The effect of transforming growth factor beta1 null mutation on murine reproductive function

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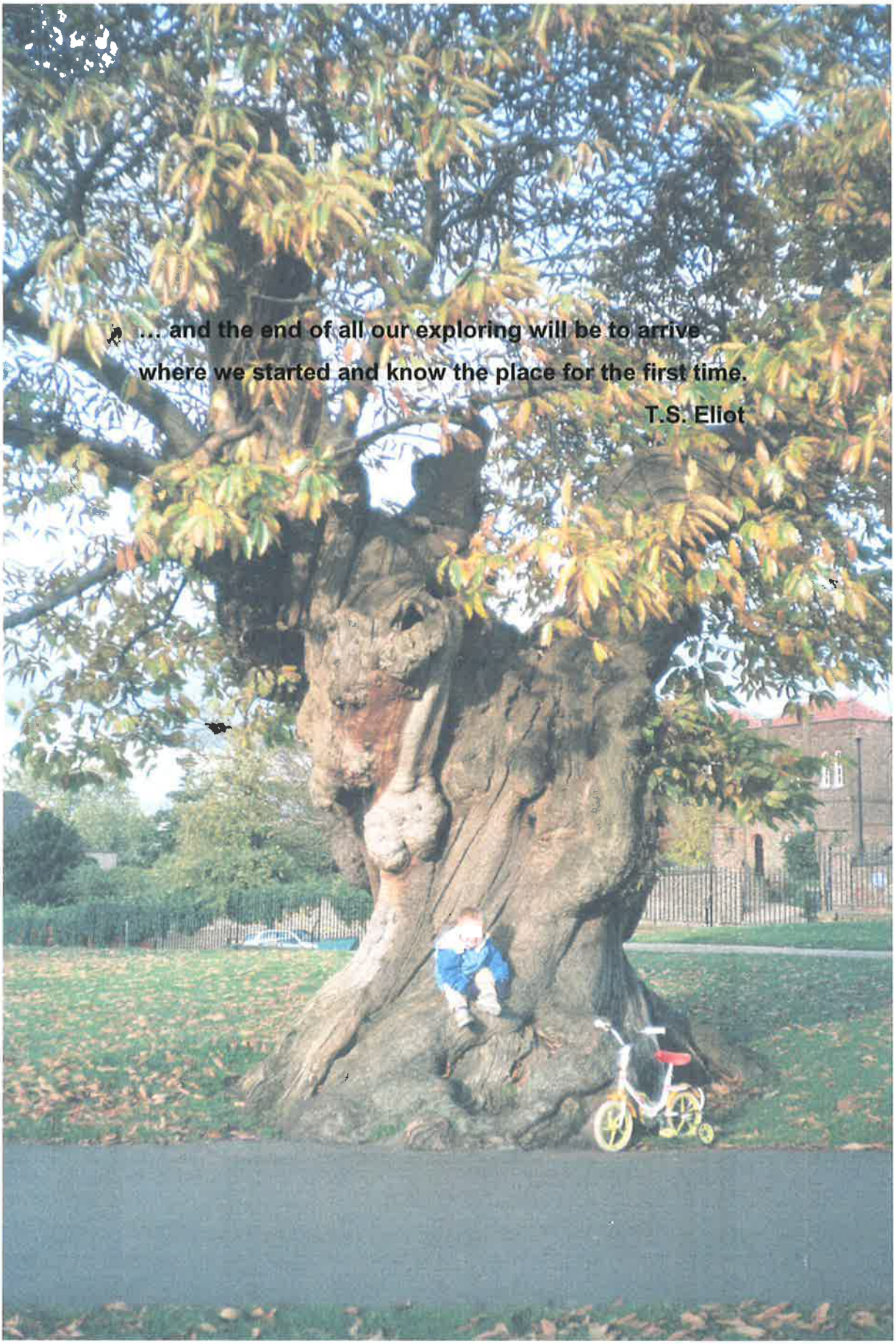
... and the end of all our exploring will be to arrive
where we started and know the place for the first time.
T.S. Eliot

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ABSTRACT

Transforming growth factor beta 1 (TGFβ1) is a multifunctional cytokine implicated in gonad and secondary sex organ development, spermatogenesis and ovarian function, immunoregulation of pregnancy, embryo implantation and placental development. The TGFβ1 null mutant mouse offers the unique opportunity to study the role of TGFβ1 in vivo. TGFβ1 null mutant males are 100% infertile. When housed with normal females they do not deposit sperm or induce pseudopregnancy. Serum testosterone levels in adult TGFβ1 null mutant mice is decreased by 75%, caused by factors upstream of testis function as testosterone production can be induced by exogenous gonadotrophins. In the majority of TGFβ1 null mice, spermatogenesis proceeds normally and in vitro fertilisation experiments have shown the sperm are viable. Behavioural studies revealed that TGFβ1 null mutant males display mounting behaviour and while some intromit, ejaculation never occurs. Nitric oxide synthase enzymes were not induced in the penis of TGFβ1 null males in response to gonadotrophin, and this may be the cause of impaired sexual performance. Neither replacement of testosterone during perinatal development and/or adulthood, nor treatment with sildenafil citrate restored sexual function. Female TGFβ1 null mice also have severe fertility deficiencies. These mice suffer three distinct reproductive lesions (1) failure of 50% of the females to mate with normal stud males, (2) in females that do mate, failure of preimplantation embryo development leading to 80% infertility and (3) failure to nurture pups in the small proportion of females that produce live litters. Ovarian function is severely impaired in TGFβ1 null mutant females and is likely to be the principle cause of reproductive failure. The number of ovulations is reduced by 40% and each corpora lutea produces less progesterone leading to a 75% decrease in serum progesterone during early pregnancy. Embryos from TGFβ1 null mutant females on day 3.5 post coitum were developmentally arrested in the morula stage. Embryos from superovulated null mutant mice fertilised with normal sperm and cultured in vitro also failed to develop to blastocysts. Together, these studies suggest that preimplantation embryo developmental failure is the result of a lesion in oocyte development in the ovary prior to ovulation. These studies demonstrate that TGFβ1 is indeed a critical factor in many aspects of murine reproductive function.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

I consent to this thesis being made available for photocopying and loan if accepted for the award of the degree.

