithelium between days 10.5 and 11.5dpc, however at this stage the ovaries and testes are indistinguishable and thus the gonad is classed as bipotential.

It has been established that the presence of a Y chromosome ultimately determines whether a bipotential gonad will develop into a testis or ovary. The specific gene responsible for testis development was found to be SRY (Sex-determining Region of Y gene), expressed in the gonad at 11.5 dpc (**Figure 1.312**) (Sinclair *et al.* 1990) (reviewed in Goodfellow & Lovell-Badge 1993; Parker *et al.* 1999). However, the exact role of SRY is currently undefined. Other factors have been identified that play crucial roles in this process (**Figure 1.312**) (Parker *et al.* 1999), such as SF-1 (Steroidogenic Factor-1) (Luo *et al.* 1994), which begins to be expressed from 9dpc in both males and females, but by 12.5dpc is only expressed in males (Ikeda *et al.* 1993). *Wt1* (Wilms' tumour suppressor gene) (Hastie 1994) which is also expressed from day 9 in the developing mouse (Pelletier *et al.* 1991) and *Sox9* (Wagner *et al.* 1994; Foster *et al.* 1994) which is highly expressed in males at day 11.5 (Morais *et al.* 1996). Recently evidence has begun to emerge that also suggests the presence of a factor able to promote ovarian development (Bardoni *et al.* 1994). This region is known as DSS (Dosage Sensitive Sex reversal) and contains such genes as DAX1 (Muscatelli *et al.* 1994).

Once these factors are expressed, the precursor Sertoli cells, which are believed to develop from the undifferentiated mesenchymal cells in the developing mesenephros, migrate into the indifferent gonad and begin proliferating, forming cell aggregates by day 11.5 in the mouse (Russell 1993c; Lovell-Badge & Hacker 1995; Hughes *et al.* 1999; Capel 2000). The germ cells, which have also migrated through the dorsal mesentery of the hindgut at 11.5 dpc, divide mitotically on arrival and are then organised into these aggregates. The Sertoli cells and germ cells are then arranged into elongated testicular cords, with the gonocytes remaining quiescent until between puberty (Vergouwen *et al.* 1991). The Sertoli

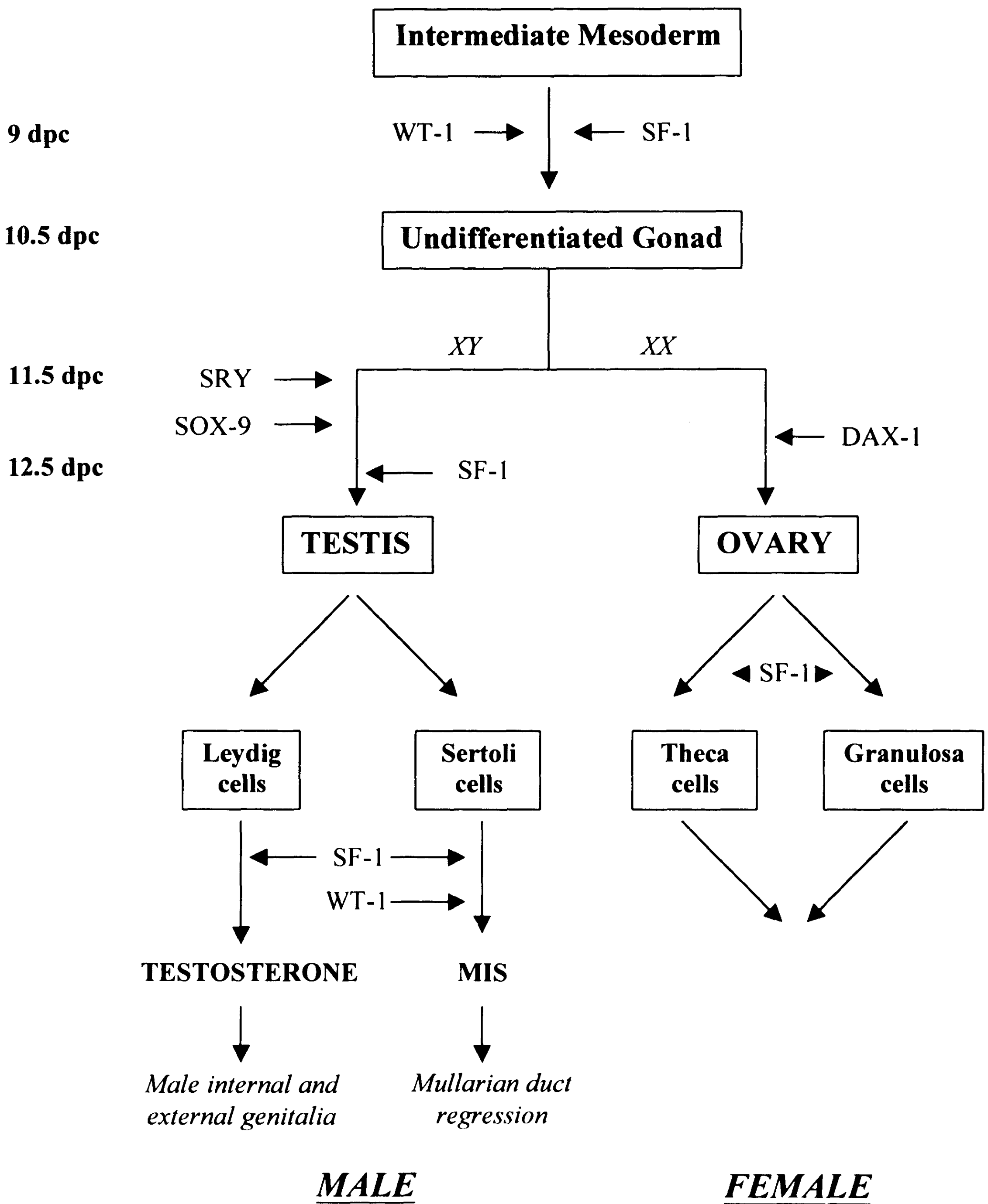


Figure 1.312 Mammalian sex determination

The series of developmental events from an undifferentiated gonad to either a testis or ovary, with the genes believed to be important for this process indicated.
Adapted from Parker *et al* (1999)

cells are crucial for gonocyte survival, with those not arranged in the cords undergoing cell death (Byskov 1986).

The Sertoli cells then have two major roles, firstly they begin to secrete anti-Müllerian hormone (MIS) to cause the regression of the Müllerian ducts, and secondly they signal the interstitial cells to differentiate into Leydig cells, to allow them to now secrete the high levels of testosterone crucial for the completion of male differentiation (Lejeune *et al.* 1998). The Leydig cells are also believed to originate from undifferentiated mesenchymal cells in the developing mesenephros (Lejeune *et al.* 1998).

After birth (day 19 in the mouse), the testis remains hormonally active as it descends from the abdomen into the scrotum. The relocation of the testes from the urogenital ridge to the inguinal abdominal wall (transabdominal descent) and the subsequent migration into the scrotum (inguinoscrotal descent) is a hormonally regulated process (Hutson & Donahoe 1986; Setchell *et al.* 1994a). It has been shown that androgens are crucial to testis migration, particularly in the development of the genital-inguinal ligament or gubernaculum that moves the testis from the internal inguinal ring to the scrotum capsule (Rajfer & Walsh 1977; van der 1992). Exogenous oestrogens administered *in utero* have also been shown to inhibit this process, increasing the incidence of intra-abdominal testes (for review see Hutson *et al.* 1997; McLachlan *et al.* 1998), however it was believed that this was an indirect oestrogenic effect via reducing LH levels and therefore androgens

In the neonatal testis, the Wolfiaan ducts will differentiate into the male associated ductal structures such as the efferent ducts, epididymis and vas deferens. Whereas the gonad is organised into two major compartments; the testicular cords, containing the immature Sertoli cells and the primordial germ cells, surrounded by the immature interstitial Leydig cells. The testes have two major functions in the mature animal, the production and storage of viable spermatozoa, and the manufacture and secretion of steroid hormones.

1.32 Spermatogenesis

The testis is surrounded by the tunica albuginea capsule and consists primarily of convoluted seminiferous tubules (**Figure 1.321**) (Berne & Levy 1993; Setchell *et al.* 1994a). The primary cell types within these tubules are the Sertoli cells and developing germ cells. Both these cells play interconnecting roles in the process of spermatogenesis, with the multifunctional epithelial Sertoli cells crucial in the coordinated maturation of immature spermatogonia through to mature elongated spermatids (**Section 1.33**) (Russell 1993a). The other major cell type in the testis are the Leydig cells, located in the surrounding interstitium and primarily responsible for the synthesis of steroids (**Section 1.34**) (Benton *et al.* 1995). Macrophages are also frequently found in the interstitium. All cells in the testis play intertwining roles in the highly coordinated maturation of the developing germ cells, which can be divided into 3 distinct phases.

1.321 Phases of spermatogenesis

i Proliferative phase

The germ cells first exist as primordial germ cells that appear as early as day 7 of gestation, populating the gonadal ridge at 11.5 dpc and then constantly proliferating until about 13.5 dpc (**Table 1.321**) (Clark J et al 1975, Eddy et al 1981, Wylie and Heasman 1993). By 19 dpc, many primordial germ cells undergo a series of coordinated cell death to reduce numbers to a level able to be supported by the Sertoli cells (**Section 1.532**). The surviving pro-spermatogonia resume proliferating 6 days after birth and are now known as undifferentiated type A spermatogonia (McCarrey 1993).

Type A spermatogonia can be grouped into three major groups; the renewing stem cells consisting of A isolated (A_{is}); undifferentiated consisting of A paired (A_{pr}) and A aligned (A_{al}); and differentiating consisting of A_1 to A_4 (Clermont 1972). Germ cells develop in a synchronous manner due to their cellular connections through cytoplasmic bridges, resulting from incomplete cytokinesis. By day 8, type A_4 spermatogonia undergo 2 mitotic steps to form an intermediate spermatogonium (In) and then a type B spermatogonium

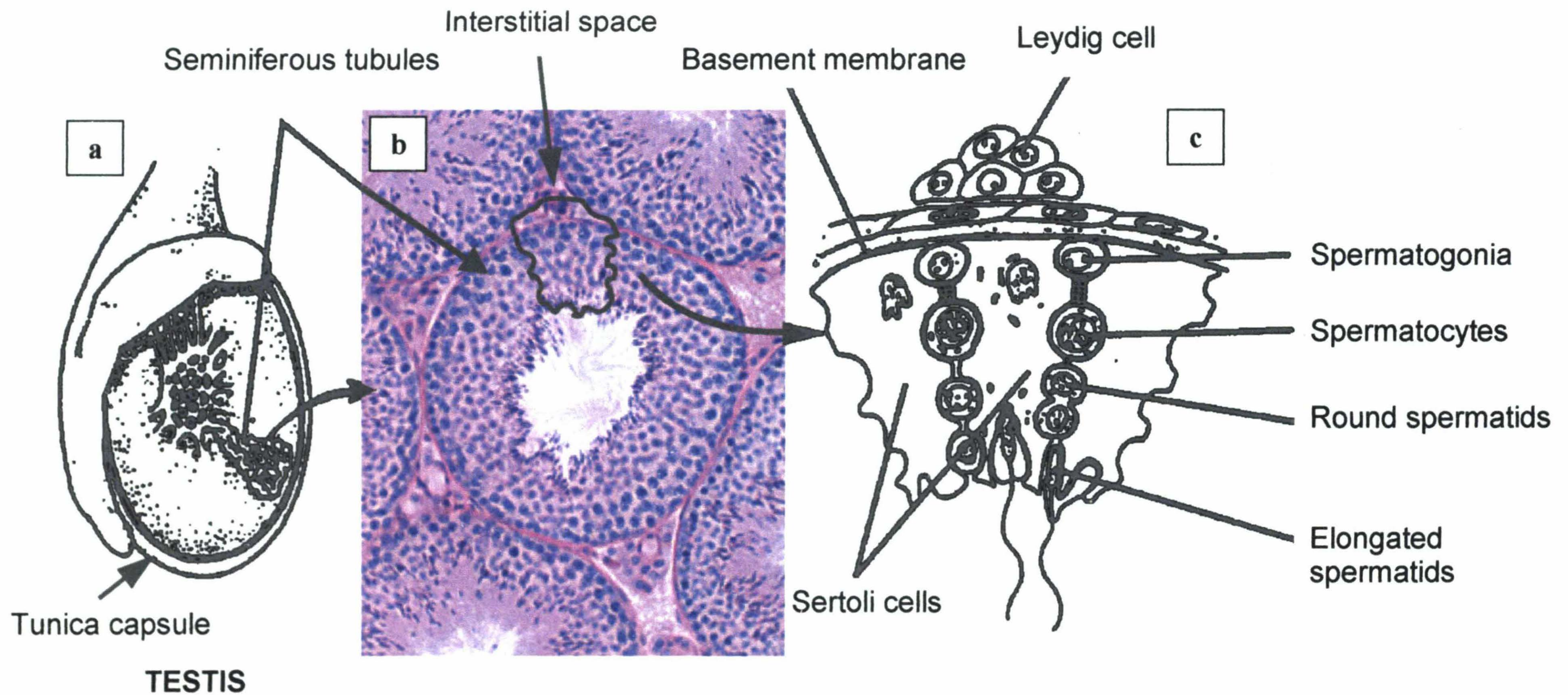


Figure 1.321 The structure of the testis

(**a**) The testis is encompassed within a capsule called the tunica albuginea. (**b**) It is comprised of convoluted tubules, surrounded by interstitial Leydig cells responsible for synthesising testosterone from an LH stimulus. (**c**) Within these seminiferous tubules, the Sertoli cells support the development of germ cells from immature spermatogonia through to an elongated spermatid.

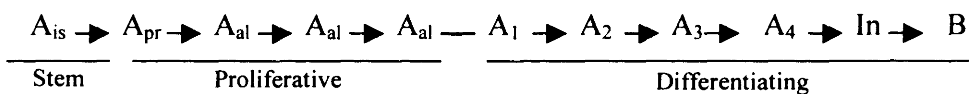
Modified from Berne and Levy (1993)

Table 1.321 The time when each germ cell type appears during pre-natal and post-natal (highlighted) development in the mouse

	Cell type	Time of first appearance
Pre-germ cells	*Zygote*	*1 dpc*
	Morula	*2 dpc*
	Inner cell mass	*3 dpc*
	Primitive ectoderm	*4 dpc*
	Epiblast	*6 dpc*
Primordial germ cells	*PGCs*	*7 dpc*
Prespermatogenesis	*M-Prospermatogonia*	*12 dpc*
	T_1-Prospermatogonia	*14 dpc*
	T2- Prospermatogonia	1 dpp
Undifferentiated spermatogonia	A_{is}-spermatogonia	6 dpp
	A_{pr}-spermatogonia	6 dpp
	A_{al}-spermatogonia	6 dpp
Differentiating spermatogonia	A_{1-4}-spermatogonia	8 dpp
	In-spermatogonia	8 dpp
	B-spermatogonia	8 dpp
Meiosis Primary spermatocytes	Pre-leptotene	10 dpp
	Leptotene	10 dpp
	Zygotene	12 dpp
	Pachytene	14 dpp
	Diplotene	17-18 dpp
	Dictyate	17-18 dpp
Meiosis II	Secondary spermatocytes	18 dpp
Spermiogenesis	Round spermatids	20 dpp
	Condensing spermatids	30 dpp
	Spermatozoa	35 dpp

Reproduced from McCarrey (1993)

(**Table 1.321**). Each spermatogonium is classified by their level of chromatin, increasing from type A to B. These large immature germ cells lie flattened against the basement membrane, until day 10 when the majority of B spermatogonia move away from the basement membrane and enter a period of meiosis to form a pre-leptotene spermatocyte (Russell *et al.* 1990). This series of mitoses can be summarised as follows, with each arrow indicating a division:



ii Meiotic phase

The first stage of meiosis in the mouse takes approximately 8 days (McCarrey 1993), with prophase consisting of the pre-leptotene spermatocyte transforming initially into a leptotene spermatocyte (Russell *et al.* 1990). Then pairing of the homologous chromosomes occurs and the resulting zygotene spermatocytes form pachytene spermatocytes, in which the chromosomes are completely paired. This is the longest stage, following which the chromosomes separate forming a diplotene spermatocyte. Each spermatocyte can be classified according to their morphological appearance, as their chromatin begins to condense, and position within the epithelium as they move towards the lumen (Hess 1990). At day 18 the primary pachytene spermatocyte undergoes its first meiotic division into two smaller secondary spermatocytes, reducing the volume of the germ cell by approximately 30-40%. These spermatocytes contain two sets of haploid chromosomes, thus then undergo a brief second meiotic division to divide for the final time to form the truly haploid germ cell at day 20. Therefore one spermatocyte gives rise to 4 round spermatids (Russell *et al.* 1990).

iii Spermiogenic phase

The final stage of spermatogenesis occurs in 16 distinct steps in the mouse and involves the differentiation of the round spermatid into an elongated spermatid with no further

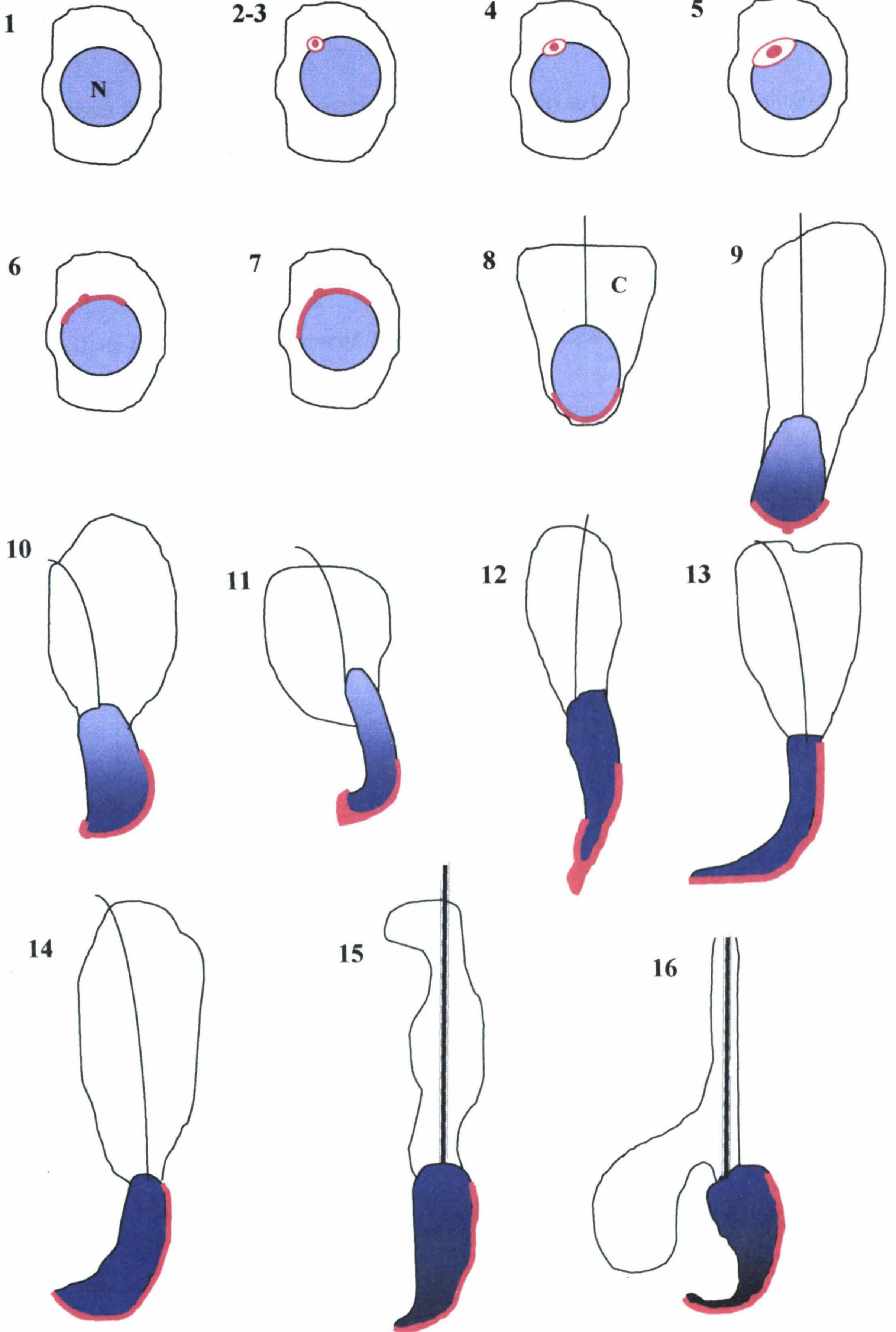
divisions. Each step is identified by the progressive development of the acrosome (**Figure 1.322**) (Russell *et al.* 1990).

Step 1 spermatids, which are newly formed from secondary spermatocytes, are clearly characterised by their reduction in nuclear volume. The Golgi apparatus then produces pro-acrosomal granules contained in pro-acrosomal vesicles. These will join to form the acrosome, which contains various substances including hyaluronidase enzyme required for penetration through the cumulus cells of the oocyte at fertilization. The pro-acrosomic vesicles can be visualised on the nucleus at step 2 (**Figure 1.322**). By step 3 these granules have fused into one, and begin to flatten and spread out over the nucleus from step 4 to 6. By step 6, the acrosome covers approximately one third of the nucleus head, increasing to half by step 7. By step 8 the round spermatid has orientated itself so that the nucleus is polarised to one side of the cytoplasm (**Figure 1.322**), and the acrosome is facing towards the basement membrane. The flagellum is visible at this stage and the process of elongation has begun. The round spermatid is now known as an elongating spermatid. At step 9 the nucleus is clearly beginning to elongate and the nuclear material is undergoing condensation (**Figure 1.322**).

As the spermatid makes its way from step 10 to stage 16 the nuclear head narrows and further elongates as the chromatin becomes tightly packed. The flagellum fully develops and there is a loss of cytoplasm. The elongating spermatid is embedded deep within the Sertoli cell and as it matures is translocated firstly towards the basement membrane and then back towards the lumen. This movement is believed to be facilitated by microtubules (Vogl *et al.* 2000). Then at step 16 the mature elongated spermatid, in a process known as spermiation, is released from the epithelium into the lumen of the tubule, leaving behind its cytoplasm in the form of the residual body which is consequently phagocytosed by the Sertoli cell (Russell 1993b). A small droplet of cytoplasm remains attached to the base of the head of the spermatozoa, persisting here as the sperm travel through the rete testis and efferent ducts into the epididymis. The cytoplasmic droplet is then lost as the sperm traverse the epididymis (Hess 2000). As the spermatozoa completes this journey it

Figure 1.322 Schematic representation of spermiogenesis, illustrating the 16 steps of spermatid development

Each of the first 7 steps of spermatid maturation are defined by the spread of the acrosome on the round spermatid nucleus (N). Step 1 spermatids are smaller than secondary spermatocytes and have no visible acrosome. The pro-acrosomic vesicle is first observed at step 2-3 (highlighted in pink). The acrosome then begins to flatten and spread out over the nucleus from step 4 (approximately 40°) to step 5 (95°), to step 6 (120°) round spermatids. By step 7 the acrosome is covering half of the nucleus (150°). Once step 1 to 7 are staged, the other cell types can be identified as appearing in each of these stages (**Figure 1.323**). Alternatively step 8 to 16 elongating spermatids can be identified by the shape of the nucleus and their position in the cell. For example, step 8 spermatids have a nucleus that has moved to the base of the cytoplasm (C) with the acrosome facing towards the basement membrane. By step 9 the nucleus has begun to elongate and at step 10 a ventral angle is formed. This continues to develop at step 11 with also a dorsal angle forming. Step 12 is most clearly defined by the presence of secondary spermatocytes, with the spermatid nucleus continuing to elongate and the chromatin condense from step 13 to 16.
Adapted from Russell *et al* (1990)



1
N
2-3
4
5
6
7
8
C
9
10
11
12
13
14
15
16

undergoes further maturation changes, acquiring the ability to move forward (motility) and fertilize oocytes by the time it reaches the cauda epididymis (Setchell *et al.* 1994a).

The estimated time for the germ cell to pass through each stage of spermatogenesis (Oakberg 1956) is summarised in **Table 1.322.**

Table 1.322 Estimation of the time to complete each stage in spermatogenesis

STAGE	DURATION (HOURS)
Spermatogonia	
Type A	Always present
Intermediate	27.3
Type B	29.4
Primary spermatocytes	
Pre-leptotene	31.0
Leptotene	31.2
Zygotene	37.5
Pachytene	175.3
Diplotene	21.4
Diakenesis + metaphase	10.4
Secondary spermatocytes	10.4
Spermatids	229.2

Adapted from Oakberg (1956)

1.322 Spermatogenic cycle

Spermatogenesis is a highly coordinated series of maturation events that in the mouse occurs in 12 highly structured stages (I-XII) (Russell *et al.* 1990). Each stage represents

one cell association depicted in any one cross section of the seminiferous epithelium (**Figure 1.323**). The cells along the bottom are the spermatogonia and immature spermatocytes that lie against the basement membrane. Type A spermatogonia, not depicted in the Figure, are found at all 12 stages. Whereas the cells at the top are those found closest to the lumen. For instance, at stage I the cells which are observed are type A spermatogonia, pachytene spermatocytes, step 1 round spermatids and step 13 elongated spermatids. Whereas it is at stage XII that zygotene and secondary spermatocytes are present, alongside step 12 elongated spermatids. The development of type A spermatogonia to a step 16 elongated spermatid occurs over 4 and a half cycles, taking aproximately 5 weeks.

1.33 Sertoli cells

The somatic Sertoli cells form the structure of the seminiferous tubules and reside on a basement membrane comprised of peritubular myoid cells (**Figure 1.331**). These cells have numerous functions that pertain to the successful completion of spermatogenesis (for review see; de Kretser & Kerr 1988; Bardin *et al.* 1988; Jegou 1992; Russell 1993a).

1.331 Development and function

The pre-Sertoli cells are the first cell type to differentiate in the developing gonad from the mesenephros (**Section 1.31**) (Russell 1993c). In the rat, Sertoli cells undergo a series of proliferations from day 16 pc until 2 days prior to birth (Orth 1982). At birth, the seminiferous cords are comprised almost entirely of Sertoli cells that number approximately 1 million, with the few gonocytes localized centrally (Wang *et al.* 1989). The Sertoli cells then rapidly proliferate again in the first 10 days after birth in the rat under the stimulus of FSH, with FSH receptors appearing on the Sertoli cells at this time (Almiron & Chemes 1988). At day 15 they number approximately 38 million, with this number remaining relatively stable throughout life, and now begin to differentiate (Wang *et al.* 1989). As Sertoli cells can only support a certain number of germ cells, the immature germ cells undergo apoptosis to reduce their number to a level that the Sertoli cells are

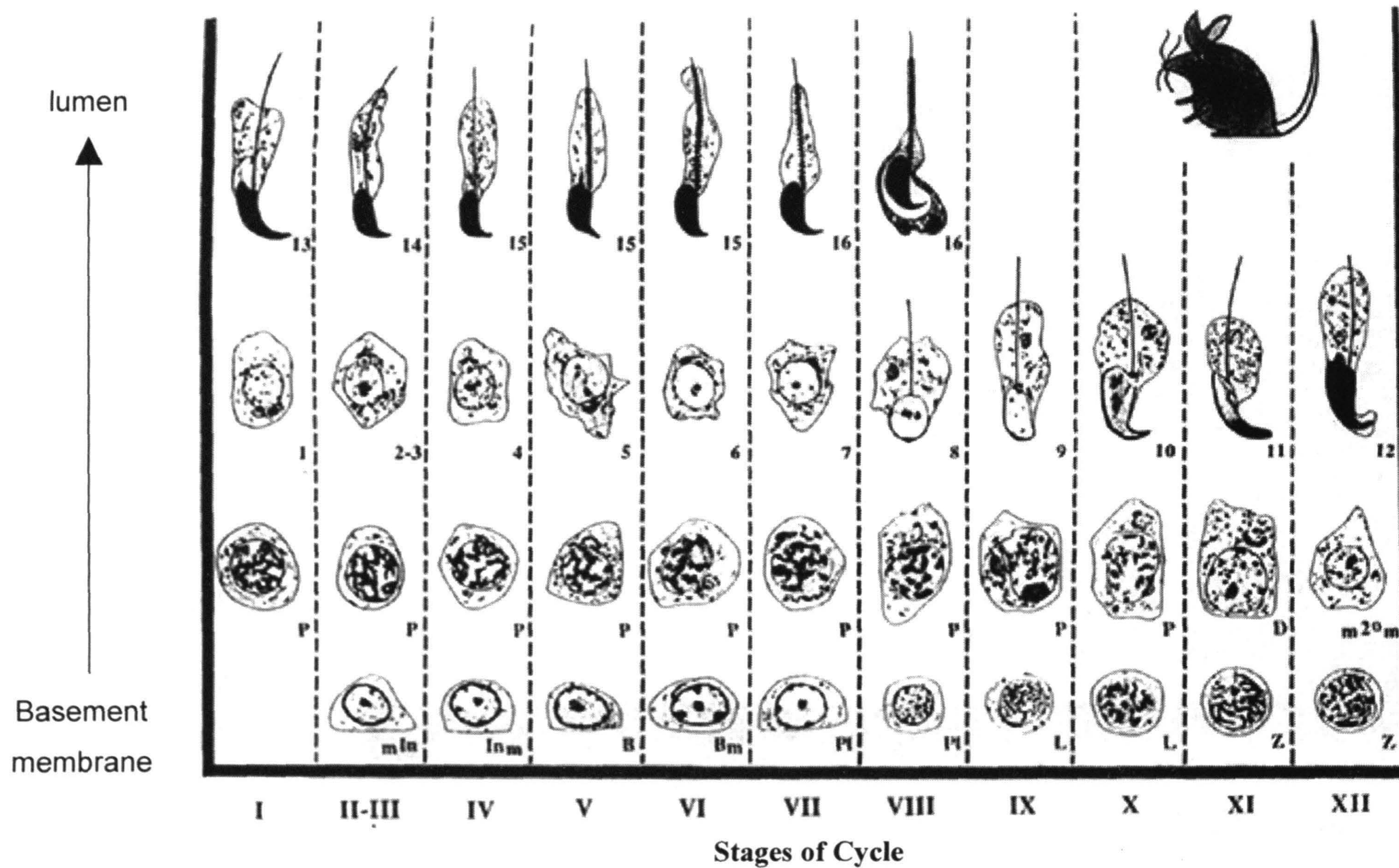


Figure 1.323 Spermatogenic cycle of the mouse

The spermatogenic cycle in the mouse contains 12 stages from I to XII. Each stage represents one cell association depicted in any one cross section of the seminiferous epithelium. The cells along the bottom are the cells that lie against the basement membrane, such as spermatogonia (In-B) and immature spermatocytes (pre-leptotene (Pl), leptotene (L) and zygotene (Z)). As the cell mature they transition towards the lumen, from pachytene (P) spermatocyte to round spermatid (step 1-8) to elongating spermatid (step 9-16). Reproduced from Russell *et al* (1990)

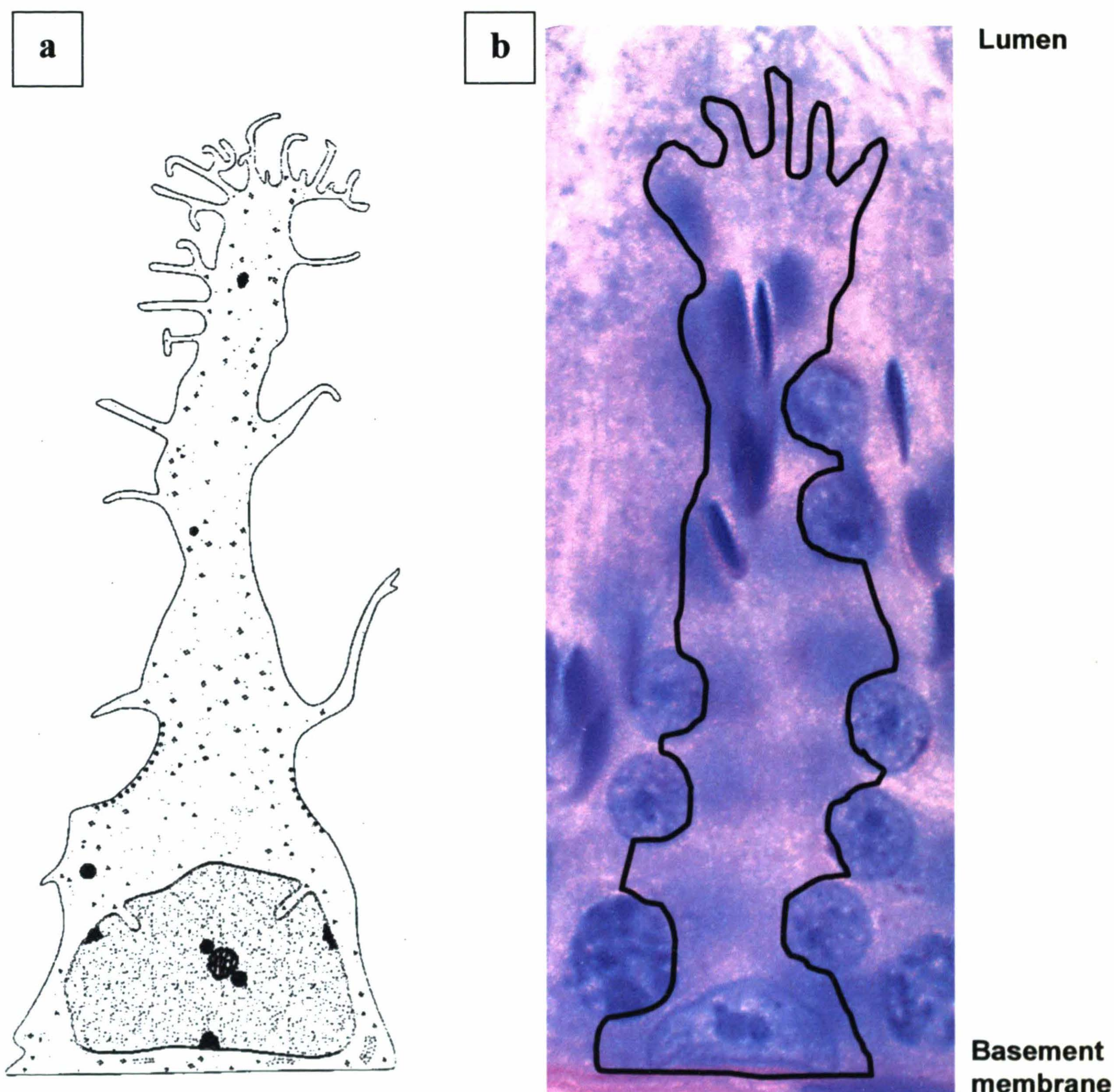


Figure 1.331 Sertoli cell structure

The Sertoli cells (**a**) depicted schematically and (**b**) in the seminiferous epithelium. They extend from the basement membrane to the lumen and are clearly characterised by their tall columnar structure, extension of cytoplasmic fingers and large cytoplasmic volume. Their nuclei are distinct in that they are irregularly shaped and contain a tripartite nucleolus. Adapted from Russell *et al* (1993)

capable of supporting (**Section 1.532**). Therefore, the relationship between the germ cells and Sertoli cell is intimate, with the number of mature sperm reliant on the number of Sertoli cells (Orth *et al.* 1988).

Once proliferation ceases, the lumen appears at day 15 in response to the formation of Sertoli cell junctional complexes (Vitale *et al.* 1973). These tight junctions divide the seminiferous epithelium into a basal compartment, which houses the immature spermatogonia and early spermatocytes, and an adluminal compartment, in which the more mature germ cells reside (**Figure 1.332**) (Hudson & Burger 1979; Jegou 1992)). This junction forms part of the blood testis barrier which allows the seminiferous epithelium to regulate the entry of certain factors from the circulation (de Kretser & Kerr 1988). In this respect, the environment surrounding the germ cells from the early spermatocyte stage onwards is tightly controlled by the Sertoli cells themselves. The early spermatocytes move through this junction into the apical compartment. This occurs by the junctional complexes breaking down and reforming behind the germ cell, thus retaining the integrity of the blood testis barrier (**Figure 1.333**) (Jegou 1992). The development of these junctional complexes may be independent of hormonal action (Chemes *et al.* 1979) and the presence of germ cells (Jegou 1992). The Sertoli cells are responsible for the movement of germ cells from the basement membrane to the lumen as they mature. Spermiation, or release of the mature spermatid, appears to be also facilitated by the Sertoli cell itself (Russell 1993a).

Once the blood testis barrier has been established, the immature Sertoli cells undergo a series of morphological transformations, such as increasing their size, increasing their cytoplasmic connections to germ cells, and various intracellular alterations (de Kretser & Kerr 1988), until maturity is reached at day 35. The cells extend from the basement membrane to the lumen and are clearly characterised by their tall columnar structure, extension of cytoplasmic fingers and large cytoplasmic volume (**Figure 1.331**). Their nuclei are distinct in that they are irregularly shaped and contain a tripartite nucleolus. The nucleus and many organelles are situated close to the basement membrane, with

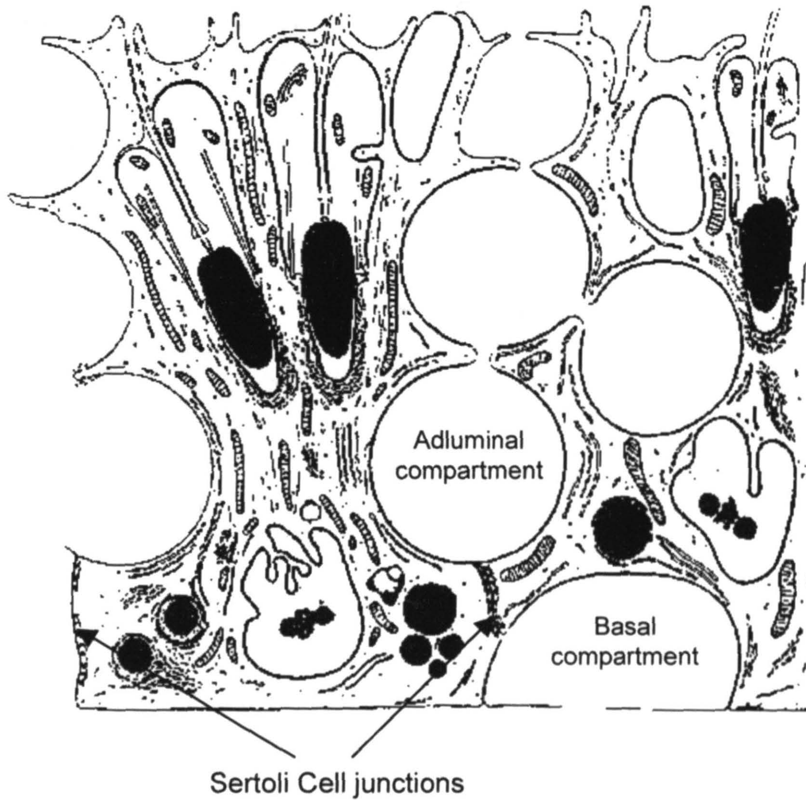


Figure 1.332 Schematic diagram illustrating the location of the Sertoli cell junctions

Tight junctions divide the seminiferous epithelium into a basal compartment, which houses the immature spermatogonia and early spermatocytes, and an adluminal compartment, in which the more mature germ cells reside. This junction forms part of the blood testis barrier which allows the seminiferous epithelium to regulate the entry of certain factors from the circulation.

Reproduced from Hudson and Burger (1979)

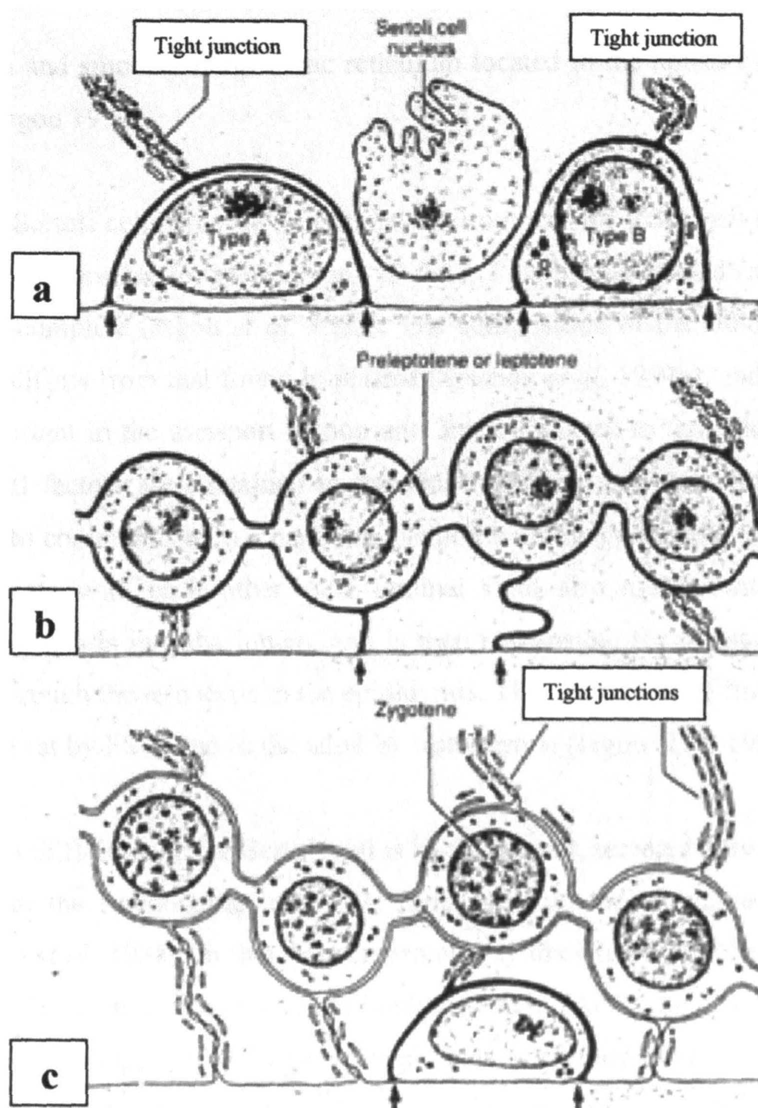


Figure 1.333 Schematic diagram illustrating the formation of the Sertoli cell junctions

(**a**) The spermatogonia lie on the basement membrane. (**b**) As they mature and move upward from the basal compartment, Sertoli cell tight junctions reform beneath the cell, forming the apical compartment. (**c**) The spermatocytes are now connected by cytoplasmic bridges.

Reproduced from Jegou (1992)

mitochondria and smooth endoplasmic reticulum located in the apical cytoplasm towards the lumen (Jegou 1992).

Not only do Sertoli cells provide an optimal environment for germ cell development, but they are also responsible for the secretion of fluid. This begins around day 20 after lumen formation is complete (Jegou *et al.* 1982). The composition of the fluid produced in the Sertoli cells differs from that found in plasma (Setchell *et al.* 1994a), and fluid production appears important in the transport of nutrients from the basal to the apical regions of the cell. Essential factors are contained in this fluid for germ cell maturation, allowing the Sertoli cells to communicate in a paracrine fashion with the germ cells, and the germ cells to communicate with each other. The luminal fluid also assists with the release of elongated spermatids into the lumen, and is then responsible for transporting the mature spermatids through the rete testis to the epididymis. The production of fluid is controlled in the immature rat by FSH, and in the adult by testosterone (Jegou *et al.* 1982).

The secretion of fluid from the Sertoli cell is bi-directional, secreted either into the luminal space, or into the surrounding interstitial fluid and into the circulation (approximately 20%) (Bardin *et al.* 1988). In this respect, proteins synthesised by the Sertoli cells can act in a paracrine fashion such as androgen binding protein (ABP), believed to play a role in the intracellular transport of androgens. Or proteins can be secreted into the circulation to act in an endocrine fashion, such as inhibin, to negatively regulate FSH production at the level of the pituitary (Bardin *et al.* 1988).

1.34 Leydig cells

The interstitium surrounds the seminiferous tubules and encompasses approximately 1/6 of the testis volume (Mori & Christensen 1980). It consists of macrophages, blood vessels and peritubular myoid cells, however the most prominent cell type are the Leydig cells, important primarily for testosterone biosynthesis (**Figure 1.341**).

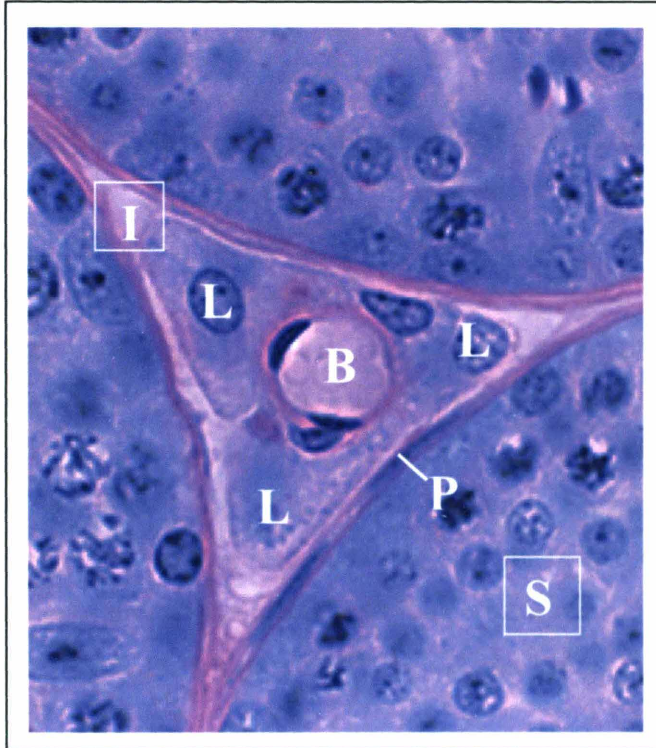


Figure 1.341 The structures within the interstitial space

The interstitial space (**I**) surrounds the seminiferous tubules (**S**) in the testis, and contains Leydig cell nuclei (**L**) surrounded by cytoplasm, blood vessels (**B**), peritubular myoid cells (**P**) and macrophages (not shown).

1.341 Morphological development

Leydig cells undergo 2 major maturational stages of development before they reach full maturity (for review see Benton *et al.* 1995; Ge *et al.* 1996; Lejeune *et al.* 1998).

i Foetal

Due to the presence of testosterone, precursor Leydig cells which have developed from mesenchymal-like stem cells are already present in the gonad as early as 11.5dpc (Merchant-Larios *et al.* 1993; Benton *et al.* 1995), and almost immediately differentiate into foetal-type Leydig cells (Byskov 1986). This occurs under the influence of factors such as IGF-1 (Rouiller-Fabre *et al.* 1998). The primary function of these Leydig cells is to synthesise androgens required for differentiation of the Wolfiaan ducts into the male gonads. Without these androgens, the Wolfiaan duct will regress and the Mullarian duct will differentiate, forming female reproductive structures. Foetal Leydig cells therefore play a crucial role in male development. However once the male urogenital tract is formed, the foetal Leydig cells are believed to regress (Habert & Picon 1982; Habert *et al.* 1992; Habert 1993), possibly by the assistance of TGFβ (Gautier *et al.* 1994).

ii Postnatal

During the neonatal period, another population of Leydig cells emerge (**Figure 1.342**). These progenitor Leydig cells are not derived from the foetal Leydig cell population, but from peritubular 'mesenchymal-like' cells, suggesting a similar origin to foetal Leydig cells (Hardy *et al.* 1989; Vergouwen *et al.* 1991). It was believed that foetal Leydig cells remained in the postnatal testis, but only numbered 0.008% of the adult, therefore were postulated not to play a significant role in androgen production (Benton *et al.* 1995). However a recent study has found that they are the primary testosterone synthesising cell until day 7 of life (Ariyaratne & Chamindrani Mendis-Handagama 2000). They remain in the testis in the adult, producing testosterone for the continued development of the male reproductive system, however compared to adult-type Leydig cells are not rapidly proliferating (Ariyaratne & Chamindrani Mendis-Handagama 2000). During postnatal

Proliferation

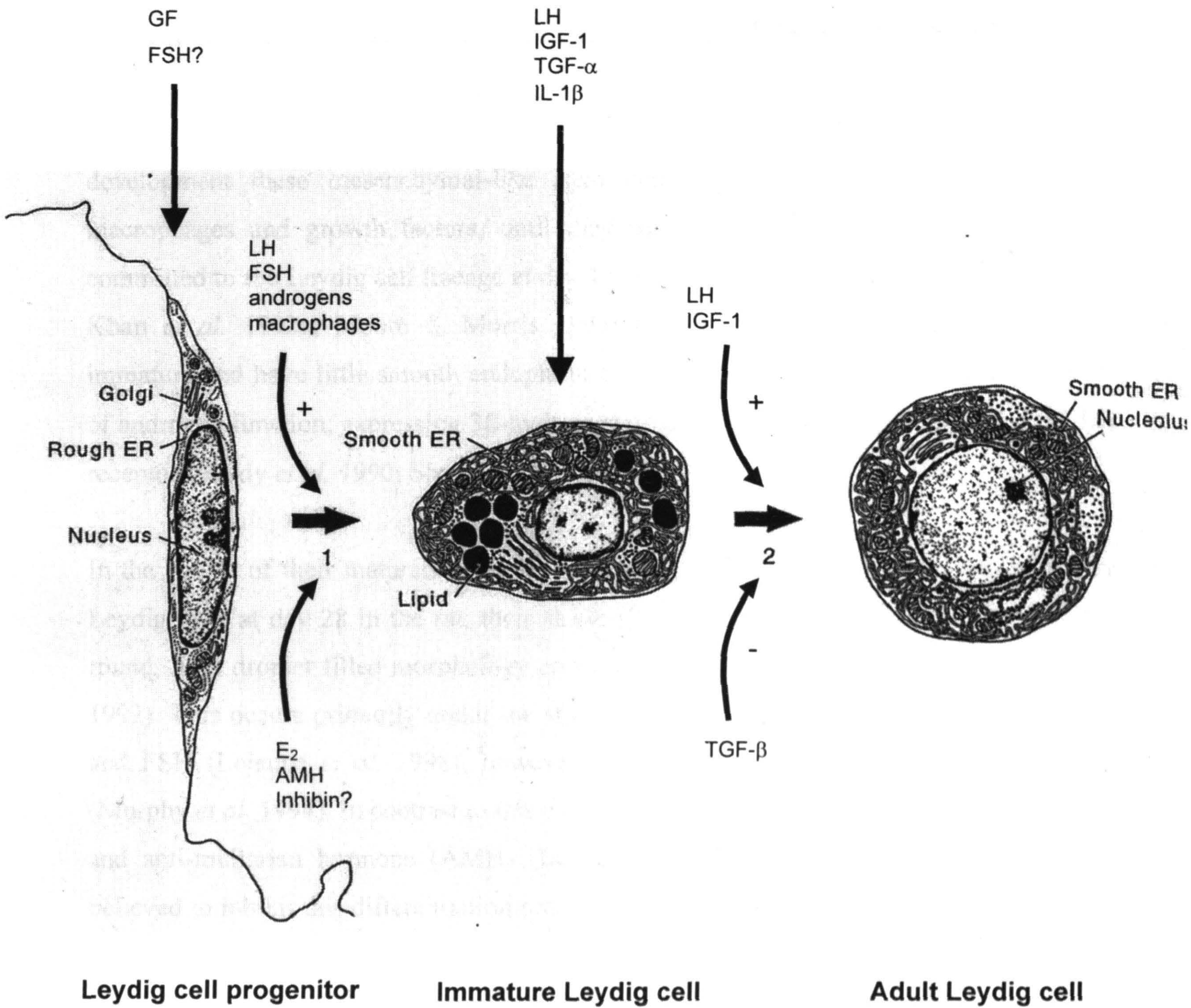


Figure 1.342 Model for the proliferation and differentation of Leydig cells

Proliferation of progenitor Leydig cells occurs through stimulation by growth factors (GF) and possibly FSH. These cells then differentiate into immature Leydig cells primarily under the influence of LH, but also by FSH, androgens and macrophages. This process can be inhibited by oestradiol and anti-mullerian hormone (AMH). Immature Leydig cells then undergo a single division to form an adult cell, stimulated again by LH, also by IGF-1, TGF-α and IL-1β. This final differentiation can be inhibited by TGFβ.

Adapted from Benton *et al* (1995) and Lejeune *et al* (1998)

development these mesenchymal-like stem cells proliferate under a stimulus from macrophages and growth factors, until they differentiate into precursor Leydig cells, committed to the Leydig cell lineage at day 14 (Hardy *et al.* 1989; Vergouwen *et al.* 1991; Khan *et al.* 1992a; Moore & Morris 1993; Gaytan *et al.* 1994). Although they are immature and have little smooth endoplasmic reticulum, these precursor cells are capable of androgen function, expressing 3β-hydroxysteroid dehydrogenase (3β-HSD) and the LH receptor (Hardy *et al.* 1990; Shan & Hardy 1992).

In the course of their maturation, precursor Leydig cells then differentiate into immature Leydig cells at day 28 in the rat, their shape altering from the spindle shape to form the round, lipid droplet filled morphology consistent with adult Leydig cells (Shan & Hardy 1992). This occurs primarily under the stimulus of LH (Teerds *et al.* 1989; Teerds 1996) and FSH (Lejeune *et al.* 1998), however androgens are also believed to be important (Murphy *et al.* 1994). In contrast to this oestrogens (see **Section 1.4** for further discussion), and anti-mullarian hormone (AMH) (Behringer *et al.* 1994; Mishina *et al.* 1996) are believed to inhibit this differentiation process. The immature Leydig cells with their larger smooth endoplasmic reticulum are increasingly steroidogenically active, however due to their high level of 5α-reductase, primarily produce 5α-reduced androgens such as androstane-3α,17β-diol (Adiol) and 3α-hydroxysteroid dehydrogenase (3α-HSD) (Murono 1989; Shan *et al.* 1993).

After a final single division by day 56 in the adult rat (age of sexual maturity), stimulated by LH and growth factors such as IGF-1, TGF-α and IL-1β (Khan *et al.* 1992a; Khan *et al.* 1992b; Baker *et al.* 1996), and inhibited by TGF-β, the immature Leydig cells finally form mature Leydig cells. This final division to form mature cells occurs alongside morphological transitions, such as increases in cell size, LH receptor, and the steroidogenic secreting capabilities of the cell, involving the enzymes 3β-HSD, P450scc, P450c17 and 17β-HSD (Shan *et al.* 1993). In the adult, the activity of 5α-reductase and 3α-HSD decline, with the primary androgen secreted in the adult now being testosterone (Shan *et*

al. 1993; Viger & Robaire 1995). Adult Leydig cells are responsible for secreting the testosterone required for the onset of spermatogenesis and for maintaining reproductive function (de Kretser & Kerr 1988). Once the Leydig cells have reached adulthood they rarely proliferate, if the population is destroyed they instead self-renew from the mesenchymal stem cell population which have persisted in the adult (Hardy *et al.* 1989; Moore *et al.* 1992).

1.342 Steroidogenesis

The Leydig cells are believed to be the primary cell in the testis capable of synthesising testosterone from the cholesterol substrate (Cooke *et al.* 1972) (for review see Saez 1994; Payne & Youngblood 1995). This process is dependant on LH, released from the pituitary under the stimulus of the hypothalamic gonadotrophin-releasing hormone (GnRH) (**Figure 1.343**). LH binds to its receptor on the surface of Leydig cells (**Figure 1.344**), activating the receptor and causing an increase in intracellular cAMP, via adenylate cyclase (for review see Dufau 1988; Cooke *et al.* 1992; Huhtaniemi & Toppari 1995). This leads to free cholesterol being transported to the inner mitochondrial membrane via the steroidogenic acute regulatory (STAR) protein (Clark *et al.* 1994; Stocco & Clark 1996). Cholesterol precursors are derived from the blood in the form of circulating lipoproteins; from stores of free cholesterol inside the Leydig cell itself; or synthesised *de novo* from acetate (Payne & Youngblood 1995).

The rate-limiting step in this process is the cholesterol side chain cleavage (P450scc) enzyme, present only in the mitochondria of the Leydig cells (Payne 1990). Once this enzyme has converted C_{27} cholesterol to C_{21} pregnenolone, pregnenolone then diffuses back across the mitochondrial membrane to the smooth endoplasmic reticulum, where the enzymes are present for further modification (Payne & Sha 1991; Greco & Payne 1994). The first step in the formation of testosterone is the conversion of pregnenolone to progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD), again this enzyme is only found in the Leydig cells (Dupont *et al.* 1990; Pelletier *et al.* 1992), suggesting that this is

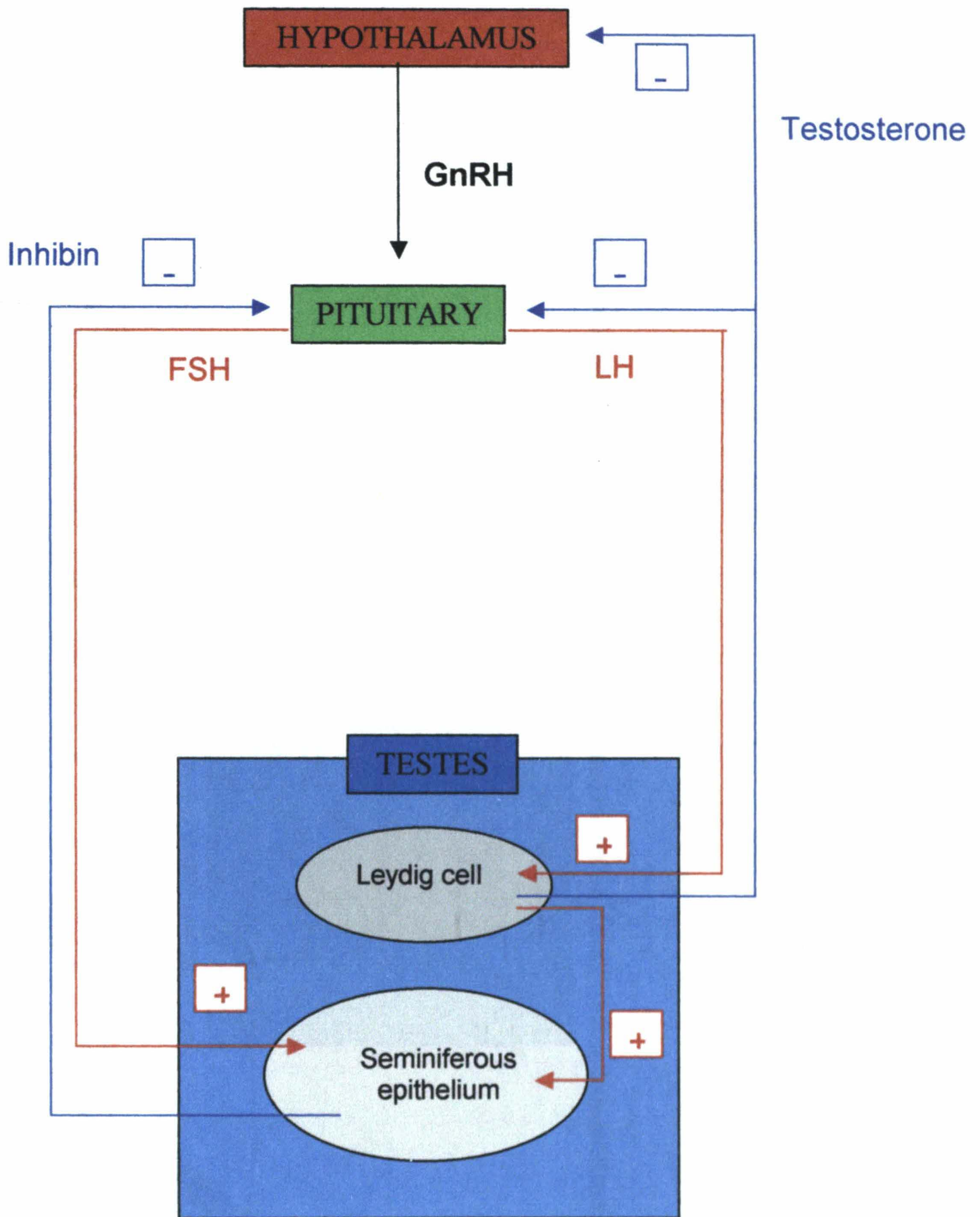


Figure 1.343 The Hypothalamus-Pituitary-Gonadal (HPG) axis

LH and FSH are released from the pituitary under the control of GnRH, and act on the Leydig cells and Sertoli cells, respectively. Testosterone, synthesised under the stimulus of LH, is able to act in a negative feedback manner at both the pituitary and hypothalamus to inhibit LH secretion. Whereas, the Sertoli cells release the peptide inhibin to inhibit FSH secretion at the pituitary.

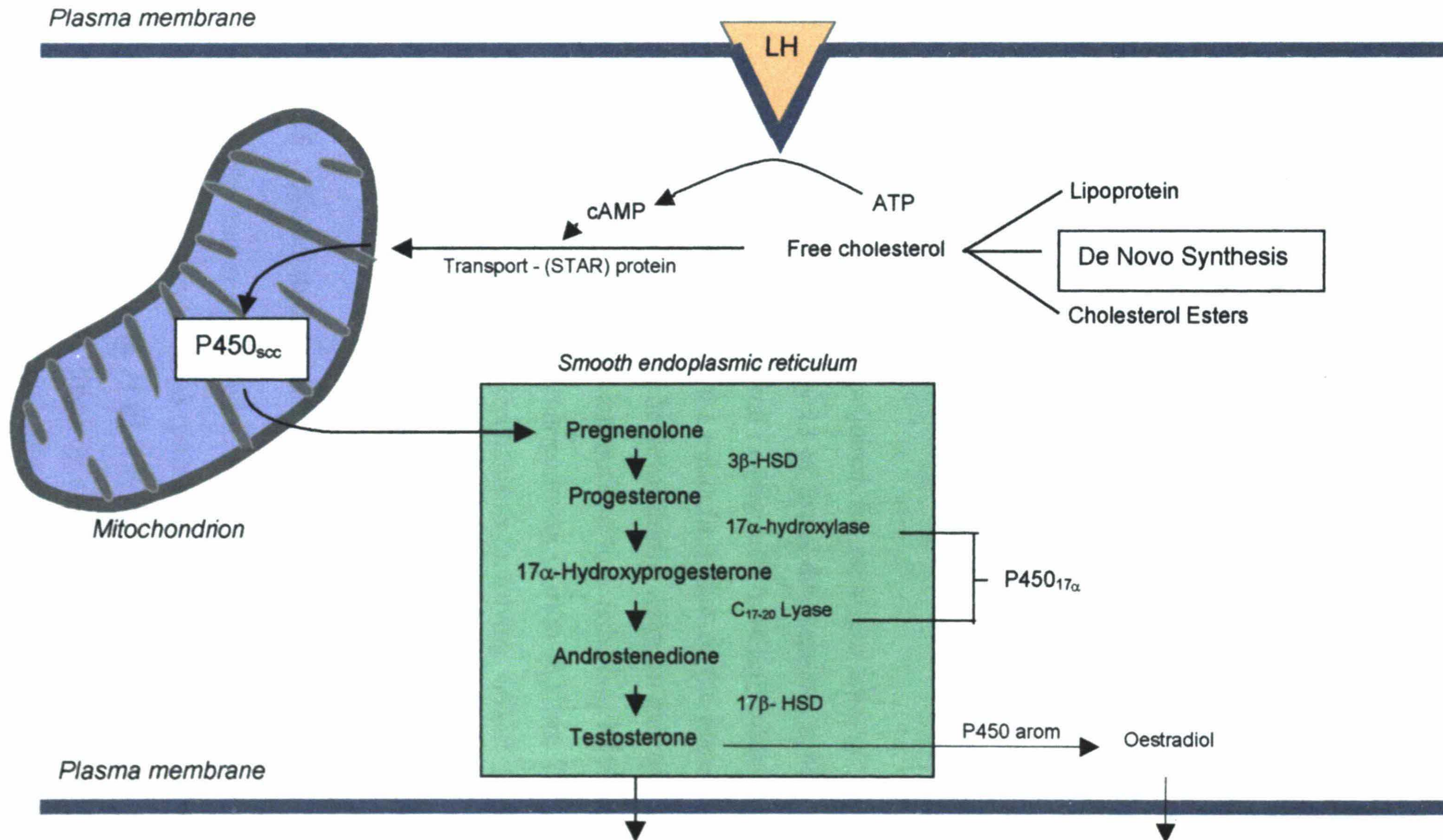


Figure 1.344 Biosynthesis of testosterone in the testicular Leydig cells

LH increases cAMP to transport free cholesterol into the inner mitochondrial membrane via the STAR protein, where the rate limiting enzyme, $P450_{scc}$ converts the cholesterol to pregnenolone. Further steps in the biosynthesis of testosterone occur in the smooth endoplasmic reticulum. Testosterone is then released from the Leydig cells either into the bloodstream or by diffusing into the seminiferous tubules, or is further metabolised in the Leydig cells via the 5α-reductase or aromatase enzymes. Adapted from Payne and Youngblood (1995)

the only cell in the testis able to convert cholesterol to progesterone. However, the seminiferous tubule epithelium is still capable of further modifying the steroids secreted by Leydig cells. After progesterone is formed (**Figure 1.344**), its C_{17} is hydroxylated by 17α-hydroxylase, and then converted to androstenedione by the actions of C_{17-20} Lyase. These 2 enzymes can be grouped together as $P450_{17\alpha}$. To form testosterone, the 17-ketone is reduced by 17-hydroxysteroid dehydrogenase type 3 (17β-HSD). Testosterone is then secreted from the Leydig cells into the bloodstream or is able to diffuse into the seminiferous tubules to maintain spermatogenesis. Androstenedione and testosterone can be converted via aromatisation into oestrone or oestradiol, respectively (**Section 1.21**).

The essential enzymes required for androgen biosynthesis ($P450_{17\alpha}$ and 3β-HSD), are expressed as early as day 13 of foetal development in the mouse (Greco & Payne 1994). This suggests that the foetal Leydig cells have the ability to synthesize androgens from this early age, important in the masculinisation of the male urogenital tract. These enzymes function throughout life, however during aging in the rodent, testosterone levels are found to decrease. This is not attributable to a reduction in serum LH levels, but rather in all aspects of the steroidogenic pathway (Zirkin & Chen 2000). Both LH receptors and the intracellular signalling mechanisms involving cAMP production are decreased, as are levels of the STAR protein, suggesting an impairment in cholesterol transport to the mitochondria, and all steroidogenic enzymes (Zirkin & Chen 2000).

1.35 Associated ducts

Once the mature sperm are released from the seminiferous epithelium during the process of spermiation, they undergo further maturational changes as they make their passage through the rete testis (Goyal *et al.* 1997). This major duct system of the testis empties into a collection of tubules, lined by simple cuboidal epithelial cells, which join to the initial segment of the epididymis through small efferent ductules (Ilio & Hess 1994). The primary function of these ductules is to reabsorb water, ions and proteins, studies showing they reabsorb 90% of the rete testis fluid. The removal of this fluid allows for more

concentrated sperm to enter the epididymis, ensuring a high concentration of sperm will be ejaculated (Ilio & Hess 1994; Clulow *et al.* 1994; Clulow *et al.* 1998; Hess 2000). The journey from the seminiferous epithelium through to the cauda epididymis also acquires the spermatozoa specific motility and fertilisation capabilities by the time they reach the cauda (tail) epididymis (Setchell *et al.* 1994b).

1.36 Hormonal regulation of spermatogenesis

The testes are not only responsible for the production and storage of viable spermatozoa, but are a major endocrine organ responsible for the manufacture and secretion of steroid hormones. Both of these functions require stimulation by the gonadotrophins, LH and FSH, released from the pituitary under the control of GnRH (**Figure 1.343**) (for review see Weinbauer & Nieschlag 1993; McLachlan *et al.* 1995b; Ulloa-Aguirre *et al.* 1995; McLachlan *et al.* 1996). LH stimulates the Leydig cells to synthesise androgens such as testosterone, which can then diffuse into the seminiferous epithelium, whereas FSH acts directly on the epithelium. It is generally believed that androgens and FSH act on receptors within the Sertoli cells rather than within the germ cells (Kangasniemi *et al.* 1990; Rannikki *et al.* 1995) (for review see Jegou 1993).

The importance of gonadotrophins is observed through numerous studies where these hormones are withdrawn and spermatogenesis is detrimentally affected. Such as GnRH immunisation (Awoniyi *et al.* 1989), GnRH agonist/antagonist treatment (Matsumoto *et al.* 1986; Rea *et al.* 1986; Huhtaniemi *et al.* 1986), hypophysectomy (Russell *et al.* 1987b) and suppression of intratesticular testosterone levels through exogenous testosterone (Kula 1988). Also the *hpg* (hypogonadal) mouse, congenitally deficient in GnRH therefore lacking FSH and LH, which displays underdeveloped testes with spermatogenesis arrested at the early stages of germ cell development (Ebling *et al.* 2000). However, the specific importance of each hormone in spermatogenesis is only recently beginning to be understood.

In the immature animal, FSH is crucial for the proliferation and structure of the Sertoli cells, thus in this regard plays an important role in controlling the sperm producing capacity of the testis (Singh & Handelsman 1996a). Further importance for FSH in Sertoli cell development can be seen with specific knockout models. Mice lacking a functional FSH Receptor (FORKO) (Dierich *et al.* 1998) have underdeveloped testes and reduced fertility, with a reduction in the epithelial diameter presumably as a consequence of inhibited Sertoli cell proliferation (Krishnamurthy *et al.* 2000). The phenotype is similar, although slightly less severe, when the FSHβ subunit gene is functionally disrupted, with the males remaining fertile, how compensatory mechanisms may be a factor (Kumar *et al.* 1997). FSH also plays a role in the mature rodent, particularly in the secretory capacity of the Sertoli cell, including factors such as inhibin (Le Gac & de Kretser 1982), transferrin (Skinner & Griswold 1982), plasminogen activator (Lacroix *et al.* 1977) and aromatase (Dorrington & Armstrong 1975). It is speculated that it is through Sertoli cell specific proteins that an intricate communicatory network exists between this cell and the germ cells (Jegou 1993). However, there is also evidence to suggest that FSH may play a direct role in spermatogenesis, particular in the maturation of spermatogonia and spermatocytes (Moudgal *et al.* 1997; Meachem *et al.* 1998) (for review see McLachlan *et al.* 1996) and in their survival following hypophysectomy (Russell *et al.* 1993) and GnRH immunisation (McLachlan *et al.* 1995a). This is further examined in the FORKO mouse, in which the absence of a functional FSH R leads to a significant decrease in the number of mature spermatids (Krishnamurthy *et al.* 2000). Thus, FSH appears to play an important role in early germ cell maturation.

Androgen receptors are also localised to Sertoli cells and highly expressed at the mid stages which are believed to be androgen dependant. As mentioned, FSH is believed to be important for progression through mitosis and meiosis, however is unable to stimulate any further maturation through spermiogenesis (Bartlett *et al.* 1989; Singh & Handelsman 1996b). Testosterone, on the other hand, is essential for a round spermatid to progress to an elongated spermatid (Sun *et al.* 1990), specifically step 7 to step 8 (O'Donnell *et al.* 1994). In the absence of this hormone, the round spermatids are unable to adhere to the epithelium

and are prematurely released, presumably as a result of a loss of testosterone dependant cell adhesion molecules (O'Donnell *et al.* 2000). Following the withdrawal of testosterone, step 7 spermatids are also susceptible to cell death, with the administration of testosterone able to increase their survival (Russell & Clermont 1977; Kerr *et al.* 1993). However, investigations into the *hpg* mouse suggest that testosterone may also be important for earlier stages of spermatogenesis, where in the absence of LH and FSH, testosterone replacement is sufficient to reinitiate quantitatively normal spermatogenesis (Singh *et al.* 1995). FSH is, however, still required for normal levels of Sertoli cells, thus in these animals total spermatogenic output is decreased unless FSH is replaced (Singh & Handelsman 1996a).

To date, it has been fairly well established that FSH and testosterone are two major hormonal players in the process of spermatogenesis. However, strong evidence is now emerging that indicates another player, oestrogen, as possibly having an as yet unidentified but crucial role in this complex process.

1.4 LOCALISATION OF AROMATASE AND ER IN THE TESTIS

Oestrogen has been recognised since the 1930's to be synthesised in the male (Zondek 1934), however the significance of finding this 'female' hormone in the male was short lived as its role was considered to be of little importance to the functioning of the testis and as such was not well studied. In fact by the 1970's knowledge on the cell types within the testes responsible for oestrogen synthesis were unclear, as was where oestrogen was acting. In this section of the review, an overview of the expression of ERα, β, and aromatase localization of mRNA and protein in the testis from the foetal rodent through to adulthood will be presented, see Abney 1999; Carreau 2000; Carreau *et al.* 1999 and Hess *et al.* 1995 for other reviews.

1.41 Foetal testis

During foetal development in the mouse, oestrogen receptors are found in all stages of testicular development. In fact, immunocytochemical studies localise the ER (no distinction was made between ERα and β) as early as day 10 in the developing mouse foetus, when the gonad is still undifferentiated, suggesting oestrogen may have a role very early in the differentiation process (Greco *et al.* 1992). Greco and colleagues (Greco *et al.* 1992) demonstrated that both the associated ducts and the gonads contained ER from day 13 of gestation, while the interstitial Leydig cells were positive at day 15 and remained so until birth. At day 17 of gestation, ER was specifically localised to both Sertoli cells and gonocytes (Greco *et al.* 1992). At birth in the mouse (day 19) ERs are found in the epididymis (Greco *et al.* 1992), and a high level of oestradiol binding sites have been demonstrated in the efferent ducts and initial segment, decreasing in abundance along the epididymis towards the cauda (Cooke *et al.* 1991; Greco *et al.* 1992).

Studies by Nielsen *et al* (2000), and Jefferson *et al* (2000), using a specific ERα antibody, readdressed the issue of ER localisation in the male reproductive structures during foetal development in the mouse (**Table 1.4**). ERα was faintly immunolocalised to the undifferentiated sex ducts at day 11.5 p.c., leading Nielson *et al* (2000) to suggest that the ER detected by Greco *et al* (1992) may in fact be ERα. At day 13.5 the gonad is recognizable as male (**Section 1.31**), with ERα only localised to the interstitial Leydig cells (Nielsen *et al.* 2000; Jefferson *et al.* 2000). Although ERα increases in the Leydig cells until birth, the testicular cords (the developing seminiferous tubules) remain negative throughout development (Nielsen *et al.* 2000). At day 15.5 the developing epididymis and efferent ductules were also immunopositive for ERα, with expression increasing in the efferent ductules (associated ducts) during late foetal development (Nielsen *et al.* 2000). Recently, Jefferson et al (2000) examined expression of ERα and β throughout development in the mouse. ERβ mRNA was found as early as day 14 in the foetal testis, and ERβ protein was immunolocalised to the germ cells (gonocytes) at day 16. At this age,

Table 1.4 Published studies investigating the localisation of ERα, ERβ and aromatase in the rodent testis throughout development

	ERα		ERβ		Aromatase	
	+	-	+	-	+	-
FOETAL						
Gonad	**R** m- (Jefferson *et al.* 2000)		**R** m- (Jefferson *et al.* 2000)		**A** r- (Weniger & Zeis 1987; Weniger & Zeis 1988)	
Gonocytes		**P** m- (Nielsen *et al.* 2000) r- (Fisher *et al.* 1997; Saunders *et al.* 1998)	**R** r- (van Pelt *et al.* 1999) **P** m- (Jefferson *et al.* 2000) r- (Saunders *et al.* 1998; van Pelt *et al.* 1999)			
Sertoli cells		**P** m- (Nielsen *et al.* 2000) r- (Fisher *et al.* 1997; Saunders *et al.* 1998)	**R** r- (van Pelt *et al.* 1999) **P** r- (van Pelt *et al.* 1999; Saunders *et al.* 1998)			
Leydig cells	**P** m- (Nielsen *et al.* 2000; Jefferson *et al.* 2000) r- (Fisher *et al.* 1997; Saunders *et al.* 1998; Sar & Welsch 2000)		**R** r- (van Pelt *et al.* 1999) **P** r- (van Pelt *et al.* 1999; Saunders *et al.* 1998)		**A** r- (Tsai-Morris *et al.* 1985)	
Associated ducts	**P** m- (Nielsen *et al.* 2000) r- (Fisher *et al.* 1997; Sar & Welsch 2000)		**P** r- (Sar & Welsch 2000)			
NEONATAL/ PUBERTAL						
Whole testis	**R** m- (Jefferson *et al.* 2000) r- (Tena-Sempere *et al.* 2000)		**R** m- (Jefferson *et al.* 2000) r- (Tena-Sempere *et al.* 2000)			
Germ cells		**P** m- (Nielsen *et al.* 2000) r- (Fisher *et al.* 1997; Saunders *et al.* 1998; Sar & Welsch 2000)				
Gonocytes/ Spermatogonia			**R** r- (van Pelt *et al.* 1999) **P** m- (Jefferson *et al.* 2000) r- (van Pelt *et al.* 1999; Saunders *et al.* 1998)			**P** r- (Kurosumi *et al.* 1985)
Spermatocytes			**R** r- (Saunders *et al.* 1998) **P** m- (Jefferson *et al.* 2000)			
Sertoli cells		**P** m- (Nielsen *et al.* 2000) r- (Fisher *et al.* 1997; Saunders *et al.* 1998; Sar & Welsch 2000)	**R** r- (van Pelt *et al.* 1999) **P**r- (van Pelt *et al.* 1999; Saunders *et al.* 1998)		**A** r- (Tsai-Morris *et al.* 1985; Rommerts *et al.* 1982; Papadopoulos *et al.* 1986)	**P** r- (Kurosumi *et al.* 1985)
Leydig cells	**P** *m- (Nielsen et al. 2000; Jefferson et al. 2000)*		**R** r- (van Pelt *et al.* 1999)		**P** r- (Kurosumi *et al.* 1985) **A** r- (Tsai-Morris *et al.* 1985;	

	r- (Fisher *et al.* 1997; Saunders *et al.* 1998; Sar & Welsch 2000)				Rommerts *et al.* 1982; Papadopoulos *et al.* 1986)	
Rete testis	**P** r- (Fisher *et al.* 1997)					
Efferent ducts	**P** m- (Nielsen *et al.* 2000) r- (Fisher *et al.* 1997)					
Epididymis	**R** m- (Jefferson *et al.* 2000) **P** m- (Jefferson *et al.* 2000; Nielsen *et al.* 2000) r- (Fisher *et al.* 1997; Sar & Welsch 2000)		**R** m- (Jefferson *et al.* 2000) **P** m- (Jefferson *et al.* 2000) r- (Sar & Welsch 2000)			
ADULT						
Whole testis	**R** m- (Couse *et al.* 1997)		**R** m- (Rosenfeld *et al.* 1998)	**R** m- (Couse *et al.* 1997) r- (Pelletier *et al.* 2000)		
Germ cells		**P** r- (Fisher *et al.* 1997; Sar & Welsch 2000)			**A** m- (Nitta *et al.* 1993)	**P** r- (Kurosumi *et al.* 1985)
Spermatogonia		**R** r- (Pelletier *et al.* 2000)	**R** r- (van Pelt *et al.* 1999) **P** r- (van Pelt *et al.* 1999; Shughrue *et al.* 1998)	**R** r- (Shughrue *et al.* 1998) **P** r- (van Pelt *et al.* 1999)		
Spermatocytes	**P** r- (Pelletier *et al.* 2000)	**R** r- (Pelletier *et al.* 2000)	**R** r- (van Pelt *et al.* 1999; Shughrue *et al.* 1998) **P** r- (van Pelt *et al.* 1999; Saunders *et al.* 1998)		**R** m- (Nitta *et al.* 1993) r- (Levallet *et al.* 1998a; Levallet *et al.* 1998b; Carreau & Levallet 1997) **P** m- (Nitta *et al.* 1993) r- (Janulis *et al.* 1998) **A** r- (Janulis *et al.* 1998)	
Round spermatids	**R** r- (Pelletier *et al.* 2000)		**R** r- (van Pelt *et al.* 1999) **P** r- (van Pelt *et al.* 1999; Saunders *et al.* 1998)		**R** m-(Nitta *et al.* 1993) r- (Levallet *et al.* 1998a; Levallet *et al.* 1998b; Carreau & Levallet 1997) **P** m- (Nitta *et al.* 1993) r- (Janulis *et al.* 1998) **A** r- (Janulis *et al.* 1998)	
Elongated spermatids			**P** m- (Rosenfeld *et al.* 1998)	**P** r- (van Pelt *et al.* 1999; Saunders *et al.* 1998)	**R** r- (Levallet *et al.* 1998a; Carreau & Levallet 1997) **P** m- (Nitta *et al.* 1993) **A** (Levallet *et al.* 1998a)	
Sertoli cells		**R** m- (Pelletier *et al.* 2000) **P** r- (Fisher *et al.* 1997; Sar & Welsch 2000)	**R** r- (van Pelt *et al.* 1999; Shughrue *et al.* 1998) **P** r- (van Pelt *et al.* 1999; Saunders *et al.* 1998; Saunders	**R** r- (Pelletier *et al.* 2000)	**R** r- (Levallet *et al.* 1998a; Carreau & Levallet 1997) **P** m- (Nitta *et al.* 1993) Activity	

			et al. 1997; Pelletier *et al.* 2000)		r- (Tsai-Morris *et al.* 1985; Rommerts *et al.* 1982; Papadopoulos *et al.* 1986)
Leydig cells	**R** r- (Pelletier *et al.* 2000) **P** r- (Fisher *et al.* 1997; Sar & Welsch 2000; Pelletier *et al.* 2000)		**R** m- (Rosenfeld *et al.* 1998) **P** m- (Rosenfeld *et al.* 1998)	**R** r- (van Pelt *et al.* 1999) **P** r- (van Pelt *et al.* 1999)	**R** r- (Levallet *et al.* 1998a; Carreau & Levallet 1997; Janulis *et al.* 1996a) **P** m- (Nitta *et al.* 1993) r- (Kurosumi *et al.* 1985; Janulis *et al.* 1998; Levallet *et al.* 1998a) **A** m- (Nitta *et al.* 1993) r- (Tsai-Morris *et al.* 1985; Rommerts *et al.* 1982; Levallet *et al.* 1998a; Papadopoulos *et al.* 1986; Valladares & Payne 1979)
Rete testis	**P** r- (Fisher *et al.* 1997)				
Efferent ducts	**R** r- (Hess *et al.* 1997) **P** r- (Fisher *et al.* 1997; Hess *et al.* 1997)		**R** r- (Hess *et al.* 1997) **P** m- (Rosenfeld *et al.* 1998)		
Epididymis	**R** m- (Couse *et al.* 1997) r- (Hess *et al.* 1997) **P** r- (Hess *et al.* 1997; Sar & Welsch 2000)	**P** r- (Fisher *et al.* 1997)	**R** m- (Couse *et al.* 1997; Rosenfeld *et al.* 1998) r- (Hess *et al.* 1997) **P** m- (Rosenfeld *et al.* 1998; Sar & Welsch 2000)		
Epididymal Sperm (cytoplasmic droplet)					**P** r- (Janulis *et al.* 1998; Janulis *et al.* 1996b) **A** r- (Janulis *et al.* 1998; Janulis *et al.* 1996b)

The demonstration of either protein (**P**), mRNA (**R**) or activity (**A**) in either rat (**r**) or mouse (**m**) is indicated in the + column. Organs/cell types were only included in the – column if a particular manuscript had specifically indicated that either the ER or aromatase was not present. Earlier studies that did not discriminate between ERα and ERβ are not included. The term 'associated ducts' in the foetus includes the Wolfiaan duct which differentiates into the epididymis and vas deferens, and also the efferent ductules and epididymis. Protein localisation was determined by immunocytochemistry or Western blot, with mRNA localisation by RNA protection assay, RT-PCR, insitus hybridisation or Northern blot.

ERα mRNA was found in whole testis and epididymis, with low levels of ERβ seen in the same tissues.

ERα and ERβ have been localised to the foetal rat testis (**Table 1.4**). Both ERβ protein and mRNA are found as early as day 16 of gestation in the gonocytes, Sertoli cells and Leydig cells, remaining in these cells until birth (Saunders *et al.* 1998; van Pelt *et al.* 1999; Sar & Welsch 2000). However, it is the gonocytes that express ERβ in higher abundance than the other testicular cells (van Pelt *et al.* 1999; Saunders *et al.* 1998), possibly suggesting a direct role in gonocyte maturation. In contrast to ERβ, ERα is only found outside of the seminiferous epithelium (Fisher *et al.* 1997; Saunders *et al.* 1998), with both ERα protein and ERβ mRNA and protein localised in foetal rat Leydig cells (Fisher *et al.* 1997; Saunders *et al.* 1998; van Pelt *et al.* 1999). In fact oestrogen receptors are expressed in the Leydig cells at a stage in development when the androgen receptor is not yet expressed (Majdic *et al.* 1995), highlighting a role for oestrogen at this stage. There is evidence for both ERα and ERβ protein in the rat associated ducts, which will eventually differentiate into the efferent ductules and adjoining epididymis (Fisher *et al.* 1997; Sar & Welsch 2000).

The foetal rat testis also has aromatase activity (Weniger & Zeis 1987; Weniger 1990; Weniger 1993), and is first expressed by day 19 (Weniger & Zeis 1987). The cell type responsible may be the Sertoli cells, as oestrogen production by foetal testes in culture is stimulated by FSH (Weniger & Zeis 1988). Interestingly, this is close to the time when FSH receptors first appear on the Sertoli cells (see Pelliniemi *et al.* 1993 for review). There is also evidence that foetal Leydig cells have the capacity for LH stimulated-aromatisation of androgens (Tsai-Morris *et al.* 1986).

1.42 Neonatal and immature testis

After birth, the testis continues to express both ER isoforms and aromatase (**Table 1.4**). Most reports suggest that ERβ protein and mRNA, rather than ERα, appears to be

localised to the rat seminiferous epithelium, in both Sertoli cells and developing germ cells (Saunders *et al.* 1998; van Pelt *et al.* 1999). In the mouse, the testis at this time also continues to abundantly express ERβ mRNA, with immunocytochemical studies localising this ER to the germ cells (Jefferson *et al.* 2000).

ERα expression is restricted to the cells which lie outside of the seminiferous epithelium (Fisher *et al.* 1997; Saunders *et al.* 1998; Nielsen *et al.* 2000), with ERα (Fisher *et al.* 1997; Saunders *et al.* 1998; Jefferson *et al.* 2000; Sar & Welsch 2000; Nielsen *et al.* 2000), along with ERβ (Saunders *et al.* 1998; van Pelt *et al.* 1999), continuing to be found in the Leydig cells of mice and rats during the neonatal period. ERα protein is also found in the rete testis (Fisher *et al.* 1997), efferent ductules (Fisher *et al.* 1997; Nielsen *et al.* 2000), and the epididymis (Fisher *et al.* 1997; Nielsen *et al.* 2000; Sar & Welsch 2000), where it has also remained since foetal development. In fact, ERα is expressed in much higher levels in the efferent ducts than the cauda epididymis (Fisher *et al.* 1997; Nielsen *et al.* 2000). ERβ is also found in the epididymis (Jefferson *et al.* 2000; Sar & Welsch 2000).

At this stage of development, basal aromatase activity is found in both the immature Leydig cells and Sertoli cells (Rommerts *et al.* 1982; Tsai-Morris *et al.* 1985; Papadopoulos *et al.* 1986), however activity is significantly induced by FSH in the Sertoli cells (Papadopoulos *et al.* 1986). In fact at this age, Sertoli cells were more active in producing oestrogen than neonatal Leydig cells and adult Sertoli cells, suggesting that these cells are an important source of oestrogen in the postnatal testis (Rommerts *et al.* 1982). At this age, germ cells have been reported not to contain aromatase (Tsai-Morris *et al.* 1985; Kurosumi *et al.* 1985)

During days 10-26 in the immature rat, Leydig cells and Sertoli cells are dividing and undergoing functional maturation (see de Kretser & Kerr 1988 for review). Again, ERα is absent from the seminiferous epithelium with only ERβ prominent in the epithelium of the immature rodent (**Table 1.4**). Specifically in the tubules of the rat, ERβ mRNA and protein

are weakly stained in the spermatogonia (Saunders *et al.* 1998; van Pelt *et al.* 1999) and in immature Sertoli cells (Saunders *et al.* 1998; van Pelt *et al.* 1999), however by day 21 it is abundantly expressed in the pachytene spermatocytes (Saunders *et al.* 1998). Other germ cells did not show ERβ staining (van Pelt *et al.* 1999). By day 12 in the mouse, ERβ is also specifically immunolocalised to the spermatocytes, however its expression then decreases and is undetectable by day 26 (Jefferson *et al.* 2000). Again, rat and mouse (van Pelt *et al.* 1999) Leydig cells express ERα (Fisher *et al.* 1997; Saunders *et al.* 1998; Sar & Welsch 2000; Jefferson *et al.* 2000) (**Table 1.4**).

During the neonatal and pubertal period of development, ERα is prominent in the rete testis, efferent ductules (Fisher *et al.* 1997) and appears to be present in the mouse epididymis (Jefferson *et al.* 2000), with low to undetectable levels in the maturing rat epididymis (Fisher *et al.* 1997). ERβ mRNA and protein is also present in the epididymis at this time (Jefferson *et al.* 2000).

Aromatase appears to have an age dependant pattern of expression. As the animal matures, the Leydig cells appear to take over the role of oestrogen production from the Sertoli cells (Tsai-Morris *et al.* 1985), with their basal aromatase activity increasing 3-4 fold (Rommerts *et al.* 1982) and now stimulated by LH (Papadopoulos *et al.* 1986). The Sertoli cells continue to express basal levels of aromatase (Papadopoulos *et al.* 1986). This cellular redistribution may be due to the immaturity of the Leydig cells in the neonatal testis, causing them to be incapable of oestrogen synthesis until they have undergone a series of postnatal maturation steps.

1.43 Adult testis-germ cells

There is now considerable evidence that germ cells contain both oestrogen receptors and aromatase. In the rat, both ERβ and aromatase co-localise in many germ cell types, with some conflicting results. One study found both ERβ mRNA and protein in type A spermatogonia (van Pelt *et al.* 1999), whereas another study did not (Shughrue *et al.* 1998).

There is also evidence for ERβ in intermediate and type B spermatogonia in some studies (Saunders *et al.* 1998), but not others (van Pelt *et al.* 1999), and absent from leptotene, zygotene, and early pachytene spermatocytes (Saunders *et al.* 1998). ERβ mRNA and protein is found in pachytene spermatocytes (Saunders *et al.* 1998; Shughrue *et al.* 1998; van Pelt *et al.* 1999), with aromatase mRNA and activity also found in these cells in rats and mice (Nitta *et al.* 1993; Shughrue *et al.* 1996; Carreau & Levallet 1997; Janulis *et al.* 1998; Levallet *et al.* 1998a).

Both ERβ and aromatase remain in the germ cells as they further mature into round spermatids (**Table 1.4**) (Nitta *et al.* 1993; Carreau & Levallet 1997; Saunders *et al.* 1998; Janulis *et al.* 1998; Levallet *et al.* 1998a; Levallet *et al.* 1998b; van Pelt *et al.* 1999). While several reports show ERβ in pachytene spermatocytes and round spermatids, see **Table 1.4**, only one study in mice found it only in elongated spermatids (Rosenfeld *et al.* 1998), with another finding no expression at all in the testes of mice (Couse *et al.* 1997), Another study in rats did not find ERβ in spermatocytes and spermatids, but did suggest the presence of ERα (Pelletier *et al.* 2000). Thus there is conflicting data on the localisation of the two receptors, presumably arising from methodological differences, although species differences may also have confounding effects on the interpretation of the data.

Aromatase appears to be present in higher levels in mature spermatids of the rat than earlier in germ cells (Carreau & Levallet 1997; Levallet *et al.* 1998a). Aromatase mRNA and activity was higher in the germ cells of the mouse and rat when compared to Leydig cells (Nitta *et al.* 1993; Janulis *et al.* 1996a; Carreau & Levallet 1997), suggesting that the germ cells are an important source of oestrogen in the testis. Aromatase localisation is observed to move from the Golgi apparatus to the cytoplasm during spermatid development (Nitta *et al.* 1993). When round spermatids begin the morphological transformation into elongated spermatids, aromatase continues to be found in these cells (Janulis *et al.* 1996a; Carreau & Levallet 1997; Levallet *et al.* 1998a; Levallet *et al.* 1998b; Janulis *et al.* 1998; van Pelt *et al.* 1999). ERβ is not present in the elongated spermatids in

the rat (Saunders *et al.* 1998; van Pelt *et al.* 1999), however there is a report that it may be present in mice (Rosenfeld *et al.* 1998). Aromatase appears to be immunolocalised to the flagella of the developing spermatid (Nitta *et al.* 1993). When elongated spermatids are released from the epithelium, during the process of spermiation, aromatase remains in the residual body that is subsequently phagocytosed by the Sertoli cell (Nitta *et al.* 1993; Janulis *et al.* 1996a). However, not all the cytoplasm is phagocytosed, and aromatase activity remains in the cytoplasmic droplet that is still attached to the flagellum as the sperm make their way through the epididymis (Janulis *et al.* 1996b; Janulis *et al.* 1998). Thus it appears as if mature sperm are able to synthesise their own oestrogen, as they traverse the efferent ducts (Hess *et al.* 1995; Janulis *et al.* 1998; Janulis *et al.* 1996b). The ability to synthesise oestrogen gradually decreases as the droplet slowly moves to the end of the tail during epididymal transit, until the droplet is finally lost (Janulis *et al.* 1996b). This is important as it suggests that the sperm themselves could control the levels of oestrogen present in the luminal fluid, directly modulating functions such as the reabsorption of fluid from the efferent ductules (Hess *et al.* 1995).