20/11/02

Wendy Ingman date

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PUBLICATIONS ARISING FROM THESE AND RELATED STUDIES

1. **Ingman WV** and Robertson SA (in press) Defining the actions of transforming growth factor beta in reproduction. *BioEssays*

2. Robertson SA, **Ingman WV**, O'Leary S, Sharkey DJ, Tremellen KP (in press) Transforming growth factor beta – a mediator of immune deviation in seminal plasma. *Journal of Reproductive Immunology*

3. **Ingman WV** and Robertson SA (in preparation) Impaired steroidogenesis and spermatogenesis in male TGFbeta1 null mutant mice.

4. **Ingman WV** and Robertson SA (in preparation) Impaired sexual performance in male TGFbeta1 null mutant mice.

5. **Ingman WV** and Robertson SA (in preparation) Ovarian dysfunction in female TGFbeta1 null mutant mice.

ABSTRACTS ARISING FROM THESE STUDIES

Presenting author underlined

1. **Ingman WV** and Robertson SA (1999) "Morphology of the testis and seminal vesicles of the male TGFbeta1 null mouse" Australian Society for Reproductive Biology, Annual Meeting, Australia (oral presentation)

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3. **Ingman WV** and Robertson SA (2000) "Reproductive performance in female TGFbeta1 deficient mice" Australian Society for Medical Research National Conference, Australia (poster presentation)

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5. **Ingman WV** and Robertson SA (2001) "Impaired ovarian function and preimplantation embryo development in transforming growth factor beta1 null mutant mice" Society for the Study of Reproduction, 34th Annual Meeting, Canada (oral presentation)

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7. **Ingman WV** and Robertson SA (2001) "Impaired ovarian function in preimplantation embryo development in TGFbeta1 null mutant mice" Society for Reproductive Biology, Annual Meeting, Australia (oral presentation)

8. **Ingman WV** and Robertson SA (2001) "Impaired ovarian function in preimplantation embryo development in TGFbeta1 null mutant mice" Australian

Society for Medical Research South Australian Conference, Australia (oral presentation)

9. **Ingman WV** and Robertson SA (2002) "Intromission failure leads to infertility in transforming growth factor beta1 null mice" Society for Reproductive Biology, Annual Meeting, Australia (oral presentation)

10. Robertson SA and **Ingman WV** (2002) "Null mutation in TGFβ1 causes oocyte incompetence and early embryo arrest" Gordon Research Conference – Reproductive Tract Biology, USA (poster presentation)

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ABBREVIATIONS

ANOVA	analysis of variance
bp	base pair
CL	corpus luteum
CSF-1	colony-stimulating factor-1
Ct	Cycle threshold
DNA	deoxyribonucleic acid
eNOS	endothelial nitric oxide synthase
FSH	follicle-stimulating hormone
g	gravity
hCG	human chorionic gonadotropin
HSD	hydroxysteroid dehydrogenase
iNOS	inducible nitric oxide synthase
IU	international unit
IVF	in vitro fertilisation
kDa	kilo Daltons
LAP	latency associated peptide
LH	luteinizing hormone
MHC	major histocompatibility complex
MMTV	mouse mammary tumour virus
MQ	milli Q
NEOR	neomycin resistance
NMS	normal mouse serum
nNOS	neural nitric oxide synthase
NOS	nitric oxide synthase
P450c17	P450 17 α-hydroxylase/C17-20-lyase
P450scc	P450 side chain cleavage
PBS	phosphate buffered saline
pc	post coitum
PCR	polymerase chain reaction
PKCS	catalytic subunit of DNA-dependent protein kinase
PMSG	pregnant mare serum gonadotropin
RIA	radioimmunoassay
RNA	ribonucleic acid
scid	severe combined immunodeficiency
SNB	spinal nucleus of the bulbocavernosus
StAR	steroidogenic acute regulatory protein
Tβ-R	transforming growth factor beta receptor
TGFβ	transforming growth factor beta
TP	testosterone propionate
UV	ultraviolet
WAP	whey acid protein

Chapter 1 Literature review

1.1 INTRODUCTION

The cytokine transforming growth factor beta (TGFβ) was first identified two decades ago by virtue of its ability to induce anchorage independent growth of fibroblasts (Moses et al., 1981; Roberts et al., 1981). It has since been found to comprise a number of alternative isoforms encoded by individual genes, which share many biological functions and characteristically influence viability and apoptosis, proliferation and differentiation, adhesion and migration in several cell lineages.

In contrast to most tissues, the reproductive tract is notable for the substantial extent of ongoing development and cyclic remodelling that occurs during pubertal and adult life, as the processes of germ cell maturation and secondary sex organ development, and the capacity to support pregnancy are acquired. Given the diverse roles of TGFβ in regulation of cell differentiation and proliferation in tissue development and repair, it seems reasonable that TGFβ family members should contribute to the molecular regulation of reproductive events. Indeed a multitude of studies implicate TGFβ1, TGFβ2 and TGFβ3 in almost every aspect of reproductive function. Considerable research effort has focused on localising TGFβ mRNA transcripts and protein, and to a lesser extent TGFβ receptors, in reproductive tissues. In vitro culture experiments have documented the actions of TGFβ on specific cell types and have demonstrated interactions between TGFβ, hormones and other growth factors. Despite this, the precise significance of these factors in individual reproductive processes is far from clear. Much of the published data in this field is limited by being simply descriptive in nature, or conducted ex vivo and hence in cells devoid of the constraints of in vivo micro-environmental context.

Identification of the precise roles of TGFβ cytokines is now being assisted by the application of sophisticated genetic approaches and novel molecular tools. Transgenic and mutant mice bearing null mutations in genes encoding TGFβ ligands, receptors and other regulatory moieties, particularly those offering the advantage of tissue- or cell-lineage-specific perturbation, are beginning to yield important insights.

The purpose of this chapter is to provide an overview of our current understanding of the physiological role of the TGFβ cytokines in reproductive events. We have drawn on selected published data, principally from rodent models, in an attempt to synthesise a coherent viewpoint and to highlight deficiencies where an increased research effort is warranted.