Studies in other species, such as in the cynomolgus monkeys, found ERβ in spermatogonia and spermatids (Pelletier *et al.* 1999), and in spermatocytes and round spermatids in humans (Enmark *et al.* 1997). In seasonal breeders, such as the bank vole, when exposed to long light, ERβ was found in spermatocytes and elongated spermatids (Bilinska *et al.* 2000). Aromatase localisation in the bank vole was also dependant on light, with aromatase in spermatocytes as well as the Leydig cells with long light (Bilinska *et al.* 2000). Similar to the rodent, ERα is not reported to be expressed in the seminiferous epithelium of the marmoset (Fisher *et al.* 1997), human (Enmark *et al.* 1997) and bank vole (Bilinska *et al.* 2000). See Carreau 2000; Carreau *et al.* 1999 for a more comprehensive review on aromatase localisation in other species.

1.44 Adult testis-Sertoli cells

Through earlier stages of development, ERα has not been immunolocalised to the seminiferous epithelium (**Table 1.4** and **Section 1.41, 1.42**). This continues to be the case in the adult with no reports of ERα in adult Sertoli cells, although there is one report of ERα in spermatids (Pelletier *et al.* 2000). However, both ERβ mRNA and protein have been localised to rat Sertoli cells from the foetus to adulthood (**Table 1.4** and **Section 1.41, 1.42**) (Saunders *et al.* 1997; Saunders *et al.* 1998; Shughrue *et al.* 1998; van Pelt *et al.* 1999; Pelletier *et al.* 2000). These immunocytochemical studies suggest that ERβ is not stage-dependent in the rat (Saunders *et al.* 1997; Saunders *et al.* 1998). In contrast to the rat, there are no reports of ERβ in mouse Sertoli cells (**Table 1.4**).

1.45 Adult testis-Leydig cells

The presence of ERα, ERβ and aromatase in the adult testis has been the subject of numerous recent studies and there appears to be some degree of species differences between rat and mouse, in terms of ERα and ERβ localisation.

Throughout development into adulthood, ERα is expressed in both mouse and rat Leydig cells (Fisher *et al.* 1997; Pelletier *et al.* 2000). In the adult mouse the ERβ protein is also localised to the Leydig cells (Rosenfeld *et al.* 1998), but this does not seem to be the case in the adult rat (van Pelt *et al.* 1999; Pelletier *et al.* 2000). The observations in the rat are similar to those obtained from humans (Enmark *et al.* 1997) and cynomolgus monkeys (Pelletier *et al.* 1999) in that ERβ is not found in mature Leydig cells (Pelletier *et al.* 2000).

By adulthood, the Leydig cells express a high level of aromatase (Kurosumi *et al.* 1985; Nitta *et al.* 1993; Levallet *et al.* 1998a; Janulis *et al.* 1998; Carreau *et al.* 1999) which is stimulated by LH (Valladares & Payne 1979a; Tsai-Morris *et al.* 1985). In fact, aromatase activity is higher in the adult than at any other age (Tsai-Morris *et al.* 1985) and is higher in the adult Leydig cells than in the Sertoli cells (Levallet *et al.* 1998a). The decrease in

aromatase activity during Sertoli cell maturation into the adult form (see Dorrington & Khan 1993 for review), may be related to the control of Sertoli cell division and adult cell function (**Section 1.33**). The Leydig cells are also the major site of oestrogen synthesis in species such as pigs (Raeside & Renaud 1983), sheep (Schmalz & Bilinska 1998; Bilinska *et al.* 1997) and humans (Payne *et al.* 1976).

1.46 Associated ducts

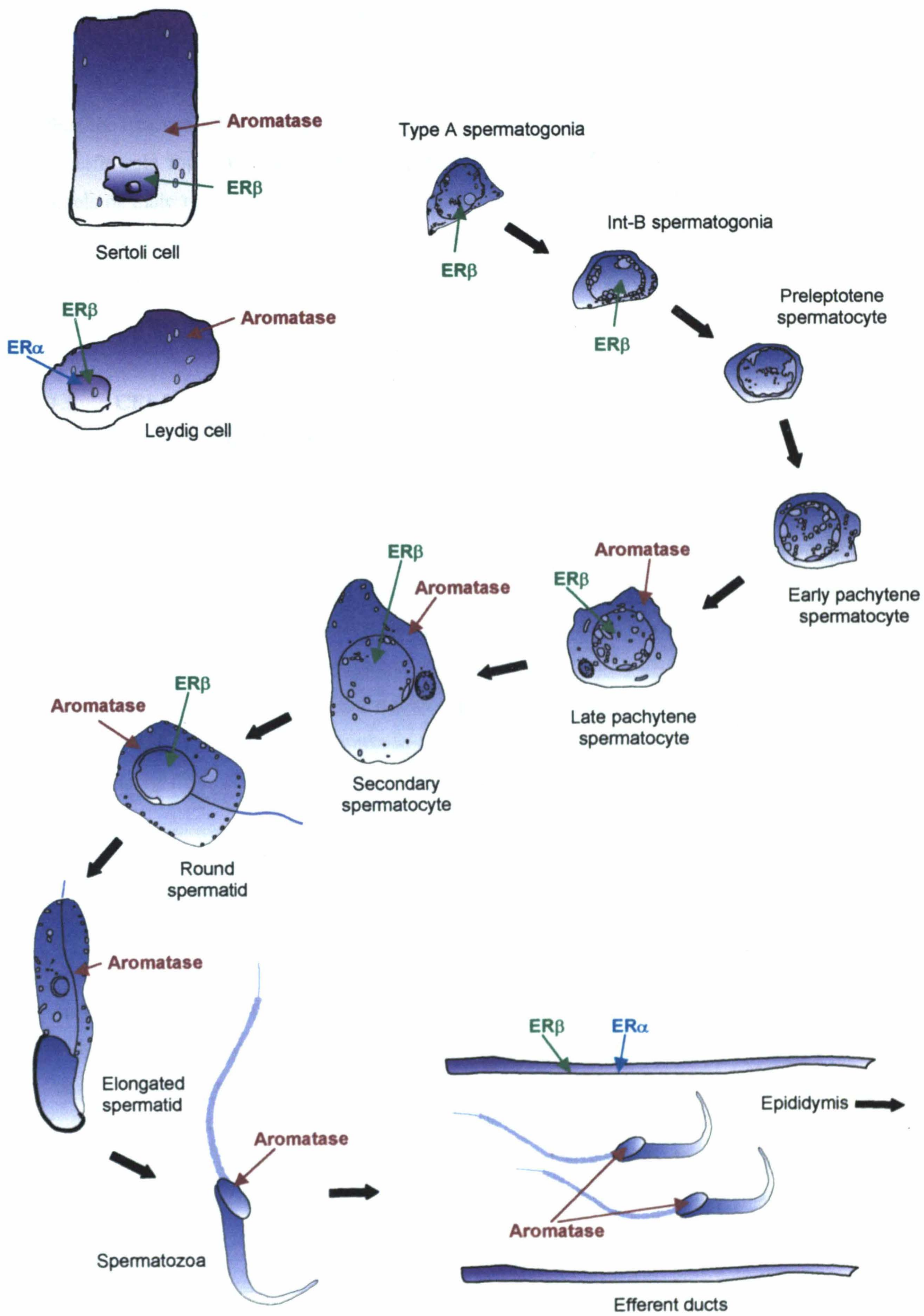
During foetal development, ERα is found in the associated ducts (**Table 1.4**) (Fisher *et al.* 1997; Sar & Welsch 2000), which differentiate into the efferent ductules and epididymis. In the adult, the expression pattern remains similar, with very high expression of ERα in the efferent ductules of the rat (Fisher *et al.* 1997; Hess *et al.* 1997b). In fact, it has been found that it is the efferent ductules which possess the highest level of ERα immunostaining, when considering the testis, excurrent ducts and epididymis, throughout life (Fisher *et al.* 1997). In addition, the efferent ductules appear to be the first male reproductive structure to express the oestrogen receptor in foetal development (Cooke *et al.* 1991), suggesting a role for oestrogen in the development of this tissue. ERα is present in the rat and mouse epididymis (Couse *et al.* 1997; Hess *et al.* 1997b; Sar & Welsch 2000), although in low levels, while another report could not detect it in rats (Fisher *et al.* 1997). Again, similar to earlier ages, both ERβ mRNA and protein are present from the efferent ducts to the epididymis of the mouse (Couse *et al.* 1997; Rosenfeld *et al.* 1998) and the rat (Hess *et al.* 1997b; Sar & Welsch 2000).

1.47 Summary

The high localisation of both ERα and ERβ in the seminiferous epithelium and associated structures suggests the testis is a direct target for the actions of oestrogen (for summary see **Figure 1.4**). As the interstitial cells, but also the germ cells, are actively synthesising oestrogen, and the caput epididymal fluid contains a concentration of oestrogen up to 25 times higher than that in plasma and higher than that found in female serum, a direct role for oestrogen is highly probable (Kumari *et al.* 1980) (for review see Hess 2000). Despite

Figure 1.4 Schematic of the postulated localisation of both aromatase and the ERs in the rodent testis.

Aromatase is found in the Sertoli cells in the immature animal, then the Leydig cells in the mature. Within the germ cells, aromatase is confined to the pachytene spermatocytes in low levels, then in the Golgi apparatus of round spermatids, the cytoplasm of elongated spermatids and then remains in the cytoplasmic droplet attached to the base of the tail as they make their journey to the epididymis. ERα is only localised outside of the seminiferous epithelium in the Leydig cells and efferent ducts. In contrast, ERβ is highly localised to the epithelium, specifically the spermatogonia, late pachytene spermatocytes, round and elongated spermatids where it co-localises with aromatase. ERβ is also found in the efferent ducts.



Sertoli cell
Aromatase
ERβ
Leydig cell
ERβ
ERα
Aromatase
Type A spermatogonia
ERβ
Int-B spermatogonia
ERβ
Preleptotene spermatocyte
Early pachytene spermatocyte
Late pachytene spermatocyte
Aromatase
ERβ
Secondary spermatocyte
ERβ
Aromatase
Round spermatid
ERβ
Aromatase
Elongated spermatid
Aromatase
Spermatozoa
Aromatase
ERβ
ERα
Epididymis
Aromatase
Efferent ducts

this, the exact role for oestrogen remains unclear. One factor hindering the elucidation of a specific role for oestrogen in spermatogenesis is that it has numerous roles at different levels of male reproduction, such as the hypothalamo-pituitary axis, Leydig cells, Sertoli cells and in the epididymis. Due to this, dissecting out a specific role for oestrogen in male fertility has been difficult.

1.5 CELL CYCLE PROGRESSION AND APOPTOSIS DURING SPERMATOGENESIS

Both cellular division and cell death, or apoptosis, are essential factors in animal development. These two mechanisms work cooperatively - the removal of excess or aged cells through organised cell death essential for the growth and the differentiation of new cells born through mitosis. As excess cell proliferation and insufficient cell death can result in major defects in foetal development and the occurrence of cancer in adulthood, both cellular division and apoptosis are tightly regulated through complex cell cycle and cell death pathways controlled by numerous checkpoints (for reviews see Elledge 1996; Jacks & Weinberg 1998; King & Cidlowski 1998; Pestell *et al.* 1999; Hengartner 2000).

Spermatogenesis is a constantly evolving process, involving the continuous progression of immature spermatogonia into mature spermatozoa under close contact and support of the surrounding Sertoli cells. The crucial first stage in spermatogenesis is the stem cell spermatogonia undergoing a series of mitotic divisions in order to increase their numbers. However, this series of divisions is somewhat uncoordinated, resulting in a vast excess of A-type spermatogonia. As the Sertoli cells can only support a specific number of developing germ cells, it is crucial that the excess spermatogonia are removed through apoptosis (Allan *et al.* 1992; Bartke 1995). Apoptosis is a normal function of spermatogenesis, occurring in both embryonic and postnatal life, to ultimately control germ cell numbers (Allan *et al.* 1992; Billig *et al.* 1995b). Both cell division and cell death

in the testis are intrinsically controlled by mitogenic and proliferative factors such as growth factors and hormones, with the removal of these observed to instigate cell cycle arrest and apoptosis (Russell *et al.* 1987a; Kiess & Gallaher 1998; de Kretser *et al.* 1998; Sinha Hikim & Swerdloff 1999; Print & Loveland 2000). The actual molecular pathways that are activated following these insults are just beginning to be elucidated.

1.51 The cell cycle

The cell cycle is a progression of events that directs the cell to divide into two identical daughter cells. Progression through this cycle is controlled by many factors which ultimately decide whether a cell proliferates or withdrawals into a state of quiescence (for review see Pestell *et al.* 1999; Hengartner 2000).

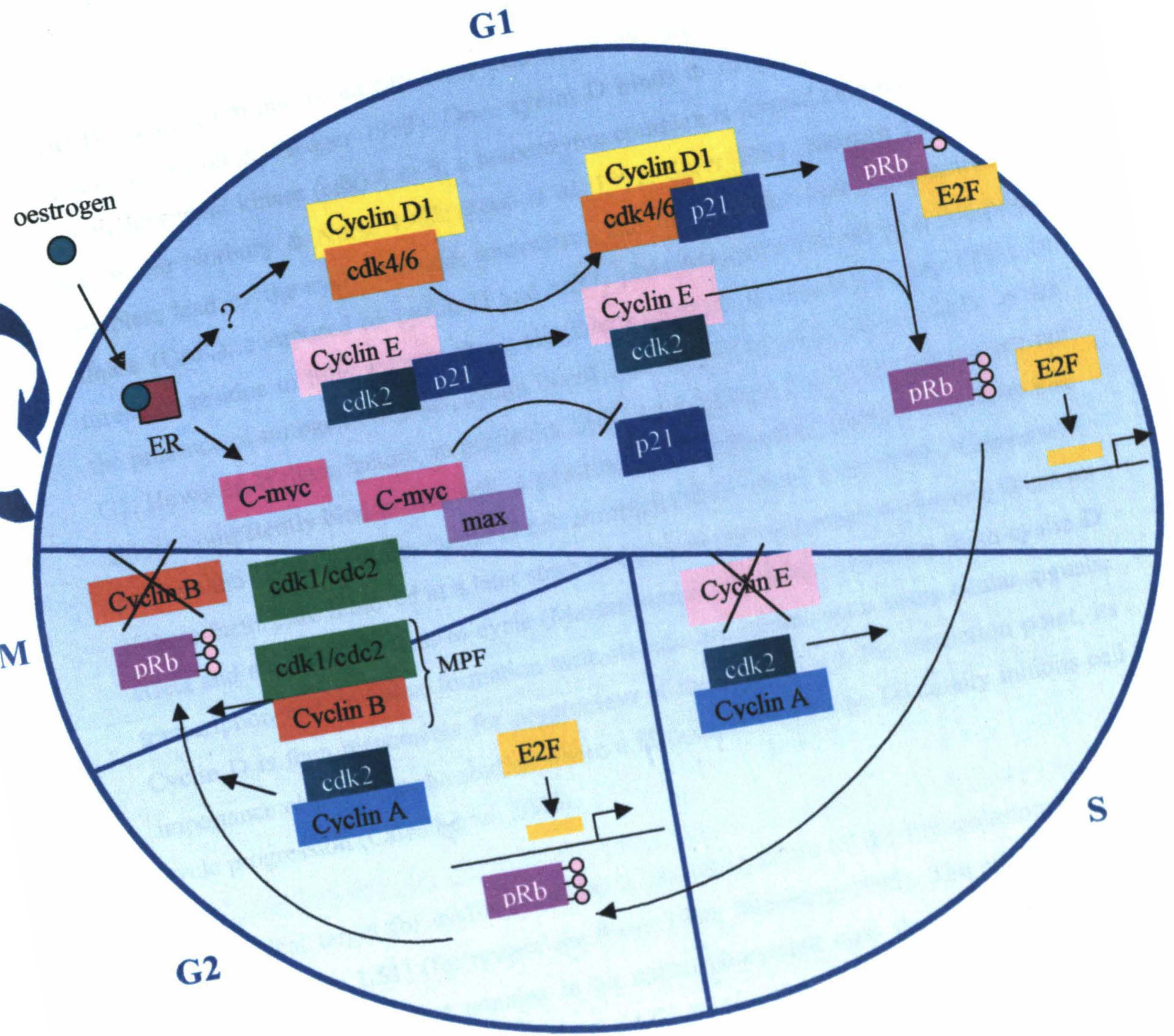
1.511 Entry into the cell cycle

Entry into the first gap phase (G1) of the cell cycle is driven by extracellular mitogenic factors, such as hormones, growth factors and differentiation factors (**Figure 1.51**) (for review see (Pestell *et al.* 1999; Jones & Kazlauskas 2001; Hulleman & Boonstra 2001)). Hormonal stimulation involves the steroid hormone binding to its receptor to activate the receptor complex (**Section 1.22**), whereas stimulation by growth factors requires activation of a signalling kinase cascade to transcriptionally activate specific genes. The hormone pathway will be discussed in **section 1.53**, with the growth factor mediated pathway briefly discussed here (for further reviews see Kelly & Rizzino 1999; Cook *et al.* 2000; Hulleman & Boonstra 2001; Jones & Kazlauskas 2001). Binding of growth factors to their specific receptors initiates G protein coupling to begin the protein kinase cascade. The Ras protein is activated through the Ras exchange factor, SOS, and is now able to bind to Raf (isoform 1-3) kinases to translocate them to the cell membrane. Active Raf then phosphorylates MEK 1 and 2, which in turn activate the downstream ERK (extracellular signal-regulated protein kinases) 1 and 2 kinases. ERK 1 and 2 can bind to and activate more than 50 substrates, including the transcription of the D (D1, D2 and D3) type cyclins (Sherr 1995; Lewis *et al.* 1998).

Figure 1.51 The cell cycle and its regulation

Numerous mitogenic factors control cell cycle progression, some via intracellular kinase cascades and others, eg. oestrogen, by binding to its cytoplasmic receptor. Once bound, oestrogen via an unknown intermediary factor, causes the transcription of early response genes, such as c-*myc,* and cyclin D1. Cyclin D1 binds to its catalytic subunit, cdk4/6, to be activated. The primary role of the cyclin D1 complex is to sequester the cdk inhibitor p21 away from the cyclin E/cdk2 complex. p21 was first determined to be an inhibitor of cdk, however once it binds to cyclin D1/cdk4/6, it further activates this complex, translocating it into the nucleus. This complex then phosphorylates the pRb, inactivating it. The removal of this p21 inhibitor activates cyclin E/cdk2, which completes this phosphorylation of pRb and causes it to release its hold of the E2F transcription factors. The free E2Fs then permit the transcription of specific genes required for S phase entry, such as cyclin A and DNA synthesis proteins.

As the cells enter the S phase, cyclin E is degraded, releasing cdk2 which then forms complexes with cyclin A, possibly transcribed by E2F. The role of cyclin A is to maintain pRb in a phosphorylated form until the cells have undergone mitosis. When the nuclei membrane degrades during the DNA synthesis stage, cyclin B is able to enter the nucleus and complex with cdk1/cdc2, forming a complex known as the M-phase promoting factor (MPF). Its role is to control entry and exit through mitosis. During anaphase cyclin B is then degraded, releasing cdk1 which inactivates the MPF and the cell leaves mitosis. For the cell to re-enter the cell cycle, mitogenic stimulatory factors must be present, otherwise the cell enters a quiescent stage known as G0.


G1
oestrogen
ER
?
Cyclin D1
cdk4/6
Cyclin D1
cdk4/6
p21
pRb
E2F
Cyclin E
cdk2
p21
Cyclin E
cdk2
p21
C-myc
C-myc
max
pRb
E2F
Cyclin B
cdk1/cdc2
M
pRb
cdk1/cdc2
Cyclin B
MPF
Cyclin E
cdk2
Cyclin A
cdk2
Cyclin A
E2F
pRb
S
G2

The D cyclins form part of an early response to positive signals, acting as growth factor sensors (Winston & Pledger 1993). Once cyclin D binds to its catalytic subunit, either cyclin dependant kinase (cdk) 4 or 6, a holoenzyme complex is formed (**Figure 1.51**) (for review see Norbury & Nurse 1992; Reed *et al.* 1992; Sherr 1993; Morgan 1995). This complex, lead by the cyclin subunit, transverses into the nucleus where a cdk-activating kinase (CAK), composed of cyclin H and cdk7, phosphorylates the cdk at a conserved threonine residue to fully activate the cyclin D/cdk complex (Fisher & Morgan 1994). In the presence of mitogenic signals, cyclin D-cdk activity will increase in the middle to late G1. However, if these factors, in particular ERK, are removed, the cyclin subunits are not able to competently bind to their cdk, suggesting that the signalling cascade are able to also promote the existence of the cyclin D-cdk complex (Matsushime *et al.* 1994). Conversely, if these factors are removed at a later stage of the cycle, the degradation of cyclin D has no effect and the cell continues to cycle (Matsushime *et al.* 1994). Therefore, both cyclin D transcription and complex formation with its cdk are reliant upon extracellular signals. Cyclin D is then responsible for progression of the cell through the restriction point, its importance observed in the studies where a 50% decline in cyclin D1 totally inhibits cell cycle progression (Carroll *et al.* 2000).

The critical target for cyclin D-cdk4/6 is phosphorylation of the retinoblastoma protein (pRb) (**Figure 1.51**) (for review see Ewen 1994; Weinberg 1995). The pRb is a tumour suppressor protein that remains in an unphosphorylated state throughout the early G1 phase, however is phosphorylated in mid G1 by two generally accepted events. Firstly, the cyclin D1-cdk4/6 complex binds directly to the pRb or pRb like proteins (p130 and p107), collectively known as pocket proteins, to phosphorylate and thus instigate the inactivation of pRb (reviewed by Dowdy *et al.* 1993; Weinberg 1995; Taya 1997). The pRb can interact with approximately 50 different proteins (Mulligan & Jacks 1998), 6 of these belonging to a family of transcription factors known as E2F (Chellappan *et al.* 1991). When the E2Fs are bound to the pRb, the Rb protein represses their ability to transcribe genes crucial for cell cycle progression by blocking their transcription activation domain (Dyson 1998). However phosphorylation of pRb by cyclin D1-cdk4/6 leads to its partial

inactivation, thus causing it to release its hold on some of the E2F's (Chellappan *et al.* 1991).

Secondly, the pRb is fully phosphorylated through to the late mitotic phase by the assistance of the cyclin E-cdk2 complex (**Figure 1.51**) (Lundberg & Weinberg 1998). It appears as if the pRB is required to be initially phosphorylated by cyclin D1/cdk4 in mid G1, as this is suggested to unmask a second cdk phosphorylation site on the Rb protein to allow for cyclinE/cdk2 interaction (Ezhevsky *et al.* 1997). The activation of cyclinE/cdk2 then leads to the complete release of E2Fs towards the end of G1. These now free E2Fs, by an as yet unknown mechanism, permit the coordinated transcription of specific genes required for S phase entry, including cell cycle proteins such as cyclin E, cyclin A and cdk2, c-myc, and B-myb, also DNA synthesis proteins such as DNA polymerase α and thymidine kinase (for review see Helin 1998; Dyson 1998). In fact, one of the genes that E2F transcribes is cyclin E, which leads to a positive feedback of cell cycle progression (Strausfeld *et al.* 1996). It is at this point that cell cycle progression is no longer under the mitogen stimulated cyclin D stimulation, but is now under cyclin E activation and is progressing independently of mitogens.

1.512 Progression through the cell cycle

Once the cell enter the S phase, cyclin E is degraded, releasing cdk2 to allow it to form a complex with cyclin A. Cyclin A and cyclin B dependant kinases then act to maintain pRb in a phosphorylated state until the cells have undergone mitosis (**Figure 1.51**) (Ludlow *et al.* 1990; Ludlow *et al.* 1993).

There exists two A type cyclins, A1 and A2, with the original cyclin A renamed A2 after the discovery of A1. Cyclin A2 is found in numerous tissues (Sweeney *et al.* 1996), whereas the expression of A1 is restricted to the testis and certain hematopoietic progenitor cells (Yang *et al.* 1997; Yang *et al.* 1999b). After progression through the S phase, cyclin E is degraded, releasing cdk2, which can now bind to cyclin A1 to form an active complex.

In fact, cyclinE/cdk2 is crucial for the activation of cyclin A1/cdk2, also playing a crucial role in the transcription of cyclin A1 through the release of free E2Fs (Xu *et al.* 1994; Krek *et al.* 1995). Cyclin A1 first appears at the G0 phase and rises gradually to reach elevated levels at the S and G2/M phases, whereas A2 appears later with a rapid increase in its levels, both then localising to the nucleus (Yang *et al.* 1999a). Both cyclin A1 and A2 promote S phase entry by binding to the pRb family members, p107 and p130 (Cao *et al.* 1992; Devoto *et al.* 1992), thus maintaining the pRb in a phosphorylated state during the S phase. As cyclin A1/A2 do not appear until the cell is about to progress through the S phase (**Figure 1.51**), it is suggested that they are also required for the transition through the G2/M (Pagano *et al.* 1992).

Cyclin B is first synthesised in the cytoplasm of the S phase, before it enters the nucleus during membrane degradation to complex with cdk1/cdc2 (**Figure 1.51**) (for reviews see Hartwell & Weinert 1989; Hunter & Pines 1994). This forms the M-phase promoting factor (MPF), whose primary role is to control both entry into and exit from the mitotic (M) phase, by maintaining the pRb in a phosphorylated state to allow for the specific transcription of M phase specific genes. Levels of cdk1/cdc2 remain constant throughout the cycle, whereas cyclin B increases to peak at the G2/M phase. Cyclin B is then rapidly degraded during anaphase, releasing cdk1/cdc2, which in turn inactivates the MPF and causes the cell to leave mitosis.

1.513 Cell cycle inhibitors

Cyclin-cdk activity can be inhibited by the presence of cdk-inhibitors (for review see Elledge & Harper 1994; Jacks & Weinberg 1998; Sherr & Roberts 1999). This family of structurally related cdk inhibitors inhibits cyclin D, E and A kinases, and includes $p21^{Cip1/Waf1/Cap20/Pic1/SDI1}$ (Harper *et al.* 1993; el Deiry *et al.* 1994; Xiong *et al.* 1993), $p27^{Kip1}$ (Polyak *et al.* 1994; Toyoshima & Hunter 1994) and $p57^{Kip2}$ (Lee *et al.* 1995). These Cip/Kip inhibitors play an important role in passage through the restriction point,

with p21 and p27 inhibiting cyclin-cdk complexes such as cyclin E-cdk2 and cyclin A-cdk2.

In a non-cycling cell, p27 is abundant, however when the cell begins to proliferate, the levels of p27 decline (Sherr & Roberts 1995). In contrast, p21 levels are low in the non-cycling cell but increase during S phase. Another significant role for cyclin D, other than to phosphorylate the pRb, is to sequester p21 and p27 inhibitors away from the cyclin E-cdk2 to allow its activation in the late G1 phase (**Figure 1.51**) (Sherr & Roberts 1995). Once the cdk inhibitors have bound to cyclin D, the cyclin D-cdk-cdk inhibitor complex is not itself inhibited, but rather further activated, stabilising the interaction between cyclin D and cdk4/6 and then translocating it into the nucleus (Cheng *et al.* 1999). Once a certain level of inhibitor has been removed from the cyclin E-cdk2 by cyclin D, this complex is now part active and is able to complete its full activation through phosphorylating and thus causing the degradation of p27 (Sheaff *et al.* 1997). Both p21 and p27 remain bound to the cyclin D-cdk for the remainder of the cell cycle (Zhang *et al.* 1994). However, when mitogenic stimulation is removed, cyclin D synthesis ceases and any residual cyclin D is degraded. This causes the release of the inhibitors from the cyclin D complex, allowing them to inhibit cyclinE-cdk2 and arrest the cell in G0.

p21 is not only activated by mitogenic signals, but is also a critical downstream repressor of cell cycle arrest in response to DNA damage. p53 is a protein not directly involved in the cell cycle, however plays an important role as a checkpoint controller (for reviews see Hinds & Weinberg 1994; Cox & Lane 1995). It recognises DNA damage to a cell's genome and halts the cell cycle at the G1 phase by activating p21 through its p53 binding site in its promoter region (el Deiry *et al.* 1993). The importance of p21 in the G1 checkpoint is observed in mice lacking a functional p21 protein. These mice presented with severe deficiencies in their ability to arrest the cell in G1 in response to DNA damage, however p53 induced apoptosis was still able to occur, suggesting the inducement of other factors (Deng *et al.* 1995). The suppression of cyclin E-cdk2 activity through p21 prevents the phosphorylation of pRb and the consequential activation of the E2F transcription

factors. This cell cycle arrest is to enable the DNA to be repaired before S phase replication. If the damage is severe and unable to be repaired, or if the levels of E2F increase even when p53 is elevated, then p53 is able to induce apoptosis (Wu & Levine 1994). This occurs through the induction of pro-apoptotic proteins such as the bcl-2 family (**Section 1.522**). This family of inhibitors can also bind directly to the A type cyclins (Hall *et al.* 1995).

A second family of inhibitors exist known as the INK4 proteins, as they are specific inhibitors of only cdk4 or cdk6. They are structurally related to the first Cip/Kip family with their activation blocking G1 progression causing cell cycle arrest. There are 4 subtypes based on molecular weight ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$) (Serrano *et al.* 1993; Sherr & Roberts 1999). How these inhibitors are induced is not well understood, however it is known that p15 is stimulated by TGF-β (Hannon & Beach 1994), p16 is only present in aging cells (Alcorta *et al.* 1996) and p18 and p19 are expressed only during foetal development (Phelps *et al.* 1998). Psossible hypotheses for how the INK4 proteins are able to inhibit cell cycle progression are as follows. Once cdk4 has undergone folding it has formed a complex with cyclin D, the Kip/Cip inhibitors may prevent translocation of this complex into the nucleus and cell cycle progression. Alternatively, the INK4 inhibitors can bind to cdk4, which prevent its interaction with cyclin D (McConnell *et al.* 1999; Parry *et al.* 1999). Due to cyclin D being unable to bind to its catalytic partner it subsequently degrades. Thus, p21 and p27 remain bound to cyclin E, are cyclin D is unable to sequester them, preventing phosphorylation of pRb and cell cycle progression.

In summary, progression through G1 is controlled ultimately by the phosphorylation of pRb, which releases E2F to promote the transcription of S phase specific genes. This can only occur if cyclin D, expressed via mitogenic stimulation, sequesters the cdk inhibitors away from the cyclin E-cdk2 complex to allow this kinase to phosphorylate pRb. Thus cyclin D has 2 functions, firstly to phosphorylate pRb, and secondly to sequester Cip/Kip inhibitors away from cyclin E-cdk2 so it can phosphorylate pRb. Once a certain level of inhibitor has been removed from the cyclin E-cdk2, and this complex is now active and

able to degrade p27, the cell cycle becomes independent of mitogens and is now committed to undergoing mitosis. As the cell progress through the S phase, cyclin E is degraded, and then A1 in the G2 phase. Now the cell requires cyclin D once again for entry into the G1 phase of the cell cycle.

1.52 Apoptosis

Apoptosis, or programmed cell death, is as essential as mitosis itself. Acting as a control system to remove the many genetic errors that occur during cell division, and also functioning to control the constant renewal of specific tissues during normal mammalian development. This process of cell death is not, as once believed, the uncontrolled destruction of a cell, but is in fact a highly ordered series of events controlled by the cell itself (for review see Wyllie *et al.* 1980; Vaux & Strasser 1996; Ameisen 1996; Guo & Hay 1999). Thus, in all cells, apoptosis is defined simply as a cell suicide program, characterised by structural changes such as cell shrinkage, blebbing of the plasma membrane, cytoplasmic vacuolisation and chromatin condensation (Wyllie 1993). Once the cell is broken down into membrane bound apoptotic bodies, they are quickly phagocytosed by scavenger macrophages or neighbouring cells, preventing an inflammatory response (for review see Savill & Fadok 2000). If a cell fails to fully die through the apoptosis process, then it may become necrotic. However, during this passive process of death the cell leaks internal contents and thus induces an undesirable inflammatory response (for review see Ameisen 1996; Hengartner 2000).

Apoptosis may be stimulated by numerous factors, including reactive oxygen species, irradiation, imprecise DNA sequences, incorrect level of cyclins, drugs, toxins, growth factors, hormones or in the immune response to viral infections (for review see Vaux & Strasser 1996). All these stimuli will activate one of two largely independent pathways; the death receptor pathway, in which apoptosis is induced by extracellular signals, or the mitochondrial pathway, which signals cell death by sensing intracellular disruptions such as DNA damage. These pathways can also interact with many other signalling pathways

such as that involving TNF, leading to NF-kappa B activation (for review see Wyllie *et al.* 1980; Vaux & Strasser 1996; Hengartner 2000; Schneider & Tschopp 2000).

1.521 Death receptor pathway

The death receptor pathway is activated by members of the death receptor superfamily (**Figure 1.52**), including CD95, TNFR1 (Baker & Reddy 1998) and FAS (Watanabe-Fukunaga *et al.* 1992). Binding of the specific ligand, for instance FASL, to its type II transmembrane receptor, FAS, induces clustering of the receptors (**Figure 1.52**) (Nagata & Golstein 1995). These form a death-induced complex that recruits FADD (FAS associated death domain) (Chinnaiyan *et al.* 1995). The formation of this complex, and thus FAS induced signal transduction, can be potentiated by FAF (FAS associated factor) or inhibited by FAP-1 (FAS associated phosphatase) (Sato *et al.* 1995). The FAS receptor is universally localised in the body, however the ligand is tissue specific, believed to be only expressed in immunologically privileged sites such as the eye, thyroid gland and the testis (Suda *et al.* 1993; French *et al.* 1996). Not only does the FAS system play an important role in the regulation of the immune system, including the maintenance of B and T cell tolerance and in the control of immune privileged sites (Griffith *et al.* 1995; Nagata & Golstein 1995), it also is crucial in maintaining homeostasis in organs such as the liver, lung and heart (Adachi *et al.* 1995), in the regulation of red blood cells (De Maria *et al.* 1999) and in the maintenance of the intestinal epithelium (Inagaki-Ohara *et al.* 1997).

This FAS membrane bound signalling complex begins the intracellular death pathway by recruiting procaspase-8. Caspases are cysteine proteases that are synthesised in an inactive form (for review see Cohen 1997). They are activated by the removal of their N-terminal pro-domain, enabling them to selectively cleave specific target proteins at aspartate-XXX sites. They can be classed into two major groups based on the length of their N terminal pro-domain. Those with a long pro-domain, or type I caspases, are initiator caspases. The simplest way to activate a caspase is to expose it to another activated one, thus type I caspases begin the apoptosis cascade by cleaving the pro-domain of, and thus activating,

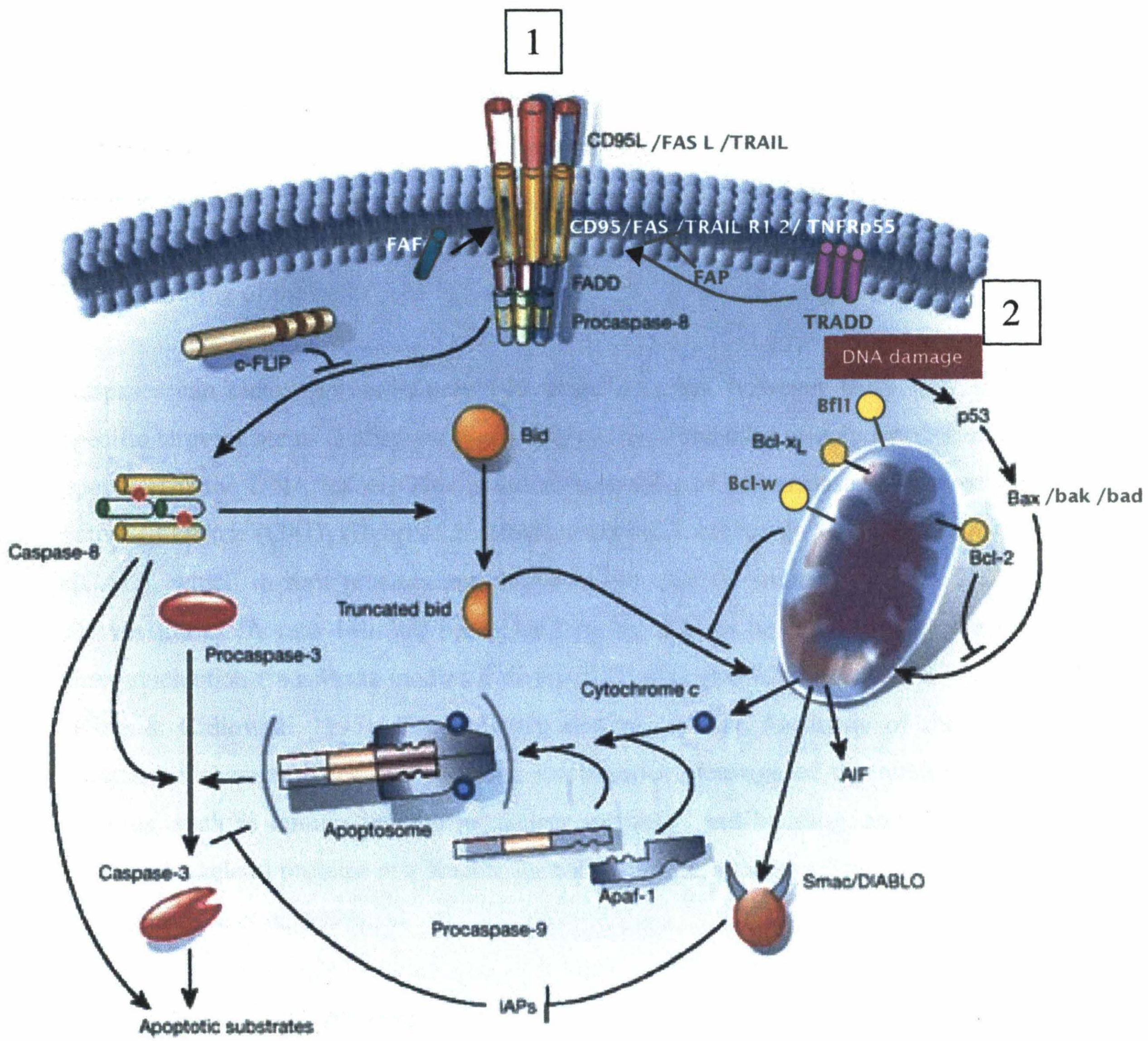
Figure 1.52 The two major apoptotic pathways in mammalian cells

(1) *The death receptor pathway.* This pathway is initiated by extracellular signals. Binding of the ligand, eg. FASL, CD95L and TRAIL (TNF related apoptosis induced ligand) to its death receptor (FAS, CD95, TRAIL R1 and R2, TNFRp55) forms a complex that recruits adaptor molecules. For eg. FAS binds FADD (FAS associated death domain) directly. FAS signal transduction can then be inhibited through FAP-1 (FAS associated phosphatase) or potentiated through FAF (FAS associated factor). The adaptor molecules recruit pro-caspase-8, which is cleaved to form active caspase-8. This begins the caspase cascade that eventually leads to the cleavage and inactivation of target proteins associated with cell survival.

(2) *The mitochondrial pathway* is activated by intracellular signals such as DNA damage. These lead to an activation of p53, a tumour suppressor protein which induces apoptosis by activating a pro-apoptotic member of the bcl-2 family eg. bax, bak or bad. Anti-apoptotic members, eg. bfl1, bcl-x, bcl-w and bcl-2, are attached to intracellular membranes. If apoptosis is to occur, then pro-apoptotic members meet at the surface of the mitochondria and cause the release of cytochrome C by an unknown mechanism. Cytochrome C then forms a complex with Apaf-1 and pro-caspase 9 to form the apoptosome.

The two pathways converge at the level of caspase 3.

Reproduced from Hengartner *et al* (2000)


1
CD95L /FAS L /TRAIL
FAF
CD95/FAS /TRAIL R1 2/ TNFRp55
FAP
FADD
Procaspase-8
TRADD
2
c-FLIP
DNA damage
Bfl1
p53
Bid
Bcl-xL
Bcl-w
Bax /bak /bad
Caspase-8
Bcl-2
Truncated bid
Procaspase-3
Cytochrome c
AIF
Apoptosome
Caspase-3
Apaf-1
Smac/DIABLO
Procaspase-9
IAPs
Apoptotic substrates

type II 'effector' caspases (for review see Thornberry 1997). In the cell, apoptosis begins with the cleavage of numerous procaspase–8, which have been recruited to the death receptor complex, to form caspase-8. Capase-8 is termed the initiator caspase in the death receptor pathway, and can be inhibited by the protein, c-FLIP (Irmler *et al.* 1997). Caspase-8 activates the 3 short pro-domain effectors caspases, 3, 6 or 7, which then begin the dismantling of the cell.

Caspases can cleave approximately 100 target proteins, however these may not all be specific target proteins (Earnshaw *et al.* 1999). One of the most widely recognised signs of apoptosis is the DNA ladder. This is due to activation of the caspase designated caspase-activated DNase (CAD) (Enari *et al.* 1998). Caspase 3 cleaves the CAD inhibitory subunit (ICAD), which in turn releases and activates the catalytic subunit (Nagata 2000). This cleaves the DNA into 180-200 base pair lengths, able to be visualised by the terminal deoxynucleotide transferase-mediated deoxy-UTP nick end labelling (TUNEL) technique (King & Cidlowski 1995). Caspases are also responsible for many of the other well recognized characteristics of apoptosis, for instance cleavage of the nuclear membrane proteins, such as lamins, leading to nuclear shrinkage and budding, and cleavage of the major cytoskeletal proteins responsible for cell structure, such as gelsolin (Kothakota *et al.* 1997; Buendia *et al.* 1999).

1.522 Mitochondrial pathway

This apoptotic pathway is activated in response to intracellular signals and damage such as DNA damage (**Figure 1.52**) (for reviews see Rich *et al.* 2000; Hengartner 2000). In response to these cues, the proto-oncogene p53 is activated. As has been described above (**Section 1.513**), p53 has been found to have 2 major roles. Firstly as a checkpoint to inhibit cell growth when required, for instance when the DNA is in want of repair, and secondly to mediate apoptosis when the DNA damage is large scale and thus unable to be repaired (Nelson & Kastan 1994). p53 promotes apoptosis by transcriptionally activating

pro-apoptotic members of the bcl-2 family of proteins through binding to its binding sites in their promoter regions (Miyashita *et al.* 1994; Miyashita & Reed 1995).

The bcl-2 (named after the first member involved in b-cell lymphoma) family can be divided into 3 groups based on structural similarities; members of group I are death protectors, whereas group II and III promote cell death (for review see Chao & Korsmeyer 1998; Adams & Cory 1998; Antonsson & Martinou 2000). All members of the bcl-2 family can homodimerise but most importantly can heterodimerize with each other (Basu & Haldar 1998). In this respect they are able to control each other's action. Pro-apoptotic members (bax, bad, bak, bim, bcl-Xs, bid, bik, hrk, bok) are free to move within the cytoplasm and all converge on the mitochondrial membrane, whereas anti-apoptotic members of this family (bcl-2, bcl-xL, bcl-w, mcl-1, A1/bfl-1) are anchored to intracellular membranes (for review see Adams & Cory 1998; Evan & Littlewood 1998). Both pro- and anti-apoptotic members meet at the surface of the mitochondria to oppose each other's action, for instance bax can directly bind to bcl-2 to neutralize its survival action (Oltvai *et al.* 1993). If the cell is to die, cytochrome *c* and various other pro-apoptotic factors are released from the mitochondria into the cytoplasm, anti-apoptotic members acting to inhibit this release (Kluck *et al.* 1997). In this respect, the mitochondria appears to play two major roles; firstly for cell survival in the process of glucose oxidation using cytochrome *c* as an electron carrier, and secondly in cell death through employing the same cytochrome *c* as a death molecule. It is a yet undetermined how cytochrome *c* manages to cross the mitochondrial membrane, however it does involve the assistance of the pro-apoptotic bcl-2 family possibly in the formation of membrane pores or anion channels (Gross *et al.* 1999). Once released, cytochrome *c* binds with the adaptor protein Apaf-1 to activate procaspase-9 and to form a holoenzyme known as the apoptosome (Zou *et al.* 1997; Cain *et al.* 1999).

1.523 Crosstalk between the two pathways

Both the death receptor and the mitochondrial pathways operate largely independent of each other, however some cross talk does exist. For instance, it has been suggested that it is the caspases that signal the demise of the cell, whereas others say it is the release of factors from the mitochondria that signals irreversible cell death. However, it has been found that the death receptor pathway often circumvents the mitochondrial pathway, with cytochrome *c* release the result of caspases breaking down the nuclear membrane, not the involvement of the bcl-2 family (Loeffler & Kroemer 2000). However, bcl-x and bcl-2 have been found to interact between these two pathways, inhibiting the loss of the mitochondrial membrane potential and thus the release of apoptotic factors induced in FAS treated cells (Boise & Thompson 1997) and in cells induced to undergo apoptosis by a variety of cancer therapy drugs (Decaudin *et al.* 1997).

The two pathways can also directly converge at the level of caspase 3 (**Figure 1.52**), with the apoptosome and caspase-8 both cleaving pro-caspase-3 to form active capase-3 (Hengartner 2000). This cleavage by the apoptosome can be inhibited by IAPs (Inhibitor of Apoptosis Proteins), present to prevent accidental activation of caspase-3. Therefore, to absolutely ensure the death of the cells, pro-apoptotic factors, other than cytochrome *c*, are released from the mitochondria. These include Smac (second mitochondria-derived-binding protein with low pI) and DIABLO (direct IAP-binding protein with low pI), which are released primarily to antagonise the inhibitory capacity of the IAP proteins, thus allowing for the activation of caspase-3 and for cell death to proceed (for review see Hengartner 2000). Bcl-x also forms part of the apoptosome with Apaf-1 and caspase-9 to promote cell death (Pan *et al.* 1998). They also converge upstream in the pathway, with caspase-8 cleaving bid, a pro-apoptotic member, which in turn further promotes cytochrome *c* release (Gross A 1999, Yin X 1999).

Mitochondrial members, such as bcl-xL have also been found to protect cells from FAS induced apoptosis in the immune system, specifically in B and T cells (Boise L *et al* 1997;

Castedo M *et al* 1997), suggesting that the pathways are more complicated than first assumed. However, even though both apoptotic pathways contain some interactions, these can only be to fully insure that a cell will either die or live.

1.53 Cell cycle progression and apoptosis in the testis

The process of spermatogenesis begins with the clonal expansion of stem spermatogonia, then a constant series of mitosis, meiosis and differentiation. However not all germ cells survive, with apoptosis crucial in order to keep a regulated balance between germ cell number and the support capacity of the Sertoli cells (for review see Kierszenbaum 2001). Constant progression through the cell cycle, and thus the prevention of apoptosis, can be induced via growth factors, cytokines and hormones. Hormones are particularly important in normal cycling, their withdrawal from hormone dependant organs of the body causing a massive wave of apoptosis. This has been well studied in female reproductive organs such as the ovary, with follicular atresia, and during the regression of the post lactational mammary gland (see Kiess & Gallaher 1998). But is also observed in the male, such as in the prostate following castration (Denmeade *et al.* 1996).

1.531 Cell cycle gene expression in the testis

For spermatogenesis to begin, stem spermatogonia must undergo a series of mitoses to build up the population of germ cells (**Section 1.32**). These cells then halve their chromatin within two meiosis steps to form the truly haploid germ cell. Division now halts and the round spermatids differentiate into the recognisable elongated spermatid. Cell cycle genes are thus an essential component of spermatogenic progression, with the removal of these severely detrimental. As would therefore be expected, all of the cyclins and their cdk partners are expressed in the testis in a cell specific fashion (**Table 1.531**) (for review see Wolgemuth *et al.* 1995).

Table 1.531 Published studies investigating the localisation of cell cycle genes in the rodent testis

	Germ cells	Spermatogonia	Spermatocytes	Round spermatids	Elongated spermatids	Sertoli cells	Leydig cells
Cyclin A1	**R** m- (Liu *et al.* 1998)		**P** m- (Ravnik & Wolgemuth 1999) **R** m- (Sweeney *et al.* 1996)				
Cyclin A2		**R** m- (Ravnik & Wolgemuth 1996) **P** m- (Ravnik & Wolgemuth 1996; Ravnik & Wolgemuth 1999)	**R** m- (Ravnik & Wolgemuth 1996) **P** m- (Ravnik & Wolgemuth 1996; Ravnik & Wolgemuth 1999)				**R** r- (Ge & Hardy 1997)
Cdc2/cdk1			**R** m-(Rhee & Wolgemuth 1995) **P** r-(Godet *et al.* 2000) m- (Chapman & Wolgemuth 1994; Ravnik & Wolgemuth 1999)			**R** m- (Rhee & Wolgemuth 1995)	
Cdk2		**P** m- (Ravnik & Wolgemuth 1999)	**P** m- (Ravnik & Wolgemuth 1999) **R** m- (Rhee & Wolgemuth 1995)			**R** m- (Rhee & Wolgemuth 1995)	
Cyclin B1			**R** r- (Gromoll *et al.* 1997) **P** m- (Chapman & Wolgemuth 1994) (Liu *et al.* 2000) r- (Godet *et al.* 2000)	**R** m- (Chapman & Wolgemuth 1992; Chapman & Wolgemuth 1994) r- (Gromoll *et al.* 1997) **P** r- (Gromoll *et al.* 1997)			
Cyclin B2			**R** m- (Chapman & Wolgemuth 1993)				
Cyclin D1		**P** m- (Beumer *et al.* 2000)				**R** m- (Ravnik *et al.* 1995) **P** r- (Musa *et al.* 1998)	
Cyclin D2		**P** m- (Beumer *et al.* 2000)	**P** m- (Beumer *et al.* 2000)	**P** m- (Beumer *et al.* 2000)			
Cyclin D3		**P** m- (Beumer *et al.*	**P** r- (Kang *et al.* 1997)	**R** m- (Ravnik *et al.*	**P** m- (Zhang *et al.*	**P** m- (Zhang *et al.*	**P** m- (Zhang *et al.*

	2000; Zhang *et al.* 1999)		1995) **P** m- (Zhang *et al.* 1999)	1999)	1999) (Beumer *et al.* 2000)	1999; Beumer *et al.* 2000) r- (Sriraman *et al.* 2000)
Cdk4	**P** r- (Kang *et al.* 1997)	**P** r- (Kang *et al.* 1997)			**R** m- (Rhee & Wolgemuth 1995)	
Cyclin E					**P** r- (Musa *et al.* 1998)	**P** r- (Musa *et al.* 1998)
P21		**P** m- (Saberan-Djoneidi *et al.* 1998; Beumer *et al.* 1997)	**P** m- (Beumer *et al.* 1997)	**P** m- (Saberan-Djoneidi *et al.* 1998)		
PRb	**P** r- (Yan *et al.* 1997)	**R** r- (Yan *et al.* 1997)	**R** r- (Yan *et al.* 1997)	**R** r- (Yan *et al.* 1997) **P** r- (Yan *et al.* 1997)	**P** r- (Yan *et al.* 1997)	
Cdk5					**P** r- (Musa *et al.* 1998)	**P** r- (Musa *et al.* 1998)

The demonstration of either protein (**P**) or mRNA (**R**) is donated by a capital letter. The species, either mouse (**m**) or rat (**r**) are denoted by a lower case letter. Protein localisation was determined by immunocytochemistry or Western blot, with mRNA localisation by RNA protection assay, RT-PCR, insitus hybridisation or Northern blot.

i Cyclin A

Continual progression through the cell cycle is highly reliant on the later expressed cyclins, A and B (**Section 1.512**). Two cyclin A subtypes exist, both with distinct localisation in the testis (Sweeney *et al.* 1996; Ravnik & Wolgemuth 1996). For example, mouse cyclin A1 mRNA and protein is present just before and during the first meiotic division, but is undetectable during the second meiosis stage of spermatogenesis (Sweeney *et al.* 1996; Ravnik & Wolgemuth 1999). Whereas, cyclin A2 mRNA and protein is expressed predominantly in earlier germ cells, the spermatogonia and preleptotene spermatocytes (**Table 1.531**) (Ravnik & Wolgemuth 1996; Ravnik & Wolgemuth 1999), and also in developing Leydig cells (Ge & Hardy 1997). The importance of cyclin A1 is observed in mice with a targeted disruption of this gene (Liu *et al.* 1998). Unlike the cyclin A2 knockout mouse, which dies *in utero*, cyclin A1 was not required for survival, but was essential for spermatogenic progression through meiosis. Therefore both cyclin A subtypes appear to play differing roles in both mitosis and meiosis.

Cyclin A1 partners both cdk1/cdc2 and cdk2, whereas cyclin A2 binds only cdk2 (Ravnik & Wolgemuth 1999). Both cdk1/cdc2 mRNA and protein are highly expressed in similar cell types to cyclin A1, specifically pachytene to diplotene spermatocytes in both rats and mice (**Table 1.531**) (Ravnik & Wolgemuth 1999; Godet *et al.* 2000), levels decreasing once they transitioned into round spermatids. The mRNA is also found in Sertoli cells (Rhee & Wolgemuth 1995). Cdk2 mRNA and protein also display similar cellular expression, with high levels in the spermatocytes, with lower in the spermatogonia and Sertoli cells (**Table 1.531**).

ii Cyclin B

Cyclin B1 functions at the G2/M phase of the cell cycle, stimulating the progression into mitosis. In the rodent testis, cyclin B1 mRNA was predominantly expressed in the late pachytene spermatocytes decreasing as they progressed into the postmeiotic round spermatids in rodents (**Table 1.531**) (Chapman & Wolgemuth 1994; Gromoll *et al.* 1997; Godet *et al.* 2000). The finding that the postmeiotic early round spermatids may also

express cyclin B1 suggests that it may play an important role in spermatid differentiation (Gromoll *et al.* 1997). Cyclin B1 protein expression is crucial for cell cycle progression, in its absence spermatogonia and early primary spermatocytes are unable to complete their progression through the cell cycle, resulting in arrested spermatogenesis (Kong *et al.* 2000). Cyclin B2 is also most abundant in the pachytene spermatocytes (Chapman & Wolgemuth 1993) with levels also found in the spermatogonia (Liu *et al.* 2000). The MPF (M-Phase promoting factor) transcripts (mouse) and complex (rat), consisting of the cyclin B1 and cdk1/cdc2, were in highest levels in spermatocytes just before the first meiotic division, decreasing at the end of meiosis (Rhee & Wolgemuth 1995; Godet *et al.* 2000). Knockout mice have been created who lack both cyclin B1 and B2 (Brandeis *et al.* 1998). Mice lacking cyclin B1 died *in utero*, whereas cyclin B2 knockout mice survived, however males appeared to have no detrimental abnormalities in their fertility. This may suggest that one of the B type cyclins may compensate for the other.

iii Cyclin D

The D-type cyclins, which initiate entry into the cell cycle, have all been localised to the testis in the mouse and rat. Specifically, cyclin D1 mRNA and protein was found in the non-dividing Sertoli cells (Ravnik *et al.* 1995; Musa *et al.* 1998), whereas the protein has only been localised to spermatogonia in mice (**Table 1.531**) (Beumer *et al.* 2000a). Cyclin D2 has also been immunolocalised in mice to the spermatogonia, but also to the spermatocytes and spermatids (Beumer *et al.* 2000a). The importance of cyclin D2 can be seen when it is disrupted, with male knockout mice displaying a severe reduction in testicular weight and a 2-3 fold decline in spermatozoa concentration (Sicinski *et al.* 1996). It has also been observed that following neonatal testis injury, such as that inflicted by DEHP (di-(2-ethylhexyl) phthalate), multinucleated cells appear, Sertoli cell proliferation is decreased, and there is a decline in the expression of cyclin D2 (Li *et al.* 2000). Cyclin D3 mRNA was found in highest levels in the round spermatids of the mouse testis (Ravnik *et al.* 1995), however the protein was detected in almost all of the germ cells, including abundant levels in the spermatogonia, lower levels in the pachytene spermatocytes with the protein level then increasing again to reach high levels in the elongated spermatids (Zhang

et al. 1999; Beumer *et al.* 2000a). Regarding expression in the rat, there has been one report where the protein was detected primarily in the early spermatocytes (Kang *et al.* 1997), therefore suggesting some species difference. In both rodents, cyclin D3 protein has been found in the proliferating and differentiated Leydig cells and in mouse Sertoli cells (**Table 1.531**). Thus, cyclin D3 appears to play its well known role during spermatogonial mitosis, but may also plays a role in chromatin remodelling in the non-proliferating spermatids, Leydig and Sertoli cells.

The catalytic subunit of the cyclin D complex, cdk4, has been located in the spermatogonia and early primary spermatocytes of rats (Kang *et al.* 1997), with its mRNA found in the Sertoli cells of mice (Rhee & Wolgemuth 1995). The importance of cdk4 in spermatogenesis can be seen when it is removed by targeted disruption, resulting in hypoplastic seminiferous tubules (Tsutsui *et al.* 1999).

iv Cyclin E

The other important cyclin for progression through G1 is cyclin E, also expressed in the adult testis. One study, using rat tissue, localised this cyclin specifically in both Sertoli and Leydig cell lines (Musa *et al.* 1998). While its catalytic partner, cdk2, was localised in mice to Sertoli cells, some spermatogonia and high levels in the meiotic spermatocytes (**Table 1.531**) (Rhee & Wolgemuth 1995; Ravnik & Wolgemuth 1999).

Before progression can occur, the cdk inhibitor p21 must be sequestered away from the cyclin E complex. p21 has been localised weakly from the pachytene spermatocytes to early step 5 round spermatids (Beumer *et al.* 1997; Saberan-Djoneidi *et al.* 1998), and in the flagella of late elongated spermatids (Saberan-Djoneidi *et al.* 1998). Once cyclin D1 and E are activated, they act to phosphorylate the retinoblastoma protein to release the E2F family of transcription factors from the hold of pRb. The pRb mRNA has been localised to the germ cells, specifically from stage V spermatocytes to step 13 spermatids (Yan *et al.* 1997). Whereas the protein is not transcribed until later in the cycle, observed in the elongated spermatids at step 14-19, the spermatogonia and Sertoli cells (**Table 1.531**). This

suggests that it may play a role in spermatogonia proliferation and in the maturation of spermatids. Once the E2F family of transcription factors are released, they are able to transcribe specific genes required for S phase entry. E2F overexpressing mice resulted in sterility through too much apoptosis (Holmberg *et al.* 1998), whereas mice lacking E2F experience testicular atrophy yet are fertile (Yamasaki *et al.* 1996).

The localisation of the cyclins and their cyclin dependant kinases in the different testicular cells suggests an important role not only in the control of both mitosis and meiosis, but also in the differentiation of both spermatids, Sertoli and Leydig cells.

1.532 Apoptosis in the testis

Germ cell apoptosis during development normally occurs in two major waves. In the mouse, the first occurs at day 13 of gestation when the primordial germ cells have populated the genital ridge and have ceased proliferating (Wang *et al.* 1998). The second wave of apoptosis occurs approximately from postnatal day 10 to 13 of development, coinciding with the first round of spermatogenesis (Mori *et al.* 1997; Wang *et al.* 1998). At this stage it is primarily spermatocytes that are undergoing cell death, with apoptosis crucial to the normal function of the testis (Huckins 1978; Bartke 1995; Rodriguez *et al.* 1997).

During adult spermatogenesis apoptosis still occurs, with A2, A3 spermatogonia, but also some A4 spermatogonia, spermatocytes and round spermatids, continuously and spontaneously undergoing apoptosis during stage I-XII (Huckins 1978; Allan *et al.* 1992; Blanco-Rodriguez & Martinez-Garcia 1996). In fact, only between 25-75% of A1 spermatogonia complete the transition through to a pre-leptotene spermatocyte, this actually being a clear representative of normal, healthy spermatogenesis. Apoptosis in the adult is important as it is believed to reduce the number of germ cells to a level able to be supported by the Sertoli cells, possibly even initiated by these cells themselves (Rodriguez *et al.* 1997; Boekelheide *et al.* 2000). However, various insults, such as toxic exposure or

hormonal withdrawal, greatly increases the incidence of apoptosis (Billig *et al.* 1995a; Shetty *et al.* 1996; Blanchard *et al.* 1996; Nandi *et al.* 1999) for review see (Sinha Hikim & Swerdloff 1999; Richburg 2000).

As the normal functioning of the testis is highly dependant on hormonal support, withdrawing androgens and pituitary gonadotrophins leads to an initiation of germ cell death. This is seen through hypophysectomy and GnRH antagonist treatment, both initiating apoptosis in pre-leptotene and pachytene spermatocytes, round and elongated spermatids at stage VII (Russell & Clermont 1977; Russell *et al.* 1987a; Tapanainen *et al.* 1993; Sinha Hikim *et al.* 1997), possibly by damage to Sertoli cell structure (Ghosh *et al.* 1992). However, removal of gonadotrophins does not cause apoptosis in the Leydig and Sertoli cells even though their development is dependant on these hormones (Kerr & Sharpe 1985; Almiron & Chemes 1988; Ghosh *et al.* 1992). FSH was found to be crucial for germ cell survival, observed when FSH was replaced following hypophysectomy and the number of degenerating cells significantly decreased (Tapanainen *et al.* 1993). Withdrawing testosterone, either by destroying the Leydig cells through EDS (ethane dimethane sulphonate) treatment (Sharpe *et al.* 1990) or by testosterone and oestradiol implants decreasing LH secretion from the pituitary (O'Donnell *et al.* 1994), also induced cell death in the germ cells. This occurred primarily in the round spermatids as they make their transition from step VII to VIII, not surprising as stages VII and VIII are known to be highly androgen dependant (**Section 1.36**).

The existence of DNA fragmentation suggested that the degeneration of the germ cells at different stages of the cycle, following the removal of hormonal survival factors, occurred almost exclusively through apoptosis (Billig *et al.* 1995a; Brinkworth *et al.* 1995). However, the molecular mechanisms behind apoptosis in the testis were still unclear.

i Mitochondrial pathway apoptotic genes

As apoptosis in the testis occurs during 2 waves of cell death, and spontaneously throughout maturation, the expression of the bcl-2 family are also developmentally

regulated. The first postnatal apoptotic wave occurs at approximately day 10 in the mouse when spermatogenesis is reinitiated. Regarding expression levels, it appears as if apoptosis may be initiated by bad, highly expressed in the spermatogonia and Sertoli cells (Meehan *et al.* 2001). The remaining germ cells may be protected by bcl-xL, also highly expressed in these cells. As the germ cells mature, by day 20, it is now bax and bak that may play an important role in reducing spermatogonial number to a level able to be supported by the Sertoli cells (Meehan *et al.* 2001). The remaining germ cells may now be protected by bcl-2, highly expressed in the spermatogonia and spermatocytes (Meehan *et al.* 2001). In the adult rodents (**Table 1.532**), germ cell apoptosis appears to involve a down regulation of bcl-2 localised to the spermatogonia (Furuchi *et al.* 1996), spermatocytes and round spermatids (Beumer *et al.* 2000b), and bcl-xL in the spermatogonia and spermatocytes (Beumer *et al.* 2000b). And an upregulation of bak, primarily found in the spermatogonia, spermatocytes (Yan *et al.* 2000) and round spermatids (Taylor *et al.* 1999). p53 is also elevated at the same time as bax (**Table 1.532**), and is important in the control of spontaneous germ cell apoptosis and removing defective germ cells from the spermatogenic cycle (Yin *et al.* 1998).

When the expression of apoptotic genes was studied in rodents in response to testosterone withdrawal, the pro-apoptotic bax was found to be upregulated (Woolveridge *et al.* 1999). As bax is expressed in abundant levels in both spermatocytes (**Table 1.532**) and round spermatids (Rodriguez *et al.* 1997; Beumer *et al.* 2000b), its upregulation may be instigating the death of these cells. A concurrent increase in the pro-survival bcl-2, localised to spermatocytes and round spermatids (Beumer *et al.* 2000b), may be in an attempt to protect the remaining germ cells (Woolveridge *et al.* 1999). However, other studies have found that withdrawing testosterone causes a decrease in the mRNA and protein expression of bcl-2 (Ohigashi *et al.* 1999). In the human testis, withdrawing androgens caused no alteration in the levels of bak, bax and p53 (Woolveridge *et al.* 1999). Therefore, hormones, such as androgens, appear to have a crucial role in the prevention of germ cell apoptosis by manipulation of the bcl-2 family of apoptotic proteins.

Table 1.532 Published studies investigating the localisation of apoptotic genes in the rodent testis

	Testis	Germ cells	Spermatogonia	Spermatocytes	Round spermatids	Elongated spermatids	Sertoli cells	Leydig cells
MITOCHONDRIAL PATHWAY GENES								
p53	**P** m- (Rodriguez *et al.* 1997) Rodriguez I et al	**P** m- (Yin *et al.* 1997)		**R** m- (Schwartz *et al.* 1993), **P** r- (Sjoblom & Lahdetie 1996; Fujisawa *et al.* 2001)				
bcl-w		**P** m- (Print *et al.* 1998)	**R** m- (Print *et al.* 1998; Meehan *et al.* 2001) r- (Yan *et al.* 2000) **P** r- (Yan *et al.* 2000) m- (Meehan *et al.* 2001)	**R** m- (Print *et al.* 1998; Meehan *et al.* 2001) r- (Yan *et al.* 2000) **P**r- (Yan *et al.* 2000)	**R** m- (Print *et al.* 1998; Meehan *et al.* 2001)	**P** m- (Ross *et al.* 1998)	**R** m- (Print *et al.* 1998) r- (Yan *et al.* 2000) **P** m- (Ross *et al.* 1998; Print *et al.* 1998; Meehan *et al.* 2001) r- (Yan *et al.* 2000)	**R** r- (Yan *et al.* 2000) **P** r- (Taylor *et al.* 1999; Yan *et al.* 2000)
bcl-2	**R** r- (Fujisawa *et al.* 2001)		**P** m- (Furuchi *et al.* 1996)	**P** m- (Beumer *et al.* 2000)	**P** m- (Beumer *et al.* 2000)			**P** m- (Beumer *et al.* 2000)
bok	**R** r - (Hsu *et al.* 1997)							
bcl-xL			**P** m- (Beumer *et al.* 2000)	**R** m- (Meehan *et al.* 2001) **P** m- (Beumer *et al.* 2000)	**R** m- (Meehan *et al.* 2001)			r- (Woolveridge *et al.* 2001)
bim			**R** m- (Meehan *et al.* 2001)	**R** m- (Meehan *et al.* 2001)	**R** m- (Meehan *et al.* 2001)	**R** m- (Meehan *et al.* 2001)	**R** m- (Meehan *et al.* 2001)	
bak			**R** m- (Meehan *et al.* 2001) **P** r- (Yan *et al.* 2000)	**R** m- (Meehan *et al.* 2001) **P** r- (Yan *et al.* 2000)	**R** m- (Meehan *et al.* 2001) **P** r- (Taylor *et al.* 1999)	**R** m- (Meehan *et al.* 2001)	**P** r- (Yan *et al.* 2000)	**P** r- (Yan *et al.* 2000; Woolveridge *et al.* 2001)
bax	**P** m- (Rodriguez *et al.* 1997)		**R** m- (Meehan *et al.* 2001) Rodriguez I et al	**P** m- (Rodriguez *et al.* 1997; Beumer *et al.* 2000)	**P** m- (Beumer *et al.* 2000)		**P** m- (Beumer *et al.* 2000) r- (Yan *et al.* 2000)	**P** m- (Beumer *et al.* 2000) r- (Yan *et al.*

			1997, **P** m- (Beumer *et al.* 2000; Rodriguez *et al.* 1997) r- (Yan *et al.* 2000)	r- (Yan *et al.* 2000)				2000)
bad				**R** m- (Meehan *et al.* 2001)	**R** m- (Meehan *et al.* 2001)			
DEATH RECEPTOR PATHWAY GENES								
FAS	**R** m- (Lee *et al.* 1999; Xu *et al.* 1999) r- (Lee *et al.* 1997; Lee *et al.* 1999) **P** m- (Xu *et al.* 1999) r- (Boekelheide *et al.* 1998)	**R** m- (Xu *et al.* 1999) **P** m- (Xu *et al.* 1999) r- (Richburg *et al.* 1999)	**P** m- (Ogi *et al.* 1998)	**R** m- (Xu *et al.* 1999) **P** m- (Ogi *et al.* 1998) r- (Lee *et al.* 1997)	**P** m- (Ogi *et al.* 1998) r- (Li *et al.* 1997)		**P** m- (Riccioli *et al.* 2000)	**R** m- (Koji *et al.* 2001)
FASL	**R** m- (Lee *et al.* 1999; Xu *et al.* 1999) r- (Lee *et al.* 1997; Lee *et al.* 1999; D'Alessio *et al.* 2001) **P** r- (Boekelheide *et al.* 1998)			**R** m- (D'Alessio *et al.* 2001) r- (D'Alessio *et al.* 2001)	**R** m- (D'Alessio *et al.* 2001) r- (D'Alessio *et al.* 2001)	**R** r- (D'Alessio *et al.* 2001) **P** m- (D'Alessio *et al.* 2001) r- (D'Alessio *et al.* 2001)	**R** m- (Xu *et al.* 1999) (Koji *et al.* 2001) **P** m- (Wang *et al.* 1998) r- (Lee *et al.* 1997; Richburg *et al.* 1999)	**P** r- (Li *et al.* 1997)
FADD	**P** r- (Boekelheide *et al.* 1998)							
FAP	**P** r- (Boekelheide *et al.* 1998)							
TRAIL	**P** r- (Boekelheide *et al.* 1998)							
TNFRp55	**P** r- (Boekelheide *et al.* 1998)							
RIP	**P** r- (Boekelheide *et al.* 1998)							
Caspase-8								

The demonstration of either protein (**P**), mRNA (**R**) is donated by a capital letter. The species, either mouse (**m**) or rat (**r**) are denoted by a lower case letter. Protein localisation was determined by immunocytochemistry or Western blot, with mRNA localisation by RNA protection assay, RT-PCR, *insitu* hybridisation or Northern blot.

Other insults, such as altering the strictly regulated temperature environment, either by inducing cryptorchidism in adults (Shikone *et al.* 1994) or incubating the testes at an increased temperature (Yamamoto *et al.* 2000), also causes the germ cells to undergo apoptosis. Chronic ethanol abuse also induces apoptosis in spermatogonia and spermatocytes, via a p53 induced mechanism (Zhu *et al.* 2000). This is not surprising considering p53 is highly abundant in both spermatogonia (**Table 1.532)** and zygotene-early pachytene spermatocytes at stages VIII-I (Rodriguez *et al.* 1997; Schwartz *et al.* 1993; Sjoblom & Lahdetie 1996). Another insult, testicular irradiation, again causes DNA damage leading to a stimulation of apoptosis via an upregulation of both p53 (Odorisio *et al.* 1998; Sjoblom & Lahdetie 1996; Hasegawa *et al.* 1998), bcl-2 and bcl-xL, however does not appear to involve bax (Beumer et al 2000; Beumer *et al.* 2000b). Suggesting that the immature germ cells were not induced to die via a bax pathway after irradiation, even though p53 directly regulates bax expression (Miyashita & Reed 1995).

The use of knockout and transgenic models has provided important information on the importance of the bcl-2 family members in regulating testicular apoptosis. Mice that lack bcl-xL die *in utero* as a consequence of massive apoptosis of hematopoietic and neuronal cells (Motoyama *et al.* 1995). However, primordial germ cell survival was studied during embryo development, showing a lack of spermatogonia. To fully examine the role of bcl-xL in the testis, this gene was specifically inactivated to create a bcl-xL hypomorphic mouse, or a gonad specific KO (Rucker, III *et al.* 2000). Mice that had two copies presented with severe infertility due to apoptosis of the primordial germ cells (PGC), suggesting that bcl-xL is required for the survival of the immature germ cells and thus the fertility of the adult. This is not surprising considering that bcl-xL is expressed in the immature spermatogonia (**Table 1.532**) (Beumer *et al.* 2000b).

To further analyse the possible importance of these survival factors, both bcl-2 (Furuchi *et al.* 1996) and bcl-xL (Rodriguez *et al.* 1997) were overexpressed. As both of these survival factors are expressed in spermatogonia (**Table 1.532**), this resulted in a massive accumulation of these cells, followed by a severe reduction in spermatocytes and

spermatids. However, there was normal proliferation of Sertoli cells and Leydig cells, consistent with these cells not expressing either of these factors (**Table 1.532**). Therefore, overexpressing pro-survival factors prevented the crucial wave of spermatogonial apoptosis, leading to an accumulation of premeiotic germ cells and an inability for spermatogenesis to be established. However, overexpression of these survival factors did not affect the sporadic apoptosis, which occurs normally in the adult spermatogonia, suggesting that this cell death is not affected by these apoptotic factors (Rodriguez *et al.* 1997).

Similar to this phenotype are mice that are deficient in the pro-apoptotic bax (Knudson *et al.* 1995). Bax is a heterodimeric partner of bcl-2, and is localised to the spermatogonia, spermatocytes and round spermatids (Rodriguez *et al.* 1997; Beumer *et al.* 2000b; Yan *et al.* 2000). However, bax is expressed only during the first postnatal wave of apoptosis, disappearing at maturity (Rodriguez *et al.* 1997). Removing bax leads to an increase in the numbers of premeiotic spermatocytes, with no mature round spermatids. They also showed multinucleated cells and disrupted seminiferous epithelium. Thus, again, removing an essential pro-death factor prevents the wave of apoptosis and the density regulation required for the progression of spermatogenesis.

Mice deficient in bcl-2 were found to develop phenotypically normally until later in life when they developed disorders such as motorneuron degeneration, lymphopenia and polycystic kidney disease (Veis *et al.* 1993; Kamada *et al.* 1995). However the testes were not examined. The only bcl-2 model in which the testis phenotype has been addressed is in mice deficient in bcl-w, of which two exist (Ross *et al.* 1998; Print *et al.* 1998). Although bcl-w is expressed in adult tissues such as salivary gland, brain, colon, liver, heart, skeletal muscle and stomach (Gibson *et al.* 1996), it appears to only have an essential function in the testis. The localisation of the bcl-w mRNA, in rodent spermatogonia, spermatocytes, round spermatids, elongated spermatids and Sertoli cells (**Table 1.532**), is consistent with the localisation of the protein, specifically in the spermatogonia, spermatocytes, step 10 to 16 elongated spermatids, Sertoli cells and Leydig cells (Ross *et al.* 1998; Print *et al.* 1998;

Yan *et al.* 2000). The bcl-w null males display infertility due to an age related degeneration of the testes (Ross *et al.* 1998). The spermatogenic phenotype is first observed in the first wave of spermatogenesis at day 19, with an increase in degenerating spermatocytes and an arrest in the formation of round spermatids. Then, as the animals' age, earlier germ cells are progressively lost until at 6 months of age only Sertoli cells remain. It is suggested that this may be a bak mediated factor (Print *et al.* 1998; Yan *et al.* 2000).

In summary, both the apoptotic waves, spontaneous apoptosis and the cell death instigated by insult, all involve members of the bcl-2 family of proteins.

***ii** The death receptor pathway apoptotic genes*

The death receptor pathway must also be considered when examining testicular apoptosis. The FAS receptor is ubiquitously expressed in the body, however the ligand is believed to be more tissue specific, expressed in highest levels in the immunologically privileged site of the testis (**Table 1.532**) (Suda *et al.* 1993; Suda *et al.* 1995). Due to this high level, the FAS signalling cascade has been implicated in germ cell apoptosis (Lee *et al.* 1997; Boekelheide *et al.* 1998; Richburg *et al.* 1999; Pentikainen *et al.* 1999). It is suggested that FAS may be important both in the apoptotic waves, particularly in the embryonic stage of development (Wang *et al.* 1998), but possibly also at the onset of the spermatogenic cycle in order to reduce the number of germ cells to a level able to be supported the Sertoli cells (Lee *et al.* 1997). The FAS pathway is also important in cases of hormonal deprivation and toxic insult and injury, where the expression of FAS and FASL increases in rodents (Ohta *et al.* 1996; Lee *et al.* 1997; Pentikainen *et al.* 1999; Lee *et al.* 1999). Germ cell apoptosis can be successfully prevented if the FAS pathway is inhibited, significantly increasing germ cell survival (Lee *et al.* 1997).

In light of this apparent role for the FAS signalling pathway in germ cell apoptosis, two transgenic mice were examined who possess an inability to initiate cell death through the FAS pathway. The *lpr* (lymphoproliferation) mice express little or no FAS due to an inserted disruption in intron 2 (Adachi *et al.* 1993; Kimura & Matsuzawa 1994), and the

gld (generalised lymphoproliferative disease) and *lpr*^{cg} (*lpr* complementing gld) mice who have both nonfunctional FAS and FASL due to a point mutation (Takahashi *et al.* 1994; Nagata & Suda 1995). The general phenotype of these mice was an inhibition of lymphocyte apoptosis, resulting in an abnormal proliferation of lymphocytes resulting in autoimmune disease. However, the *gld* mice were fertile, presenting with an increase in testicular weight and elongated spermatids, and actually appearing to have a small increase in the level of spontaneous apoptosis (Richburg *et al.* 2000). This and other studies (Koji *et al.* 2001), have suggested that the FAS pathway may not be crucial in the spontaneous apoptosis that occurs normally throughout spermatogenesis.

The FAS signalling pathway has been shown to be important in regard to cell death initiated through other types of toxic insult. For example, when Sertoli cell injury was induced through exposure to the Sertoli cell toxicant MEHP (mono-(2-ethylhexyl) phthalate), the *gld* mice were unable to remove the germ cells through apoptosis, suggesting the importance of FAS in this regard (Richburg *et al.* 2000). The application of the FAS pathway appeared to be dependant on the nature of the insult, with germ cell injury initiated through radiation exposure, causing an increase only in the FAS receptor (Lee *et al.* 1999) and normal levels of cell death (Richburg *et al.* 2000). As the differing toxic agents target certain germ cells, the role of FAS in the testis may be germ cell specific with it playing a role in the more mature germ cells, but not in the stem spermatogonia (Richburg *et al.* 2000). Inducing cryptorchidism also leads to infertility with an increase in apoptosis primarily in the spermatocytes and spermatids (Ohta *et al.* 1996) which coincided with an increase in FAS mRNA. Nevertheless, the cells did not appear to use this pathway to die, as apoptosis still occurs in the *gld* and *lpr* mice that lack FAS action (Ohta *et al.* 1996).

Recently there is a great deal of speculation as to the localisation of both FAS and FASL in the testis. It has been generally accepted that the FAS ligand was constitutively expressed on rodent Sertoli cells (**Table 1.532**) (Lee *et al.* 1997; Sugihara *et al.* 1997; Wang *et al.* 1998; Xu *et al.* 1999; Richburg *et al.* 1999; Koji *et al.* 2001), whereas FAS was localised

to the germ cells, particularly the spermatocytes and round spermatids (Lee *et al.* 1997; Ogi *et al.* 1998; Xu *et al.* 1999). This suggested the existence of a paracrine interaction between the Sertoli cells and the germ cells, whereby the individual germ cells may specifically target themselves for destruction by the Sertoli cells by becoming FAS positive. However recent studies have contradicted many of these initial publications. To clarify the expression distribution of both FAS and FASL in the testis, D'Alessio *et al* (2001) undertook an extensive study using Northern blot and *insitu* hybridization, with control and germ cell depleted testes. They discovered that FAS receptor mRNA was present on the Sertoli cells, with the FASL mRNA in fact localised to the spermatocytes, round and elongated spermatids. Other studies have confirmed this, immuno-localising FASL to the germ cells (Pentikainen *et al.* 1999) and the receptor to the Sertoli cells (Sugihara *et al.* 1997; Riccioli *et al.* 2000), suggesting that the FAS-FASL system may operate independently of the Sertoli cells. A commentary recently published in *Nature* by Restifo (2000) suggested possible reasons for these conflicting results, including RT-PCR performed on purified Sertoli cell cultures where it is known that they contain contaminating germ cells, and non specific antibodies leading to numerous false positives. It is suggested that the high level of FAS constitutively expressed by the testis must play an important role, not just in killing FAS positive infiltrating macrophages to maintain this immune privileged site

In summary, the death receptor pathway also appears to play a role in the control of testicular apoptosis during development and also in response to injury or toxic insult.

1.6 HUMAN MUTATIONS AND KNOCKOUT MICE

Oestrogen plays well-characterised important roles in numerous physiological functions. Unlike the androgen receptor which contains mutations resulting in the testicular feminised male (Tfm) in rodents (Bullock 1986) and androgen insensitivity syndrome (AIS) in

humans (Patterson *et al.* 1994), or the thyroid (McDermott & Ridgway 1993) and glucocorticoid receptor (Chrousos *et al.* 1993) mutants which also contain structural defects leading to resistance against their ligands, by 1994 no mutation had been reported either in aromatase or in the oestrogen receptor. Therefore at this time oestrogen was believed to be crucial for survival.

1.61 Human mutations

1.611 Oestrogen receptors

The first human mutation in the ER was reported in 1994. A 28 year old man was described to possess a non-functional ERα (**Table 1.61**) (Smith *et al.* 1994). When first identified he had a normal appearance, including the normal onset of puberty and genitalia development, but displayed persistent liner growth reaching the 95th percentile due to the failure of epiphyseal fusion. He presented a marked bone phenotype, including delayed bone age, low mineralisation and osteoporosis. Hormonal analysis found that serum testosterone was normal, while oestrogens and gonadotrophins were elevated. His testes were of normal size (20-25ml), with semen analysis showing a normal sperm count (25×10^6 ml), although motility was reduced by half of normal. After high dose treatment with ethinyl oestradiol failed to lower his serum oestrogen and gonadotrophins or improve his bone physiology, genetic analysis discovered a homozygous mutation in exon 2 of his ERα. This resulted in a premature stop codon which translated into a receptor containing only the N terminal domain, missing the vital DBD and LBD. His parents (cousins), both phenotypically normal, were heterozygous for the mutation.

At this time, very little was known regarding oestrogen in the male, therefore this man provided crucial information, particularly regarding the importance of oestrogen on the skeleton. However, all that can be concluded regarding his fertility is that he is heterosexual and has normal sexual function and sperm count, although motility is below normal.

Table 1.61 ***Comparison of the reproductive phenotypes between the ERα and the 2 aromatase mutated adult male patients***

	ERα mutated male (Smith *et al.* 1994)	**Aromatase mutated male** (Morishima *et al.* 1995)	**Aromatase mutated male** (Carani *et al.* 1997)
Male genitalia	Normal	Normal	Normal
Descended testes	Yes	Yes	Yes
Testes size	Normal	Large	Small
Gender identity	Normal	Normal	Normal
FSH	Elevated	Elevated	Elevated
LH	Elevated	Elevated	Normal
T	Normal	Elevated	Normal
DHT	Normal	Elevated	N/D
E2	Elevated	Undetectable	Undetectable

(N/D- not described)

1.612 Aromatase

There have been nine documented cases of aromatase-mutated humans; of which only 3 are males, two adults and one infant (not described here, see Deladoey *et al.* 1999).

The first adult male was characterised in 1995 (**Table 1.61**) (Morishima *et al.* 1995), and the second in 1997 (Carani *et al.* 1997). Similar to the ERα mutated male, they both presented with tall stature, delayed skeletal maturation, unfused epiphyses and osteopenia. However, one male had elevated levels of FSH, LH, DHT, T and androstenedione (Morishima *et al.* 1995), whereas the other had elevated FSH but normal levels of LH, T and androstenedione (Carani *et al.* 1997). In comparison to the ER mutated man, oestrogen treatment normalised the bone phenotype and serum hormonal levels. When the reproductive phenotype of these men was examined, both men had normal genital development and onset of puberty, with normal nocturnal emissions and ejaculations. The first male had large testes, but as no serum analysis was undertaken and at this time he was

a virgin, his fertility was undetermined (Morishima *et al.* 1995). The second man had small testes, severe oligospermia (20 fold decrease) with all sperm immotile. A testicular biopsy showed low spermatogenesis and germ cell arrest at the level of the primary spermatocytes, and hence he was infertile. The administration of oestrogens normalised the bone phenotype, however spermatogenesis was not restored. This patient's brother was also found to display azoospermia but did not have the aromatase mutation (Carani *et al.* 1997). Therefore, the effect of abolishing oestrogen synthesis on male reproductive function at this stage was unknown.

Genetic analysis found that the aromatase deficiency in both males was a consequence of single base pair mutations in exon IX of the CYP19 gene, resulting in a structurally defunct protein with only 0.2-0.4% of normal activity.

The human cases of natural oestrogen deficiency brought to our attention the role of oestrogen in many aspects of human physiology, and possibly hinted at a role for oestrogen in male fertility. However to fully study oestrogen action in the male, and complement the human studies, mouse models were generated.

1.62 Knockout mice

1.621 αERKO

The oestrogen receptor knockout (αERKO) mouse was created in 1993, the year before the discovery of a human with a non functional ERα, by the insertion of a neomycin resistant gene into exon 2 (Lubahn *et al.* 1993). This mutation, in the same exon as the ERα null patient, was not embryo leathal, but αERKO males and females were both infertile. Only the male reproductive phenotypes will be briefly discussed in this review, for information regarding the females and other characteristics of the αERKO mice see review by Couse and Korach *et al.* (1999).

Studies performed with the αERKO mouse vastly increased our knowledge regarding the possible role of oestrogens in male reproduction, with the removal of a functional ERα resulting in age related male infertility. Prior to puberty, the testicular morphology of the αERKO mice appeared to be normal, however a grossly dilated seminiferous tubule lumen was observed as early as 20 days. By 90 days the testes in these mice were atrophic with major disruptions to the seminiferous epithelium. The sperm had abnormal morphology and severely compromised fertility, illustrated in a fertility mating study (Eddy *et al.* 1996). Studies demonstrated that ERα had a crucial role in the reabsorption of luminal fluid in the efferent ductules, such that the αERKO mice had an excess of fluid accumulated in the testis. This subsequently resulted in a backpressure of fluid on the seminiferous tubules, adversely affecting the seminiferous epithelium and spermatogenesis. Therefore, it was suggested that the disruption to spermatogenesis in the αERKO mice was an indirect effect caused by abnormal fluid reabsorption. ERα was also found to play a role in the development of sexual behaviour, with αERKO mice show a reduction in the number of mounts and failure to ejaculate (Ogawa *et al.* 1997).

As expected, oestrogen plays a negative feedback role at the level of the hypothalamo-pituitary with a slight elevation in LH (Eddy *et al.* 1996; Lindzey *et al.* 1998). The testes also showed evidence of Leydig cell hyperplasia (Rosenfeld *et al.* 1998) and increased serum testosterone levels (Eddy *et al.* 1996; Lindzey *et al.* 1998). Serum FSH levels did not alter (Eddy *et al.* 1996; Lindzey *et al.* 1998), however inhibin was not measured. The serum hormone levels are different from those observed in the ERα null patient.

Examining the reproductive phenotype of the αERKO male mice highlighted the importance of ERα signalling in maintaining the integrity of the testis fluid environment, and thus the normal maturation of developing sperm, and in the development of sexual behaviour.

1.622 βERKO

With the discovery of ERβ, a possible second oestrogen-signalling pathway was indicated. To investigate the importance of this receptor subtype in oestrogen action, the ERβ null mouse (βERKO) was created in 1998 through insertion of a neomycin cassette into exon 3 of the ERβ gene (Krege *et al.* 1998). This resulted in a protein with a nonfunctional first zinc finger, therefore inhibited receptor binding to the ERE.

However, βERKO mice show no disruption to spermatogenesis and testicular morphology. This is surprising given the localisation of ERβ to many cell within the efferent ducts and seminiferous epithelium (**Section 1.4**). It was postulated that ERα may play a compensatory role. These mice are also fertile and show no deficits concerning their development of sexual behaviour.

The two ER null mice brought to our attention the apparent contrasting roles of each ER subtype in the testis and in other target organs.

1.623 αβERKO

The crossing over of both the ERα and ERβ null mice generated a mouse that lacked both ERα and β (αβERKO). These mice are developmentally normal, confirming that both ER are not crucial to survival (Couse *et al.* 1999). However, they are infertile, with a severe reduction in the concentration and motility of spermatozoa. In fact, these mice display a phenotype more reminiscent of the αERKO males, demonstrating disruptions to the seminiferous epithelium that result in a loss of maturing germ cells (Dupont *et al.* 2000).

1.624 Limitations with the ER null models

To create the αERKO mouse, exon 2 was disrupted by the addition of a neomycin resistant gene. However, this produced a truncated transcript that till contained the DBD, LBD and therefore still possessed transcriptional activity, although this was considerably reduced

when compared to the wildtype (Couse *et al.* 1995). Although the αERKO mice were deemed insensitive to oestrogen when well known uterine markers failed to be induced following oestrogen treatment, too fully investigate the function of the oestrogen receptors in the reproductive phenotype of mice, particularly with respect to their AF-1 and AF-2 transactivation domains, new null mutants were generated for ERα, ERβ and ERαβ (Dupont *et al.* 2000). The ERα null mutant lacked regions C to F, while the ERβ null mutant lacked not only the first zinc finger of the DBD, but also exon 3, 6 and 7, in comparison to the βERKO that was only missing exon 3 (Krege *et al.* 1998). Similar to the previous αERKO and βERKO mice these mice presented with very similar if not identical characteristics (Dupont *et al.* 2000). Particularly regarding the testis and male reproductive tract (**Table 7.1**).

It is also possible that compensatory factors come into play in the absence of a functional ER. These include non-genomic actions, as described in **Section 1.222**. Oestrogen is not only able to bind to its nuclear ER, but also to a cell membrane receptor, activating the cell via signal transduction pathways. In this respect, oestrogen may still promote a cell specific response in these ER null models.

1.63 The Aromatase Knockout (ArKO) Mouse

To fully investigate the specific role for oestrogens in males and females, the P450 aromatase enzyme was disrupted, generating mice unable to synthesise endogenous oestrogens. In this regard it is the hormone itself that is removed, not the possible means of its action.

1.631 Generation of the ArKO mouse

The strategy used for targeted disruption of the *Cyp19* gene and a preliminary description of the phenotype have already been discussed (see Fisher *et al.* 1998). Briefly, exon IX of the mouse *Cyp19* gene was selected for disruption as the coding region between the *Eco*RV (bp 1047) and *Xho*I (bp 1210) sites present in this exon is highly conserved

amongst all aromatase cDNAs reported thus far and is important for many structural features of the enzyme such as the K helices and the β-sheet region (Simpson *et al.* 1997). Insertion of the targeting plasmid (pPollshort-neoPA-HSVTK), containing the neomycin (*neo)* resistant gene and 2 herpes simplex virus-thymidine kinase (HSC-TK) genes, between these two restriction sites in exon IX deleted 163bp of coding region (amino acid residues 349-403) (**Figure 1.63**).

The targeting plasmid was transfected by electroporation into undifferentiated embryonic stem (ES) cells, and successful clones selected by neomycin resistance. As the neo gene also contained an *Eco*R1 (E) restriction site, the DNA from these clones was analysed by *Eco*R1 digestion and Southern blot analysis. **Figure 1.63** illustrates the position of the probe, hybridising to the 3' end of intron IX to the 5' end of exon X. The WT gave a band of 7.4kb, whereas the KO allele resulted in a band of size 3.0kb (due to the neomycin insert containing the additional *Eco*R1 restriction site). The successful clones were injected into C57BL/6 blastocysts and then transplanted into pseudopregnant ICR females, to produce chimeric mice with the disrupted gene. The mice that specifically transmitted the mutated aromatase gene into their germ line were selected (F1). Breeding of mice heterozygote for the aromatase disruption produced F2 offspring with the normal Mendelian frequency; 2 heterozygote, 1 knockout and 1 wildtype (WT). Each mouse was genotyped by Southern procedure. Aromatase inactivation was confirmed by a tritiated water release assay (Ackerman *et al.* 1981) performed on ArKO and WT ovaries at 12 weeks of age (Fisher *et al.* 1998), with no detectable aromatase activity.

1.632 Preliminary phenotype

The ArKO mice were born phenotypically normal in the correct sex ratio, suggesting oestrogen was not crucial for sex determination (Fisher *et al.* 1998). Both males and females presented with undetectable serum oestradiol levels. A brief overview of the male and female phenotypes, initially examined by Fisher *et al.* (1998), is presented below.