1.2 REGULATION OF TGFβ ACTION

The synthesis of TGFβ ligand and receptors appears to be almost ubiquitous in reproductive tissues, so a better understanding of the true physiological functions of this cytokine family in reproductive processes requires analysis of the full range of determinants governing TGFβ responsiveness. Temporal and spatial regulation and isoform specific production of TGFβ may contribute to restricting its sphere of influence. However the effects of TGFβ are constrained not only by ligand expression, but also by activation from latent precursor moieties, the local availability of soluble and transmembrane binding proteins, and expression of the various components of cognate receptors. In addition, the intracellular signalling pathways activated by ligand binding are complex and offer further opportunity for modulation. The consequences of ligand binding on downstream cellular processes is dependent on a number of regulatory transcription factors, some of which interact with other receptor mediated activation pathways, and others that are restricted to specific cell lineages. Thus the effect of TGFβ on a target cell is the net consequence of a large number of regulatory processes, summarised in Figure 1.1.

1.2.1 TGFβ isoforms

The TGFβ superfamily consists of more than thirty proteins sharing 30-80% sequence homology (Lawrence, 1996; Massague, 1998). The best characterised members include the five isoforms of TGFβ, activin, inhibin, Mullerian inhibitory substance, the bone morphogenic proteins and the growth/differentiation factors. TGFβ1, TGFβ2 and TGFβ3 are expressed in mammals, while TGFβ4 and TGFβ5 are expressed in birds and Xenopus laevis respectively. Biologically active TGFβ1, TGFβ2 and TGFβ3 are 25 kDa homodimers linked by disulfide bonds sharing a high

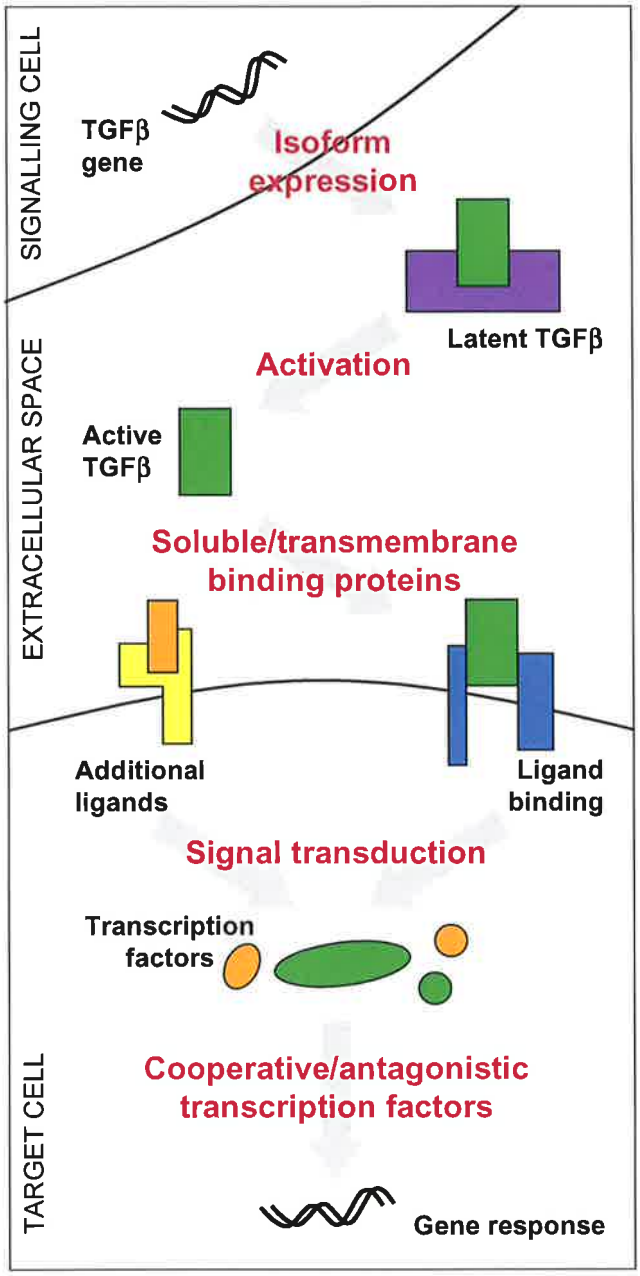

FIGURE 1.1 Regulation of TGFβ action is complex and multifactorial. TGFβ signalling at the target cell surface is the net result of regulation at the level of expression of TGFβ isoform mRNA expression, activation of latent TGFβ, the presence of soluble or transmembrane binding proteins and decoy receptors, and finally coupling with cognate Tβ-RI and Tβ-RII receptors. Upon signal transduction, the effect on target cell gene expression will depend on the abundance of several cooperative or antagonistic transcription factors (see Table 1.1), some of which are regulated by binding of other ligands at the cell surface.

level of sequence conservation and almost identical tertiary structure (Lawrence, 1996).

Since each of the TGFβ isoforms employ the same receptors for signal transduction and are interchangeable in many in vitro systems, it has been suggested that the isoforms are functionally redundant. However, the promoter sequence within each gene show distinguishing features and differential expression is evident in vivo. TGFβ2 and TGFβ3 tend to be hormonally and developmentally regulated, while transcription of TGFβ1 is selectively responsive to the products of oncogenes and immediate early genes, as well as autocrine regulation.

1.2.2 Regulation of TGFβ activation

TGFβ is secreted in an inactive form, non-covalently linked to a latency associated peptide (LAP) which in turn is covalently bound to a large latent TGFβ binding protein, together comprising a 225-260 kDa complex (Lawrence, 1996). Latent TGFβ binding protein is implicated in assisting secretion, storage in extracellular matrix depots and in facilitating activation of TGFβ. Release of active TGFβ from the latent precursor is clearly an important limiting factor in its biological activity.