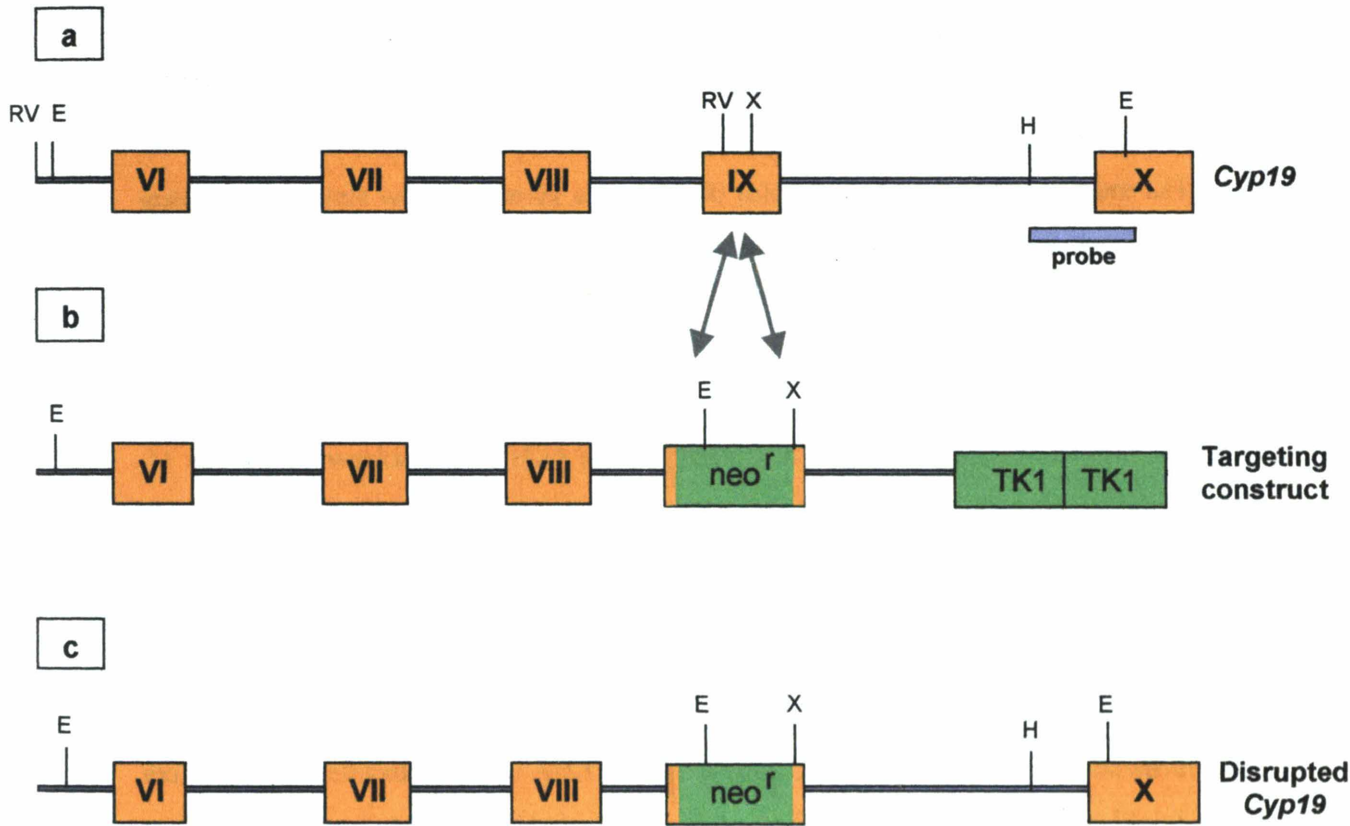


Figure 1.63 The targeting construct used to generate the ArKO mouse

Schematic illustrating the (**a**) wildtype mouse *Cyp19* gene (from exon VI to X), (**b**) the targeting vector containing the neomycin resistant gene and 2 herpes simplex virus thymidine kinase (TK1) genes and (**c**) the disrupted *Cyp19* gene. Also shown are the *Eco*RV (RV), *Xho*I (X), *Hind*III (H), *Eco*RI (E) sites, and the *Hind*III-*Eco*RI fragment used to probe the Southern blots for genotyping analysis.

Reproduced from Fisher et al (1998)

i Female

The female ArKO mice at 12 weeks of age presented with underdeveloped uteri, ovarian follicles possibly up to the antral stage, however an absence of corpora lutea suggesting a failure to ovulate. The gonadal fat pads were significantly increased in weight. Serum testosterone levels were markedly elevated (10 fold) compared to WT, as were LH (2-10 fold) and FSH (3-4 fold). Preliminarily oestradiol replacement studies were found to increase uterine weights to WT weight.

ii Male

At age 12-14 weeks male ArKO mice appeared to be phenotypically normal, however showed differences in the reproductive system, including an increase in the weight of their seminal vesicles and urinary bladder/prostate, possibly due to an accumulation of fluid. No difference was observed in the testicular weights, with the morphology of the testis structure appearing normal including the presence of mature sperm in the testis and epididymis. Serum testosterone and androstenedione levels were variable but a trend existed for an elevation in the ArKO mice. Serum LH was also increased, however FSH was not examined. Male ArKO mice were initially fertile (8-11 weeks), capable of siring normal sized litters. However fertility progressively declined with age, 2 males infertile at age 14-17 weeks, with another able to sire litters to age 6 months (when the paper was published). This cause for this decline in fertility was unknown.

1.633 Two other ArKO mice

Since the generation of the ArKO mouse described above by Fisher *et al* (1998), two additional ArKO mice have been generated. Honda *et al* (1998) replaced exon I and II with a neomycin resistant gene. As these regions of the *Cyp19* gene contain transcriptional and translational initiation codons these mice expressed no aromatase mRNA. The phenotype of these mice is similar to that described above, with testis weight not differing between ArKO and WT at 12-16 weeks with sperm present in the testis and epididymis. These mice were sub fertile, reported to be due to decreased number of mounts. The third ArKO mouse

was generated by Toda and Shizuta (1999), by disrupting exon IX, the same exon as Fisher *et al* (1998), by homologous recombination. The preliminary phenotype matched that described above, with only 20% of males reported to be fertile, however age is unknown. No detailed examination of the testes of either of these mice was undertaken.

1.64 Aims

From initial studies employing the αERKO and the ArKO mice, and the specific localisation of the oestrogen receptors and aromatase in the testis, it is hypothesised that oestrogen plays an important and possibly direct role in male reproduction; specifically at the level of testicular development, spermatogenesis and fertility. Hence, a comprehensive examination of the male ArKO mouse is warranted to elucidate the role of oestrogens in male reproduction.

This thesis describes a thorough investigation into the role of oestrogens in male reproductive function, particularly with respect to fertility. Firstly, an initial characterisation of the testicular phenotype was carried out, including the effect of withdrawing oestrogens on testicular structure, such as seminiferous epithelial volume, luminal volume and tubule diameter. Then spermatogenesis was explored in young and mature ArKO mice, in particular the specific effect of withdrawing oestrogens in germ cell development. Serum gonadotrophins and testosterone levels were also measured. Following this, an *in vitro* study characterising the mature spermatozoa, with respect to motility and *in vitro* fertilization capacity, was carried out. As was the capacity for normal sexual behaviour, such as mounting. Following these initial characterisation studies, an investigation was conducted into the role that dietary phytoestrogens, which are found in soy, a common constituent of standard mouse chow, was playing in the ArKO phenotype. Finally, a more specific examination at the role of oestrogens in spermatogenesis was undertaken with various molecular analytical techniques.

Chapter Two

Materials and Methods

2.1 GENERAL REAGENTS

ABC complex	Vectastain Elite;Vector Laboratories, Burlingame CA
Acetic acid glacial	BDH Laboratory supplies, Poole, England
30% Acrylamide/Bis solution 29:1	BioRad Laboratories, Hercules, CA
Agarose LE	Boehringer Mannheim, Mannheim, Germany
Ammonium persulfate (APS)	Sigma chemical Co, St Louis, Mo
AMV enzyme and buffer	Boehringer Mannheim, Mannheim, Germany
Boric acid	Ajax chemicals, NSW, Australia
Bromphenol blue	Sigma chemical Co, St Louis, Mo
BSA	Sigma chemical Co, St Louis, Mo
Butanol-1	Sigma chemical Co. St Louis, Mo
C.therm enzyme	Roche Diagnostics, Mannheim, Germany
Carbon	Norite A, JT Baker
CAS Block	Zymed, South San Francisco, CA
Calcium chloride ($CaCl_2$)	Sigma chemical Co., St Louis, Mo
Chloroform	BDH Laboratory supplies, Poole, England
Citric acid	Sigma chemical Co., St Louis, Mo
Dextran (leuconostoc mesentrioides)	Sigma Diagnostics, St Louis, Mo
DAB	Sigma chemical Co., St Louis, Mo
DEPC (diethyl pyrocarbonate)	Sigma chemical Co., St Louis, Mo
Diethyl ether	Ajax chemicals, NSW, Australia
Digoxigenin labelled dideoxy-dUTP	Boehringer Mannheim, Mannheim, Germany
DNA ladder (1.5kb)	GibCo BRL, Life technologies, Paisley, UK
Dextran sulphate	Sigma chemical Co, St Louis, Mo
DNTPs	Boehringer Mannheim, Mannheim, Germany
DPX	BDH Laboratory supplies, Poole, England
DL-DTT (dithiothreitol)	Sigma chemical Co, St Louis, Mo
EDTA	Sigma chemical Co, St Louis, Mo

Ethanol	CSR Limited, Yarraville, Vic, Australia
Ethidium bromide	Sigma chemical Co, St Louis, Mo
Ficoll	Sigma chemical Co, St Louis, Mo
Formaldeyde	BDH Laboratory supplies, Poole, England
Formamide	Ajax chemicals, NSW, Australia
Forward reaction buffer (5x)	GibCo BRL, Life technologies, Paisley, UK
Gluteraldehyde	ProSciTech
Glycerol	Sigma Diagnostics, St Louis, Mo
Glucose-6-phosphate dehydrogenase	Sigma Diagnostics, St Louis, Mo
Haemotoxylin Mayers	Sigma Diagnostics, St Louis, Mo
Histosol	Australian Biostain Pty Ltd, Traralgon Vic, Australia
Iodogen reagent	Sigma Diagnostics, St Louis, Mo
Isopropanol (Propan-2-ol)	BDH Laboratory supplies, Poole, England
Magnesium (Light cycler)	Roche Diagnostics, Mannheim, Germany
Methacrylate resin	Technovit 3040 &7100, Kulzer, Wehrheim, Germany
Mineral oil	Sigma chemical Co, St Louis, Mo
MOPS	Sigma chemical Co, St Louis, Mo
Normal rabbit serum	Australian Lab Services
Normal sheep serum	Australian Lab Services
PCR (10x) buffer	Boehringer Mannheim, Mannheim, Germany
Periodic acid	Unilab, APS Ajax Finechem
Picric acid	BDH Laboratory supplies, Poole, England
Potassium cacodylate	Boehringer Mannheim, Mannheim, Germany
Potassium chloride	Sigma chemical Co, St Louis, Mo
Potassium phosphate	Sigma chemical Co, St Louis, Mo
Polyvinylpyrrolidone,	Sigma chemical Co, St Louis, Mo
Radioactive labels (^{32}P, ^{35}S)	Amersham pharmacia biotech
Random hexamer primer	GibCo BRL, Life technologies, Paisley, UK
RNase inhibitor	Boehringer Mannheim, Mannheim, Germany
Salmon sperm	GibCo BRL, Life technologies, Paisley, UK

SDS (Lauryl sulfate)	Sigma chemical Co, St Louis, Mo
Schiffs reagent	Amber scientific, Belmont, Western Australia
Sigmacote	Sigma chemical Co, St Louis, Mo
Sodium acetate	Sigma chemical Co, St Louis, Mo
Sodium azide (NaAz)	Aldrich chemical Co, WI, USA
Sodium cacodylic acid	ProSciTech
Sodium chloride (NaCl)	Sigma chemical Co, St Louis, Mo
Sodium citrate	Sigma chemical Co, St Louis, Mo
Sucrose	Sigma chemical Co, St Louis, Mo
SYBER green master mix	Roche Diagnostics, Mannheim, Germany
T4 polynucleotide kinase	GibCo BRL, Life technologies, Paisley, UK
Taq enzyme and PCR buffer	Boehringer Mannheim, Mannheim, Germany
TCA (trichloroacetic acid)	Sigma chemical Co, St Louis, Mo T-9159
TdT	Boehringer Mannheim, Mannheim, Germany
TEMED	ICN Biomedicals Inc, Ohio
Terminal transferase enzyme	Roche Diagnostics, Mannheim, Germany
Terminal transferase buffer	Roche Diagnostics, Mannheim, Germany
Tris HCl	Boehringer Mannheim, Mannheim, Germany
Trizma base	Sigma chemical Co, St Louis, Mo
Triton X-114	Sigma chemical Co, St Louis, Mo
Trypsin	GibCo BRL, Life technologies, Paisley, UK
Urea	Sigma chemical Co, St Louis, Mo
Xylene cyanole FF	Sigma chemical Co, St Louis, Mo

2.2 METHODS

2.21 Animals

2.211 Genotyping

All primers used in the following studies were designed as follows. The mRNA sequence of interest was retrieved through ENTREZ (NCBI nucleotide website) and the primers selected by DNASTAR Primer Select, having a length of between 17-25 nucleotides and a location of 200-400 bp. Once selected, their specificity was reaffirmed by a basic BLAST search (NCBI website), then their ability to form primer dimers and hairpins was analysed by Net Primer (Prmierbiosoft website). To genotype the ArKO colony, PCR specific primers were designed to anneal to both exon IX and the neomycin resistant insert of the *Cyp19* gene (**Figure 2.211, Table 2.211**). Originally only exon IX primers were used, however the neo insert is approximately 1.5 kb and thus resulted in 2 PCR products of exceedingly different lengths.

Genomic DNA was extracted from 0.5 cm of mouse tail by incubating each tail with 100 μl K buffer (20 μl 10 x PCR buffer, 1 μl Tween-20, 20 μl proteinase K (10 mg/ml) and sterile water to 100 μl) at 56°C for 45 min. To inactivate the proteinase K, the temperature was increased to 99°C for 15 min. A PCR reaction was set up, containing 0.5 μl extracted DNA in a 25 μl reaction. The reaction mix contained 2.5 μl 10 X PCR Buffer with Mg^{2+}, 1 μl dNTP's (10 mM), 0.5 μl (10 pmol/μl) neo forward and reverse primers, and 0.5 μl (10 mol/μl) exon 9 forward and reverse primers, 0.5 μl (5 units/μl) *Taq* polymerase and sterile water to 25 μl. The reaction mixes were then loaded into a PCR machine (PCR Express) with the following program. The first stage was a single denaturing step for 1 min at 94°C, followed by the second stage of 35 cycles involving the denaturing step for 15 s, the annealing step at 55°C for 30 s and the extension step for 45 s at 72°C. The third stage was a final extension step for 6 min.

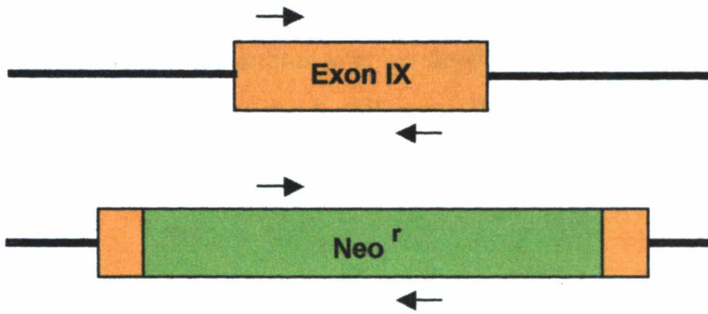


Figure 2.211 The location of the primers (arrows) used to genotype the ArKO mice.

Both exon IX forward and reverse, and neo forward and reverse primers generated PCR products of 220bp and 180bp, respectively.

Table 2.211 Genotyping PCR primer sequences

Gene	Forward primer	Reverse primer
Neo	5'- atc agg atg atc tgg acg aag a -3'	5'- cca cag tcg atg aat cca gaa -3'
Exon IX	5'- gtg aca gag aca taa aga tcg -3'	5'- gta aat tca ttg ggc tta ggg -3'

As the two PCR products only differ in length by 40bp, a 4% agarose gel (2% low melting temperature agarose, 2% LE agarose) was prepared with 5 µl/100 ml ethidium bromide (4 mg/ml), for accurate genotyping. DNA loading buffer, 4x, was then added to the PCR product and loaded into the gel in a BioRad sub cell with a 1.5 plus kb molecular marker. The gel was run for 60 min at 80 V using the Hoefer power supply in 0.5 x TBE buffer (10x: 108 g Tris base, 55 g Boric acid, 40 ml 0.5M EDTA, pH 8.0, water to 1 L). The DNA was visualised with a UV transilluminator contained in a Gel Doc 2000 apparatus (BioRad Laboratories).

If a single band existed at 220 bp this ascertained the presence of two alleles containing neomycin inserts and thus this animal was genotyped an ArKO (**Figure 2.212**). If 2 bands occurred, these corresponded to the 2 different alleles and the mouse was pronounced a Heterozygote (Het), and finally if a single band occurred at 180 bp then it was pronounced a Wildtype (WT).

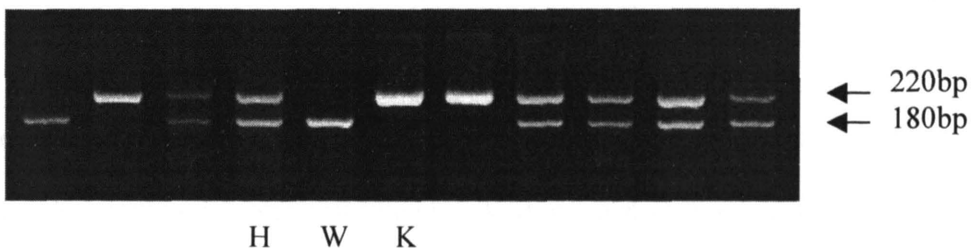


Figure 2.212 Genotyping

The lane with a single band at 220 bp represents the ArKO animal (K) with the neo insert, the lane with 2 bands is the heterozygote (H; 1 of each allele) and wildtype with an undisrupted exon IX represented by one band at 180 bp (W).

2.212 Husbandry

WT, ArKO and Het mice were housed under a 12 h light/dark cycle in the Specific Pathogen Free (SPF) facility at Monash Medical Centre Animal House, and were fed mouse chow and water *ad libitum*. For breeding purposes, generally 2 Het animals were placed together, the mendelian frequency of the offspring being 50% Het, 25% ArKO and 25% WT. Weaning occurred at 3 wks, when females and males were placed in separate cages. Mice were ear tagged an identification number by the routine method. All experiments were approved by the Animal Experimentation Ethics Committee at Monash Medical Centre.

2.213 Mouse chow constituents

Glen Forrest Stockfeeders (Glen Forrest, Western Australia, Australia) manufactured the two gamma-irradiated mouse chows used in these studies. Both were standard mouse chows containing wheat, lupins, blood meal, fish meal, meat meal, bran, pollard, vegetable oil, pure amino acids and vitamin mineral premix. However, one contained the standard 10% soya meal, and one had the soy replaced with additional protein supplements. In 1999, 1 year after the ArKO mouse colony was established in Australia, 80% of the colony was raised on the soy free mouse chow.

2.214 Collection of tissue

WT and ArKO male mice were sacrificed by cervical dislocation and blood collected by beheading. The blood was spun at 3000 RPM for 10 min to separate the serum, which was then snap frozen on dry ice and stored at -20°C for hormone analysis (**Section 2.27**). The testis were dissected out, the epididymis and gonadal fat removed, and all tissue weighed. One testis and epididymis was immediately snap frozen in liquid nitrogen for storage at –80°C, the other was fixed (see below).

2.215 *Fixation of tissue*

Whole testes with the epididymides still attached were either placed in paraformaldehyde (4% paraformaldehyde in PBS) or Bouins fixative (approximately 9 g picric acid per 750 ml water until saturated, 250 ml formaldehyde, 50 ml glacial acetic acid) for immersion fixation. After 4-5 h the testes were washed and stored in 70% ethanol, at 4°C.

2.22 Fertility

2.221 Mating study

WT and ArKO mice were placed with WT females at age 50 days (Het females were also used if no WT mice were available). The number of litters, number of pups per litter and time until the first litter were monitored for 50 days.

2.222 Sexual behaviour

WT female mice were ovariectomized and injected intra-peritoneally with 10 μg oestradiol 48 h prior to test, then 500 μg progesterone 4-7 h before test (Ogawa *et al.* 1998b). All tests were performed during the dark phase. Male WT and ArKO mice were placed in a viewing cage and left for 5 min to adjust to their new surroundings before the female was introduced. An infra-red illuminator using 10 infra Red Light Emitting Diodes (MS-1570B) mounted on a Perspex strip (assembled by ourselves) was used to illuminate across the cage. This was attached to a MOD-BW miniature video camera (Govideo N266, Tandy's Electronics, Perth, WA) that was used to film the mice for 20 min. For each male the number of mounts, their mount latency and general behaviour was recorded, the tapes viewed if further examination was required. If the female appeared to be unresponsive another replaced her in the viewing cage.

2.223 Quantitation of epididymal spermatozoa

Epididymides stored at –80°C (**Section 2.214**) were thawed to room temperature. After weighing, each was homogenised for 30 s in 1 ml 0.05% Triton (in PBS). As the chromosomes in the elongated spermatids from step 12 onwards have condensed, the sperm heads are protected against rupturing, allowing spermatozoa numbers to be quantified. The number of spermatozoa per epididymis was quantified using a haemocytometer, multiplying the number counted per square by 10^4 and the dilution factor.

2.224 In vitro analysis of spermatozoa

The caudae epididymides were removed from WT and ArKO males, and the spermatozoa allowed to swim out of a small incision made in each epididymis into 0.5 ml modified Tyrode's medium (MT6) under mineral oil, and capacitated for 2 h at 37°C in an atmosphere of 5% CO_2 in air. Spermatozoa were then analysed for concentration and motility. Droplets of 20 μl sperm solution (2 x 10^6 spermatozoa/ml) under mineral oil were used for insemination. Oocytes were examined 6 h after insemination for fertilisation as

determined by the presence of 2 pronuclei and the extrusion of the second polar body, then every 10-14 h for development to the blastocyst stage. These experiments were carried out by Dr Orly Lacham-Kaplan at the Institute of Reproduction and Development.

2.23 Histology

Testes fixed in Bouins were sectioned into 3 segments; top, middle and bottom, and each of these into half again resulting in 6 sections. Epididymides and each testis segment (one representative from top, middle and bottom) were embedded in methacrylate resin, with the remaining testis segments stored in 70% ETOH to be embedded in paraffin.

2.231 Methacrylate Resin

i Embedding

Each testis segment and epididymides were dehydrated in a spinning rotator (Ratek Instruments, Boronia, Vic, Australia), each wash of increasing alcohol concentration: 70% ETOH (1 h), 100% ETOH (3 x 1 h), butanol (1 h) and butanol (3 h). After the final dehydration step, approximately 1 ml infiltration solution (1g hardner I in 100 ml 7100 solution; Technovit 7100, Kulzer, Wehrheim, Germany) was added to each tissue and left to spin slowly overnight. Each tissue was then placed in a mould and numbered on a paper label with pencil. Then a hardner solution (1 ml hardner II in 15 ml infiltration solution; Technovit 7100, Kulzer, Wehrheim, Germany) was pipetted into the moulds to completely cover the tissue and left for approximately 2 days to harden.

The backing blocks (10 g Technovit 3040 powder and 5 ml Technovit 3040 liquid; Kulzer, Wehrheim, Germany) were constructed using the same moulds as above, taking approximately 5-10 min to set. Each block was attached to the methacrylate embedded tissue using a small quantity of the backing block mixture. The blocks were removed and stored long term with silca gel moisture indicator crystals (Chem-supply, South Australia).

ii Histology

Sections, 2μm and 25μm, were cut on a supercut microtome (Leica LM 2055, Reichart) with a glass knife formed by the knife cutter (2078 Histo Knifemaker, Leica KB Bromma). Each section was placed on an uncoated slide (HD Scientific) and left for 2 h (2 μm) or 24 h (25 μm) at 37°C to adhere. All sections, unless specified, were dipped in Periodic acid (1:100 w/v in water) for 30 min, then 2 water dips. This was followed by either 1 h (2 μm) or 45 min (25 μm) in Schiffs reagent at room temperature, then running tap water for 10 min to initiate the Schiff reaction, staining the carbohydrate moieties pink. To visualise the nuclei, the sections were counterstained with haemotoxylin (2 μm for 10 min and the 25 μm for 35 min), and Scotts tap water (10x; Sigma Diagnostics, St Louis, Mo) for 2-5 min until blue, one water dip halting the Scotts reaction. Sections were then dried at 37°C for either 1 h (2 μm) or overnight (25 μm). They were then mounted under glass with DPX.

*2.232 **Paraffin***

WT and ArKO testes were embedded in paraffin wax (Paraplast tissue embedding medium; Oxford Labware, Sherwood Medical, St Lois, MO) by the standard method for imunocytochemistry. Either 3 μm or 5 μm sections were cut with a microtome (Leica Reicheit-Jung) and mounted on Superfrost slides (Menzel-glaser, Germany), 2 per slide. Each slide was dewaxed using decreasing alcohol concentrations: histosol 2 x 10 min, 100% ETOH 2 x 10 min and 70% ETOH 1 x 10 min. A wax pen (Pap pen, Zymed Laboratories) was used to trace around each section.

2.24 Stereology

Stereological analyses of the testes were performed on both 2 μm and 25 μm methacrylate embedded sections. Sections were examined under a BX-50 Olympus microscope (Tokyo). The image was captured by a Pulnix TMC-6 video camera (Pulnix America, Sunnyvale, CA) coupled to an Amiga 2000 Pentium computer, and projected onto the computer screen by using a Screen Machine II fast multimedia video adapter (Hamburg, Germany). The

computer program CASTGRID VI (Olympus, Munich) was used to generate point grids and unbiased counting frames

2.241 Testicular Volumes

To calculate the volume of the testis taken up by the lumen, interstitium and epithelium, 2 μm sections were examined under x 10 magnification. A point grid was created consisting of 4 major and 16 fine points (**Figure 2.241**). The major points were counted if they landed on seminiferous tubules, as most of the testicular volume is comprised of epithelium, whereas the number of fine points landing on lumen, interstitium and tunica were noted. Approximately 10-20 counting frames were chosen, where one frame directly followed the previous in order to determine the volume of the entire testis section. To calculate the volumes, the total points landing on each compartment from testis top, middle and bottom were calculated. The points landing on seminiferous epithelium were multiplied by 16 as each major point was a representative of 16 fine points. The total number of points landing on each compartment was divided by the total points per testis then multiplied by the testis weight (g) to give the volume (mm^3) of each compartment.

2.242 Cell numbers

The optical disector approach was used to quantitate the number of cells per testis. Germ cells were classified into four major groups: spermatogonia, spermatocytes, round spermatids and elongated spermatids (Russell *et al.* 1990). Two counting frames were constructed directly on the screen (**Figure 2.242**). One was 2,302 μm^2, in which spermatogonia were counted as they occurred less frequently, and enclosed in this was a 230 μm^2 frame in which the remaining germ cells were counted. The germ cells were counted if their nuclei came into focus on the screen when focusing in on the section to a depth of 10μm. At least 100 fields per section, selected by a systematic uniform random sampling scheme using a motorised stage (Wreford 1995). Sertoli cell nuclei were counted in a frame of 1.381 μm^2 in 150 fields per section. The cells were only counted if their

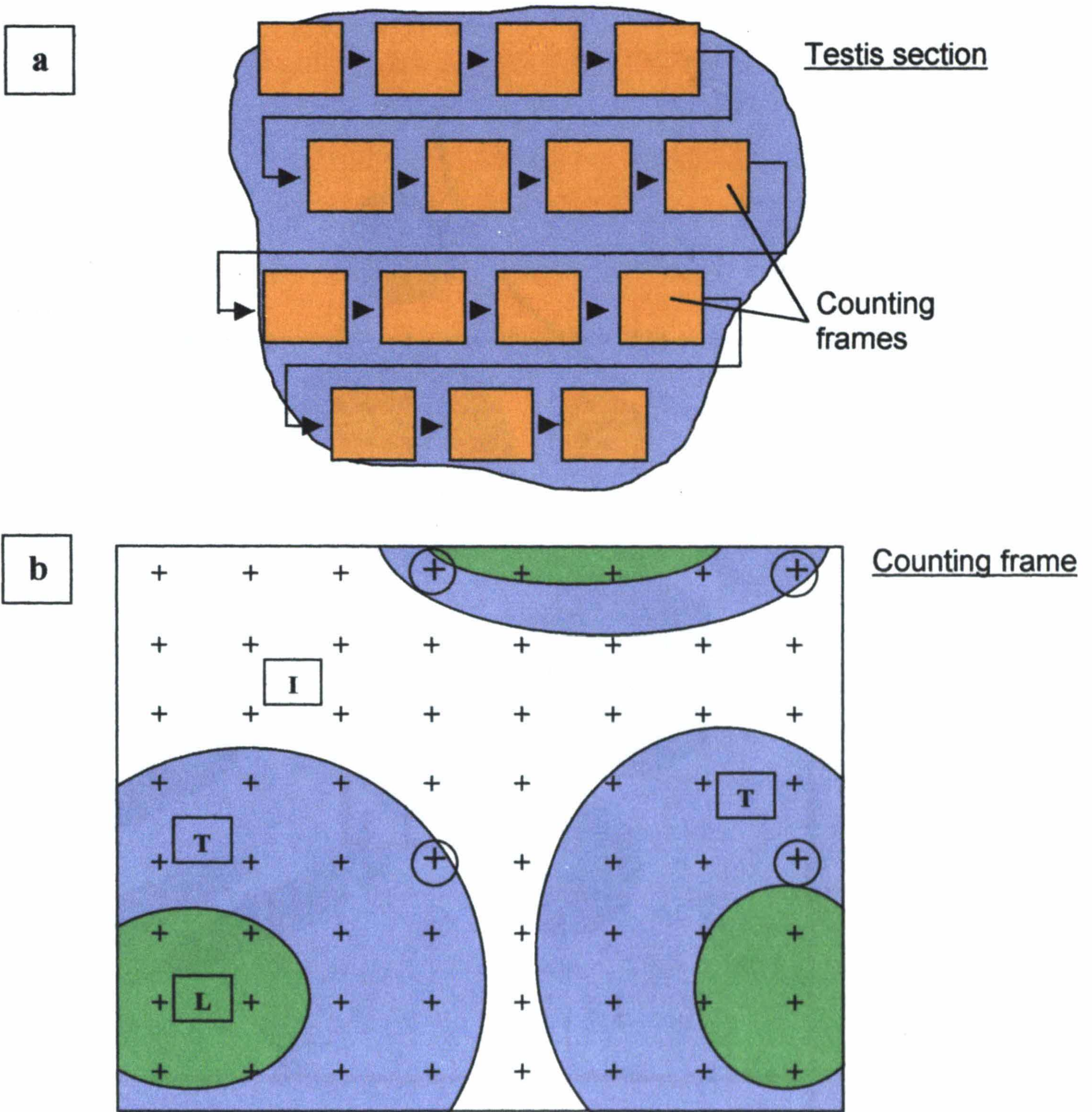


Figure 2.241 Calculating the volumes of the testicular compartments

(**a**) To determine the volume of the entire testis section, approximately 10-20 counting frames were randomly chosen using a motorised stage, each following the other. (**b**) A point grid was created consisting of 4 major ⊕ and 16 fine + points. The major points were counted if they landed on seminiferous tubules (T), and the fine points if they landed on lumen (L), interstitium (I) and tunica (not shown). For example, in this figure 4 major points are on the tubules, 12 fine on lumen and 25 on interstitium. The total number of points landing on each compartment was divided by the total points per testis then multiplied by the testis weight (g) to then give the volume (mm^3) of each compartment.

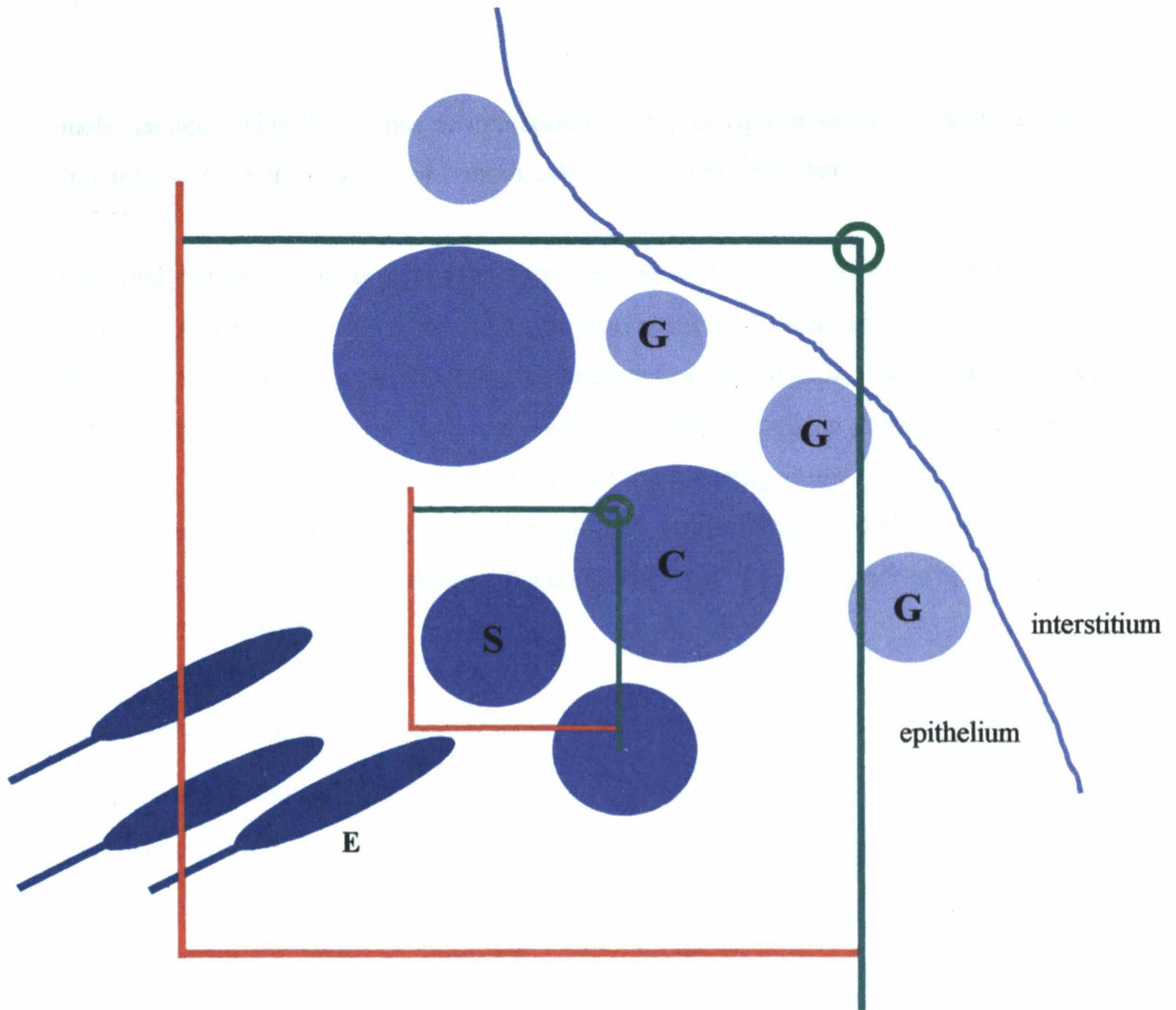


Figure 2.242 The optical disector method for quantifying germ cells

Two counting frames were constructed directly on the computer screen, with 100 fields per testis section chosen by a systematic uniform random sampling scheme. Spermatogonia (G) were counted if they landed within the boundaries (green) of the larger counting frame (2,302μm^2) as they occurred less frequently. Enclosed in this was a 230μm^2 frame in which spermatocytes (C), round spermatids (S) and elongated spermatids (E) were counted. In this example, one spermatocyte and one round spermatid intersected the small frame, whereas 3 spermatogonia fell in the larger frame.

nuclei landed inside the frames or intersected the top or right boundary (**Figure 2.242**). If they intersected either the left or bottom side of the frame they were discounted.

The total number of cells per testis were calculated by the following formula. Firstly, volume = 10 μm x TF x A, where TF is the total number of fields and A is the frame area. Then N = NC/Volume, were NC is the total number of cells. Finally Total number cells per testis (millions) = N x Wt x 10^{12}, were Wt is the testis weight in grams. Whilst this was occurring, where the top right point (circle) of the counting frame (**Figure 2.242**) landed was also noted, for example, either interstitium or seminiferous epithelium, either in stage I-VII or stage VIII-XII. This allowed the composition of the seminiferous epithelium to be preliminarily analysed.

2.243 Diameter of seminiferous tubules

Cross sections of the seminiferous tubules were measured using the stereological system at x10 magnification. Approximately 100 tubules were measured per animal, the average diameter (μm) then calculated. From this, the average tubule length could also be calculated with the following equations. Firstly, tubule volume: TV= (PE + PL/TP) x Wt, where PE is the number of points landing on the seminiferous epithelium, PL the points on the lumen and TP being the total number of points landing on the testis, multiplied by the weight of the testis in mg. Then tubule area was then calculated using the following equation: $TA = \pi x (d/2)^2$, where d equals the average tubule diameter in μm. And finally, the tubule length in metres (TL) which equals: $TL = TV \times 10^{-3}/TA \times 10^{-6}$.

2.244 Hourly production rate

The hourly production rate of each germ cell type, per testis, was calculated using published time divisors, whereby the number of sperm per testis was divided by the particular time in hours: 351.2 h for spermatogonia, 328.8 h for spermatocytes, 138.4 h for round spermatids and 212.8 h for elongated spermatids (Russell *et al.* 1990). This allowed

the hourly production rate for each germ cell type per testis to be compared between WT and ArKO.

2.25 Electron microscopy

WT and ArKO testes were immersion fixed in 0.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). After 1 h they were removed, cut in half and replaced into the buffer for a further 2 h to ensure complete fixation. After fixing, the testes were removed, cut into 10ths with 3 random pieces from the top middle and bottom removed and stored in 0.01 M cacodylate buffer only at 4°C. These were embedded in Epon araldite, then 1 μm sections were prepared, stained with toluidine blue, and 3-4 tubules of interest chosen when examined under light microscopy. Ultrathin (90nm) sections were then prepared using a Reichert OMU3 microtome. These were prepared for electron microscopy by incubating with uranyl acetate in distilled water, and stained with Reynolds lead citrate stain as per standard electron microscopy procedures.

2.26 Immunocytochemistry

2.261 3β-hydroxysteroid dehydrogenase (3β HSD)

In order to visualise and quantify the Leydig cells, they were stained with a 3β-HSD (3β-hydroxysteroid dehydrogenase) antibody. Dewaxed 5μm paraffin sections were washed in PBS (0.01 M Phosphate Buffer, 0.015 M NaCl, pH 7.5). Trypsin (0.02% in 0.1% $CaCl_2$) was used as the antigen retrieval step, sections incubated for 40 min and the reaction terminated by a 10 min water wash. Endogenous peroxidase activity was removed by treating the sections with hydrogen peroxide (H_2O_2, 6% v/v) for 30 min at room temperature, with a wash in PBS halting this reaction. The sections were then incubated for 20 min with CAS block containing 10% normal sheep serum (diluted in PBS), to block non-specific antibody binding. After removal of the serum, one section on each slide was immediately incubated with 1:300 polyclonal rabbit anti-ovine 3β-HSD antiserum (diluted in PBS, kindly supplied by Gail Risbridger from the Institute of Reproduction and

Development) and the other in 1:300 normal rabbit serum (NRS, diluted in PBS) as a negative control, at 4°C overnight.

After removal of the primary antibody, the secondary antibody, IgG-biotinylated sheep anti-rabbit (1:100; Silenus) was incubated with the 2 sections for 1 h at room temperature. The avidin biotinylated conjugate (ABC) was made up 30 min prior to use by following manufacturers instructions. After the secondary antibody was removed (2 PBS washes), the sections were incubated with the ABC reagent for 1 h. This incubation allowed the avidin and horseradish peroxidase (HRP) to attach to the biotin located on the secondary antibody, thus labelling the antibody. DAB (3,3'-diaminobenzidine tetrahydrochloride) was prepared according to manufacturers instructions and incubated with the sections for 40 s -1 min. The HRP caused the hydrogen peroxidase to react with the DAB, the Leydig cells visualised by the resulting brown colour reaction. Mayers haemotoxylin for 3 min, then Scotts tap water (10x; Sigma Diagnostics, St Louis, Mo) for 40 s counterstained the cells, a water wash terminating these reactions. The sections were dehydrated in 1 x 70% ETOH, 2 x 100% ETOH and 2 x histosol dips. They remained in the final histosol dip for 15 min prior to being mounted under glass with DPX. The volume of the Leydig cells was determined stereologically as described (**Section 2.241**).

2.262 *TUNEL assay*

Apoptotic cells were visualised using the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) technique, kindly performed by Dr Sarah Meachem, Prince Henry's Institute of Medical Research, Clayton, Vic. Once again, endogenous peroxidase activity was removed by treating the 5μm sections with hydrogen peroxide (H_2O_2, 3%v/v in methanol) for 30 min at room temperature. A PBS wash halted this reaction. The reaction mix for labelling the 3' end of the fragmented DNA was as follows: 5 x terminal transferase buffer (potassium cacodylate, 1 mol/L; Tris HCL, 125 mmol/L; BSA 1.25 mg/ml, pH 6.6 at 25°C), $CoCl_2$ (25 mM), digoxigenin labelled dideoxy-dUTP (5 μM) and TdT (25 IU/μl). The second section on the slide was used as a

control, with distilled water replacing the TdT. After the reaction mix was applied, the sections were coverslipped and sealed using nail varnish, the reaction then proceeding for 30 min at 37°C in a humid chamber. After removal of the coverslips the reaction was halted by 2 PBS washes (5 min). To block non-specific binding of the antibody, the sections were then incubated for 10 min at room temperature with 20% NRS (diluted in PBS). The incorporated DIG was detected with a sheep anti digoxigenin-peroxidase (1:500 in 20% NRS in PBS) at room temperature in a humidified chamber for 30 min. Sections were washed twice for 5 min in Tris Buffer (0.5 M Tris, 0.15 M NaCl, pH 7.5) to remove excess antibody. The secondary antibody applied was IgG-biotinylated rabbit anti sheep (1:500 in 20% NRS in Tris Buffer) for 30 min at 37°C. This reaction was terminated by 2 washes in PBS (5 min). The sections were then incubated in a streptavidin-biotinylated horseradish complex (1:100 in Tris Buffer) at room temperature for 30 min, and then washed twice with Tris Buffer. The apoptotic cells were visualised with 3,3'-diaminobenzidine tetrahydrochloride (DAB) for 10-15 min according to manufacturers instructions, this reaction halted by a Tris Buffer wash. Sections were counterstained with Mayers haemotoxylin for 10 s, then dehydrated in 1 x 70% ETOH, 2 x 100% ETOH and 2 x histosol dips. They remained in the final histosol dip for 15 min prior to being mounted under glass with DPX.

Each section, 1 per animal, were examined for the presence of TUNEL positive cells. Under 10 x magnification using an Olympus microscope (CBH-2), each tubule was initially classed as being at either at the early (I-IV), mid (V-VIII) or late (X-XII) stage of development. Then the number of apoptotic cells per tubule and the type of cell that had stained brown were recorded. Also noted were the total number of tubules counted. This allowed the number of apoptotic cells to be calculated per tubule providing an initial characterisation of the tubules.

2.27 Serum Hormone Assays

2.271 Testosterone

Serum testosterone (T) was detected using a double antibody radio-immuno assay (RIA). The assay used an acidic citrate buffer (0.05 M citric acid, 0.01% sodium azide, 0.02% γ globulin, pH 5.1) to release T from its binding proteins in the serum. The tracer used was iodinated histamine-testosterone (10,000 cpm/ml, purchased from Clinical Biochemistry, Monash Medical Centre, Australia) with a rabbit anti-T primary antibody (TAS 0811 in 1:60 normal rabbit serum (NRS) (Sirosera, Sydney, Australia)). Each assay consisted of a standard curve from 333 pg/100µl to 1.3 pg/100µl, against which the unknowns were measured. Samples, 5 µl, were added in duplicate to a total volume of 500 µl. After 24 h incubation at 4°C, the goat anti-rabbit secondary antibody (1:10, GAR#12; Silenus) and 4% polyethylene glycol were added to facilitate pelleting. As a control, the same quantity of charcoal stripped normal mouse serum was used. All samples were measured in the single assay with a gamma counter (Wallac 1470, Turku, Finland). The detection limit of the assay was 0.4 ng/ml and the within assay variation was 4.6%. Both dose response curves were parallel with the stripped serum standard, with this curve repeated if new serum was used. The primary antibody is known to cross react with 5α dihydrotestosterone (DHT) 39%.

2.272 *FSH and LH*

The following assays were undertaken by Anne O'Connor from the Institute of Reproduction and Development, Clayton, Vic. Both LH and FSH assays were determined by RIA with the following iodinated preparations (iodinated using Iodogen reagent) and antisera: rFSH 1-8 and anti-rFSH-S-11; and rLH-1-9 and anti-rLH-S-10 rFSH1-8 (NIDDK, Bethesda, MD, USA). The secondary antibody used in both RIA's was goat anti-rabbit IgG (GAR #12), and the assay buffer 0.01 M PBS containing 0.5% BSA. All samples were assayed in 20 µl duplicates with 20 µl assay buffer and 20 µl antiserum. After incubating overnight at room temperature, 5000 cpm/20µl tracer was added. Following a second

overnight incubation, 20 μl of the second antibody was added and the total serum concentration in each tube equalised using normal mouse serum. The next day, 0.9 % saline (4°C) was added, centrifuged and pellets counted on a gamma counter (Wallac 1470, Turku, Finland). The data was log transformed and calculated using straight line statistics. Again, all samples were measured in the single assay. The sensitivity limits of the assay were 1.05 ng/ml (FSH) and 0.08 ng/ml (LH), whereas the within assay variations were 5.3% (FSH) and 9.2% (LH).

2.28 RNA isolation

RNA was isolated from whole testes using the Ultraspec RNA isolation system (Fisher Biotec) according to manufacturers instructions. Briefly, each frozen testis was cut into half and homogenised with a hand held homogeniser in 1ml Ultraspec reagent. After incubating on ice for 5 min, 200 μl chloroform was added, vortexed to mix, then the homogenate placed back on ice for a further 5 min. The samples were centrifuged at 12,000 x *g* for 30 min at 4°C, following which the upper aqueous phase containing the RNA was removed and an equal volume of isopropanol added. The samples were inverted to mix and left on ice for 10 min. Following centrifugation at 12,000 x *g* for 20 min at 4°C, the supernatant was discarded. The pellet was washed twice with 75% ethanol by vortexing, spun at 7,500 x *g* for 5 min at 4°C, and left to dry for 10 min before dissolving in 50 μl DEPC water (0.1% DEPC in sterile water, stir until dissolved and autoclave) at 65°C for 10 min.

To obtain the final RNA concentration, 2 μl of RNA in 50 μl DEPC water was analysed with a spectrophotometer (Beckman, DU 530) at 260/280nm absorbance according to the following formula:

$$[RNA]\mu g/ml = A_{260/280} \times \text{dilution factor} \times 40$$

To visualise the extracted RNA, 100 ng was loaded into a 1% agarose gel and run at 100 V for 30 min. If the RNA was not degraded, the 28S and 18S rRNA were clearly visible with

the Gel Doc UV transilluminator (BioRad Laboratories, Hercules, CA). The remaining total RNA was snap frozen on dry ice and stored at –80°C until further use.

2.29 Restriction fragment differential display PCR

Differential display PCR was carried out using the displayPROFILE kit (Display Systems Biotech) according to manufacturers instructions. Briefly, this involved reverse transcribing 10 μg total testis RNA, then phenol/chloroform extracting the cDNA and ethanol precipitating it overnight at –20°C. Gel electrophoresis (0.8%) was performed on 5 μl of the cDNA to check the quality of the cDNA, a smear should have been visible. The following day the cDNA was restriction digested with *TaqI*, a 4-base cutter which left a 5'-overhang. Then two specifically constructed DNA adaptors were ligated to the ends of the cDNA fragment. A ^{33}P labelled 5'-primer then annealed to these adaptors with 3 bases of the primer extending into the cDNA sequence. A total of 64 primer combinations were required to amplify all 3 base combinations. The differentially expressed genes in the testes samples were separated by 6% polyacrylamide gel electrophoresis.

2.291 Polyacrylamide gel

One of the two 40 x 55cm glass plates was treated with Sigmacote, then the polyacrylamide gel solution (35.82 g urea, 24.8 ml 30% Bis/acrylamide, 900 μl 10% APS and 120μl TEMED in 149 ml 1 x TBE) pipetted in between the two glass plate. The shark toothcomb was then inserted and left for approximately 1 h to set. After flushing the wells with 0.5 x TBE, the plates were secured into the vertical apparatus (BioRad) and 5 μl of 6 x loading buffer (0.125 g bromophenol blue, 0.125 g xylene cyanol, 20 g sucrose and DEPC water to 50 ml) added into each lane. The gel was pre-run at 250 V for 45 min to visualise each lane and to preheat the gel to retain the samples in a denatured state. The samples were then loaded and the gel run until the dye had moved approximately 75% of the plate distance. After removal of the gel onto Whatmann paper it was vacuum dried (Biorad Model 483 Slab Dryer) for approximately 90 min at 80°C, then exposed overnight

to phosphorimager plate (Molecular Dynamics), then phosphorimager (Storm, Molecular Dynamics). The dried gel was also exposed for approximately 5 days to an autoradiograph film (Kodak Biomax) at room temperature.

2.292 Reamplification of genes

Any differentially expressed genes were cut out of the gel and placed in 50μl sterile water. This was then heated at 95°C for 15 min, vortexed to mix and spun down at 12,000 x *g* for 10 min. Following this, a PCR reaction was set up containing 5 μl of the fragment containing solution and 8 μl of the display probe, as per manufacturers instructions. The PCR products were then loaded into a 1.5% agarose gel and run at 100 V for 30 min. Then the PCR products of the appropriate size were cut out of the agarose gel and purified using the QIAEX II Gel Extraction Kit (QIAGEN) according to the manufacturers instructions. As the DNA concentration was very low, it was ethanol precipitated by adding 2.5 vol 100% ethanol and $1/10^{th}$ vol sodium acetate, vortexed and incubated on dry ice for 15 min. The tubes were spun at 12,000 x *g* for 30 min at 4°C, the pellet washed with 75% cold ethanol and then spun for at 12,000 x *g* for 5 min at 4°C. The pellet was air dried then resuspended in 10 μl sterile water to a final concentration of 10–40 ng/μl for sequence analysis.

2.293 Northern blot analysis

Northern blot analysis was used to confirm if these genes of interest were differentially expressed between WT and ArKO mice.

i Formaldehyde gel preparation

A 1.2% agarose-formaldehyde gel was prepared with 10 ml 10 x MOPS (100 g MOPS, 16.67 g sodium acetate, 24.5 ml 0.5 ml EDTA and sterile water to 1222.5 ml, filter sterilised and stored in the dark), 17.5 ml acid free formaldehyde and DEPC water to 50 ml at 60°C. This was then slowly added to the molten agarose (0.48 g of agarose in 50 ml

DEPC water at 60°C) and carefully poured into the RNase free (soaked in 1.0 M NaOH) gel tray.

***ii** RNA preparation*

WT and ArKO total testis RNA, 15 μg in 5 μl, was added to 7 μl deionised formamide (5 g deionising resin (Biorad) /100 ml formamide shaken for 1 h then filtered), 2 μl 5 x MOPS, 3 μl formaldehyde and 4.2 μl 6x bromophenol blue, and the reaction heated for 10 min at 60°C. The total RNA was then loaded into the gel and run at 50 V until the samples had penetrated the gel, then was run at 40 V for approximately 4 h until the bromophenol blue had progressed 2 cm into the gel.

***iii** Transfer of RNA to membrane*

Following electrophoresis, the gel was washed twice in DEPC treated water to remove the formaldehyde. Northern transfer was carried out using 20 x SSC (3 M NaCl, 0.3 M Na_3citrate, pH 7.0) as the transfer buffer for 18 h, using the transfer set up as described by Sambrook *et al* (1989). The Hybond C membrane (Amersham Pharmacia Biotech) was carefully removed from the gel and crosslinked using a GS gene linker UV chamber (BioRad Laboratories) for 120 s. After washing in 2 x SSC, the blot was baked for 1-2 h at 80°C.

***iv** Pre-hybridisation*

The membrane was pre-hybridised with 25 ml of hybridisation buffer (5 x SSC, 5 x Denhardt's Reagent (1 g Ficoll 400, 1 g polyvinylpyrrolidone, 1 g BSA, water to 50 ml, then filter sterilised), 5% SDS, 50μl (10 mg/ml) of salmon sperm) at 65°C for a minimum of 1 h in a rotating hybridisation oven (Mini hybridisation oven, Hybaid).

***v** Labelling of probe*

To use the PCR product (**Section 2.292**) as a probe, 25 ng was added to 45 μl of sterile water, then incubated at 100°C for 5 min and immediately cooled on ice. This was then

added to a tube containing the RTS RadPrime DNA labelling system (Gibco BRL), and vortexed to mix. The DNA was labelled by adding 5 μl (10 mCi/ml) α-^{32}P-dCTP and incubating at 37°C for a minimum of 10 min. The reaction was halted by the addition of 5 μl 0.5 M EDTA. Before the probe was added to the membrane, any unbound label ws removed using the NucTRap purification columns (Stratagene) according to manufacturers instructions, to remove any unbound label. Once purified the probe was denatured at 100°C for 5 min, immediately cooled on ice, and added to the membrane and pre-hybridisation buffer. The membrane was then incubated at 65°C for 18 h.

vi Washes

Following hybridisation, the membrane was transferred to 500 ml of a low stringency wash (2 x SSC, 0.1% SDS) and washed twice by gentle shaking for 10 min at room temperature. This was followed by two 500 ml washes in a higher stringency wash (1 x SSC, 0.1% SDS) prewarmed to 65°C for 10 min, then two washes in a high stringency wash (0.1 x SSC, 0.1% SDS) again prewarmed to 65°C for 10 min. The stringency of the washes could be varied depending on the background counts. The membranes were then sealed in plastic and exposed to phosphorimager plates (Molecular Dynamics) overnight, and then autoradiograph film (Kodak Biomax) for approximately 5 days.

2.30 Gene array

A mouse cell cycle GEarray kit (mGEA1012010, Superarray) comprised of the following cell cycle genes: cyclins (cyclin A, cyclin B, cyclin C, cyclin D1, D2, D3, cyclin E1, E2), cyclin dependant kinases (cdk1, cdk2L, cdk4, cdk6), cdk inhibitors (p15^{ink4b}, p16^{ink4a}, p18, p19, p21^{waf1}, p27^{kip1}, p57^{kip2}) and various other cell cycle specific genes (retinoblastoma protein, E2F1, Skp1 and Skp2), was used to examine the expression of cell cycle genes between WT and ArKO testes. The GEarray was carried out according to the manufacturers instructions. Briefly, total testes RNA from one WT and one ArKO at 18 wks of age were used as a template to create two ^{32}P labelled cDNA probes. These were

hybridised overnight to the membranes provided, washed and exposed overnight to phosphoimager plate. Each membrane was analysed as described (**Section 2.34**).

2.31 Real-time PCR

2.311 Primers

Specific primers for a selection of cell cycle regulated genes (**Table 2.311**) were designed (**Section 2.211**). A 20-35 bp sequence in between the two PCR primer pairs was chosen as a probe for Southern blotting (**Table 2.312**). These again were checked through a BLAST search.

Table 2.311 PCR Primer sequences

Gene	Forward primer	Reverse primer
18s	5'-cgg cta cca cat cca agg aa-3'	5'-gct gga att acc gcg gct-3'
Cyclin A1	5'-ccg tgc tag ggg tgt tga ctg aa-3'	5'-tcc tct gac tgg gca tgg gtg gtg-3'
Cyclin B1	5'-tga ccc aaa cct ctg tag tga ata-3'	5'-tgg tgc tta gtg tac gtg ttg tta-3'
Cyclin D1	5'-cgc cat gac tcc cca cga ttt c-3'	5'-ggc gca ggc ttg act cca g-3'
Cyclin E	5'-tat ggt gtc ctc gct gct tct gct-3'	5'-tgc tcg ctg ctc tgc ctt ctt act-3'
p21 waf1	5'-atg tcc aat cct ggt gat gt-3'	5'-tgc agc agg gca gag gaa gt-3'
p53	5'-gaa att tgt atc ccg agt atc t-3'	5'-ggg gca gtt cag ggc aaa gga c-3'
pRb	5'-gca tct tta tcg cag cag tt-3'	5'-att acc agg tca tct tcc atc t-3'
E2F1	5'-gct gca act gct ttc gga gga ct-3'	5'-agg ctg ggg atg tgg agg gag gtg-3'

2.312 Reverse transcription

Standards, used to measure the unknown samples against in the Real-time PCR reactions, were constructed for each gene using 1 μg total testis RNA and 50 ng random hexamer primer in a 20 μl volume. The reaction mix contained 5 μl 5 x AMV buffer, 0.5 μl (0.1 M) DTT, 1 μl 20 nM dNTP, 0.63 μl (25 units/μl) RNase inhibitor and 1.7 μl (40 units/μl)

AMV RT enzyme (Boehringer Mannheim) and DEPC water to 20 μl. A negative control was included with DEPC water replacing the RNA. The reactions were incubated at room temperature for 5 min, 42°C for 60 min, 65°C for 5 min and 95°C for 5 min.

Table 2.312 Probe sequences

Gene	Probe
Cyclin A1	5'-ccc tgc ggc tgg aaa gaa ag-3'
Cyclin B1	5'-cta ata cag gtt cag atg aa-3'
Cyclin D1	5'-ttg tgg ccc tct gtg cca ca-3'
Cyclin E	5'-cat gga aga ctc cca caa cat cca gac cca cac ca-3'
p21 waf1	5'-ccg ctg gag ggc aac ttc gtc tgg gag cgc gtt cg-3'
p53	5'-cca cca tcc act aca agt aca tgt gta ata gct cc-3'
pRb	5'-ctc tac agc aaa tta gaa cgg acg tgt gaa ctt at-3'
E2F	5'-ttg atg ttt tcc tgt gcc cgg agg aga gtg cag ac-3'

2.313 PCR

A 50 μl PCR reaction was set up, containing 2 μl cDNA (**Section 2.312**) and 1 μl of each forward and reverse primer (10 pmol/μl). The reaction mix contained 5 μl 10x PCR buffer, 1 μl 20 nM dNTP, 0.14 μl (5 units/μl) Taq polymerase enzyme (Boehringer Mannheim) and sterile water to 50 μl. Two negative controls were included, one using 2 μl of the control reverse transcriptase (RT) reaction and another replacing the 2 μl cDNA with sterile water. The following program was applied to all PCR reactions in the block PCR machine (PCR Express, Hybaid), with only the annealing temperature varying accordingly (**Table 2.313**). The first stage was a single denaturing step for 1 min at 94°C, followed by the second stage of 35 cycles of the denaturing stage for 45 s, the annealing step for 20 s and the extension step for 30 s at 72°C. The 30 s extension step was based on products less than 500 bp. The third stage is a final extension step of 1 min.

Table 2.313 The annealing temperature for each primer pair

Gene	Size of product (bp)	Annealing temperature (°C)
18s	180	55
Cyclin A1	346	60
Cyclin B1	357	55
Cyclin D1	323	60
Cyclin E	336	65
p21 waf1	301	55
p53	306	55
pRb	337	54
E2F	335	60

2.314 cDNA purification

The PCR products were loaded into a standard 1% agarose gel and run for 30 min at 100 V in 1 x TBE buffer and the DNA visualised with an UV transilluminator (Gel doc, Biorad). The cDNA's of the appropriate size were excised from the agarose gel and purified using the QIAEX II Gel Extraction Kit (QIAGEN) according to the manufacturers instructions. The DNA was then quantified using the spectrophotomoter (Beckman, DU530) and 5μl run on a 1% agarose gel to check the size and purity of the extracted product. If the band was faint, suggesting a low concentration of product, then the PCR was repeated.

2.315 Reverse transcription for Real-time PCR

For the Light cycler PCR method, the *C.therm* reverse transcriptase enzyme (Roche) was used in place of the AMV as it was found to be more efficient when transcribing mRNA with secondary structures. One μg of RNA and 100 ng random hexamer primer in a 20 μl volume was added to the reagents supplied in the kit: 4 μl 5 x reverse transcriptase buffer,

1 μl (0.1 M) DTT, 0.6 μl (100%) DMSO, 1.5 μl *C.therm* polymerase (4 units/μl) enzyme, 0.64 μl dNTP (20 nM) and DEPC water to 20 μl. The reactions were incubated at room temperature for 5 min, heated to 42°C for 15 min, 60°C for 60 min, 70°C for 15 min then 95°C for 2 min.

2.316 Optimisation of $MgCl_2$ concentrations for Real-time PCR

As the Real Time PCR are very sensitive to the concentration of magnesium chloride, this was first optimised. One to five mM of Mg^{2+} and 2 μl of the SYBER green master mix (10 x), contained in the FastStart DNA master SYPR green 1 kit (Roche), were added to 1 μl of the cDNA, 0.5 μl of 10 pmol/μl forward and reverse primer and sterile water (to 20 μl) in a Light Cycler capillary (Roche). The following program (**Table 2.315**) was applied to all PCR reactions, with the annealing temperature and extension time varying accordingly (**Table 2.314**).

Table 2.314 Light Cycler program variables

Gene	$MgCl_2$ (mM)	Annealing temperature (°C)	Extension time (s)	Read at (°C)
18s	3	60	10	80
Cyclin A1	3	62	14	83
Cyclin B1	5	58	15	80
Cyclin D1	4	63	15	87
Cyclin E	3	65	13	82
p21 waf1	3	56	15	87
p53	3	56	13	83
pRb	4	54	14	78
E2F	3	65	14	85

Table 2.315 LightCycler Experimental Protocol.

Denature

Cycles	1						
Type:	None		Florescence Display Mode =			F/1	
Segment Number	**Temperature Target (°C)**	**Hold Time (sec)**	**Slope (C°/sec)**	**2° Target Temp (°C)**	**Step Size (°C)**	**Step Delay (Cycles)**	**Acquisition Mode**
1	95	600	20	0	0	0	None

PCR

Cycles	40						
Type:	Quantification		Florescence Display Mode =			F/1	
Segment Number	**Temperature Target (°C)**	**Hold Time (sec)**	**Slope (C°/sec)**	**2° Target Temp (°C)**	**Step Size (°C)**	**Step Delay (Cycles)**	**Acquisition Mode**
1	95	600	20	0	0	0	None
2	60	5	20	0	0	0	None
3	72	10	20	0	0	0	None
4	80	1	20	0	0	0	Single

Melting Curve

Cycles	1						
Type:	Melting Curves		Florescence Display Mode =			F/1	
Segment Number	**Temperature Target (°C)**	**Hold Time (sec)**	**Slope (C°/sec)**	**2° Target Temp (°C)**	**Step Size (°C)**	**Step Delay (Cycles)**	**Acquisition Mode**
1	95	0	20	0	0	0	None
2	55	10	20	0	0	0	None
3	95	0	0.2	0	0	0	Continuous

Cool

Cycles	1						
Type:	None		Florescence Display Mode =			F/1	
Segment Number	**Temperature Target (°C)**	**Hold Time (sec)**	**Slope (C°/sec)**	**2° Target Temp (°C)**	**Step Size (°C)**	**Step Delay (Cycles)**	**Acquisition Mode**
1	40	60	20	0	0	0	None

This protocol was used for each LightCycler PCR run, with the annealing temperature, the extension time and the temperature the florescence was read at (shaded in grey) varied accordingly (**Table 2.314**).

The first stage was a 10 min incubation at 95°C to release the *Taq* polymerase from an antibody designed to prevent non-specific amplification. The second stage was a denaturing step of 95°C for 0.1 s, followed by an annealing step for 5 s and the extension step at 72°C for approximately 40 cycles. The extension time was calculated by the product size divided by a factor of 25. In order to determine the melting temperature in the amplicons, the third stage consisted of increasing the temperature to 95°C then slowly cooling to 55°C in order to create a melting curve. The final stage cooled the capillaries to 40°C for their removal.

In each run, a standard curve was created with which to compare the unknown WT and ArKO samples against. For example the first 5 capillaries in the 18S run were dilutions of the 18S standard **(Section 2.312)**; 1ng/µl, 100pg/µl, 10pg/µl, 1 pg/µl and 0.1pg/µl (these altered depending on the expression level of the gene), followed by 6 WT samples (2µl cDNA), 6 ArKO samples and finally 2 negative controls.

2.317 Southern blot analysis

To confirm that the amplified cDNA was the correct product, it was run on a 1% agarose gel in 1 x TBE, then transferred overnight for Southern blot analysis using the procedure according to Sambrook *et al* (1989). Briefly, the DNA was transferred onto a Hybond N+ membrane (Amersham pharmacia biotech) using a denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 18 h. The membrane was UV crosslinked for 2 min, washed in 2 x SSC, and air-dried.

i Hybridisation

Each membrane was pre-hybridised with the hybridisation buffer (5 x SSC, 5 x Denhardt's Reagent, 1% SDS, 50 µl (10 mg/ml) salmon sperm and DEPC water to 5 ml) at 42°C for 1 h in a rotating hybridisation oven (Hybaid).

ii Labelling of probe

To label the oligonucleotide (**Section 2.311**), 5 μl 5 x forward reaction buffer, 1 μl T4 polynucleotide kinase (GibCo BRL), 16 μl ddH_2O and 2.5 μl (10 mCi/ml) [γ-^{32}P]ATP was added to 50 pmol of the oligonucleotide and incubated at 37°C for a minimum of 10 min. Before the probe was added to the membrane, any unbound label was removed using the NucTrap purification columns (Stratagene). Once purified, the probe was added to the membrane and hybridised in a rotating hybridisation oven (Hybaid) at 42°C for 18 h.

iii Washes

The membrane was transferred to 500 ml of low stringency wash (2 x SSC) and washed by gentle shaking for 3 x 10 min at 42°C. This was followed by 3 further 500 ml washes in a high stringency wash (0.2 x SSC) at 42°C, each for 10 min. After sealing the membranes in plastic, they were exposed to autoradiograph (Kodak Biomax) for approximately 10 min.

2.32 *Insitu* hybridisation

2.321 Oligonucleotide labelling

To label the 35bp sense and antisense oligonucleotides (**Table 2.321**), 4 μl 5x terminal transferase buffer, 1 μl terminal transferase, 3 μl $CoCl_2$ (diluted 1 in 5), 5.5 μl ddH_2O and 5 μl (10 mCi/ml) [α-^{35}S]dATP were added to 1.5 μl of the oligonucleotide (30 ng/μl) and incubated at 37°C for 2 h. The labelled oligonucleotide was purified using the NucTrap purification columns (Stratagene), according to manufacturers instructions. To quantify the radioactivity, 1 μl was added to 3 μl scintillation fluid and quantified with a liquid scintillation analyser (2500 TR, Packard) with a count between 100,000 and 300,000 cpm/μl indicating successful labelling. The probe could be stored at this time at –20°C by adding 1-2 μl 1 M DTT.

Table 2.321 Insitu probe sequences

Gene	Probe
***p21^{Waf1}* - sense**	5'- ccg ctg gag ggc aac ttc gtc tgg gag cgc gtt cg-3'
- antisense	5'-cga acg cgc tcc cag acg aag ttg cat cca gcg g-3'

2.322 Hybridisations

The slides were incubated in 1 x SSC for 2 min, then dehydrated in 70%, 95% and 2 x 100% ethanol each for 3 min, then 100% histosol for 15 min followed by a final 100% ethanol for 3 min. The slides were then left to air dry for 2 h to overnight. A humid chamber was constructed consisting of a slide box (Kartell) with 4 x 15 mm petri dish at each corner (**Figure 2.231**). These were filled with buffer (12.5 ml 4 x SSC, 25 ml 50% formamide and DEPC water to 50 ml) to retain the chamber in a humid state for the 18 h.

To approximately 1 ml hybridisation buffer (25 ml deionised formamide, 10 ml 20 x SSC, 4 g dextran sulphate and DEPC water to 40 ml, shaken for 2-4 h to dissolve the dextran), 250 µl 1 M DTT was added. Then the labelled oligonucleotide was diluted to 3 x 10^6 cpm/ml in this buffer. Approximately 120 µl of the diluted probe was pipetted onto the section, covered with a coverslip and placed into the humid chamber sealed with parafilm (American National Can, Wi). To optimise the hybridisation conditions, slides examining p21^{Waf1} were either incubated at 42°C, 50°C or 55°C for 18 h.

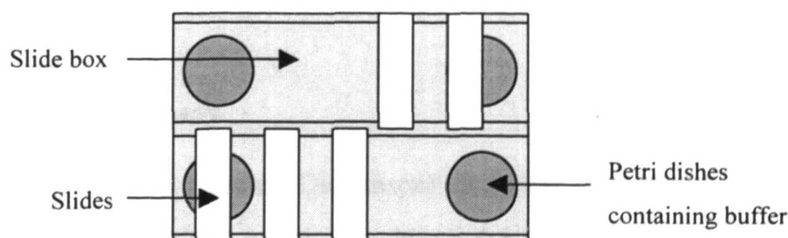


Figure 2.231 Insitu Hybridisation Humid Chamber

After hybridisation the slides were washed in 1 x SSC for 1 min, then incubated in 1 x SSC at 55°C for 60 min, followed by another 1 x SSC wash for 1 min. This was followed by dehydration washes in 70%, 95% and 2 x 100% ethanol each for 3 min. After leaving to dry for a minimum of 2 h at room temperature, each slide was exposed overnight to phosphoimager slide to indicate the time required for exposure to autoradiograph film.

2.323 Dipping slides

The slides were dipped in emulsion (10 ml in 10 ml distilled water at 40°C; Ilford Scientific Product) and left to dry for 2-4 h in the dark. Two blank slides were also dipped, one exposed straight away to light and the other used for background grain counts. Once dry, the slides were transferred to a light sealed slide box and stored for 3 wks at 4°C.

2.324 Developing emulsion dipped slides

The slides were placed in the Developer solution (diluted 1:4 distilled water; Ilford Phenisol Xray Developer (CP4277)) for 3 min then placed directly in the Acid stop bath (diluted 1:40 distilled water; Ilford IN-1 Indicator Stop Bath (CP4701)) for 2 min. Following this, the slides were left to briefly drain before placing in the final fixative solution (diluted 1:4 water) for 4 min, then rinsed with running tap water for 20-30 min.

To stain the testis sections, the slides were placed in haematoxylin for approximately 20 s, followed by the following dehydration steps each for 1 min; distilled water, 50%, 70%, 95% and 2 x 100% ethanol, then 2 x bioclear each for 7 min. Each slide was then mounted directly under glass using DPX.

2.33 RNase protection assay

The RNase protection assay (Becton Dickensen) was used to examine the expression of differentially expressed apoptotic genes. Two templates were applied, mAPO-2 containing the bcl-2 family of proteins (bcl-2, bfl1, bcl-xS, bcl-xL, bak, bax and bad) and mAPO-3 containing the death receptor pathway genes (Caspase-8, FASL, FAS, FADD, FAP, FAF,

TRAIL, TNFRp55, TRADD and RIP). Each assay also contained 2 housekeeping genes, L32 and GAPDH. The assay was performed according to manufacturers instructions (**Figure 2.331** for summary) with the following amendments. For the synthesis of the probe, 2 μl of the template and T7 RNA polymerase was added into the reaction, along with 15 μl (10 mCi/ml) [α-^{32}P]UTP as it contained a dye which lowers the T7 RNA polymerase efficiency. To generate the probe the reaction mix was incubated for 2 h. During the phenol chloroform extraction, approximately 10 μl of the yeast tRNA was added to allow for easier visualisation of the pellet. Instead of incubating at –70°C when ethanol precipitating, the tubes were placed in dry ice for 30 min. To assist in solubilising the pellet it was heated at 65°C for 10 min prior to vortexing. To quantitate the probe, the tip containing 1 μl of probe was placed into a scintillation vial without scintillation fluid. Then, 10 μg of RNA was used, with both the negative control (yeast tRNA) and the mouse positive control RNA provided. The polyacrylamide gel was prepared as described in **section 2.291**.

2.34 Phosphorimaging analysis

The gene array and RNase protection assays were exposed overnight to a phosphorimager plate (Molecular Dynamics), then phosphorimager (Storm, Molecular Dynamics). Each was analysed by calculating the pixels of each blot, subtracting the background and dividing the pixels by the area. Each value was normalised to beta actin, as GAPDH is believed to be regulated by factors such as oestrogen.

2.35 Statistical analysis

Data was analysed using SPSS 10.0 for Windows. Analyses in Chapter 3, 5 and 6 were performed using a one-way analysis of variance (ANOVA) to determine whether a significant difference existed between WT and ArKO mice. In Chapter 4, statistical analysis was performed using a two-way analysis of variance (ANOVA) to determine whether a significant difference existed between genotype and diet. If a positive result was

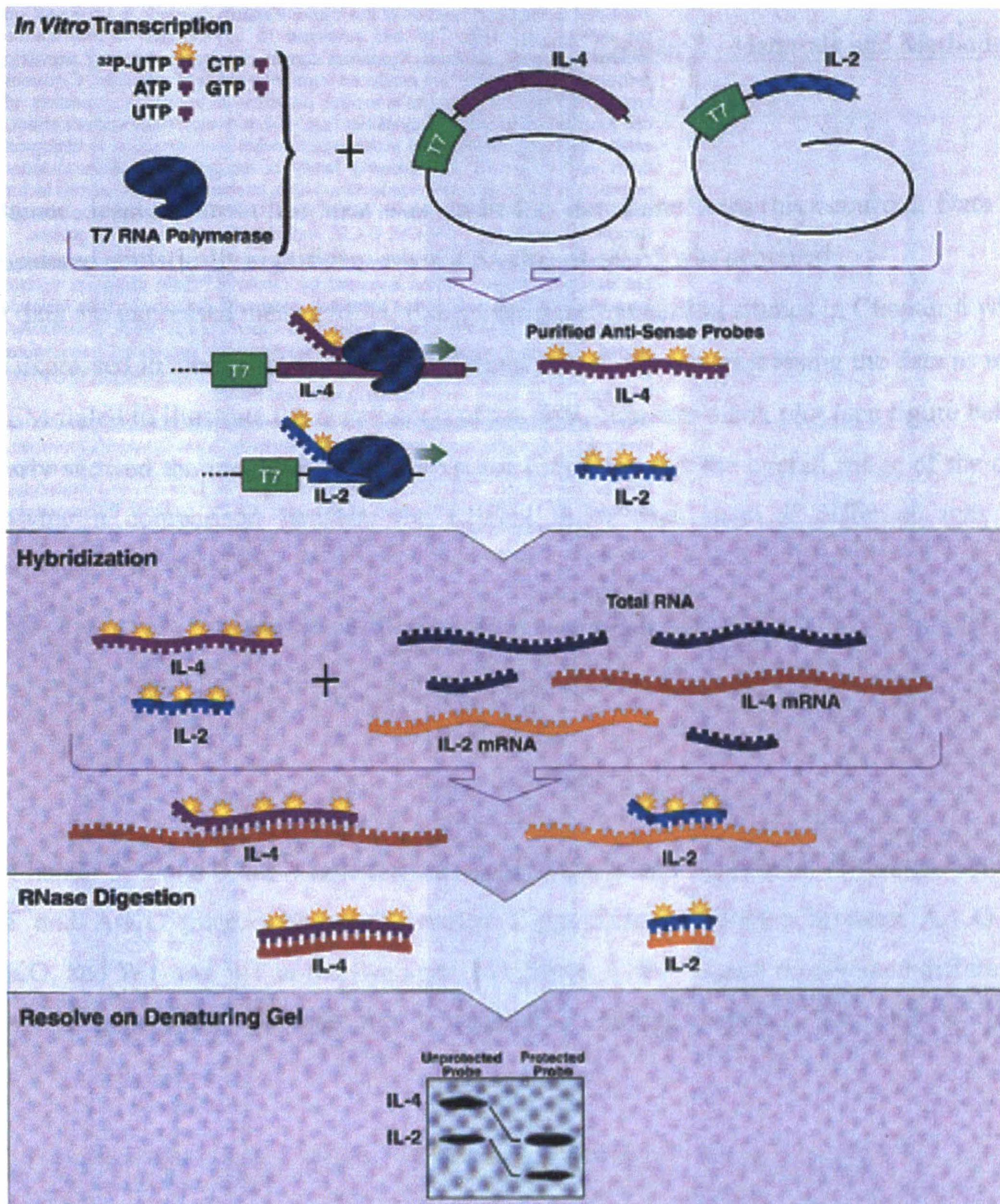


Figure 2.331 Overview of Riboquant RNase protection assay method

The cDNAs contained in the plasmid, supplied by the manufacturer, were *in vitro* transcribed with T7 polymerase and labelled with [α-^{32}P]UTP. Following purification of the anti-sense probes, they were hybridised overnight to total RNA. Then the free single stranded RNA and any unbound probe were digested with RNase A, leaving the protected double stranded RNA. The protected ^{32}P labeled probes were then run on a denaturing gel and quantitated by phosphorimaging.

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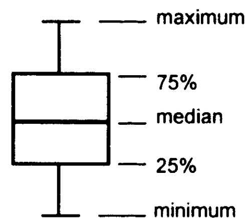
obtained, least squares difference was applied to determine were this occurred. Data was considered statistically significant when a p value of <0.05 was achieved.

All data are expressed mean ±SEM. Except the gene expression studies in Chapter 6 which are expressed in a box plot format. This format was chosen, as expressing the data as mean ±SEM failed to illustrate the distribution of the data. Whereas a box plot (see figure below) clearly showed the median values, the spread (25-75%) and the overall range of the data, allowing a comparison between the expression of each gene at different ages and genotypes to be made.



In Chapter 3, 5 and 6, the * notation was used to illustrate a significant difference between WT and ArKO mice, whereas # denoted a significant difference between ArKO and ArKO, and WT and WT at the two ages. In Chapter 4, the * again represented differences between genotypes, whereas the † denoted a change between S+ and S- diets.

Chapter Three

Characterisation of the Testicular Phenotype of the ArKO Mouse

3.1 INTRODUCTION

It is well known that androgens and gonadotrophins play crucial roles in spermatogenesis and in the development and function of the male reproductive system (**Section 1.36**). However, the role of oestrogens in male reproduction still remains to be elucidated. An indirect role for oestrogen in spermatogenesis was demonstrated in adult mice lacking a functional oestrogen receptor α isoform, αERKO (**Section 1.621**). The most prominent characteristic of the testicular phenotype in these mice was a grossly dilated seminiferous tubule lumen, observed as early as 20 days (Eddy *et al.* 1996). Subsequent studies on the efferent ductules of αERKO mice (Hess *et al.* 1997a) demonstrated that ERα played a crucial role in the reabsorption of luminal fluid in the efferent ductules, such that in the αERKO mice an excess of fluid accumulated in the testis. Therefore, it was suggested that the disruption to spermatogenesis in the ERKO mice was an indirect effect caused by abnormal fluid reabsorption (Hess *et al.* 1997a).

It has been recognised that ERα is only expressed outside of the seminiferous epithelium, present in the Leydig cells and efferent ductules of the rat (**Section 1.4, Table 1.4**). Oestrogen receptor β (ERβ), however, has been demonstrated in rat Sertoli cells, late spermatocytes and early round spermatids, and in mouse Leydig cells and elongated spermatids (**Section 1.4, Table 1.4**). While aromatase activity in Sertoli cells and Leydig cells is well documented, although at different stages of development, aromatase has also been demonstrated in spermatocytes and spermatids of the mouse and rat (**Section 1.4, Table 1.4**). This localisation of ERβ and aromatase within the seminiferous epithelium raised the possibility that oestrogen may actually play a direct role in spermatogenesis that was yet to be resolved.

Spermatogenesis was therefore investigated in the male ArKO mice that lack a functional aromatase enzyme due to targeted disruption of the *Cyp19* gene (**Section 1.63**). The testicular phenotype was evaluated using stereological techniques to quantify changes in

volumes of testicular compartments, and the optical disector approach used to enumerate Sertoli and germ cells.

3.2 RESULTS

3.21 General characteristics

The ArKO mice in our colony continued to consist of the correct Mendelian frequency, with litter numbers no different from their WT colony mates. The ArKO males were born phenotypically normal and appeared to develop normally in subsequent months.

3.22 Weight

Wildtype (WT) and ArKO mice colony mates were killed at ages 18 weeks (WT, n=6; ArKO, n=5) and 1 year (WT, n=4; ArKO, n=7), then body, testis and epididymal weight obtained. Body weight did not change between WT and ArKO at either 18 weeks or 1 year of age, however both WT (p=0.031) and ArKO (p=0.001) body weights did increase as the animals aged (**Table 3.221**). The testes in the ArKO animals all appeared to have descended normally (personal observation). There was no change in the weight of the ArKO testis at 18 weeks, however by one year of age the testis was significantly reduced in size (p=0.021) compared to WT mice (**Table 3.221**). This alteration in testis weight may indicate a possible disruption to the testis morphology.

3.23 Testis morphology

3.231 Qualitative observations on testicular morphology

To further examine the morphology of the testes they were immersion fixed in Bouins fluid, sectioned and stained (**Section 2.23**).

Table 3.221 Weights of WT and ArKO males

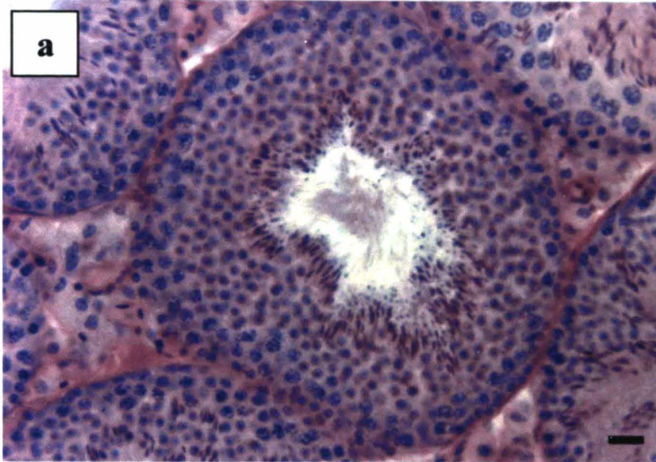
	Body weight (g)	Testis weight (mg)	Epididymis (mg)
WT 18 wks (n=6)	31.83 ± 2.1	137.38 ± 2.9	41.90 ± 2.8
ArKO 18 wks (n=5)	28.40 ± 1.6	113.38 ± 16.9	37.88 ± 2.6
WT 1 year (n=4)	43.25# ± 4.5	134.5 ± 6.4	40.13 ± 2.8
ArKO 1 year (n=7)	41.71# ± 2.1	101.54* ± 8.2	49.43 ± 3.8

Both WT (#$p<0.05$) and ArKO (#$p<0.001$) males increased their body weight as they aged. At 1 year of age, the ArKO mice have decreased testis weight compared to WT (*$p<0.05$). The notation * represents a significant difference between genotypes, # a difference between age. Data expressed as mean ± SEM.

At 18 weeks of age, there were no apparent histological differences between WT and 4 out of the 5 ArKO testes (**Figure 3.231a**). Illustrated is only the WT mouse, the ArKO appearing identical to this. However one ArKO mouse at age 18 weeks displayed grossly dysmorphic seminiferous tubules and disrupted spermatogenesis with no elongated spermatids present, indicating spermiogenic arrest (**Figure 3.231b**).

By 1 year of age, all 4 WT animals showed normal testicular morphology (**Figure 3.232a**), however removing endogenous oestrogens resulted in grossly dysmorphic seminiferous tubules with severe abnormalities in spermatogenesis in all 7 ArKO mice. The site of the spermatogenic disruption appeared to be early spermiogenesis with no elongating spermatids present, suggesting round spermatids failed to complete elongation and spermiation (**Figure 3.232b**). Six of the seven 1-year-old ArKO mice had tubules displaying spermiogenic arrest, but some normal tubules were also present (**Figure 3.232c**), suggesting a heterogeneity in the disruption. However one 1-year-old ArKO mouse had no elongated spermatids present in the testis, indicative of complete spermiogenic failure in the testes of these animals. When the germ cells were more closely examined, few cells more mature than step 4-5 round spermatids were observed. The seminiferous epithelium was frequently disrupted, with vacuoles in the epithelium (**Figure 3.232c**), and degenerating often multinucleated cells in the lumen (**Figure 3.232b**). Tubules were found to exist with an entire class of germ cells absent, for instance early and late spermatocytes and elongated spermatids were present, but no round spermatids, or tubules with only elongated spermatids (**Figure 3.232d**), or with just round spermatids. Tubules with no germ cells, only Sertoli cells, were also observed.

Further examination found specific disruptions occurring within the spermatogenic cycle. For instance step 1-7 spermatids were developing in a tubule with pachytene spermatocytes and early spermatocytes suggesting that the round spermatids should be in fact at a maturity stage of step 10-11 (**Figure 3.233c**). This may due to oestrogen having a significant role in round spermatid maturation or possibly even a survival function, evidenced by high levels of round spermatid degeneration (**Figure 3.233b**).



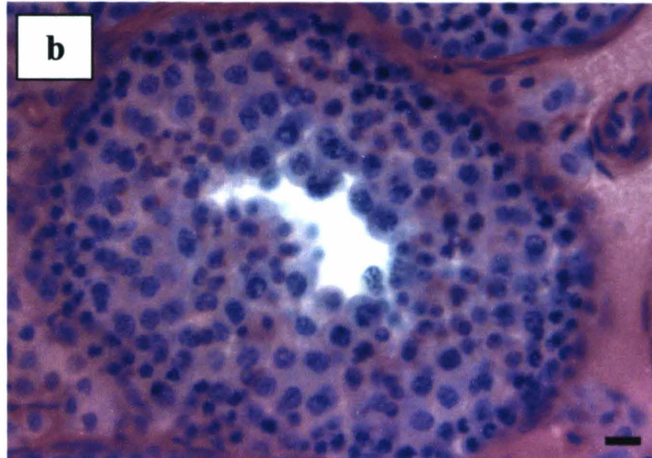
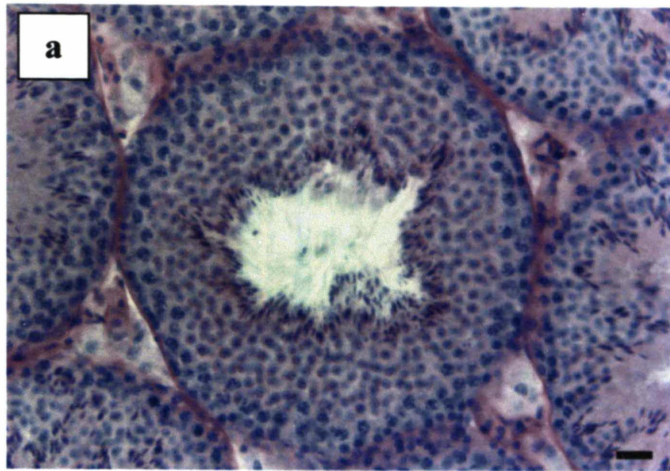


Figure 3.231 Testicular morphology: 18 weeks of age

Light microscopic analysis of 25μm methacrylate embedded testes from WT and ArKO animals at 18 weeks of age. Sections were stained with periodic acid-Schiff and counterstained with haemotoxylin (**a**) All 6 WT and 4 out of the 5 ArKO males at 18 weeks of age had testes with normal morphology. (**b**) One ArKO mouse had severe disruptions to spermatogenesis with no elongating spermatids present, suggesting spermiogenic arrest. Scale bars 20μm.

Figure 3.232 Testicular morphology: 1 year of age

Light microscopic analysis of 25μm methacrylate embedded testes from WT and ArKO animals at 1 year of age. Sections were stained with periodic acid-Schiff and counterstained with haemotoxylin. (**a**) All four 1-year-old WT animals had testes with normal morphology. (**b**) Major disruptions were evident in the testes of 1-year-old ArKO mice, the site of disruption appearing to be early spermiogenesis, with no elongating spermatids present. Symplasts (arrowhead) and degenerating early spermatids were visible. (**c**) Most animals also had some tubules with normal morphology (n) adjacent to tubules with spermiogenic arrest (s). (**d**) Some tubules were missing entire classes of germ cells, this one displaying only elongating spermatids. Scale bars 20μm.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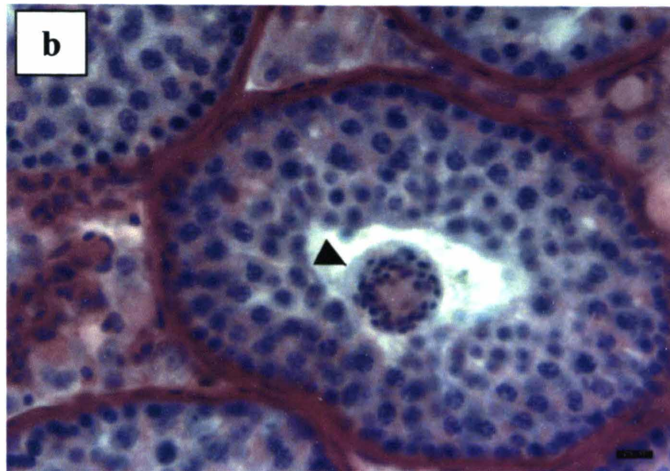
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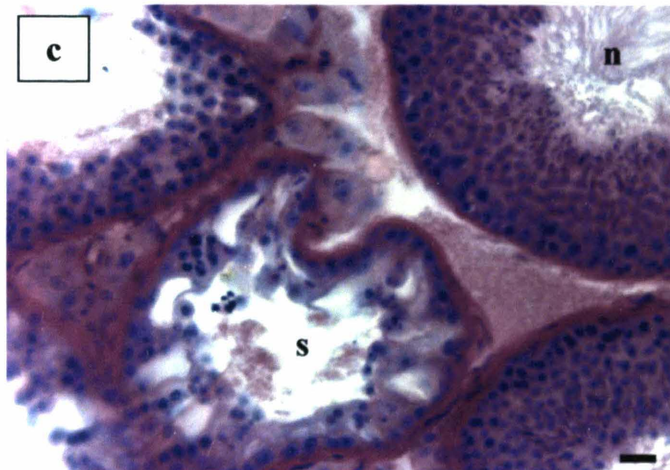
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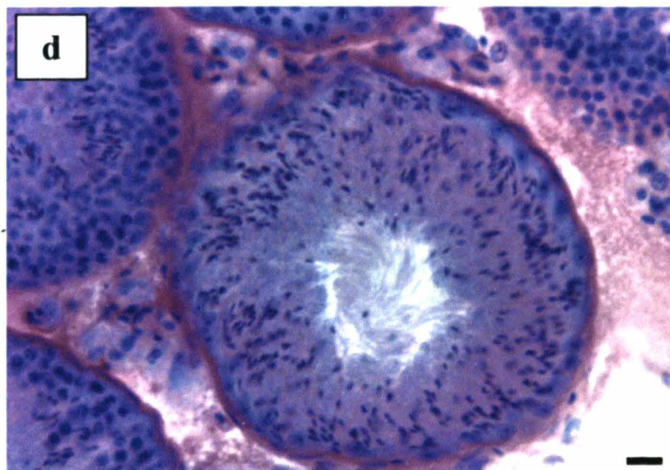
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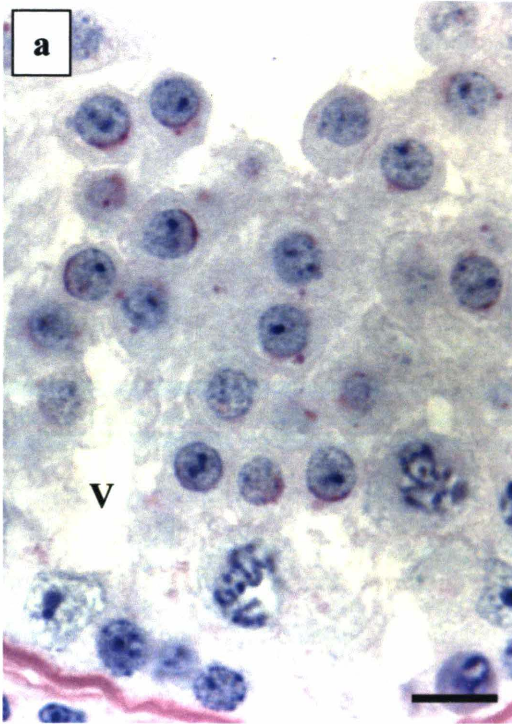
Figure 3.233 Seminiferous epithelial morphology: 1 year of age

Light microscopic analysis of 25µm methacrylate embedded testes from WT and ArKO animals at 1 year of age. Sections were stained with periodic acid-Schiff and counterstained with haemotoxylin. (**a**) The disrupted seminiferous epithelium of the ArKO mouse showing the absence of elongating spermatids and vacuoles (V) within the epithelium. (**b**) Degenerating round spermatids were often observed (arrowheads). (**c**) Round spermatids appeared to be failing to mature into elongated spermatids, seen by the presence of stage I-VII round spermatids (R) in this stage X-XI tubule, as determined by the presence of leptotene/zygotene spermatocytes (C) (**Figure 1.323**). Scale bars 10µm.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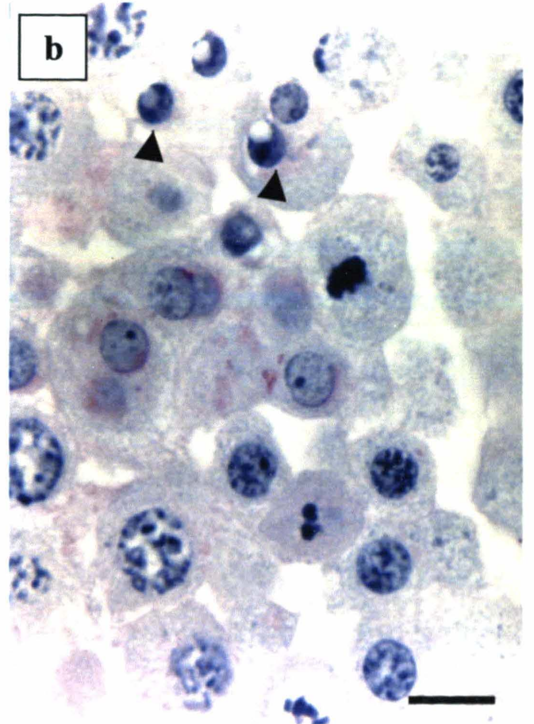
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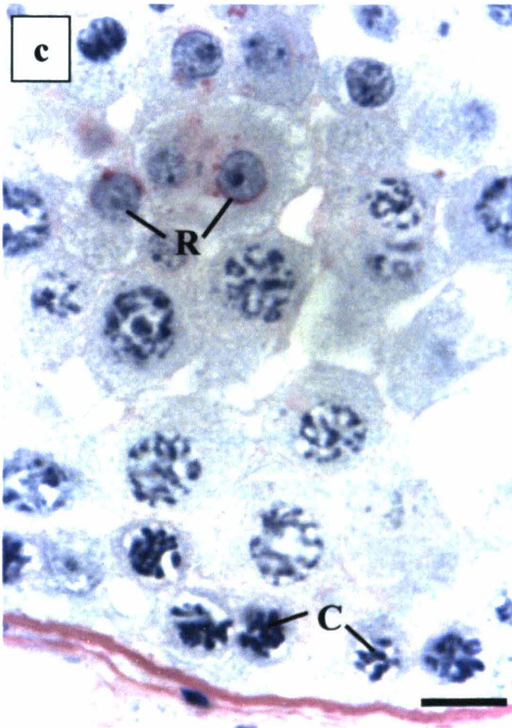
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Further analysis was performed on the testis sections in order to more closely examine the morphological structure of the testis and spermatogenesis.