TGFβ can be artificially released from its latent form by activation with acid, heat, urea or SDS but activation in vivo more probably occurs enzymatically (Brown et al., 1990; Lawrence, 1996). Association of latent TGFβ with the mannose-6-phosphate / insulin-like growth factor II receptor has been shown to activate urokinase plasminogen activator, cleaving plasminogen to plasmin, which frees TGFβ from its latent precursor (Dennis and Rifkin, 1991; Godar et al., 1999). Physiological release of biologically active TGFβ can be achieved by proteases including plasmin, cathepsins B and D, and calpain and glycosidases including endoglycosidase F, sialidase and neuraminidase (Khalil, 1999). The glycoprotein thrombospondin-1 is attributed a major activating role in vivo through its interaction with the LAP of latent TGFβ which causes a conformational change exposing TGFβ receptor binding sites (Schultz Cherry and Murphy Ullrich, 1993). The physiological

importance of this mode of activation is demonstrated by the inflammatory condition seen after null mutation of the thrombospondin-1 gene . In epithelial cells, association between αvβ6 and αvβ8 integrin and RGD sequences in the LAP domain of L-TGFβ may also induce TGFβ receptor binding competence without LAP release (Munger et al., 1999; Mu et al., 2002). Similarly, reactive oxygen intermediates have been implicated in the generation of active TGFβ through their effects on LAP stability (Barcellos-Hoff and Dix, 1996). It is likely that the physiological significance of each of these enzymes will vary between tissues and even within local microenvironments, so identifying their relative importance in achieving TGFβ activation in vivo will be a complex task.

1.2.3 TGFβ signalling

Two transmembrane receptors (termed Tβ-RI and Tβ-RII) with serine/threonine protein kinase cytosolic domains mediate TGFβ binding and signal transduction in target cells (Massague, 1998). A heterotetrameric complex of Tβ-RI and Tβ-RII in association with TGFβ ligand is required for signal transduction. Tβ-RII binds active TGFβ1 and 3 with high affinity, and Tβ-RI is subsequently recruited (Massague, 1998). In contrast, TGFβ2 has low affinity for Tβ-RII and requires co-localisation of additional transmembrane docking molecules to facilitate receptor binding. Other receptors related to Tβ-RI, specifically ALK1 and TSK 7L (the mouse homologue of activin type I receptor A), also bind TGFβ and have the potential to modulate TGFβ signalling in vivo (Attisano et al., 1993; Ebner et al., 1993; ten Dijke et al., 1994).

Three additional transmembrane proteins have now been identified which bind TGFβ but are not capable of signal transduction. Betaglycan, also known as the type III TGFβ receptor, appears to localise TGFβ1, TGFβ2 and TGFβ3 at the plasma membrane, and is particularly important for stabilising the interaction between TGFβ2 and Tβ-RII (Massague, 1998) and enhances TGFβ signalling through interaction with the cytoplasmic domain of Tβ-RII (Blobe et al., 2001). Endoglin is a transmembrane protein that binds TGFβ1 and TGFβ3 and forms complexes with TGFβ receptors and other receptors of the TGFβ superfamily (Barbara et al., 1999).

Endoglin is suggested to be an accessory molecule for TGFβ family receptor binding, although its precise role may be target cell lineage dependent. For example, endoglin may facilitate TGFβ1 and TGFβ3 inhibition of human trophoblast differentiation (Caniggia et al., 1997), but in contrast, it is reported to inhibit the effects of TGFβ1 on human endothelial cells (Li et al., 2000) and rat myoblasts (Letamendia et al., 1998). The TGFβ pseudoreceptor BAMBI, cloned from Xenopus laevis, inhibits signalling by TGFβ as well as activin and bone morphogenic protein, presumably by competing for ligand binding (Onichtchouk et al., 1999). A number of soluble proteins also bind TGFβ (Miyazono et al., 1993) and may act to sequester TGFβ away from the cell surface. Together this array of receptors and binding proteins offer considerable scope for regulating TGFβ binding to signal transducing receptor complexes.

Signalling events downstream of the cell membrane provide further opportunity for micro-environmental context and cell lineage to impact on the target cell response to TGFβ. Tβ-RII phosphorylates Tβ-RI, causing activation of intracellular transcription factors (Table 1.1), most notably the Smad family. However, the ensuing pattern of transcription of response elements is dependent on the concurrent activity of several synergising and inhibitory cofactors, regulated by other receptor signalling pathways (ten Dijke et al., 2000). Thus the precise effect of TGFβ signalling on gene expression in a given target cell is the net consequence of the integrated effects of several modulating forces (Figure 1.1).

1.3 GENETIC MODELS FOR TGFβ DISRUPTION

Targeted mutations or overexpression of TGFβ isoforms in mice has made it possible to analyse the role of TGFβ proteins in vivo. That the three mammalian isoforms of TGFβ have vital roles in development and immune regulation has been clearly demonstrated by findings that null mutations in any one of these genes is lethal (Shull et al., 1992; Kulkarni et al., 1993; Kaartinen et al., 1995; Proetzel et al., 1995; Sanford et al., 1997). Moreover, the striking differences between the three phenotypes underscores the existence of isoform-specific activities in vivo and

Receptor regulated factors	Cooperative factors	Antagonistic factors	Interaction with other signalling pathways	Response genes
SMAD2	SMAD4	SMAD6	JAK/STAT (IFNγ induces SMAD7)	Cyclin dependent kinase inhibitors
SMAD3	SARA	SMAD7		COL1A1, COL1A2
	FAST1	TGIF	NF-κB (TNFα induces SMAD7)	c-myc
	AP-1 family	Ubiquitin dep degradation		perlecan
	TFE3	Calmodulin	MAPK (EGF and HGF interact with SMAD2)	PAI-1
	VDR	Evi-1		IgA
	CBF/PEBP2	GR		c-Fos
	CBP/p300	c-Ski		Goosecoid
	ATF-2	ER		
	Menin			

TABLE 1.1 Transcription factors implicated in TGFβ signalling. Transcription factors activated by ligand dependent TGFβ receptor phosporylation, and factors known to enhance or inhibit receptor-regulated transcription factor activity, are shown. Also shown are other intracellular signalling pathways known to interact with TGFβ signalling, and genes known to be regulated by TGFβ ligand binding. References: (Christian and Nakayama, 1999; Massague, 2000; Miyazono, 2000; ten Dijke et al., 2000; Zimmerman and Padgett, 2000)

provides evidence that the three isoforms are not functionally redundant.

Detailed evaluation of phenotypes in genetic mutants can provide important and convincing information on gene function. However, a degree of caution must be exercised in interpreting this data, particularly when attempting to reconcile in vivo phenotypes with in vitro findings. In many cases, null mutants show that specific TGFβ isoforms are not an absolute requirement for development and function in a given tissue where in vitro studies have suggested a critical role. However, such a finding does not necessarily support the conclusion of no function at all, since overlapping functions between growth factors and cytokines might well compensate for TGFβ deficiency. Strategies that permit temporal deletion of function will be important tools in overcoming the possibility of adaptive compensation in ontogeny. Moreover, detailed studies of tissue function are required to detect subtle defects, many of which might be less evident in the rarefied environment of an animal house than after exposure to challenges typical of the wild, such as nutritional deprivation or infection.

A further problem in in vivo genetic experiments is distinguishing between effects of local gene perturbation versus systemic effects. This is particularly the

case with TGFβ, where blood-borne and even diet-derived cytokine clearly contributes to net availability in peripheral tissues. The reproductive system is additionally problematic because of the hierarchical relationship between neuroendocrine tissues and reproductive organs. For example, lesions in hypothalamic, pituitary or steroidogenic tissues would be reflected downstream in other tissues and will complicate attempts to pinpoint specific lesions. Tissue specific knockouts and strategies involving organ transplantation between null mutants and cytokine replete animals can partially surmount these limitations.

Notwithstanding these considerations, a more thorough analysis of structure and function of individual tissue systems in TGFβ null mutant mice, especially undertaken in concert with tissue specific knockouts, will help to elucidate redundant and non-redundant functions of TGFβ isoforms. To date, this approach has been highly informative in unravelling the roles of TGFβ in the mammary gland and in fetal development, and is beginning to have an impact in other aspects of reproduction (Table 1.2).

1.4 THE ROLE OF TGFβ IN MALE REPRODUCTIVE FUNCTION

1.4.1 Testis function and spermatogenesis

There is differential production of TGFβ and TGFβ receptors in the testis during development, and in vitro reports suggest that TGFβ participates in the cell-cell communication responsible for appropriate development and prevention of premature onset of spermatogenesis and steroidogenesis. Messenger RNA encoding all three TGFβ isoforms and both TGFβ receptors are expressed in embryonic and adult testes (Watrin et al., 1991; Mullaney and Skinner, 1993; Le Magueresse Battistoni et al., 1995). TGFβ1 is expressed by both somatic cells (Sertoli cells, peritubular myoid cells and macrophages) (Mullaney and Skinner, 1993) and germ cells (Watrin et al., 1991) while TGFβ2 and TGFβ3 are expressed only by somatic cells. Tβ-RI and II are expressed in greatest abundance in the immature testis (Le Magueresse Battistoni et al., 1995), suggesting that TGFβ has a key role in early testicular development. Differential TGFβ expression also occurs

Genetic manipulation	Phenotype
TGFβ1 null mutation	Defective yolk sac vasculogenesis and haematopoeisis in 50% (embryo mortality) (Dickson et al., 1995)
	Preimplantation development failure in 50% (embryo mortality) (Kallapur et al., 1999)
TGFβ2 null mutation	Lung and cardiovascular abnormalities (perinatal mortality) (Sanford et al., 1997)
TGFβ3 null mutation	Palate abnormalities (perinatal mortality) (Proetzel et al., 1995)
	Impaired mammary gland involution (Nguyen and Pollard, 2000)
Tβ-RII dominant negative receptor transgenic	Defective yolk sac vasculogenesis and haematopoeisis in 100% (embryo mortality) (Goumans et al., 1999)
Tβ-RII dominant negative receptor transgenic targeted to prostate with C3(1) promoter	Increased number of epithelial cells and reduced apoptosis in prostate (Kundu et al., 2000)
Tβ-RII dominant negative receptor transgenic targeted to mammary with MMTV promoter	Mammary epithelial cell hyperplasia, inappropriate milk protein expression (Gorska et al., 1998)
TGFβ1 transgenic targeted to mammary with MMTV promoter	Reduced ductal growth in virgin mammary gland (Pierce et al., 1993)
TGFβ1 transgenic targeted to mammary with WAP promoter	Lactation defect in postpartum mammary gland (Jhappan et al., 1993)
Tβ-RII dominant negative receptor transgenic under control of matallothionein promoter	Increased mammary epithelial growth (Joseph et al., 1999)

TABLE 1.2 Reproductive phenotypes of mice with null mutations or transgenic over-expression of TGFβ or TGFβ receptor genes. Additional details are given in the text.

during post-natal development of the testis and onset of spermatogenesis (Mullaney and Skinner, 1993), and at least in organ culture, is capable of regulating the number of germ cells in developing testes by inducing apoptosis (Olaso et al., 1998).

The actions of TGFβ in testicular target cells are influenced by endocrine hormones and sex steroids. Production of TGFβ1 by rat Sertoli cells can be upregulated by the presence of estradiol (Dorrington et al., 1993) which is expected to be part of a negative feedback loop regulating estradiol synthesis in the testis as TGFβ1 inhibits follicle-stimulating hormone (FSH) induced aromatase activity (Morera et al., 1992). Interestingly it has recently come to light that ligand bound estrogen receptor inhibits Smad3 activity in human kidney carcinoma cells, effectively switching off TGFβ action (Matsuda et al., 2001). There is still much to be understood as to the nature of the relationship between TGFβ and estrogen. FSH

negatively regulates TGFβ2 synthesis by cultured rat peritubular cells (Konrad et al., 2000) and pubertal Sertoli cells (Mullaney and Skinner, 1993), but together with luteinizing hormone (LH), enhances secretion of TGFβ1 in fetal testes (Gautier et al., 1997). TGFβ1 reduces responsiveness to LH/human chorionic gonadotropin (hCG), and inhibits steroidogenesis in cultured rat Leydig cells (Avallet et al., 1987). Moreover, TGFβ3 may influence the events guiding the passage of preleptotene spermatocytes from the basal to the adluminal compartment of the seminiferous epithelium, since in vitro culture experiments show that TGFβ3 inhibits expression of proteins mediating formation of tight junctions between Sertoli cells (Lui et al., 2001).