3.232 Quantitative assessment of testicular histology

To quantify changes in the composition of the testis of ArKO mice, quantitative histomorphometry was performed using stereological methods (**Section 2.241**). No difference was observed in the absolute volume of the interstitium, seminiferous epithelium and the luminal space between WT and ArKO testes at 18 weeks (**Table 3.232**). By 1-year of age, the volume of seminiferous epithelium was markedly decreased in the 1-year-old ArKO animals as compared to WT (p=0.013). No changes in seminiferous tubule luminal volume or interstitium were seen. The only difference in tubule length and diameter was observed in WT mice, whose tubule length significantly declined with age (p=0.05).

3.233 Leydig cell hypertrophy

Although there was no difference in the volume of the interstitial space, ArKO mice at 1 year of age appeared to have an abundance of Leydig cells (**Figure 3.234b**). To further examine this, 5 μm paraffin sections from 1 year old WT and ArKO testes (the same animals as studied above) were incubated with a polyclonal rabbit antiovine 3β-HSD antibody, then counterstained with haematoxylin (**Section 2.261**). This allowed for the clear visualisation of the Leydig cells, staining brown. WT mice showed few Leydig cells in the interstitial space (**Figure 3.234c,e**). In contrast, ArKO mice clearly had an increase in the population of Leydig cells (**Figure 3.234d,f**).

To quantify changes in the volume of these cells between genotypes, quantitative histomorphometry was performed as described (**Section 2.241**). The ArKO mice were found to have a significant increase in their volume of Leydig cells when compared to WT mice (p=0.049) (**Table 3.233**), this is shown with an increase in the volume of interstitium reanalysed on the paraffin sections (**Table 3.232, 3.233**).

Table 3.232 Morphometric data on testicular compartments

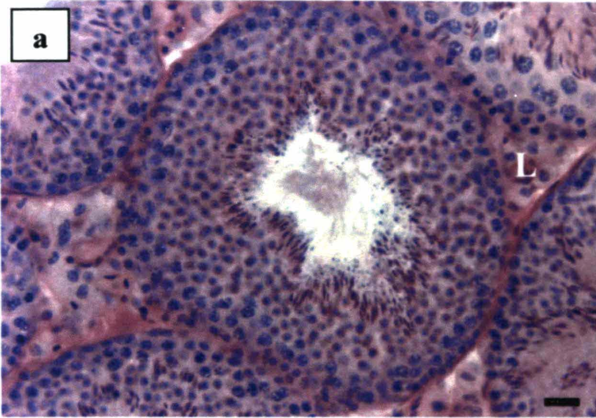
	Volume Lumen (mm^3)	**Volume Epithelium (mm^3)**	**Volume Interstitium (mm^3)**	**Tubule Diameter (μm)**	**Tubule Length (m)**
WT 18 wks (n=6)	10.24 ±1.0	106.10 ±3.2	16.64 ±1.6	236.10 ±2.9	2.77 ±0.1
ArKO 18 wks (n=5)	8.21 ±1.3	85.08 ±13.3	15.19 ±3.4	229.12 ±15.8	2.44 ±0.5
WT 1 year (n=4)	16.84 ±3.7	95.97 ±5.1	15.81 ±0.7	247.20 ±6.0	$2.36^{\#}$ ±0.2
ArKO 1year (n=7)	12.38 ±2.7	63.58 * ±7.4	21.40 ±3.2	213.95 ±12.0	2.04 ±0.3

Quantitative histomorphometry was performed on 10μm methacrylate sections from WT and ArKO testes to quantify changes in the composition of the testes. There were no differences in the absolute volumes of the various compartments of the testis in the 18-week ArKO mice compared to WT. In 1-year old ArKO mice the absolute volume of seminiferous epithelium was decreased by 34% (*p<0.05) compared to WT. The tubule length decreased in the WT mice as they age (#p<0.05). The notation * represents a difference between genotypes, # a difference between age. Data expressed as mean ±SEM.

Figure 3.234 Leydig cell morphology

Light microscopic analysis of 25μm methacrylate embedded testes from WT and ArKO animals at 1 year of age. Sections were stained with periodic acid-Schiff and counterstained with haemotoxylin. (**a**) WT animals at 1 year of age had normal appearing Leydig cells (L). (**b**) All ArKO animals showed evidence of what appeared to be Leydig cell hyperplasia/hypertrophy.

Analysis of 3βHSD immunolocalisation (brown) in 5μm paraffin sections from WT and ArKO 1 year old testes. Sections were incubated with a polyclonal rabbit antiovine 3β-HSD antibody, to visualise the Leydig cells, then counterstained with haemotoxylin. (**c**) WT testes had few Leydig cells (arrowheads) residing in the interstitial space surrounding the seminiferous tubules. (**e**) WT testes at higher power. (**d**) In contrast, all ArKO animals had a vast increase in Leydig cell numbers and possibly volume. (**f**) ArKO testes at higher power. WT and ArKO sections incubated with the secondary antibody alone showed no staining (not shown). Scale bars 20μm.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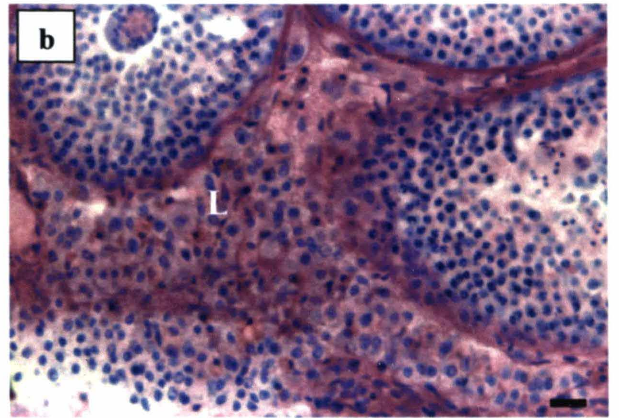
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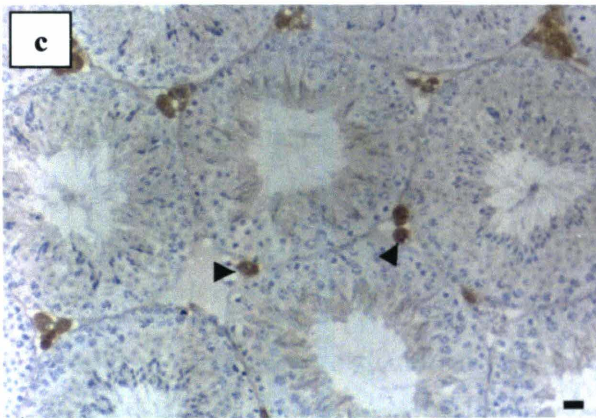
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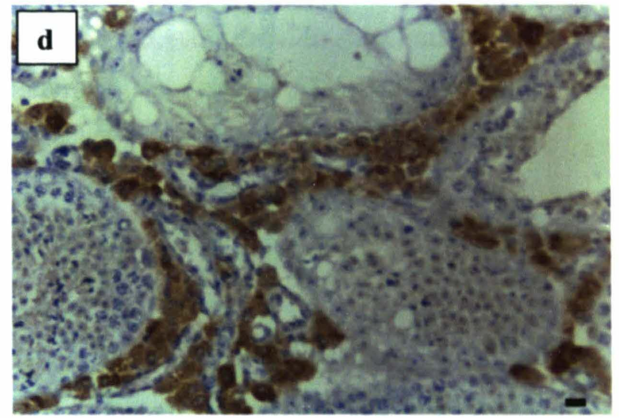
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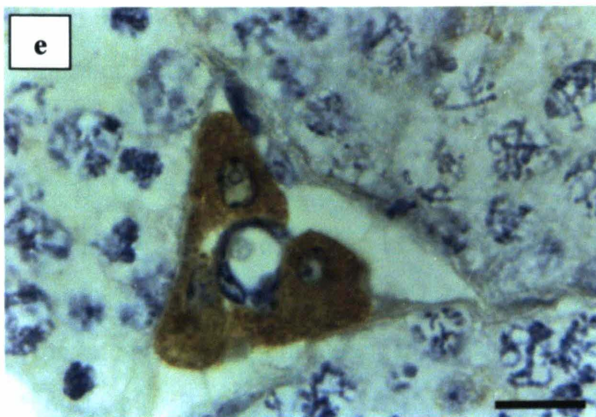
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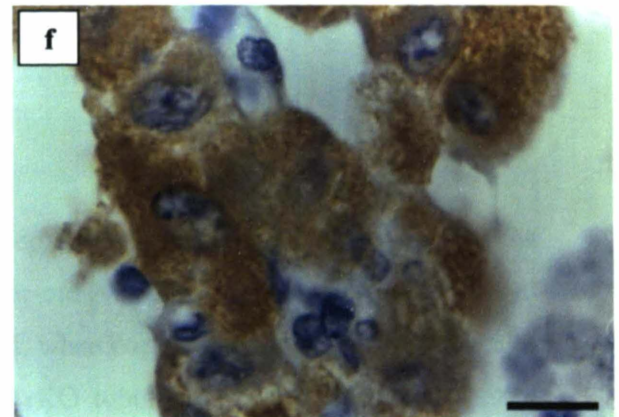
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Table 3.233 Leydig cell volumes

	Volume Epithelium (mm^3)	Volume Interstitium (mm^3)	Volume Leydig cells (mm^3)
WT* *1 year (n=4)	124.51 ± 8.4	6.27 ± 2.1	3.78 ± 0.2
ArKO* *1 year (n=4)	72.21 * ± 14.1	19.72 * ± 5.4	7.45 * ± 1.5

Quantitative histomorphometry was performed on 5μm paraffin sections from 1 year old WT and ArKO testes to quantify changes in the volume of Leydig cells. In ArKO mice the absolute volume of the seminiferous epithelium was decreased by 58% (*$p<0.01$) with the interstitium (*$p<0.05$) and Leydig cell volume (*$p<0.05$) increased, suggesting that the Leydig cells are hypertrophic. Data expressed as mean ±SEM.

3.234 Sertoli cell numbers

Sertoli cell numbers were quantified with the optical disector (**Section 2.242**) to elucidate a possible cause for the decreased seminiferous epithelium volume in the 1-year-old ArKO animals. The numbers were found to be unchanged between WT and ArKO animals at both 18 weeks and 1 year of age (**Figure 3.235**). Sertoli cells did also not decrease in number as the animals aged.

3.235 Stage assessment

To analyse the composition of the seminiferous epithelium, the spermatogenic stage of the epithelium was noted (**Section 2.242**). The 1-year-old ArKO mice showed fewer tubules at the late stage of spermatogenesis, stage VIII-XII, when compared to younger ArKO males ($p=0.011$) (**Table 3.234**). Also, 5.4% of the ArKO tubules at 1 year of age contained tubules with no germ cells, only Sertoli cells.

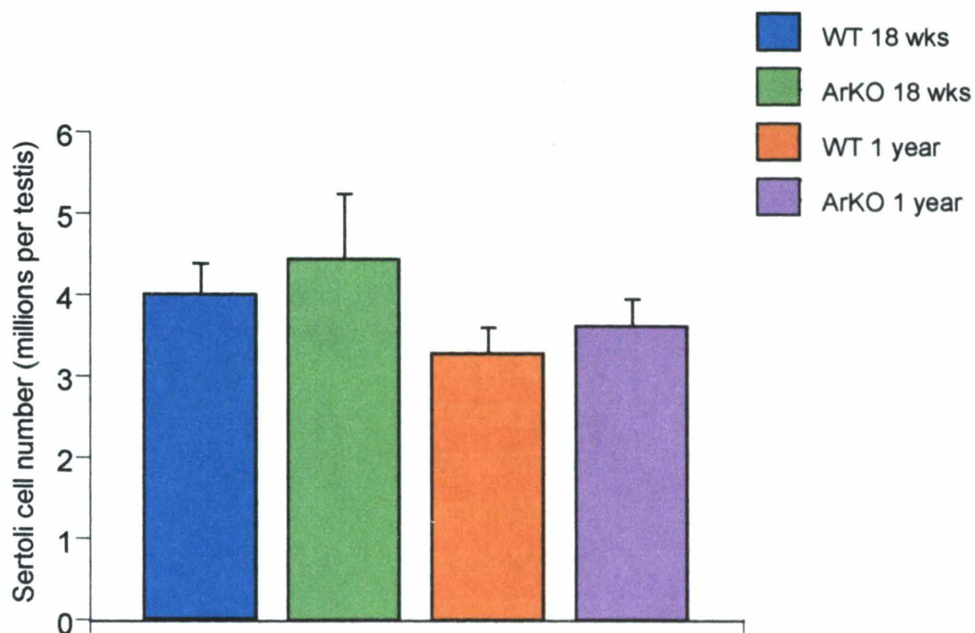


Figure 3.235 Sertoli cell numbers

The optical disector approach was used to quantitate Sertoli cell number (millions) per testis. There was no difference in cell numbers between ArKO and WT mice at 18 weeks and 1 year of age. There was also no age related effect, Sertoli cell numbers not decreasing between 18 weeks and 1 year. Data expressed as mean ±SEM.

Table 3.234 Stage of the spermatogenic cycle

	I-VII	**VIII-XII**	**Sertoli cell only**
WT 18 wks (n=6)	59.06 ± 2.4	28.18 ± 2.3	0
ArKO 18 wks (n=5)	56.41 ± 4.5	28.84 ± 2.1	0
WT 1 year (n=4)	55.81 ± 2.6	25.35 ± 2.6	0
ArKO 1 year (n=7)	48.93 ± 5.5	20.2[#] ± 1.8	3.96 ± 2.4

The proportion of the seminiferous epithelium at the beginning (stage I-VII) or the end of the spermatogenic cycle (VIII-XII) was determined between WT and ArKO through stereological analysis. The ArKO mice at 1 year of age had less tubules in the later stages of spermatogenesis when compared to ArKO males at 18 weeks (#$p<0.05$), however no significance was observed between ArKO and WT and 1 year. In these 1-year-old ArKO mice, 5.4% of tubules contained only Sertoli cells. The notation # represents significant difference between age. Data expressed as mean ±SEM.

3.236 Germ cells

***i** Numbers*

When the testes were examined histologically (**Section 3.231**) it indicated specific abnormalities in the process of spermatogenesis. Therefore to further investigate this, the germ cells were quantified using the optical disector (**Section 2.242**).

Germ cells were counted in 4 major categories: spermatogonia, spermatocytes, round spermatids and elongated spermatids (**Section 1.32**). At 18 weeks no differences existed in the numbers of these germ cells when compared to WT (**Figure 3.236**). However, by one year of age disruptions were clearly apparent with significantly fewer round (p=0.001) and elongated spermatids (p=0.004). As the animals aged, the ArKO mice also showed a significant decline in spermatogonia (p=0.008), and a trend for a decrease in spermatocytes (p=0.056) and round spermatids (p=0.065). It is pertinent to note that no differences existed between the numbers of round and elongated spermatids in the ArKO mice at 1 year, suggesting that if the germ cell was able to progress through to the round spermatid stage then it would continue to develop into a mature spermatozoa.

***ii** Hourly production rate*

To further investigate the decline in germ cell number at the round spermatid level, the hourly production rate of each germ cell type was calculated (**Section 2.244**). As expected, no differences were observed at 18 weeks (**Table 3.235**). However at 1 year of age, there was a significant reduction in the number of round (p=0.001) and elongated spermatids (p=0.004) produced per hour when compared to WT. This correlates with the decline observed in round and elongated spermatid numbers (**Figure 3.236**). Also observed were fewer spermatogonia being produced as the ArKO animals aged, with a significant reduction by 1 year of age (p=0.008). This again mirrored the decline in spermatogonia number (**Figure 3.236**). Once again there existed a trend for fewer spermatocytes (p=0.056) and round spermatids to be produced per hour (p=0.065).

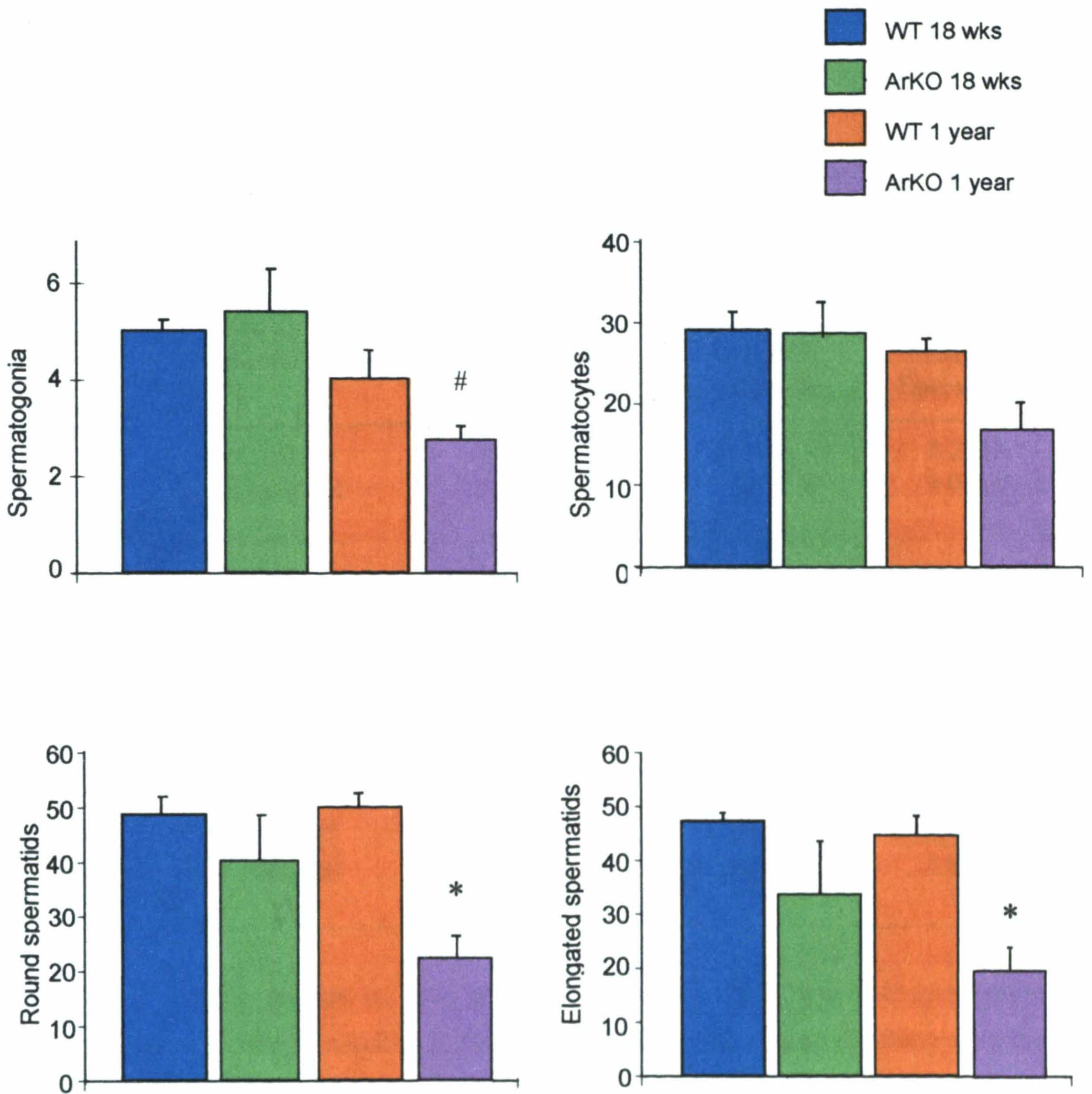


Figure 3.236 Germ cell numbers

The optical disector approach was used to quantitate germ cell number (millions) per testis. There was no difference in germ cell numbers between WT and ArKO at 18 weeks. However at 1 year there were significantly less round spermatids (*$p<0.001$) and elongated spermatids (*$p<0.05$) in the ArKO testis when compared to WT. ArKO mice at 1 year also had significantly fewer spermatogonia (#$p<0.05$) than at 18 weeks. The notation * represents a significant difference between genotypes, # a difference between age. Data expressed as mean ± SEM.

Table 3.235 Hourly production rates

	Spermatogonia	Spermatocytes	Round Spermatids	Elongated Spermatids
WT* *18 wks* *(n=6)	0.0143 ± 0.001	0.0885 ± 0.008	0.3618 ± 0.031	0.2218 ± 0.009
ArKO* *18 wks* *(n=5)	0.0154 ± 0.002	0.0869 ± 0.011	0.2991 ± 0.054	0.1581 ± 0.039
WT* *1 year* *(n=4)	0.0115 ± 0.002	0.0802 ± 0.005	0.3742 ± 0.020	0.1675 ± 0.031
ArKO* *1 year* *(n=7)	0.008# ± 0.001	0.0512 ± 0.011	0.2091* ± 0.018	0.0924* ± 0.021

The hourly production rate of each germ cell type, per testis, was calculated using published time divisors (Russell *et al* 1990). At 18 weeks, there was no difference in the hourly production rate of all germ cells between WT and ArKO males. However, at 1 year of age, less round (*$p<0.001$) and elongated spermatids (*$p<0.05$) were being produced per hour when compared to WT. Also, fewer spermatogonia were produced per hour in the ArKO animals at 1 year of age compared to 18 weeks (#$p<0.05$). The notation * represents a significant difference between genotypes, # a difference between age. Data expressed as mean ± SEM.

These results suggest that the germ cells were developing to the late spermatocytes/early round spermatid stage of development, but then ceased to complete the spermatogenic cycle. Further examination was conducted to decipher at what stage oestrogen is required to enable spermatogenesis to proceed and what possible role it could be playing.

3.237 Acrosome abnormalities

***i** Acrosome morphology*

One interesting feature that was often observed in the ArKO seminiferous tubules were acrosome abnormalities. The acrosome, which surrounds the head of the mature sperm, contains crucial enzymes required for penetration of the oocyte membrane during fertilization (**Section 1.32**). Further investigation reported round spermatids with incomplete acrosome spreading over the nuclear membrane, failure of the acrosome to uniformly spread, and instances were more than one acrosomal granule was visible (**Figure 3.237a**). As the level of acrosomal spread is crucial for staging, these abnormalities made this task increasingly difficult.

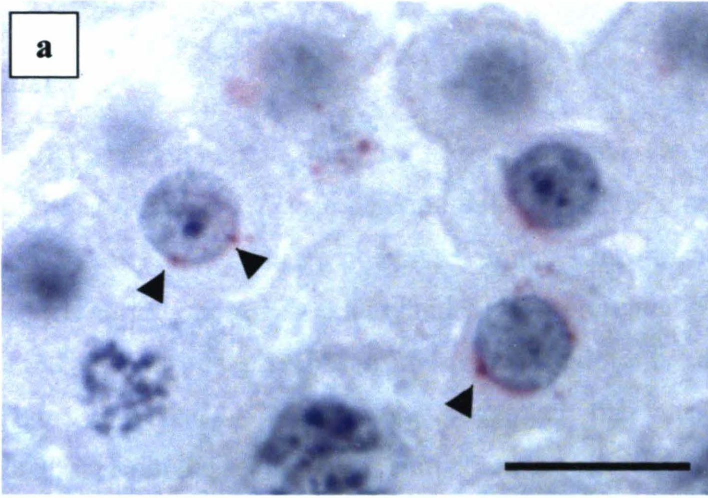
***ii** Electron microscopy*

To further examine the acrosome abnormalities 4 WT and ARKO 1-year-old testes were fixed for electron microscopy analysis (**Section 2.25**). Preliminarily examination with the 1μm sections under light microscopy revealed no noticeable abnormalities, however the germ cells were difficult to visualise as the Toll blue staining was very faint. Further examination using the electron microscope by Dr Liza O'Donnell and myself revealed most spermatids to possess normal developing acrosomes (**Figure 3.237b,c**), suggesting these tubules did not possess the phenotype. Further exploration was out of the scope of this initial characterisation study.

Figure 3.237 Acrosome morphology

(**a**) In tubules in which spermiogenic disruption was evident, there appeared to be impaired acrosomal development. An example is shown in which spermatids with abnormal acrosome development are seen in a stage IV-V tubule. Multiple acrosomal vesicles were noted (arrowheads), and in some cases acrosomes failed to uniformly spread over the spermatid nuclei (arrowhead).

Electron microscopy was performed to analyse round spermatid acrosomes in WT and ArKO animals. (**b**) Shown is a step 5 round spermatid (st) in the ArKO illustrating the acrosomic vesicle (av). (**c**), and a step 6 round spermatid (st) with the acrosome spread over one third of the round spermatid nucleus. Also shown is the plasma membrane (p). No abnormalities appeared to be present on the 4 ArKO animals studied. WT not shown. Scale bars (a) 10μm, (b) 200nm, (c) 500nm.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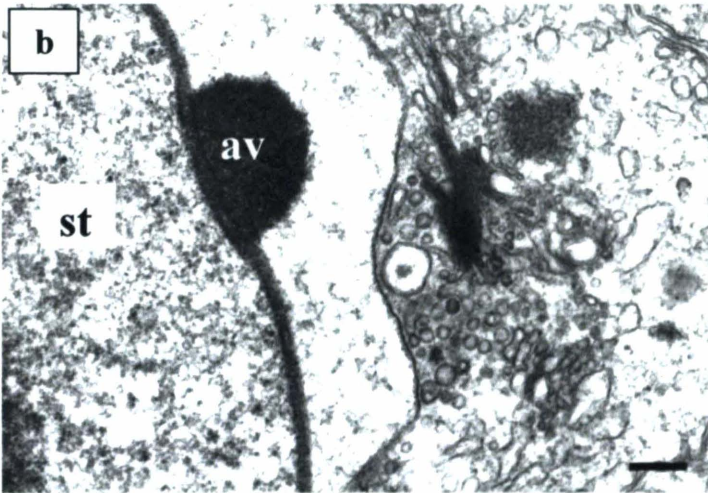
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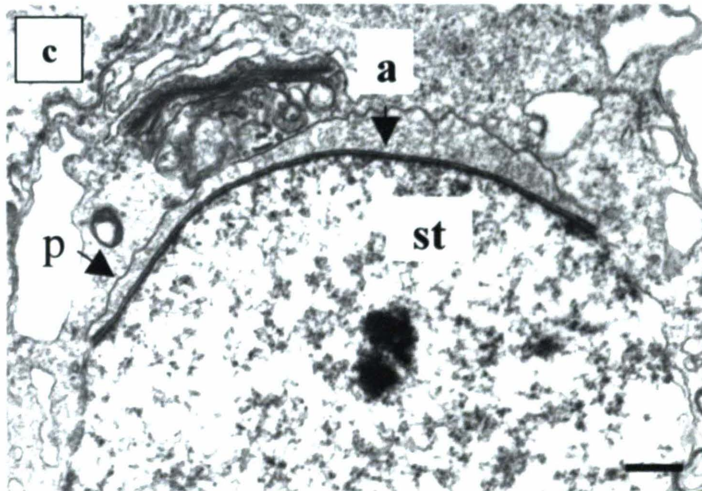
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3.24 Epididymis

3.241 Morphology

The WT and ArKO epididymides, from the testes examined above, were fixed and sectioned as described (**Section 2.23**), to determine the presence of mature spermatozoa. At 1 year of age, the WT epididymides possessed an abundance of mature spermatozoa (**Figure 3.241a**). In contrast, the ArKO epididymides either presented with some sperm, no sperm (**Figure 3.241b**) or the presence of what appeared to be degenerating round spermatids (**Figure 3.241c,d**). These results suggested that the germ cells were prematurely sloughing from the seminiferous epithelium to collect in the epididymis.

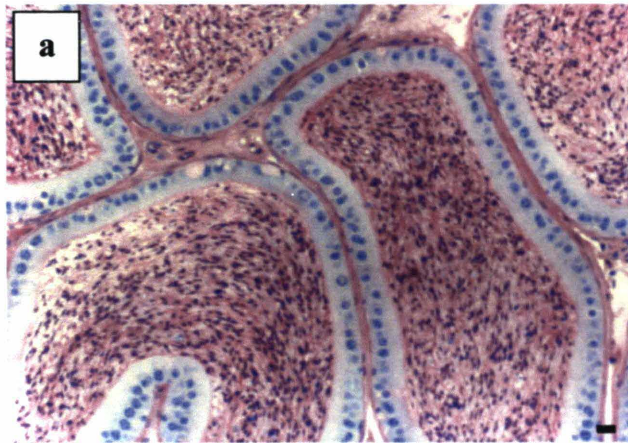
As the extent of spermatogenic disruption varied between ArKO animals, the sperm content in the epididymides of ArKO animals was examined as a comparison to germ cell number (**Table 3.241**). Almost all ArKO germ cells are reduced in number compared to the WT mean. When this is compared to the epididymal morphology, relationships are seen. For instance, the one animal (4723) that showed a significant decline in round spermatid number and no elongated spermatids also possessed no mature sperm in the epididymis. Similar reductions in mature sperm were observed if round and elongated spermatid number were decreased. Although one animal (4693) did have markedly reduced germ cell numbers but had mature spermatozoa present in the cauda epididymis. The two animals (4723, 5319) that showed degenerating round spermatids in their epididymis both had severely reduced round spermatid number.

3.242 Quantitation of spermatozoa

To quantitate spermatozoa number in each epididymis, sperm were extracted from corresponding WT and ArKO epididymides at 1 year of age (**Section 2.223**). As expected, ArKO mice had significantly fewer sperm present in their epididymides when compared to WT (*p=0.023) (**Table 3.242**).

Figure 3.241 Cauda epididymis morphology: 1 year of age

Light microscopic analysis of 25μm methacrylate embedded epididymides from WT and ArKO animals at 1 year of age. Sections were stained with periodic acid-Schiff and counterstained with haemotoxylin. (**a**) WT cauda epididymis with an abundance of sperm. (**b**) The lumen of the ArKO mice showing an absence of sperm; 3 out of the 7 have no elongated spermatids visible in the cauda. (**c**) The ArKO animals that have undergone spermiogenic arrest show evidence of degenerating germ cells (arrowheads). (**d**) These are presumably round spermatids, some obviously degenerating (drst), some having relatively normal morphology (rst). Scale bars (a-c) 20μm, (d) 10μm.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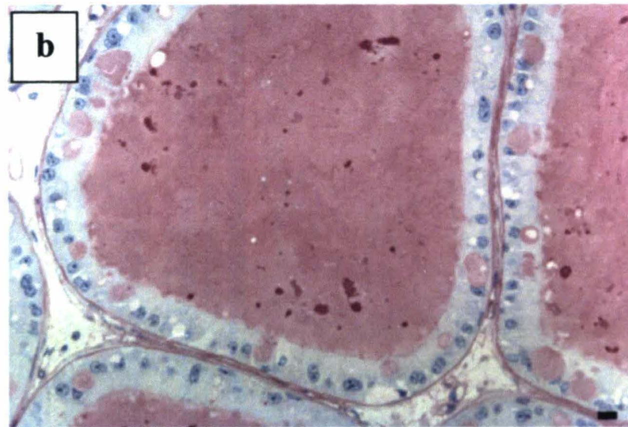
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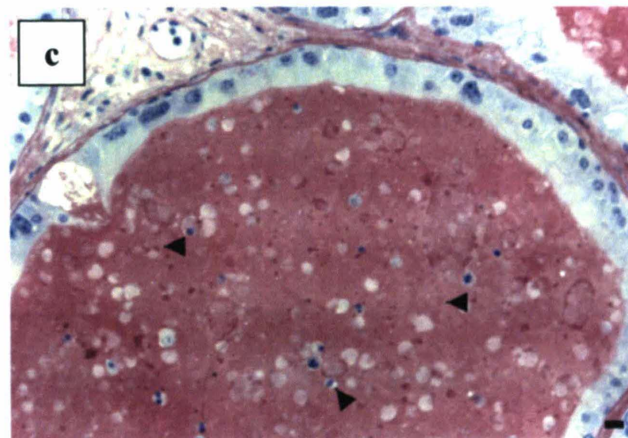
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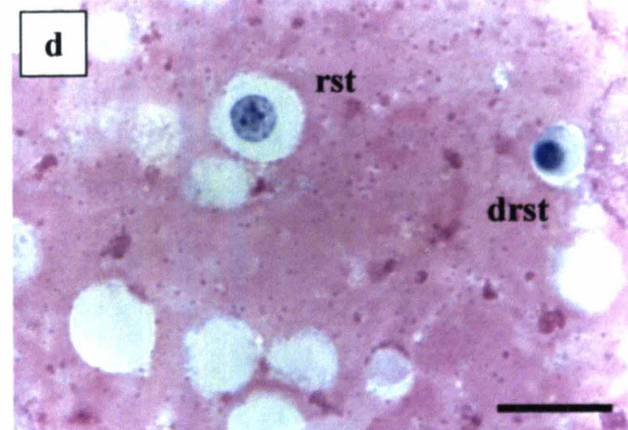
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Table 3.241 Epididymal sperm content in relation to germ cell number: 1 year of age

		Spermato-gonia	**Spermato-cytes**	**Round spermatids**	**Elongated spermatids**	**Epididymal morphology**
WT (n=4)	*Mean ± SEM*	4.03 ± 1.2	26.38 ±3.4	50.30 ±5.5	44.49 ±7.5	Sperm present
ArKO *(animal no.)*	*4723*	4.06 =	34.12 ↑	12.61 ↓↓↓	0 ↓↓↓↓	No sperm Degenerating round spermatids
	4695	2.04 ↓↓	6.6 ↓↓↓	12.5 ↓↓↓	9.2 ↓↓↓	No sperm
	4724	2.24 ↓↓	15.25 ↓	28.78 ↓↓	25.70 ↓↓	Sperm in approx.1% of epididymis
	5319	2.47 ↓↓	15.09 ↓	19.57 ↓↓↓	21.23 ↓↓	Very little sperm Degenerating round spermatids
	4693	2.04 ↓↓	8.60 ↓↓	12.77 ↓↓↓	15.59 ↓↓	Sperm present
	4694	3.5 ↓	19.3 ↓	31.8 ↓↓	31.6 ↓	Sperm present
	4655	2.9 ↓↓	18.82 ↓	39.6 ↓	31.3 ↓	Sperm present

The sperm content in the epididymides of ArKO animals was examined as a comparison to germ cell number (millions per testis). The arrows indicate the proportional decrease or increase in that particular germ cell in relation to the WT mean, 3 arrows indicating a substantial decline. As is clearly observed, almost all germ cells are reduced in number compared to the mean WT. When this is compared to the epididymal morphology and sperm content, relationships can be observed.

Table 3.242 Quantitation of spermatozoa: 1year of age

	WT (n=4)	*ArKO* (n=5)
Epididymal sperm (millions per epididymis)	2.21 ±0.24	0.88 * ±0.36

Elongated spermatids were extracted from epididymides through homogenisation and quantified using a haemocytometer. The ArKO males at 1 year had significantly fewer mature spermatozoa present in their epididymis compared to WT (*$p<0.05$).

3.25 TUNEL assay

To determine whether the decline in round spermatid number was attributable to an increase in the death of these cells through apoptosis, the TUNEL assay was performed by Sarah Meachem (Prince Henry's Institute of Medical Research, Clayton, Vic) (**Section 2.262**). Paraffin testicular sections were examined for the presence of apoptotic cells, with the ArKO tubules that were disrupted appearing have a marked increase in the number of dying cells in their seminiferous epithelium than WT (**Figure 3.251b**). The cells undergoing apoptosis were localised to the adluminal compartment, suggesting they were round spermatids.

To further examine the tubules, the number of cells were quantified noting the stage of the cycle (early, mid or late) and the position in the epithelium in which the apoptotic cells appeared (**Section 2.262**). The only differences in the levels of apoptosis were observed in the one ArKO animal (4723) who presented with the complete spermiogenic arrest (**Table 3.251**). This animal was clearly undergoing massive cell death, with 3.327 apoptotic cells per tubule. The phenotype of the other ArKO mice is quite variable, with most containing disrupted tubules situated next to what appeared to be normal tubules. Thus, although they contained increased dying cells in the disrupted tubules, overall no difference was seen. However as the TUNEL technique only pinpoints cells in a particular stage of death there may be an abundance of degenerating cells past the apoptotic detection stage.

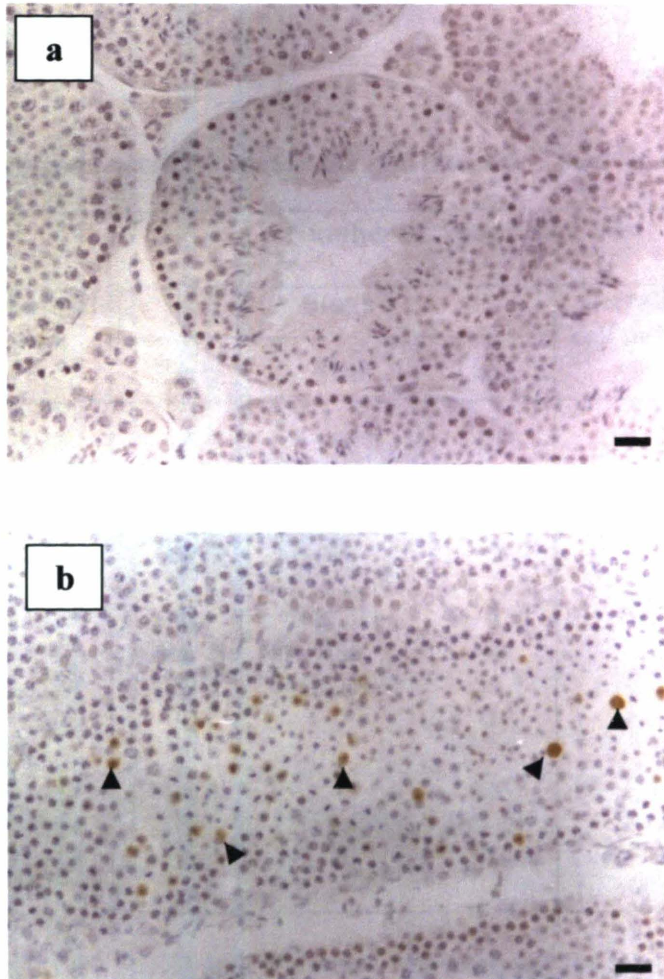


Figure 3.251 TUNEL staining: 1 year of age

TUNEL staining (brown) for apoptosis in 5µm testes paraffin sections from WT and ArKO animals at 1 year of age. Sections were counterstained with haematoxylin (**a**) A WT stage XII-I tubule with no evidence of apoptotic cells. (**b**) ArKO stage XII-I tubule with multiple stained germ cells in the adluminal compartment (arrowheads), indicating high level of spermatid cell death. Scale bars 20µm.

Table 3.251 Apoptotic cells present in the ArKO testes: 1 year of age

	Animal no.	Number of Apoptotic Cells					No. of cell /tubule
		Stage			Sertoli cell only	Elongated spermatids only	
		Early	Mid	Late			
WT 18 wks	*32*	2a	0	4b	-	-	0.811
ArKO 18 wks	*58*	13a, 2b	6a	4a	-	-	0.305
	5	7a, 1b	2a	1a	-	-	0.117
WT 1 year	*5319*	2b	3a, 3el	4a, 4 el	1b	1b	0.254
	4723	99a, 2b	38a	34a	-	-	3.327
	4724	0	4a, 5b	5a	-	-	0.212
	4693	0	0	0	-	-	0
ArKO 1 year	*4654*	11a, 4b	0	1a	-	-	0.254
	4664	6b	1a	10a, 3b	-	-	0.244
	4656	2a	0	0	-	-	0.021

TUNEL staining in the ArKO and WT testes was analysed for number of apoptotic cells, their position in the epithelium (next to the basement membrane (b) or in the adluminal compartment (a), or are elongated spermatids (el)) and the stage (mid-late) in which they have occurred. When expressed as the total number of apoptotic cells present per tubule (final column), the only animal showing an increase in apoptosis is animal number 4724, with an average of 3.327 apoptotic cells per tubule.

3.26 Serum hormone levels

To analyse hormone levels, serum hormonal assays were performed (**Section 2.27**). Serum testosterone levels in the 18 week and 1 year old animals were variable (**Table 3.261**), with no significant increase or decrease apparent. This differs from the female mice who have a significant increase in T levels (**Section 1.632**), and may suggest sexual dimorphism. FSH levels were unchanged in the ArKO mice compared to the WT at both age groups, suggesting that changes in the levels of FSH did not contribute to the spermatogenic disruption observed in the ArKO animals. Levels of LH were also unchanged, however due to limited amounts of serum these results are not conclusive (**Table 3.261**). Serum oestrogen levels were not analysed at this stage as an assay sensitive enough to detect the low level of oestrogen in the male was not available.

3.3 DISCUSSION

The findings presented here indicate a direct role for oestrogen in male germ cell development. The ArKO animals show a specific defect in the development of spermatids during spermiogenesis evident from 18 weeks of age, with all seven 1-year old ArKO mice showing evidence of disrupted spermatogenesis. Quantitation of cell numbers demonstrated that Sertoli cell and earlier germ cell populations were unchanged compared to wildtype, whereas significant decreases were seen in round and elongated spermatid number in ArKO animals. In addition, ArKO animals displayed an increased frequency of germ cell apoptosis, some abnormalities in early acrosome development, Leydig cell hyperplasia/hypertrophy, as evidenced by an increase in the absolute volume of Leydig cells per testis and a marked reduction in mature epididymal spermatozoa. These findings suggest that oestrogen plays a hitherto unsuspected role in spermatid differentiation and spermatogenesis.

Table 3.261 Serum hormone levels

	Testosterone (ng/ml)	FSH (ng/ml)	LH (ng/ml)
***WT* 18 weeks**	27.55 ± 22.1 (n=6)	8.98 ± 0.7 (n=6)	0.12 ± 0.0 (n=6)
***ArKO* 18 weeks**	50.03 ± 25.9 (n=5)	9.85 ± 0.9 (n=3)	1.15 (n=2)
***WT* 1 year**	20.95 ± 16.5 (n=3)	10.41 ± 0.3 (n=4)	1.49 (n=2)
***ArKO* 1 year**	14.73 ± 0.8 (n=6)	10.08 ± 0.3 (n=7)	0.99 ± 0.4 (n=5)

Serum hormone levels were measured using double antibody RIAs. There were no significant differences in serum testosterone levels between WT and ArKO animals at 18 weeks and 1 year of age. There were also no differences in FSH levels. Due to serum restraints, LH levels were unable to be fully measured in this study, however appear to also show no differences. Data expressed as mean ± SEM.

The disruptions to spermatogenesis in the ArKO animals is not surprising given the localisation of aromatase in the seminiferous epithelium. Rodent spermatocytes, round and elongated spermatids, Sertoli and Leydig cells all express the aromatase enzyme (**Section 1.4**). With studies by Nitta *et al* (1993) appearing to demonstrate that spermatids had more intense immunostaining and higher aromatase activity than any other testicular cells. The localisation of aromatase within the spermatids of the rodent seminiferous epithelium are in line with the findings presented here, that mice deficient in aromatase show a loss of round and elongated spermatids. There is also good evidence to suggest that oestrogen receptors are present within the seminiferous epithelium. ERβ has been reportedly localised to rodent Sertoli cells, late spermatocytes and early round spermatids, Leydig cells and elongated spermatids (**Section 1.4**). Whereas ERα is primarily expressed outside of the seminiferous epithelium, observed in the Leydig cells but not the germ cells (**Section 1.4**). I, and others (Nitta *et al.* 1993) hypothesised that oestrogen may be synthesised by germ cells which may act in a paracrine fashion on Sertoli cells causing them to release factors to specifically regulate germ cell development. Aromatase activity in the Leydig cells could also contribute to this (Nitta *et al.* 1993; Janulis *et al.* 1996a). Alternatively, oestrogen could be synthesized by the germ cells and act in an 'intracrine' fashion, providing a local source of oestrogen involved in controlling the complex process of spermatogenesis. The increase in cell death and degenerating spermatids present in the cauda epididymis of ArKO animals also reaffirms oestrogens role as a cell survival factor, however this needs to be further investigated.

The finding that ArKO animals show disruptions to spermiogenesis is further supported in studies were oestrogen synthesis was inhibited. For instance, significant reductions in round and elongated spermatid populations, but not earlier germ cells, were observed in adult male bonnet monkeys administered an aromatase inhibitor for 150 days (Shetty *et al.* 1998). Furthermore, disruptions to spermatogenesis were also noted in adult male rats administered an aromatase inhibitor for 19 weeks (Turner *et al.* 2000b). Also, adult rats injected with a partially purified ovarian protein, that was isolated due to its ability to inhibit granulosa cell aromatase activity, showed a reduction in elongated spermatid

number and round spermatid degeneration (Tsutsumi *et al.* 1987a; Tsutsumi *et al.* 1987b). These studies all lend further support to the observed ArKO phenotype.

The phenotype of the male ArKO mouse differs from that of the αERKO (ERα knockout) mouse in which infertility is evident from the onset of puberty (**Section 1.621**) (Hess *et al.* 1997a; Eddy *et al.* 1996). Moreover, in the αERKO mouse, the lumina of the seminiferous tubules are extremely dilated, in contrast to the ArKO mouse in which the luminal volume is unchanged. In ArKO animals there was no evidence of abnormal fluid reabsorption by the efferent ductules. It is interesting to note that Hess and colleagues demonstrated that wildtype isolated efferent ducts treated with an antagonist of both ERα and β did not swell like tissues isolated from αERKO animals (Hess *et al.* 1997a). Since the antagonism of ERα and β in wildtype tissues could be considered analogous to the absence of oestrogen in ArKO animals, this observation is consistent with the apparent lack of an effect on fluid reabsorption in ArKO mice.

ArKO animals showed evidence of Leydig cell hyperplasia/hypertrophy, which may be attributed to increased circulating LH levels, as has been noted in previous studies in rats (Mendis-Handagama *et al.* 1998). Due to serum restrictions, the LH levels were unable to be conclusively measured in the ArKO mice, however in the preliminary examination of the ArKO mice by Fisher *et al* (1998), LH levels were elevated. Since Leydig cells have high levels of aromatase which may be important for Leydig cell function (Levallet *et al.* 1998a; Mendis-Handagama *et al.* 1998), it is possible that the Leydig cells in ArKO animals may over- or under-express factors which contribute to the disruption of spermatogenesis. Therefore, the spermatogenic disruption in ArKO mice may be due to a deficiency of aromatase in the Leydig cell, the seminiferous epithelium, or both. The disruption to spermatogenesis in ArKO animals is not believed to be a direct consequence of high LH/testosterone or LH-induced Leydig cell hyperplasia. Previous studies have shown that mice given a 5α-reductase inhibitor for 83 weeks (Prahalada *et al.* 1994) have similar Leydig cell hypertrophy/hyperplasia, and similar serum LH and testosterone levels

to ArKO animals. However the 5α-reductase inhibitor treated animals showed normal seminiferous epithelial morphology and spermatogenesis. These observations support the contention that spermatogenic disruption in ArKO animals is not directly due to increased androgens or hypertrophic Leydig cells.

In the ArKO mice evidence of abnormal acrosomal development was also observed, such as multiple acrosomal vesicles and uneven spreading over the nuclear surface. It is interesting to note that immunocytochemical localisation of aromatase P450 shows strong positive labelling of early round spermatids, particularly in the Golgi (Nitta *et al.* 1993). Therefore local aromatase expression could be important for proper acrosomal formation, however this requires further investigation.

Two other aromatase knockout mice have also been described (**Section 1.633**) (Honda *et al.* 1998; Toda *et al.* 2001a). These ArKO mice were initially sub-fertile and had sperm present in their epididymis, however exhibited modified sexual behaviour. No behavioural abnormalities were noted in 12 week old male ArKO mice generated by our group (Fisher *et al.* 1998), however a full characterisation has yet to occur. The phenotypes of the ArKO mice appear similar, such as the late onset infertility, however only a complete testicular/spermatogenic characterisation has been completed on our ArKO mice. Recently the phenotype of the ERβ knockout (βERKO) mouse was reported (Krege *et al.* 1998). Fertility of males was assessed between 6 and 12 weeks of age and no changes in breeding rates were seen (Ogawa *et al.* 1999). As indicated previously (Fisher *et al.* 1998), there is no change in testicular morphology in ArKO mice at this age. Given the distribution of ERβ in the seminiferous epithelium, it was believed that some parallels between the ERβ and the ArKO testicular phenotype would exist. However the βERKO mice show no abnormalities related to their reproductive morphology and function up to 18 months of age. Initial evaluation of the fertility of the double KO (αβERKO) animals indicated that they may have a phenotype similar to that of the αERKO mice (Couse *et al.* 1999), however further examination is awaited.

It was unknown why the disruption to spermiogenesis in the ArKO testes took months to develop, or why all tubules were not affected. With regards to the latter, heterogeneity in spermatogenic disruption is apparent in other knockout models (Eddy *et al.* 1996; Print *et al.* 1998). Furthermore, this variability of response following administration of an aromatase inhibitor, has also been noted (Turner *et al.* 2000a). The progressive nature of the phenotype may be intrinsic to the mechanisms of action of oestrogen in the adult seminiferous epithelium. It is interesting to note that female ArKO mice also show a progressive phenotype (Britt *et al.* 2000). Alternative endogenous ligands, which may bind to and activate the oestrogen receptor could also be important in the delayed phenotype (**Section 1.222**). In addition, alternative ligands for ERs could contribute to the difference in the phenotype between ArKO and ERαKO mice. It is believed that growth factors, such as epidermal growth factor (EGF), can activate the ER, thus participating in 'cross talk' between the growth factor signalling pathways and steroid receptors (Smith 1998). An alternative hypothesis to explain the delayed phenotype is that the diet of the mice in this study may contribute sufficient exogenous oestrogens to maintain normal spermatogenesis in young animals. Standard mouse chow contains as much as 10% of soy meal, and thus contains significant quantities of isoflavones, phytoestrogens with potential oestrogenic activity. Mice are currently being raised on a soy free diet to investigate whether this potential source of phytoestrogens is a contributory factor in the slow onset and variability of the phenotype.

In conclusion, disruption of the *Cyp19* gene leads to a progressive disruption of spermatogenesis. The lesion in spermatogenesis appears to be due to a direct effect on germ cell development, rather than an indirect effect as is the case in the αERKO mouse (Hess *et al.* 1997a). Since aromatase and oestrogen receptor-β co-exist in the seminiferous epithelium, it is suggested that the actions of oestrogen on male germ cell development are a consequence of paracrine and indeed 'intracrine' interactions. These results highlight the hitherto unsuspected role of oestrogen as a locally-acting male hormone.

Chapter Four

An Investigation into the Late Onset Spermatogenic Phenotype

4.1 INTRODUCTION

The importance of oestrogen in the adult testis was highlighted by the testicular phenotype of the ArKO mouse (**Chapter 3**), where the inhibition of oestrogen biosynthesis resulted in severe spermatogenic abnormalities. The mice are initially fertile, but at around 18 weeks of age showed a specific postmeiotic defect in early spermiogenesis coinciding with an elevation in apoptosis and a reduction in fertility (Robertson *et al.* 1999).

As the oestrogen receptors are expressed in the developing reproductive tract from foetal life through to adulthood, and ERβ is predominant in the seminiferous epithelium (**Section 1.4**), oestrogen may act directly on the seminiferous tubules to mediate spermatogenesis. The foetal development of the male reproductive tract is clearly under strict endocrine control, and thus compounds that are potentially able to disrupt this hormonal homeostasis are of increasing concern. Included are numerous environmental compounds that are postulated to possess oestrogenic-like biological activity (Degen & Bolt 2000), including agricultural and industrial by-products, organochlorine pesticides and insecticides, and phytoestrogens (Turner & Sharpe 1997). Although many of these have a low potency, it has been hypothesized that prolonged environmental exposure may result in severe malformations in reproductive systems and subsequent decreased fertility (Jensen *et al.* 1995; Cummings 1997; Harrison *et al.* 1997).

Phytoestrogens are weakly oestrogenic plant derived non-steroidal compounds, comprised of three classes, the isoflavonoids, coumestans and lignans (Murkies 1998; Tham *et al.* 1998; Davis *et al.* 1999). As many of these environmental oestrogens are significantly less potent than other oestrogenic substances, exposure to them has been regarded as non-harmful, even beneficial (Whitten & Naftolin 1998). For instance, isoflavones, such as genistein, occur in high quantities in beans such as the soy bean, and it is the ingestion of this soy meal, the staple diet of many Asian communities, that has been suggested by numerous epidemiological and experimental studies to cause a protective effect against

hormone dependent cancers, such as breast and prostate in these populations (for review see Adlercreutz 1990; Tham *et al.* 1998; Setchell & Cassidy 1999).

Limited studies completed in the male have suggested a role for these compounds in male reproductive processes (Santti *et al.* 1998; Whitten & Naftolin 1998; Jefferson & Newbold 2000). The phytoestrogens, particularly genistein and coumesterol, are able to bind to oestrogen receptors in an agonistic form with high affinity, in particular to ERβ (Kuiper *et al.* 1998; Brzezinski & Debi 1999). Given that both ER isoforms, but particularly ERβ, are localized at sites important for male reproduction (Carreau *et al.* 1999; O'Donnell *et al.* 2001), it is reasonable to speculate that these agonistic ligands may have direct effects on male reproductive function. It has been determined that virtually all commercial rodent diets that include soy as a source of protein have detectable levels of oestrogenic isoflavones (Boettger-Tong *et al.* 1998; Thigpen *et al.* 1999) and therefore such diets may be capable of sustaining oestrogen-like effects.

The ArKO mouse, which lacks aromatase products, is an ideal model to test whether the dietary soy protein in rodent diets has agonistic oestrogenic effects on the male reproductive system, particularly in terms of the maintenance of spermatogenesis. We previously hypothesised that the late onset of the spermatogenic phenotype in male ArKO mice (**Section 3.3**) (Robertson *et al.* 1999) was due to the fact that their diet may contain oestrogen-like substances which have an agonistic effect on spermatogenesis. This study aimed to investigate the effects of a soy free diet and a natural rodent soy-containing diet on spermatogenesis in ArKO mice to understand whether dietary soy can have an action on the testis. Also investigated were the effects of a soy-free and soy-containing diet on wildtype mice to evaluate the effects of dietary soy in normal mice.

4.2 RESULTS

4.21 Diet

The soy containing diet (S+) (**Section 2.213**) contained approximately 146µg/g isoflavones, whereas in the soy free diet (S-) no isoflavones were detected by HPLC analysis.

4.22 Fertility study

A fertility study was performed to characterise the effect of dietary soy on the reproductive potential of both WT and ArKO mice between 50 and 100 days of age. The female's chosen were known to be fertile. Diet did not influence litter number with either WT or ArKO males (**Table 4.221**), although more pups were sired per litter when soy was absent from the diet ($p<0.05$). ArKO mice on either diet showed a significant decline in fertility when compared to WT males at 14 weeks, with only 20% of the mating pairs on the S+ diet ($p<0.001$) and 50% ($p<0.05$) of the mating pairs on the S- diet siring litters

4.23 Weights

A soy-free diet was associated with an increase in body weight in WT ($p=0.001$) and ArKO ($p=0.02$) mice at 14 weeks of age (**Table 4.231**) and in ArKO mice ($p=0.021$) at 1 year of age (**Table 4.232**) compared to colony mates raised on a diet containing soy meal.

The presence or absence of soy in the diet did not alter testis weight in either ArKO or WT mice at 14 weeks of age (**Table 4.231**), nor in WT mice at 1 year of age (**Table 4.232**). However, ArKO mice had decreased testis weights compared to WT mice at 1 year of age, regardless of diet (**Table 4.232**), and this was further decreased in ArKO mice consuming a soy-free diet ($p=0.003$).

Table 4.221 Fertility of WT and ArKO males on either a soy containing or soy free diet at 14 weeks of age

	Number of litters/animal	**Number of pups**	**Days to first litter**
WT S+ (n=8)	1.75 ± 0.2	6.50 ± 0.5	25.38 ± 2.4
WT S- (n=8)	1.75 ± 0.4	8.63† ± 0.5	33.88 ± 5.0
ArKO S+ (n=5)	0.2 * ± 0.2	2	47
ArKO S- (n= 10)	0.6 * ± 0.2	7.6 † ± 0.8	28.2 ± 3.3

ArKO and WT males on the S+ and S- diet were placed with WT females for 50 days and their fertility monitored. At 14 weeks, ArKO males on the S+ ($p<0.001$) and S- ($p<0.05$) diets sired significantly fewer litters when compared to their WT colony mates, with only 1 mating pair on the S+ diet siring a litter. Both WT (†$p<0.05$) and ArKO (†$p<0.05$) males sired significantly more pups per litter when soy was removed in their diet. The notation * represents significant difference between genotypes, † between diets. Results are shown as mean ± SEM

Table 4.231 Weights of ArKO and WT males at 14 weeks of age

	Body weight, (g)	Testis weight, (mg)
WT S+ ***(n=8)***	25.22 ± 1.2	119.14 ± 5.6
WT S- ***(n=12)***	33.36† ± 1.6	121.14 ± 5.2
ArKO S+ ***(n= 6)***	26.0 ± 2.5	120.88 ± 3.0
ArKO S- ***(n=11)***	31.36† ± 1.1	118.07 ± 2.8

The WT and ArKO animals at 14 weeks on a diet containing no soy meal (S-) were heavier than the WT (†$p<0.001$) and ArKO (†$p<0.05$) mice, respectively, on the diet that contained soy. There was no difference in testes weight. The notation † represents a significant difference between diet. Data expressed as mean ±SEM.

Table 4.232 Weights of ArKO and WT males at 1 year of age

	Body weight, (g)	Testis weight, (mg)
WT S+ (n=4)	43.25 ± 1.2	134.50 ± 6.4
WT S- (n=4)	49.25 ± 3.2	140.78 ± 6.0
ArKO S+ (n= 7)	41.71 ± 2.1	101.54* ± 8.2
ArKO S- (n=4)	52.0† ± 1.8	53.58*† ± 7.2

At 1 year of age, ArKO mice on a S- diet were heavier than ArKO mice on a S+ diet (†$p<0.05$) and showed a reduction in testicular weight (†$p<0.05$). As shown before, ArKO mice on the S+ diet have reduced testis weight compared to WT on the corresponding diet (*$p<0.05$). The notation * represents significant difference between genotypes, † between diets. Data expressed as mean ±SEM.

4.24 Testis morphology

4.241 Qualitative observations on testicular morphology

Histological examination at 14 weeks showed no noticeable difference in testicular morphology in WT animals consuming a S- compared to a S+ diet. However, differences were clearly apparent when comparing ArKO males raised on the S+ or S- diets (data not shown). Animals raised on a S+ diet showed normal testicular morphology, however 3 out of the 10 S- ArKO animals showed evidence of spermatogenic disruption. The most notable feature was spermiogenic abnormalities such that elongated spermatids were lacking in all tubules, however the remainder of the epithelium appeared normal. This suggested that the ArKO phenotype may arise earlier than 18 weeks when soy is removed, however further investigations were required.

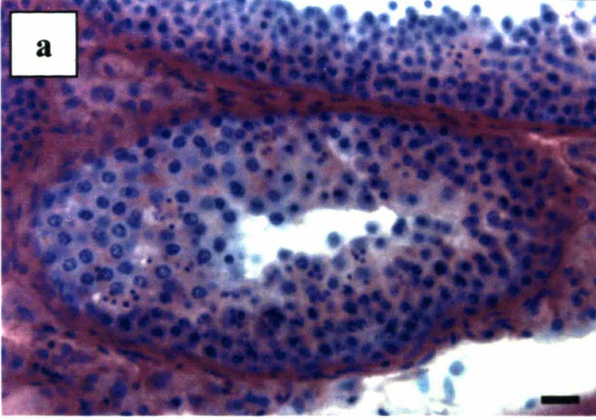
Testes from 1-year-old WT animals raised on either a S+ or S- diets showed no noticeable changes in morphology. As described previously (**Chapter 3**), ArKO animals raised on a soy-containing diet showed a predominant phenotype of spermiogenic arrest with most tubules showing evidence of round spermatid apoptosis and/or reductions in more mature spermatids (**Figure 4.241a**). However spermatogenic disruption was far more pronounced when ArKO animals were raised on a soy-free diet. Three animals showed heterogeneity in tubule morphology with some tubules containing only Sertoli cells, other tubules with marked epithelial disruption (**Figure 4.241c,d**) and noticeable reductions in germ cells (**Figure 4.241e**), and tubules in which spermiogenic arrest was evident (**Figure 4.241b**). The majority of tubules in two of these animals showed marked epithelial disruption. One animal had a complete Sertoli cell only phenotype (**Figure 4.241f**). All had marked increases in interstitial volume.

4.242 Quantitative assessment of testicular morphology

To further analyse the testicular structure, quantitative histomorphometry was performed using stereological methods. At 14 weeks of age, diet had no effect on the absolute volumes of the testicular lumen, epithelium and interstitium in either WT or ArKO animals

Figure 4.241 Testicular morphology: 1 year of age

Light microscopic analysis of 25μm methacrylate embedded testes from ArKO animals at 1 year of age on the soy-containing or soy-free diet. Sections were stained with periodic acid-Schiff and counterstained with haemotoxylin. (**a**) The disrupted seminiferous epithelium of the ArKO mouse on the soy containing diet. (**b**) The predominant phenotype when soy was removed was an absence of elongating spermatids with epithelial vacuoles (V) and Leydig cells hypertrophy (L). (**c**) Male ArKO mice on the soy-free diet showed far more pronounced disruptions to spermatogenesis with major epithelial damage, illustrated by the reduced seminiferous epithelium height (arrow). (**d**) Degenerating germ cells were clearly evident in the damaged epithelium (arrowheads). (**e**) Most tubules contained a marked reduction in germ cells, with this tubule only containing spermatogonia (g), spermatocytes (c) and elongated spermatids (e), no round spermatids. (**f**) One animal presented with a Sertoli cell only phenotype, characterised by a complete absence of germ cells. Vacuoles (V) are also clearly present in the seminiferous epithelium. Scale bars (a-d,f) 10μm, (e) 20μm.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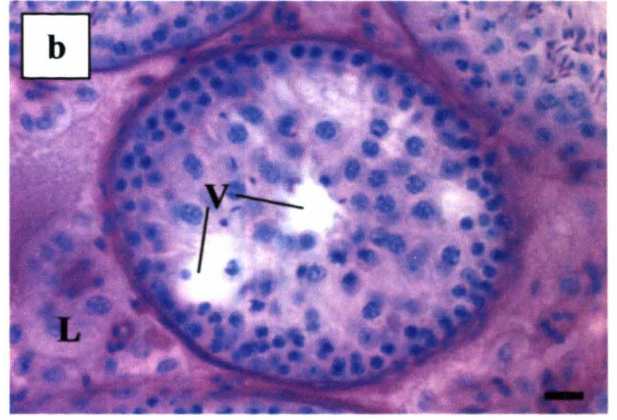
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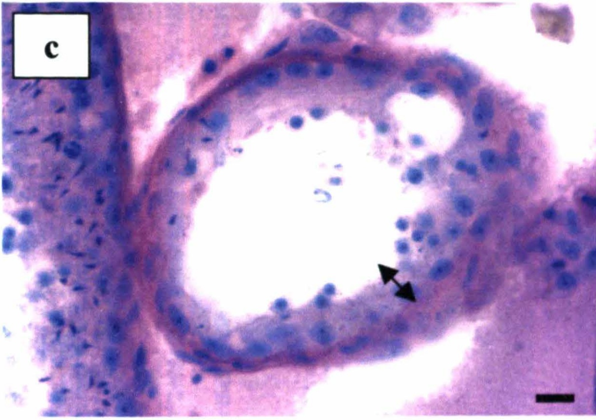
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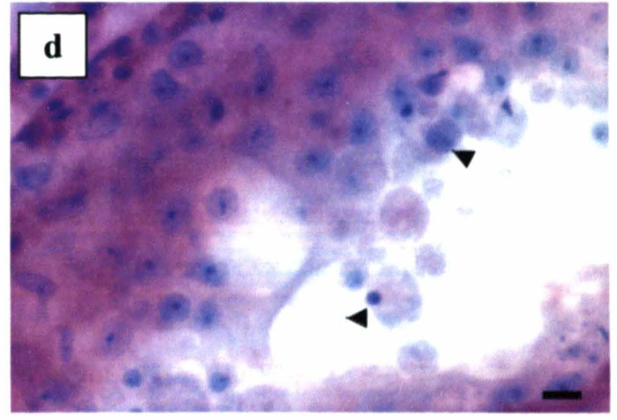
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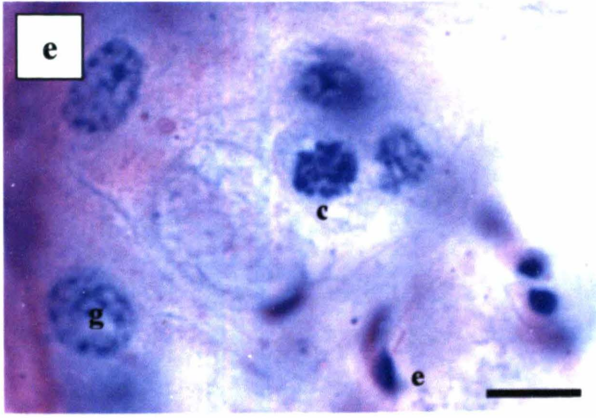
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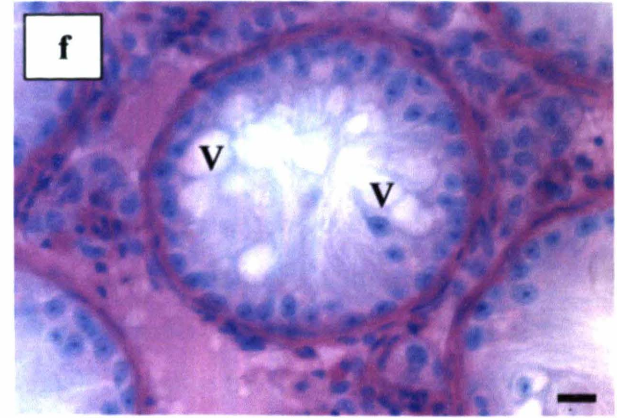
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(**Table 4.241**). However, the tubule diameter decreased and the length increased in both genotypes when soy was absent from the diet. At this age no differences were noted between WT and ArKO animals on the same diet.

By 1 year of age, diet promoted significant changes in various morphometric parameters in WT testes. WT animals reared on a soy-free diet (**Table 4.242**) had an increase in the volume of the epithelium (p=0.017), a decrease in the volume of interstitium (p=0.002) and an increase in the length of the tubules (p=0.038) compared to animals raised on a diet containing soy.

The disruptions to testicular morphology in 1 year old ArKO males raised on a diet containing soy (**Chapter 3**) were markedly exacerbated when ArKO animals were raised on a soy free diet (**Table 4.242**). The absence of dietary soy promoted marked decreases in the volume of the testis taken up by seminiferous epithelium (p=0.007) and lumen (p=0.02), being 2.3 and 6.3 fold lower than S+ ArKOs respectively, tubule diameter was also markedly decreased (p=0.02), however the volume of interstitium remained unchanged. When comparing 1 year old WT and ArKO animals on the soy free diet, ArKO males had decreased luminal (p=0.003) and epithelial (p<0.001) volume, an increase in interstitial volume (p=0.004) and a decrease in tubule diameter (p<0.001) and length (p<0.001). In contrast, the only difference between WT and ARKO animals on a soy containing diet was a decreased epithelial volume (p=0.013) in ArKO testes.

4.243 Germ cells

To further investigate the role soy may have in spermatogenesis, germ cell numbers were quantified using the optical disector. At 14 weeks of age, germ cell numbers were not altered by diet in WT animals (**Figure 4.242**). Whereas, ArKO animals raised on a S+ diet had fewer spermatogonia (p=0.029) than WT animals, which is in contrast to the previous chapter. However no changes were noted in spermatocyte numbers in any groups. In contrast to the apparent inhibitory effect of dietary soy on spermatogonia, ArKO animals

Table 4.241 Morphometric data on testicular compartments at 14 weeks of age

	Volume Lumen (mm^3)	Volume Epithelium, (mm^3)	Volume Interstitium, (mm^3)	Tubule Diameter (μm)	Tubule Length (m)
WT S+ (n=8)	10.44 ± 0.7	96.74 ± 5.0	11.53 ± 1.2	256.48 ± 4.7	2.08 ± 0.1
WT S- (n=12)	11.12 ± 0.6	97.08 ± 3.8	12.50 ± 0.9	243.56† ± 2.8	2.35† ± 0.1
ArKO S+ (n=6)	9.60 ± 0.5	94.17 ± 4.2	11.07 ± 0.6	257.15 ± 4.4	2.04 ± 0.8
ArKO S- (n=11)	9.90 ± 0.7	93.73 ± 2.5	13.30 ± 1.3	239.24† ± 5.4	2.33† ± 0.1

In both WT (†$p<0.05$) and ArKO (†$p<0.05$) mice there was a decrease in the diameter of the seminiferous tubules and an increase in tubule length when S- diet was consumed. The notation † represents significant difference between diet. Data expressed as mean ±SEM.

Table 4.242 Morphometric data on testicular compartments at 1 year of age

	Volume Lumen (mm^3)	Volume Epithelium (mm^3)	Volume Interstitium (mm^3)	Tubule Diameter (μm)	Tubule Length (m)
WT S+ ***(n=4)***	16.84 ± 3.7	95.97 ± 5.1	15.81 ± 0.7	247.20 ± 6.0	2.36 ± 0.2
WT S- ***(n=4)***	9.84 ± 1.2	119.85† ± 5.3	10.70† ± 0.7	242.10 ± 4.1	2.82† ± 0.1
ArKO S+ ***(n=7)***	12.38 ± 2.7	63.58* ± 7.4	21.40 ± 3.2	213.95 ± 12.0	2.05 ± 0.3
ArKO S- ***(n= 4)***	1.98*† ± 1.1	27.69*† ± 5.0	23.03* ± 2.6	162.23*† ± 10.6	1.40* ± 0.1

At 1 year of age, WT mice on a S- diet had an increase in their epithelial volume (†$p<0.05$) and tubule length (†$p<0.05$) and a decrease in interstitial volume (†$p<0.05$) when compared to WT on a S+ diet. The ArKO animals on a S- diet showed a decrease in lumen (†$p<0.05$) and epithelium volume (†$p<0.05$), and in tubule diameter (†$p<0.05$) compared to ArKO mice on a S+ diet. In comparison to WT on a S- diet, the ArKO mice had significantly lower luminal (*$p<0.05$) and epithelial (*$p<0.001$) volume, and an increased interstitial (*$p<0.05$) volume, plus smaller tubule diameter (*$p<0.001$) and shorter tubule length (*$p<0.001$). The only parameter than differed between WT and ArKO on a S+ diet was a decreased epithelial volume in the ArKO mice (*$p<0.05$). The notation * represents significant difference between genotypes, † between diets. Data expressed as mean ± SEM

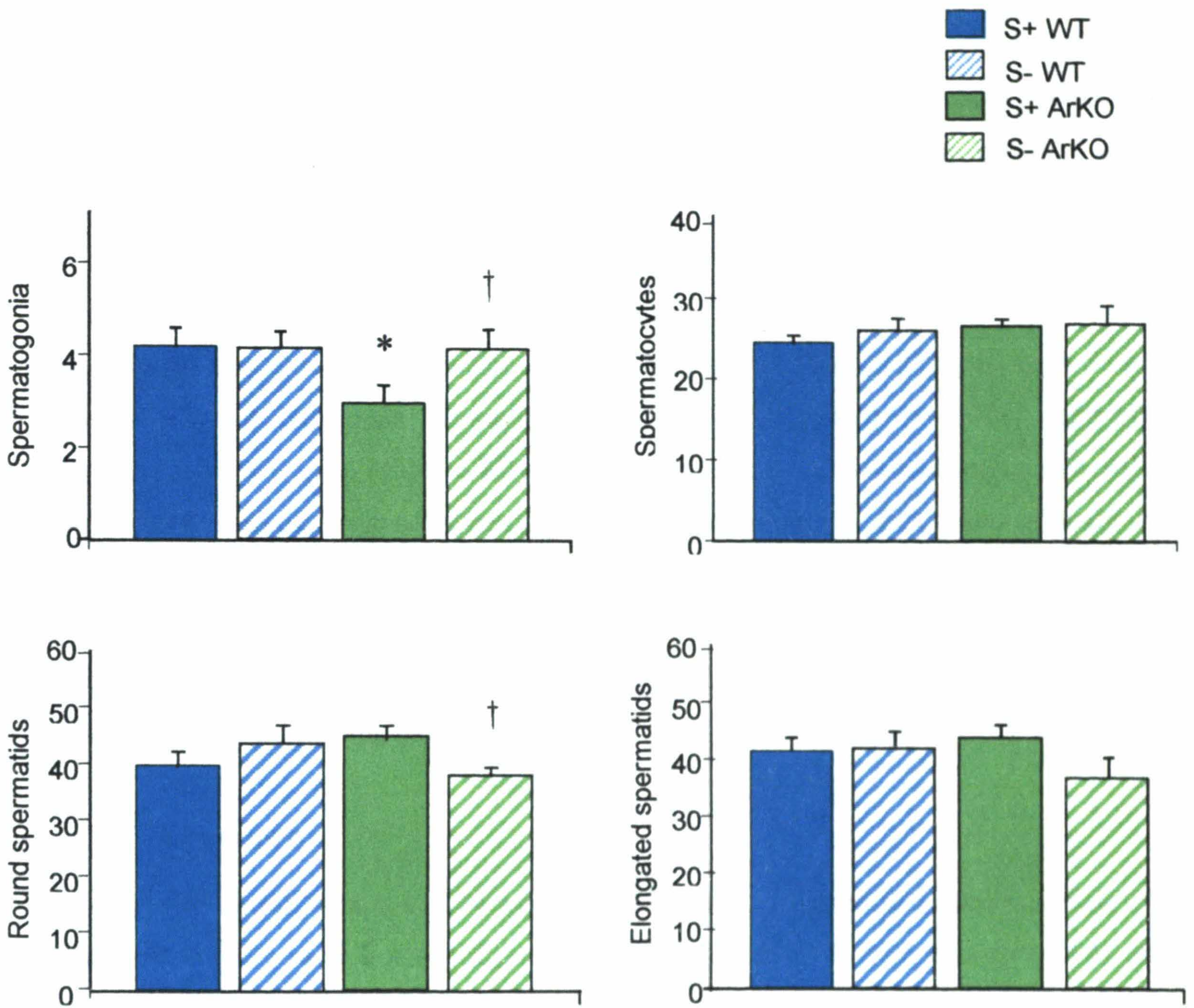


Figure 4.242 Germ cell numbers at 14 weeks of age

The optical disector approach was used to quantitate germ cell numbers (millions) per testis in ArKO and WT mice on the S+ and S- diet. ArKO animals on the S- diet had more spermatogonia when compared to those consuming a S+ diet (†$p<0.05$). ArKO animals on the S+ diet also have less spermatogonia when compared to the corresponding WT animals on the S+ diet (*$p<0.05$). ArKO animals on the S- diet had fewer round spermatids when compared to ArKO mice on the soy containing diet (†$p<0.05$). There were no differences in the number of spermatocytes or elongated spermatids.. The notation * represents significant difference between genotypes, † between diets. Data expressed as mean ± SEM

raised on a S- diet had significantly fewer round spermatids (p=0.004) when compared to ArKO animals on the S+ diet (**Figure 4.242**), suggesting agonistic effects of dietary soy on these germ cells in the absence of aromatase.

At 1 year of age there was no effect of diet on germ cell numbers in WT mice (**Figure 4.243**). As was observed previously (**Section 3.236**), ArKO animals raised on a S+ diet had significant reductions in round (p=0.001) and elongated (p=0.004) spermatids compared to WT mice (**Figure 4.243**). These disruptions to spermatogenesis in ArKO mice were markedly enhanced when the mice were reared on a S- diet. Although there was variability in the phenotype between mice (**Section 4.241**), analysis of germ cell numbers showed that ArKO mice on a S- diet had significant decreases in spermatocytes (p<0.001), round (p=0.001) and elongated (p<0.001) spermatids to 25, 20 and 24 % of ArKO males raised on a diet containing soy.

4.244 Sertoli cells

Analysis of Sertoli numbers in 14-week-old WT animals showed that mice raised on a S- diet had slightly more but significantly higher numbers of Sertoli cells (p=0.006) than those raised on a S+ diet (**Figure 4.244a**), but this was not evident in 1 year old animals. Diet did not affect Sertoli cell number in ArKO animals at either age (**Figure 4.244a,b**). Interestingly, a significant increase in Sertoli cell numbers in ArKO mice on a S+ diet was evident when compared to WT on the same diet at 14 weeks (p=0.018), suggesting an inhibitory action of oestrogenic substances on Sertoli cell numbers. Similar trends were observed in one year old animals, however no statistically significant differences were observed (**Figure 4.244b**).

i Germ cells per Sertoli cell

Since differences in Sertoli cell numbers were observed, the capacity of each Sertoli cell to support germ cells was examined between diets (**Figure 4.245**). There were no significant differences between any group at 14 weeks of age. However at 1 year of age, the ArKO

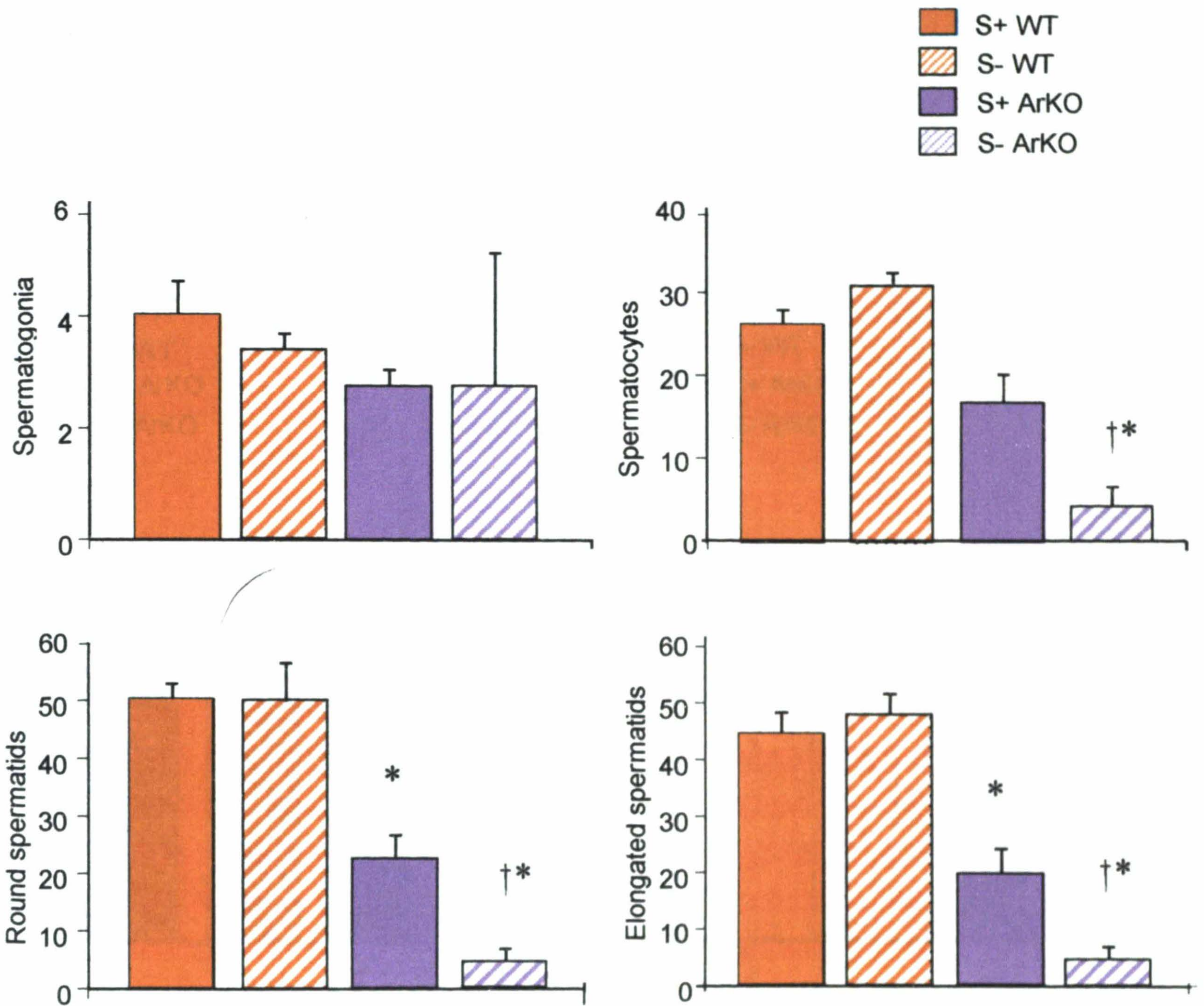


Figure 4.243 Germ cell numbers at 1 year of age

The optical disector approach was used to quantitate germ cell numbers (millions) per testis in ArKO and WT mice on the S+ and S- diet. ArKO animals on the S- diet had less spermatocytes (†$p<0.05$), round spermatids (†$p<0.05$) and elongated spermatids (†$p<0.05$) when compared to ArKO animals raised on a S+ diet. ArKO mice on a S- diet also had fewer spermatocytes (*$p<0.001$), round spermatids (*$p<0.001$) and elongated spermatids (*$p<0.001$) when compared to WT animals raised on the corresponding diet. Whereas, ArKO mice on the S+ diet had less round spermatids (*$p<0.001$) and elongated spermatids (*$p<0.05$) compared to WT on the corresponding diet. The notation * represents significant difference between genotypes, † between diets. Data expressed as mean ± SEM.

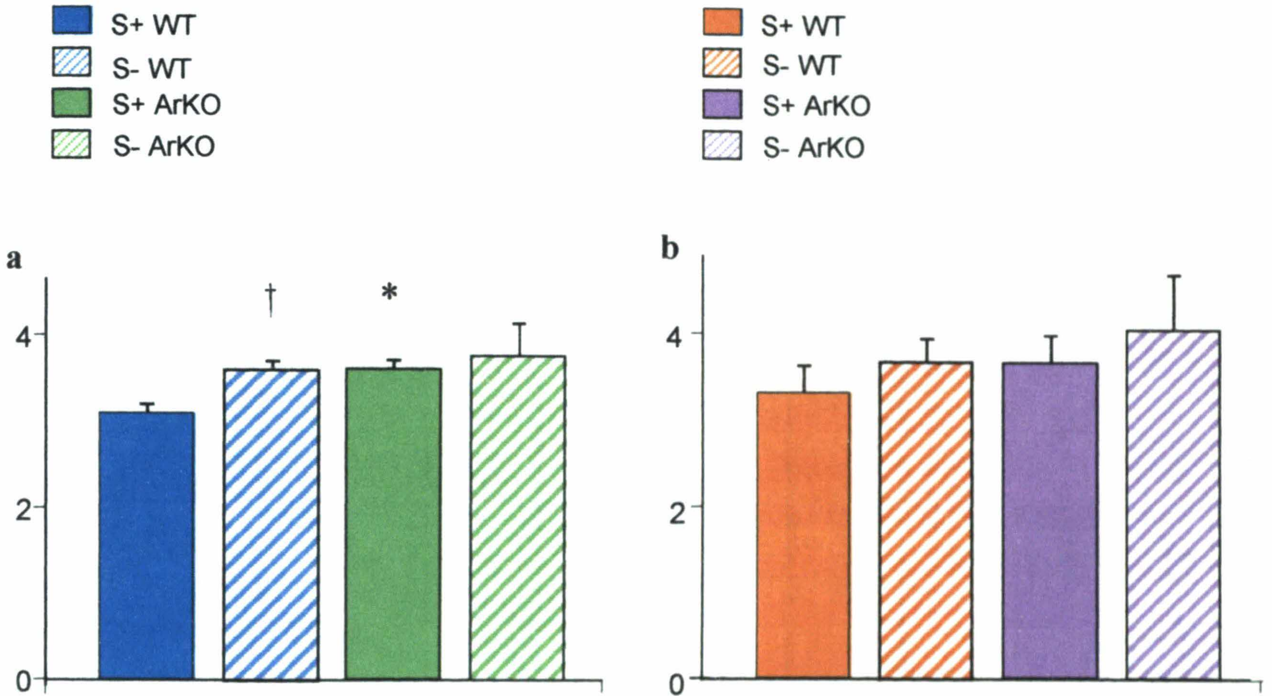
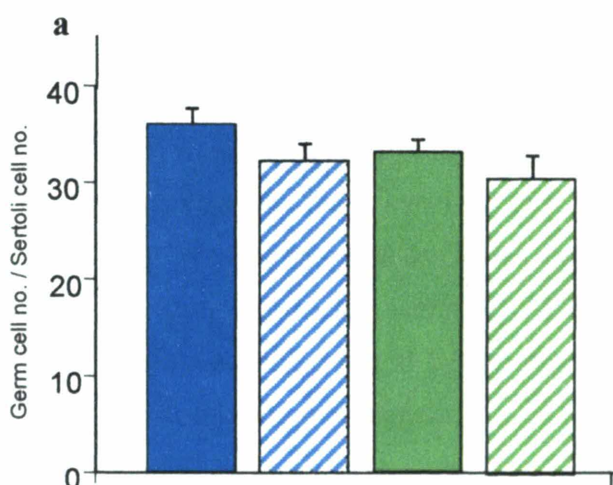


Figure 4.244 Sertoli cell numbers

The optical disector approach was used to quantitate Sertoli cell numbers (millions) per testis in ArKO and WT mice on the S+ and S- diet. (**a**) At 14 weeks, WT mice on a S- diet have significantly more Sertoli cells compared to WT on a S+ diet (†p<0.005). The WT on the S+ diet also have significantly fewer Sertoli cells number than the ArKO on the corresponding diet (*p<0.05). (**b**) There were no differences between all the groups at 1 year of age. The notation * represents significant difference between genotypes, † between diets. Data expressed as mean ± SEM



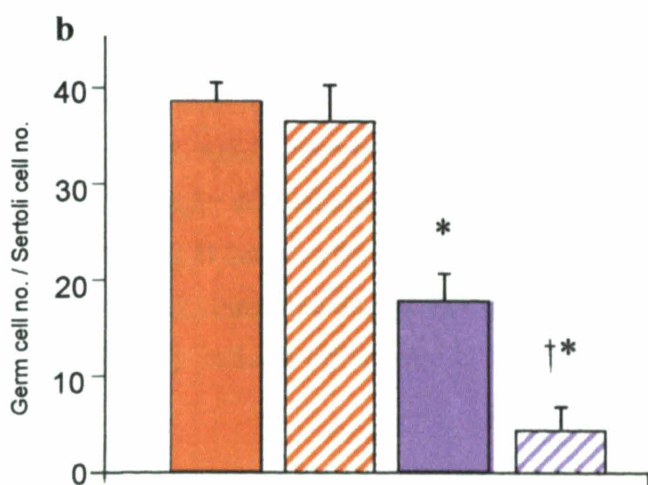


Figure 4.245 Germ cells per Sertoli cell

Total germ cell number was expressed as a proportion of Sertoli cell number at (**a**) 14 weeks of age and (**b**) 1 year of age. There were no significant differences between diet and genotype in the number of germ cells each Sertoli cell was able to support at 14 weeks. However at 1 year of age, the Sertoli cells in the ArKO mice were found to support less germ cells when soy was removed from their diet (†$p<0.05$). The ArKO mice on the soy free diet (*$p<0.001$) and on the soy containing diet (*$p<0.001$) were also able to support less germ cells than the WT on the corresponding diet. The notation * represents significant difference between genotypes, † between diets. Data expressed as mean± SEM.

mice on both the S+ (p=0.001) and S- (p<0.001) diets showed less germ cells per Sertoli cell compared to WT mice on the corresponding diet. Also, Sertoli cells in ArKO males supported fewer germ cells when soy was absent compared to S+ ArKO mice (p=0.013), reflecting the fact that disruption to spermatogenesis and testicular morphology is markedly enhanced by the absence of soy in the diet.

4.25 Serum hormone assays

There was no effect of diet on the levels of serum LH and FSH in male WT and ArKO animals at 14 weeks (**Table 4.251**), nor were there differences in ArKO mice compared to WT comsuming the same diet. However at 1 year of age, ArKO mice on the S- diet had increased FSH levels compared to ArKO males raised on a S+ diet, suggesting an agonistic role of soy at the pituitary. There was no change in serum LH between the groups sampled, however, insufficient serum only allowed the analysis to be completed on two WT mice on a soy containing diet at 1 year. A larger sample size may indicate differences in LH not observed with two animals.

4.4 DISCUSSION

The ArKO mouse was used to test the hypothesis that dietary soy protein has a biological effect on the testis, possibly maintaining spermatogenesis in the absence of endogenous oestrogen. The disruptions to spermatogenesis appeared to be induced slightly earlier when soy was removed from the diet, with a significant decrease in round spermatids, however this effect was miminal. The data does however clearly indicate that soy has agonistic effects on spermatogenesis in the absence of oestrogen, particularly in terms of the maintenance of germ cell development. This agonistic effect was not associated with an increased gonadotropic stimulus to the testis, suggesting that dietary soy can have direct effects on receptors within the male reproductive tract. Dietary soy was also able to

Table 4.251 Serum hormone Levels

	FSH (ng/ml)		LH (ng/ml)	
	S+	**S-**	**S+**	**S-**
WT 14-18wks	10.88 ± 1.2 (n=6)	9.73 ± 0.6 (n=6)	0.37 ± 0.07 (n=6)	0.47 ± 0.15 (n=6)
ArKO 14-18wks	8.47 ± 2.6 (n=3)	11.13 ± 0.9 (n=6)	0.52 ± 0.2 (n=3)	0.45 ± 0.1 (n=6)
WT 1 year	10.41 ± 0.3 (n=4)	13.92 ± 2.0 (n=6)	1.49 (n=2)	0.27 ± 0.1 (n=6)
ArKO 1 year	9.86 ± 0.2 (n=7)	16.55† ± 1.2 (n=4)	0.99 ± 0.4 (n=4)	0.38 ± 0.1 (n=4)

Serum hormone levels were measured using double antibody RIAs. There was no difference in FSH levels between the two diets at 14 weeks. However, at a year of age, there was a significant elevation in FSH levels in the ArKO mice fed a diet containing no soy when compared to those fed a soy diet (†$p<0.001$). Examining LH, there was no difference between the WT and ArKO on either a S+ or S- diet at 14 weeks and at 1 year of age. The notation † represents a significant between diets. Data expressed as mean ± SEM.

produce slight changes in testicular histology in WT animals, highlighting the fact that soy found in commercial rodent chow has an action on the testis.

In the absence of endogenous oestrogen, soy consumption clearly has a beneficial effect on the testis, by partially maintaining testis weight, germ cell development and seminiferous tubule epithelial and luminal volume. In general, the testes are morphologically less impaired when soy was consumed, exhibiting reduced epithelial and structural damage. These effects on the testis and spermatogenesis could not be explained by increases in circulating LH and FSH, and are thus likely to be mediated by direct effects on the testis. The phytoestrogens genistein and daidzein, both present in soy, are agonists at both ERs but particularly ERβ (Casanova *et al.* 1999). Given the localisation of ERs in both Leydig and Sertoli cells in the adult testis, and of both aromatase and ERβ in meiotic and post-meiotic germ cells (**Section 1.4**), agonistic effects on spermatogenesis are therefore not unexpected. The defects in germ cell development in ArKO mice, in the absence of decreases in gonadotrophins or androgens (**Chapter 3**), clearly suggest a direct effect of oestrogen on germ cell development. Here I show that this agonistic effect can be mimicked in part by dietary oestrogens, with a soy diet preventing the marked decline in spermatocyte, round and elongated spermatid numbers caused by oestrogen withdrawal. Previous studies show that genistein, in doses equal to those concumed in a soy based diet (2.5 mg s.c./kg of body weight), are able to induce squamous epithelial metaplasia, a sign of oestrogen action, in mouse accessory sexual organs such as the prostate (Strauss *et al.* 1998), and also produce a high oestrogenic response in the uterus of immature rats consuming a standard phytoestrogen containing diet (210 mg/kg) (Boettger-Tong *et al.* 1998), providing further evidence for an agonistic role of phytoestrogens in the testis.

Comparison of 1 year old ArKO mice raised on a soy-free diet with those raised on a soy-containing diet not only demonstrates a biological action of dietary soy, but reveals hitherto unrecognised roles of aromatase/oestrogen in spermatogenesis. The demonstration of reduced numbers of spermatocytes in soy-free ArKO males suggest that spermatocyte development may also be dependent on aromatase. This is not unexpected, given the

localisation of aromatase and ERβ in certain spermatocyte populations (**Section 1.4**). Previous studies have shown that seminiferous tubules lacking ERα secrete less fluid (Hess *et al.* 1997a), suggesting that oestrogen action is important for the maintenance of Sertoli cell fluid secretion. This is supported by these findings which show that 1 year old ArKO mice raised on a soy-free diet have a marked reduction in seminiferous tubule luminal volume and a significant increase in FSH levels, suggesting that Sertoli cell function, and probably inhibin B secretion, is disrupted when there is no oestrogenic stimulus. A further observation for dietary soy assisting in the maturation of Sertoli cell function is the markedly decreased seminiferous epithelium volume and tubule diameter in the ArKO males. Thus, oestrogen production is likely to be important for the maintenance of pre and post-meiotic germ cell development and Sertoli cell function.

It is possible that the elevation in FSH, when soy is removed from the diet, is also a result of soy inhibiting gonadotrophin secretion from the pituitary. Endogenous oestrogen has a homeostatic feedback role at the hypothalamus and pituitary, potentially through a direct action on the ERs localised specifically in these tissues (Shughrue *et al.* 1997). As dietary soy prevents the increase in FSH caused by the removal of oestrogen, it suggests an oestrogenic action of soy at the level of the hypothalamus/pituitary (Couse *et al.* 1997; Mitchner *et al.* 1998; Laflamme *et al.* 1998).

In WT animals, dietary soy had minor but measureable effects on the testis as evidenced by a reduction in seminiferous epithelium volume. A surprising finding in this study was a slight decrease in the number of Sertoli cells in ArKO animals on the soy-containing diet compared to WT anmals at 14 weeks, which is in contrast to the findings presented earlier (**Chapter 3**). Similar trends were also observed in the ArKO mice on a soy-free diet, however statistical differences were not observed. It is thus possible that an absence of aromatase during neonatal development could result in changes in Sertoli cell proliferation, such that more Sertoli cells are produced when aromatase is absent. This is perhaps not surprising, given that exposure to pharmacological levels of oestrogen can interfere with Sertoli cell proliferation, either directly or via effects on FSH, leading to reduced Sertoli

cell numbers (Atanassova *et al.* 1999). However, further studies on the effects of dietary soy during the neonatal period of Sertoli cell proliferation are required to determine the mechanism of this effect. Exogenous oestrogens can also interfere with Sertoli cell maturation, as evidenced by decreases in Sertoli cell volume (Atanassova *et al.* 1999; Perez-Martinez *et al.* 1996) and protein secretion (Sharpe *et al.* 1998). In the WT mice, it is possible that dietary soy may synergise with the endogenous oestrogen already present in these animals (Casanova *et al.* 1999) to promote higher levels of oestrogenic ligands that have slight inhibitory effects on Sertoli cells.

It is also possible that phytoestrogens may in fact antagonise the action of endogenous ostrogens to prevent oestrogen action (Makela *et al.* 1995; Whitten *et al.* 1995b; Tansey *et al.* 1998), or prevent oestrogen biosynthesis through the inhibition of enzymes such as aromatase (Adlercreutz *et al.* 1993; Campbell & Kurzer 1993). These functions of phytoestrogens may provide an explanation as to the increase in interstitial volume when a soy diet is consumed, particularly as Leydig cell proliferation and differentiation has been shown to be inhibited by oestrogens (Dhar & Setty 1976; Abney & Carswell 1986; Perez-Martinez *et al.* 1996; Perez-Martinez *et al.* 1997). Also, the specific actions of phytoestrogens in the presence of endogenous oestrogen may be concentration dependant, with an agonist function observed at low doses and an antagonistic at high concentrations in genistein treated MCF-7 cells (Wang & Kurzer 1998).

The consumption of soy by WT mice was associated with a reduction in the number of pups sired in each litter. I have no explanation for this finding, however it is possible that changes in their epididymal maturation, sperm-zona pellucida binding or female reproductive physiology could be modulated by phytoestrogens (Burroughs *et al.* 1990; Whitten *et al.* 1995a; Hinsch *et al.* 2000).

ArKO mice have been reported to exhibit an increase in body weight and fat deposition (Jones *et al.* 2000), suggesting a direct role for oestrogen on adipocytes via ERs. Interestingly, in ArKO mice at both 14 weeks and 1 year of age, dietary soy prevented this

increase in body weight. Consumption of a phytoestrogen containing diet also decreased body weight in WT mice, which is in agreement with recent studies in normal adult rats (Weber *et al.* 2001). It is well known that exogenous oestrogens can reduce body weight at birth (Levy *et al.* 1995; Flynn *et al.* 2000), weaning (Whitten *et al.* 1995a) and maturity (Atanassova *et al.* 2000; Flynn *et al.* 2000).

The finding that spermatogenesis appears to progress almost undisrupted in the younger ArKO males was puzzling, however is not unique to our ArKO mouse. Both ArKO mice generated by Honda *et al* (1998) and Toda *et al* (2001a) observed no histological or spermatogenic abnormalities up to 16 weeks of age, with active sperm present in the cauda epididymis. In addition to this, rats treated with an aromatase inhibitor presented with minimal disruptions up to 19 weeks of treatment, with more pronounced disruptions after 1 year, suggesting a time related effect and possible compensatory mechanisms (Turner *et al.* 2000a).

It was previously suggested that the late onset of the spermatogenic phenotype in male ArKO mice could be due to oestrogenic substances present in their diet that are capable of agonistic effects on spermatogenesis (**Section 3.3**). As round spermatid number significantly decreases at 14 weeks when soy is removed, dietary soy may be contributing in maintaining spermatogenesis when endogenous oestrogen synthesis is prevented. However, as withdrawing soy on spermatogenesis does not produce spermatogenic disruptions during the initial spermatogenic cycle, and at 14 weeks disruptions are minimal, it suggests the importance of other alternative ligands. These may include growth factors, such as insulin-like growth factor (IGF) (Aronica & Katzenellenbogen 1993) and epidermal growth factor (EGF) (Ignar-Trowbridge *et al.* 1992), capable of activating the ER via the activation of intracellular MAPK pathways (Smith 1998). Importantly, two androgen metabolites; 5α-androstane-3β,17β-diol and 5-androstene-3β,17β-diol, can also activate the ER, particularly ERβ, when present in high concentrations (**Section 1.222**). This adds to the complexity of oestrogen action, particularly as our unpublished findings suggest high levels of these androgen metabolites in the young male testes (personal

communication, Wah Chin Boon, Priunce Henry's Institute). Thus non-aromatase derived endogenous oestrogenic ligands may maintain spermatogenesis in ArKO mice until approximately 14 weeks of age. It is worth noting that two other ArKO mice lines (Honda *et al* 1998; Toda *et al* 2001) also show spermatogenesis at 16 weeks of age. Further studies in my laboratory are persuing the mechanisms responsible for the late onset phenotype in the ArKO male.

In conclusion, these studies reveal an action of relatively low levels of dietary phytoestrogens on male reproduction. Measurable changes in testicular compartments were seen in WT mice, however the effect of dietary phytoestrogens was most marked in ArKO mice which lack a functional aromatase enzyme. The data suggests that oestrogen production is important for the maintenance of germ cell development and Sertoli cell function and shows that dietary phytoestrogens can mimic the action of endogenous oestrogen within the seminiferous epithelium. These findings are important as they clearly highlight the significance of these environmental oestrogens in testicular development and function.

Chapter Five

Further Examination of Male ArKO Fertility

5.1 INTRODUCTION

It has already been established the ArKO males have markedly reduced fertility, siring significantly fewer litters when compared to WT (**Section 4.22**), and that this is presumably due to a significant decrease in the number of round and elongated spermatids (**Section 3.236**). However, it needed to be established if an impairment in sexual behaviour also contributes to this reduction in fertility.

In the male, spermatogenesis would be futile without the ability to copulate. Unlike many male reproductive structures, whose development and maturation are dependant upon androgen stimulation, the development of male specific sexual behaviours requires these androgen precursors to be aromatised to oestrogens (Meisel & Sachs 1994). In fact, oestrogen is synthesised locally in the sexually dimorphic neurons of the rodent MPOA, the site implicated in the control of sexual behaviour, in higher levels in the male than the female (for review see Hutchison 1997). This is demonstrated by the discrete localisation and activity of the enzyme aromatase cytochrome P-450 in rat (Tobet *et al.* 1985; Lephart *et al.* 1992; Roselli & Resko 1993a) mouse (Beyer *et al.* 1993; Karolczak *et al.* 1998), ram (Roselli *et al.* 1998) and quail brain (Balthazart *et al.* 1990b; Balthazart & Surlemont 1990).

Oestrogen biosynthesis is vital in the perinatal stage of development if masculine type sexual behaviour is to be exhibited in adulthood (Roselli & Resko 1993b; Negri-Cesi *et al.* 1996; Hutchison *et al.* 1997). Treatment with an aromatase inhibitor (Balthazart *et al.* 1990a; Gonzalez & Leret 1992; Houtsmuller *et al.* 1994), or removing the ability to synthesise oestrogens during this period (Honda *et al.* 1998), results in a significant reduction in copulatory behaviour in adulthood, explicable in terms of preventing this testosterone conversion. However, oestrogen synthesis is also required in adulthood for the continual display of sexual behaviour in response to female stimuli. Castrating mature males, or treating them with an aromatase inhibitor, leads to a failure to mount in many

species; including quail (Alexandre & Balthazart 1986; Watson & Adkins-Regan 1989; Balthazart *et al.* 1990a) mice (Wallis & Luttge 1975; Clemens & Pomerantz 1982; Wee *et al.* 1988), rat (Clancy *et al.* 1995), rams (D'Occhio & Brooks 1980), boar (Parrott & Booth 1984) and ferret (Carroll *et al.* 1988). Treatment with either oestrogens, or aromatisable androgens in the case of castration, restores this behaviour.

It was also important that the fertilisation capacity of mature spermatozoa from the ArKO males was examined to determine if the cause of the decreased fertility was also at the level of fertilisation. (Hess *et al.* 1997a; Lee *et al.* 2000). Therefore young ArKO mice were characterised with respect to the concentration and motility of mature spermatozoa, IVF capacity and sexual behaviour.

5.2 RESULTS

5.21 Sperm viability

To determine whether the viability of the mature spermatozoa was compromised in the absence of endogenous oestrogens, an *in vitro* analysis of the sperm was performed (**Section 2.224**). At 15 weeks of age ArKO males had normal sperm concentration but decreased motility (p=0.008) (**Table 5.211**). At this age, sperm from 6 out of 7 ArKO mice were able to fertilize oocytes *in vitro* (data not shown). This is in contrast to 1-year-old ArKO males whose sperm were unable to fertilize *in vitro*. This may be associated with the significant decrease observed in the sperm concentration (p=0.05) and motility (p=0.008) (**Table 5.211**). It is pertinent to note that between 15 weeks and 1 year of age, the ArKO mice sperm concentration (p=0.001) and motility (p=0.007) further declines.

Table 5.211 Sperm data

	Sperm concentration (number/ml x10^6)	Sperm motility (%)
WT* *15 wks	59.3 ± 19.1 (n=4)	81.0 ± 4.1 (n=4)
ArKO* *15 wks	65.1 ± 7.5 (n= 7)	67.5 ± 1.7 * (n=6)
WT* *1 year	41.6 ± 10.6 (n=5)	72.0 ± 2.0 (n=5)
ArKO* *1 year	10.75 ± 5.6 *# (n=5)	24.0 ± 13.4 *# (n=5)

Epididymal sperm was extracted from WT and ArKO mice and analysed for concentration and motility. At 15 weeks, ArKO sperm concentration is unaffected, however they have significantly decreased motility (*p<0.05). By 1 year of age ArKO sperm is present in a much lower concentration (*p<0.05) and the motility is more severely compromised (*p<0.05). As the ArKO animals aged their sperm concentration (#p<0.001) and motility (#p<0.05) significantly declined. The notation * represents a significant difference between genotypes, # a difference between age. Data expressed as mean ± SEM.

5.22 Sexual behaviour

To investigate sexual behaviour, WT and ArKO males were placed with hormonally primed females and behaviour recorded in the dark phase. Sexual behaviour appeared normal in the WT male animals at age 12-14 weeks, in that they approached the female, repeatedly sniffing the anogenital region, and then mounted an average of 11 (±1.9) times in 20 min (**Figure 5.221**). There was only 4.41 (±1.3) min until the first mount occurred and each subsequent mount occurred regularly, with a latency period of 1.3 (±0.2) min. In contrast, the ArKO mice demonstrated impaired mounting behavior. When placed with the females, males immediately interacted with the female, appearing to sniff her genital area, similar to the WT. However, this soon ceased and the male withdrew. No attempt to mount was initiated from any of the 4 males.

One-year-old males were also studied, with again no ArKO males (n=4) mounting in the 20 min time period. However there was also an impairment in the ability of the 1-year-old WT males (n=4) to mount with only 2 mounting. There was also a decline in the number of mounts the WT mice initiated at 1 year, with an increased time until the first mount.

The males used in the behavioural study were the same as those used in the previous study (**Chapter 4**), therefore comparisons were drawn to further analyse the fertility of the ArKO males. The 4 ArKO males at 12-14 weeks tested above did not attempt to mount females. When this was compared to their litter numbers (**Table 4.221**), only 1 out of the 4 sired no litters, the other 3 appearing to be fertile. Further comparisons found that none of these 4 males presented with the spermatogenic phenotype (**Section 4.24**). The one animal at 14 weeks who did present with the severe disruptions to spermatogenesis (**Section 4.24**) did not sire any litters (**Table 4.221**). Therefore, the fertility of the male ArKO mice may be in part attributable to abnormalities in either mounting or spermatogenesis, but most probably not both.

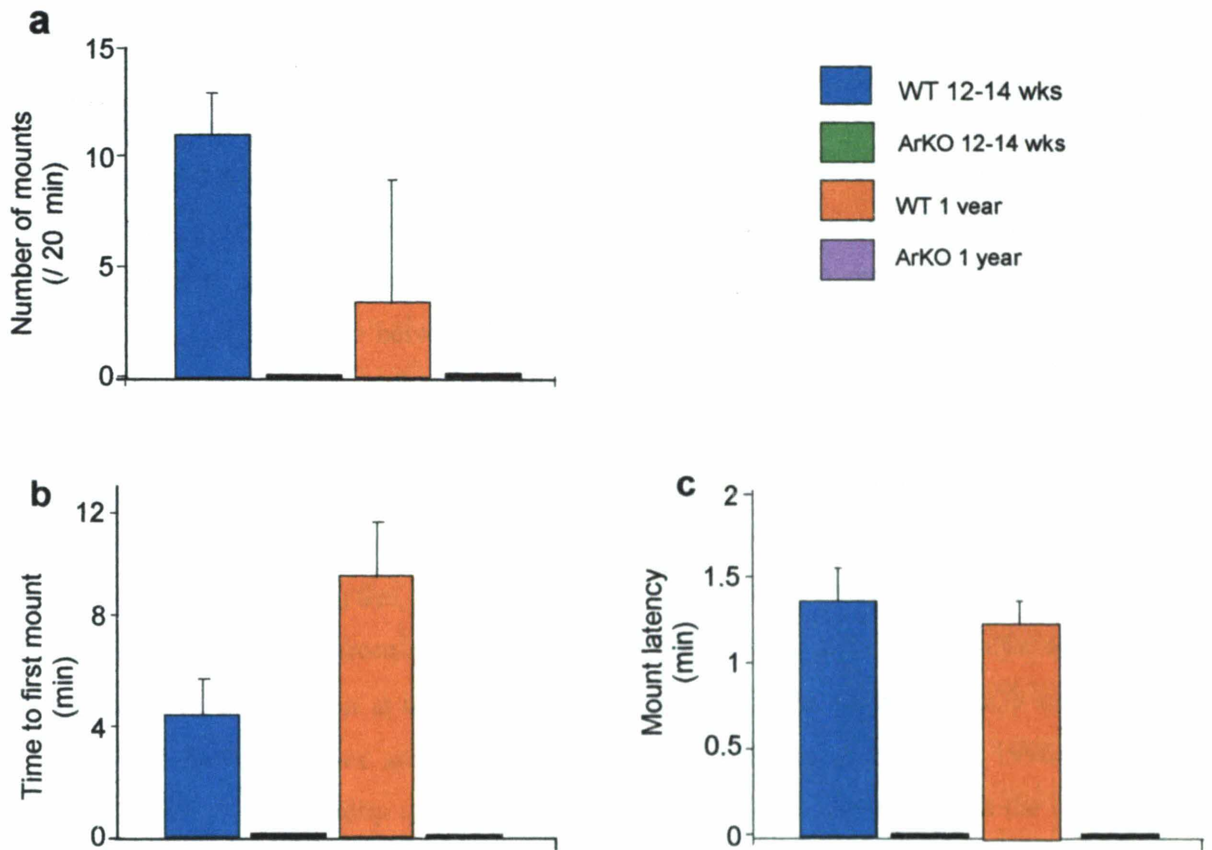


Figure 5.221 Behavioural data

WT and ArKO males (n=4) were placed with hormonally primed ovariectomised (OVX) females and sexual behaviour recorded in the dark phase. The WT males at 12-14 weeks of age mounted an average of 11 times with 4 min until the first mount and 1½ min latency between mounts. This ability to mount declined as the WT aged, with only 2 out of the 4 mounting with a reduction in the number of mounts and time to first mount. In comparison, no ArKO males were observed to mount. Results are shown as mean ± SEM.

5.3 DISCUSSION

Previous studies have established that aromatase appears to have a specific role in spermatogenesis (**Chapter 3**), specifically in the ability of germ cells to mature into round spermatids. In this study, mature spermatozoa from ArKO mice were further analysed for their ability to fertilise oocytes *in vitro* and instigate development past the zygote stage. There was no difference between 15-week-old WT and ArKO animals in this respect. However, sperm from 1-year-old animals was compromised, with decreased concentration and motility and an inability to fertilise oocytes. These findings may be due to impairments in their maturation whilst in the seminiferous epthelium (**Chapter 3**), however it is also possible that the spermatozoa may be failing to properly mature in the transition to the epididymis. The finding that spermatozoa from 15 week old ArKO males can still fertilise occytes *in vitro* differs from those observed with the αERKO mice in which the sperm are incapable of fertilisation at this young age. However, similar to the αERKO males, the sperm of the ArKO mice are less motile at 15 weeks of age (Eddy *et al.* 1996). As the ArKO mice age, the ability to fertilise declines, which may correspond with the late onset disruptions observed in spermatogenesis.

One explanation for the significant decrease in fertility observed with the young ArKO males (**Section 4.22**) could be an impairment in copulatory behavior. In fact, preliminary examinations of the sexual behaviour of these mice, and that of the ArKO mouse generated by Honda *et al* (1998), found that they appeared to have an inability to initiate mounting. This compared to WT males who mated readily with sexually receptive females. Considering past literature on male sexual behaviour, which emphasises the importance of oestrogen synthesis locally in the medial preoptic area during development, this inability to display masculine type sexual behaviour is not unexpected (Meisel & Sachs 1994). However, a more specific study is required to fully determine which aspect of sexual behaviour may be disrupted in the ArKO mice. When observing their behaviour, following being placed with a hormonally primed female, they were found to initially approach the

receptive females, however compared to the WT they appeared more hesitant and may have been curious rather than sexually motivated. The importance of aromatase in sexual behaviour is observed when aromatase inhibitors are utilised. Following administration either shortly after birth or in adulthood, copulatory behaviour is inhibited in quails, rats, mice and ferrets (Clemens & Pomerantz 1982; Carroll *et al.* 1988; Balthazart *et al.* 1990a; Gonzalez & Leret 1992; Clancy *et al.* 1995). However this importance of aromatase may have species specificity. A study employing adult Syrian hamsters showing that administering aromatase inhibitors systemically for 5 to 8 weeks failed to have any effect on their mounting behaviour (Cooper *et al.* 2000).

In comparison to the ArKO mice, male sexual behaviour in knockout mice that lack the gene for either ERα (αERKO) or ERβ (βERKO) individually (**Section 1.62**) (Lubahn *et al.* 1993; Krege *et al.* 1998), is only partially disrupted. Two further studies indicated that the αERKO mice, although infertile and rarely ejaculate, showed either normal levels of mounts and just reduced levels of intromissions (Ogawa *et al.* 1997), or a reduction in both mounting and intromissions (Wersinger *et al.* 1997). Administration of testosterone or dopamine restored mounts and intromissions in gonadectomised αERKO mice, however ejaculation was not restored, suggesting that it is regulated through pathways other than ERα (Ogawa *et al.* 1998b; Wersinger & Rissman 2000). In contrast all three components of sexual behaviour are present and robust in the βERKO mice (Ogawa *et al.* 1999). On the other hand, the double knockout (αβERKO) males do not show any component of sexual behaviour, including simple mounting behavior (Ogawa *et al.* 2000). This is similar to the ArKO mice and suggests that ERα and ERβ can complement one another in regard to this behaviour.

In summary, the decrease in fertility exhibited by the ArKO males (**Section 4.22**) may be attributable to spermatogenic disruptions that appear to arise randomly with increasing age (**Chapter 3**). It is also possible that oestrogen plays a role in the maturation of spermatozoa during the transition to the epididymis, its absence causing a decline in the

fertilisation potential of mature spermatozoa. Conversely, the reduction in fertility could be attributable to a severe impairment in copulatory behaviour. In conclusion, these results indicate that oestrogen appears to play a crucial role in many areas of male reproduction.

Chapter Six

The Role of Oestrogens in Testicular Cell Cycle and Apoptotic Gene Expression

6.1 INTRODUCTION

The importance of oestrogen in spermatogenesis was highlighted in Chapter 3, where utilisation of the ArKO males demonstrated a possible direct action of oestrogens on male germ cell development. Removing endogenous oestrogens resulted in severely dysfunctional spermatogenesis, including a post-meiotic developmental block that resulted in a significant decline in the number of mature germ cells (**Section 3.2**) and thus the infertility of the mice (**Section 4.22**). As oestrogen is synthesised by almost all of the testicular cell types, including pachytene spermatocytes to elongated spermatids, Sertoli cells and Leydig cells, and the ERs are also present in these cells (**Section 1.4**), this importance of oestrogen in spermatogenesis was not surprising.

Spermatogenesis consists of a highly coordinated series of spermatogonial proliferations to vastly increase cell number, followed by two distinctive spermatocyte meiosis stages to produce the truly haploid germ cell (**Section 1.32**). Progression through mitosis and meiosis is driven by extracellular mitogenic factors, such as hormones, growth factors and differentiation factors (**Section 1.531**). Not only are cells continuously proliferating, but A2, A3 and A4 spermatogonia, and also some spermatocytes and round spermatids, are spontaneously undergoing apoptosis (**Section 1.532**). In fact, this is a clear indication of normal, healthy spermatogenesis. However, many studies have shown that if the testis suffers toxic insult or hormonal withdrawal, the incidence of apoptosis greatly increases.

Through preliminarily TUNEL assays (**Section 3.25**), withdrawing oestrogen appeared to increase the incidence of apoptosis within the germ cell population in the ArKO testis. As oestrogen and the ERs are localised to the seminiferous epithelium, and this hormone is recognised to have a protective effect in many tissues in the body, it was hypothesised that the absence of oestrogen would instigate a halt in cell cycle progression and/or upregulate apoptotic initiator genes. The effect of removing oestrogen on the expression of cell cycle regulated and apoptotic genes in the testis was therefore investigated.

6.2 RESULTS

All animals used in the following studies were raised on the soy free diet.

6.21 Differential display

To investigate the disruption to spermatogenesis, total RNA was isolated from one 18-week WT and ArKO testis (**Section 2.28**), and restriction fragment differential display PCR (ddPCR) performed to analyse the expression of all differentially genes in the testis (**Section 2.29**). This would allow a direct comparison of gene expression between ArKO and WT testis.

6.211 Sequencing

From the polyacrylamide gel, 24 differentially expressed genes which appeared to be either clearly present or absent were extracted from the gel (**Figure 6.211**) and reamplified (**Figure 6.212**) as described (**Section 2.29**). Both the extracted DNA and the original PCR product were sent for sequencing analysis, however the concentration appeared to be too low in the extracted DNA and the PCR product contained too many non-specific bands to enable this to be successfully completed.

6.212 Northern blots

To determine if the above genes were in fact differentially displayed between WT and ArKO, Northern blot analysis was performed on those which appeared to be either clearly present or absent (**Section 2.293**). Each selected gene was detected in relatively equal amounts in both genotypes by Northern blot (**Figure 6.213**), thus concluding that these were all false positives. This is a common limitation of the ddPCR technique, with increasing the number of samples in each run able to overcome this. Also, accurately extracting the band is difficult as they run very close together. Due to this, other methods

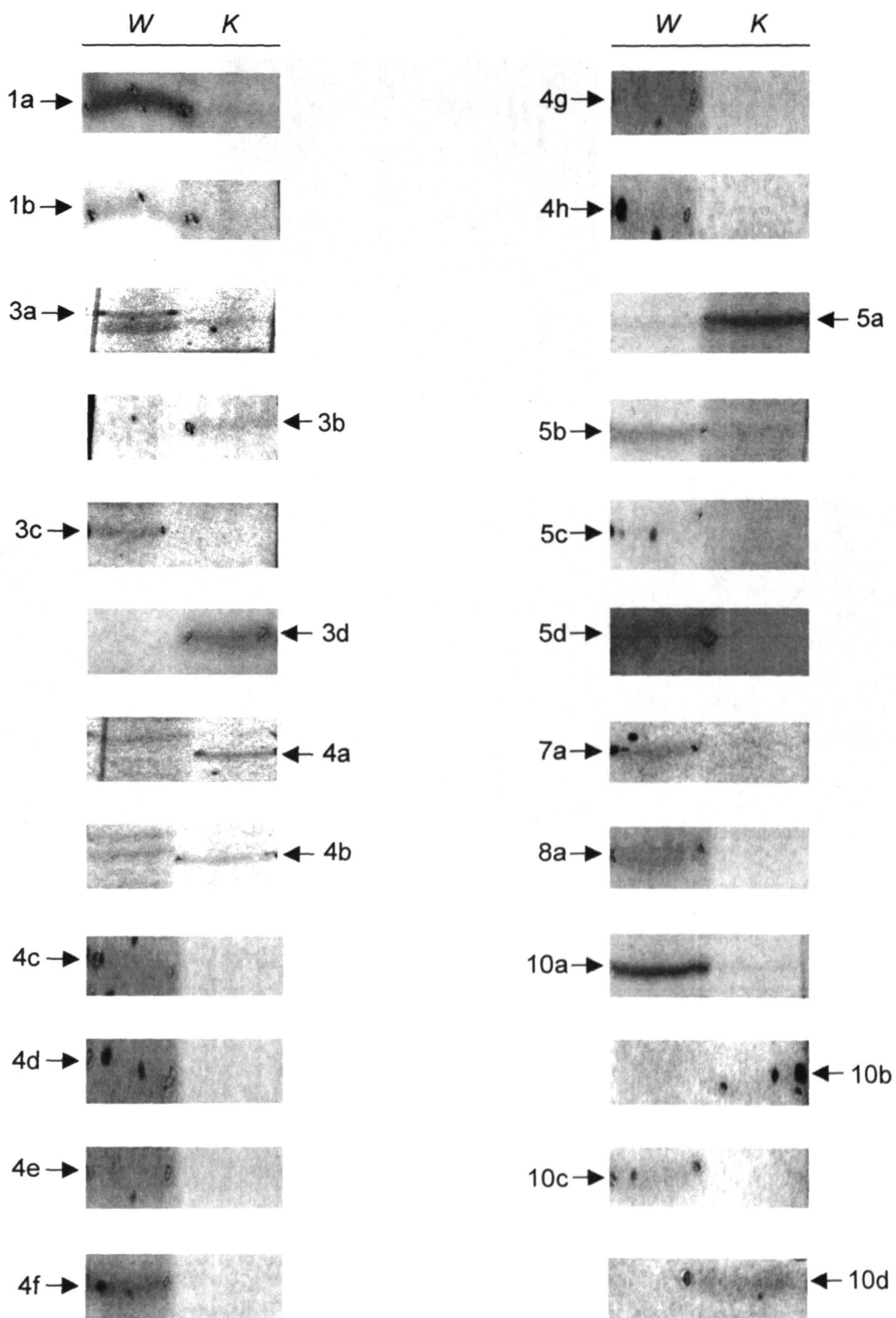
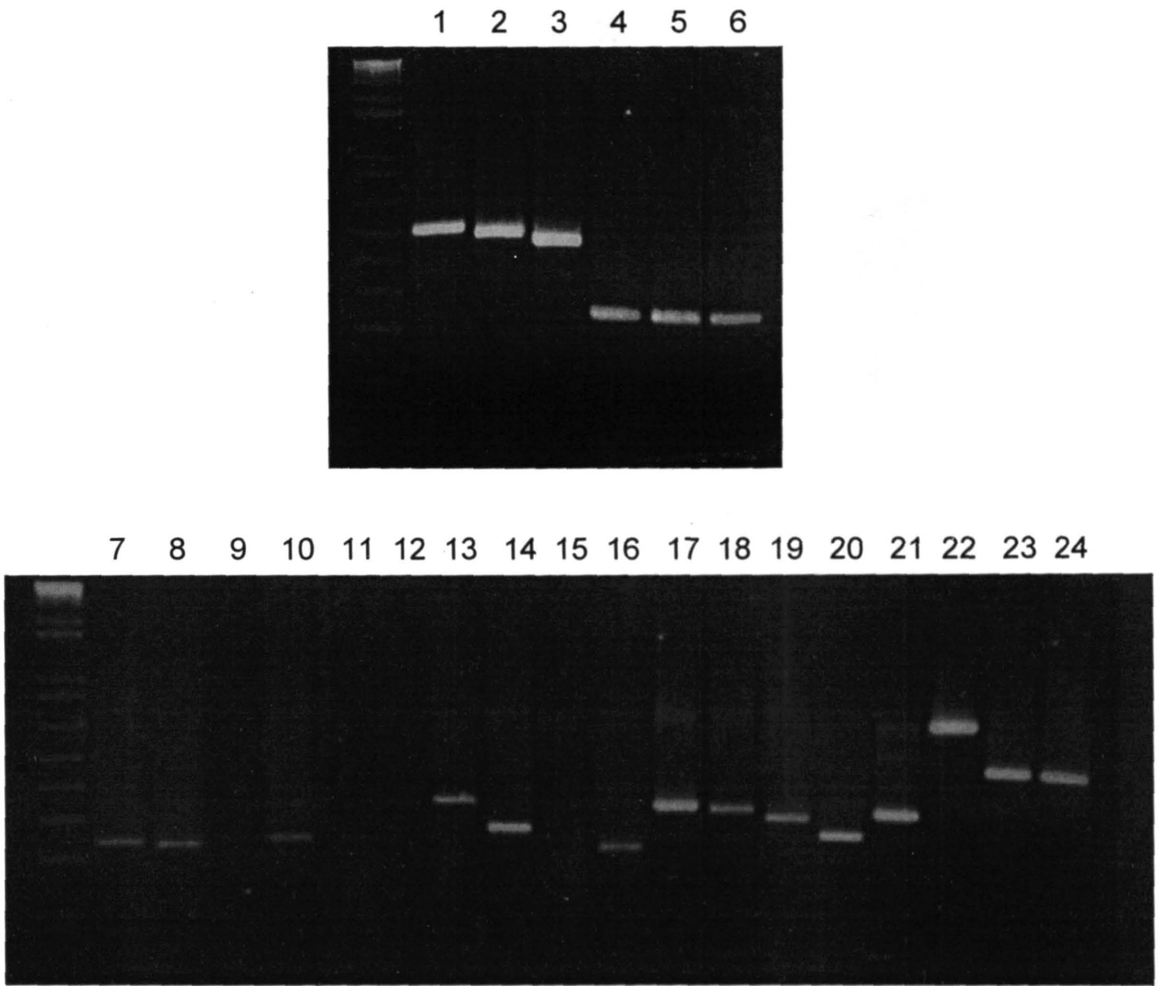


Figure 6.211 Differential display PCR results

Differentially expressed genes in testes from 18 week WT (W) and ArKO (K) were separated by polyacrylamide gel electrophoresis. Each square represents the differentially expressed gene, with the appropiate primer pair.



	Primer pair	*Genotype*		*Primer pair*	*Genotype*		*Primer pair*	*Genotype*
1	3a	WT	**9**	4h	WT	**17**	5a	KO
2	4a	KO	**10**	3b	KO	**18**	5b	WT
3	4b	KO	**11**	1a	WT	**19**	5c	WT
4	4c	WT	**12**	1b	WT	**20**	7a	WT
5	4d	WT	**13**	3c	WT	**21**	8a	WT
6	4e	WT	**14**	3d	KO	**22**	10a	WT
7	4f	WT	**15**	5d	WT	**23**	10b	KO
8	4g	KO	**16**	10d	KO	**24**	10c	WT

Figure 6.212 The re-amplified genes of interest

From the polyacrylamide gel 24 differentially expressed genes were extracted, reamplified and sent for sequence analysis.

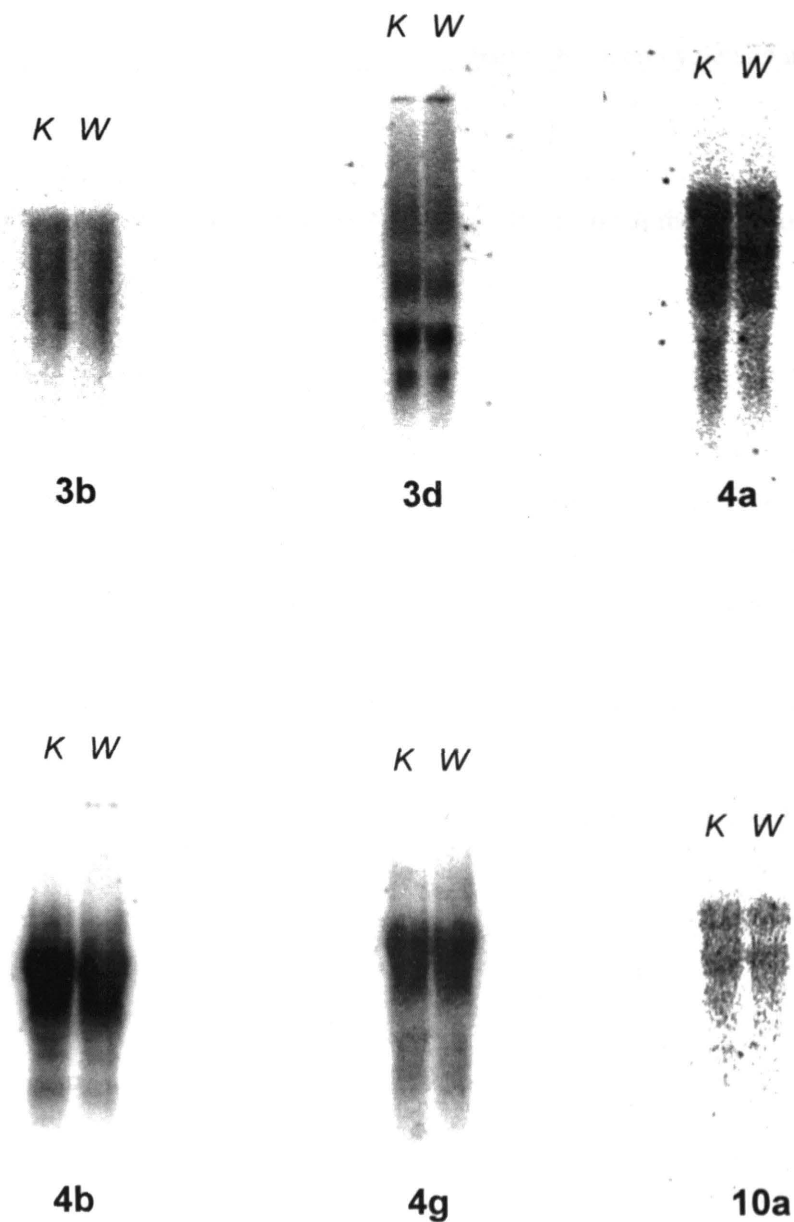


Figure 6.213 Northern Blot analysis

Northern blot analysis was performed using the PCR products from the ddPCR to probe the blots. Six genes either clearly absent or present were chosen. Both WT (W) and ArKO (K) testes had almost equal expression of each gene, therefore it was concluded that the differential expression of each gene were false postives.

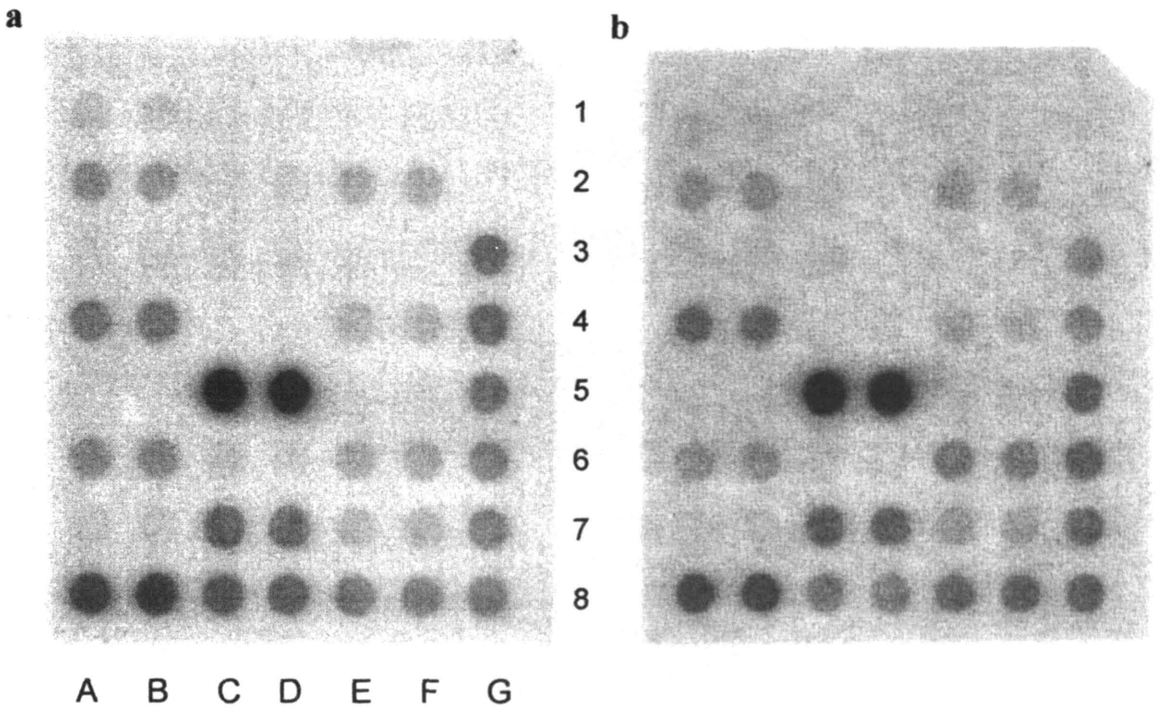
of examining the differences between the ArKO and WT testis at the molecular level were applied.

6.22 Cell cycle genes

One possible cause for the massive decrease in round and elongated spermatid numbers in the ArKO mice is that the germ cells are failing to progress through the cell cycle. If this is the case, then it is hypothesised that there would be a decrease in the expression of the cyclins and cyclin dependant kinases (cdks), and possibly an upregulation in the cdk inhibitors. To investigate this, a gene array, comprised of 24 cell cycle related genes and 2 house keeping genes, β-actin and GADPH, was used (**Section 2.30**). RNA was extracted from 1 WT and 1 ArKO testes at 18 weeks of age (**Section 2.28**). The ArKO animal used was the 1 out of the 5 who exhibited the spermatogenic phenotype (**Section 3.231**).

Each ^{32}P labelled cDNA probes was hybridised overnight to the membranes (**Figure 6.221**), with each signal analysed by phosphorimaging. The expression of each gene was normalized to β-actin, as GAPDH was suggested to be regulated by oestrogen, and then plotted as a ratio to WT expression (**Figure 6.222**).

A decrease was observed in the expression of cyclins in the ArKO testes, particularly cyclin D1 (2 fold) and cyclin A (1.6 fold). Cyclin D2 and E2 were both undetectable. However, there was no decrease in the expression of the cyclin dependent kinases. Instead cdk6, 2L and 1 all increased about 3-7 fold, with cdk4 undetectable. In contrast, the majority of the cyclin dependent kinase inhibitors did increase, particularly the $p21^{Waf1}$ (4.5 fold) and $p15^{ink4b}$ (3.2 fold), suggesting a possibly inhibition of cell cycle progression through this means. Lastly, a 6 fold decrease was observed in the expression of the E2F1 family of transcription factors, which are essential for S phase entry. p27 remained unchanged. The following genes were not expressed in the testis, cyclin D2, cyclin E2 and cdk4.



Gene Name	*GEA Location*	*Gene Name*	*GEA Location*
cdk2L	**1A, 1B**	p19	6C, 6D
cdk1	**2A, 2B**	**p57 kip2**	**7C, 7D**
cyclin C	3A, 3B	skp2	8C, 8D
cyclin D3	**4A, 4B**	plasmid	1G, 2G
E2F1	5A, 5B	beta actin	3G, 4G
p18	6A, 6B	GAPDH	5G, 6G, 7G, 8G, 8E, 8F
p27 kip1	7A, 7B	**cdk6**	**1E, 1F**
Skp1	8A, 8B	**cyclin B**	**2E, 2F**
cdk4	1C, 1D	cyclin D2	3E, 3F
cyclin A	2C, 2D	cyclin E2	4E, 4F
cyclin D1	3C, 3D	p16 ink4a	5E, 5F
cyclin E1	4C, 4D	**p21 waf1**	**6E, 6F**
p15 ink4b	**5C, 5D**	pRb	7E, 7F

Figure 6.221 Mouse Cell cycle-1 GEarray Kit.

GEarray membranes were hybridised overnight to RNA from 18 week old (**a**) WT and (**b**) ArKO testes. Each signal was analysed by phosphorimaging and normalised to beta actin. Highlighted in bold are those genes with altered expression between WT and ArKO.

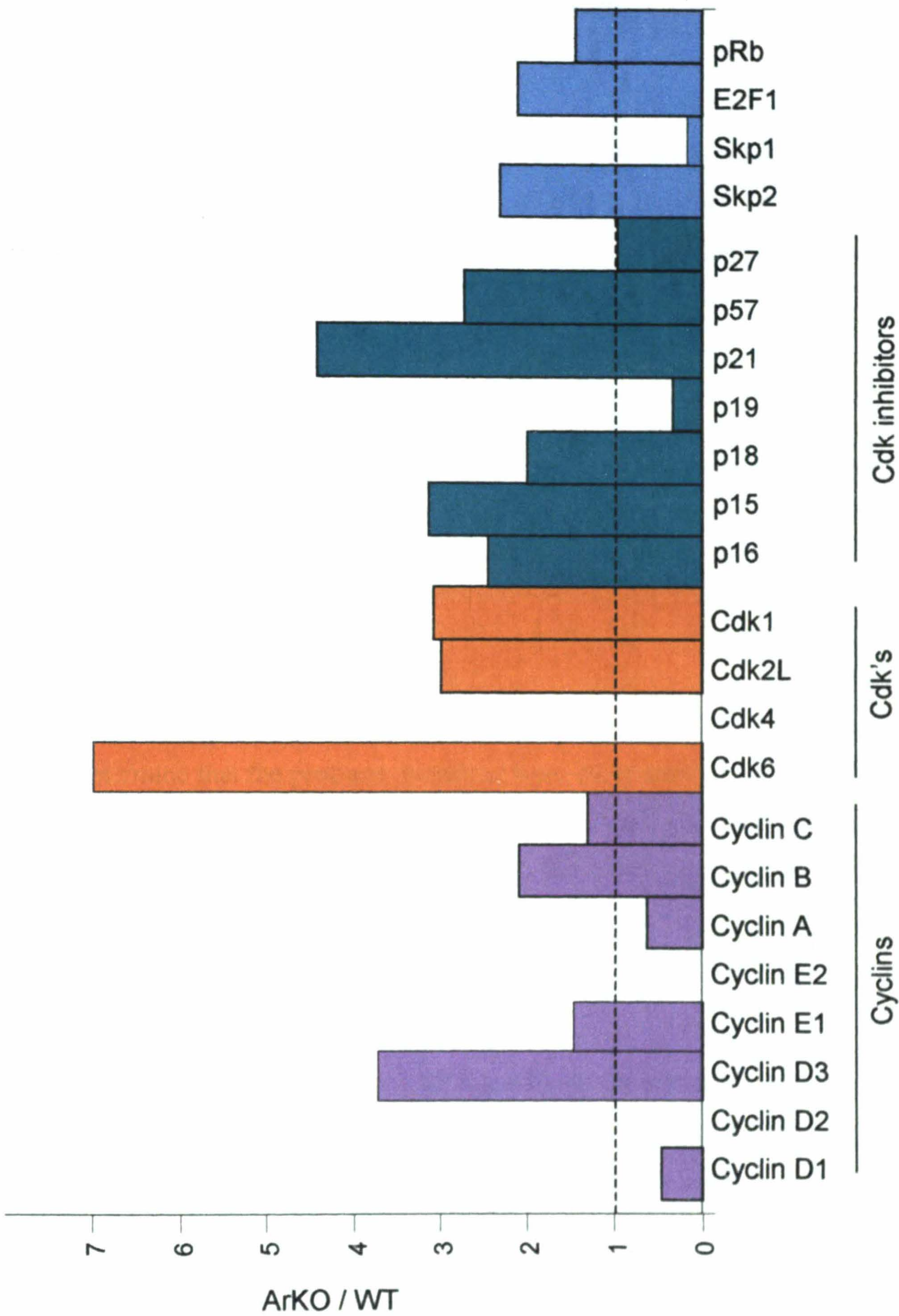


Figure 6.222 GEarray expression levels

A gene array, comprised of 24 cell cycle related genes and 2 house keeping genes, β-actin and GADPH, was used to examine the expression of cell cycle related genes. Each value was normalised to beta actin and then each ArKO value was expressed as a ratio to the wildtype. The values higher than 1 indicate that the knockout testis is expressing a higher level of that gene.

These results suggest that removing oestrogen from the testis altered the expression of crucial cell cycle specific genes, possibly leading to cell cycle arrest in the developing germ cells. In order to confirm these findings, the genes in question were reexamined using Real Time PCR.

6.23 Real Time PCR

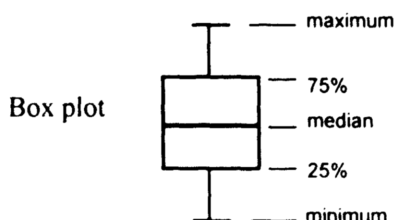
Specific PCR primers were designed for a selection of cell cycle related genes (**Section 2.311**). RNA was extracted from 6 WT and 6 ArKO at 18 weeks, and 6 WT and 6 ARKO at 1 year (**Section 2.28**). The expression of these genes in the testes of ArKO mice was compared to WT using Real Time PCR.

6.231 Southern blot analysis

To first determine that the products amplified were those anticipated, the amplicons of the 5 standard dilutions (**Section 2.316**) were run on a 1% gel and analysed by Southern blot analysis (**Section 2.317**). The results indicated that every product was the expected one (**Figure 6.231**).

6.232 Results

Each Real Time PCR reaction, either with or without the standards, was run on a standard 1% agarose gel (**Appendices 1iii - 8iii**) to determine PCR product size and confirm the amplification of one product (**Appendices 1i - 8i**). Each result was then normalised to 18S and expressed in a box plot format. This format was chosen as expressing the data as mean ±SEM failed to illustrate the distribution of the data, whereas a box plot (see figure below) clearly shows the median values, the spread (25-75%) and the overall range of the data, allowing a comparison between the expression of each gene at different ages and genotypes to be made.



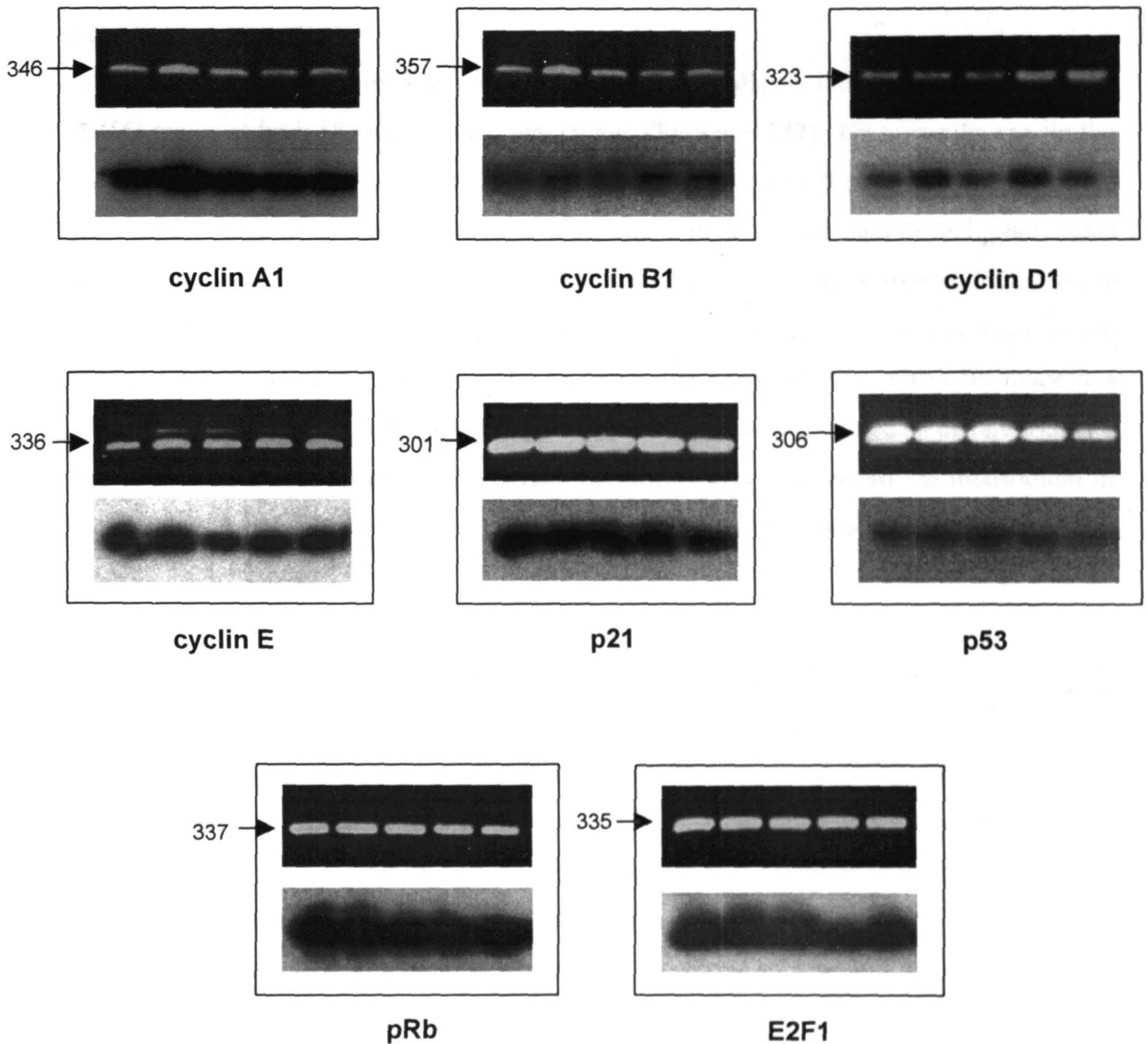


Figure 6.231 Light cycler standards

Light cycler standards (**Section 2.316**) were run on a 1% agarose gel, then transferred to nylon membrane overnight and probed with a specific ^{32}P labeled oligonucleotide (**Table 2.312**) to confirm that the product amplified was the correct product. As is apparent, the PCR primers designed were very specific. Also illustrated are the product sizes (bp).

i Cyclins

At 18 weeks of age, the median value of cyclin A1 and distribution of data was lower in ArKO males at both 18 weeks and 1 year of age (**Figure 6.232**). These results are similar to that observed with the gene array (**Section 6.22**). At 18 weeks there was no difference in the median value of cyclin B1, however there was a trend for the data to be higher, again similar to the GEarray results, whereas at 1 year the levels were markedly lower in the ArKO testes. Examining cyclin D1, the median was unchanged at 18 weeks however the range of data was lower (**Figure 6.232**). At 1 year, the ArKO median value was lower than the WT, however the data range was higher. Cyclin E, however, showed a difference at 18 weeks with a lower median in the ArKO males, however once again the distribution of data tended to be higher, whereas at 1 year although the median was higher, the data range showed no difference.

All the results at 18 weeks showed the same trends at the GEarray results (also at 18 weeks), although the testes used for these studies did not yet show the spermatogenic disruptions. The gene expression levels of the cyclins appeared to vary quite markedly between animals. The cause for this is unknown but may be related to the genetic strain of the ArKO mice who are still on the C57/BL6 background. These mice are currently being crossed onto the FVB/N background, a purer strain which may provide more consistant results. More animals per group may also reduce the variation.

ii Cdk inhibitor

The median values of the cdk inhibitor $p21^{Waf1}$ were increased at both 18 weeks and 1 year of age ArKO testes compared to WT (**Figure 6.233**). These results are similar to those observed with the gene array where the expression of $p21^{Waf1}$ was found to have increased in the ArKO compared to WT (**Section 6.22**). As p53 transcriptionally activates $p21^{Waf1}$, this gene was also examined. However, the median values of p53 at 18 weeks and 1 year were both lower in the ArKO testes (**Figure 6.233**).

cyclin A1

Relative densitometric units / 18s

.0008 .0006 .0004 .0002 .0000 -.0002

1 year

18 weeks | 1 year
WT (n=6) | ArKO (n=6) | WT (n=6) | ArKO (n=5)

cyclin B1

Relative densitometric units / 18s

.0005 .0004 .0003 .0002 .0001 0.0000 -.0001

1 year

18 weeks | 1 year
WT (n=5) | ArKO (n=6) | WT (n=6) | ArKO (n=4)

cyclin D1

Relative densitometric units / 18s

.0000016 .0000012 .0000008 .0000004 .0000000

18 weeks | 1 year
WT (n=4) | ArKO (n=3) | WT (n=4) | ArKO (n=3)

cyclin E1

Relative densitometric units / 18s

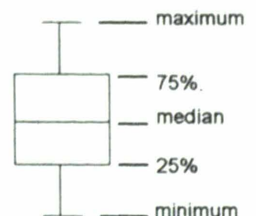
.0000012 .0000008 .0000004 .0000000

1 year

18 weeks | 1 year
WT (n=5) | ArKO (n=4) | WT (n=5) | ArKO (n=4)

Figure 6.232 Cell cycle gene expression

Expression of the cyclin A1, B1, D1 and E1 in the WT and ArKO testes analysed by Real Time PCR at 18 weeks and 1 year of age. Data expressed as a box plot (see explanation), showing distribution of data from SPSS statistical computer program.



p21

Relative densitometric units / 18s

.000003 .000002 .000001 .000000

18 weeks: WT (n=4), ArKO (n=4); 1 year: WT (n=6), ArKO (n=3)

p53

Relative densitometric units / 18s

.0000005 .0000004 .0000003 .0000002 .0000001 .0000000

18 weeks: WT (n=6), ArKO (n=4); 1 year: WT (n=4), ArKO (n=5)

pRb

Relative densitometric units / 18s

.000004 .000003 .000002 .000001 .000000 -.000001

1 year

18 weeks: WT (n=4), ArKO (n=5); 1 year: WT (n=5), ArKO (n=4)

E2F1

Relative densitometric units / 18s

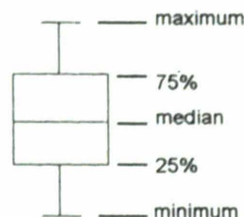
.0000014 .0000010 .0000006 .0000002 -.0000002

1 year

18 weeks: WT (n=5), ArKO (n=4); 1 year: WT (n=4), ArKO (n=4)

Figure 6.233 Cell cycle gene expression

Expression of p21, p53, pRb and E2F in the WT and ArKO testes analysed by Real Time PCR at 18 weeks and 1 year of age. Data expressed as a box plot (see explanation), showing distribution of data from SPSS statistical computer program.



iii Cell cycle related genes

In order for the cell to progress through G1, it relies almost exclusively on the phosphorylation of the retinoblastoma protein (pRb). The median level of pRb expression is decreased in the ArKO testes at 18 weeks of age, however the distribution of data strongly overlaps. At 1 year the median is slightly higher in the WT however the data distribution is very similar (**Figure 6.233**). Once this protein is inactivated it dissociates from the E2F transcription factors, which then transcribe essential genes for G1 progression. At 18 weeks of age, the median level of E2F expression is higher in the ArKO compared to WT. By one year there is a decrease in the median value, and it should be noted that the expression of E2F was undetectable in 2 ArKO animals at this age.

6.24 *Insitu* hybridisation

The localisation of p21^{Waf1} in 1-year-old WT and ArKO testes was investigated by *insitu* hybridisation as described (**Section 2.32**). To optimise the hybridisation temperature, the sections were incubated at 42°C, 50°C and 55°C for 18 hours, with 50°C producing the strongest and clearest signal with the least background. At this temperature, p21^{Waf1} appeared to show specific binding to the seminiferous tubules with a stronger signal visible in the ArKO than WT (**Figure 6.241c,d**). This signal is also stronger than the sense probe (**Figure 6.241f**).

6.25 Apoptotic genes

Another possible reason for the decrease in germ cell number when oestrogens are removed may be an increase in the incidence of apoptosis, either as a consequence of cell cycle arrest or independent of this. Therefore, RNase protection assays were undertaken to examine the expression of specific apoptotic genes, followed by phosphorimager analysis (**Section 2.33**).

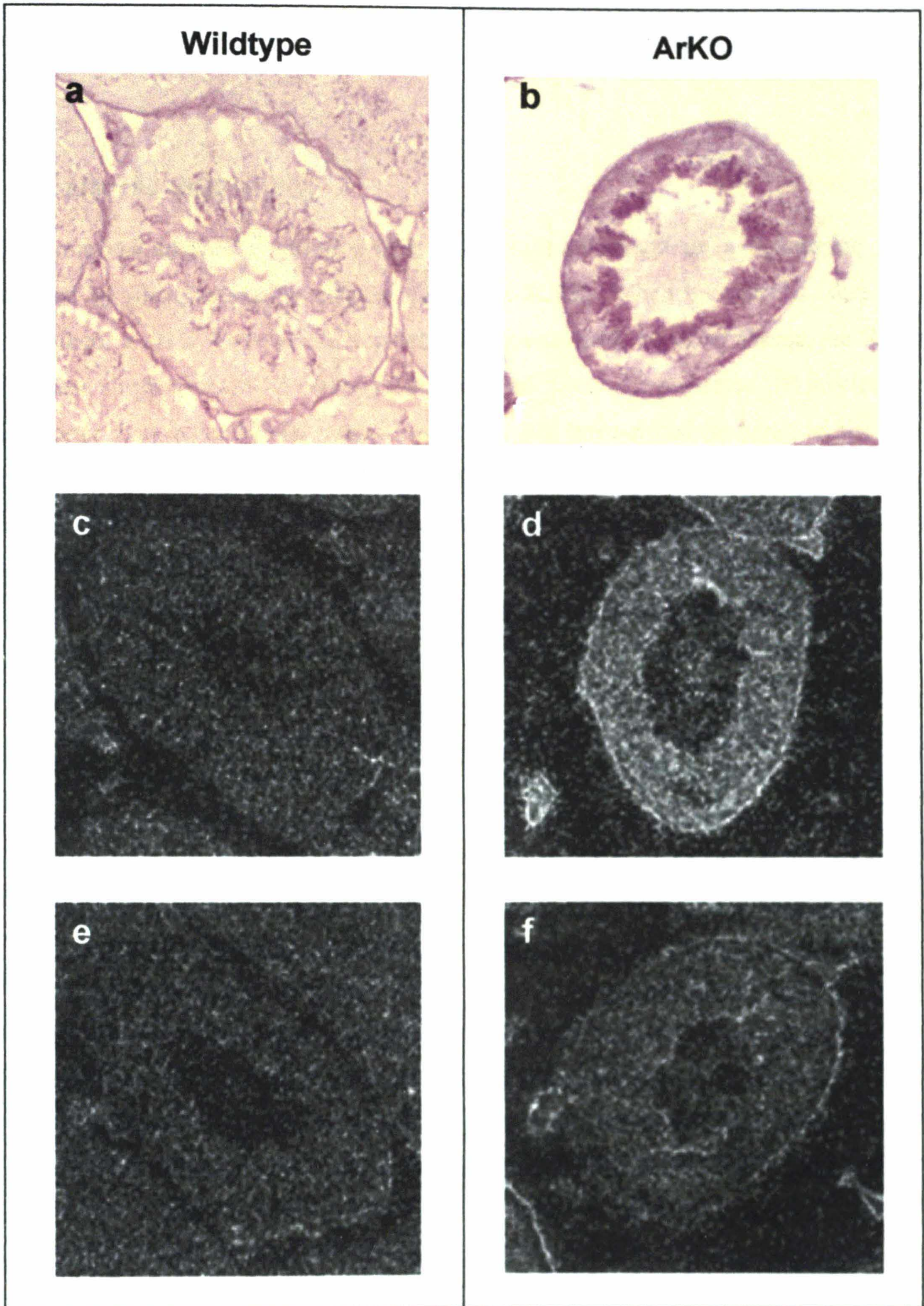


Figure 6.241 Localisation of p21^{Waf1} mRNA in the testes of WT and ARKO mice

(**a** and **b**) Testes sections stained in haemotoxylin. (**c** and **d**) *Insitu* hybridisation analysis of 1 year old WT and ArKO testes sections incubated with a ^{35}S labelled 35bp p21^{Waf1} oligonucleotide, (**e** and **f**) and a sense control.

6.251 Mitochondrial pathway

The protected apoptosis related probes were electrophoresed on a denaturing gel and analysed by phosphorimager (**Figure 6.251** and **6.252**). When the cDNAs were *in vitro* transcribed, a section of the plasmid was also transcribed, however during the RNase A step these single stranded overhangs were digested. Thus, the protected ^{32}P labeled probes are shorter than the originally transcribed probe. The intensity of the band, ie. the level of protected probe, is directly correiated to the expression level of the gene. Each gene expression level was normalised to the ribosomal protein L32 expression level. As can be seen from the gel, the highest expressed genes in the testis are bcl-xL, followed by bcl-w and bad. Genes such as bfl-1 were not found to be expressed in the testis.

Anti-apoptotic genes

No significant difference was observed in the expression of bcl-w, bcl-xL and bcl-2 between WT and ArKO at either 18 weeks or 1 year of age (**Figure 6.253**). Both bcl-w and bcl-xL did have significantly reduced expression at 1 year of age, but there was no change between genotypes.

Pro-apoptotic genes

When the death genes of the bcl-2 family were examined, there was an increase in the expression of the pro-apoptotic bax in the ArKO testes when compared with WT at 18 weeks ($p=0.03$) (**Figure 6.254**). The expression then drastically decreased in both genotypes by 1 year of age, with no difference detected. There was no significant change in the expression of the other pro-apoptotic genes, bak and bad (**Figure 6.254**) at either age group. As the expression level of bax is higher than the other 2 pro-apoptotic genes, it suggests that bax is the main apoptosis player in the testis.

Ratio of survival/death

As both the pro and anti-apoptotic members of the bcl-2 family are not only able to homodimerize, but also heterodimerise with each other, they are able to neutralize the

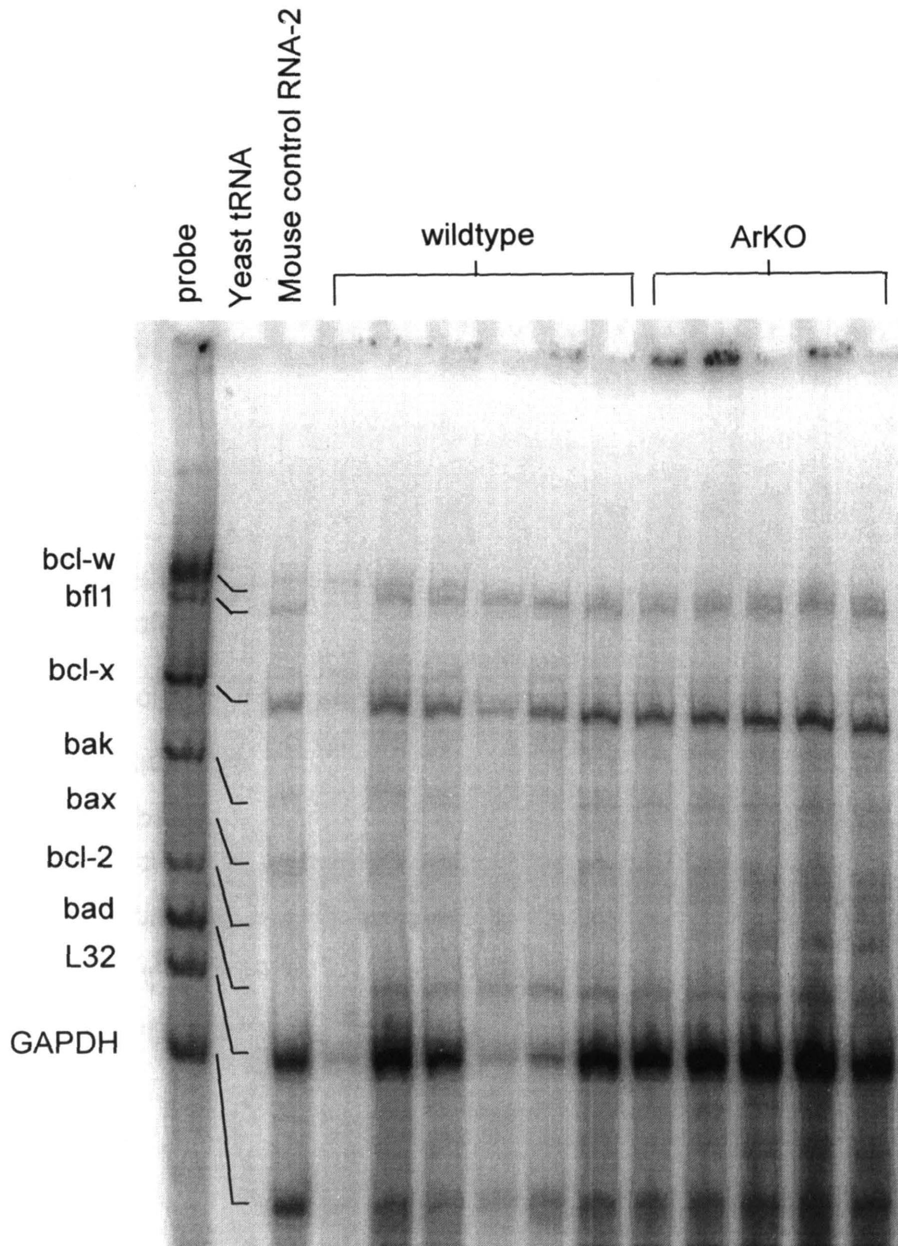


Figure 6.251 RNase protection assay (mAPO-2 template): 18 weeks

The protected apoptosis related probes were electrophoresed on a denaturing gel and analysed by phosphorimager. The intensity of the band, ie. the level of protected probe, is directly correlated to the expression level of the gene.

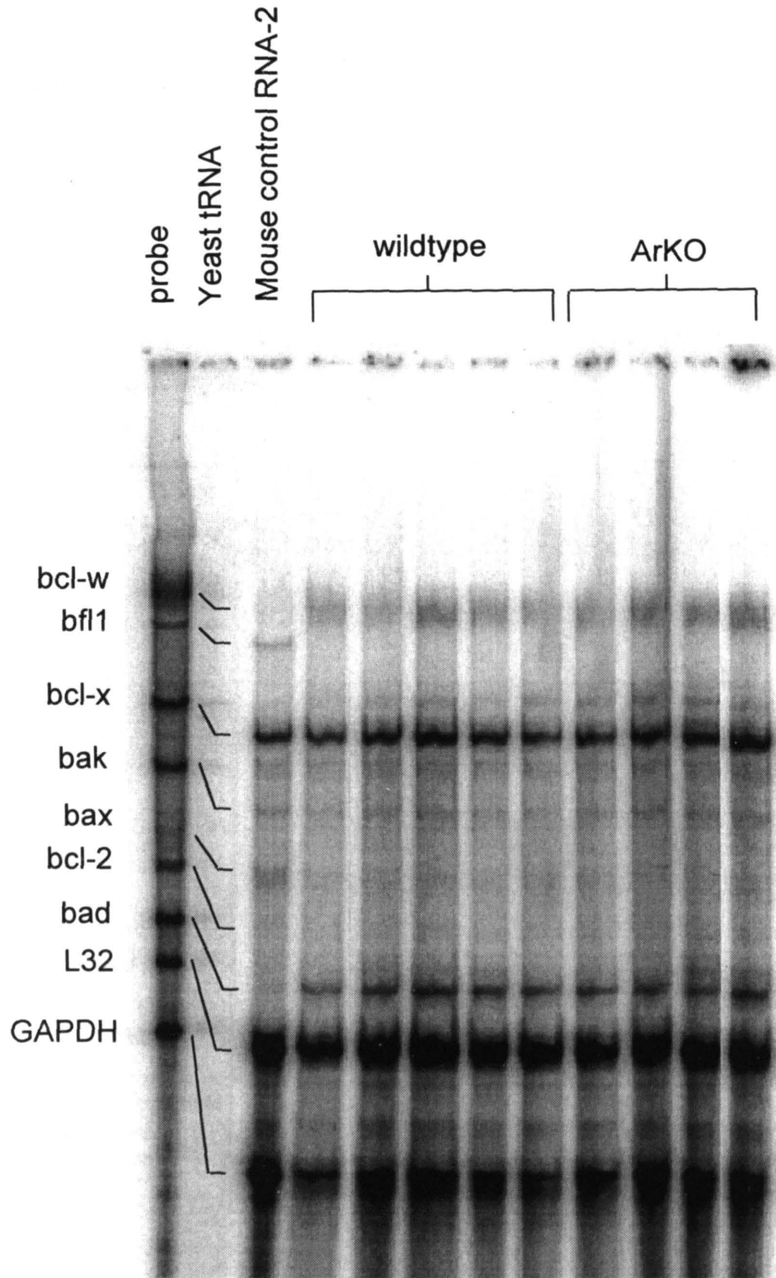
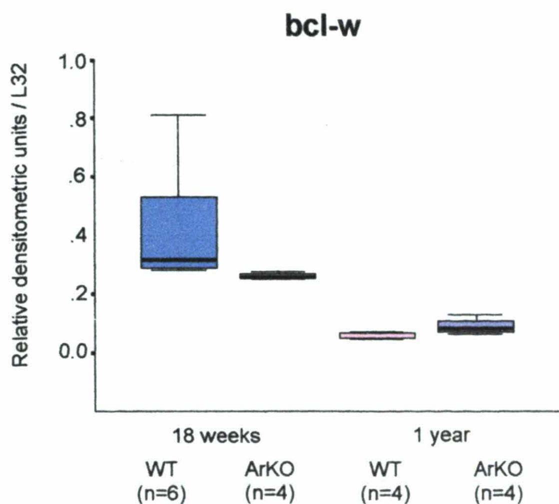
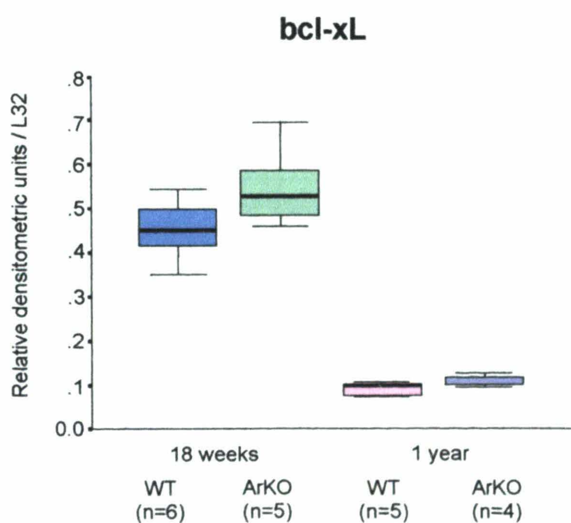


Figure 6.252 RNase protection assay (mAPO-2 template): 1 year

The protected apoptosis related probes were electrophoresed on a denaturing gel and analysed by phosphorimager. The intensity of the band, ie. the level of protected probe, is directly correlated to the expression level of the gene.





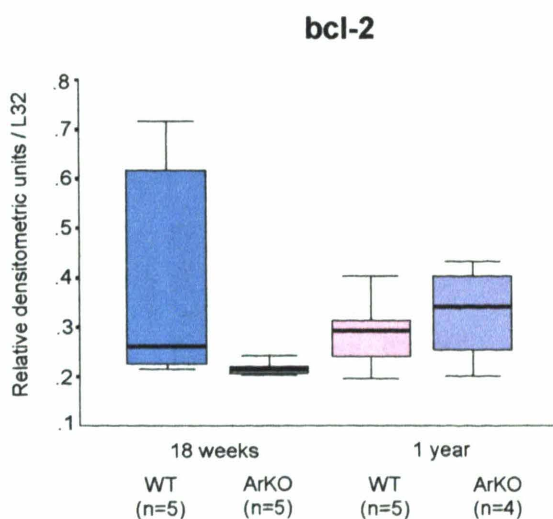
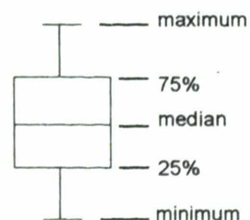
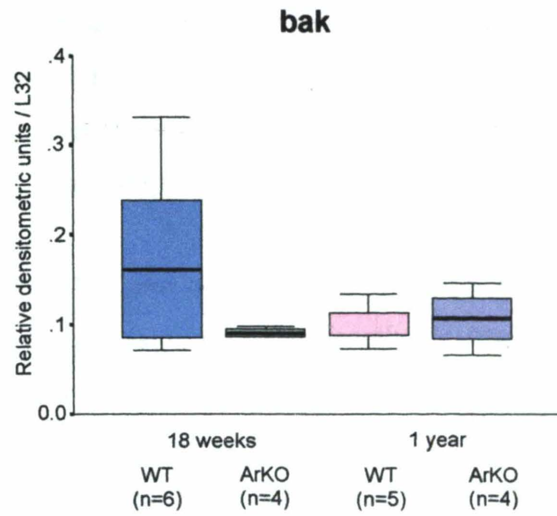
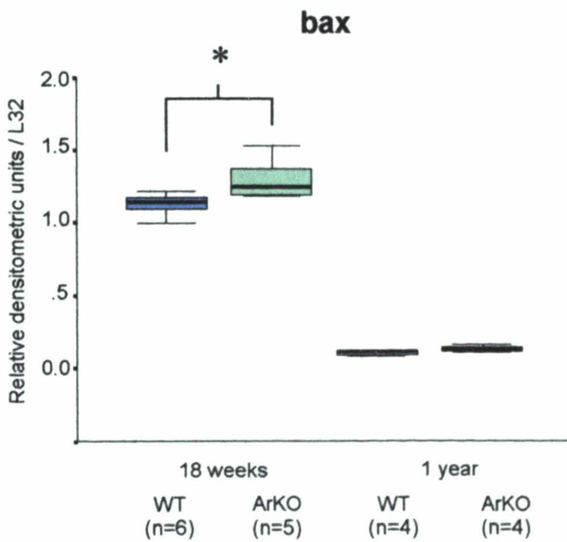


Figure 6.253 Anti-apoptotic gene expression from RNase protection assay

Expression of bcl-w, bcl-xL and bcl-2 at 18 weeks and 1 year of age in the WT and ArKO testes. Data expressed as a box plot (see explanation), showing distribution of data from SPSS statistical computer program.







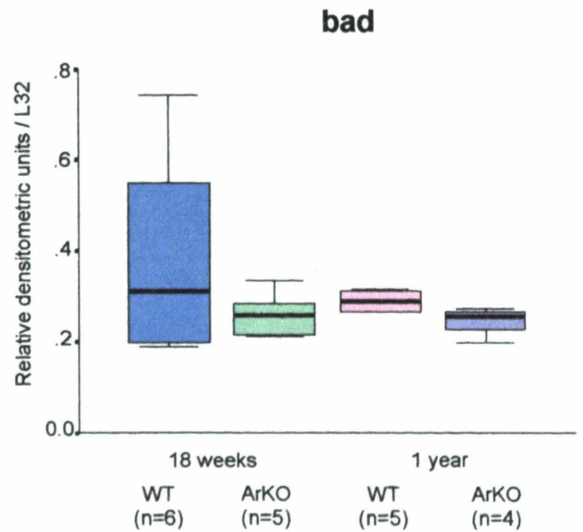
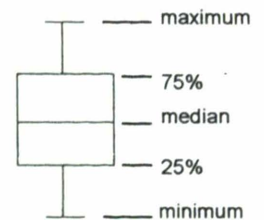


Figure 6.254 Pro-apoptotic gene expression from RNase protection assay

Expression of bak, bax and bad at 18 weeks and 1 year of age in the WT and ArKO testes. Data expressed as a box plot (see explanation), showing distribution of data from SPSS statistical computer program, *$p<0.05$.



function of each other. In this respect, it is the ratio that will determine whether or not that cell will undergo apoptosis. Therefore, the bcl-2 members that have been previously demonstrated to heterodimerize (**Section 1.522**) were expressed as a ratio between anti and pro apoptotic expression (**Figure 6.255**).

The ratio of bax to bcl-w expression showed that at 18 weeks of age the ratio of bax expression was significantly elevated in the ArKO testis (p=0.01), with no difference at 1 year of age (**Figure 6.255**). Furthermore, the ratio of bax to bcl-xL again showed a significant elevation in the expression of bax (p=0.024) at 18 weeks of age. However no difference was detected between bax:bcl-2 and bak:bcl-w between ArKO at WT at either age (**Figure 6.255**). These are the only members of the bcl-2 family demonstrated to heterodimerise.

This suggested that at 18 weeks of age in the ArKO testes, when the spermatogenic phenotype is first arising, that there was an upregulation of the pro-apoptotic bax.

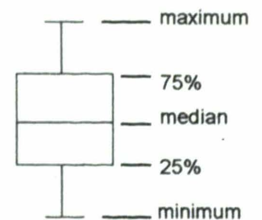
6.252 Death receptor pathway

Differentially expressed genes from the death receptor pathway were also run on a denaturing gel and analysed by phosphoimager (**Figure 6.256 and 6.257**). Each gene was again normalized to the ribosomal protein L32. In the testis, the FAF (FAS Associated Factor) was the highest expressed member of this pathway, with also expression observed of caspase-8, FASL, FADD (FAS Associated Death Domain), FAP (FAS Associated Phosphatase), RIP and TRADD.

At 18 weeks of age there was no difference in the expression of the death receptor pathway genes, except for a decrease in FAF in the ArKO testes compared to WT. By 1 year of age there was a significant upregulation in the expression of caspase-8 (p=0.021), FASL (p=0.025) and FAS (p=0.02) in the ArKO testes when compared to the WT (**Figure 6.258**). There was no difference in the expression of FADD (p=0.065), FAP (p=0.068) or

Figure 6.255 Ratio pro-apoptotic: anti-apoptotic gene expression

The ratio of bax:bcl-w, bax:bcl-xL, bax:bcl-2 and bak:bcl-w expression at 18 weeks and 1 year of age in the WT and ArKO testes. Data expressed as a box plot (see explanation), showing distribution of data from SPSS statistical computer program., *p<0.05.



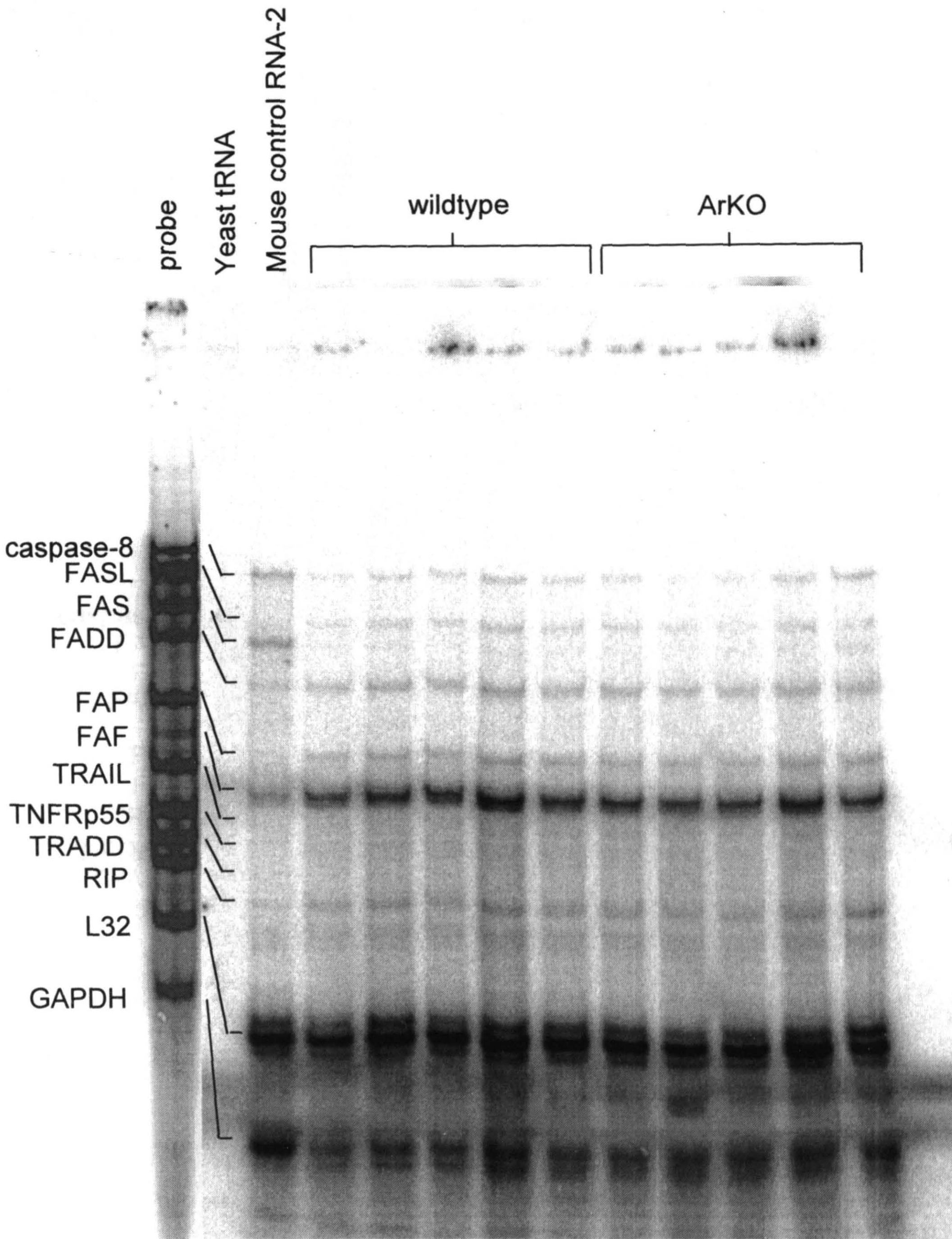


Figure 6.256 RNase protection assay (mAPO-3 template): 18 weeks

The protected apoptosis related probes were electrophoresed on a denaturing gel and analysed by phosphorimager. The intensity of the band, ie. the level of protected probe, is directly correlated to the expression level of the gene.

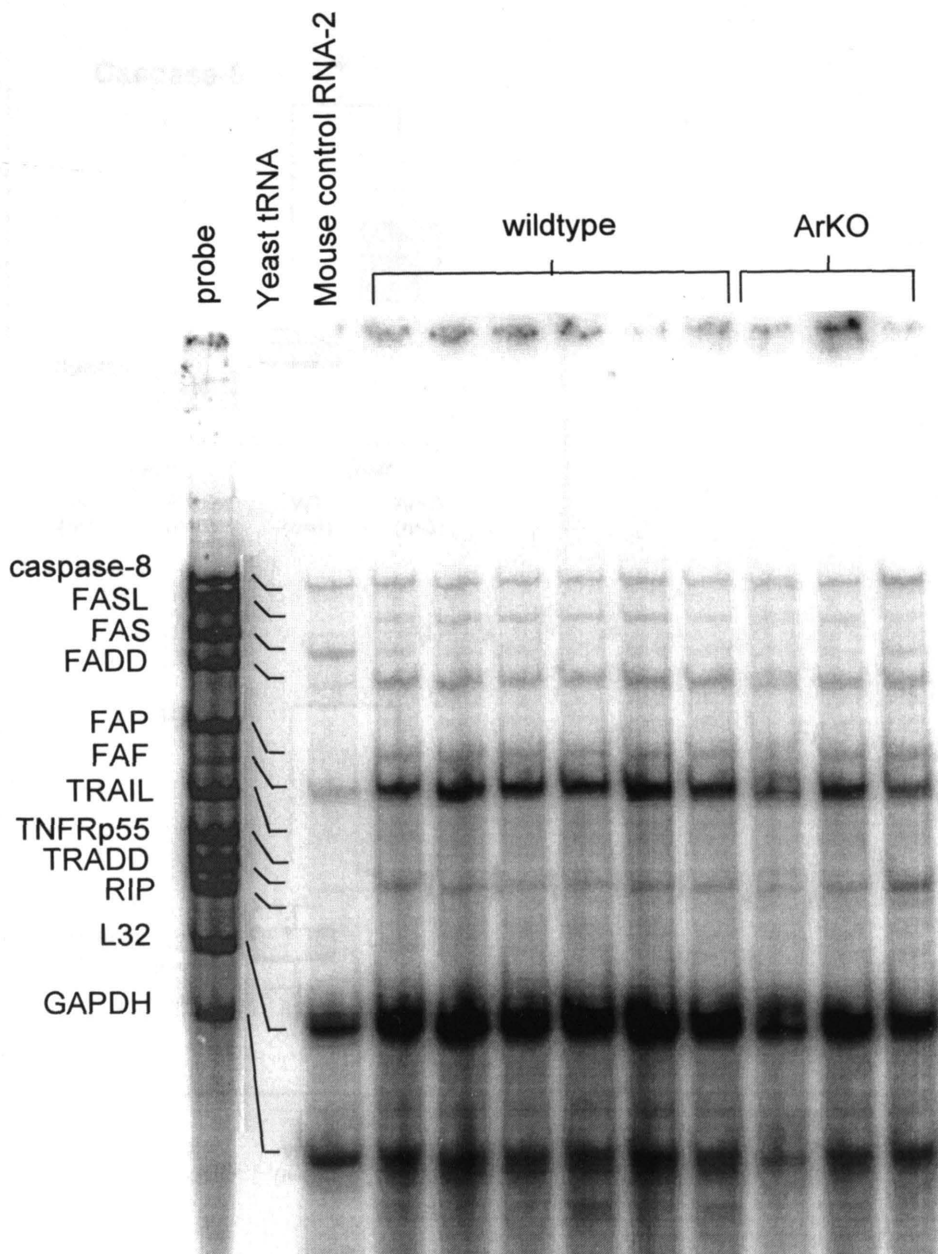
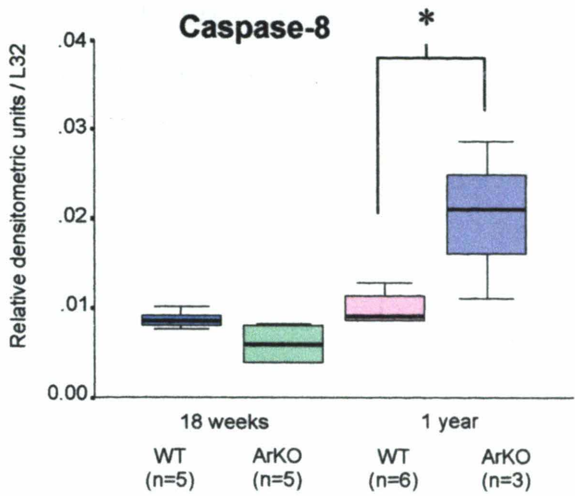
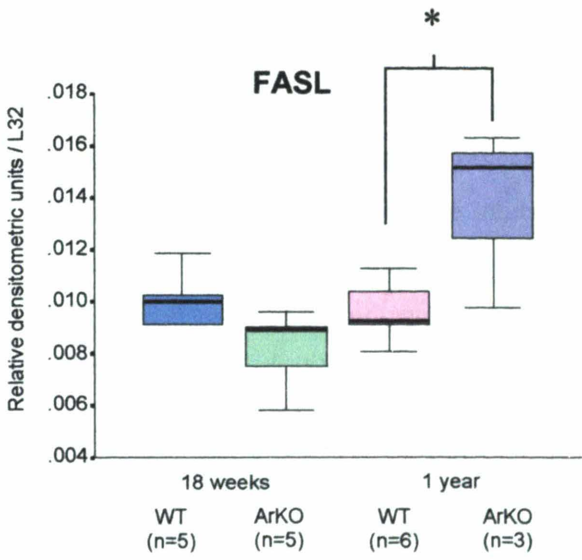


Figure 6.257 RNase protection assay (mAPO-3 template): 1 year

The protected apoptosis related probes were electrophoresed on a denaturing gel and analysed by phosphorimager. The intensity of the band, ie. the level of protected probe, is directly correlated to the expression level of the gene.





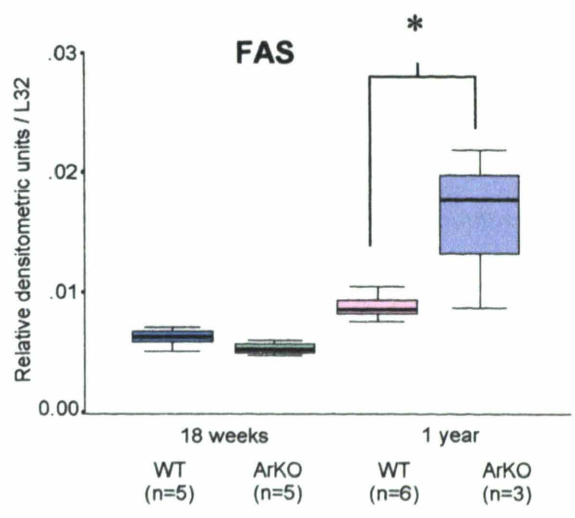
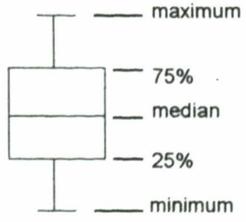


Figure 6.258 Death receptor pathway gene expression from RNase protection assay

Expression of Caspase-8, FASL and FAS at 18 weeks and 1 year of age in the WT and ArKO testes. Data expressed as a box plot (see explanation), showing distribution of data from SPSS statistical computer program, *$p<0.05$.



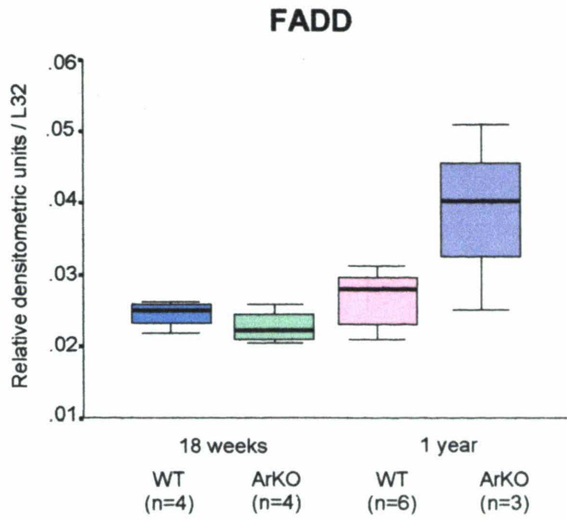
FAF (**Figure 6.259**). No difference also existed between TRADD and RIP between ArKO and WT (data not shown).