1.4.2 Penis, seminal vesicle and prostate growth and development

A role for TGFβ1 in male secondary sex organ development is suggested by analyses of TGFβ1 protein levels in male rats during and following puberty. It has been shown that TGFβ1 in the rat penis increases two- to three-fold in abundance between 3 and 7 weeks of age, coincident with accelerated penile growth (Gelman et al., 1998). This together with previous findings that TGFβ1 stimulates collagen synthesis by smooth muscle cells from human corpora cavernosa in vitro (Moreland et al., 1995) might be interpreted to imply a role for TGFβ in promoting penile growth. Indeed, TGFβ is suggested to contribute to penile fibrosis observed in aging men (Moreland et al., 1995), and aberrant TGFβ1 protein synthesis is evident in Peyronie's disease (el Sakka et al., 1997), a connective tissue disorder of penile tissue characterised by calcified fibrous plaques. However, experiments by Gelman et al. (1998) suggest that TGFβ1 may act as an inhibitory signal to limit growth towards the end of puberty. TGFβ1 administered directly to the corpora cavernosa of immature male rats for a period of 7 days using miniosmotic pumps was shown to significantly decrease penile shaft weight. Although androgens are implicated in terminating penile growth at the onset of adulthood and TGFβ1 has been found to reduce androgen receptor levels in rat penile smooth muscle cell cultures (Freedman et al., 1992), a mechanism operating independently of androgens cannot be excluded since exogenous administration of TGFβ1 to the rat penis during puberty does not alter androgen receptor protein synthesis (Gelman et al., 1998).

Interactions between androgens and TGFβ have also been investigated in the prostate and seminal vesicle. TGFβ1, TGFβ2 and TGFβ3 mRNAs are detected in rat ventral prostate (Itoh et al., 1998). Expression of prostatic TGFβ appears to be kept in check by sex steroids as castration causes an increase in TGFβ mRNA levels (Kyprianou and Isaacs, 1989). Furthermore, androgen ablation is found to induce expression of each of the three TGFβ isoforms in an isoform and tissue-specific manner in the seminal vesicle and epididymis (Desai and Kondaiah, 2000). Whole organ culture of neonatal seminal vesicles with TGFβ1 inhibits androgen induced epithelial branching and suppresses protein and mRNA synthesis, however TGFβ1 does not affect seminal vesicle growth in the absence of androgens (Tanji et al., 1994).

Interest in the role of TGFβ in the prostate stems from its likely involvement in prostate cancer (Lee et al., 1999). Administration of TGFβ1 to the prostate of mature rats decreases prostatic cell DNA synthesis (Martikainen et al., 1990) and induces apoptosis. This concurs with the finding of epithelial hyperplasia in the prostate of mice carrying a dominant negative Tβ-RII transgene targeted to the prostate with the C3(1) promoter (Kundu et al., 2000). Prostate cancer cells lose responsiveness to TGFβ as a growth inhibitory factor and produce large quantities presumably to evade immune recognition, promote angiogenesis and extracellular matrix production (Lee et al., 1999).

Together, these studies support a role for TGFβ in regulating the development of male accessory sex organs. TGFβ is likely to have differential activities dependent on the developmental stage of the animal and the target cell lineage, but it is notable that the patterns of expression and function suggest a predominantly constraining role, where TGFβ limits cell growth and proliferation.

1.5 THE ROLE OF TGFβ IN FEMALE REPRODUCTIVE FUNCTION

1.5.1 Ovarian function

Folliculogenesis and oocyte maturation are the processes comprising complex networks of cell-cell interactions, in which growth factors feature commonly as messengers to regulate proliferation and differentiation of ovarian cells. The three isoforms of TGFβ are each detectable in the theca and granulosa cells of the ovary and their localisation fluctuates over the course of gonadotropin induced follicle maturation (Ghiglieri et al., 1995). As in the male, there is an interaction between sex hormones, gonadotrophins and both the expression and function of TGFβ. TGFβ is believed to augment several of the effects of FSH including induction of aromatase activity in murine granulosa cells (Adashi et al., 1989) and promotion of DNA synthesis in hamster follicular cells (Roy, 1993). While it is not believed that TGFβ1 facilitates either of these effects by increasing cell sensitivity to FSH, TGFβ1 and TGFβ2 have both been reported to stimulate FSH receptor expression by rat granulosa cells (Dunkel et al., 1994).

Ovarian TGFβ aids folliculogenesis and production of mature oocytes, at least partially through amplifying the effects of FSH and by stimulating production of estradiol. This view is supported by the positive correlation between the TGFβ1 content of follicular fluid and pregnancy success in human in vitro fertilization (Fried and Wramsby, 1998). Inhibition of ovulation by TGFβ may play a role in the kinetics of ovulation, preventing premature follicle rupture (Juneja et al., 1996), by a mechanism potentially involving inhibition of macrophage activation (Van der Hoek et al., 2000), thereby allowing completion of oocyte cytoplasmic maturation.

After ovulation, TGFβ is likely to be involved in promoting the development and function of the corpus luteum, where TGFβ1 and TGFβ2 are produced by luteal cells (Ghiglieri et al., 1995) and macrophages (Matsuyama and Takahashi, 1995). The role of these TGFβ isoforms in luteal function may be to mediate the luteotropic effects of prolactin (Matsuyama et al., 1990) and subsequently inhibit apoptosis of luteinized cells (Matsubara et al., 2000).

1.5.2 Endometrial remodelling

The actions of steroid hormones in the endometrium during the ovarian cycle are mediated by locally expressed growth factors, amongst which TGFβ is prominent. Fluctuations in TGFβ1, TGFβ2 and TGFβ3 as well as Tβ-RII in the endometrium occur over the course of the cycle (Godkin and Dore, 1998). Estradiol treatment of human endometrial stromal cells upregulates TGFβ1 and TGFβ3 mRNA expression (Arici et al., 1996), while TGFβ2 production by these cells is inhibited by progesterone, testosterone and estradiol treatment (Kanzaki et al., 1995).

TGFβ is overexpressed in women with endometriosis, however this appears to be a rescue response as TGFβ is capable of mediating the suppressive effects of progesterone in preventing experimental endometriosis (Bruner et al., 1999), particularly through down-regulating matrix metalloproteinase synthesis.