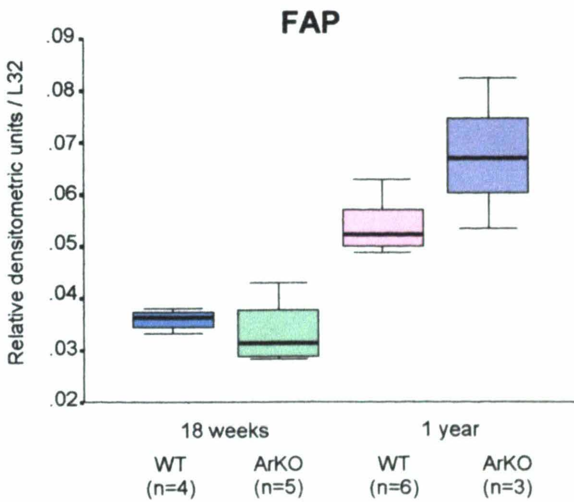
These results suggest that the germ cells at one year of age in the ArKO testis may be undergoing cell death via the FAS signalling pathway.

6.3 DISCUSSION

The findings presented here illustrate an important role for oestrogen in germ cell survival. The study detailed in Chapter 3 illustrated abnormalities surrounding the spermatocyte-round spermatid transition when endogenous oestrogens were absent, such that round spermatid numbers were severely reduced (**Section 3.23**). This study investigated the mechanism of this reduction, revealing a trend for the expression of cell cycle specific genes to decline and cell cycle inhibitors to increase (for summary see **Figure 6.3**). It is thus possible that germ cells are undergoing cell cycle arrest followed by apoptosis-mediated cell death, indicated by the upregulation in the expression of apoptotic genes. This study clearly highlights the growing importance of oestrogens in spermatogenesis.

In 18-week-old ArKO animals, germ cell maturation appears to be progressing normally, with only 1 out of the 5 mice analysed exhibiting spermatogenic disruptions. However, the lack of oestrogen may already be exerting an effect in the younger animals with trends for the expression of key cell cycle genes to be altered. At 18 weeks of age, the median expression level for cyclin A1 has decreased, as has that of cyclin E1. The spread of data for cyclin D1 also shows a decreased expression in the ArKO however no change is apparent in the median value. The expression of the retinoblastoma protein (pRb) is also decreased in the ArKO testes at 18 weeks of age.





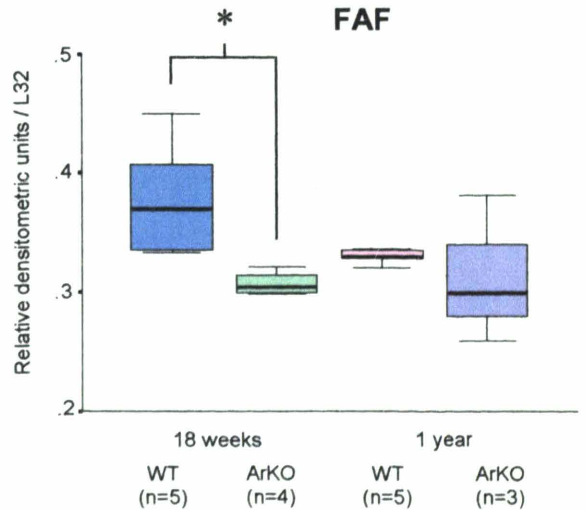


Figure 6.259 Death receptor pathway gene expression from RNase protection assay

Expression of FADD, FAP and FAF at 18 weeks and 1 year of age in the WT and ArKO testes. Data expressed as a box plot (see explanation), showing distribution of data from SPSS statistical computer program, *$p<0.05$.

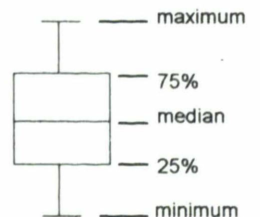
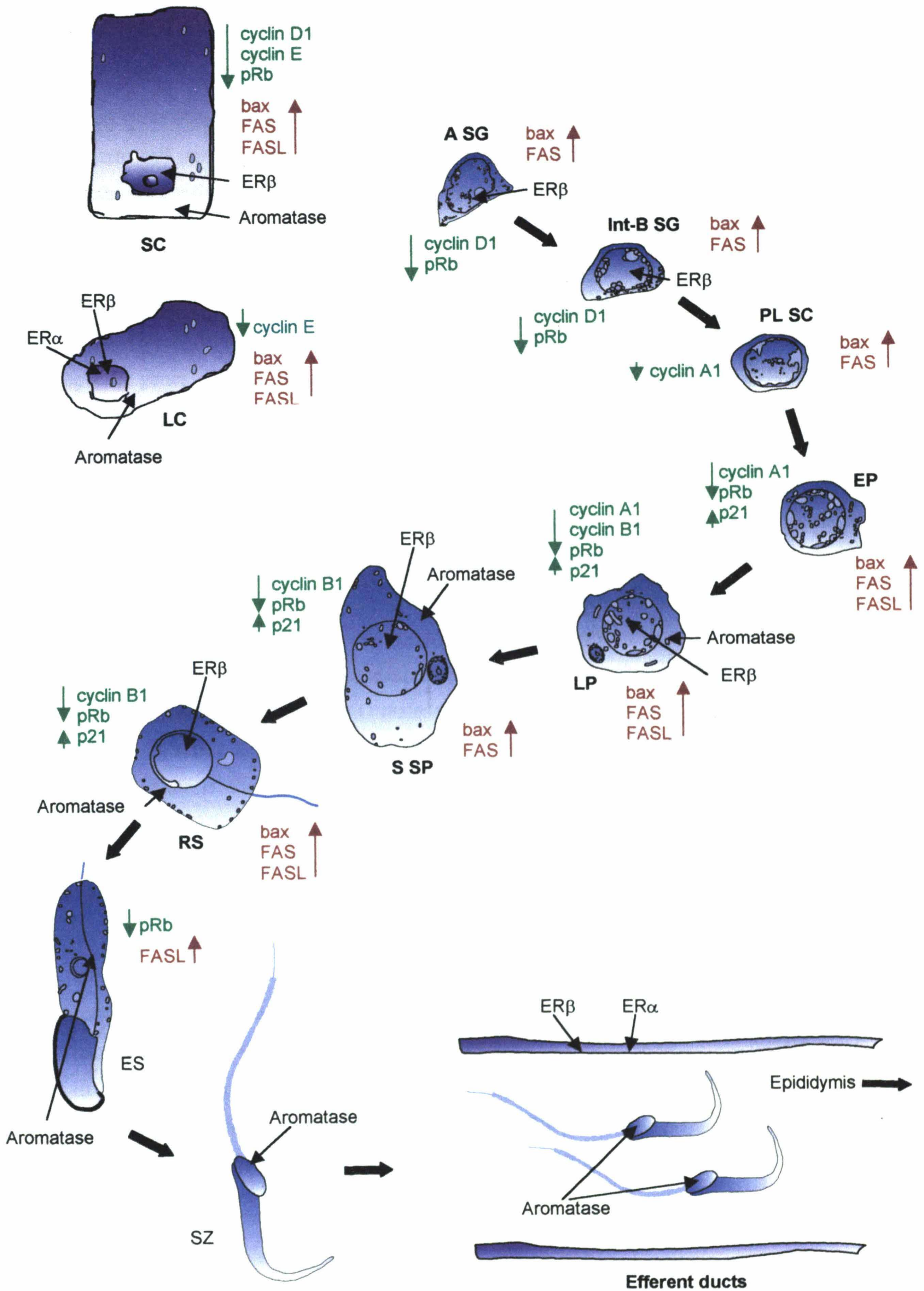


Figure 6.3 Schematic illustrating the maturation of the germ cells, showing the localisation of cell cycle genes/proteins (**Table 1.531**) and apoptotic genes/proteins (**Table 1.532**) and their up or down regulation in the ArKO testis (arrows).

SC, Sertoli cell; LC, Leydig cell; A SG, type A spermatogonia; Int-B SG, intermediate-B type spermatogonia; PL SC, pre-leptotene spermatocyte; EP, early pachytene spermatocyte; LP, late pachytene spermatocyte; S SP, secondary spermatocytes; RS, round spermatid; ES, elongated spermatid; SZ, spermatozoa.


cyclin D1
cyclin E
pRb
bax
FAS
FASL
ERβ
Aromatase
SC
A SG
bax
FAS
ERβ
cyclin D1
pRb
Int-B SG
bax
FAS
ERβ
cyclin D1
pRb
PL SC
cyclin A1
bax
FAS
ERβ
ERα
cyclin E
bax
FAS
FASL
LC
Aromatase
cyclin A1
pRb
p21
EP
bax
FAS
FASL
cyclin A1
cyclin B1
pRb
p21
Aromatase
ERβ
LP
bax
FAS
FASL
ERβ
Aromatase
cyclin B1
pRb
p21
S SP
bax
FAS
ERβ
cyclin B1
pRb
p21
Aromatase
RS
bax
FAS
FASL
pRb
FASL
ES
Aromatase
Aromatase
SZ
ERβ
ERα
Epididymis
Aromatase
Efferent ducts

By one year of age the testis is severely dysmorphic, presenting with marked abnormalities to germ cell progression. At this age, alterations still exist in cell cycle gene expression. Cyclin A1 continues to be decreased, with the median level of cyclin D1 also indicating a decline. Cyclin B1 is also decreased, as is E2F. The cdk inhibitor p21 was again increased.

These findings may suggest that the germ cells in the ArKO are undergoing cell cycle arrest. Given that oestradiol has been shown to influence the expression of cell cycle specific genes, promoting progression through the cell cycle (for review see Sutherland *et al.* 1998), these findings are not surprising. This includes cyclin D1, whose expression is upregulated following oestrogen administration in breast cancer cell lines (Foster & Wimalasena 1996; Altucci *et al.* 1996; Planas-Silva & Weinberg 1997; Prall *et al.* 1997) and in uterine epithelial cells (Zhang *et al.* 1998), possibly directly via the oestrogen responsive region in the cyclin D1 promoter region (Altucci *et al.* 1996). However, as cyclin D1 is not in the germ cells of interest, but in the spermatogonia and Sertoli cells (**Section 1.531**), it is possible that this may be an indirect effect. Oestrogen may also exert its effect at the level of spermatogonial proliferation, since numbers of spermatogonia have a trend to be reduced in the ArKO males. The demonstration that differentiated cells with little proliferation activity, such as Sertoli cells and round spermatids, continue to express cyclins suggests alternative roles for cell cycle proteins in the function of these cells (Ravnik *et al.* 1995).

In contrast, cyclin A1, important in the later stages of the cell cycle and specific to the testes, is localised to the spermatocytes during meiosis I (**Section 1.531, Table 1.531**). Its importance in the testes is clearly outlined in the cyclin A1 knockout mouse where spermatogenesis is impaired at meiosis I (Liu *et al.* 1998). Although no role for oestrogen has been revealed, withdrawing oestrogen does causes a decline in cyclin A2 mRNA levels in breast cancer cells lines (MCF-7 and T47D) (Maas *et al.* 1995), with the addition of oestrogen increasing cyclin A2 expression in uterine epithelial cells (Tong & Pollard 1999). In the present study, we have shown that there is a trend for cyclin A1 expression to

be decreased in the ArKO testes. Since cyclin A1 is expressed in the same cells as aromatase and ERβ, a role for oestrogen in cell cycle progression may be indicated.

Cyclin B1 is also important in the G2/M phase of the cell cycle. Although the cell is now progressing independently of mitogenic stimulus, oestrogen may still be important, with a trend for the decrease in cyclin B1 expression in the ArKO testes at 1 year of age. The importance of oestrogen in the expression of this cyclin is observed with an upregulation in cyclin B1 expression in MCF-7 cells 16-36 hours following exposure to oestrogen, coinciding with entry into G2/M (Thomas & Thomas 1994; Zhang *et al.* 1998). As this cyclin is found in spermatogonia, spermatocytes and round spermatids (**Section 1.531, Table 1.531**), a role for oestrogen in the G2/M phase may be possible.

Oestrogen may also decrease the expression of cell cycle inhibitory proteins, such as $p21^{Waf1}$, to facilitate progression through G1. The expression of this inhibitor appears to be increased in the ArKO testes, shown both by Real Time PCR and *insitu* analysis. Although the specific localisation of p21 was unable to be visualised, previous studies have localised the protein to the spermatocytes, round and elongated spermatids (**Section 1.531, Table 1.531**). As these are the cells that specifically synthesise oestrogens, and oestrogen withdrawal has been observed to increase the mRNA (Maas *et al.* 1995) and protein levels of $p21^{Waf1}$ in breast cancer cells (Truchet *et al.* 2000), with an increase in its inhibitory binding to cyclin E/cdk2 (Cariou *et al.* 2000), a role for $p21^{Waf1}$ in contributing to cell cycle arrest in the ArKO testes is likely.

Cell cycle progression occurs by the phosphorylation and inactivation of the pRb (**Section 1.51**). Although the median level of pRb expression is decreased at 18 weeks in the ArKO testes, the range of data are similar, suggesting no change in this transcript levels. As oestrogen is observed to act posttranslationally, hyperphosphorylating the Rb protein (Foster & Wimalasena 1996; Hurd *et al.* 1997; Planas-Silva & Weinberg 1997; Moudgil *et al.* 2001), the failure to observe changes in expression is perhaps not surprising.

The apparent role of oestrogen in cell cycle progression, observed by the alteration of cell cycle specific genes in the ArKO testis (for summary see **Figure 6.3**), suggests that in the absence of this hormone germ cells fail to complete their progression through the cell cycle. As the spermatogenic cycle is a constant progression of germ cell maturations, it is essential that these germ cells be removed from the seminiferous epithelium to provide space for the next population of cells. In this respect, it is likely that the late spermatocytes and/or early round spermatids will undergo programmed cell death, particularly as it has been shown that specific hormonal stimuli are essential for the maturation and successful transition from a spermatocyte through to an elongated spermatid (**Section 1.32**), and if these hormones are withdrawn then apoptosis occurs (**Section 1.532**).

Although TUNEL assays failed to detect apoptotic cells at 18 weeks of age (**Section 3.25**) (Robertson *et al.* 1999), at this age there is an upregulation in the expression of one of the pro-apoptotic bcl-2 family members, namely bax. Bax is normally only expressed in the first wave of apoptosis during puberty, localised to the spermatogonia, spermatocytes, round spermatids, the Sertoli and Leydig cells (for summary see **Figure 6.3**) then disappears at maturity (**Section 1.532**). However, the finding that bax is upregulated when oestrogen is withdrawn is consistent with its upregulation in the ArKO ovary (Toda *et al.* 2001b), and following testicular insults, such as cryptorchidism (Xu *et al.* 2000) and androgen withdrawal (Woolveridge *et al.* 1999). The increase in this apoptotic gene may indicate the presence of spermatogenic abnormalities at this early age, although morphologically the testes appear unchanged.

The protective role of oestrogen is well characterised in numerous cell culture and rodent models, particularly involving the bcl-2 members. For instance, in neuronal and breast cancer cell cultures, oestrogen increases the expression and activity of both the anti-apoptotic bcl-2 (Singer *et al.* 1998; Garcia-Segura *et al.* 1998; Dong *et al.* 1999; Leung & Wang 1999) and bcl-xL (Gollapudi & Oblinger 1999; Pike 1999). As members of the bcl-2 family can not only homodimerise, but can also heterodimerise, in this respect it is the ratio between pro- and anti-apoptotic proteins that will ultimately determine if the cell is

destined to die. When the expression of bax in the ArKO testis was re-examined as a ratio to bcl-w and bcl-xL, its expression was elevated in both instances. Bcl-xL contains a putative oestrogen response element in its promoter region (Dong *et al.* 1999), and is upregulated in the mammary glands of transgenic mice overexpressing aromatase (Kirma *et al.* 2001). It is also localised specifically to the spermatogonia, spermatocytes and round spermatids, therefore the suggestion that withdrawing oestrogen may stimulate cell death by decreasing the expression of this survival factor while also increasing bax, is reasonable.

One well recognised initiator of mitochondrial mediated apoptosis is via p53. This tumour suppressor protein recognises DNA damage and in the case of excessive damage initiates cell death, possibly via an upregulation of bax (Fujisawa M et al 2001). The absence of oestrogens had no effect on p53 expression in the ArKO testes, despite a previously reported role for oestradiol in p53 expression (Hurd *et al.* 1997; Moudgil *et al.* 2001) and its importance in meiosis indicated by its high and specific localisation to the spermatocytes (**Section 1.532**). However, p53 is believed to undergo many posttranslational modifications that may be influenced by oestrogen, possibly increasing active protein levels.

By one year of age, TUNEL assays indicated a substantial elevation of cell death present in the disrupted epithelium (**Section 3.25**) (Robertson *et al.* 1999). However, at this age, the bcl-2 family appears not to be the central player in instigating germ cell apoptosis when oestrogen is withdrawn, but a second death receptor mediated pathway. The use of the FAS pathway to initiate apoptosis in the ArKO testes is not surprising, given that the FASL and FASR are highly expressed in the spermatocytes and round spermatids, where the lesion is occurring during spermatogenesis (**Section 1.531, Table 1.531**).

Although it is possible that apoptosis occurs as a result of cell cycle arrest, the fact that the spermatocytes and round spermatids also contain aromatase and ERβ, and an element that

recognises oestrogen has been recently identified in the promoter region of the FASL (Mor *et al.* 2000), suggests that oestrogen may also directly protect against apoptosis.

In the absence of oestrogen, the spermatocytes and round spermatids may upregulate their expression of FAS to target themselves for apoptosis by the FASL constitutively expressed by the Sertoli cells (**Section 1.532**). Or, in addition, the FASL, also expressed by the germ cells, may target these or neighbouring cells to instigate an apoptosis induced cell death (for summary see **Figure 6.3**). As the FAS pathway plays an important role in the cell death initiated following insult by Sertoli cells and germ cell toxicants (Lee *et al.* 1997; Lee *et al.* 1999; Richburg 2000; Zhu *et al.* 2000), ischemia (Koji *et al.* 2001; Lysiak *et al.* 2000) and following androgen withdrawal (Nandi *et al.* 1999), a role for FASL following oestrogen withdrawal is not surprising.

Once FASL binds to its specific FAS receptor, it forms a complex that orders the dismantling of the cell by cleaving pro-caspase 8 into its active form, caspase 8 (**Section 1.521**). Further evidence for the involvement of the FAS pathway in initiating cell death in the germ cells of the ArKO mice is evidenced by the upregulation of caspase-8. However, it should be noted that overlap between the two apoptotic pathways does exist, such that the initiation of the caspase cascade can alter the membrane potential of the mitochondria and cause the release of cytochrome *c*, in a bcl-2 independent manner.

It is possible that the removal of oestrogen inhibits the germ cells cycling through the cell cycle, or prevents their full maturation with the cyclins postulated to have important roles in cell differentiation. One difficulty in analysing gene expression in the ARKO testes in this study has been the marked decline in the round spermatid population of cells, in that a decrease in gene expression may be a true effect or a result of the absence of that cell type. Also, as it is the whole testis that is used in the gene expression studies, it may be possible that the alterations in expression levels are occurring in either the supportive Sertoli cells or Leydig cells. However, as Sertoli cell numbers do not alter between WT and ARKO,

and Leydig cell numbers actually appear to increase, I believe that this it is a germ cell factor.

These findings indicate that in the absence of oestrogen, the successful progression of germ cells through the cell cycle may be impaired. However, as heterogeneity exists in the level of spermatogenic disruption in the ArKO mouse, the involvement of other factors allowing some level of spermatogenic progression to occur is suggested. As many cell cycle-specific genes are postulated to play a role in spermatid maturation, oestrogen may have additional roles following the final stage of meiosis. If the development of spermatocytes/round spermatids is inhibited, it is likely these cells consequently undergo an apoptotic-mediated cell death. As aromatase is present in the cells of interest, and oestrogen is a known proliferation inducer and also cell protective factor, these results strongly support an important role for oestrogen in spermatogenesis.

Chapter Seven

General Discussion and Conclusions

7.1 DISCUSSION

7.11 Summary

The role of oestrogens in male reproduction, in particular spermatogenesis and fertility potential, has only recently begun to be appreciated. Despite both aromatase and the oestrogen receptors (α and β) being localised to the testis, particularly ERβ in the germ cells themselves, the role of oestrogen was presumed to be of little significance with androgens remaining the dominant hormonal influence. Through gene targeting technology, models have been designed to elucidate oestrogen action, with the significance of this 'female' hormone in the process of male fertility beginning to be realised. One of these models, developed in our laboratory, is the aromatase knockout (ArKO) mouse (Fisher *et al.* 1998). The ArKO mouse lacks a functional aromatase cytochrome P450 enzyme, thus is unable to catalyse the conversion of C19 steroids (androgens) to C18 steroids (oestrogens). These mice have been the key to the elucidation of oestrogen's function during spermatogenesis.

In the studies conducted in this thesis, it was initially found that withdrawing endogenous oestrogens resulted in severe disruptions to spermatogenesis between 18 weeks and 1 year of age. Quantitation of germ cell numbers indicated that spermatogonia and spermatocyte numbers were unchanged, however there were significantly fewer round and elongated spermatids, some animals showing complete spermiogenic arrest; this occurring with no change in Sertoli cell number. In addition, abnormalities were observed in early acrosome development and Leydig cell hypertrophy. The decline in germ cell number by 1 year of age is suggested to be due to an increase in germ cell apoptosis, characterised by an increase in the expression of apoptosis genes, particularly the FAS pathway. Due to these abnormalities in developmental spermatogenesis, the mature spermatozoa contained in the epididymis are severely reduced in concentration and present with abnormalities in their functional motility. As such they are unable to fertilize oocytes *in vitro*, resulting in a marked reduction in the ability to sire litters. As the ArKO mice also have impairments in

their sexual behaviour, failing to mount receptive females, the reduced fertility may also be a consequence of behavioural abnormalities. Male mice are considered sexually mature by 6-7 weeks of age, however the onset of the spermatogenic disruptions occurs between 18 weeks and 1 year of age. This was puzzling, with one hypothesis being that the consumption of dietary soy by these mice was providing them with an alternative exogenous oestrogen source able to maintain spermatogenesis. The removal of the dietary soy severely exacerbated the phenotype of the ArKO mice, suggesting that the phytoestrogenic constituents of soy were exerting agonistic effects to prevent the severity of the phenotype arising. As animals on the soy free diet presented with the disruptions to spermatogenesis no earlier than 14 weeks, it is possible that the soy partially contributed to the late onset phenotype, however other factors such as androgens and growth factors would appear to be important. The ArKO mice on the diet containing no soy represent a completely oestrogen free model, and as such, all ArKO mice are currently raised on this diet.

Thus, disruption of the aromatase *Cyp19* gene leads to a progressive disruption of spermatogenesis. Oestrogen appears to have a direct effect on the development and survival of the germ cells, with male mice having severely reduced fertility in its absence. As both ERβ and aromatase co-exist in the germ cells where the lesion is observed, it is postulated that the actions of oestrogen are a result of paracrine and/or intracrine interactions within the cells of the seminiferous epithelium. The comprehensive studies of the ArKO male reproductive phenotype in this thesis have brought to our attention the hitherto unsuspected role of oestrogen as a crucial male hormone.

7.12 Testicular development

Although a specific role for oestrogen in the development of the foetal reproductive tract has not yet been defined, the demonstration that rodent foetal testicular cells synthesise oestrogen and express both ERα and β (**Section 1.41**) strongly suggested a role for oestrogen in the foetal male reproductive tract and in gonocyte differentiation (O'Donnell

et al. 2001). In contrast, the importance of androgens was well established with either an inactive androgen receptor (testicular feminized mouse (*tfm*)) (Bullock 1986) or mutation in SF-1 (Parker 1998), both resulting in the failure of male reproductive structures to develop.

Previous experiments have postulated a role for oestrogen in the developing male foetus, as abnormal oestrogen exposure is able to cause reproductive dysfunctions (Arai *et al.* 1983; Toppari *et al.* 1996; McLachlan *et al.* 1998). It is also possible that environmental oestrogens may have agonistic effects to impede this development. However, it was initially presumed that these were primarily indirect hypothalamo-pituitary effects, with the increase in oestrogen feedback decreasing LH and thus androgen levels (Bartke *et al.* 1978; Bellido *et al.* 1990). As the distribution of ERs and aromatase in the developing reproductive tract support a direct role for oestrogen, elucidating the role of oestrogen's at this time is crucial (**Section 1.41**).

Although the male ArKO mice were not studied in any depth during foetal development in this studies, the findings do suggest that they develop normally with abnormalities arising as they age (**Section 3.2**). This may indicate that oestrogen is not important at this time, but it is possible that oestrogen derived from the mother or neighbouring littermates, as suggested by Toda *et al* (2001), is sufficient to ensure that the male reproductive tract develops normally. Feeding the WT a diet containing phytoestrogens or removing this source of oestrogenic substances in the ArKO mice had no effect on this development.

One of the major stages in foetal reproductive development is testicular descent. It has been shown that androgens are crucial to testis migration (**Section 1.31**). However, as hypogonadal (*hpg*) mice, who lack gonadotrophins and therefore androgens, have normal development of the gubernaculum except when they too were treated with oestrogen (Grocock *et al.* 1988), cryptorchidism may in fact be a direct effect of excess oestrogen.

As oestrogen can potentially act to inhibit testes descent, probably via local ERs, it suggests a role for oestrogen in this process. However, the ArKO mice studied in this thesis, either on a soy containing or soy free diet, showed no evidence of undescended testes. This is similar to that of the αERKO, however further examination of these mice found that their cremaster sac was undeveloped and its muscle thicker, suggesting oestrogen was important for the proper development of the gubernaculum and associated structures (Donaldson *et al.* 1996). A more in-depth examination of the ArKO reproductive tract may also indicate such abnormalities. To coincide with these findings, there were no published reports of undescended testes in the three published aromatase mutated male patients (**Section 1.61**) (Morishima *et al.* 1995; Carani *et al.* 1997; Deladoey *et al.* 1999) and the ER mutated human (Smith *et al.* 1994). Interestingly a fourth aromatase mutated male, who is currently being characterised by our laboratory, is cryptorchid (Murata *et al.* 2001). Therefore it is possible that oestrogen is important, with oestrogen withdrawal having a similar effect to excess oestrogen, however more studies need to be completed to fully ascertain if this is an oestrogen mediated process.

7.13 Testicular morphology

In adulthood, this thesis has clearly demonstrated that the removal of endogenous oestrogen does cause testicular disruptions, with a significant decrease in testicular weight in the ArKO males by 1 year of age (**Section 3.22**), with a further exacerbation when exogenous oestrogenic substances were also removed (**Section 4.23**). These findings indicated that oestrogen is important in the structural growth of the testis.

7.131 Germ cells

i Development

The process of spermatogenesis involves a complex series of maturational events, forming spermatozoa capable of fertilisation from an immature spermatogonium, with both FSH and testosterone playing important roles in this process (**Section 1.36**). In addition, a function for oestrogen in germ cell maturation and survival had been postulated (Hess *et*

al. 1995; Carreau *et al.* 1999; Carreau 2000). Dissecting out a direct role for oestrogen has been difficult, considering the influence of oestrogen on Sertoli cell and Leydig cell functions (**Section 7.132, 7.133**) and the negative feedback of oestrogen at the pituitary. Considering that germ cells not only express ERβ but also synthesise high levels of oestrogens throughout development (**Section 1.4**) (O'Donnell *et al.* 2001), a direct role for oestrogen in spermatogenesis was reasonable.

Initial examinations of the αERKO mice (**Section 1.62**) failed to reveal a direct role for oestrogen in spermatogenesis. As no cells within the seminiferous tubules express ERα (**Section 1.4**), this indirect effect is not surprising, particularly as the αERKO germ cells are able to successfully mature if transplanted into a WT mouse which has normal ERα function (Mahato *et al.* 2000). Considering that ERβ is expressed quite extensively within the seminiferous epithelium, particularly the developing germ cells and Sertoli cells, studies examining the βERKO mice have been quite disappointing, with no reproductive phenotype as a consequence of inactivating this receptor. It is postulated that ERα may actually compensate for the removal of ERβ thus altering its tissue specific expression (Rosenfeld *et al.* 1998), or oestrogen may act via a nongenomic mechanism (**Section 1.222**). The action of oestrogen through its membrane bound ER is well documented and suggests a plausible route for oestrogen action in the absence of a nuclear ER (Revelli *et al.* 1998). In this respect, the αERKO, βERKO and αβERKO mice do not provide a complete ER null model, with the question of the role of oestrogen at the level of the germ cell being decidedly unanswered.

The examination of the ArKO testes in this thesis has revealed a direct role for of oestrogen in germ cell development and survival (Robertson *et al.* 1999). There is a trend for a decline in the number of spermatogonia, suggesting a possible role for oestrogen in mitosis (**Section 3.236**). As aromatase is not present in these cells (**Section 1.43**), it is possible that oestrogen is derived from outside the seminiferous epithelium, such as the Leydig cells. Examination of the later type germ cells revealed a lesion either in the

transition from a spermatocyte to a round spermatid, or in early round spermatid development (**Section 3.236**). It was then shown that this agonistic effect can be mimicked by dietary oestrogens, with a soy diet preventing the massive decline in spermatocyte, round and elongated spermatid numbers caused by oestrogen withdrawal. This affect on spermatocyte numbers, not shown when a soy diet was consumed, suggests that spermatocyte development may depend on oestrogen. Thus, when a substantial source of phytoestrogens is removed from the diet of the ArKO mice, so producing a complete oestrogen free model, germ cell maturation is much more severely impaired by 1 year of age, with very little spermatogenic activity.

As it is the spermatocyte and round spermatids that contain high levels of aromatase (**Section 1.43**), it is possible that the germ cells are locally synthesising a level of oestrogen proportional to the number of developing sperm, vital for spermatogenic processes. For instance, oestrogen may be an essential factor for their transition to the next developmental stage. Other studies have also revealed a role for oestrogen in spermiogenesis, observed in adult bonnet monkeys administered an aromatase inhibitor which show a reduction in round and elongated spermatids (Shetty *et al.* 1998). Similar disruptions were also observed in rats administered an aromatase inhibitor for 19 weeks (Turner *et al.* 2000b). Injection of an ovarian protein, which inhibits aromatase activity, into male rats also caused round spermatid degeneration and reduced elongated spermatids (Tsutsumi *et al.* 1987a; Tsutsumi *et al.* 1987b). As FSH is at normal levels in our ArKO mice, possible via an elevation in inhibin B levels, it suggests that this detrimental effect of withdrawing oestrogen on spermatogenesis is a direct oestrogen effect.

Not all seminiferous tubules in the ArKO testes presented with the same level of spermatogenic disruption. This heterogeneity is also apparent in the αERKO (Eddy *et al.* 1996) and in rats administered an aromatase inhibitor (Turner *et al.* 2000b). One hypothesis put forward by Toda *et al* 2001 (Toda *et al.* 2001a), who generated the third aromatase knockout mouse (**Section 1.633**), is that the developing ArKO males may be

exposed to various levels of oestrogens whilst *in utero* via heterozygote, WT or female littermates.

The finding that spermatogenesis appears to progress undisrupted in the younger ArKO males, was puzzling, however it is not unique to the ArKO mouse described in this thesis. Both ArKO mice generated by Honda *et al* (1998) and Toda *et al* (2001a) observed no histological or spermatogenic abnormalities up to 16 weeks of age, with active sperm present in the cauda epididymis. In addition to this, rats treated with an aromatase inhibitor presented with minimal disruptions up to 19 weeks of treatment, with more pronounced disruptions after 1 year, suggesting a time related effect and possible compensatory mechanisms (Turner *et al.* 2000a).

It was hypothesised that the late onset of the spermatogenic phenotype in male ArKO mice (Robertson *et al.* 1999) could be due to oestrogenic substances present in their diet that are capable of agonistic effects on spermatogenesis. However, as withdrawing soy on spermatogenesis does not produce spermatogenic disruptions during the initial spermatogenic cycle, and at 14 weeks disruptions are minimal, it suggests the importance of other alternative ligands. These may include growth factors, such as insulin-like growth factor (IGF) (Aronica & Katzenellenbogen 1993) and epidermal growth factor (EGF) (Ignar-Trowbridge *et al.* 1992), capable of activating the ER via the activation of intracellular MAPK pathways (Smith 1998). Importantly, two androgen metabolites; 5α-androstane-3β,17β-diol and 5-androstene-3β,17β-diol, can also activate the ER, particularly ERβ, when present in high concentrations (Garcia & Rochefort 1979; Kuiper *et al.* 1997). This adds to the complexity of oestrogen action, particularly as unpublished findings in the ArKO males suggest high levels of these androgen metabolites in the young male testes (personal communication, Wah Chin Boon).

***ii** Cell death*

In the absence of oestrogen, round spermatids were found to undergo cell death. An initial examination of the cauda epididymis in this thesis indicated that these germ cells were

prematurely sloughing from the seminiferous epithelium and abnormally collecting in the epididymis (**Section 3.24**). Apoptosis occurs frequently in the normal adult testis, and in fact is a clear indication of healthy spermatogenesis (**Section 1.532**). However, various insults, such as toxic exposure or hormonal withdrawal, greatly increase the incidence of apoptosis (**Section 1.532**). Oestradiol has also been found to have a direct role in germ cell survival, shown when human seminiferous tubules were cultured in an environment devoid of serum survival factors, with oestradiol treatment able to sufficiently inhibit apoptosis (Pentikainen *et al.* 2000). Oestrogen appears to be acting in this instance through the classical receptors as ICI 182,780, an ERα and β antagonist, blocks this protection.

When the spermatogenic phenotype first arises in the ArKO testes, at 14-18 weeks, an examination of gene expression showed upregulation in the pro-apoptotic bax (**Section 6.25**). Bax is a member of the bcl-2 family, which contain both pro and anti- apoptotic actions. While the role of oestrogens during spermatogenesis has not yet been fully addressed, the protective role of oestrogen has been well characterised in numerous cell culture and rodent models, particularly involving the bcl-2 members (Singer *et al.* 1998; Garcia-Segura *et al.* 1998; Dong *et al.* 1999; Leung & Wang 1999; Gollapudi & Oblinger 1999; Pike 1999). Bax has been found to be upregulated in the testis of mice following androgen withdrawal (**Section 1.532**), the expression of bcl-xL, the heterodimeric partner of bax, found to decline. As bcl-xL contains a putative oestrogen response element in its promoter region (Dong *et al.* 1999), withdrawing oestrogen may stimulate cell death by decreasing the expression of this survival factor while also increasing the pro-apoptotic bax. This is also possible as bcl-xL is localised specifically to the spermatogonia, spermatocytes and round spermatids, and therefore may be required for their survival (**Table 1.532**). The alterations in the expression levels of these apoptotic genes indicates the presence of spermatogenic abnormalities at 18 weeks, even though morphologically the testes appear no different from WT.

By one year of age, the testes of the ArKO mice are severely dysmorphic. The germ cells are highly degenerative, with a significant increase in apoptosis appearing to occur via

activation of the FAS death receptor pathway, with an upregulation in both the FAS receptor and ligand, and components of the signalling pathway such as caspase-8, (**Section 6.25**). The use of this pathway to initiate apoptosis in the ArKO testes is not surprising, given that the FASL and FAS are highly expressed in the spermatocytes and round spermatids, the same germ cells where aromatase and ERβ are highly localised and those decreased in number in the absence of oestrogen (**Section 1.532, Table 1.532**). Oestrogen may also potentially play a role in FAS induced apoptosis, as an element that recognises oestrogen has been recently identified in the promoter region of the FASL in breast tissue (Mor *et al.* 2000). This suggests that oestrogen could directly regulate its expression. As it is ERβ that is highly expressed within the seminiferous epithelium, specifically the germ cells, this suggests that ERβ may be mediating cell death via the FAS pathway. In the absence of oestrogen, the spermatocytes and round spermatids may upregulate the expression of FAS to target themselves for apoptosis by the FASL constitutively expressed by the Sertoli cells (**Section 1.532**). Or, in addition, the FASL, also expressed by the germ cells, may target themselves or neighbouring cells to instigate an apoptosis induced cell death.

iii *Cell cycle*

There are several instigators of cellular apoptosis, including cell cycle arrest. The testis is unique in that it contains a population of differentiated cells, but also cells that are constantly undergoing periods of meiosis and mitosis. In this respect, cell cycle specific genes are postulated to play a critical role in the progression of spermatogenesis (**Section 1.531**). Oestrogen is able to recruit non-cycling growth arrested cells back into the cell cycle, through directly stimulating the transcription of early cell cycle genes such as *c-myc* and cyclin D1 through their oestrogen responsive regions (Altucci *et al.* 1996). Therefore, it is possible that withdrawing oestrogen maintains these cells in a quiescent state, possibly even inducing their death. When spermatogenesis is first beginning to be disrupted, at 18 weeks, following the absence of oestrogen, a decline is observed in the expression of cell cycle specific genes, such as cyclin A1, E1, the retinoblastoma protein and an increase in the cdk inhibitor p21 (**Section 6.23**). By one year of age, when spermatogenesis is severely

disrupted, cyclin A1, B1, D1 and E2F are decreased, with $p21^{Waf1}$ continuing to be elevated.

An elevation of Cyclin D1 is the crucial first step for the cell to enter into the cell cycle, with oestrogen having a well recognised mitogenic role at this stage (Foster & Wimalasena 1996; Altucci *et al.* 1996; Planas-Silva & Weinberg 1997; Prall *et al.* 1997; Zhang *et al.* 1998). Although progression through later stages of the cell cycle occurs in a mitogenic independent manner, the decrease in cyclin A1, B1 and E2F expression suggests that oestrogen could be acting at various stages of the cell cycle machinery. Many cell cycle genes, such as cyclin D1 and cyclin E, are also localised to differentiated cells including the Sertoli cells. This suggests that these cell cycle proteins may have alternative roles in the function of these cells.

An interesting observation regarding the ArKO seminiferous epithelium was the abnormalities in acrosomal development (**Section 3.237**). The acrosome vesicle contains the enzymes required for penetration of the oocyte at fertilisation. It originates from the Golgi apparatus in the developing round spermatids and moves to the nucleus, flattening over the nuclear surface as the round spermatids mature (**Section 1.32**). In the ArKO spermatids, there appeared to be multiple acrosomal vesicles and uneven spreading over the nucleus (**Section 3.327**). It is interesting that aromatase is contained in the Golgi apparatus in high amounts during this process, suggesting a possible involvement of oestrogen (Nitta *et al.* 1993). Another possibility is that the acrosomes develop normally, however the germ cells are halting their development through the spermatogenic cycle, such that stage IV spermatids are located in the epithelium alongside stage VIII spermatogonia and spermatocytes. Then as the germ cells begin to degenerate, the acrosome is also affected.

In summary, oestrogen is synthesised by the germ cells at a level higher than that of the Leydig cells (**Section 1.43**). Oestrogen synthesised by the germ cells may act back upon the germ cells in an autocrine or intracrine fashion or adjoining germ cells in a paracrine

manner to regulate their own developmental maturation. This may occur via ERβ, although oestrogen has also been found to act through membrane bound oestrogen receptors (**Section 1.22**). In addition to this, oestrogen could also act on neighbouring Sertoli cells (via their ERβ), to influence the secretion of specific germ cell maturational factors. It is possible that oestrogen could be synthesised from the Leydig cells to act directly on the germ cells, or indirectly via the Sertoli cells. Alternatively, oestrogen from the germ cells could act on the Leydig cells to regulate their secretion of factors such as androgens required for their development.

These studies in the testis of the ArKO mouse have demonstrated that local oestrogen synthesis and response to oestrogens by the germ cells is important throughout spermatogenesis. As the germ cells are capable of oestrogen synthesis and ERβ is highly localised to the seminiferous epithelium, even co-localising in the same germ cells, it suggests that they may regulate their own development. However, as the βERKO males present with no testicular phenotype, this may highlight the intricate and complex roles for oestrogens in the testis, possibly through ER independent actions.

7.132 Sertoli cells

The somatic Sertoli cells form the structure of the seminiferous tubules and have numerous functions that pertain to the successful completion of spermatogenesis (**Section 1.33**). The Sertoli cells and germ cells have such an intimate communication that if the function of the Sertoli cells is impaired, then spermatogenesis will consequently fail to occur.

Sertoli cell proliferation during neonatal life is suggested to be an oestrogen dependant event (Dorrington *et al.* 1993). In addition, it is also well known to be regulated by FSH (**Section 1.36**). Aromatase levels are high in these cells during this period, responsible for the majority of oestrogen production in the neonatal testis (Pelissero *et al.* 1996; Dorrington & Khan 1993). In the immature Sertoli cells, FSH is principally responsible for stimulating the aromatisation of androgens to oestrogen (**Section 1.331**). Correlations have

been made between FSH-induced aromatisation and mitotic activity, with oestradiol causing granulosa cell proliferation (Dorrington *et al.* 1993; Dorrington & Khan 1993). The growth factor, TGF-β was also found to promote granulosa cell division, synergistically mediating the mitogenic effects of oestrogen (Bendell & Dorrington 1991). As granulosa cells and Sertoli cells are in fact derived from the same precursor, it is not surprising that FSH and TGF-β also stimulate Sertoli cell division, with oestrogen interacting to stimulate the secretion of TGF-β (Bendell & Dorrington 1991). Therefore, evidence points to oestrogen mediating the proliferation of Sertoli cells during this neonatal period of development, either directly through the ERβ, or indirectly via Sertoli cell-specific factors such as TGF-β.

Despite this apparent role for oestrogen in Sertoli cell proliferation, in the present studies there was no effect of oestrogen withdrawal on Sertoli cell number. It should be noted that initially the ArKO animals showed no increase in FSH (**Section 3.26**), which could stimulate the proliferation of these cells. No effect was reported when functional oestrogen receptors were removed, but Sertoli cell numbers were not examined.

Further investigation into the influence of dietary phytoestrogens on Sertoli cell proliferation in the ArKO testes found no effect. This is despite ArKO mice raised on a soy free diet having a significant elevation in FSH levels. It is possible that this elevation is a result of soy inhibiting gonadotrophin secretion from the pituitary. Endogenous oestrogen has a homeostatic feedback role at the hypothalamus and pituitary, potentially through a direct action on the ERs localised specifically in these tissues (Shughrue *et al.* 1997). As dietary soy prevents the increase in FSH caused by the removal of oestrogen, it suggests an oestrogenic action of soy at the level of the hypothalamus/pituitary (Couse *et al.* 1997; Mitchner *et al.* 1998; Laflamme *et al.* 1998).

However dietary soy appeared to have minor but measureable effects on the WT testis, as evidenced by a slight decrease in Sertoli cell number (**Section 4.244**). This is perhaps not

surprising, given that exposure to pharmacological levels of oestrogen can interfere with Sertoli cell proliferation, either directly or via effects on FSH, leading to reduced Sertoli cell numbers (Atanassova *et al.* 1999). In the WT mice, it is possible that dietary soy may synergise with the endogenous oestrogen already present in these animals (Casanova *et al.* 1999) to promote higher levels of oestrogenic ligands that have slight inhibitory effects on Sertoli cells.

Once the Sertoli cells cease to proliferate, they undergo a series of maturational steps to form a fully mature adult Sertoli cell (**Section 1.33**). Oestrogen has been postulated to have an inhibitory role in regards to Sertoli cell maturation (Dorrington & Khan 1993). One problem in studying the potential effects of oestrogen on the reproductive tract has been the inhibition of gonadotrophins. To dissect out the actual role of oestrogen, a study compared GnRH antagonist treated and DES treated rats, illustrating that exogenous oestrogens may have a direct effect on Sertoli cell differentiation (Sharpe *et al.* 1998). Exposing neonatal rats to DES lead not only to a reduction in FSH, and consequently decreased Sertoli cells numbers and an inhibition of the normal initiation of spermatogenesis (similar to GnRH antagonist treated animals), but also markedly inhibited their functional maturation, as determined by their secretion of specific Sertoli cell proteins such as inhibin α and SGP-1. This significantly affected the ability of Sertoli cells to function normally through to adulthood. As Sertoli cells express ERβ (**Section 1.44**) this apparent direct inhibitory effect of oestrogens on Sertoli cell maturation is not surprising.

The findings presented in this thesis, and investigations into the αERKO males, have illustrated that oestrogen does appear to be important in adult Sertoli cell function. Initially, efferent duct ligation experiments illustrated that the Sertoli cells were not secreting excess fluid, but actually secreting less fluid in the αERKO males (Hess *et al.* 1997a). This suggests that oestrogen action was important for the maintenance of Sertoli cell fluid secretion. However as the Sertoli cells in mice only contain ERβ (**Section 1.44**), it may be an indirect effect. These findings are supported by observations in the ArKO

mouse, raised on the soy free diet, which show that 1 year old ArKO mice have a marked reduction in seminiferous tubule luminal volume and a marked increase in FSH levels (**Section 4.242, 4.25**). It is possible that Sertoli cell function, and probably inhibin B secretion, is disrupted when there is no oestrogenic stimulus. As these effects are less drastic when soy is consumed, it highlights the oestrogenic action of soy in Sertoli cell function.

A decrease in oestrogen production by the Sertoli cells, to allow the Sertoli cell to begin to differentiate, can be inhibited by other factors which may have a role in the ArKO mouse. These include thyroid hormone, which has an inhibitory effect on aromatase activity and thus oestradiol production by the immature Sertoli cells, and in this way stimulates Sertoli cell differentiation (Ulisse *et al.* 1994; Cooke *et al.* 1994; Bunick *et al.* 1994; Panno *et al.* 1996). Also, factors secreted by the developing germ cells inhibit Sertoli cell oestradiol production (Le Magueresse & Jegou 1988), as do androgens synthesised from the surrounding Leydig cells either directly (Verhoeven & Cailleau 1988a; Hardy *et al.* 1991) or indirectly by stimulating peritubular cells to produce factors that also have this inhibitory function (Verhoeven & Cailleau 1988b).

Oestrogens may act in several ways to control the testicular environment. Not only may the oestrogen specifically synthesised by the Sertoli cells act to impair their own differentiation in an autocrine or intracrine manner but it has also been suggested that Sertoli cell oestrogen may actually have a negative effect on Leydig cell function, decreasing their ability to synthesise androgens until spermatogenesis can competently be initiated and maintained by the Sertoli cells (Dorrington & Khan 1993).

7.133 Leydig cells

The Leydig cells, present in the interstitial space that surrounds the seminiferous tubules, play a primary role in testosterone biosynthesis (**Section 1.34**). During postnatal development, precursor Leydig cells differentiate into immature Leydig cells, primarily

under the stimulus of LH and FSH, however androgens are also believed to be important. This process, essential for the maturation of the Leydig cells, can be inhibited by factors such as oestradiol (for review see Abney 1999).

Exposing the Leydig cells to oestrogen during periods of proliferation, either *in utero* (Perez-Martinez *et al.* 1996; Perez-Martinez *et al.* 1997) or in the neonatal period (Dhar & Setty 1976; Abney & Carswell 1986), results in either a severe diminishment in their number and volume, or a complete abolishment in adulthood (Limanowski *et al.* 1999). Oestradiol appears to be acting at the level of precursor differentiation, as following adult EDS treatment, an alkylating agent that selectively destroys Leydig cells within 24 hours, the LH controlled regeneration process was arrested if oestrogen was given 2 weeks after treatment (Edwards *et al.* 1988; Abney & Myers 1991). In this respect, oestrogen in believed to act as a cell cycle blocker, halting the ability of the Leydig cell precursors to proliferate (Ge & Hardy 1997).

The finding that ERα is highly expressed in the Leydig cells at this time (**Section 1.45**) suggested a direct action of oestrogen. In fact, knocking out ERα results in Leydig cell hyperplasia (Donaldson *et al.* 1996; Rosenfeld *et al.* 1998). Therefore it may be presumed that oestrogen, produced by the Leydig cells, may act upon themselves via their ERα to regulate their proliferation. In the absence of ERα or indeed oestrogen, as seen with the ArKO mice in this thesis, the Leydig cells appear to proliferate uncontrolled (**Section 3.233**). However, as the ArKO mice do not show an increase in the number of Leydig cells until 1 year of age, it is possible that the hyperplasia and hypertrophy are a result of elevated LH levels.

Not only do oestrogens regulate Leydig cell proliferation during foetal and neonatal life, but Leydig cell steroidogenesis is directly inhibited by oestrogen treatment (Bartke *et al.* 1977; Sairam & Berman 1979; Kalla *et al.* 1980; Brinkmann *et al.* 1980). Oestrogens have been found to inhibit steroidogenic enzymes in the pubertal and adult animal, in particular,

P450 side chain cleavage ($P450_{scc}$), the rate limiting enzyme catalysing the first reaction of cholesterol to pregnenolone (Akingbemi *et al.* 2000), as well as cytochrome P450 17α hydroxylase/c17-20 lyase (P450c17) mRNA and protein expression (Onoda & Hall 1981; Li 1991) and its activity (Majdic *et al.* 1996). These enzymes are crucial in the testosterone biosynthesis pathway, therefore in their absence testosterone synthesis is dramatically impaired, leading to severe repercussions in adult fertility potential (Abney & Keel 1986; Gray *et al.* 1989; Limanowski *et al.* 1999). The exact mechanism by which oestrogen instigates this inhibition is unknown, but is believed not to be through a restriction in cholesterol availability (Akingbemi *et al.* 2000) or an inhibition in the expression of the StAR protein (Akingbemi *et al.* 2000).

Further evidence for oestrogen affecting Leydig cell steroidogenesis may be observed in both the ArKO (Fisher *et al.* 1998) and αERKO (Eddy *et al.* 1996) mice by the elevation in serum testosterone. Although, this may also be a consequence of the Leydig cell hyperplasia/hypertrophy, possibly caused by an elevation in LH (Lindzey *et al.* 1998; Fisher *et al.* 1998). Although these studies do not examine LH levels at 1 year of age due to serum restrictions, preliminarily studies into the ArKO males do find an increase in this hormone (Fisher *et al.* 1998).

Although oestrogen has negative effects on both Leydig cell development and function, aromatase is highly expressed by these cells, with oestrogen synthesis by the Leydig cells during development perhaps providing a source of oestrogen for many male reproductive tract functions, for example at puberty it may diffuse into the seminiferous tubule to regulate germ cell development through their oestrogen receptors present (**Section 1.4**). If this oestrogen synthesis is inhibited by treatment with a non-steroidal aromatase inhibitor such as letrozole (Junker & Nogues 1994), or absent in the ArKO mice, it leads to either hyperplastic or atrophied cells, suggesting that oestrogen is important in Leydig cell morphology and function. However the exact role that oestrogen is playing is unknown and needs to be fully explored, particularly regarding the potential effects that abnormal exposure of oestrogens, such as environmental toxicants and pollutants, synthetic

oestrogen such as DES and endogenous oestradiol, may have on Leydig cell development (for review see LeBlanc *et al.* 1997).

7.14 Associated ducts

As the excurrent ducts and epididymis possess the highest level of ERα throughout life (**Section 1.4**), and are the first male reproductive structure to express the oestrogen receptor in foetal development (Cooke *et al.* 1991), it is not surprising that the removal of a functional ERα results in impaired efferent duct function (**Table 7.1**). Oestrogen was found to act through this ER to regulate the reabsorption of luminal fluid in the efferent ducts (Hess *et al.* 1997a). Sperm maturation is believed to depend primarily upon the surrounding luminal fluid environment, controlled by the efferent ducts themselves. As the efferent ducts also contain ERβ, it was postulated that oestrogen might also act through this ER. However, the βERKO mice did not have the same phenotype as the αERKO, with no evidence of dilated epididymal lumens. In this respect, it may be possible that ERβ has a secretory role in the efferent ducts, in contrast to the obvious reabsorptive role of the ERα. The ArKO mice studied in this thesis also do not exhibit an increase in luminal fluid, with the seminiferous tubule lumens actually decreasing as the animal aged. A possible reason may be that the inhibition of both receptors, by removing the natural ligand, may lessen the severity of the phenotype. This is observed when ICI 182,780, an ERα and β antagonist, was given to WT mice and was unable to produce a similar effect to that of the αERKO (Hess *et al.* 1997a). Another possible explanation is that the still functional ERs in the ArKO efferent ducts, are activated either in a ligand independent means or by alternative ER ligands (**Section 1.22**). The initial characterisation of the double ER knockout mouse, the αβERKO, suggests a phenotype similar to that of the αERKO, not the ArKO, with the possibility that ERβ may not have an important role in the normal functioning of the efferent ducts. However further characterisation is required.

The importance of oestrogens at the level of the efferent ducts is not surprising, given that this hormone is present in higher concentrations in the luminal fluid (Ganjam & Amann

Table 7.1 ***Comparison of the male phenotype of the ER and aromatase knockout mouse models***

	αERKO	βERKO	αβERKO	ArKO
FERTILITY	Age related infertile (Lubahn *et al.* 1993; Eddy *et al.* 1996; Dupont *et al.* 2000)	Fertile (Krege *et al.* 1998; Dupont *et al.* 2000)	Infertile (Couse & Korach 1999; Dupont *et al.* 2000)	Initially sub-fertile (Fisher *et al.* 1998; Robertson *et al.* 2001; Toda *et al.* 2001), then declines with age
EXTERNAL GENITALIA	Normal (Lubahn *et al.* 1993)	Normal (Krege *et al.* 1998; Dupont *et al.* 2000)	Normal (Couse & Korach 1999)	Normal (Fisher *et al.* 1998; Honda *et al.* 1998; Toda *et al.* 2001)
DESCENDED TESTES	Some (Donaldson *et al.* 1996)	Not described	Not described	Yes
TESTIS WEIGHT	Initially increase then decreased by day 185 (Lubahn *et al.* 1993; Donaldson *et al.* 1996; Eddy *et al.* 1996; Hess *et al.* 1997)	Not described	Not described	Unchanged 9-18 wks (Toda *et al.* 2001; Robertson *et al.* 1999; Honda *et al.* 1998) reduced 1 year (Robertson *et al.* 1999)
TESTIS MORPHOLOGY				
epithelium	Atrophied and degenerating (Eddy *et al.* 1996; Hess *et al.* 1997)	Not described	Atrophied and reduction in epithelial height (Dupont *et al.* 2000; Couse *et al.* 1999)	Decreased volume (Robertson *et al.* 1999)
lumen	Dilated (Eddy *et al.* 1996)	Not described	Dilated (Dupont *et al.* 2000; Couse *et al.* 1999)	Normal (Robertson *et al.* 1999; Toda *et al.* 2001)
germ cells	Germ cell loss due to increase fluid buildup (Mahato *et al.* 2000; Dupont *et al.* 2000)	Not described	Various stages of spermatogenesis, but possible loss of germ cells (Dupont *et al.* 2000; Couse *et al.* 1999)	Normal 18 wks, 1 year reduction in round and elongated spermatids (Robertson *et al.* 1999)
Sertoli cells	Possible increase in fluid secretion (Hess *et al.* 1997)	Not described	Not described	Normal numbers (Robertson *et al.* 1999)
Leydig cells	Hyperplasia (Donaldson *et al.* 1996; Rosenfeld *et al.* 1998)	Not described	Not described	18 wks normal, 1 year hyperplasia/hypertrophy (Robertson *et al.* 1999)
ASSOCIATED DUCTS				
rete testis	Dilated (Eddy *et al.* 1996; Hess *et al.* 1997; Dupont *et al.* 2000)	Not described	Dilated (Dupont *et al.* 2000)	Not described
efferent ducts	Dilated, reduction in epithelial height (Hess *et al.* 1997; Lee *et al.* 2000; Hess *et al.* 2000)	Not described	Not described, but possibly abnormalities similar to αERKO (Couse *et al.* 1999)	Not described, but lacks dilation (Robertson *et al.* 1999)
epididymis	Initial segment dilated, some epithelial abnormalities, no sperm	Normal morphology (Krege *et al.* 1998)	Reduction in epididymal numbers (Couse *et al.* 1999)	Varies between normal sperm content, absence of mature sperm, degenerating

1976; Free & Jaffe 1979) than in the serum of the male (Kumari *et al.* 1980) or even female (Smith *et al.* 1975). The source of this oestrogen is believed to be the germ cells themselves. When mature elongated spermatids are released from the seminiferous epithelium, they leave behind their cytoplasm. However, a small droplet of cytoplasm, possibly containing remnants of the Golgi apparatus, remains attached to the base of the tail, and it is in this that aromatase is actively synthesising oestrogens (Janulis *et al.* 1996a; Janulis *et al.* 1998). Thus, as the spermatozoa make their journey through the ductal system into the epididymis, they are producing a concentration of oestrogen correlated to the concentration of sperm. The aromatase activity is highest at the area of the efferent ducts, then declines as the sperm traverses towards the cauda epididymis (Janulis *et al.* 1996b). So in this sense, the sperm themselves may be capable of regulating the reabsorptive function of the efferent ducts

The oestrogen synthesised by the spermatozoa may also control their own maturation in the epididymal ducts, with oestradiol shown to increase their motility, oxidative metabolism, longevity, and oocyte penetrative capabilities (Idaomar *et al.* 1989; Mbizvo *et al.* 1990). However, it is possible this also occurs through non-genomic oestrogen receptors located on the sperm membrane, via a cAMP induced mechanism (Luconi *et al.* 1999) (**Section 1.222**). As both ERs are believed to occur on mature sperm, the classical nuclear and the putative membrane bound forms, both may have defined roles in communicating with the uterine environment and in oocyte binding.

The mature epididymal spermatozoa in the αERKO mice are present in reduced concentration, often being completely absent by 20-24 weeks of age, and are characterised by marked morphology abnormalities, such as sperm heads separated from the flagellum, a severe reduction in their motility and an incapability of successful fertilisation (**Table 7.1**) (Lubahn *et al.* 1993; Eddy *et al.* 1996; Couse *et al.* 1999). It is possible that this failure to mature is a result of the increased luminal fluid diluting the essential factors present in the fluid for maturation (Sharpe 1997). It is also possible that oestrogen acts through ERα to

	by 20 wks (Eddy *et al.* 1996) (Hess *et al.* 2000)			immature sperm (Robertson *et al.* 1999)
SPERMATOZOA CHARACTERISTICS				
morphology	Abnormal (Eddy *et al.* 1996)	Not described	Not described	Functional (Toda *et al.* 2001)
concentration	Reduced (Lubahn *et al.* 1993; Eddy *et al.* 1996; Couse *et al.* 1999)	Normal (Couse *et al.* 1999)	Severely reduced (Couse & Korach 1999)	Normal 9-16 wks (Toda *et al.* 2001), reduced with age (Robertson *et al.* 2001)
motility	Reduced (Eddy *et al.* 1996)	Not described	normal (Couse *et al.* 1999)	Normal 9-16 wks (Toda *et al.* 2001), reduced with age (Robertson *et al.* 2001)
IVF capacity	Unable to fertilise (Eddy *et al.* 1996)	Not described	Not described	Able to fertilise 6-18 wks (Toda *et al.* 2001),unable to fertilise 1 year (Robertson *et al.* 2001)
SEXUAL BEHAVIOUR	Normal ability to mount, however reduced intromissions and no ejaculations (Ogawa *et al.* 1997; Wersinger *et al.* 1997)	Normal (Ogawa *et al.* 1999)	Fail to mount, no intromission, no ejaculations (Ogawa *et al.* 2000)	Increased mount latency and decreased number of mounts, no intromissions (Honda *et al.* 1998) (Toda *et al.* 2001; Robertson *et al.* 2001)
HORMONAL ANALYSIS				
Serum FSH	Normal (Eddy *et al.* 1996) Elevated (Lindzey *et al.* 1998)	Not described	Not described	Normal (Robertson *et al.* 1999)
Serum LH	Normal (Eddy *et al.* 1996) Elevated (Lindzey *et al.* 1998)	Not described	Not described	Elevated (Fisher *et al.* 1998)
Serum T	Elevated (Eddy *et al.* 1996)	Not described	Not described	Elevated (Fisher *et al.* 1998; Toda *et al.* 2001)
Serum E_2	Normal (Couse & Korach 1999)	Not described	Not described	Undetectable (Fisher *et al.* 1998)
GENERAL TESTIS MORPHOLOGY	Age related disruptions to seminiferous tubules- degenerating and atrophic tubules, decreased sperm counts, motility and fertilization capacity (Eddy *et al.* 1996). Presumably as a consequence of abnormal fluid content (Mahato *et al.* 2000)	Normal testicular morphology, sperm output and fertilization capacity	Infertile, possibly due to phenotype similar to αERKO	Age related disruptions to spermiogenesis and germ cell maturation, resulting in increased apoptosis

secrete vital maturation factors, and in the absence of a functional ERα this fails to occur, resulting in immature spermatozoa (Mahato *et al.* 2000).

The ArKO mice studied here also have an age dependant reduction in concentration and motility and are also unable to successfully fertilise oocytes (**Section 5.21, Table 7.1**) (Robertson *et al.* 2001b). These observations may indicate that oestrogen has a direct effect on the maturation of the spermatozoa as they transition through to the epididymis. However, as the germ cells are impaired in both knockout animals whilst maturing in the seminiferous epithelium, it is difficult to determine if oestrogen is also acting in the epididymal ducts.

7.15 Sexual behaviour

Successful spermatogenesis would be futile without the ability to copulate. Oestrogen biosynthesis is important during the perinatal stage of development if masculine type sexual behavior is to be exhibited in adulthood (Negri-Cesi *et al.* 1996; Hutchison *et al.* 1997; Roselli & Resko 1993b). In this respect, removing the ability to synthesise oestrogens during this period, such as in the ArKO mice described here (**Section 5.22**) (Robertson *et al.* 2001b) and the other two ArKO mice that have been generated (Honda *et al.* 1998; Toda *et al.* 2001a), resulted in a significant reduction in copulatory behavior in adulthood, explicable in terms of preventing this testosterone conversion. When observing their behaviour after being placed with a hormonally primed female, ArKO males failed to initiate mounting (**Section 5.22**). Replacing oestrogen in the ArKO mouse created by Toda *et al* (2001) within 7 days of birth, was found to restore some fertility of the ArKO males, however few effects were observed regarding normal mounting behaviour suggesting that the critical period is probably prenatally or in the few days following birth (Lephart 1996). In comparison to the ArKO mice, male sexual behaviour in the αERKO is only partially disrupted (**Table 7.1**) (Krege *et al.* 1998). These mice, although infertile and rarely ejaculating, showed either WT levels of mounts and intromissions (Ogawa *et al.* 1997), or a significant reduction in both mounting and intromissions (Wersinger *et al.* 1997). In

contrast, all three components of sexual behaviour are present in the βERKO (Ogawa *et al.* 1999). On the other hand, the double knockout (αβERKO) males do not show any component of sexual behaviour (**Table 7.1**) (Ogawa *et al.* 2000). This is similar to the ArKO mice and suggests that ERα and ERβ may be able to complement one another in regard to this behaviour.

It is possible that humans may also have a similar importance for oestrogen in adult sexual behaviour. Although this is more difficult to assess, the psychosexual and sexual behaviours of one of the men possessing a mutation in his aromatase gene (**Section 1.612**) was analysed before and after oestrogen replacement (Carani *et al.* 1999). Oestrogen was observed to increase male sexual behaviours such as libido, frequency of sexual intercourse, masturbation and erotic fantasies. This suggests that oestrogen may also have a role in human sexual behaviour, however this needs to be more fully explored.

7.16 Fertility

The male reproductive functions discussed above all exist for one reason, successful propagation of the species. Oestrogen, once considered to be a female hormone with no useful function in the male, in fact plays a crucial role in almost all of these different areas. When oestrogen is withdrawn, or oestrogen action is impaired through the inactivation of its receptors, fertility is severely affected (**Table 7.1**). Although the ArKO mice described in preliminary studies (Fisher *et al.* 1998), in this thesis (Robertson *et al.* 2001a), and in other groups (Toda *et al.* 2001a), are all initially sub-fertile, siring few litters, this declines with age. A similar affect is observed with the αERKO and αβERKO mice. The decline in fertility is probably due to the action of oestrogen at numerous levels in male reproduction; including spermatogenic development, Sertoli cell and Leydig cell function, efferent duct function, maturation of spermatozoa, and sexual behaviour. Hence, these results reveal that oestrogen, acting a various points in male reproduction, is crucial for male fertility.

7.2 CONCLUSIONS

The process of spermatogenesis involves a coordinated series of developmental events to form a mature spermatozoon capable of fertilization from an immature spermatogonium. These maturation steps occur within the seminiferous tubules of the testis, in close communication with the supportive Sertoli cells and surrounding interstitium, and then in the epididymal ducts. This process has been well characterised with respect to androgens and gonadotrophins. Oestrogens were also recognised to be synthesised in many of these reproductive structures. Although abnormal levels of oestrogens at various stages of development were shown to result in abnormalities in spermatogenesis, suggesting the testis was a possible oestrogenic site of action, the exact role of oestrogen remained unclear. A specific and direct role for oestrogen was not unreasonable given that both oestrogen receptors, predominantly ERβ, and aromatase, were localised in the testis.

The phenotypes of the αERKO, βERKO, αβERKO mice have all illustrated specific roles for oestrogen during this process. However it is the ArKO mouse that has demonstrated a direct role for oestrogen in spermatogenesis, detailed in this thesis. The developing germ cells not only contain ERβ but also synthesise high amounts of oestrogen in the seminiferous epithelium. This provides a local source of oestrogen evidently important for germ cell progression, acrosome maturation and ultimately round spermatid survival (For summary see **Figure 7.2**). Local synthesis of oestrogen is also essential for other important aspects of male reproduction including Leydig cell proliferation and function, Sertoli cell function, spermatozoa maturation and fertilisation capabilities and sexual behaviour. Until now, three independent ArKO mice have been generated, all the males exhibiting very similar phenotypes, however these studies in this thesis are unique in their intricate examination in the progression of spermatogenesis, the molecular basis for the elevation in germ cell apoptosis and the role that exogenous oestrogens play in the ArKO phenotype. The ArKO mouse has also revealed that the testis is a direct target for the actions of commonly consumed dietary phytoestrogens, oestrogenic compounds that have a

beneficial agonistic role in preventing testicular abnormalities caused by the absence of endogenous oestrogens. This highlights the possibility that other environmental oestrogens may also have such effects.

In conclusion, this thesis has clearly demonstrated the importance of oestradiol, once thought to be a purely female hormone, in many aspects of male reproduction, and indicates that in this context it should be considered to be an androgen.

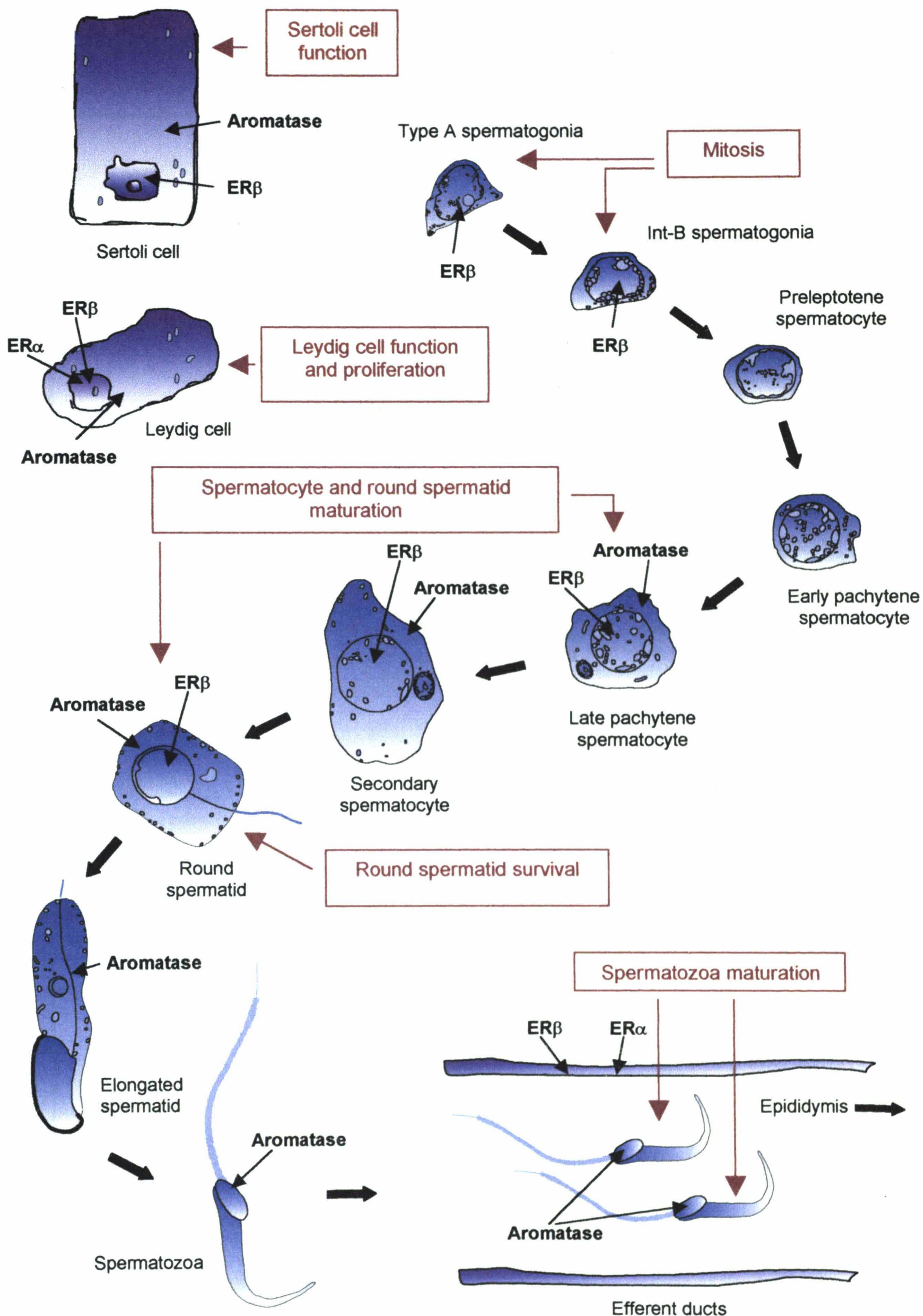


Figure 7.2 The postulated effects of oestrogen on spermatogenesis.

Bibliography

BIBLIOGRAPHY

Abney TO 1999 The potential roles of estrogens in regulating Leydig cell development and function: a review. *Steroids* **64** 610-617.

Abney TO & Carswell LS 1986 Gonadotropin regulation of Leydig cell DNA synthesis. *Mol.Cell Endocrinol.* **45** 157-165.

Abney TO & Keel BA 1986 Temporal effects of diethylstilbestrol administration in vivo on testosterone production in Leydig cells. *Arch.Androl* **17** 79-86.

Abney TO & Myers RB 1991 17 beta-estradiol inhibition of Leydig cell regeneration in the ethane dimethylsulfonate-treated mature rat. *J.Androl* **12** 295-304.

Ackerman, G. E, Smith, M. E, Mendelson, C. R., MacDonald, P. C, and Simpson, E. R. 1981 Aromatisation of androstenedione by human adipose tissue stromal cells in monolayer culture. *J.Clin.Endocrinol.Metab* **53**, 412-417.

Adachi M, Suematsu S, Kondo T, Ogasawara J, Tanaka T, Yoshida N & Nagata S 1995 Targeted mutation in the Fas gene causes hyperplasia in peripheral lymphoid organs and liver. *Nat.Genet.* **11** 294-300.

Adachi M, Watanabe-Fukunaga R & Nagata S 1993 Aberrant transcription caused by the insertion of an early transposable element in an intron of the Fas antigen gene of lpr mice. *Proc.Natl.Acad.Sci.U.S.A* **90** 1756-1760.

Adams JM & Cory S 1998 The Bcl-2 protein family: arbiters of cell survival. *Science* **281** 1322-1326.

Adlercreutz H 1990 Western diet and Western diseases: some hormonal and biochemical mechanisms and associations. *Scand.J.Clin.Lab Invest Suppl* **201** 3-23.

Adlercreutz H, Bannwart C, Wahala K, Makela T, Brunow G, Hase T, Arosemena PJ, Kellis JT, Jr. & Vickery LE 1993 Inhibition of human aromatase by mammalian lignans and isoflavonoid phytoestrogens. *J.Steroid Biochem.Mol.Biol* **44** 147-153.

Akingbemi BT, Ge RS, Klinefelter GR, Gunsalus GL & Hardy MP 2000 A metabolite of methoxychlor, 2,2-bis(p-hydroxyphenyl)-1,1, 1- trichloroethane, reduces testosterone biosynthesis in rat leydig cells through suppression of steady-state messenger ribonucleic acid levels of the cholesterol side-chain cleavage enzyme. *Biol Reprod.* **62** 571-578.

Alcorta DA, Xiong Y, Phelps D, Hannon G, Beach D & Barrett JC 1996 Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts. *Proc.Natl.Acad.Sci.U.S.A* **93** 13742-13747.

Alexandre C & Balthazart J 1986 Effects of metabolism inhibitors, antiestrogens and antiandrogens on the androgen and estrogen induced sexual behavior in Japanese quail. *Physiol Behav.* **38** 581-591.

Allan DJ, Harmon BV & Roberts SA 1992 Spermatogonial apoptosis has three morphologically recognizable phases and shows no circadian rhythm during normal spermatogenesis in the rat. *Cell Prolif.* **25** 241-250.

Almiron I & Chemes H 1988 Spermatogenic onset. II. FSH modulates mitotic activity of germ and Sertoli cells in immature rats. *Int.J.Androl* **11** 235-246.

Altucci L, Addeo R, Cicatiello L, Dauvois S, Parker MG, Truss M, Beato M, Sica V, Bresciani F & Weisz A 1996 17beta-Estradiol induces cyclin D1 gene transcription, p36D1-p34cdk4 complex activation and p105Rb phosphorylation during mitogenic stimulation of G(1)-arrested human breast cancer cells. *Oncogene* **12** 2315-2324.

Ameisen JC 1996 The origin of programmed cell death. *Science* **272** 1278-1279.

Antonsson B & Martinou JC 2000 The Bcl-2 protein family. *Exp.Cell Res.* **256** 50-57.

Arai Y, Mori T, Suzuki Y & Bern HA 1983 Long-term effects of perinatal exposure to sex steroids and diethylstilbestrol on the reproductive system of male mammals. *Int.Rev.Cytol.* **84** 235-268.

Ariyaratne HB & Chamindrani Mendis-Handagama S 2000 Changes in the testis interstitium of Sprague Dawley rats from birth to sexual maturity. *Biol Reprod.* **62** 680-690.

Aronica SM & Katzenellenbogen BS 1993 Stimulation of estrogen receptor-mediated transcription and alteration in the phosphorylation state of the rat uterine estrogen receptor by estrogen, cyclic adenosine monophosphate, and insulin-like growth factor-I. *Mol.Endocrinol.* **7** 743-752.

Atanassova N, McKinnell C, Turner KJ, Walker M, Fisher JS, Morley M, Millar MR, Groome NP & Sharpe RM 2000 Comparative effects of neonatal exposure of male rats to potent and weak (environmental) estrogens on spermatogenesis at puberty and the relationship to adult testis size and fertility: evidence for stimulatory effects of low estrogen levels [In Process Citation]. *Endocrinology* **141** 3898-3907.

Atanassova N, McKinnell C, Walker M, Turner KJ, Fisher JS, Morley M, Millar MR, Groome NP & Sharpe RM 1999 Permanent effects of neonatal estrogen exposure in rats on reproductive hormone levels, Sertoli cell number, and the efficiency of spermatogenesis in adulthood. *Endocrinology* **140** 5364-5373.

Awoniyi CA, Santulli R, Chandrashekar V, Schanbacher BD & Zirkin BR 1989 Quantitative restoration of advanced spermatogenic cells in adult male rats made azoospermic by active immunization against luteinizing hormone or gonadotropin-releasing hormone. *Endocrinology* **125** 1303-1309.

Baker J, Hardy MP, Zhou J, Bondy C, Lupu F, Bellve AR & Efstratiadis A 1996 Effects of an Igf1 gene null mutation on mouse reproduction. *Mol.Endocrinol.* **10** 903-918.

Baker SJ & Reddy EP 1998 Modulation of life and death by the TNF receptor superfamily. *Oncogene* **17** 3261-3270.

Balthazart J, Evrard L & Surlemont C 1990a Effects of the nonsteroidal inhibitor R76713 on testosterone-induced sexual behavior in the Japanese quail (Coturnix coturnix japonica). *Horm.Behav.* **24** 510-531.

Balthazart J, Foidart A & Harada N 1990b Immunocytochemical localization of aromatase in the brain. *Brain Res.* **514** 327-333.

Balthazart J & Surlemont C 1990 Androgen and estrogen action in the preoptic area and activation of copulatory behavior in quail. *Physiol Behav.* **48** 599-609.

Bardin CW, Cheng CY, Musto NA & Gunsalus GL 1988 The Sertoli Cell. In *The Physiology of Reproduction*, pp 933-974. Eds E Knobil & J Neill. New York: Raven Press.

Bardoni B, Zanaria E, Guioli S, Floridia G, Worley KC, Tonini G, Ferrante E, Chiumello G, McCabe ER, Fraccaro M & . 1994 A dosage sensitive locus at chromosome Xp21 is involved in male to female sex reversal. *Nat.Genet.* **7** 497-501.

Barkhem T, Carlsson B, Nilsson Y, Enmark E, Gustafsson J & Nilsson S 1998 Differential response of estrogen receptor alpha and estrogen receptor beta to partial estrogen agonists/antagonists. *Mol.Pharmacol.* **54** 105-112.

Bartke A 1995 Apoptosis of male germ cells, a generalized or a cell type-specific phenomenon? *Endocrinology* **136** 3-4.

Bartke A, Mason M, Dalterio S & Bex F 1978 Effects of tamoxifen on plasma concentrations of testosterone and gonadotrophins in the male rat. *J.Endocrinol.* **79** 239-240.

Bartke A, Williams KI & Dalterio S 1977 Effects of estrogens on testicular testosterone production in vitro. *Biol Reprod.* **17** 645-649.

Bartlett JM, Weinbauer GF & Nieschlag E 1989 Differential effects of FSH and testosterone on the maintenance of spermatogenesis in the adult hypophysectomized rat. *J.Endocrinol.* **121** 49-58.

Basu A & Haldar S 1998 The relationship between Bcl2, Bax and p53: consequences for cell cycle progression and cell death. *Mol.Hum.Reprod.* **4** 1099-1109.

Behringer RR, Finegold MJ & Cate RL 1994 Mullerian-inhibiting substance function during mammalian sexual development. *Cell* **79** 415-425.

Bellido C, Pinilla L, Aguilar R, Gaytan F & Aguilar E 1990 Possible role of changes in post-natal gonadotrophin concentrations on permanent impairment of the reproductive system in neonatally oestrogenized male rats. *J.Reprod.Fertil.* **90** 369-374.

Bendell JJ & Dorrington J 1991 Estradiol-17 beta stimulates DNA synthesis in rat granulosa cells: action mediated by transforming growth factor-beta. *Endocrinology* **128** 2663-2665.

Benton L, Shan LX & Hardy MP 1995 Differentiation of adult Leydig cells. *J.Steroid Biochem.Mol.Biol* **53** 61-68.

Berne RM & Levy MN 1993 *Physiology*. Missouri: Mosby Company.

Beumer TL, Roepers-Gajadien HL, Gademan IS, Kal HB & de Rooij DG 2000a Involvement of the D-type cyclins in germ cell proliferation and differentiation in the mouse. *Biol Reprod.* **63** 1893-1898.

Beumer TL, Roepers-Gajadien HL, Gademan IS, Lock TM, Kal HB & de Rooij DG 2000b Apoptosis regulation in the testis: involvement of Bcl-2 family members. *Mol.Reprod.Dev.* **56** 353-359.

Beumer TL, Roepers-Gajadien HL, Gademan LS, Rutgers DH & de Rooij DG 1997 P21(Cipl/WAF1) expression in the mouse testis before and after X irradiation. *Mol.Reprod.Dev.* **47** 240-247.

Beyer C, Wozniak A & Hutchison JB 1993 Sex-specific aromatization of testosterone in mouse hypothalamic neurons. *Neuroendocrinology* **58** 673-681.

Bilinska B, Lesniak M & Schmalz B 1997 Are ovine Leydig cells able to aromatize androgens? *Reprod.Fertil.Dev.* **9** 193-199.

Bilinska B, Schmalz-Fraczek B, Sadowska J & Carreau S 2000 Localization of cytochrome P450 aromatase and estrogen receptors alpha and beta in testicular cells--an immunohistochemical study of the bank vole [In Process Citation]. *Acta Histochem.* **102** 167-181.

Billig H, Furuta I, Rivier C, Tapanainen J, Parvinen M & Hsueh AJ 1995b Apoptosis in testis germ cells: developmental changes in gonadotropin dependence and localization to selective tubule stages. *Endocrinology* **136** 5-12.

Billig H, Furuta I, Rivier C, Tapanainen J, Parvinen M & Hsueh AJ 1995a Apoptosis in testis germ cells: developmental changes in gonadotropin dependence and localization to selective tubule stages. *Endocrinology* **136** 5-12.

Blanchard KT, Allard EK & Boekelheide K 1996 Fate of germ cells in 2,5-hexanedione-induced testicular injury. I. Apoptosis is the mechanism of germ cell death. *Toxicol.Appl.Pharmacol.* **137** 141-148.

Blanco-Rodriguez J & Martinez-Garcia C 1996 Spontaneous germ cell death in the testis of the adult rat takes the form of apoptosis: re-evaluation of cell types that exhibit the ability to die during spermatogenesis. *Cell Prolif.* **29** 13-31.

Boccuzzi G, Brignardello E, Di Monaco M, Forte C, Leonardi L & Pizzini A 1992 Influence of dehydroepiandrosterone and 5-en-androstene-3 beta, 17 beta- diol on the growth of MCF-7 human breast cancer cells induced by 17 beta-estradiol. *Anticancer Res.* **12** 799-803.

Boekelheide K, Fleming SL, Johnson KJ, Patel SR & Schoenfeld HA 2000 Role of Sertoli cells in injury-associated testicular germ cell apoptosis. *Proc.Soc.Exp.Biol Med.* **225** 105-115.

Boekelheide K, Lee J, Shipp EB, Richburg JH & Li G 1998 Expression of Fas system-related genes in the testis during development and after toxicant exposure. *Toxicol.Lett.* **102-103** 503-508.

Boettger-Tong H, Murthy L, Chiappetta C, Kirkland JL, Goodwin B, Adlercreutz H, Stancel GM & Makela S 1998 A case of a laboratory animal feed with high estrogenic activity and its impact on in vivo responses to exogenously administered estrogens. *Environ.Health Perspect.* **106** 369-373.

Boise LH & Thompson CB 1997 Bcl-x(L) can inhibit apoptosis in cells that have undergone Fas-induced protease activation. *Proc.Natl.Acad.Sci.U.S.A* **94** 3759-3764.

Brandeis M, Rosewell I, Carrington M, Crompton T, Jacobs MA, Kirk J, Gannon J & Hunt T 1998 Cyclin B2-null mice develop normally and are fertile whereas cyclin B1- null mice die in utero. *Proc.Natl.Acad.Sci.U.S.A* **95** 4344-4349.

Brinkmann AO, Leemborg FG, Roodnat EM, de Jong FH & van der Molen HJ 1980 A specific action of estradiol on enzymes involved in testicular steroidogenesis. *Biol Reprod.* **23** 801-809.

Brinkworth MH, Weinbauer GF, Schlatt S & Nieschlag E 1995 Identification of male germ cells undergoing apoptosis in adult rats. *J.Reprod.Fertil.* **105** 25-33.

Britt KL, Drummond AE, Cox VA, Dyson M, Wreford NG, Jones ME, Simpson ER & Findlay JK 2000 An age-related ovarian phenotype in mice with targeted disruption of the Cyp 19 (aromatase) gene. *Endocrinology* **141** 2614-2623.

Brzezinski A & Debi A 1999 Phytoestrogens: the "natural" selective estrogen receptor modulators? *Eur.J.Obstet.Gynecol.Reprod.Biol.* **85** 47-51.

Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O, Ohman L, Greene GL, Gustafsson JA & Carlquist M 1997 Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* **389** 753-758.

Buendia B, Santa-Maria A & Courvalin JC 1999 Caspase-dependent proteolysis of integral and peripheral proteins of nuclear membranes and nuclear pore complex proteins during apoptosis. *J.Cell Sci.* **112 (Pt 11)** 1743-1753.

Bullock BC, Newbold RR & McLachlan JA 1988 Lesions of testis and epididymis associated with prenatal diethylstilbestrol exposure. *Environ.Health Perspect.* **77** 29-31.

Bullock LP 1986 Animal models of androgen insensitivity. *Adv.Exp.Med.Biol* **196** 269-278.

Bulun SE, Rosenthal IM, Brodie AM, Inkster SE, Zeller WP, DiGeorge AM, Frasier SD, Kilgore MW & Simpson ER 1994 Use of tissue-specific promoters in the regulation of aromatase cytochrome P450 gene expression in human testicular and ovarian sex cord tumors, as well as in normal fetal and adult gonads. *J.Clin.Endocrinol.Metab* **78** 1616-1621.

Bunick D, Kirby J, Hess RA & Cooke PS 1994 Developmental expression of testis messenger ribonucleic acids in the rat following propylthiouracil-induced neonatal hypothyroidism. *Biol Reprod.* **51** 706-713.

Burroughs CD, Mills KT & Bern HA 1990 Long-term genital tract changes in female mice treated neonatally with coumestrol [published erratum appears in Reprod Toxicol 1990;4(3):243]. *Reprod.Toxicol.* **4** 127-135.

Byskov AG 1986 Differentiation of mammalian embryonic gonad. *Physiol Rev.* **66** 71-117.

Cain K, Brown DG, Langlais C & Cohen GM 1999 Caspase activation involves the formation of the aposome, a large (approximately 700 kDa) caspase-activating complex. *J.Biol Chem.* **274** 22686-22692.

Campbell DR & Kurzer MS 1993 Flavonoid inhibition of aromatase enzyme activity in human preadipocytes. *J.Steroid Biochem.Mol.Biol* **46** 381-388.

Cao L, Faha B, Dembski M, Tsai LH, Harlow E & Dyson N 1992 Independent binding of the retinoblastoma protein and p107 to the transcription factor E2F. *Nature* **355** 176-179.

Capel B 2000 The battle of the sexes. *Mech.Dev.* **92** 89-103.

Carani C, Qin K, Simoni M, Faustini-Fustini M, Serpente S, Boyd J, Korach KS & Simpson ER 1997 Effect of testosterone and estradiol in a man with aromatase deficiency. *N.Engl.J.Med.* **337** 91-95.

Carani C, Rochira V, Faustini-Fustini M, Balestrieri A & Granata AR 1999 Role of oestrogen in male sexual behaviour: insights from the natural model of aromatase deficiency. *Clin.Endocrinol.(Oxf)* **51** 517-524.

Cariou S, Donovan JC, Flanagan WM, Milic A, Bhattacharya N & Slingerland JM 2000 Down-regulation of p21WAF1/CIP1 or p27Kip1 abrogates antiestrogen- mediated cell cycle arrest in human breast cancer cells. *Proc.Natl.Acad.Sci.U.S.A* **97** 9042-9046.

Carreau S 2000 Estrogens and male reproduction. *Folia Histochem.Cytobiol.* **38** 47-52.

Carreau S, Drosdowsky MA & Courot M 1984 Androgen-binding proteins in sheep epididymis: age-related effects on androgen-binding protein, cytosolic androgen receptor and testosterone concentrations. Correlations with histological studies. *J.Endocrinol.* **103** 281-286.

Carreau S, Genissel C, Bilinska B & Levallet J 1999 Sources of oestrogen in the testis and reproductive tract of the male. *Int.J.Androl* **22** 211-223.

Carreau S & Levallet J 1997 Cytochrome P450 aromatase in male germ cells. *Folia Histochem.Cytobiol.* **35** 195-202.

Carreau S, Papadopoulos V & Drosdowsky MA 1988 Stimulation of adult rat Leydig cell aromatase activity by a Sertoli cell factor. *Endocrinology* **122** 1103-1109.

Carroll JS, Prall OW, Musgrove EA & Sutherland RL 2000 A pure estrogen antagonist inhibits cyclin E-Cdk2 activity in MCF-7 breast cancer cells and induces accumulation of p130-E2F4 complexes characteristic of quiescence. *J.Biol Chem.* **275** 38221-38229.

Carroll RS, Weaver CE & Baum MJ 1988 Evidence implicating aromatization of testosterone in the regulation of male ferret sexual behavior. *Physiol Behav.* **42** 457-460.

Casanova M, You L, Gaido KW, Archibeque-Engle S, Janszen DB & Heck HA 1999 Developmental effects of dietary phytoestrogens in Sprague-Dawley rats and interactions of genistein and daidzein with rat estrogen receptors alpha and beta in vitro. *Toxicol.Sci.* **51** 236-244.

Chang GW & Kam PC 1999 The physiological and pharmacological roles of cytochrome P450 isoenzymes. *Anaesthesia* **54** 42-50.

Chao DT & Korsmeyer SJ 1998 BCL-2 family: regulators of cell death. *Annu.Rev.Immunol.* **16** 395-419.

Chapman DL & Wolgemuth DJ 1993 Isolation of the murine cyclin B2 cDNA and characterization of the lineage and temporal specificity of expression of the B1 and B2 cyclins during oogenesis, spermatogenesis and early embryogenesis. *Development* **118** 229-240.

Chapman DL & Wolgemuth DJ 1994 Regulation of M-phase promoting factor activity during development of mouse male germ cells. *Dev.Biol* **165** 500-506.

Chellappan SP, Hiebert S, Mudryj M, Horowitz JM & Nevins JR 1991 The E2F transcription factor is a cellular target for the RB protein. *Cell* **65** 1053-1061.

Chemes HE, Dym M & Raj HG 1979 The role of gonadotropins and testosterone on initiation of spermatogenesis in the immature rat. *Biol Reprod.* **21** 241-249.

Cheng M, Olivier P, Diehl JA, Fero M, Roussel MF, Roberts JM & Sherr CJ 1999 The p21(Cip1) and p27(Kip1) CDK 'inhibitors' are essential activators of cyclin D-dependent kinases in murine fibroblasts. *EMBO J.* **18** 1571-1583.

Chinnaiyan AM, O'Rourke K, Tewari M & Dixit VM 1995 FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell* **81** 505-512.

Cho H & Katzenellenbogen BS 1993 Synergistic activation of estrogen receptor-mediated transcription by estradiol and protein kinase activators. *Mol.Endocrinol.* **7** 441-452.

Chrousos GP, Detera-Wadleigh SD & Karl M 1993 Syndromes of glucocorticoid resistance. *Ann.Intern.Med.* **119** 1113-1124.

Clancy AN, Zumpe D & Michael RP 1995 Intracerebral infusion of an aromatase inhibitor, sexual behavior and brain estrogen receptor-like immunoreactivity in intact male rats. *Neuroendocrinology* **61** 98-111.

Clark BJ, Wells J, King SR & Stocco DM 1994 The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR). *J.Biol Chem.* **269** 28314-28322.

Clemens LG & Pomerantz SM 1982 Testosterone acts as a prohormone to stimulate male copulatory behavior in male deer mice (peromyscus maniculatus bairdi). *J.Comp Physiol Psychol.* **96** 114-122.

Clermont Y 1972 Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. *Physiol Rev.* **52** 198-236.

Clulow J, Jones RC & Hansen LA 1994 Micropuncture and cannulation studies of fluid composition and transport in the ductuli efferentes testis of the rat: comparisons with the homologous metanephric proximal tubule. *Exp.Physiol* **79** 915-928.

Clulow J, Jones RC, Hansen LA & Man SY 1998 Fluid and electrolyte reabsorption in the ductuli efferentes testis. *J.Reprod.Fertil.Suppl* **53** 1-14.

Cohen GM 1997 Caspases: the executioners of apoptosis. *Biochem.J.* **326 (Pt 1)** 1-16.

Colborn T, vom Saal FS & Soto AM 1993 Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ.Health Perspect.* **101** 378-384.

Cook SJ, Balmanno K, Garner A, Millar T, Taverner C & Todd D 2000 Regulation of cell cycle re-entry by growth, survival and stress signalling pathways. *Biochem.Soc.Trans.* **28** 233-240.

Cooke BA, Choi MC, Dirami G, Lopez-Ruiz MP & West AP 1992 Control of steroidogenesis in Leydig cells. *J.Steroid Biochem.Mol.Biol* **43** 445-449.

Cooke BA, de Jong FH, van der Molen HJ & Rommerts FF 1972 Endogenous testosterone concentration in rat testes interstitial tissue and seminiferous tubules during *in vitro* incubation. *Nature New Biol* **237** 255-256.

Cooke PS, Young P, Hess RA & Cunha GR 1991 Estrogen receptor expression in developing epididymis, efferent ductules, and other male reproductive organs. *Endocrinology* **128** 2874-2879.