1.5.3 Mammary gland development

In situ hybridisation studies have identified mRNA encoding all three isoforms of TGFβ in the mouse mammary gland. Several studies demonstrate conclusively a major role for each isoform in mammary morphogenesis, and this work has attracted a great deal of attention since a paradoxical role for TGFβ as both an anti-tumour agent and in tumour progression in breast cancer is suggested (Reiss and Barcellos Hoff, 1997). TGFβ2 is transcribed only during pregnancy, and all three are almost completely absent during lactation (Robinson et al., 1991). Understanding the role of TGFβ in the mouse mammary gland has been greatly facilitated by in vivo experimental approaches including administration of exogenous TGFβ, genetic manipulation of TGFβ expression levels and transplantation strategies.

Implants containing TGFβ1 placed adjacent to the developing terminal end buds of the mammary of peripubertal virgin mice impair ductal elongation (Silberstein and Daniel, 1987), without affecting pregnant mammary tissue (Daniel et al., 1989). The pattern of TGFβ1 protein deposition around the budding region of the

mammary ducts but not the alveolus is consistent with TGFβ1 acting to inhibit ductal growth without affecting alveolar bud formation during pregnancy (Silberstein et al., 1992). Further confirmation of a constraining role for TGFβ in ductal morphogenesis is provided by transgenic models that elicit overexpression of TGFβ1 in the mammary gland. When TGFβ1 transcription is targeted using the mouse mammary tumour virus (MMTV) promoter, a reduction in total ductal volume is seen in adult virgin mice, while pregnancy-associated alveolar development proceeds normally, resulting in normal lactation in the post partum animal (Pierce et al., 1993). Interestingly, similar results are not obtained when TGFβ1 is overexpressed in the latent form, suggesting that TGFβ activity in the mammary gland is constrained by the activation event (Pierce et al., 1993).

In contrast, TGFβ1 overexpression under the control of the pregnancy-responsive whey acid protein (WAP) gene promoter to elicit transgene expression in the pregnant and lactating mouse causes sufficient inhibition of lobuloaveolar development to prevent lactation (Jhappan et al., 1993). It is likely that greater transgene expression under the WAP promoter explains the disparity between these two studies (Pierce et al., 1993). In a later study, WAP promoter-driven overexpression of TGFβ1 was found to cause early senescence of lobuloalveloar progenitor cells (Kordon et al., 1995). These data are consistent with the finding that TGFβ inhibits the production of milk caseins in explants of mammary tissue from pregnant mice (Robinson et al., 1993).

An inhibitory role for TGFβ in ductal morphogenesis is further supported by studies in mice carrying defective TGFβ receptors causing reduced sensitivity to its ligand. A truncated Tβ-RII, targeted to the mammary gland with the MMTV promoter, caused mammary epithelial hyperplasia at the age of 20 weeks, with virgin animals exhibiting inappropriate alveolar development and expression of milk protein β-casien (Gorska et al., 1998). Dominant negative Tβ-RII under the control of a metallothionein-derived promoter and induced by zinc also demonstrated increased ductal epithelial activity (Joseph et al., 1999).

Examining the role of TGFβ2 and TGFβ3 in mammary morphogenesis is complicated by the lethality of null mutations in these genes. However, transplantation of neonatal mammary tissue has proven informative in analysing the differential roles of TGFβ produced systemically versus locally in the mammary gland. Using this technique, together with TGFβ3 expression targeted to the mammary of lactating mice using the β-lactoglobulin promoter, TGFβ3 has been shown to be a critical cytokine involved in alveolar epithelial apoptosis which occurs after lactation ceases (Nguyen and Pollard, 2000). Transplanted mammary tissue carrying defective TGFβ3 confirmed that this isoform has no specific role in virgin mammary development, perhaps as a consequence of overlapping functions of TGFβ1 and 2 in limiting ductal growth.

1.6 THE ROLE OF TGFβ IN PREGNANCY

1.6.1 Embryo and fetal development

Both TGFβ1 and 2 protein are found in the developing oocyte and embryo (Schmid et al., 1994; Ghiglieri et al., 1995), with TGFβ2 being most abundant prior to fertilisation and diminished in four and eight cell embryos (Schmid et al., 1994). Expression of each TGFβ isoform increases after this stage, as the preimplantation embryo develops (Paria et al., 1992). In the blastocyst, TGFβ1, TGFβ2 and TGFβ3 are each found in both the inner cell mass and the trophectoderm, while TGFβ binding occurs primarily in trophectoderm cells (Paria et al., 1992).

Null mutations in each of the three mammalian TGFβ isoforms are embryonic lethal, but the different phenotypes provide conclusive evidence for unique roles of each isoform in fetal development, as well as an essential function for TGFβ1 in immunological homeostasis. Preimplantation embryo development, haematopoiesis and yolk sac vasculogenesis are defective in a percentage of TGFβ1 deficient conceptuses carried by TGFβ1 replete mothers, and the penetrance of these defects is strain dependent (Dickson et al., 1995; Kallapur et al., 1999). Of those TGFβ1 deficient progeny that survive, severe autoimmune destruction of peripheral

tissues leads to death at weaning (Shull et al., 1992), when maternal milk-derived TGFβ is withdrawn. This role for TGFβ1 in fetal development is supported by studies in mice with a mutation generating Tβ-RII kinase deficiency (Goumans et al., 1999), and is reflected in the severe deviation in the predicted Mendelian ratios of offspring born through heterozygous matings (Shull and Doetschman, 1994). Deficiency in TGFβ2 results in lung abnormalities and cardiovascular defects which cause neonatal death within 20 hours after birth (Sanford et al., 1997). Developmental abnormalities also stem from TGFβ3 deficiency, with null mutant mice having defective palatal shelf fusion and failing to survive for longer than a day after birth (Kaartinen et al., 1995; Proetzel et al., 1995).

1.6.2 Implantation and placental development

Implantation of the conceptus is a complex and finely orchestrated process, the success of which depends on achieving the appropriate extent of outgrowth and invasion of trophoblast cells into maternal tissues. Detection of TGFβ1, TGFβ2 and TGFβ3, Tβ-RI and II, betaglycan and endoglin at the fetal maternal interface, together with the known roles of TGFβ in regulation of proliferation, differentiation, extracellular matrix production and migration, clearly implicate each of the TGFβ isoforms in the cellular events underlying implantation.

Successful implantation and placental morphogenesis occurs through a programmed sequence of trophoblast cell differentiation and proliferation, accompanied by changes in expression of several genes most notably encoding adhesion molecules and extracellular matrix proteins. Several studies imply an essential role for regulated TGFβ activity both in promoting and limiting placental development through tightly controlled temporal, spatial and isoform specific activities.