Cooke PS, Zhao YD & Bunick D 1994 Triiodothyronine inhibits proliferation and stimulates differentiation of cultured neonatal Sertoli cells: possible mechanism for increased adult testis weight and sperm production induced by neonatal goitrogen treatment. *Biol Reprod.* **51** 1000-1005.

Cooper TT, Clancy AN, Karom M, Moore TO & Albers HE 2000 Conversion of testosterone to estradiol may not be necessary for the expression of mating behavior in male Syrian hamsters (Mesocricetus auratus) [In Process Citation]. *Horm.Behav.* **37** 237-245.

Couse JF, Curtis SW, Washburn TF, Lindzey J, Golding TS, Lubahn DB, Smithies O & Korach KS 1995 Analysis of transcription and estrogen insensitivity in the female mouse after targeted disruption of the estrogen receptor gene. *Mol.Endocrinol.* **9** 1441-1454.

Couse JF, Hewitt SC, Bunch DO, Sar M, Walker VR, Davis BJ & Korach KS 1999 Postnatal sex reversal of the ovaries in mice lacking estrogen receptors alpha and beta. *Science* **286** 2328-2331.

Couse JF & Korach KS 1999 Estrogen receptor null mice: what have we learned and where will they lead us? *Endocr.Rev.* **20** 358-417.

Couse JF, Lindzey J, Grandien K, Gustafsson JA & Korach KS 1997 Tissue distribution and quantitative analysis of estrogen receptor- alpha (ERalpha) and estrogen receptor-beta (ERbeta) messenger ribonucleic acid in the wild-type and ERalpha-knockout mouse. *Endocrinology* **138** 4613-4621.

Cowley SM, Hoare S, Mosselman S & Parker MG 1997 Estrogen receptors alpha and beta form heterodimers on DNA. *J.Biol Chem.* **272** 19858-19862.

Cox LS & Lane DP 1995 Tumour suppressors, kinases and clamps: how p53 regulates the cell cycle in response to DNA damage. *Bioessays* **17** 501-508.

Cummings AM 1997 Methoxychlor as a model for environmental estrogens. *Crit Rev.Toxicol.* **27** 367-379.

D'Alessio A, Riccioli A, Lauretti P, Padula F, Muciaccia B, De Cesaris P, Filippini A, Nagata S & Ziparo E 2001 Testicular FasL is expressed by sperm cells. *Proc.Natl.Acad.Sci.U.S.A* **98** 3316-3321.

D'Occhio MJ & Brooks DE 1980 Effects of androgenic and oestrogenic hormones on mating behaviour in rams castrated before and after puberty. *J.Endocrinol.* **86** 403-411.

Davis SR, Dalais FS, Simpson ER & Murkies AL 1999 Phytoestrogens in health and disease. *Recent Prog.Horm.Res.* **54** 185-210.

de Kretser D & Kerr JB 1988 The cytology of the testis. In *The Physiology of Reproduction*, pp 837-932. Eds E Knobil & J Neill. New York: Raven Press Ltd.

de Kretser DM, Loveland KL, Meinhardt A, Simorangkir D & Wreford N 1998 Spermatogenesis. *Hum.Reprod.* **13 Suppl 1** 1-8.

De Maria R, Testa U, Luchetti L, Zeuner A, Stassi G, Pelosi E, Riccioni R, Felli N, Samoggia P & Peschle C 1999 Apoptotic role of Fas/Fas ligand system in the regulation of erythropoiesis. *Blood* **93** 796-803.

Decaudin D, Geley S, Hirsch T, Castedo M, Marchetti P, Macho A, Kofler R & Kroemer G 1997 Bcl-2 and Bcl-XL antagonize the mitochondrial dysfunction preceding nuclear apoptosis induced by chemotherapeutic agents. *Cancer Res.* **57** 62-67.

Degen GH & Bolt HM 2000 Endocrine disruptors: update on xenoestrogens. *Int.Arch.Occup.Environ.Health* **73** 433-441.

Deladoey J, Fluck C, Bex M, Yoshimura N, Harada N & Mullis PE 1999 Aromatase deficiency caused by a novel P450arom gene mutation: impact of absent estrogen production on serum gonadotropin concentration in a boy. *J.Clin.Endocrinol.Metab* **84** 4050-4054.

Deng C, Zhang P, Harper JW, Elledge SJ & Leder P 1995 Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. *Cell* **82** 675-684.

Denmeade SR, Lin XS & Isaacs JT 1996 Role of programmed (apoptotic) cell death during the progression and therapy for prostate cancer. *Prostate* **28** 251-265.

Devoto SH, Mudryj M, Pines J, Hunter T & Nevins JR 1992 A cyclin A-protein kinase complex possesses sequence-specific DNA binding activity: p33cdk2 is a component of the E2F-cyclin A complex. *Cell* **68** 167-176.

Dhar JD & Setty BS 1976 Epididymal response to exogenous testosterone in rats sterilized neonatally by estrogen. *Endokrinologie.* **68** 14-21.

Dierich A, Sairam MR, Monaco L, Fimia GM, Gansmuller A, LeMeur M & Sassone-Corsi P 1998 Impairing follicle-stimulating hormone (FSH) signaling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. *Proc.Natl.Acad.Sci.U.S.A* **95** 13612-13617.

Donaldson KM, Tong SY, Washburn T, Lubahn DB, Eddy EM, Hutson JM & Korach KS 1996 Morphometric study of the gubernaculum in male estrogen receptor mutant mice. *J.Androl* **17** 91-95.

Dong L, Wang W, Wang F, Stoner M, Reed JC, Harigai M, Samudio I, Kladde MP, Vyhlidal C & Safe S 1999 Mechanisms of transcriptional activation of bcl-2 gene expression by 17beta-estradiol in breast cancer cells. *J.Biol Chem.* **274** 32099-32107.

Dorrington JH & Armstrong DT 1975 Follicle-stimulating hormone stimulates estradiol-17beta synthesis in cultured Sertoli cells. *Proc.Natl.Acad.Sci.U.S.A* **72** 2677-2681.

Dorrington JH, Bendell JJ & Khan SA 1993 Interactions between FSH, estradiol-17 beta and transforming growth factor-beta regulate growth and differentiation in the rat gonad. *J.Steroid Biochem.Mol.Biol* **44** 441-447.

Dorrington JH & Khan SA 1993 Steroid production, metabolism and release by Sertoli cells. In *The Sertoli Cell*, pp 538-549. Eds LD Russell & MD Griswold. Clearwater, Florida: Cache River Press.

Dowdy SF, Hinds PW, Louie K, Reed SI, Arnold A & Weinberg RA 1993 Physical interaction of the retinoblastoma protein with human D cyclins. *Cell* **73** 499-511.

Dufau ML 1988 Endocrine regulation and communicating functions of the Leydig cell. *Annu.Rev.Physiol* **50** 483-508.

Dupont E, Zhao HF, Rheaume E, Simard J, Luu-The V, Labrie F & Pelletier G 1990 Localization of 3 beta-hydroxysteroid dehydrogenase/delta 5-delta 4- isomerase in rat gonads and adrenal glands by immunocytochemistry and in situ hybridization. *Endocrinology* **127** 1394-1403.

Dupont S, Krust A, Gansmuller A, Dierich A, Chambon P & Mark M 2000 Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. *Development* **127** 4277-4291.

Dutertre M & Smith CL 2000 Molecular mechanisms of selective estrogen receptor modulator (SERM) action. *J.Pharmacol.Exp.Ther.* **295** 431-437.

Dyson N 1998 The regulation of E2F by pRB-family proteins. *Genes Dev.* **12** 2245-2262.

Earnshaw WC, Martins LM & Kaufmann SH 1999 Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu.Rev.Biochem.* **68** 383-424.

Ebling FJ, Brooks AN, Cronin AS, Ford H & Kerr JB 2000 Estrogenic induction of spermatogenesis in the hypogonadal mouse. *Endocrinology* **141** 2861-2869.

Eddy EM, Washburn TF, Bunch DO, Goulding EH, Gladen BC, Lubahn DB & Korach KS 1996 Targeted disruption of the estrogen receptor gene in male mice causes alteration of spermatogenesis and infertility. *Endocrinology* **137** 4796-4805.

Edwards G, Robertson WR & Morris ID 1988 Characterization of the regenerated Leydig cell population of the rat after destruction by ethylene-1,2-dimethanesulphonate. *J.Endocrinol.* **117** 11-18.

el Deiry WS, Harper JW, O'Connor PM, Velculescu VE, Canman CE, Jackman J, Pietenpol JA, Burrell M, Hill DE, Wang Y & . 1994 WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Res.* **54** 1169-1174.

el Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW & Vogelstein B 1993 WAF1, a potential mediator of p53 tumor suppression. *Cell* **75** 817-825.

Elledge SJ 1996 Cell cycle checkpoints: preventing an identity crisis. *Science* **274** 1664-1672.

Elledge SJ & Harper JW 1994 Cdk inhibitors: on the threshold of checkpoints and development. *Curr.Opin.Cell Biol* **6** 847-852.

Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A & Nagata S 1998 A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* **391** 43-50.

Enmark E, Pelto-Huikko M, Grandien K, Lagercrantz S, Lagercrantz J, Fried G, Nordenskjold M & Gustafsson JA 1997 Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. *J.Clin.Endocrinol.Metab* **82** 4258-4265.

Evan G & Littlewood T 1998 A matter of life and cell death. *Science* **281** 1317-1322.

Evans RM 1988 The steroid and thyroid hormone receptor superfamily. *Science* **240** 889-895.

Ewen ME 1994 The cell cycle and the retinoblastoma protein family. *Cancer Metastasis Rev.* **13** 45-66.

Ezhevsky SA, Nagahara H, Vocero-Akbani AM, Gius DR, Wei MC & Dowdy SF 1997 Hypo-phosphorylation of the retinoblastoma protein (pRb) by cyclin D:Cdk4/6 complexes results in active pRb. *Proc.Natl.Acad.Sci.U.S.A* **94** 10699-10704.

Fisher CR, Graves KH, Parlow AF & Simpson ER 1998 Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene. *Proc.Natl.Acad.Sci.U.S.A* **95** 6965-6970.

Fisher JS, Millar MR, Majdic G, Saunders PT, Fraser HM & Sharpe RM 1997 Immunolocalisation of oestrogen receptor-alpha within the testis and excurrent ducts of the rat and marmoset monkey from perinatal life to adulthood. *J.Endocrinol.* **153** 485-495.

Fisher RP & Morgan DO 1994 A novel cyclin associates with MO15/CDK7 to form the CDK-activating kinase. *Cell* **78** 713-724.

Flynn KM, Ferguson SA, Delclos KB & Newbold RR 2000 Effects of genistein exposure on sexually dimorphic behaviors in rats. *Toxicol.Sci.* **55** 311-319.

Foster JS & Wimalasena J 1996 Estrogen regulates activity of cyclin-dependent kinases and retinoblastoma protein phosphorylation in breast cancer cells. *Mol.Endocrinol.* **10** 488-498.

Foster JW, Dominguez-Steglich MA, Guioli S, Kowk G, Weller PA, Stevanovic M, Weissenbach J, Mansour S, Young ID, Goodfellow PN & . 1994 Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. *Nature* **372** 525-530.

Free MJ & Jaffe RA 1979 Collection of rete testis fluid from rats without previous efferent duct ligation. *Biol Reprod.* **20** 269-278.

French LE, Hahne M, Viard I, Radlgruber G, Zanone R, Becker K, Muller C & Tschopp J 1996 Fas and Fas ligand in embryos and adult mice: ligand expression in several immune-privileged tissues and coexpression in adult tissues characterized by apoptotic cell turnover. *J.Cell Biol* **133** 335-343.

Furuchi T, Masuko K, Nishimune Y, Obinata M & Matsui Y 1996 Inhibition of testicular germ cell apoptosis and differentiation in mice misexpressing Bcl-2 in spermatogonia. *Development* **122** 1703-1709.

Ganjam VK & Amann RP 1976 Steroids in fluids and sperm entering and leaving the bovine epididymis, epididymal tissue, and accessory sex gland secretions. *Endocrinology* **99** 1618-1630.

Garcia-Segura LM, Cardona-Gomez P, Naftolin F & Chowen JA 1998 Estradiol upregulates Bcl-2 expression in adult brain neurons. *Neuroreport* **9** 593-597.

Garcia M & Rochefort H 1979 Evidence and characterization of the binding of two 3H-labeled androgens to the estrogen receptor. *Endocrinology* **104** 1797-1804.

Gardner RL, Lyon MF, Evans EP & Burtenshaw MD 1985 Clonal analysis of X-chromosome inactivation and the origin of the germ line in the mouse embryo. *J.Embryol.Exp.Morphol.* **88** 349-363.

Gautier C, Levacher C, Avallet O, Vigier M, Rouiller-Fabre V, Lecerf L, Saez J & Habert R 1994 Immunohistochemical localization of transforming growth factor-beta 1 in the fetal and neonatal rat testis. *Mol.Cell Endocrinol.* **99** 55-61.

Gaytan F, Bellido C, Aguilar E & van Rooijen N 1994 Requirement for testicular macrophages in Leydig cell proliferation and differentiation during prepubertal development in rats. *J.Reprod.Fertil.* **102** 393-399.

Ge RS, Shan LX & Hardy MP 1996 Pubertal development of Leydig cells. In *The Leydig Cell*, pp 159-174. Eds AH Payne, MP Hardy & LD Russell. Vienna: Cache River Press.

Ge RS & Hardy MP 1997 Decreased cyclin A2 and increased cyclin G1 levels coincide with loss of proliferative capacity in rat Leydig cells during pubertal development. *Endocrinology* **138** 3719-3726.

Genissel C, Levallet J & Carreau S 2001 Regulation of cytochrome P450 aromatase gene expression in adult rat Leydig cells: comparison with estradiol production. *J.Endocrinol.* **168** 95-105.

Ghosh S, Bartke A, Grasso P, Reichert LE, Jr. & Russell LD 1992 Structural manifestations of the rat Sertoli cell to hypophysectomy: a correlative morphometric and endocrine study. *Endocrinology* **131** 485-497.

Gibson L, Holmgreen SP, Huang DC, Bernard O, Copeland NG, Jenkins NA, Sutherland GR, Baker E, Adams JM & Cory S 1996 bcl-w, a novel member of the bcl-2 family, promotes cell survival. *Oncogene* **13** 665-675.

Gill WB, Schumacher GF, Bibbo M, Straus FH & Schoenberg HW 1979 Association of diethylstilbestrol exposure in utero with cryptorchidism, testicular hypoplasia and semen abnormalities. *J.Urol.* **122** 36-39.

Godet M, Thomas A, Rudkin BB & Durand P 2000 Developmental changes in cyclin B1 and cyclin-dependent kinase 1 (CDK1) levels in the different populations of spermatogenic cells of the post- natal rat testis. *Eur.J.Cell Biol* **79** 816-823.

Gollapudi L & Oblinger MM 1999 Estrogen and NGF synergistically protect terminally differentiated, ERalpha-transfected PC12 cells from apoptosis. *J.Neurosci.Res.* **56** 471-481.

Gonzalez MI & Leret ML 1992 Role of monoamines in the male differentiation of the brain induced by androgen aromatization. *Pharmacol.Biochem.Behav.* **41** 733-737.

Goodfellow PN & Lovell-Badge R 1993 SRY and sex determination in mammals. *Annu.Rev.Genet.* **27** 71-92.

Goyal HO, Bartol FF, Wiley AA & Neff CW 1997 Immunolocalization of receptors for androgen and estrogen in male caprine reproductive tissues: unique distribution of estrogen receptors in efferent ductule epithelium. *Biol.Reprod.* **56** 90-101.

Goyal HO, Hutto V & Maloney MA 1994 Effects of androgen deprivation in the goat epididymis. *Acta Anat.(Basel)* **150** 127-135.

Gray LE, Ostby J, Ferrell J, Rehnberg G, Linder R, Cooper R, Goldman J, Slott V & Laskey J 1989 A dose-response analysis of methoxychlor-induced alterations of reproductive development and function in the rat. *Fundam.Appl.Toxicol.* **12** 92-108.

Greco TL, Furlow JD, Duello TM & Gorski J 1992 Immunodetection of estrogen receptors in fetal and neonatal male mouse reproductive tracts. *Endocrinology* **130** 421-429.

Greco TL & Payne AH 1994 Ontogeny of expression of the genes for steroidogenic enzymes P450 side- chain cleavage, 3 beta-hydroxysteroid dehydrogenase, P450 17 alpha- hydroxylase/C17-20 lyase, and P450 aromatase in fetal mouse gonads. *Endocrinology* **135** 262-268.

Green PS & Simpkins JW 2000 Neuroprotective effects of estrogens: potential mechanisms of action. *Int.J.Dev.Neurosci.* **18** 347-358.

Green S, Walter P, Kumar V, Krust A, Bornert JM, Argos P & Chambon P 1986 Human oestrogen receptor cDNA: sequence, expression and homology to v- erb-A. *Nature* **320** 134-139.

Greene GL, Gilna P, Waterfield M, Baker A, Hort Y & Shine J 1986 Sequence and expression of human estrogen receptor complementary DNA. *Science* **231** 1150-1154.

Griffith TS, Brunner T, Fletcher SM, Green DR & Ferguson TA 1995 Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science* **270** 1189-1192.

Grocock CA, Charlton HM & Pike MC 1988 Role of the fetal pituitary in cryptorchidism induced by exogenous maternal oestrogen during pregnancy in mice. *J.Reprod.Fertil.* **83** 295-300.

Gromoll J, Wessels J, Rosiepen G, Brinkworth MH & Weinbauer GF 1997 Expression of mitotic cyclin B1 is not confined to proliferating cells in the rat testis. *Biol Reprod.* **57** 1312-1319.

Gross A, McDonnell JM & Korsmeyer SJ 1999 BCL-2 family members and the mitochondria in apoptosis. *Genes Dev.* **13** 1899-1911.

Gu Q, Korach KS & Moss RL 1999 Rapid action of 17beta-estradiol on kainate-induced currents in hippocampal neurons lacking intracellular estrogen receptors. *Endocrinology* **140** 660-666.

Guengerich FP 1992 Characterization of human cytochrome P450 enzymes. *FASEB J.* **6** 745-748.

Guo M & Hay BA 1999 Cell proliferation and apoptosis. *Curr.Opin.Cell Biol* **11** 745-752.

Habert R 1993 In vivo acute testicular testosterone response to injection of luteinizing hormone in the rat fetus. *Acta Endocrinol.(Copenh)* **128** 268-273.

Habert R & Picon R 1982 Control of testicular steroidogenesis in foetal rat: effect of decapitation on testosterone and plasma luteinizing hormone-like activity. *Acta Endocrinol.(Copenh)* **99** 466-473.

Habert R, Rouiller-Fabre V, Lecerf L, Levacher C & Saez JM 1992 Developmental changes in testosterone production by the rat testis in vitro during late fetal life. *Arch.Androl* **29** 191-197.

Hall JM & McDonnell DP 1999 The estrogen receptor beta-isoform (ERbeta) of the human estrogen receptor modulates ERalpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. *Endocrinology* **140** 5566-5578.

Hall M, Bates S & Peters G 1995 Evidence for different modes of action of cyclin-dependent kinase inhibitors: p15 and p16 bind to kinases, p21 and p27 bind to cyclins. *Oncogene* **11** 1581-1588.

Hannon GJ & Beach D 1994 p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. *Nature* **371** 257-261.

Hanstein B, Liu H, Yancisin MC & Brown M 1999 Functional analysis of a novel estrogen receptor-beta isoform. *Mol.Endocrinol.* **13** 129-137.

Harada N, Yamada K, Saito K, Kibe N, Dohmae S & Takagi Y 1990 Structural characterization of the human estrogen synthetase (aromatase) gene. *Biochem.Biophys.Res.Commun.* **166** 365-372.

Hardy MP, Gelber SJ, Zhou ZF, Penning TM, Ricigliano JW, Ganjam VK, Nonneman D & Ewing LL 1991 Hormonal control of Leydig cell differentiation. *Ann.N.Y.Acad.Sci.* **637** 152-163.

Hardy MP, Kelce WR, Klinefelter GR & Ewing LL 1990 Differentiation of Leydig cell precursors in vitro: a role for androgen. *Endocrinology* **127** 488-490.

Hardy MP, Zirkin BR & Ewing LL 1989 Kinetic studies on the development of the adult population of Leydig cells in testes of the pubertal rat. *Endocrinology* **124** 762-770.

Hardy SP & Valverde MA 1994 Novel plasma membrane action of estrogen and antiestrogens revealed by their regulation of a large conductance chloride channel. *FASEB J.* **8** 760-765.

Harper JW, Adami GR, Wei N, Keyomarsi K & Elledge SJ 1993 The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin- dependent kinases. *Cell* **75** 805-816.

Harrison PT, Holmes P & Humfrey CD 1997 Reproductive health in humans and wildlife: are adverse trends associated with environmental chemical exposure? *Sci.Total Environ.* **205** 97-106.

Hartwell LH & Weinert TA 1989 Checkpoints: controls that ensure the order of cell cycle events. *Science* **246** 629-634.

Hasegawa M, Zhang Y, Niibe H, Terry NH & Meistrich ML 1998 Resistance of differentiating spermatogonia to radiation-induced apoptosis and loss in p53-deficient mice. *Radiat.Res.* **149** 263-270.

Hastie ND 1994 The genetics of Wilms' tumor--a case of disrupted development. *Annu.Rev.Genet.* **28** 523-558.

Hayes FJ, DeCruz S, Seminara SB, Boepple PA & Crowley WF, Jr. 2001 Differential regulation of gonadotropin secretion by testosterone in the human male: absence of a negative feedback effect of testosterone on follicle-stimulating hormone secretion. *J.Clin.Endocrinol.Metab* **86** 53-58.

Helin K 1998 Regulation of cell proliferation by the E2F transcription factors. *Curr.Opin.Genet.Dev.* **8** 28-35.

Hengartner MO 2000 The biochemistry of apoptosis. *Nature* **407** 770-776.

Henriksen K, Hakovirta H & Parvinen M 1995 Testosterone inhibits and induces apoptosis in rat seminiferous tubules in a stage-specific manner: in situ quantification in squash preparations after administration of ethane dimethane sulfonate. *Endocrinology* **136** 3285-3291.

Hess RA 1990 Quantitative and qualitative characteristics of the stages and transitions in the cycle of the rat seminiferous epithelium: light microscopic observations of perfusion-fixed and plastic-embedded testes. *Biol Reprod.* **43** 525-542.

Hess RA 2000 Oestrogen in fluid transport in efferent ducts of the male reproductive tract. *Rev.Reprod.* **5** 84-92.

Hess RA, Bunick D & Bahr JM 1995 Sperm, a source of estrogen. *Environ.Health Perspect.* **103 Suppl 7** 59-62.

Hess RA, Bunick D, Lee KH, Bahr J, Taylor JA, Korach KS & Lubahn DB 1997a A role for oestrogens in the male reproductive system [see comments]. *Nature* **390** 509-512.

Hess RA, Gist DH, Bunick D, Lubahn DB, Farrell A, Bahr J, Cooke PS & Greene GL 1997b Estrogen receptor (alpha and beta) expression in the excurrent ducts of the adult male rat reproductive tract. *J.Androl* **18** 602-611.

Hinds PW & Weinberg RA 1994 Tumor suppressor genes. *Curr.Opin.Genet.Dev.* **4** 135-141.

Hinsch KD, Aires V, Hagele W & Hinsch E 2000 In vitro tests for essential sperm functions using the phyto-oestrogen genistein as a test substance. *Andrologia* **32** 225-231.

Holmberg C, Helin K, Sehested M & Karlstrom O 1998 E2F-1-induced p53-independent apoptosis in transgenic mice. *Oncogene* **17** 143-155.

Honda S, Harada N, Ito S, Takagi Y & Maeda S 1998 Disruption of sexual behavior in male aromatase-deficient mice lacking exons 1 and 2 of the cyp19 gene. *Biochem.Biophys.Res.Commun.* **252** 445-449.

Houtsmuller EJ, Brand T, de Jonge FH, Joosten RN, van de Poll NE & Slob AK 1994 SDN-POA volume, sexual behavior, and partner preference of male rats affected by perinatal treatment with ATD. *Physiol Behav.* **56** 535-541.

Huckins C 1978 The morphology and kinetics of spermatogonial degeneration in normal adult rats: an analysis using a simplified classification of the germinal epithelium. *Anat.Rec.* **190** 905-926.

Hudson B & Burger H 1979 Physiology and function of the testis. In *Human Reproductive Physiology*, edn 2nd, Ed R Shearman. Melbourne: Blackwell Scientific Publications.

Hughes IA, Coleman N, Faisal AS, Ng KL, Cheng A, Lim HN & Hawkins JR 1999 Sexual dimorphism in the neonatal gonad. *Acta Paediatr.Suppl* **88** 23-30.

Huhtaniemi I & Toppari J 1995 Endocrine, paracrine and autocrine regulation of testicular steroidogenesis. *Adv.Exp.Med.Biol* **377** 33-54.

Huhtaniemi IT, Nevo N, Amsterdam A & Naor Z 1986 Effect of postnatal treatment with a gonadotropin-releasing hormone antagonist on sexual maturation of male rats. *Biol Reprod.* **35** 501-507.

Hulleman E & Boonstra J 2001 Regulation of G1 phase progression by growth factors and the extracellular matrix. *Cell Mol.Life Sci.* **58** 80-93.

Hunter T & Pines J 1994 Cyclins and cancer. II: Cyclin D and CDK inhibitors come of age. *Cell* **79** 573-582.

Hurd C, Khattree N, Dinda S, Alban P & Moudgil VK 1997 Regulation of tumor suppressor proteins, p53 and retinoblastoma, by estrogen and antiestrogens in breast cancer cells. *Oncogene* **15** 991-995.

Hutchison JB 1997 Gender-specific steroid metabolism in neural differentiation. *Cell Mol.Neurobiol.* **17** 603-626.

Hutchison JB, Beyer C, Hutchison RE & Wozniak A 1997 Sex differences in the regulation of embryonic brain aromatase. *J.Steroid Biochem.Mol.Biol.* **61** 315-322.

Hutson JM, Baker M, Terada M, Zhou B & Paxton G 1994 Hormonal control of testicular descent and the cause of cryptorchidism. *Reprod.Fertil.Dev.* **6** 151-156.

Hutson JM & Donahoe PK 1986 The hormonal control of testicular descent. *Endocr.Rev.* **7** 270-283.

Hutson JM, Hasthorpe S & Heyns CF 1997 Anatomical and functional aspects of testicular descent and cryptorchidism. *Endocr.Rev.* **18** 259-280.

Idaomar M, Guerin JF, Lornage J & Czyba JC 1989 Stimulation of motility and energy metabolism of spermatozoa from asthenozoospermic patients by 17 beta-estradiol. *Arch.Androl* **22** 197-202.

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

Ikeda Y, Lala DS, Luo X, Kim E, Moisan MP & Parker KL 1993 Characterization of the mouse FTZ-F1 gene, which encodes a key regulator of steroid hydroxylase gene expression. *Mol.Endocrinol.* **7** 852-860.

Ilio KY & Hess RA 1994 Structure and function of the ductuli efferentes: a review. *Microsc.Res.Tech.* **29** 432-467.

Inagaki-Ohara K, Nishimura H, Sakai T, Lynch DH & Yoshikai Y 1997 Potential for involvement of Fas antigen/Fas ligand interaction in apoptosis of epithelial cells by intraepithelial lymphocytes in murine small intestine. *Lab Invest* **77** 421-429.

Irmler M, Thome M, Hahne M, Schneider P, Hofmann K, Steiner V, Bodmer JL, Schroter M, Burns K, Mattmann C, Rimoldi D, French LE & Tschopp J 1997 Inhibition of death receptor signals by cellular FLIP. *Nature* **388** 190-195.

Jacks T & Weinberg RA 1998 The expanding role of cell cycle regulators. *Science* **280** 1035-1036.

Janulis L, Bahr JM, Hess RA & Bunick D 1996a P450 aromatase messenger ribonucleic acid expression in male rat germ cells: detection by reverse transcription-polymerase chain reaction amplification. *J.Androl* **17** 651-658.

Janulis L, Bahr JM, Hess RA, Janssen S, Osawa Y & Bunick D 1998 Rat testicular germ cells and epididymal sperm contain active P450 aromatase. *J.Androl* **19** 65-71.

Janulis L, Hess RA, Bunick D, Nitta H, Janssen S, Asawa Y & Bahr JM 1996b Mouse epididymal sperm contain active P450 aromatase which decreases as sperm traverse the epididymis. *J.Androl* **17** 111-116.

Jefferson WN, Couse JF, Banks EP, Korach KS & Newbold RR 2000 Expression of estrogen receptor beta is developmentally regulated in reproductive tissues of male and female mice. *Biol.Reprod.* **62** 310-317.

Jefferson WN & Newbold RR 2000 Potential endocrine-modulating effects of various phytoestrogens in the diet. *Nutrition* **16** 658-662.

Jegou B 1992 The Sertoli cell. *Baillieres Clin.Endocrinol.Metab* **6** 273-311.

Jegou B 1993 The Sertoli-germ cell communication network in mammals. *Int.Rev.Cytol.* **147** 25-96.

Jegou B, Le Gac F & de Kretser DM 1982 Seminiferous tubule fluid and interstitial fluid production. I. Effects of age and hormonal regulation in immature rats. *Biol Reprod.* **27** 590-595.

Jenkins C, Michael D, Mahendroo M & Simpson E 1993 Exon-specific northern analysis and rapid amplification of cDNA ends (RACE) reveal that the proximal promoter II (PII) is responsible for aromatase cytochrome P450 (CYP19) expression in human ovary. *Mol.Cell Endocrinol.* **97** R1-R6.

Jensen EV & DeSombre ER 1973 Estrogen-receptor interaction. *Science* **182** 126-134.

Jensen EV & Jacobson H 1962 Basic guides to the mechanism of estrogen action. *Recent Prog.Horm.Res.* **18** 387-414.

Jensen TK, Toppari J, Keiding N & Skakkebaek NE 1995 Do environmental estrogens contribute to the decline in male reproductive health? *Clin.Chem.* **41** 1896-1901.

Jones ME, Thorburn AW, Britt KL, Hewitt KN, Wreford NG, Proietto J, Oz OK, Leury BJ, Robertson KM, Yao S & Simpson ER 2000 Aromatase-deficient (ArKO) mice have a phenotype of increased adiposity [In Process Citation]. *Proc.Natl.Acad.Sci.U.S.A* **97** 12735-12740.

Jones SM & Kazlauskas A 2001 Growth factor-dependent signaling and cell cycle progression. *FEBS Lett.* **490** 110-116.

Junker WU & Nogues V 1994 Changes induced by treatment with aromatase inhibitors in testicular Leydig cells of rats and dogs. *Exp.Toxicol.Pathol.* **46** 211-213.

Kalla NR, Nisula BC, Menard R & Loriaux DL 1980 The effect of estradiol on testicular testosterone biosynthesis. *Endocrinology* **106** 35-39.

Kamada S, Shimono A, Shinto Y, Tsujimura T, Takahashi T, Noda T, Kitamura Y, Kondoh H & Tsujimoto Y 1995 bcl-2 deficiency in mice leads to pleiotropic abnormalities: accelerated lymphoid cell death in thymus and spleen, polycystic kidney, hair hypopigmentation, and distorted small intestine. *Cancer Res.* **55** 354-359.

Kang MJ, Kim MK, Terhune A, Park JK, Kim YH & Koh GY 1997 Cytoplasmic localization of cyclin D3 in seminiferous tubules during testicular development. *Exp.Cell Res.* **234** 27-36.

Kangasniemi M, Kaipia A, Toppari J, Perheentupa A, Huhtaniemi I & Parvinen M 1990 Cellular regulation of follicle-stimulating hormone (FSH) binding in rat seminiferous tubules. *J.Androl* **11** 336-343.

Karolczak M, Kuppers E & Beyer C 1998 Developmental expression and regulation of aromatase- and 5alpha- reductase type I mRNA in the male and female mouse hypothalamus. *J.Neuroendocrinol.* **10** 267-274.

Karthikeyan N & Thampan RV 1996 Plasma membrane is the primary site of localization of the nonactivated estrogen receptor in the goat uterus: hormone binding causes receptor internalization. *Arch.Biochem.Biophys.* **325** 47-57.

Kato S, Kitamoto T, Masuhiro Y & Yanagisawa J 1998 Molecular mechanism of a cross-talk between estrogen and growth-factor signaling pathways. *Oncology* **55 Suppl 1** 5-10.

Kato SS 2001 Estrogen receptor-mediated cross-talk with growth factor signaling pathways. *Breast Cancer* **8** 3-9.

Katzenellenbogen BS, Montano MM, Ediger TR, Sun J, Ekena K, Lazennec G, Martini PG, McInerney EM, Delage-Mourroux R, Weis K & Katzenellenbogen JA 2000 Estrogen receptors: selective ligands, partners, and distinctive pharmacology. *Recent Prog.Horm.Res.* **55** 163-193.

Kelly DL & Rizzino A 1999 Growth regulatory factors and carcinogenesis: the roles played by transforming growth factor beta, its receptors and signaling pathways. *Anticancer Res.* **19** 4791-4807.

Kerr JB, Millar M, Maddocks S & Sharpe RM 1993 Stage-dependent changes in spermatogenesis and Sertoli cells in relation to the onset of spermatogenic failure following withdrawal of testosterone. *Anat.Rec.* **235** 547-559.

Kerr JB & Sharpe RM 1985 Stimulatory effect of follicle-stimulating hormone on rat Leydig cells. A morphometric and ultrastructural study. *Cell Tissue Res.* **239** 405-415.

Khan S, Teerds K & Dorrington J 1992a Growth factor requirements for DNA synthesis by Leydig cells from the immature rat. *Biol Reprod.* **46** 335-341.

Khan SA, Khan SJ & Dorrington JH 1992b Interleukin-1 stimulates deoxyribonucleic acid synthesis in immature rat Leydig cells in vitro. *Endocrinology* **131** 1853-1857.

Kierszenbaum AL 2001 Apoptosis during spermatogenesis: the thrill of being alive. *Mol.Reprod.Dev.* **58** 1-3.

Kiess W & Gallaher B 1998 Hormonal control of programmed cell death/apoptosis. *Eur.J.Endocrinol.* **138** 482-491.

Kimura M & Matsuzawa A 1994 Autoimmunity in mice bearing lprcg: a novel mutant gene. *Int.Rev.Immunol.* **11** 193-210.

King KL & Cidlowski JA 1995 Cell cycle and apoptosis: common pathways to life and death. *J.Cell Biochem.* **58** 175-180.

King KL & Cidlowski JA 1998 Cell cycle regulation and apoptosis. *Annu.Rev.Physiol* **60** 601-617.

Kirma N, Gill K, Mandava U & Tekmal RR 2001 Overexpression of aromatase leads to hyperplasia and changes in the expression of genes involved in apoptosis, cell cycle, growth, and tumor suppressor functions in the mammary glands of transgenic mice. *Cancer Res.* **61** 1910-1918.

Kluck RM, Bossy-Wetzel E, Green DR & Newmeyer DD 1997 The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* **275** 1132-1136.

Knudson CM, Tung KS, Tourtellotte WG, Brown GA & Korsmeyer SJ 1995 Bax-deficient mice with lymphoid hyperplasia and male germ cell death. *Science* **270** 96-99.

Koji T, Hishikawa Y, Ando H, Nakanishi Y & Kobayashi N 2001 Expression of Fas and Fas ligand in normal and ischemia-reperfusion testes: involvement of the Fas system in the induction of germ cell apoptosis in the damaged mouse testis. *Biol Reprod.* **64** 946-954.

Kong WH, Zheng G, LU JN & Tso JK 2000 Temperature dependent expression of cdc2 and cyclin B1 in spermatogenic cells during spermatogenesis. *Cell Res.* **10** 289-302.

Kothakota S, Azuma T, Reinhard C, Klippel A, Tang J, Chu K, McGarry TJ, Kirschner MW, Koths K, Kwiatkowski DJ & Williams LT 1997 Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. *Science* **278** 294-298.

Kraichely DM, Sun J, Katzenellenbogen JA & Katzenellenbogen BS 2000 Conformational changes and coactivator recruitment by novel ligands for estrogen receptor-alpha and estrogen receptor-beta: correlations with biological character and distinct differences among SRC coactivator family members. *Endocrinology* **141** 3534-3545.

Krege JH, Hodgin JB, Couse JF, Enmark E, Warner M, Mahler JF, Sar M, Korach KS, Gustafsson JA & Smithies O 1998 Generation and reproductive phenotypes of mice lacking estrogen receptor beta. *Proc.Natl.Acad.Sci.U.S.A* **95** 15677-15682.

Krek W, Xu G & Livingston DM 1995 Cyclin A-kinase regulation of E2F-1 DNA binding function underlies suppression of an S phase checkpoint. *Cell* **83** 1149-1158.

Krishnamurthy H, Danilovich N, Morales CR & Sairam MR 2000 Qualitative and quantitative decline in spermatogenesis of the follicle- stimulating hormone receptor knockout (FORKO) mouse. *Biol Reprod.* **62** 1146-1159.

Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S & Gustafsson JA 1997 Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* **138** 863-870.

Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S & Gustafsson JA 1996 Cloning of a novel receptor expressed in rat prostate and ovary. *Proc.Natl.Acad.Sci.U.S.A* **93** 5925-5930.

Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der BB & Gustafsson JA 1998 Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology* **139** 4252-4263.

Kula K 1988 Induction of precocious maturation of spermatogenesis in infant rats by human menopausal gonadotropin and inhibition by simultaneous administration of gonadotropins and testosterone. *Endocrinology* **122** 34-39.

Kumar TR, Wang Y, Lu N & Matzuk MM 1997 Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. *Nat.Genet.* **15** 201-204.

Kumari GL, Allag IS, Das RP & Datta JK 1980 Regional differences in steroidogenesis and hormone levels in the epididymis and vas deferens of adult rats. *Int.J.Androl* **3** 267-281.

Kurosumi M, Ishimura K, Fujita H & Osawa Y 1985 Immunocytochemical localization of aromatase in rat testis. *Histochemistry* **83** 401-404.

Lacroix M, Smith FE & Fritz IB 1977 Secretion of plasminogen activator by Sertoli cell enriched cultures. *Mol.Cell Endocrinol.* **9** 227-236.

Laflamme N, Nappi RE, Drolet G, Labrie C & Rivest S 1998 Expression and neuropeptidergic characterization of estrogen receptors (ERalpha and ERbeta) throughout the rat brain: anatomical evidence of distinct roles of each subtype. *J.Neurobiol.* **36** 357-378.

Lanzino M, Catalano S, Genissel C, Ando S, Carreau S, Hamra K & McPhaul MJ 2001 Aromatase messenger RNA is derived from the proximal promoter of the aromatase gene in Leydig, Sertoli, and germ cells of the rat testis. *Biol Reprod.* **64** 1439-1443.

Le Bail JC, Marre-Fournier F, Nicolas JC & Habrioux G 1998 C19 steroids estrogenic activity in human breast cancer cell lines: importance of dehydroepiandrosterone sulfate at physiological plasma concentration. *Steroids* **63** 678-683.

Le Gac F & de Kretser DM 1982 Inhibin production by Sertoli cell cultures. *Mol.Cell Endocrinol.* **28** 487-498.

Le Magueresse B & Jegou B 1988 In vitro effects of germ cells on the secretory activity of Sertoli cells recovered from rats of different ages. *Endocrinology* **122** 1672-1680.

LeBlanc GA, Bain LJ & Wilson VS 1997 Pesticides: multiple mechanisms of demasculinization. *Mol.Cell Endocrinol.* **126** 1-5.

Lee J, Richburg JH, Shipp EB, Meistrich ML & Boekelheide K 1999 The Fas system, a regulator of testicular germ cell apoptosis, is differentially up-regulated in Sertoli cell versus germ cell injury of the testis. *Endocrinology* **140** 852-858.

Lee J, Richburg JH, Younkin SC & Boekelheide K 1997 The Fas system is a key regulator of germ cell apoptosis in the testis. *Endocrinology* **138** 2081-2088.

Lee KH, Hess RA, Bahr JM, Lubahn DB, Taylor J & Bunick D 2000 Estrogen Receptor alpha Has a Functional Role in the Mouse Rete Testis and Efferent Ductules. *Biol.Reprod.* **63** 1873-1880.

Lee MH, Reynisdottir I & Massague J 1995 Cloning of p57KIP2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. *Genes Dev.* **9** 639-649.

Lejeune H, Habert R & Saez JM 1998 Origin, proliferation and differentiation of Leydig cells. *J.Mol.Endocrinol.* **20** 1-25.

Lephart ED 1996 A review of brain aromatase cytochrome P450. *Brain Res.Brain Res.Rev.* **22** 1-26.

Lephart ED, Simpson ER, McPhaul MJ, Kilgore MW, Wilson JD & Ojeda SR 1992 Brain aromatase cytochrome P-450 messenger RNA levels and enzyme activity during prenatal and perinatal development in the rat. *Brain Res.Mol.Brain Res.* **16** 187-192.

Leung LK & Wang TT 1999 Paradoxical regulation of Bcl-2 family proteins by 17beta-oestradiol in human breast cancer cells MCF-7. *Br.J.Cancer* **81** 387-392.

Levallet J, Bilinska B, Mittre H, Genissel C, Fresnel J & Carreau S 1998a Expression and immunolocalization of functional cytochrome P450 aromatase in mature rat testicular cells. *Biol.Reprod.* **58** 919-926.

Levallet J & Carreau S 1997 [In vitro gene expression of aromatase in rat testicular cells]. *C.R.Acad.Sci.III* **320** 123-129.

Levallet J, Mittre H, Delarue B & Carreau S 1998b Alternative splicing events in the coding region of the cytochrome P450 aromatase gene in male rat germ cells. *J.Mol.Endocrinol.* **20** 305-312.

Levy JR, Faber KA, Ayyash L & Hughes CL, Jr. 1995 The effect of prenatal exposure to the phytoestrogen genistein on sexual differentiation in rats. *Proc.Soc.Exp.Biol.Med.* **208** 60-66.

Lewis TS, Shapiro PS & Ahn NG 1998 Signal transduction through MAP kinase cascades. *Adv.Cancer Res.* **74** 49-139.

Li LH, Jester WF, Jr., Laslett AL & Orth JM 2000 A single dose of Di-(2-ethylhexyl) phthalate in neonatal rats alters gonocytes, reduces sertoli cell proliferation, and decreases cyclin D2 expression. *Toxicol.Appl.Pharmacol.* **166** 222-229.

Li PS 1991 In vitro effects of estradiol, diethylstilbestrol and tamoxifen on testosterone production by purified pig Leydig cells. *Chin J.Physiol* **34** 287-301.

Limanowski A, Miskowiak B, Otulakowski B & Partyka M 1999 Morphometric studies on the testes of rats treated neonatally with oestrogen and subsequently with gonadotrophins and testosterone. *Andrologia* **31** 225-231.

Lindzey J, Wetsel WC, Couse JF, Stoker T, Cooper R & Korach KS 1998 Effects of castration and chronic steroid treatments on hypothalamic gonadotropin-releasing hormone content and pituitary gonadotropins in male wild-type and estrogen receptor-alpha knockout mice. *Endocrinology* **139** 4092-4101.

Liu D, Liao C & Wolgemuth DJ 2000 A role for cyclin A1 in the activation of MPF and G2-M transition during meiosis of male germ cells in mice. *Dev.Biol* **224** 388-400.

Liu D, Matzuk MM, Sung WK, Guo Q, Wang P & Wolgemuth DJ 1998 Cyclin A1 is required for meiosis in the male mouse. *Nat.Genet.* **20** 377-380.

Loeffler M & Kroemer G 2000 The mitochondrion in cell death control: certainties and incognita. *Exp.Cell Res.* **256** 19-26.

London WP 1993 Male sexual development in "a sea of oestrogen". *Lancet* **342** 124.

Lovell-Badge R & Hacker A 1995 The molecular genetics of Sry and its role in mammalian sex determination. *Philos.Trans.R.Soc.Lond B Biol Sci.* **350** 205-214.

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

Luconi M, Muratori M, Forti G & Baldi E 1999 Identification and characterization of a novel functional estrogen receptor on human sperm membrane that interferes with progesterone effects. *J.Clin.Endocrinol.Metab* **84** 1670-1678.

Ludlow JW, Glendening CL, Livingston DM & DeCarprio JA 1993 Specific enzymatic dephosphorylation of the retinoblastoma protein. *Mol.Cell Biol* **13** 367-372.

Ludlow JW, Shon J, Pipas JM, Livingston DM & DeCaprio JA 1990 The retinoblastoma susceptibility gene product undergoes cell cycle- dependent dephosphorylation and binding to and release from SV40 large T. *Cell* **60** 387-396.

Lundberg AS & Weinberg RA 1998 Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. *Mol.Cell Biol* **18** 753-761.

Luo X, Ikeda Y & Parker KL 1994 A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. *Cell* **77** 481-490.

Lysiak JJ, Turner SD & Turner TT 2000 Molecular pathway of germ cell apoptosis following ischemia/reperfusion of the rat testis. *Biol Reprod.* **63** 1465-1472.

Maas RA, Bruning PF, Top B, Breedijk AJ & Peterse HL 1995 Growth arrest associated changes of mRNA levels in breast cancer cells measured by semi-quantitative RT-PCR: potential early indicators of treatment response. *Cancer Lett.* **97** 107-116.

Maggiolini M, Donze O, Jeannin E, Ando S & Picard D 1999 Adrenal androgens stimulate the proliferation of breast cancer cells as direct activators of estrogen receptor alpha. *Cancer Res.* **59** 4864-4869.

Mahato D, Goulding EH, Korach KS & Eddy EM 2000 Spermatogenic cells do not require estrogen receptor-alpha for development or function [see comments]. *Endocrinology* **141** 1273-1276.

Mahendroo MS, Mendelson CR & Simpson ER 1993 Tissue-specific and hormonally controlled alternative promoters regulate aromatase cytochrome P450 gene expression in human adipose tissue. *J.Biol Chem.* **268** 19463-19470.

Majdic G, Millar MR & Saunders PT 1995 Immunolocalisation of androgen receptor to interstitial cells in fetal rat testes and to mesenchymal and epithelial cells of associated ducts. *J.Endocrinol.* **147** 285-293.

Majdic G, Sharpe RM, O'Shaughnessy PJ & Saunders PT 1996 Expression of cytochrome P450 17alpha-hydroxylase/C17-20 lyase in the fetal rat testis is reduced by maternal exposure to exogenous estrogens. *Endocrinology* **137** 1063-1070.

Makela SI, Pylkkanen LH, Santti RS & Adlercreutz H 1995 Dietary soybean may be antiestrogenic in male mice. *J.Nutr.* **125** 437-445.

Makita K, Falck JR & Capdevila JH 1996 Cytochrome P450, the arachidonic acid cascade, and hypertension: new vistas for an old enzyme system. *FASEB J.* **10** 1456-1463.

Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P & . 1995 The nuclear receptor superfamily: the second decade. *Cell* **83** 835-839.

Matsumoto AM, Karpas AE & Bremner WJ 1986 Chronic human chorionic gonadotropin administration in normal men: evidence that follicle-stimulating hormone is necessary for the maintenance of quantitatively normal spermatogenesis in man. *J.Clin.Endocrinol.Metab* **62** 1184-1192.

Matsushime H, Quelle DE, Shurtleff SA, Shibuya M, Sherr CJ & Kato JY 1994 D-type cyclin-dependent kinase activity in mammalian cells. *Mol.Cell Biol* **14** 2066-2076.

Mbizvo MT, Thomas S, Fulgham DL & Alexander NJ 1990 Serum hormone levels affect sperm function. *Fertil.Steril.* **54** 113-120.

McCarrey J 1993 Development of the germ cell. In *Cell and Molecular Biology of the Testis*, pp 58-89. Ed C Ewing. New York: University Press.

McConnell BB, Gregory FJ, Stott FJ, Hara E & Peters G 1999 Induced expression of p16(INK4a) inhibits both. *Mol.Cell Biol* **19** 1981-1989.

McDermott MT & Ridgway EC 1993 Thyroid hormone resistance syndromes. *Am.J.Med.* **94** 424-432.

McDonnell DP 1999 The Molecular Pharmacology of SERMs. *Trends Endocrinol.Metab* **10** 301-311.

McKenna NJ, Lanz RB & O'Malley BW 1999 Nuclear receptor coregulators: cellular and molecular biology. *Endocr.Rev.* **20** 321-344.

McLachlan JA, Newbold RR & Bullock B 1975 Reproductive tract lesions in male mice exposed prenatally to diethylstilbestrol. *Science* **190** 991-992.

McLachlan JA, Newbold RR, Li S & Negishi M 1998 Are estrogens carcinogenic during development of the testes? *APMIS* **106** 240-242.

McLachlan RI, Wreford N, Robertson DM & de Kretser D 2001 Hormonal control of spermatogenesis. *TEM* **6** 95-101.

McLachlan RI, Wreford NG, de Kretser DM & Robertson DM 1995a The effects of recombinant follicle-stimulating hormone on the restoration of spermatogenesis in the gonadotropin-releasing hormone- immunized adult rat. *Endocrinology* **136** 4035-4043.

McLachlan RI, Wreford NG, O'Donnell L, de Kretser DM & Robertson DM 1996 The endocrine regulation of spermatogenesis: independent roles for testosterone and FSH. *J.Endocrinol.* **148** 1-9.

McLachlan R, Wreford N, Robertson D & de Kretser D 1995b Hormonal control of spermatogenesis. *TEM* **6** 95-101.

Meachem SJ, Wreford NG, Stanton PG, Robertson DM & McLachlan RI 1998 Follicle-stimulating hormone is required for the initial phase of spermatogenic restoration in adult rats following gonadotropin suppression. *J.Androl* **19** 725-735.

Means GD, Mahendroo MS, Corbin CJ, Mathis JM, Powell FE, Mendelson CR & Simpson ER 1989 Structural analysis of the gene encoding human aromatase cytochrome P- 450, the enzyme responsible for estrogen biosynthesis. *J.Biol Chem.* **264** 19385-19391.

Meehan T, Loveland KL, de Kretser D, Cory S & Print CG 2001 Developmental regulation of the bcl-2 family during spermatogenesis: Insights into the sterility of bcl-w-/- male mice. *Cell Death.Differ.* **8** 225-233.

Meisel R & Sachs B 1994 The Physiology of Male Sexual Behavior. In *The Physiology of Reproduction*, edn II, pp 3-106. Eds E Knobil & J Neill. New York: Raven Press.

Mendis-Handagama SM, Watkins PA, Gelber SJ & Scallen TJ 1998 The effect of chronic luteinizing hormone treatment on adult rat Leydig cells. *Tissue Cell* **30** 64-73.

Merchant-Larios H, Moreno-Mendoza N & Buehr M 1993 The role of the mesonephros in cell differentiation and morphogenesis of the mouse fetal testis. *Int.J.Dev.Biol* **37** 407-415.

Michael MD, Kilgore MW, Morohashi K & Simpson ER 1995 Ad4BP/SF-1 regulates cyclic AMP-induced transcription from the proximal promoter (PII) of the human aromatase P450 (CYP19) gene in the ovary. *J.Biol Chem.* **270** 13561-13566.

Michael MD, Michael LF & Simpson ER 1997 A CRE-like sequence that binds CREB and contributes to cAMP-dependent regulation of the proximal promoter of the human aromatase P450 (CYP19) gene. *Mol.Cell Endocrinol.* **134** 147-156.

Minami T, Oomura Y, Nabekura J & Fukuda A 1990 17 beta-estradiol depolarization of hypothalamic neurons is mediated by cyclic AMP. *Brain Res.* **519** 301-307.

Mishina Y, Rey R, Finegold MJ, Matzuk MM, Josso N, Cate RL & Behringer RR 1996 Genetic analysis of the Mullerian-inhibiting substance signal transduction pathway in mammalian sexual differentiation. *Genes Dev.* **10** 2577-2587.

Mitchner NA, Garlick C & Ben Jonathan N 1998 Cellular distribution and gene regulation of estrogen receptors alpha and beta in the rat pituitary gland. *Endocrinology* **139** 3976-3983.

Miura T, Miura C, Ohta T, Nader MR, Todo T & Yamauchi K 1999 Estradiol-17beta stimulates the renewal of spermatogonial stem cells in males. *Biochem.Biophys.Res.Commun.* **264** 230-234.

Miyashita T, Harigai M, Hanada M & Reed JC 1994 Identification of a p53-dependent negative response element in the bcl- 2 gene. *Cancer Res.* **54** 3131-3135.

Miyashita T & Reed JC 1995 Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* **80** 293-299.

Moore A, Findlay K & Morris ID 1992 In-vitro DNA synthesis in Leydig and other interstitial cells of the rat testis. *J.Endocrinol.* **134** 247-255.

Moore A & Morris ID 1993 The involvement of insulin-like growth factor-I in local control of steroidogenesis and DNA synthesis of Leydig and non-Leydig cells in the rat testicular interstitium. *J.Endocrinol.* **138** 107-114.

Mor G, Kohen F, Garcia-Velasco J, Nilsen J, Brown W, Song J & Naftolin F 2000 Regulation of fas ligand expression in breast cancer cells by estrogen: functional differences between estradiol and tamoxifen. *J.Steroid Biochem.Mol.Biol* **73** 185-194.

Morais dS, Hacker A, Harley V, Goodfellow P, Swain A & Lovell-Badge R 1996 Sox9 expression during gonadal development implies a conserved role for the gene in testis differentiation in mammals and birds. *Nat.Genet.* **14** 62-68.

Morgan DO 1995 Principles of CDK regulation. *Nature* **374** 131-134.

Mori C, Nakamura N, Dix DJ, Fujioka M, Nakagawa S, Shiota K & Eddy EM 1997 Morphological analysis of germ cell apoptosis during postnatal testis development in normal and Hsp 70-2 knockout mice. *Dev.Dyn.* **208** 125-136.

Mori H & Christensen AK 1980 Morphometric analysis of Leydig cells in the normal rat testis. *J.Cell Biol.* **84** 340-354.

Morishima A, Grumbach MM, Simpson ER, Fisher C & Qin K 1995 Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens. *J.Clin.Endocrinol.Metab* **80** 3689-3698.

Morley P, Whitfield JF, Vanderhyden BC, Tsang BK & Schwartz JL 1992 A new, nongenomic estrogen action: the rapid release of intracellular calcium. *Endocrinology* **131** 1305-1312.

Moss RL, Gu Q & Wong M 1997 Estrogen: nontranscriptional signaling pathway. *Recent Prog.Horm.Res.* **52** 33-68.

Mosselman S, Polman J & Dijkema R 1996 ER beta: identification and characterization of a novel human estrogen receptor. *FEBS Lett.* **392** 49-53.

Motoyama N, Wang F, Roth KA, Sawa H, Nakayama K, Nakayama K, Negishi I, Senju S, Zhang Q, Fujii S & . 1995 Massive cell death of immature hematopoietic cells and neurons in Bcl-x- deficient mice. *Science* **267** 1506-1510.

Moudgal NR, Sairam MR, Krishnamurthy HN, Sridhar S, Krishnamurthy H & Khan H 1997 Immunization of male bonnet monkeys (M. radiata) with a recombinant FSH receptor preparation affects testicular function and fertility. *Endocrinology* **138** 3065-3068.

Moudgil VK, Dinda S, Khattree N, Jhanwar S, Alban P & Hurd C 2001 Hormonal regulation of tumor suppressor proteins in breast cancer cells. *J.Steroid Biochem.Mol.Biol* **76** 105-117.

Mulligan G & Jacks T 1998 The retinoblastoma gene family: cousins with overlapping interests. *Trends Genet.* **14** 223-229.

Murata, Y, Gong, E, Clyne, C, Aranda, C, Vasquez, M, Tubert, G, Simpson, E, and Maffei, L. Point mutation in the CYP19 gene and its consequences. 2001: Personal Communication

Murkies A 1998 Phytoestrogens--what is the current knowledge? *Aust.Fam.Physician* **27 Suppl 1** S47-S51.

Murono EP 1989 Maturational changes in steroidogenic enzyme activities metabolizing testosterone and dihydrotestosterone in two populations of testicular interstitial cells. *Acta Endocrinol.(Copenh)* **121** 477-483.

Murphy L, Jeffcoate IA & O'Shaughnessy PJ 1994 Abnormal Leydig cell development at puberty in the androgen-resistant Tfm mouse. *Endocrinology* **135** 1372-1377.

Musa FR, Tokuda M, Kuwata Y, Ogawa T, Tomizawa K, Konishi R, Takenaka I & Hatase O 1998 Expression of cyclin-dependent kinase 5 and associated cyclins in Leydig and Sertoli cells of the testis. *J.Androl* **19** 657-666.

Muscatelli F, Strom TM, Walker AP, Zanaria E, Recan D, Meindl A, Bardoni B, Guioli S, Zehetner G, Rabl W & . 1994 Mutations in the DAX-1 gene give rise to both X-linked adrenal hypoplasia congenita and hypogonadotropic hypogonadism. *Nature* **372** 672-676.

Nagata S 2000 Apoptotic DNA fragmentation. *Exp.Cell Res.* **256** 12-18.

Nagata S & Golstein P 1995 The Fas death factor. *Science* **267** 1449-1456.

Nagata S & Suda T 1995 Fas and Fas ligand: lpr and gld mutations. *Immunol.Today* **16** 39-43.

Nandi S, Banerjee PP & Zirkin BR 1999 Germ cell apoptosis in the testes of Sprague Dawley rats following testosterone withdrawal by ethane 1,2-dimethanesulfonate administration: relationship to Fas? *Biol Reprod.* **61** 70-75.

Negri-Cesi P, Poletti A & Celotti F 1996 Metabolism of steroids in the brain: a new insight into the role of 5alpha-reductase and aromatase in brain differentiation and functions. *J.Steroid Biochem.Mol.Biol.* **58** 455-466.

Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, Waterman MR, Gotoh O, Coon MJ, Estabrook RW, Gunsalus IC & Nebert DW 1996 P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* **6** 1-42.

Nelson WG & Kastan MB 1994 DNA strand breaks: the DNA template alterations that trigger p53- dependent DNA damage response pathways. *Mol.Cell Biol* **14** 1815-1823.

Neubauer BL, Best KL, Goode RL, Heiman ML, Hoover DM, Robertson DW, Sarosdy MF, Shaar CJ, Tanzer LR & Merriman RL 1992 Comparative antitumor effects of hormonal ablation, estrogen agonist, estrogen cytotoxic derivative, and antiestrogen in the PAIII rat prostatic adenocarcinoma. *Cancer Res.* **52** 4663-4671.

Neuman E, Ladha MH, Lin N, Upton TM, Miller SJ, DiRenzo J, Pestell RG, Hinds PW, Dowdy SF, Brown M & Ewen ME 1997 Cyclin D1 stimulation of estrogen receptor transcriptional activity independent of cdk4. *Mol.Cell Biol* **17** 5338-5347.

Nielsen M, Bjornsdottir S, Hoyer PE & Byskov AG 2000 Ontogeny of oestrogen receptor alpha in gonads and sex ducts of fetal and newborn mice. *J.Reprod.Fertil.* **118** 195-204.

Nilsen J, Mor G & Naftolin F 2000 Estrogen-regulated developmental neuronal apoptosis is determined by estrogen receptor subtype and the Fas/Fas ligand system. *J.Neurobiol.* **43** 64-78.

Nitta H, Bunick D, Hess RA, Janulis L, Newton SC, Millette CF, Osawa Y, Shizuta Y, Toda K & Bahr JM 1993 Germ cells of the mouse testis express P450 aromatase. *Endocrinology* **132** 1396-1401.

Norbury C & Nurse P 1992 Animal cell cycles and their control. *Annu.Rev.Biochem.* **61** 441-470.

O'Donnell L, McLachlan RI, Wreford NG & Robertson DM 1994 Testosterone promotes the conversion of round spermatids between stages VII and VIII of the rat spermatogenic cycle. *Endocrinology* **135** 2608-2614.

O'Donnell L, Robertson KM, Jones ME & Simpson ER 2001 Estrogen and spermatogenesis. *Endocr.Rev.* **22** 289-318.

O'Donnell L, Stanton PG, Bartles JR & Robertson DM 2000 Sertoli cell ectoplasmic specializations in the seminiferous epithelium of the testosterone-suppressed adult rat. *Biol Reprod.* **63** 99-108.

O'Malley BW, Schrader WT, Mani S, Smith C, Weigel NL, Conneely OM & Clark JH 1995 An alternative ligand-independent pathway for activation of steroid receptors. *Recent Prog.Horm.Res.* **50** 333-347.

Oakberg EP 1956 *Am.J.Anat.* **99** 391-413.

Odorisio T, Rodriguez TA, Evans EP, Clarke AR & Burgoyne PS 1998 The meiotic checkpoint monitoring synapsis eliminates spermatocytes via p53-independent apoptosis. *Nat.Genet.* **18** 257-261.

Ogawa S, Chan J, Chester AE, Gustafsson JA, Korach KS & Pfaff DW 1999 Survival of reproductive behaviors in estrogen receptor beta gene- deficient (betaERKO) male and female mice. *Proc.Natl.Acad.Sci.U.S.A* **96** 12887-12892.

Ogawa S, Chester AE, Hewitt SC, Walker VR, Gustafsson JA, Smithies O, Korach KS & Pfaff DW 2000 From the cover: abolition of male sexual behaviors in mice lacking estrogen receptors alpha and beta (alpha beta ERKO). *Proc.Natl.Acad.Sci.U.S.A* **97** 14737-14741.

Ogawa S, Inoue S, Watanabe T, Orimo A, Hosoi T, Ouchi Y & Muramatsu M 1998a Molecular cloning and characterization of human estrogen receptor betacx: a potential inhibitor ofestrogen action in human. *Nucleic Acids Res.* **26** 3505-3512.

Ogawa S, Lubahn DB, Korach KS & Pfaff DW 1997 Behavioral effects of estrogen receptor gene disruption in male mice. *Proc.Natl.Acad.Sci.U.S.A* **94** 1476-1481.

Ogawa S, Washburn TF, Taylor J, Lubahn DB, Korach KS & Pfaff DW 1998b Modifications of testosterone-dependent behaviors by estrogen receptor- alpha gene disruption in male mice. *Endocrinology* **139** 5058-5069.

Ogi S, Tanji N, Yokoyama M, Takeuchi M & Terada N 1998 Involvement of Fas in the apoptosis of mouse germ cells induced by experimental cryptorchidism. *Urol.Res.* **26** 17-21.

Ohigashi T, Ueno M, Iida M, Hirata R, Nakanoma T & Deguchi N 1999 Suppression of default apoptosis in androgen-dependent cells by testosterone-mediated bcl-2 expression. *Int.J.Urol.* **6** 149-155.

Ohta Y, Nishikawa A, Fukazawa Y, Urushitani H, Matsuzawa A, Nishina Y & Iguchi T 1996 Apoptosis in adult mouse testis induced by experimental cryptorchidism. *Acta Anat.(Basel)* **157** 195-204.

Oltvai ZN, Milliman CL & Korsmeyer SJ 1993 Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* **74** 609-619.

Onate SA, Tsai SY, Tsai MJ & O'Malley BW 1995 Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science* **270** 1354-1357.

Onoda M & Hall PF 1981 Inhibition of testicular microsomal cytochrome P-450 (17 alpha- hydroxylase/C-17,20-lyase) by estrogens. *Endocrinology* **109** 763-767.

Orth JM 1982 Proliferation of Sertoli cells in fetal and postnatal rats: a quantitative autoradiographic study. *Anat.Rec.* **203** 485-492.

Orth JM, Gunsalus GL & Lamperti AA 1988 Evidence from Sertoli cell-depleted rats indicates that spermatid number in adults depends on numbers of Sertoli cells produced during perinatal development. *Endocrinology* **122** 787-794.

Orti E, Bodwell JE & Munck A 1992 Phosphorylation of steroid hormone receptors. *Endocr.Rev.* **13** 105-128.

Pagano M, Pepperkok R, Verde F, Ansorge W & Draetta G 1992 Cyclin A is required at two points in the human cell cycle. *EMBO J.* **11** 961-971.

Pan G, O'Rourke K & Dixit VM 1998 Caspase-9, Bcl-XL, and Apaf-1 form a ternary complex. *J.Biol Chem.* **273** 5841-5845.

Panno ML, Sisci D, Salerno M, Lanzino M, Pezzi V, Morrone EG, Mauro L, Palmero S, Fugassa E & Ando S 1996 Thyroid hormone modulates androgen and oestrogen receptor content in the Sertoli cells of peripubertal rats. *J.Endocrinol.* **148** 43-50.

Papadopoulos V, Carreau S, Szerman-Joly E, Drosdowsky MA, Dehennin L & Scholler R 1986 Rat testis 17 beta-estradiol: identification by gas chromatography-mass spectrometry and age related cellular distribution. *J.Steroid Biochem.* **24** 1211-1216.

Papadopoulos V, Kamtchouing P, Drosdowsky MA, Hochereau de Reviers MT & Carreau S 1987 Adult rat Sertoli cells secrete a factor or factors which modulate Leydig cell function. *J.Endocrinol.* **114** 459-467.

Pappas TC, Gametchu B & Watson CS 1995 Membrane estrogen receptors identified by multiple antibody labeling and impeded-ligand binding. *FASEB J.* **9** 404-410.

Parker KL 1998 The roles of steroidogenic factor 1 in endocrine development and function. *Mol.Cell Endocrinol.* **145** 15-20.

Parker KL, Schedl A & Schimmer BP 1999 Gene interactions in gonadal development. *Annu.Rev.Physiol* **61** 417-433.

Parrott RF & Booth WD 1984 Behavioural and morphological effects of 5 alpha-dihydrotestosterone and oestradiol-17 beta in the prepubertally castrated boar. *J.Reprod.Fertil.* **71** 453-461.

Parry D, Mahony D, Wills K & Lees E 1999 Cyclin D-CDK subunit arrangement is dependent on the availability of competing INK4 and p21 class inhibitors. *Mol.Cell Biol* **19** 1775-1783.

Patterson MN, McPhaul MJ & Hughes IA 1994 Androgen insensitivity syndrome. *Baillieres Clin.Endocrinol.Metab* **8** 379-404.

Payne AH 1990 Hormonal regulation of cytochrome P450 enzymes, cholesterol side-chain cleavage and 17 alpha-hydroxylase/C17-20 lyase in Leydig cells. *Biol Reprod.* **42** 399-404.

Payne AH, Kelch RP, Musich SS & Halpern ME 1976 Intratesticular site of aromatization in the human. *J.Clin.Endocrinol.Metab* **42** 1081-1087.

Payne AH & Sha LL 1991 Multiple mechanisms for regulation of 3 beta-hydroxysteroid dehydrogenase/delta 5----delta 4-isomerase, 17 alpha-hydroxylase/C17-20 lyase cytochrome P450, and cholesterol side-chain cleavage cytochrome P450 messenger ribonucleic acid levels in primary cultures of mouse Leydig cells. *Endocrinology* **129** 1429-1435.

Payne AH & Youngblood GL 1995 Regulation of expression of steroidogenic enzymes in Leydig cells. *Biol Reprod.* **52** 217-225.

Pelissero C, Lenczowski MJ, Chinzi D, Davail-Cuisset B, Sumpter JP & Fostier A 1996 Effects of flavonoids on aromatase activity, an in vitro study. *J.Steroid Biochem.Mol.Biol.* **57** 215-223.

Pelletier G, Dupont E, Simard J, Luu-The V, Belanger A & Labrie F 1992 Ontogeny and subcellular localization of 3 beta-hydroxysteroid dehydrogenase (3 beta-HSD) in the human and rat adrenal, ovary and testis. *J.Steroid Biochem.Mol.Biol.* **43** 451-467.

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

Pelletier G, Luu-The V, Charbonneau A & Labrie F 1999 Cellular localization of estrogen receptor beta messenger ribonucleic acid in cynomolgus monkey reproductive organs. *Biol.Reprod.* **61** 1249-1255.

Pelletier J, Schalling M, Buckler AJ, Rogers A, Haber DA & Housman D 1991 Expression of the Wilms' tumor gene WT1 in the murine urogenital system. *Genes Dev.* **5** 1345-1356.

Pelliniemi LJ, Frojdman K & Paranko J 1993 Embryological and prenatal development and function of Sertoli cells. In *The Sertoli Cell*, pp 87-113. Eds LD Russell & MD Griswold. Clearwater: Cache River Press.

Pentikainen V, Erkkila K & Dunkel L 1999 Fas regulates germ cell apoptosis in the human testis in vitro. *Am.J.Physiol* **276** E310-E316.

Pentikainen V, Erkkila K, Suomalainen L, Parvinen M & Dunkel L 2000 Estradiol acts as a germ cell survival factor in the human testis in vitro [In Process Citation]. *J.Clin.Endocrinol.Metab* **85** 2057-2067.

Perez-Martinez C, Ferreras-Estrada MC, Garcia-Iglesias MJ, Bravo-Moral AM, Espinosa-Alvarez J & Escudero-Diez A 1997 Effects of in utero exposure to nonsteroidal estrogens on mouse testis. *Can.J.Vet.Res.* **61** 94-98.

Perez-Martinez C, Garcia-Iglesias MJ, Ferreras-Estrada MC, Bravo-Moral AM, Espinosa-Alvarez J & Escudero-Diez A 1996 Effects of in-utero exposure to zeranol or diethylstilboestrol on morphological development of the fetal testis in mice. *J.Comp Pathol.* **114** 407-418.

Pestell RG, Albanese C, Reutens AT, Segall JE, Lee RJ & Arnold A 1999 The cyclins and cyclin-dependent kinase inhibitors in hormonal regulation of proliferation and differentiation. *Endocr.Rev.* **20** 501-534.

Pettersson K & Gustafsson JA 2001 Role of estrogen receptor beta in estrogen action. *Annu.Rev.Physiol* **63** 165-192.

Phelps DE, Hsiao KM, Li Y, Hu N, Franklin DS, Westphal E, Lee EY & Xiong Y 1998 Coupled transcriptional and translational control of cyclin-dependent kinase inhibitor p18INK4c expression during myogenesis. *Mol.Cell Biol* **18** 2334-2343.

Pike CJ 1999 Estrogen modulates neuronal Bcl-xL expression and beta-amyloid-induced apoptosis: relevance to Alzheimer's disease. *J.Neurochem.* **72** 1552-1563.

Planas-Silva MD & Weinberg RA 1997 Estrogen-dependent cyclin E-cdk2 activation through p21 redistribution. *Mol.Cell Biol* **17** 4059-4069.

Polyak K, Lee MH, Erdjument-Bromage H, Koff A, Roberts JM, Tempst P & Massague J 1994 Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell* **78** 59-66.

Power RF, Mani SK, Codina J, Conneely OM & O'Malley BW 1991 Dopaminergic and ligand-independent activation of steroid hormone receptors. *Science* **254** 1636-1639.

Prahalada S, Majka JA, Soper KA, Nett TM, Bagdon WJ, Peter CP, Burek JD, MacDonald JS & van Zwieten MJ 1994 Leydig cell hyperplasia and adenomas in mice treated with finasteride, a 5 alpha-reductase inhibitor: a possible mechanism. *Fundam.Appl.Toxicol.* **22** 211-219.

Prall OW, Sarcevic B, Musgrove EA, Watts CK & Sutherland RL 1997 Estrogen-induced activation of Cdk4 and Cdk2 during G1-S phase progression is accompanied by increased cyclin D1 expression and decreased cyclin-dependent kinase inhibitor association with cyclin E- Cdk2. *J.Biol Chem.* **272** 10882-10894.

Pratt WB & Toft DO 1997 Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocr.Rev.* **18** 306-360.

Print CG & Loveland KL 2000 Germ cell suicide: new insights into apoptosis during spermatogenesis. *Bioessays* **22** 423-430.

Print CG, Loveland KL, Gibson L, Meehan T, Stylianou A, Wreford N, de Kretser D, Metcalf D, Kontgen F, Adams JM & Cory S 1998 Apoptosis regulator bcl-w is essential for spermatogenesis but appears otherwise redundant. *Proc.Natl.Acad.Sci.U.S.A* **95** 12424-12431.

Raeside JI & Renaud RL 1983 Estrogen and androgen production by purified Leydig cells of mature boars. *Biol Reprod.* **28** 727-733.

Rajfer J & Walsh PC 1977 Hormonal regulation of testicular descent: experimental and clinical observations. *J.Urol.* **118** 985-990.

Rannikki AS, Zhang FP & Huhtaniemi IT 1995 Ontogeny of follicle-stimulating hormone receptor gene expression in the rat testis and ovary. *Mol.Cell Endocrinol.* **107** 199-208.

Ravnik SE, Rhee K & Wolgemuth DJ 1995 Distinct patterns of expression of the D-type cyclins during testicular development in the mouse. *Dev.Genet.* **16** 171-178.

Ravnik SE & Wolgemuth DJ 1996 The developmentally restricted pattern of expression in the male germ line of a murine cyclin A, cyclin A2, suggests roles in both mitotic and meiotic cell cycles. *Dev.Biol* **173** 69-78.

Ravnik SE & Wolgemuth DJ 1999 Regulation of meiosis during mammalian spermatogenesis: the A-type cyclins and their associated cyclin-dependent kinases are differentially expressed in the germ-cell lineage. *Dev.Biol* **207** 408-418.

Razandi M, Pedram A, Greene GL & Levin ER 1999 Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ERalpha and ERbeta expressed in Chinese hamster ovary cells. *Mol.Endocrinol.* **13** 307-319.

Rea MA, Marshall GR, Weinbauer GF & Nieschlag E 1986 Testosterone maintains pituitary and serum FSH and spermatogenesis in gonadotrophin-releasing hormone antagonist-suppressed rats. *J.Endocrinol.* **108** 101-107.

Reed SI, Dulic V, Lew DJ, Richardson HE & Wittenberg C 1992 G1 control in yeast and animal cells. *Ciba Found.Symp.* **170** 7-15.

Restifo NP 2000 Not so Fas: Re-evaluating the mechanisms of immune privilege and tumor escape. *Nat.Med.* **6** 493-495.

Revelli A, Massobrio M & Tesarik J 1998 Nongenomic actions of steroid hormones in reproductive tissues. *Endocr.Rev.* **19** 3-17.

Rhee K & Wolgemuth DJ 1995 Cdk family genes are expressed not only in dividing but also in terminally differentiated mouse germ cells, suggesting their possible function during both cell division and differentiation. *Dev.Dyn.* **204** 406-420.

Riccioli A, Starace D, D'Alessio A, Starace G, Padula F, De Cesaris P, Filippini A & Ziparo E 2000 TNF-alpha and IFN-gamma regulate expression and function of the Fas system in the seminiferous epithelium. *J.Immunol.* **165** 743-749.

Rich T, Allen RL & Wyllie AH 2000 Defying death after DNA damage. *Nature* **407** 777-783.

Richburg JH 2000 The relevance of spontaneous- and chemically-induced alterations in testicular germ cell apoptosis to toxicology. *Toxicol.Lett.* **112-113** 79-86.

Richburg JH, Nanez A & Gao H 1999 Participation of the Fas-signaling system in the initiation of germ cell apoptosis in young rat testes after exposure to mono-(2- ethylhexyl) phthalate. *Toxicol.Appl.Pharmacol.* **160** 271-278.

Richburg JH, Nanez A, Williams LR, Embree ME & Boekelheide K 2000 Sensitivity of testicular germ cells to toxicant-induced apoptosis in gld mice that express a nonfunctional form of Fas ligand. *Endocrinology* **141** 787-793.

Rigaudiere N, Grizard G & Boucher D 1989 Aromatase activity in purified Leydig cells from adult rat. Comparative effects of insulin, IGF-I, and hCG. *Acta Endocrinol.(Copenh)* **121** 677-685.

Robaire B & Viger RS 1995 Regulation of epididymal epithelial cell functions. *Biol Reprod.* **52** 226-236.

Robertson KM, O'Donnell L, Jones ME, Meachem SJ, Boon WC, Fisher CR, Graves KH, McLachlan RI & Simpson ER 1999 Impairment of spermatogenesis in mice lacking a functional aromatase (cyp 19) gene. *Proc.Natl.Acad.Sci.U.S.A* **96** 7986-7991.

Robertson, K. M., Simpson, E. R., Lacham-Kaplan, O., and Jones, M. E. Characterization of the fertility of male aromatase knockout (ArKO) mice. J.Androl . 2001a.
Ref Type: In Press

Robertson KM, Simpson ER, Lacham-Kaplan O & Jones ME 2001b Characterization of the fertility of male aromatase knockout mice. *J.Androl* **22** 825-830.

Rodriguez I, Ody C, Araki K, Garcia I & Vassalli P 1997 An early and massive wave of germinal cell apoptosis is required for the development of functional spermatogenesis. *EMBO J.* **16** 2262-2270.

Rommerts FF, de Jong FH, Brinkmann AO & van der Molen HJ 1982 Development and cellular localization of rat testicular aromatase activity. *J.Reprod.Fertil.* **65** 281-288.

Roselli CE & Resko JA 1993a Aromatase activity in the rat brain: hormonal regulation and sex differences. *J.Steroid Biochem.Mol.Biol.* **44** 499-508.

Roselli CE & Resko JA 1993b Aromatase activity in the rat brain: hormonal regulation and sex differences. *J.Steroid Biochem.Mol.Biol.* **44** 499-508.

Roselli CE, Stormshak F & Resko JA 1998 Distribution and regulation of aromatase activity in the ram hypothalamus and amygdala. *Brain Res.* **811** 105-110.

Rosenfeld CS, Ganjam VK, Taylor JA, Yuan X, Stiehr JR, Hardy MP & Lubahn DB 1998 Transcription and translation of estrogen receptor-beta in the male reproductive tract of estrogen receptor-alpha knock-out and wild-type mice. *Endocrinology* **139** 2982-2987.

Ross AJ, Waymire KG, Moss JE, Parlow AF, Skinner MK, Russell LD & MacGregor GR 1998 Testicular degeneration in Bclw-deficient mice. *Nat.Genet.* **18** 251-256.

Rouiller-Fabre V, Lecref L, Gautier C, Saez JM & Habert R 1998 Expression and effect of insulin-like growth factor I on rat fetal Leydig cell function and differentiation. *Endocrinology* **139** 2926-2934.

Rucker EB, III, Dierisseau P, Wagner KU, Garrett L, Wynshaw-Boris A, Flaws JA & Hennighausen L 2000 Bcl-x and Bax regulate mouse primordial germ cell survival and apoptosis during embryogenesis. *Mol.Endocrinol.* **14** 1038-1052.

Russell LD 1993a Form, dimensions and cytology of mammalian Sertoli cells. In *The Sertoli Cell*, pp 1-38. Eds LD Russell & MD Griswold. Clearwater, Florida: Cache River Press.

Russell LD 1993b Role in spermiation. In *The Sertoli cell*, Eds LD Russell & MD Griswold. Clearwater, Florida: Cache River Press.

Russell LD 1993c Sertoli cell embryology. In *The Sertoli Cell*, Eds LD Russell & MD Griswold. Clearwater, Florida: Cache River Press.

Russell LD, Alger LE & Nequin LG 1987a Hormonal control of pubertal spermatogenesis. *Endocrinology* **120** 1615-1632.

Russell LD, Alger LE & Nequin LG 1987b Hormonal control of pubertal spermatogenesis. *Endocrinology* **120** 1615-1632.

Russell LD & Clermont Y 1977 Degeneration of germ cells in normal, hypophysectomized and hormone treated hypophysectomized rats. *Anat.Rec.* **187** 347-366.

Russell LD, Corbin TJ, Borg KE, de Franca LR, Grasso P & Bartke A 1993 Recombinant human follicle-stimulating hormone is capable of exerting a biological effect in the adult hypophysectomized rat by reducing the numbers of degenerating germ cells. *Endocrinology* **133** 2062-2070.

Russell L, Ettlin R, Sinha Hikim AP & Clegg ED 1990 *Histological and histopathological evaluation of the testis.* Clearwater, Florida: Cache River Press.

Saberan-Djoneidi D, Picart R, Escalier D, Gelman M, Barret A, Tougard C, Glowinski J & Levi-Strauss M 1998 A 21-kDa polypeptide belonging to a new family of proteins is expressed in the Golgi apparatus of neural and germ cells. *J.Biol Chem.* **273** 3909-3914.

Saez JM 1994 Leydig cells: endocrine, paracrine, and autocrine regulation. *Endocr.Rev.* **15** 574-626.

Sairam MR & Berman MI 1979 Direct inhibitory effects of estrogens on rat Leydig cells in vitro. *Steroids* **33** 233-241.

Sambrook J, Fritsch EF & Maniatis T 1989 *Molecular cloning. A laboratory manual.* Cold Spring Laboratory Press.

Santti R, Makela S, Strauss L, Korkman J & Kostian ML 1998 Phytoestrogens: potential endocrine disruptors in males. *Toxicol.Ind.Health* **14** 223-237.

Sar M & Welsch F 2000 Oestrogen receptor alpha and beta in rat prostate and epididymis. *Andrologia* **32** 295-301.

Sato T, Irie S, Kitada S & Reed JC 1995 FAP-1: a protein tyrosine phosphatase that associates with Fas. *Science* **268** 411-415.

Saunders PT, Fisher JS, Sharpe RM & Millar MR 1998 Expression of oestrogen receptor beta (ER beta) occurs in multiple cell types, including some germ cells, in the rat testis. *J.Endocrinol.* **156** R13-R17.

Saunders PT, Maguire SM, Gaughan J & Millar MR 1997 Expression of oestrogen receptor beta (ER beta) in multiple rat tissues visualised by immunohistochemistry. *J.Endocrinol.* **154** R13-R16.

Savill J & Fadok V 2000 Corpse clearance defines the meaning of cell death. *Nature* **407** 784-788.

Schmalz B & Bilinska B 1998 Immunolocalization of aromatase and estrogen receptors in ram Leydig cells. *Ginekol.Pol.* **69** 512-516.

Schneider P & Tschopp J 2000 Apoptosis induced by death receptors. *Pharm.Acta Helv.* **74** 281-286.

Schwartz D, Goldfinger N & Rotter V 1993 Expression of p53 protein in spermatogenesis is confined to the tetraploid pachytene primary spermatocytes. *Oncogene* **8** 1487-1494.

Serrano M, Hannon GJ & Beach D 1993 A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* **366** 704-707.

Setchell BP, Maddocks S & Brooks DE 1994a Anatomy, vasculature, innervation, and fluids of the male reproductive tract. In *The Physiology of Reproduction*, edn 2nd, pp 1063-1143. Eds E Knobil & J Neill. New York: Raven Press Ltd.

Setchell BP, Maddocks S & Brooks DE 1994b Efferent ducts, epididymis, and vas deferens: structure, function and their regulation. In *The Physiology of Reproduction*, edn second, pp 999-1080. Eds E Knobil & J Neill. New York: Raven Press.

Setchell KD & Cassidy A 1999 Dietary isoflavones: biological effects and relevance to human health. *J.Nutr.* **129** 758S-767S.

Shan LX & Hardy MP 1992 Developmental changes in levels of luteinizing hormone receptor and androgen receptor in rat Leydig cells. *Endocrinology* **131** 1107-1114.

Shan LX, Phillips DM, Bardin CW & Hardy MP 1993 Differential regulation of steroidogenic enzymes during differentiation optimizes testosterone production by adult rat Leydig cells. *Endocrinology* **133** 2277-2283.

Sharpe RM 1997 Do males rely on female hormones? *Nature* **390** 447-448.

Sharpe, R. M. The roles of oestrogen in the male. Trends Endocrinol.Metab 9, 371-377. 1998. Ref Type: Journal (Full)

Sharpe RM, Atanassova N, McKinnell C, Parte P, Turner KJ, Fisher JS, Kerr JB, Groome NP, Macpherson S, Millar MR & Saunders PT 1998 Abnormalities in functional development of the Sertoli cells in rats treated neonatally with diethylstilbestrol: a possible role for estrogens in Sertoli cell development. *Biol.Reprod.* **59** 1084-1094.

Sharpe RM, Maddocks S & Kerr JB 1990 Cell-cell interactions in the control of spermatogenesis as studied using Leydig cell destruction and testosterone replacement. *Am.J.Anat.* **188** 3-20.

Sharpe RM & Skakkebaek NE 1993 Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? [see comments]. *Lancet* **341** 1392-1395.

Sheaff RJ, Groudine M, Gordon M, Roberts JM & Clurman BE 1997 Cyclin E-CDK2 is a regulator of p27Kip1. *Genes Dev.* **11** 1464-1478.

Sherr CJ 1993 Mammalian G1 cyclins. *Cell* **73** 1059-1065.

Sherr CJ 1995 D-type cyclins. *Trends Biochem.Sci.* **20** 187-190.

Sherr CJ & Roberts JM 1995 Inhibitors of mammalian G1 cyclin-dependent kinases. *Genes Dev.* **9** 1149-1163.

Sherr CJ & Roberts JM 1999 CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev.* **13** 1501-1512.

Shetty G, Krishnamurthy H, Krishnamurthy HN, Bhatnagar AS & Moudgal NR 1998 Effect of long-term treatment with aromatase inhibitor on testicular function of adult male bonnet monkeys (M. radiata). *Steroids* **63** 414-420.

Shetty J, Marathe GK & Dighe RR 1996 Specific immunoneutralization of FSH leads to apoptotic cell death of the pachytene spermatocytes and spermatogonial cells in the rat. *Endocrinology* **137** 2179-2182.

Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA & Greene GL 1998 The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* **95** 927-937.

Shibata H, Spencer TE, Onate SA, Jenster G, Tsai SY, Tsai MJ & O'Malley BW 1997 Role of co-activators and co-repressors in the mechanism of steroid/thyroid receptor action. *Recent Prog.Horm.Res.* **52** 141-164.

Shikone T, Billig H & Hsueh AJ 1994 Experimentally induced cryptorchidism increases apoptosis in rat testis. *Biol Reprod.* **51** 865-872.

Shozu M, Zhao Y, Bulun SE & Simpson ER 1998 Multiple splicing events involved in regulation of human aromatase expression by a novel promoter, I.6. *Endocrinology* **139** 1610-1617.

Shughrue PJ, Lane MV & Merchenthaler I 1996 In situ hybridization analysis of the distribution of neurokinin-3 mRNA in the rat central nervous system. *J.Comp Neurol.* **372** 395-414.

Shughrue PJ, Lane MV & Merchenthaler I 1997 Comparative distribution of estrogen receptor-alpha and -beta mRNA in the rat central nervous system. *J.Comp Neurol.* **388** 507-525.

Shughrue PJ, Lane MV, Scrimo PJ & Merchenthaler I 1998 Comparative distribution of estrogen receptor-alpha (ER-alpha) and beta (ER-beta) mRNA in the rat pituitary, gonad, and reproductive tract. *Steroids* **63** 498-504.

Shughrue PJ & Merchenthaler I 2000 Estrogen is more than just a "sex hormone": novel sites for estrogen action in the hippocampus and cerebral cortex. *Front Neuroendocrinol.* **21** 95-101.

Sicinski P, Donaher JL, Geng Y, Parker SB, Gardner H, Park MY, Robker RL, Richards JS, McGinnis LK, Biggers JD, Eppig JJ, Bronson RT, Elledge SJ & Weinberg RA 1996 Cyclin D2 is an FSH-responsive gene involved in gonadal cell proliferation and oncogenesis. *Nature* **384** 470-474.

Simpson ER, Mahendroo MS, Means GD, Kilgore MW, Corbin CJ & Mendelson CR 1993 Tissue-specific promoters regulate aromatase cytochrome P450 expression. *J.Steroid Biochem.Mol.Biol* **44** 321-330.

Simpson ER, Zhao Y, Agarwal VR, Michael MD, Bulun SE, Hinshelwood MM, Graham-Lorence S, Sun T, Fisher CR, Qin K & Mendelson CR 1997 Aromatase expression in health and disease. *Recent Prog.Horm.Res.* **52** 185-213.

Sinclair AH, Berta P, Palmer MS, Hawkins JR, Griffiths BL, Smith MJ, Foster JW, Frischauf AM, Lovell-Badge R & Goodfellow PN 1990 A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. *Nature* **346** 240-244.

Singer CA, Rogers KL & Dorsa DM 1998 Modulation of Bcl-2 expression: a potential component of estrogen protection in NT2 neurons. *Neuroreport* **9** 2565-2568.

Singh J & Handelsman DJ 1996a Neonatal administration of FSH increases Sertoli cell numbers and spermatogenesis in gonadotropin-deficient (hpg) mice. *J.Endocrinol.* **151** 37-48.

Singh J & Handelsman DJ 1996b The effects of recombinant FSH on testosterone-induced spermatogenesis in gonadotrophin-deficient (hpg) mice. *J.Androl* **17** 382-393.

Singh J, O'Neill C & Handelsman DJ 1995 Induction of spermatogenesis by androgens in gonadotropin-deficient (hpg) mice. *Endocrinology* **136** 5311-5321.

Sinha Hikim AP, Rajavashisth TB, Sinha H, I, Lue Y, Bonavera JJ, Leung A, Wang C & Swerdloff RS 1997 Significance of apoptosis in the temporal and stage-specific loss of germ cells in the adult rat after gonadotropin deprivation. *Biol Reprod.* **57** 1193-1201.

Sinha Hikim AP & Swerdloff RS 1999 Hormonal and genetic control of germ cell apoptosis in the testis. *Rev.Reprod.* **4** 38-47.

Sjoblom T & Lahdetie J 1996 Expression of p53 in normal and gamma-irradiated rat testis suggests a role for p53 in meiotic recombination and repair. *Oncogene* **12** 2499-2505.

Skinner MK & Griswold MD 1982 Secretion of testicular transferrin by cultured Sertoli cells is regulated by hormones and retinoids. *Biol Reprod.* **27** 211-221.

Slaughter RL & Edwards DJ 1995 Recent advances: the cytochrome P450 enzymes. *Ann.Pharmacother.* **29** 619-624.

Smith CL 1998 Cross-talk between peptide growth factor and estrogen receptor signaling pathways. *Biol.Reprod.* **58** 627-632.

Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB & Korach KS 1994 Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. *N.Engl.J.Med.* **331** 1056-1061.

Smith MS, Freeman ME & Neill JD 1975 The control of progesterone secretion during the estrous cycle and early pseudopregnancy in the rat: prolactin, gonadotropin and steroid levels associated with rescue of the corpus luteum of pseudopregnancy. *Endocrinology* **96** 219-226.

Spiteri-Grech J & Nieschlag E 1993 Paracrine factors relevant to the regulation of spermatogenesis--a review. *J.Reprod.Fertil.* **98** 1-14.

Stillman RJ 1982 In utero exposure to diethylstilbestrol: adverse effects on the reproductive tract and reproductive performance and male and female offspring. *Am.J.Obstet.Gynecol.* **142** 905-921.

Stocco DM & Clark BJ 1996 Role of the steroidogenic acute regulatory protein (StAR) in steroidogenesis. *Biochem.Pharmacol.* **51** 197-205.

Strausfeld UP, Howell M, Descombes P, Chevalier S, Rempel RE, Adamczewski J, Maller JL, Hunt T & Blow JJ 1996 Both cyclin A and cyclin E have S-phase promoting (SPF) activity in Xenopus egg extracts. *J.Cell Sci.* **109 (Pt 6)** 1555-1563.

Strauss L, Makela S, Joshi S, Huhtaniemi I & Santti R 1998 Genistein exerts estrogen-like effects in male mouse reproductive tract. *Mol.Cell Endocrinol.* **144** 83-93.

Suda T, Okazaki T, Naito Y, Yokota T, Arai N, Ozaki S, Nakao K & Nagata S 1995 Expression of the Fas ligand in cells of T cell lineage. *J.Immunol.* **154** 3806-3813.

Suda T, Takahashi T, Golstein P & Nagata S 1993 Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell* **75** 1169-1178.

Sugihara A, Saiki S, Tsuji M, Tsujimura T, Nakata Y, Kubota A, Kotake T & Terada N 1997 Expression of Fas and Fas ligand in the testes and testicular germ cell tumors: an immunohistochemical study. *Anticancer Res.* **17** 3861-3865.

Sukovich DA, Mukherjee R & Benfield PA 1994 A novel, cell-type-specific mechanism for estrogen receptor-mediated gene activation in the absence of an estrogen-responsive element. *Mol.Cell Biol* **14** 7134-7143.

Sun YT, Wreford NG, Robertson DM & de Kretser DM 1990 Quantitative cytological studies of spermatogenesis in intact and hypophysectomized rats: identification of androgen-dependent stages. *Endocrinology* **127** 1215-1223.

Sutherland RL, Prall OW, Watts CK & Musgrove EA 1998 Estrogen and progestin regulation of cell cycle progression. *J.Mammary.Gland.Biol Neoplasia.* **3** 63-72.

Sweeney C, Murphy M, Kubelka M, Ravnik SE, Hawkins CF, Wolgemuth DJ & Carrington M 1996 A distinct cyclin A is expressed in germ cells in the mouse. *Development* **122** 53-64.

Takahashi T, Tanaka M, Brannan CI, Jenkins NA, Copeland NG, Suda T & Nagata S 1994 Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. *Cell* **76** 969-976.

Tansey G, Hughes CL, Jr., Cline JM, Krummer A, Walmer DK & Schmoltzer S 1998 Effects of dietary soybean estrogens on the reproductive tract in female rats. *Proc.Soc.Exp.Biol.Med.* **217** 340-344.

Tapanainen JS, Tilly JL, Vihko KK & Hsueh AJ 1993 Hormonal control of apoptotic cell death in the testis: gonadotropins and androgens as testicular cell survival factors. *Mol.Endocrinol.* **7** 643-650.

Taya Y 1997 RB kinases and RB-binding proteins: new points of view. *Trends Biochem.Sci.* **22** 14-17.

Taylor MF, Boer-Brouwer M, Woolveridge I, Teerds KJ & Morris ID 1999 Leydig cell apoptosis after the administration of ethane dimethanesulfonate to the adult male rat is a Fas-mediated process. *Endocrinology* **140** 3797-3804.

Teerds KJ 1996 Regeneration of Leydig cells after depletion by EDS: A model for postnatal Leydig cell renewal. In *The Leydig Cell*, pp 203-219. Eds AH Payne, MP Hardy & LD Russell. Vienna, IL: Cache River Press.

Teerds KJ, de Rooij DG, Rommerts FF, van den HR & Wensing CJ 1989 Stimulation of the proliferation and differentiation of Leydig cell precursors after the destruction of existing Leydig cells with ethane dimethyl sulphonate (EDS) can take place in the absence of LH. *J.Androl* **10** 472-477.

Tham DM, Gardner CD & Haskell WL 1998 Clinical review 97: Potential health benefits of dietary phytoestrogens: a review of the clinical, epidemiological, and mechanistic evidence. *J.Clin.Endocrinol.Metab* **83** 2223-2235.

Thigpen JE, Setchell KD, Ahlmark KB, Locklear J, Spahr T, Caviness GF, Goelz MF, Haseman JK, Newbold RR & Forsythe DB 1999 Phytoestrogen content of purified, open- and closed-formula laboratory animal diets. *Lab Anim Sci.* **49** 530-536.

Thomas T & Thomas TJ 1994 Regulation of cyclin B1 by estradiol and polyamines in MCF-7 breast cancer cells. *Cancer Res.* **54** 1077-1084.

Thornberry NA 1997 The caspase family of cysteine proteases. *Br.Med.Bull.* **53** 478-490.

Tobet SA, Baum MJ, Tang HB, Shim JH & Canick JA 1985 Aromatase activity in the perinatal rat forebrain: effects of age, sex and intrauterine position. *Brain Res.* **355** 171-178.

Toda K, Okada T, Takeda K, Akira S, Saibara T, Shiraishi M, Onishi S & Shizuta Y 2001a Oestrogen at the neonatal stage is critical for the reproductive ability of male mice as revealed by supplementation with 17beta- oestradiol to aromatase gene (Cyp19) knockout mice. *J.Endocrinol.* **168** 455-463.

Toda K, Takeda K, Okada T, Akira S, Saibara T, Kaname T, Yamamura K, Onishi S & Shizuta Y 2001b Targeted disruption of the aromatase P450 gene (Cyp19) in mice and their ovarian and uterine responses to 17beta-oestradiol. *J.Endocrinol.* **170** 99-111.

Toda K, Terashima M, Kawamoto T, Sumimoto H, Yokoyama Y, Kuribayashi I, Mitsuuchi Y, Maeda T, Yamamoto Y, Sagara Y & . 1990 Structural and functional characterization of human aromatase P-450 gene. *Eur.J.Biochem.* **193** 559-565.

Tong W & Pollard JW 1999 Progesterone inhibits estrogen-induced cyclin D1 and cdk4 nuclear translocation, cyclin E- and cyclin A-cdk2 kinase activation, and cell proliferation in uterine epithelial cells in mice. *Mol.Cell Biol* **19** 2251-2264.

Toppari J, Larsen JC, Christiansen P, Giwercman A, Grandjean P, Guillette LJ, Jegou B, Jensen TK, Jouannet P, Keiding N, Leffers H, McLachlan JA, Meyer O, Muller J, Rajpert-De Meyts E, Scheike T, Sharpe R, Sumpter J & Skakkebaek NE 1996 Male reproductive health and environmental xenoestrogens. *Environ.Health Perspect.* **104 Suppl 4** 741-803.

Toyoshima H & Hunter T 1994 p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. *Cell* **78** 67-74.

Tremblay A, Tremblay GB, Labrie F & Giguere V 1999 Ligand-independent recruitment of SRC-1 to estrogen receptor beta through phosphorylation of activation function AF-1. *Mol.Cell* **3** 513-519.

Tremblay GB, Tremblay A, Copeland NG, Gilbert DJ, Jenkins NA, Labrie F & Giguere V 1997 Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor beta. *Mol.Endocrinol.* **11** 353-365.

Troiano L, Fustini MF, Lovato E, Frasoldati A, Malorni W, Capri M, Grassilli E, Marrama P & Franceschi C 1994 Apoptosis and spermatogenesis: evidence from an in vivo model of testosterone withdrawal in the adult rat. *Biochem.Biophys.Res.Commun.* **202** 1315-1321.

Trowbridge JM, Rogatsky I & Garabedian MJ 1997 Regulation of estrogen receptor transcriptional enhancement by the cyclin A/Cdk2 complex. *Proc.Natl.Acad.Sci.U.S.A* **94** 10132-10137.

Truchet I, Jozan S, Guerrin M, Mazzolini L, Vidal S & Valette A 2000 Interconnections between E2-dependent regulation of cell cycle progression and apoptosis in MCF-7 tumors growing on nude mice. *Exp.Cell Res.* **254** 241-248.

Tsai-Morris CH, Aquilano DR & Dufau ML 1985 Cellular localization of rat testicular aromatase activity during development. *Endocrinology* **116** 38-46.

Tsai-Morris CH, Knox G, Luna S & Dufau ML 1986 Acquisition of estradiol-mediated regulatory mechanism of steroidogenesis in cultured fetal rat Leydig cells. *J.Biol.Chem.* **261** 3471-3474.

Tsutsui T, Hesabi B, Moons DS, Pandolfi PP, Hansel KS, Koff A & Kiyokawa H 1999 Targeted disruption of CDK4 delays cell cycle entry with enhanced p27(Kip1) activity. *Mol.Cell Biol* **19** 7011-7019.

Tsutsumi I, Fugimori K, Nakamura RM, Mather JP, Ono T & diZerega GS 1987a Disruption of seminiferous epithelial function in the rat by ovarian protein. *Biol.Reprod.* **36** 451-461.

Tsutsumi I, Toppari J, Campeau JD & diZerega GS 1987b Reduction of fertility in the male rat by systemic treatment with follicle regulatory protein. *Fertil.Steril.* **47** 689-695.

Turner KJ, Morley M, Atanassova N, Swanston ID & Sharpe RM 2000b Effect of chronic administration of an aromatase inhibitor to adult male rats on pituitary and testicular function and fertility. *J.Endocrinol.* **164** 225-238.

Turner KJ, Morley M, Atanassova N, Swanston ID & Sharpe RM 2000a Effect of chronic administration of an aromatase inhibitor to adult male rats on pituitary and testicular function and fertility. *J.Endocrinol.* **164** 225-238.

Turner KJ & Sharpe RM 1997 Environmental oestrogens--present understanding. *Rev.Reprod.* **2** 69-73.

Turner TT 1984 Resorption versus secretion in the rat epididymis. *J.Reprod.Fertil.* **72** 509-514.

Ulisse S, Jannini EA, Carosa E, Piersanti D, Graziano FM & D'Armiento M 1994 Inhibition of aromatase activity in rat Sertoli cells by thyroid hormone. *J.Endocrinol.* **140** 431-436.

Ulloa-Aguirre A, Midgley AR, Jr., Beitins IZ & Padmanabhan V 1995 Follicle-stimulating isohormones: characterization and physiological relevance. *Endocr.Rev.* **16** 765-787.

Valladares LE & Payne AH 1979a Acute stimulation of aromatization in Leydig cells by human chorionic gonadotropin in vitro. *Proc.Natl.Acad.Sci.U.S.A* **76** 4460-4463.

Valladares LE & Payne AH 1979b Induction of testicular aromatization by luteinizing hormone in mature rats. *Endocrinology* **105** 431-436.

van der SP 1992 Disturbed testicular descent in the rat after prenatal exposure to the antiandrogen flutamide. *J.Reprod.Fertil.* **96** 483-496.

van Pelt AM, de Rooij DG, van der BB, van der Saag PT, Gustafsson JA & Kuiper GG 1999 Ontogeny of estrogen receptor-beta expression in rat testis. *Endocrinology* **140** 478-483.

Vaux DL & Strasser A 1996 The molecular biology of apoptosis. *Proc.Natl.Acad.Sci.U.S.A* **93** 2239-2244.

Veis DJ, Sorenson CM, Shutter JR & Korsmeyer SJ 1993 Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. *Cell* **75** 229-240.

Vergouwen RP, Jacobs SG, Huiskamp R, Davids JA & de Rooij DG 1991 Proliferative activity of gonocytes, Sertoli cells and interstitial cells during testicular development in mice. *J.Reprod.Fertil.* **93** 233-243.

Verhoeven G & Cailleau J 1988a Prolonged exposure to androgens suppresses follicle-stimulating hormone- induced aromatase activity in rat Sertoli cell cultures. *Mol.Cell Endocrinol.* **57** 61-67.

Verhoeven G & Cailleau J 1988b Testicular peritubular cells secrete a protein under androgen control that inhibits induction of aromatase activity in Sertoli cells. *Endocrinology* **123** 2100-2110.

Viger RS & Robaire B 1995 Steady state steroid 5 alpha-reductase messenger ribonucleic acid levels and immunocytochemical localization of the type 1 protein in the rat testis during postnatal development. *Endocrinology* **136** 5409-5415.

Vitale R, Fawcett DW & Dym M 1973 The normal development of the blood-testis barrier and the effects of clomiphene and estrogen treatment. *Anat.Rec.* **176** 331-344.

Vogl AW, Pfeiffer DC, Mulholland D, Kimel G & Guttman J 2000 Unique and multifunctional adhesion junctions in the testis: ectoplasmic specializations. *Arch.Histol.Cytol.* **63** 1-15.

Vornberger W, Prins G, Musto NA & Suarez-Quian CA 1994 Androgen receptor distribution in rat testis: new implications for androgen regulation of spermatogenesis. *Endocrinology* **134** 2307-2316.

Wagner T, Wirth J, Meyer J, Zabel B, Held M, Zimmer J, Pasantes J, Bricarelli FD, Keutel J, Hustert E & . 1994 Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9. *Cell* **79** 1111-1120.

Wallis CJ & Luttge WG 1975 Maintenance of male sexual behavior by combined treatment with oestrogen and dihydrotestosterone in CD-1 mice. *J.Endocrinol.* **66** 257-262.

Wang C & Kurzer MS 1998 Effects of phytoestrogens on DNA synthesis in MCF-7 cells in the presence of estradiol or growth factors. *Nutr.Cancer* **31** 90-100.

Wang RA, Nakane PK & Koji T 1998 Autonomous cell death of mouse male germ cells during fetal and postnatal period. *Biol Reprod.* **58** 1250-1256.

Wang ZX, Wreford NG & de Kretser DM 1989 Determination of Sertoli cell numbers in the developing rat testis by stereological methods. *Int.J.Androl* **12** 58-64.

Watanabe-Fukunaga R, Brannan CI, Itoh N, Yonehara S, Copeland NG, Jenkins NA & Nagata S 1992 The cDNA structure, expression, and chromosomal assignment of the mouse Fas antigen. *J.Immunol.* **148** 1274-1279.

Watson CS & Gametchu B 1999 Membrane-initiated steroid actions and the proteins that mediate them. *Proc.Soc.Exp.Biol Med.* **220** 9-19.

Watson JT & Adkins-Regan E 1989 Testosterone implanted in the preoptic area of male Japanese quail must be aromatized to activate copulation. *Horm.Behav.* **23** 432-447.

Webb P, Lopez GN, Uht RM & Kushner PJ 1995 Tamoxifen activation of the estrogen receptor/AP-1 pathway: potential origin for the cell-specific estrogen-like effects of antiestrogens. *Mol.Endocrinol.* **9** 443-456.

Weber KS, Setchell KDR, Stocco DM & Lephart ED. Dietary soy-phytoestrogens decrease testosterone levels and prostate weight without altering LH, prostate 5αreductase or testicular steroidogenic acute regulatory peptide levels in adult male Sprague-Dawley rats. *J. Endocrinol* **170** 591-599.

Wee BE, Weaver DR & Clemens LG 1988 Hormonal restoration of masculine sexual behavior in long-term castrated B6D2F1 mice. *Physiol Behav.* **42** 77-82.

Wehling M 1997 Specific, nongenomic actions of steroid hormones. *Annu.Rev.Physiol* **59** 365-393.

Weinbauer GF & Nieschlag E 1993 Hormonal control of spermatogenesis. In pp 99-142. San Diego: Academic press.

Weinberg RA 1995 The retinoblastoma protein and cell cycle control. *Cell* **81** 323-330.

Weniger JP 1990 Aromatase activity in fetal gonads of mammals. *J.Dev.Physiol* **14** 303-306.

Weniger JP 1993 Estrogen production by fetal rat gonads. *J.Steroid Biochem.Mol.Biol.* **44** 459-462.

Weniger JP & Zeis A 1987 Oestrogen synthesis by the foetal rat testis in organ culture. *J.Steroid Biochem.* **28** 307-310.

Weniger JP & Zeis A 1988 Stimulation of aromatase activity in the fetal rat testis by cyclic AMP and FSH. *J.Endocrinol.* **118** 485-489.

Wersinger SR & Rissman EF 2000 Dopamine activates masculine sexual behavior independent of the estrogen receptor alpha. *J.Neurosci.* **20** 4248-4254.

Wersinger SR, Sannen K, Villalba C, Lubahn DB, Rissman EF & De Vries GJ 1997 Masculine sexual behavior is disrupted in male and female mice lacking a functional estrogen receptor alpha gene. *Horm.Behav.* **32** 176-183.

Whitten PL, Lewis C, Russell E & Naftolin F 1995a Phytoestrogen influences on the development of behavior and gonadotropin function. *Proc.Soc.Exp.Biol.Med.* **208** 82-86.

Whitten PL, Lewis C, Russell E & Naftolin F 1995b Potential adverse effects of phytoestrogens. *J.Nutr.* **125** 771S-776S.

Whitten PL & Naftolin F 1998 Reproductive actions of phytoestrogens. *Baillieres Clin.Endocrinol.Metab* **12** 667-690.

Winston JT & Pledger WJ 1993 Growth factor regulation of cyclin D1 mRNA expression through protein synthesis-dependent and -independent mechanisms. *Mol.Biol Cell* **4** 1133-1144.

Wolgemuth DJ, Rhee K, Wu S & Ravnik SE 1995 Genetic control of mitosis, meiosis and cellular differentiation during mammalian spermatogenesis. *Reprod.Fertil.Dev.* **7** 669-683.

Woolveridge I, Boer-Brouwer M, Taylor MF, Teerds KJ, Wu FC & Morris ID 1999 Apoptosis in the rat spermatogenic epithelium following androgen withdrawal: changes in apoptosis-related genes. *Biol Reprod.* **60** 461-470.

Wreford NG 1995 Theory and practice of stereological techniques applied to the estimation of cell number and nuclear volume in the testis. *Microsc.Res.Tech.* **32** 423-436.

Wu X & Levine AJ 1994 p53 and E2F-1 cooperate to mediate apoptosis. *Proc.Natl.Acad.Sci.U.S.A* **91** 3602-3606.

Wyllie A, Kerr J & Currie A 1980 Cell Death: The Significance of Apoptosis. In *Pharmacology*, edn 2nd, pp 251-306. Eds Rang & Dale. UK: Churchill Livington.

Wyllie AH 1993 Apoptosis (the 1992 Frank Rose Memorial Lecture). *Br.J.Cancer* **67** 205-208.

Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R & Beach D 1993 p21 is a universal inhibitor of cyclin kinases. *Nature* **366** 701-704.

Xu J, Xu Z, Jiang Y, Qian X & Huang Y 2000 Cryptorchidism induces mouse testicular germ cell apoptosis and changes in bcl-2 and bax protein expression. *J.Environ.Pathol.Toxicol.Oncol.* **19** 25-33.

Xu JP, Li X, Mori E, Guo MW, Matsuda I, Takaichi H, Amano T & Mori T 1999 Expression of Fas-Fas ligand in murine testis. *Am.J.Reprod.Immunol.* **42** 381-388.

Xu M, Sheppard KA, Peng CY, Yee AS & Piwnica-Worms H 1994 Cyclin A/CDK2 binds directly to E2F-1 and inhibits the DNA-binding activity of E2F-1/DP-1 by phosphorylation. *Mol.Cell Biol* **14** 8420-8431.

Yamamoto CM, Sinha Hikim AP, Huynh PN, Shapiro B, Lue Y, Salameh WA, Wang C & Swerdloff RS 2000 Redistribution of Bax is an early step in an apoptotic pathway leading to germ cell death in rats, triggered by mild testicular hyperthermia. *Biol Reprod.* **63** 1683-1690.

Yamasaki L, Jacks T, Bronson R, Goillot E, Harlow E & Dyson NJ 1996 Tumor induction and tissue atrophy in mice lacking E2F-1. *Cell* **85** 537-548.

Yan W, Samson M, Jegou B & Toppari J 2000 Bcl-w forms complexes with Bax and Bak, and elevated ratios of Bax/Bcl- w and Bak/Bcl-w correspond to spermatogonial and spermatocyte apoptosis in the testis. *Mol.Endocrinol.* **14** 682-699.

Yan W, West A, Toppari J & Lahdetie J 1997 Stage-specific expression and phosphorylation of retinoblastoma protein (pRb) in the rat seminiferous epithelium. *Mol.Cell Endocrinol.* **132** 137-148.

Yang R, Morosetti R & Koeffler HP 1997 Characterization of a second human cyclin A that is highly expressed in testis and in several leukemic cell lines. *Cancer Res.* **57** 913-920.

Yang R, Muller C, Huynh V, Fung YK, Yee AS & Koeffler HP 1999a Functions of cyclin A1 in the cell cycle and its interactions with transcription factor E2F-1 and the Rb family of proteins. *Mol.Cell Biol* **19** 2400-2407.

Yang R, Nakamaki T, Lubbert M, Said J, Sakashita A, Freyaldenhoven BS, Spira S, Huynh V, Muller C & Koeffler HP 1999b Cyclin A1 expression in leukemia and normal hematopoietic cells. *Blood* **93** 2067-2074.

Yin Y, Stahl BC, DeWolf WC & Morgentaler A 1998 p53-mediated germ cell quality control in spermatogenesis. *Dev.Biol* **204** 165-171.

Young M & McPhaul MJ 1997 Definition of the elements required for the activity of the rat aromatase promoter in steroidogenic cell lines. *J.Steroid Biochem.Mol.Biol* **61** 341-348.

Young M & McPhaul MJ 1998 A steroidogenic factor-1-binding site and cyclic adenosine 3',5'- monophosphate response element-like elements are required for the activity of the rat aromatase promoter in rat Leydig tumor cell lines. *Endocrinology* **139** 5082-5093.

Zhang H, Hannon GJ, Casso D & Beach D 1994 p21 is a component of active cell cycle kinases. *Cold Spring Harb.Symp.Quant.Biol* **59** 21-29.

Zhang Q, Wang X & Wolgemuth DJ 1999 Developmentally regulated expression of cyclin D3 and its potential in vivo interacting proteins during murine gametogenesis. *Endocrinology* **140** 2790-2800.

Zhang Z, Laping J, Glasser S, Day P & Mulholland J 1998 Mediators of estradiol-stimulated mitosis in the rat uterine luminal epithelium. *Endocrinology* **139** 961-966.

Zhu Q, Meisinger J, Emanuele NV, Emanuele MA, LaPaglia N & Van Thiel DH 2000 Ethanol exposure enhances apoptosis within the testes. *Alcohol Clin.Exp.Res.* **24** 1550-1556.

Zirkin BR & Chen H 2000 Regulation of Leydig cell steroidogenic function during aging. *Biol Reprod.* **63** 977-981.

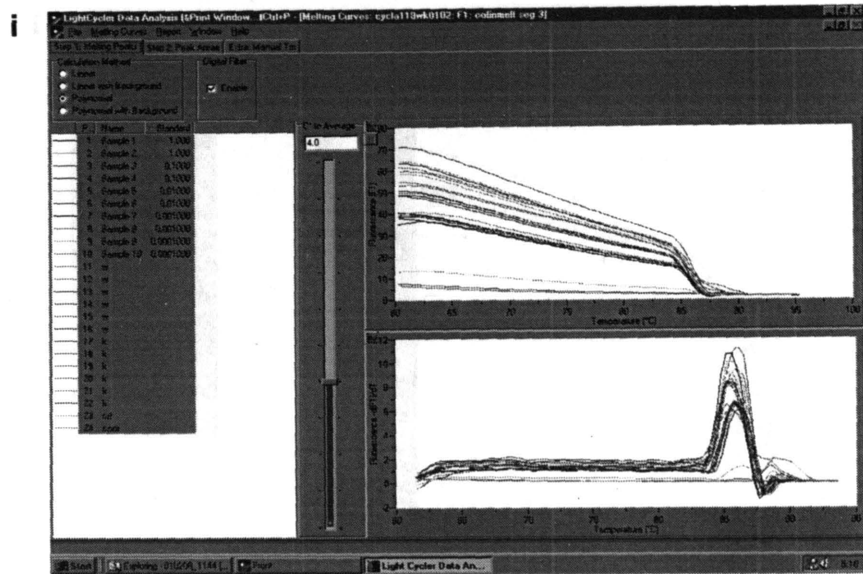
Zondek B 1934 Mass excretion of estrogenic hormone in the urine of the stallion. *Nature* **33** 209-210.

Zou H, Henzel WJ, Liu X, Lutschg A & Wang X 1997 Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell* **90** 405-413.

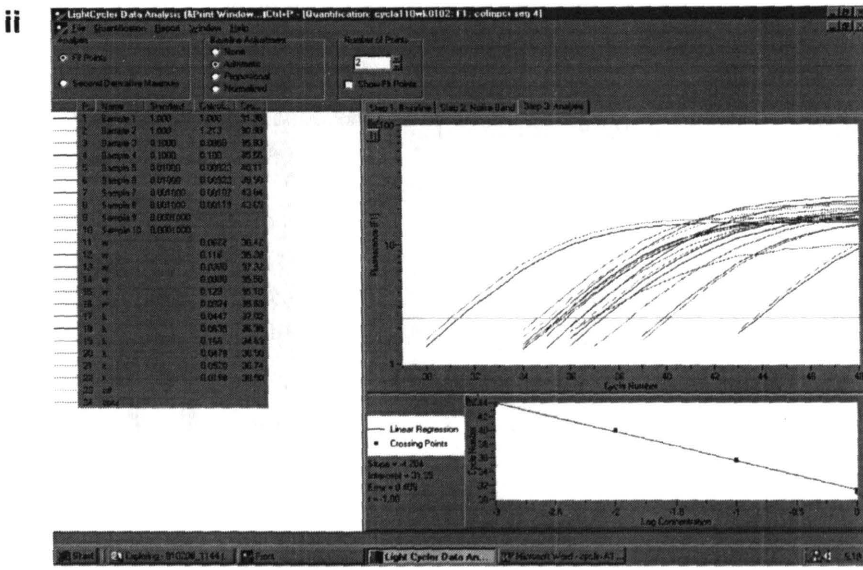
Zwijsen RM, Wientjens E, Klompmaker R, van der SJ, Bernards R & Michalides RJ 1997 CDK-independent activation of estrogen receptor by cyclin D1. *Cell* **88** 405-415.

Appendices

i



ii



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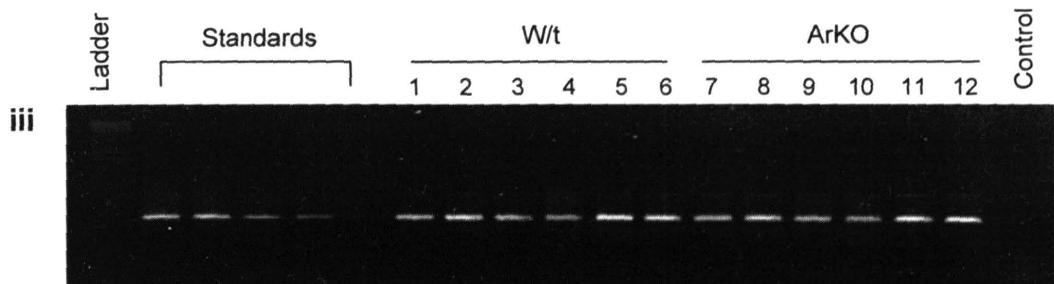
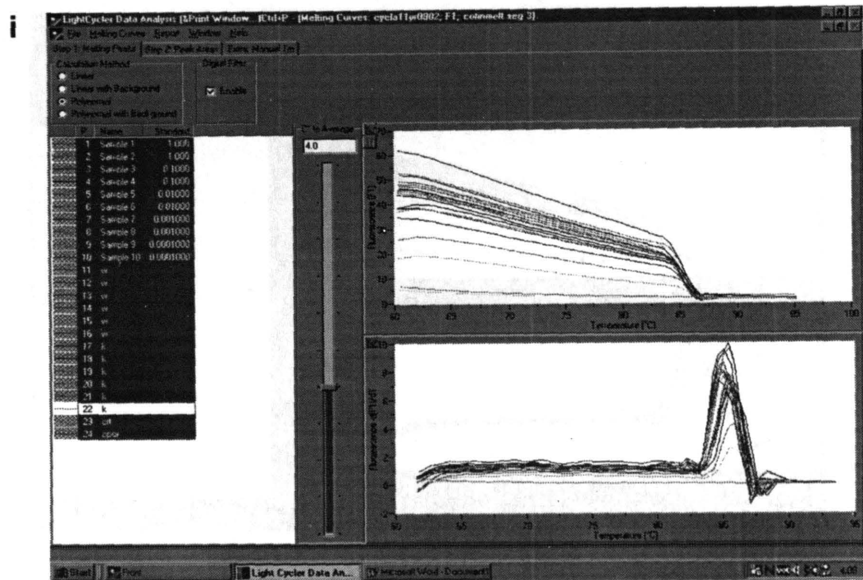


Appendix 1a Cyclin A1 18 week (i) melting curve and (ii) quantification, illustrating the standards, WT and KO animals. Also shown are the (iii) PCR products run on a 1% agarose gel.

i



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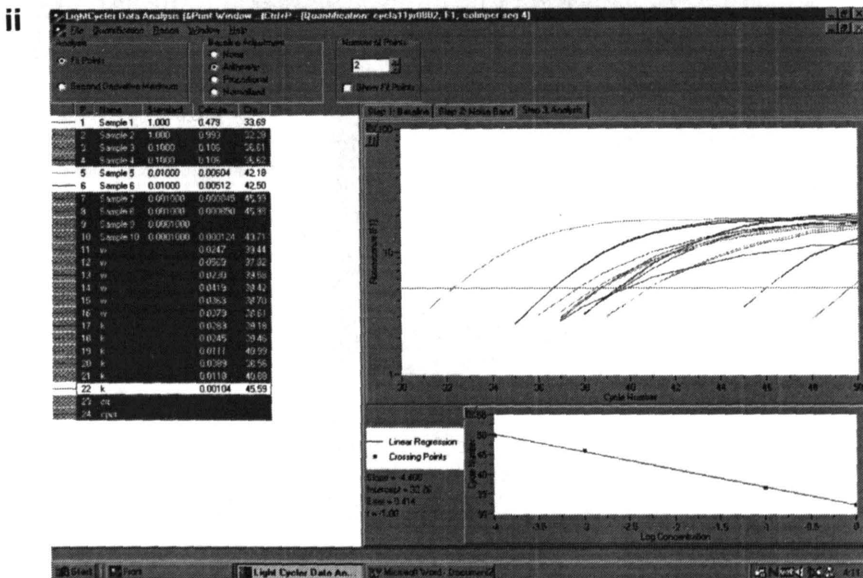

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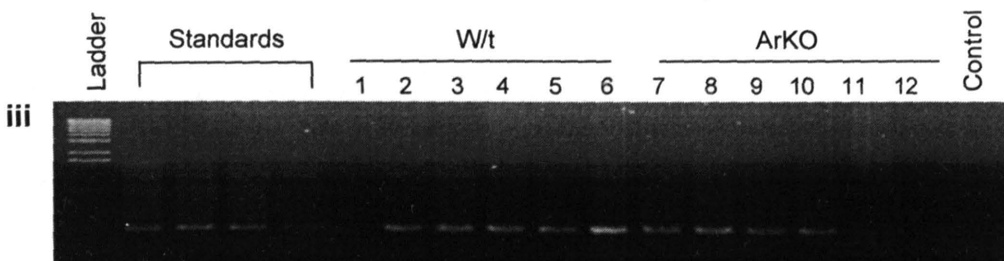
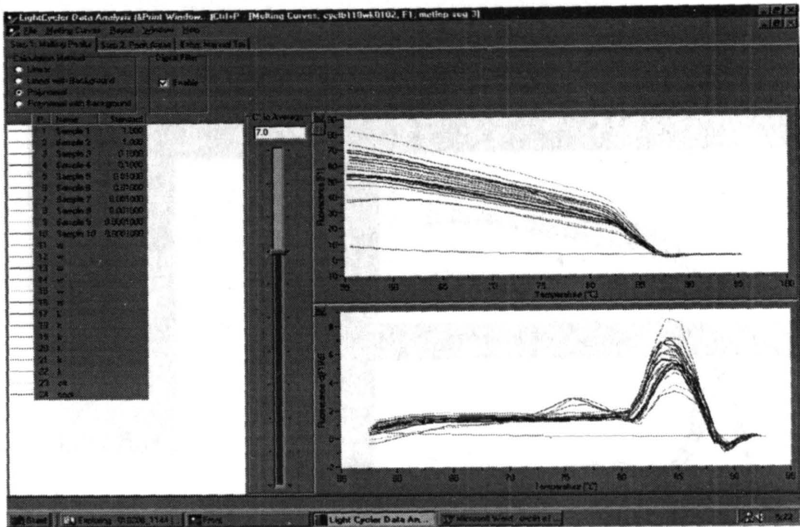


Appendix 1b Cyclin A1 1 year (i) melting curve and (ii) quantification, illustrating the standards, WT and KO animals. Also shown are the (iii) PCR products run on a 1% agarose gel.

i



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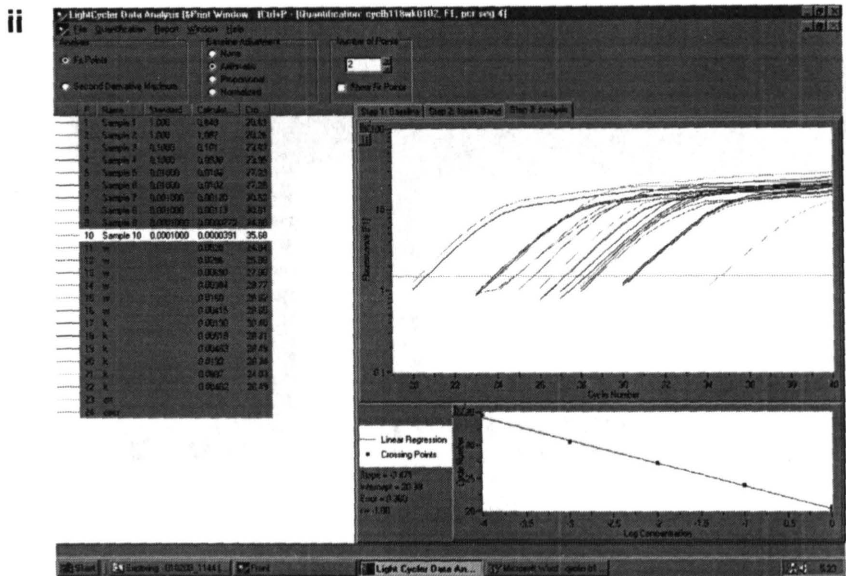

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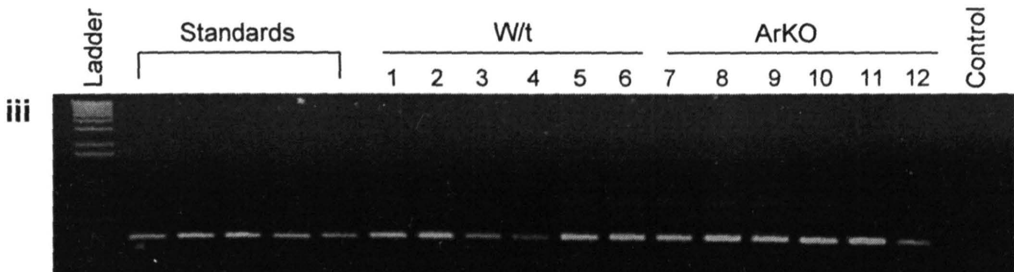
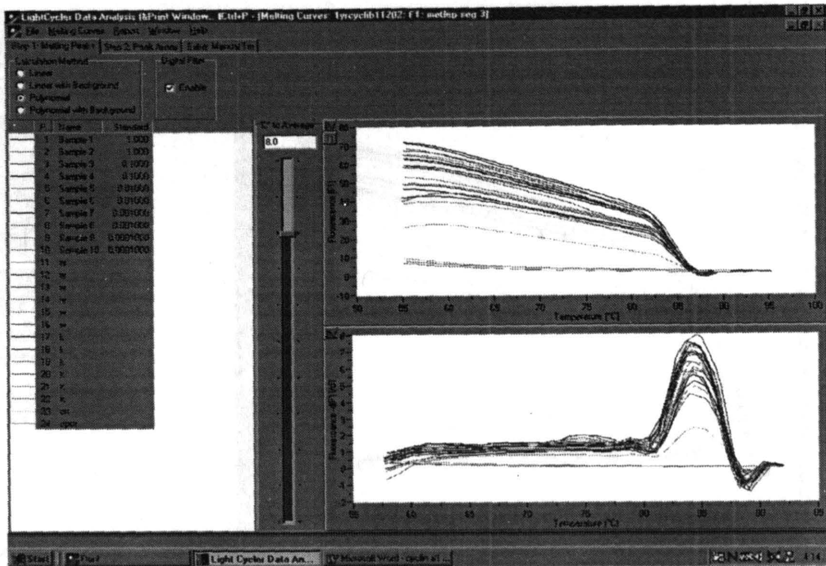


Appendix 2a Cyclin B1 18 week (i) melting curve and (ii) quantification, illustrating the standards, WT and KO animals. Also shown are the (iii) PCR products run on a 1% agarose gel.

i



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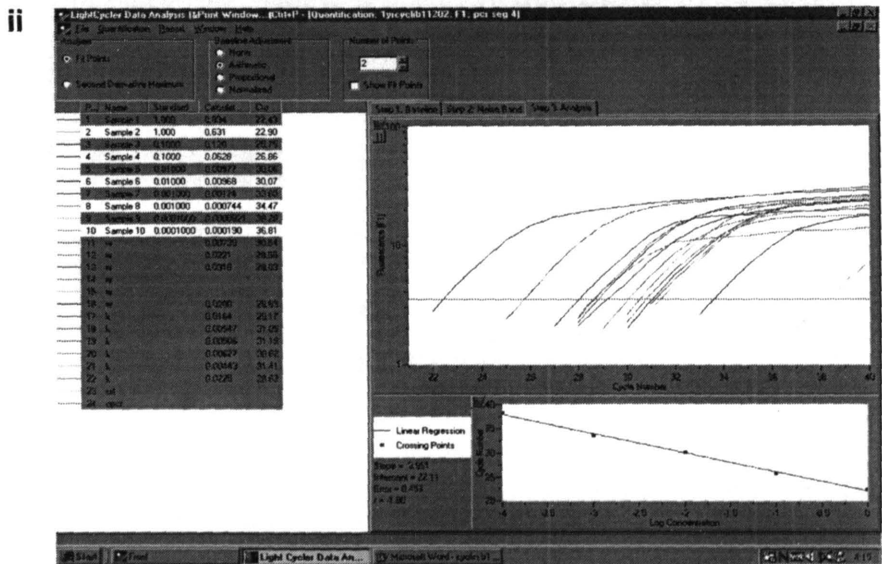

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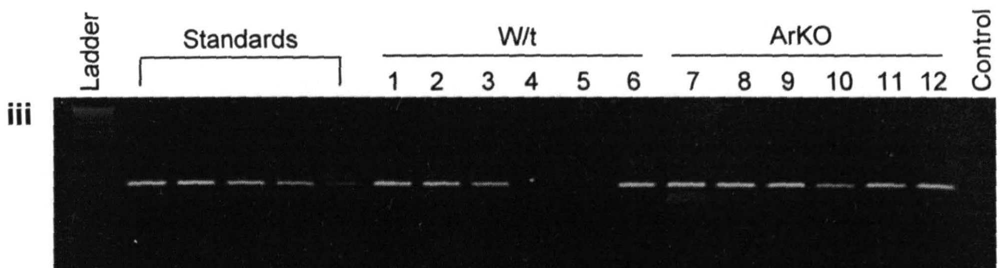
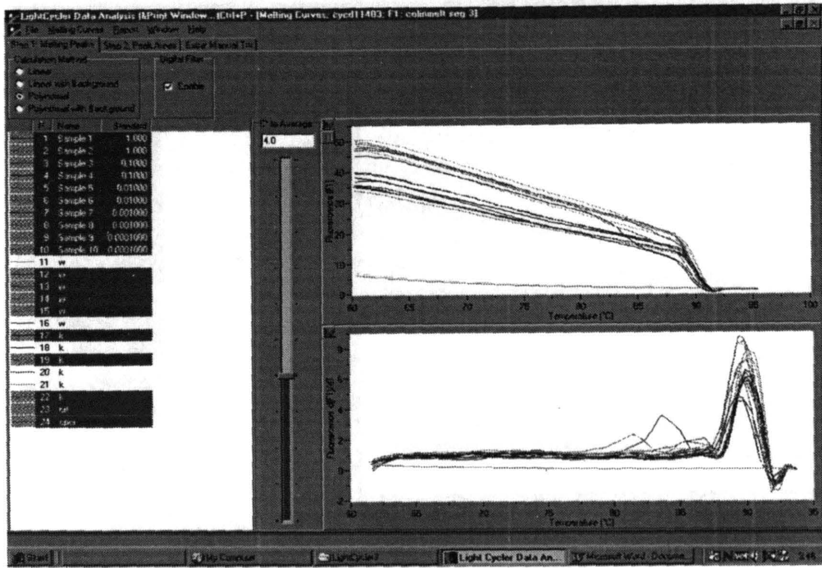


Appendix 2b Cyclin B1 1 year (i) melting curve and (ii) quantification, illustrating the standards, WT and KO animals. Also shown are the (iii) PCR products run on a 1% agarose gel.

i



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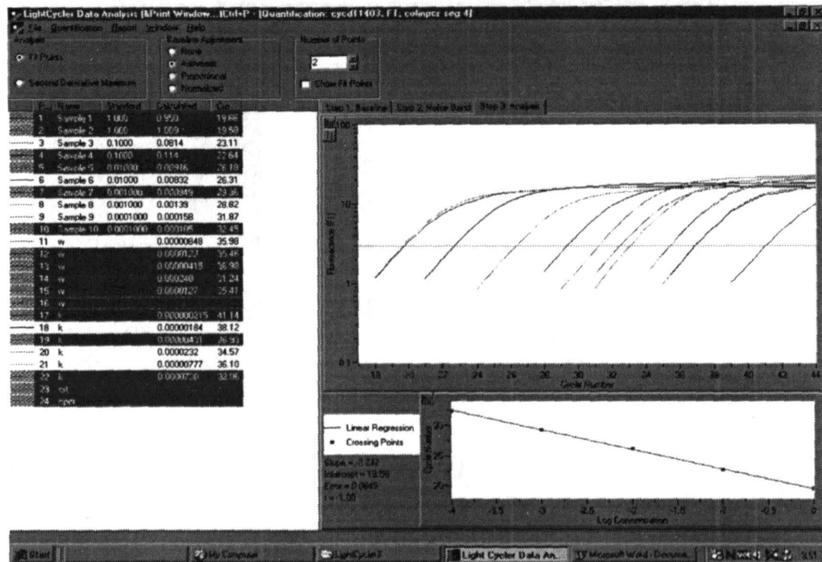

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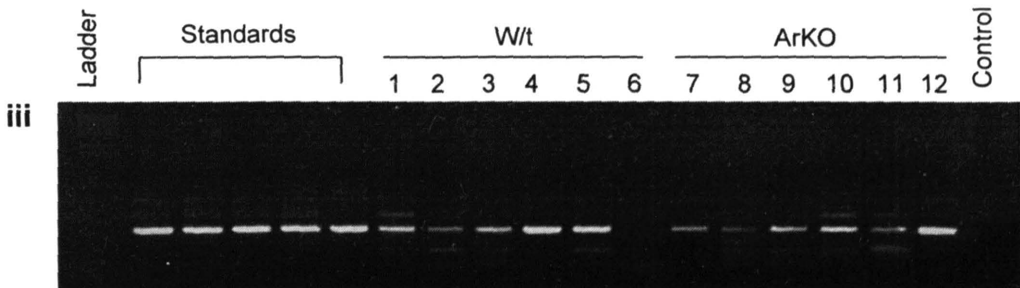
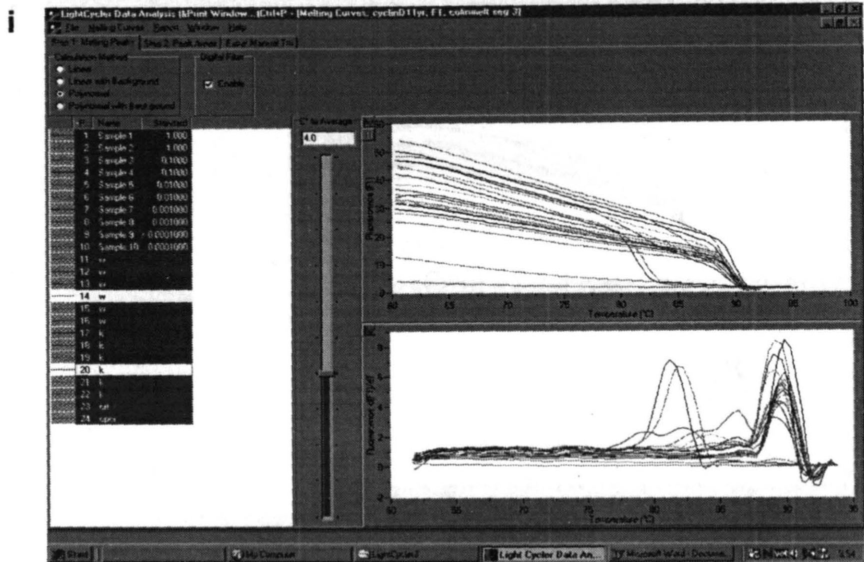


Appendix 3a Cyclin D1 18 week (i) melting curve and (ii) quantification, illustrating the standards, WT and KO animals. Also shown are the (iii) PCR products run on a 1% agarose gel.

i



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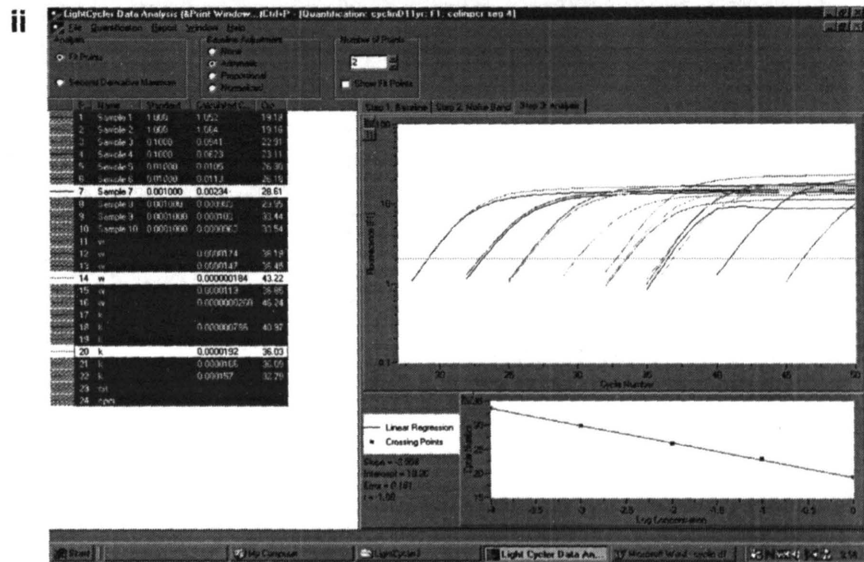

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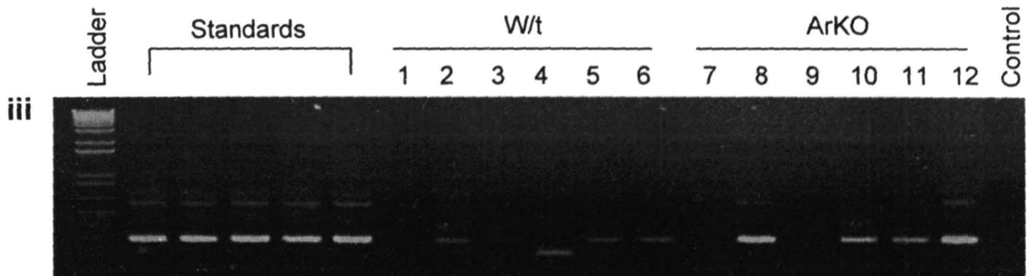
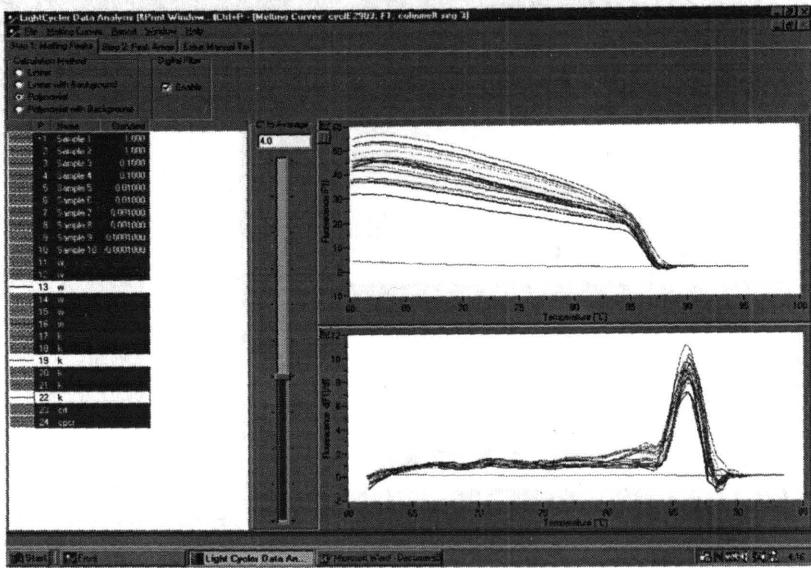


Appendix 3b Cyclin D1 1 year (i) melting curve and (ii) quantification, illustrating the standards, WT and KO animals. Also shown are the (iii) PCR products run on a 1% agarose gel.

i



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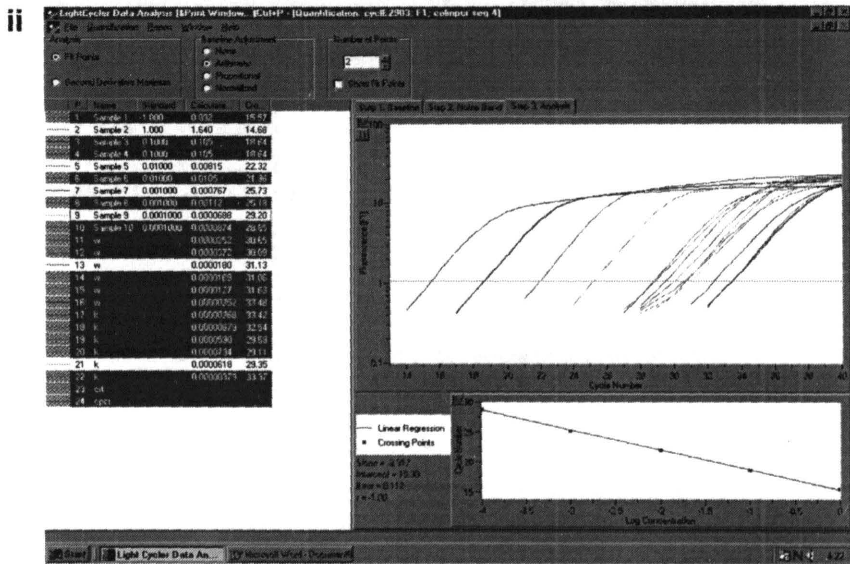

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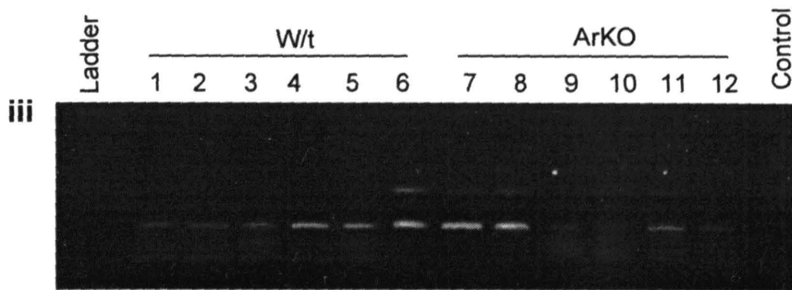
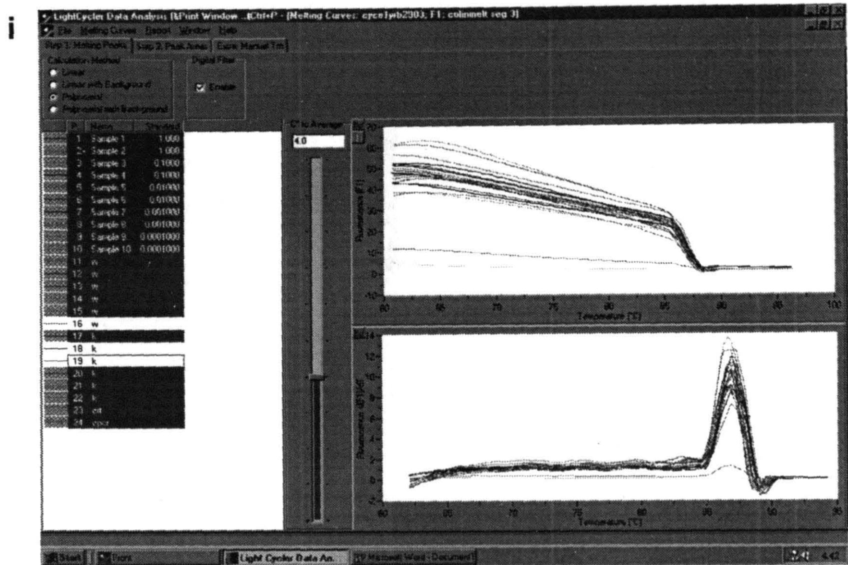


Appendix 4a Cyclin E 18 week (i) melting curve and (ii) quantification, illustrating the standards, WT and KO animals. Also shown are the (iii) PCR products run on a 1% agarose gel.

i



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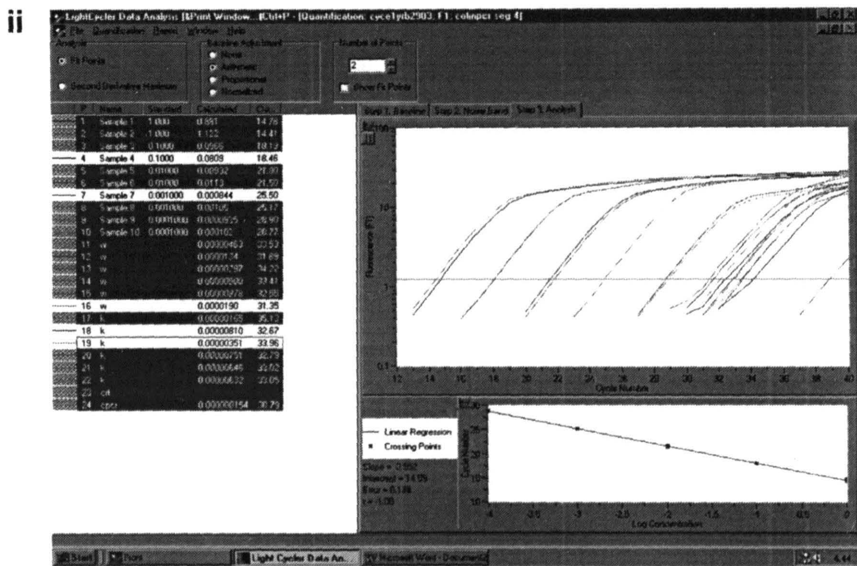

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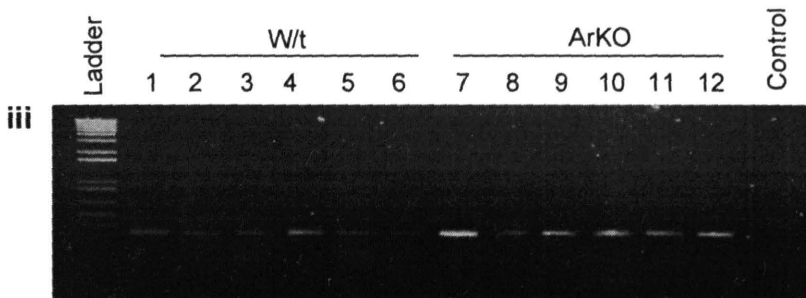
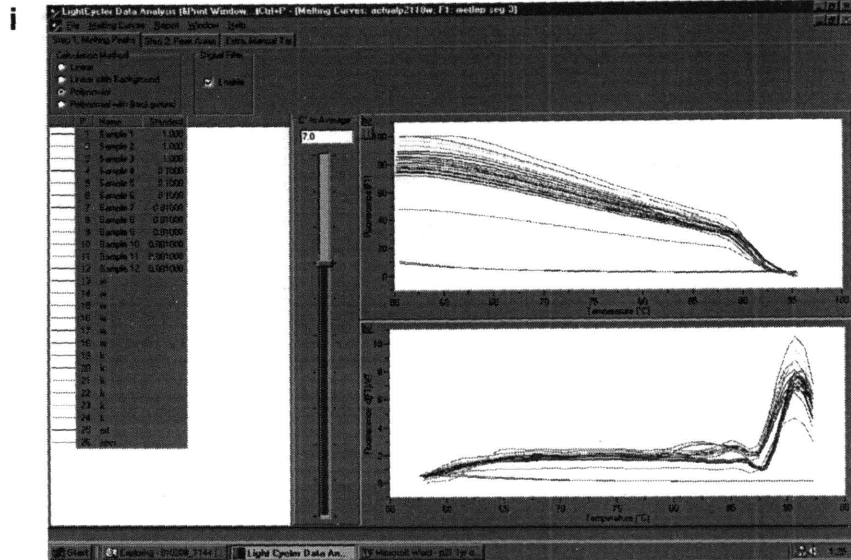


Appendix 4b Cyclin E 1 year (i) melting curve and (ii) quantification, illustrating the standards, WT and KO animals. Also shown are the (iii) PCR products run on a 1% agarose gel.

i



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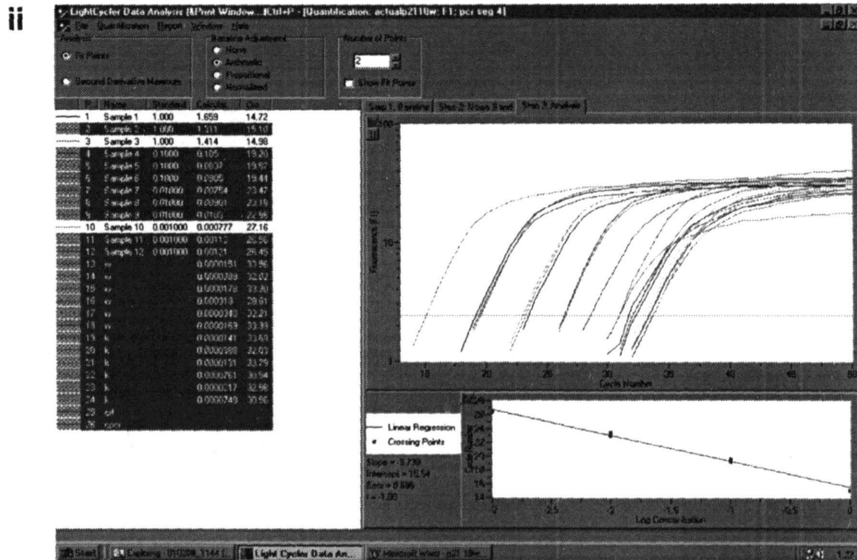

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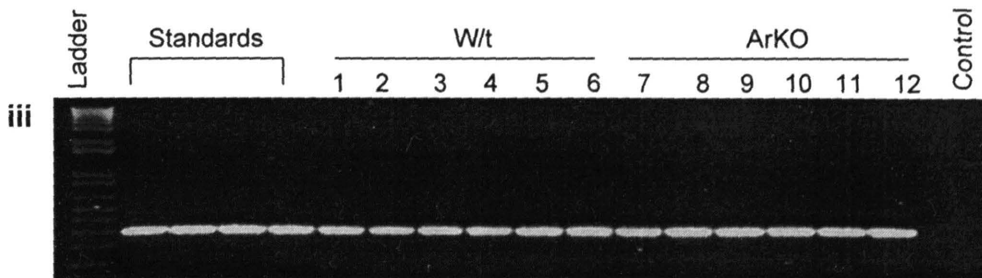
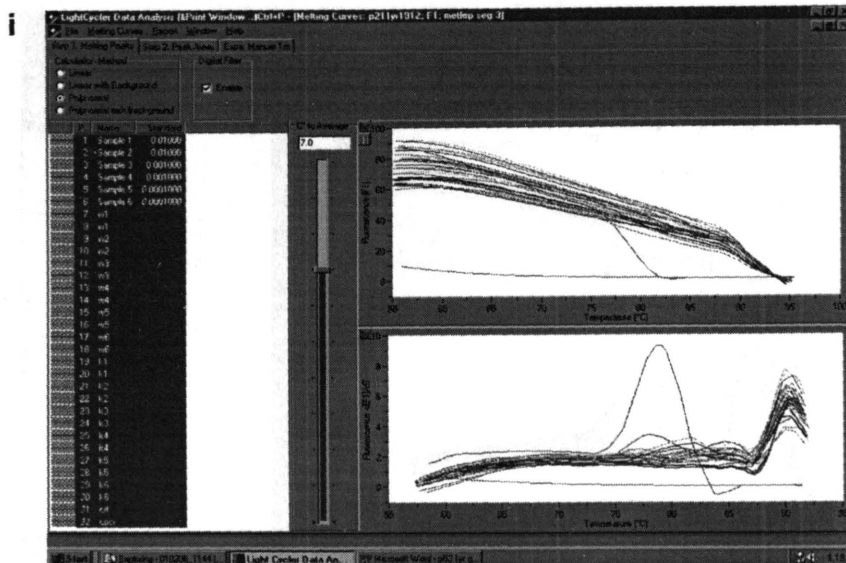


Appendix 5a p21^{Waf1} 18week (i) melting curve and (ii) quantification, illustrating the standards, WT and KO animals. Also shown are the (iii) PCR products run on a 1% agarose gel.

i



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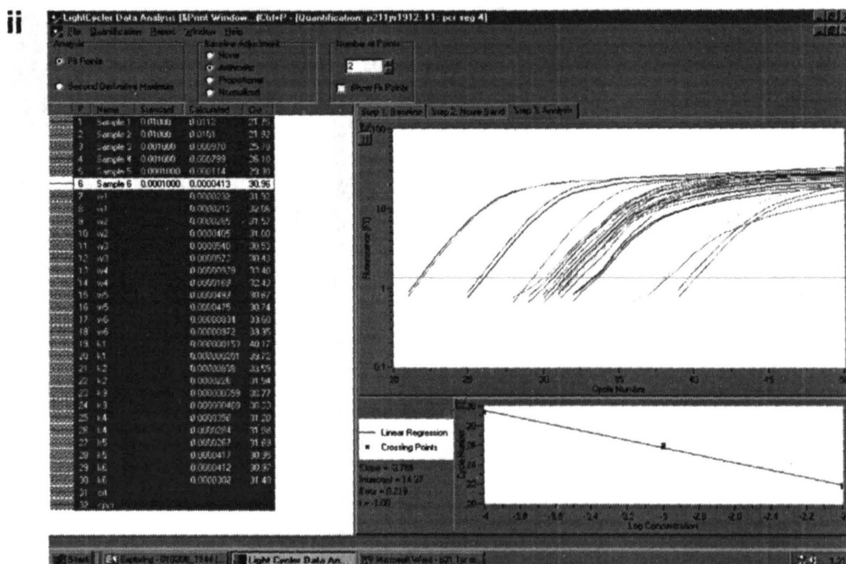

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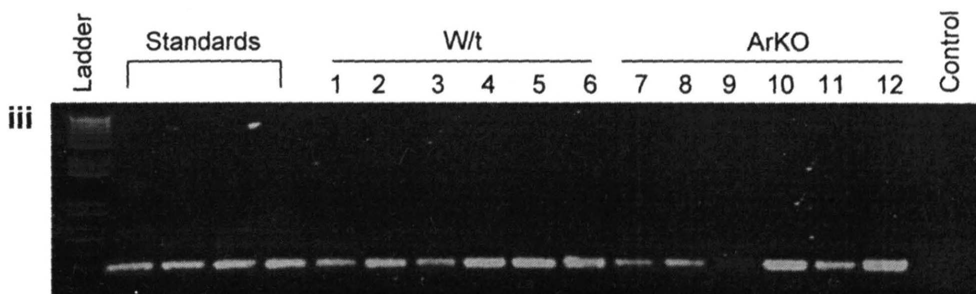
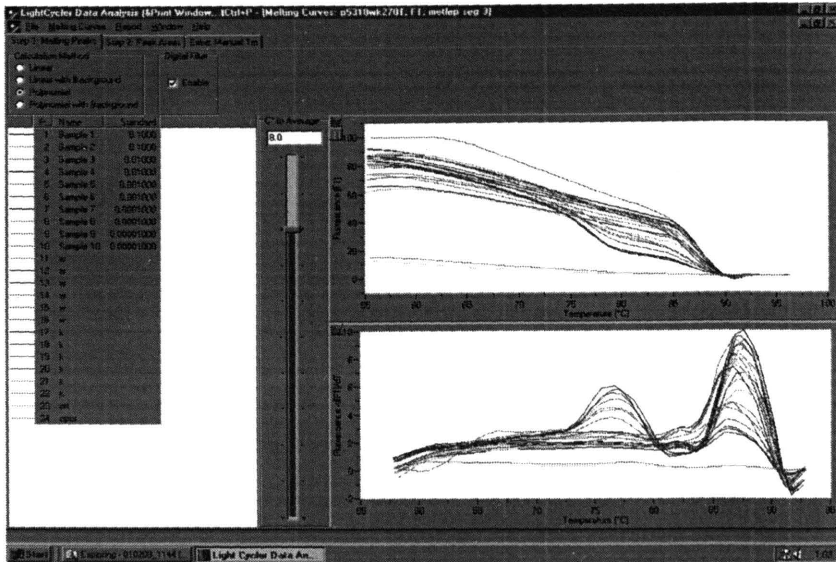


Appendix 5b p21^{Waf1} 1year (i) melting curve and (ii) quantification, illustrating the standards, WT and KO animals (in duplicate). Also shown are the (iii) PCR products run on a 1% agarose gel.

i



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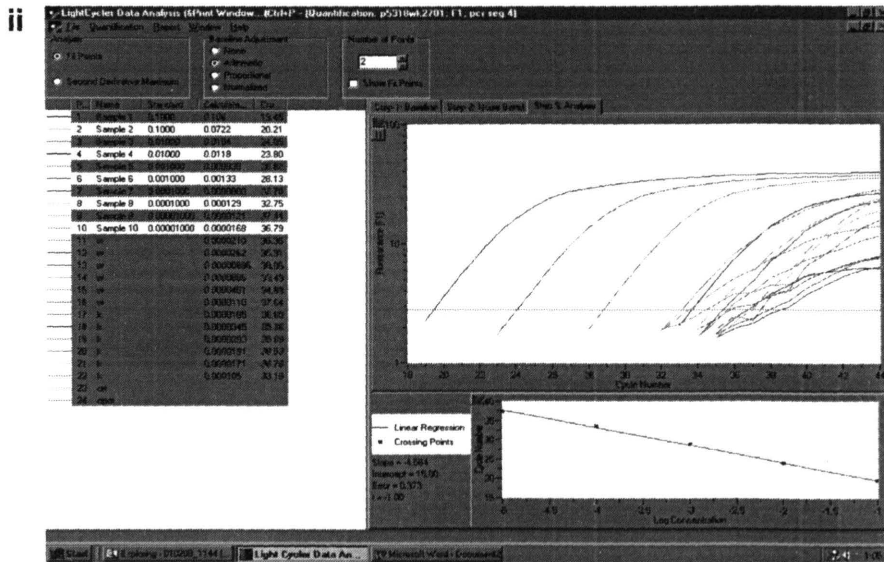

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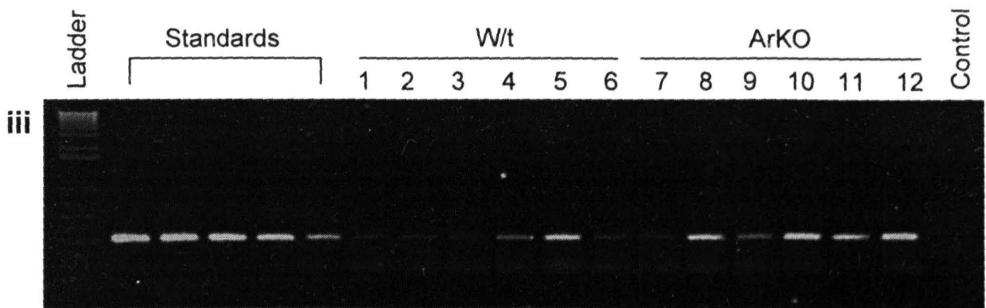
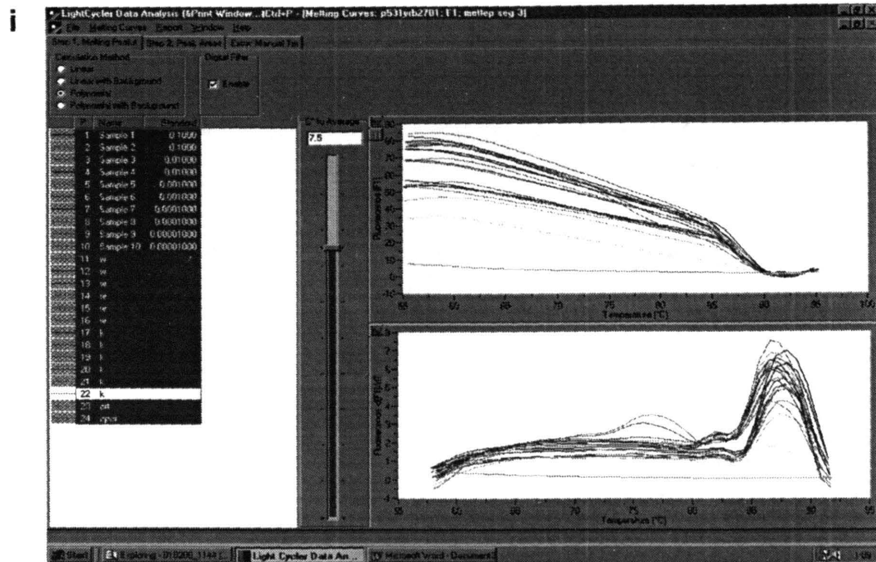


Appendix 6a p53 18 week (i) melting curve and (ii) quantification, illustrating the standards, WT and KO animals. Also shown are the (iii) PCR products run on a 1% agarose gel.

i



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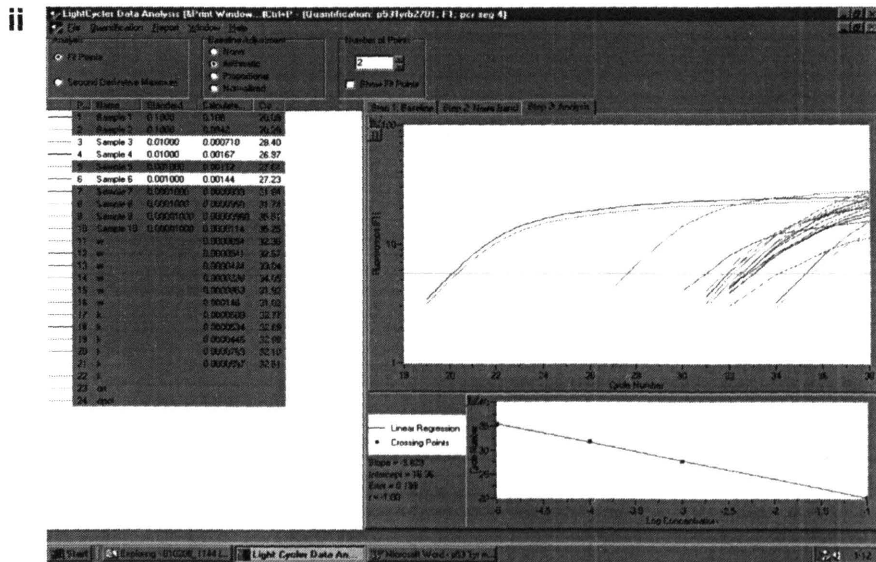

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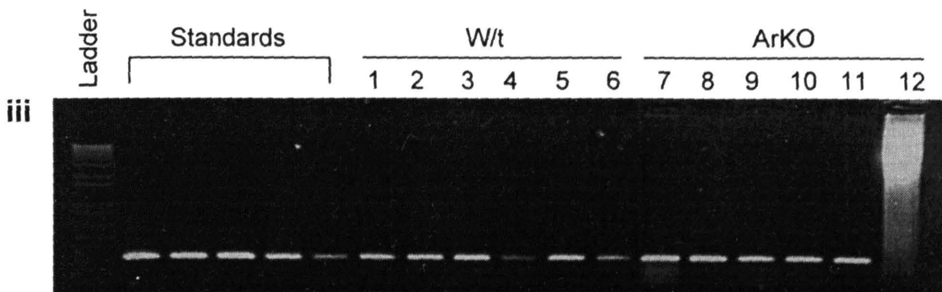
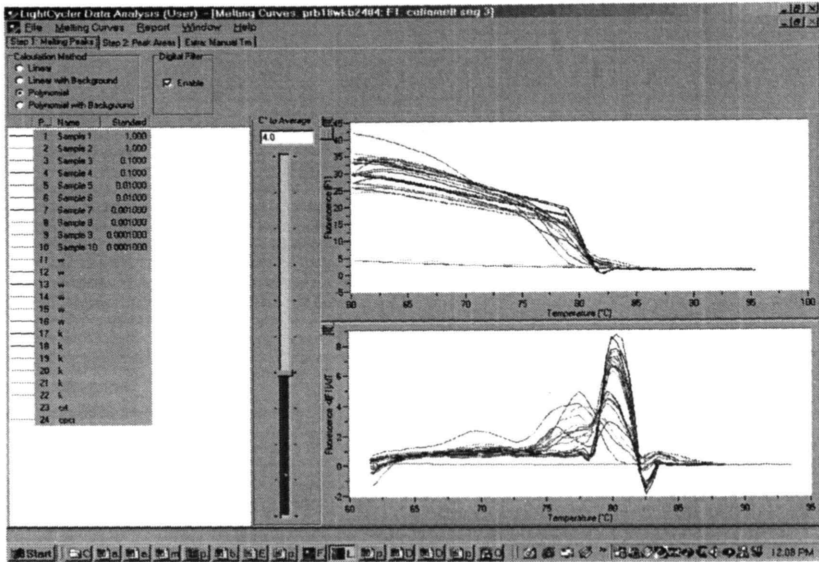


Appendix 6b p53 1 year (i) melting curve and (ii) quantification, illustrating the standards, WT and KO animals. Also shown are the (iii) PCR products run on a 1% agarose gel.

i



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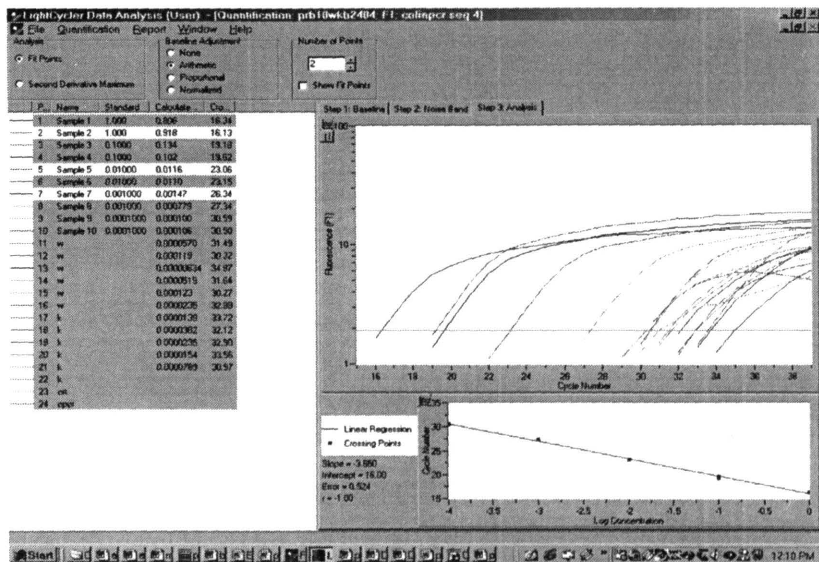

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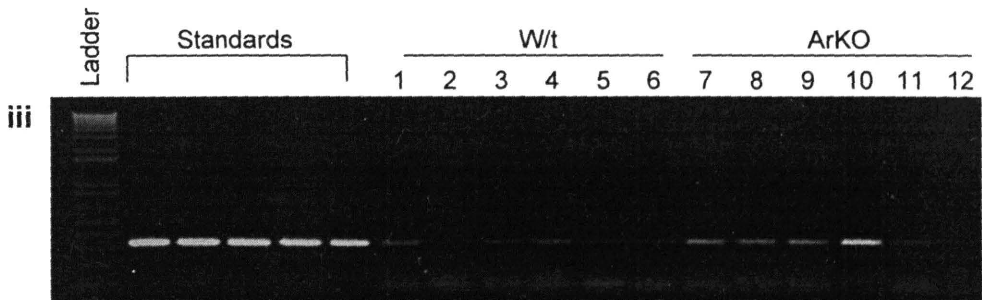
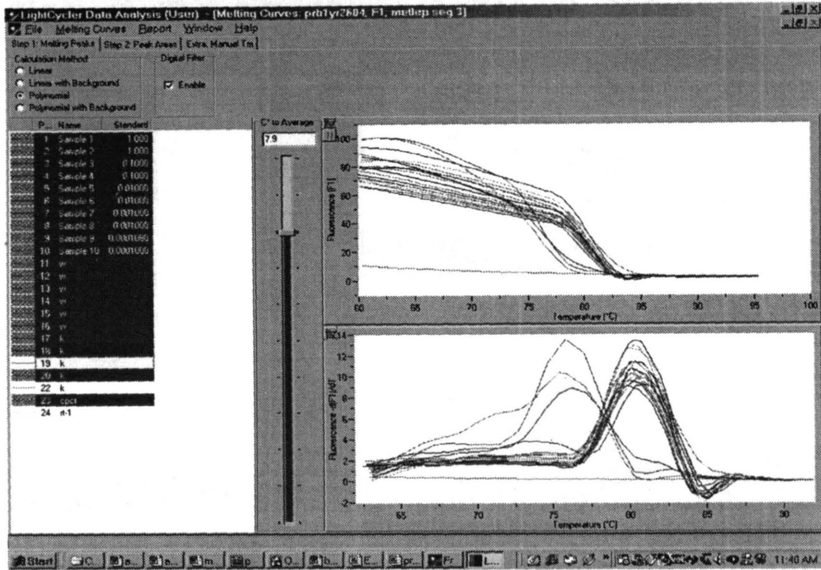


Appendix 7a prb 18 week (i) melting curve and (ii) quantification, illustrating the standards, WT and KO animals. Also shown are the (iii) PCR products run on a 1% agarose gel.

i



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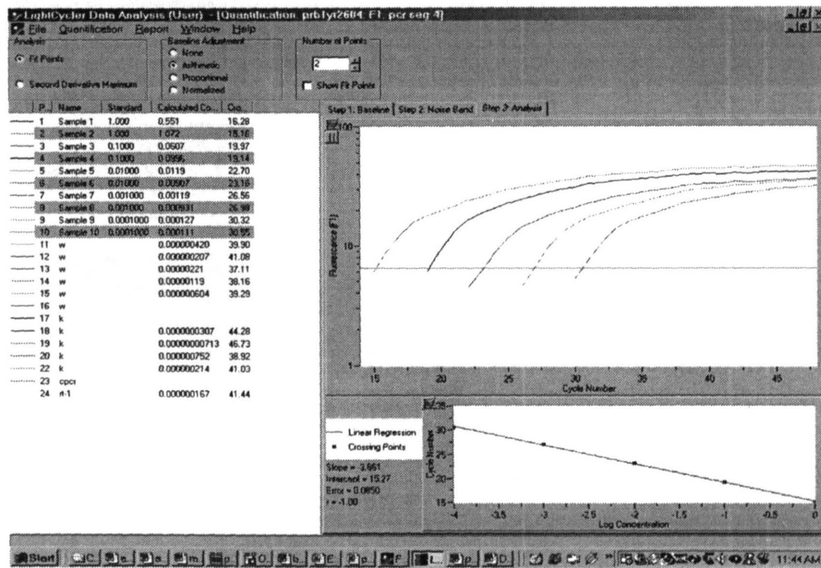
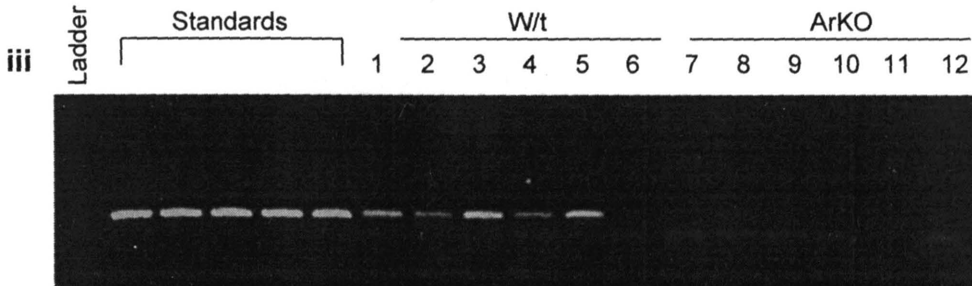
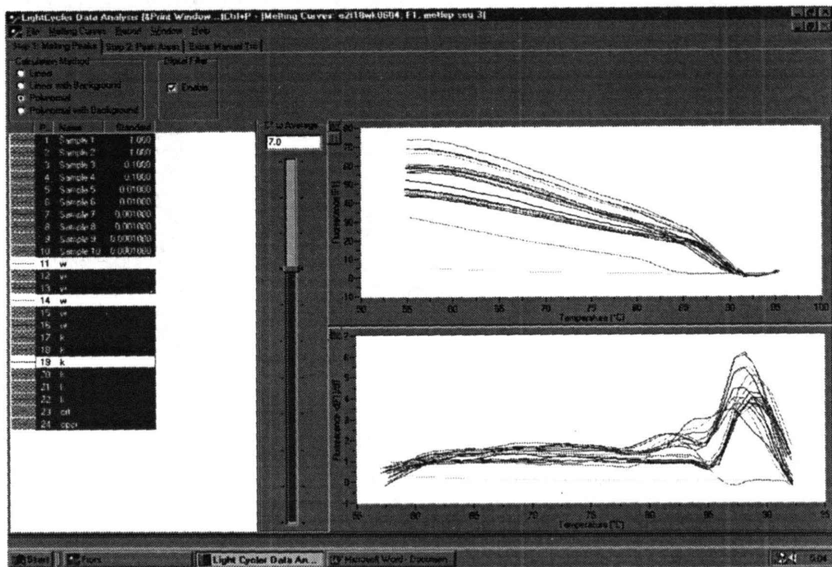


iii



Appendix 7b pRb 1 year (i) melting curve and (ii) quantification, illustrating the standards, WT and KO animals. Also shown are the (iii) PCR products run on a 1% agarose gel.

i



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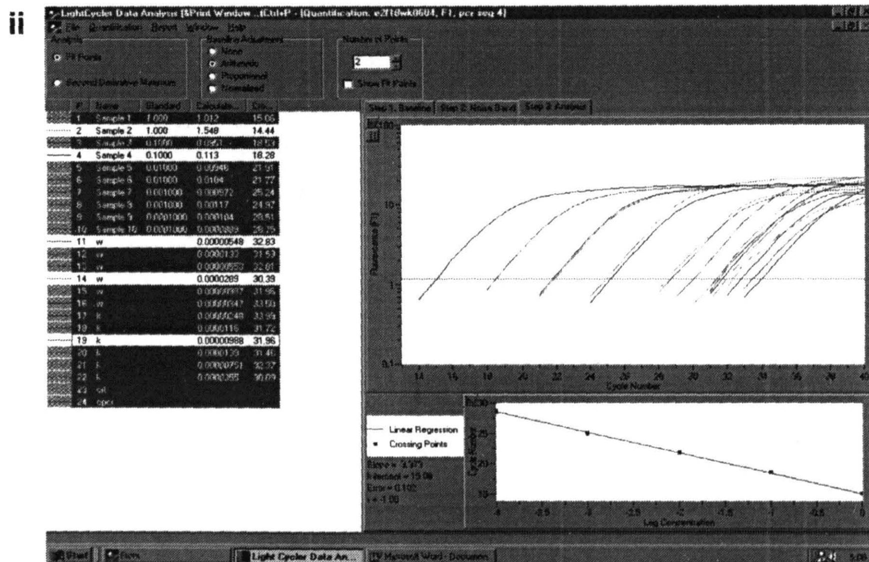

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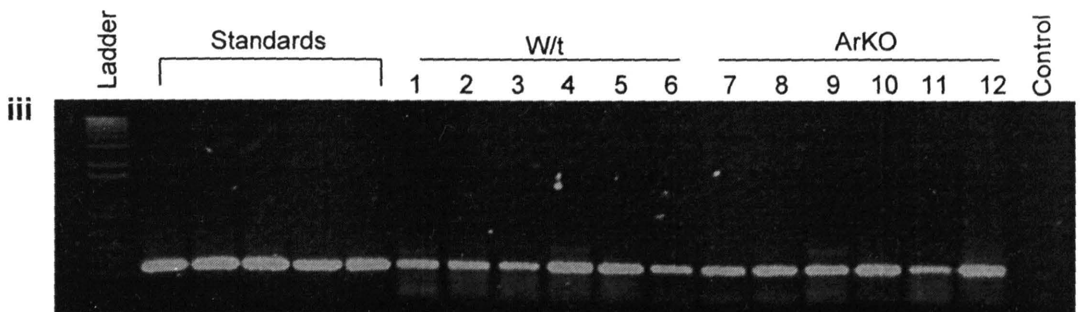
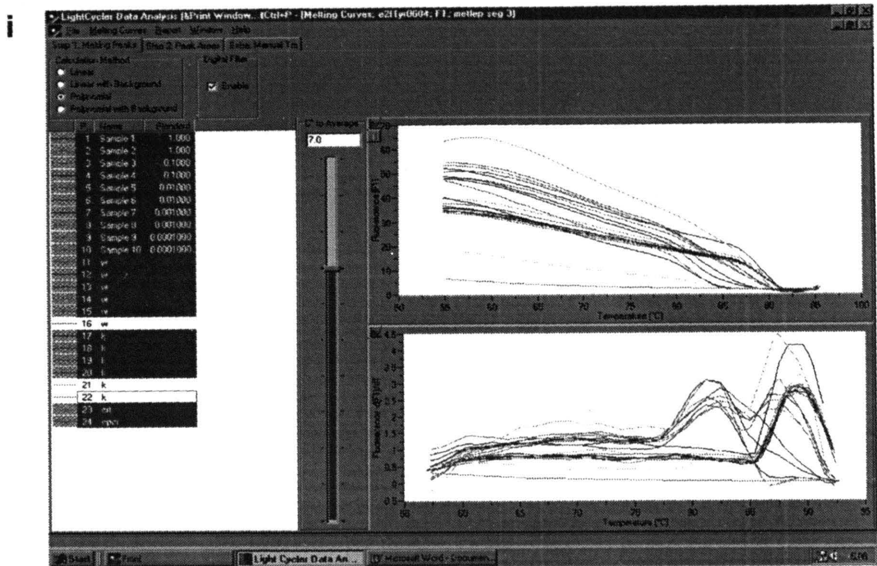


Appendix 8a E2F 18 week (i) melting curve and (ii) quantification, illustrating the standards, WT and KO animals. Also shown are the (iii) PCR products run on a 1% agarose gel.

i



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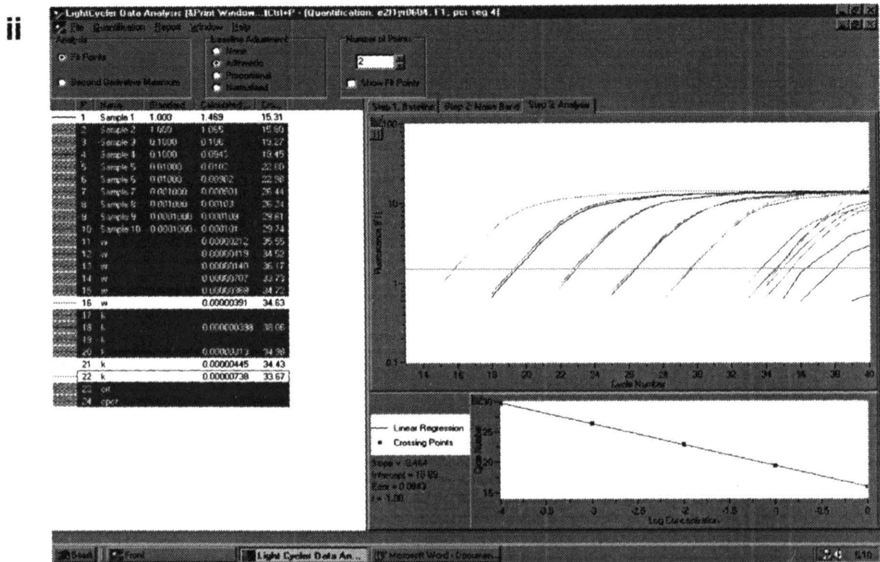

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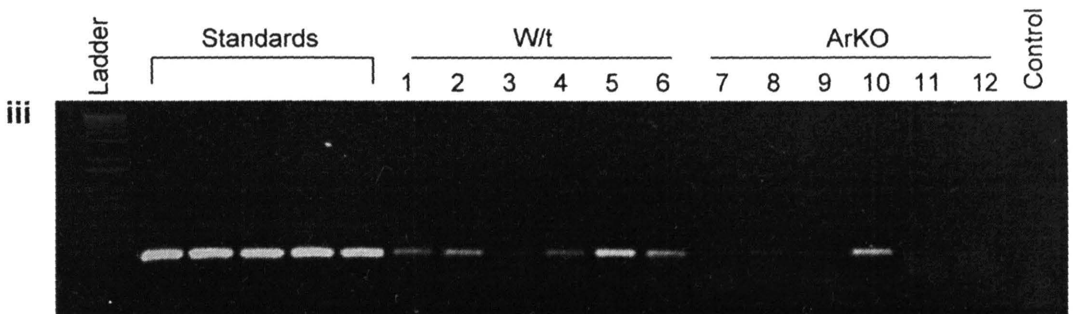

Appendix 8b E2F 1 year (i) melting curve and (ii) quantification, illustrating the standards, WT and KO animals. Also shown are the (iii) PCR products run on a 1% agarose gel.

Reference List

Couse JF, Hewitt SC, Bunch DO, Sar M, Walker VR, Davis BJ & Korach KS 1999 Postnatal sex reversal of the ovaries in mice lacking estrogen receptors alpha and beta. *Science* **286** 2328-2331.

Couse JF & Korach KS 1999 Estrogen receptor null mice: what have we learned and where will they lead us? *Endocr.Rev.* **20** 358-417.

Donaldson KM, Tong SY, Washburn T, Lubahn DB, Eddy EM, Hutson JM & Korach KS 1996 Morphometric study of the gubernaculum in male estrogen receptor mutant mice. *J.Androl* **17** 91-95.

Dupont S, Krust A, Gansmuller A, Dierich A, Chambon P & Mark M 2000 Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. *Development* **127** 4277-4291.

Eddy EM, Washburn TF, Bunch DO, Goulding EH, Gladen BC, Lubahn DB & Korach KS 1996 Targeted disruption of the estrogen receptor gene in male mice causes alteration of spermatogenesis and infertility. *Endocrinology* **137** 4796-4805.

Fisher CR, Graves KH, Parlow AF & Simpson ER 1998 Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene. *Proc.Natl.Acad.Sci.U.S.A* **95** 6965-6970.

Hess RA, Bunick D, Lee KH, Bahr J, Taylor JA, Korach KS & Lubahn DB 1997 A role for oestrogens in the male reproductive system [see comments]. *Nature* **390** 509-512.

Hess RA, Bunick D, Lubahn DB, Zhou Q & Bouma J 2000 Morphologic changes in efferent ductules and epididymis in estrogen receptor-alpha knockout mice. *J.Androl* **21** 107-121.

Honda S, Harada N, Ito S, Takagi Y & Maeda S 1998 Disruption of sexual behavior in male aromatase-deficient mice lacking exons 1 and 2 of the cyp19 gene. *Biochem.Biophys.Res.Commun.* **252** 445-449.

Krege JH, Hodgin JB, Couse JF, Enmark E, Warner M, Mahler JF, Sar M, Korach KS, Gustafsson JA & Smithies O 1998 Generation and reproductive phenotypes of mice lacking estrogen receptor beta. *Proc.Natl.Acad.Sci.U.S.A* **95** 15677-15682.

Lee KH, Hess RA, Bahr JM, Lubahn DB, Taylor J & Bunick D 2000 Estrogen Receptor alpha Has a Functional Role in the Mouse Rete Testis and Efferent Ductules. *Biol.Reprod.* **63** 1873-1880.

Lindzey J, Wetsel WC, Couse JF, Stoker T, Cooper R & Korach KS 1998 Effects of castration and chronic steroid treatments on hypothalamic gonadotropin-releasing hormone content and pituitary gonadotropins in male wild-type and estrogen receptor-alpha knockout mice. *Endocrinology* **139** 4092-4101.

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

Mahato D, Goulding EH, Korach KS & Eddy EM 2000 Spermatogenic cells do not require estrogen receptor-alpha for development or function [see comments]. *Endocrinology* **141** 1273-1276.

Ogawa S, Chan J, Chester AE, Gustafsson JA, Korach KS & Pfaff DW 1999 Survival of reproductive behaviors in estrogen receptor beta gene- deficient (betaERKO) male and female mice. *Proc.Natl.Acad.Sci.U.S.A* **96** 12887-12892.

Ogawa S, Chester AE, Hewitt SC, Walker VR, Gustafsson JA, Smithies O, Korach KS & Pfaff DW 2000 From the cover: abolition of male sexual behaviors in mice lacking estrogen receptors alpha and beta (alpha beta ERKO). *Proc.Natl.Acad.Sci.U.S.A* **97** 14737-14741.

Ogawa S, Lubahn DB, Korach KS & Pfaff DW 1997 Behavioral effects of estrogen receptor gene disruption in male mice. *Proc.Natl.Acad.Sci.U.S.A* **94** 1476-1481.

Robertson KM, O'Donnell L, Jones ME, Meachem SJ, Boon WC, Fisher CR, Graves KH, McLachlan RI & Simpson ER 1999 Impairment of spermatogenesis in mice lacking a functional aromatase (cyp 19) gene. *Proc.Natl.Acad.Sci.U.S.A* **96** 7986-7991.

Robertson, K. M., Simpson, E. R., Lacham-Kaplan, O., and Jones, M. E. Characterization of the fertility of male aromatase knockout (ArKO) mice. J.Androl . 2001.
Ref Type: In Press

Rosenfeld CS, Ganjam VK, Taylor JA, Yuan X, Stiehr JR, Hardy MP & Lubahn DB 1998 Transcription and translation of estrogen receptor-beta in the male reproductive tract of estrogen receptor-alpha knock-out and wild-type mice. *Endocrinology* **139** 2982-2987.

Toda K, Okada T, Takeda K, Akira S, Saibara T, Shiraishi M, Onishi S & Shizuta Y 2001 Oestrogen at the neonatal stage is critical for the reproductive ability of male mice as revealed by supplementation with 17beta- oestradiol to aromatase gene (Cyp19) knockout mice. *J.Endocrinol.* **168** 455-463.

Wersinger SR, Sannen K, Villalba C, Lubahn DB, Rissman EF & De Vries GJ 1997 Masculine sexual behavior is disrupted in male and female mice lacking a functional estrogen receptor alpha gene. *Horm.Behav.* **32** 176-183.