TGFβ1 is expressed by uterine epithelium from early in pregnancy (Tamada et al., 1990) and during the implantation process is believed to enhance trophoblast attachment to the endometrium through eliciting production of the extracellular matrix component oncofetal fibronectin (Feinberg et al., 1994) and promoting

adhesion of trophoblast cells to extracellular matrix (Irving and Lala, 1995). Trophoblast invasion in vitro can be inhibited by treatment with TGFβ1 (Graham and Lala, 1991), an effect believed to be partially mediated by indirect inhibition of matrix metalloproteinases (Graham and Lala, 1991) and plasmin (Graham, 1997) necessary for uterine tissue remodelling. Interestingly, expression of the TGFβ transmembrane binding protein endoglin also inhibits human trophoblast outgrowth and appears to be required for the inhibitory effects of TGFβ1 and TGFβ3, but not TGFβ2 (Caniggia et al., 1997). However, there may be differentiation stage and/or species specificity in this effect, as in vitro studies of murine blastocysts show that TGFβ1 can facilitate trophoblast cell outgrowth, through a mechanism involving autocrine release of parathyroid hormone-related protein (Nowak et al., 1999).

TGFβ3 has been implicated as a key regulator of early differentiation events, acting to mediate the inhibition of differentiation driven by low oxygen tension (Caniggia et al., 2000). When overproduced by the placenta in the pathological condition preeclampsia, TGFβ3 inhibits differentiation of trophoblast and thereby compromises trophoblast invasion (Caniggia et al., 1999).

Vascular endothelial growth factor production by a human first trimester trophoblast cell line is positively regulated by TGFβ1, a mechanism which may promote vascular development during placentation (Chung et al., 2000). Vasculogenesis is known to be dependent on TGFβ1 as endothelial differentiation is disrupted in 50% of fetal mice deficient in TGFβ1, leading to a defective capillary network and death at day 10.5 of gestation (Dickson et al., 1995).

1.7 TGFβ AND IMMUNE REGULATION IN REPRODUCTIVE TISSUES

Studies in TGFβ1 null mutant mice highlight the essential role for this cytokine in immunological events, particularly in immune tolerance. The effects of TGFβ in the immune system are mediated most commonly through the antigen presenting cells; macrophages and dendritic cells. In macrophages, TGFβ is recognised to have both stimulatory and inhibitory effects, in a differentiation-state and environmental context-dependent manner, on expression of several cytokines and

soluble mediators (Bogdan and Nathan, 1993). This has important implications in the testis, ovary, uterus and mammary gland where macrophages are abundant and are identified as key cells in the regulation of steroidogenesis, and tissue growth and remodelling as well as immune events.

Perhaps the most important immunological function of TGFβ in gametogenesis and pregnancy is likely to be in tolerance of the developing spermatozoa and oocytes, and most notably the semi-allogeneic conceptus. In each case the antigens involved are not encountered until puberty or adult life, so the conventional process of deletion of potentially harmful T-lymphocyte clones during fetal and neonatal life does not occur. In contrast, the consensus view is that immune mechanisms for coping with reproductive antigens necessitate a process of active suppression of T-lymphocyte subsets reactive with reproductive antigens. Induction of active immune tolerance is contingent on a sequence of events involving specialised antigen-processing cells, usually macrophages or dendritic cells, that favour a tolerogenic outcome through processing antigen in the context of specific cytokine environments. Studies in several mucosal and epithelial tissues heavily implicate TGFβ in the inductive and elicitation phase of immune responses deviated towards tolerance (Letterio and Roberts, 1998). Most notably, antigen presentation occurring in the context of TGFβ1 leads to the activation and expansion of T-lymphocytes with a 'Th3' phenotype, characterised by secretion of abundant TGFβ and the capacity to suppress any further immune activation in adjacent tissues (Weiner, 2001).

An immune response skewed towards type 2 or Th3 immunity appears to be a fundamental requirement for pregnancy success, while type 1 responses are associated with the limitation of placental growth and function that underlies recurrent spontaneous abortion and other pathologies of pregnancy (Piccinni and Romagnani, 1996; Raghupathy, 1997). Synthesis of TGFβ in decidual tissues is thus likely to contribute to the induction and maintenance of a maternal immune response conducive to optimal placental function and fetal growth. Indeed, lymphocyte populations secreting TGFβ are causally linked with pregnancy success in mice (Arck et al., 1999) and are diminished in the event of miscarriage.

The determinants of the quality and strength of the maternal immune response to pregnancy are not understood, but it is reasonable to focus on early events when paternal antigens would first be perceived in the maternal tract. In this regard, it is notable that seminal plasma has been identified as a potent source of TGFβ1 and TGFβ2 and that these cytokines appear to mediate the pro-inflammatory cascade elicited in the female tract at insemination (Tremellen et al., 1998). We and others have suggested a key role for these cytokines in the suppression of anti-sperm immune responses and potentially in the inductive phase of immune events leading to appropriate recognition and response to paternal antigens on the conceptus (Robertson and Sharkey, 2001).

Very little is known about the immune processes protecting developing gametes in the testes and ovary, although experiments in which mice are immunised with zona pellucida antigens implicate a process of gender-specific active tolerance (Tung et al., 2001). The abundance of TGFβ in both the ovary and testes is consistent with an immune deviating role. While convincing intervention studies are yet to be reported, observations of testicular macrophages show that they have specific qualities consistent with contributing to immune-privilege of the testis (Hedger, 2002).

1.8 CONCLUSION

A diverse range of activities for each of the TGFβ isoforms in many aspects of reproduction is suggested by the hundreds of published studies linking these cytokines with hormone induced gonad development, gamete production, steroidogenesis, invasion of the trophoblast at implantation and fetal and placental development (Figure 1.2). There is also mounting evidence implicating TGFβ in the development and function of secondary sex organs such as the penis, prostate, seminal vesicle, uterus and mammary gland. Furthermore, TGFβ present in semen and produced by the embryo and decidua may have a role in creating and maintaining an immunotolerant environment for the conceptus. Several activities of TGFβ, particularly those involving immune tolerance of gamete or conceptus

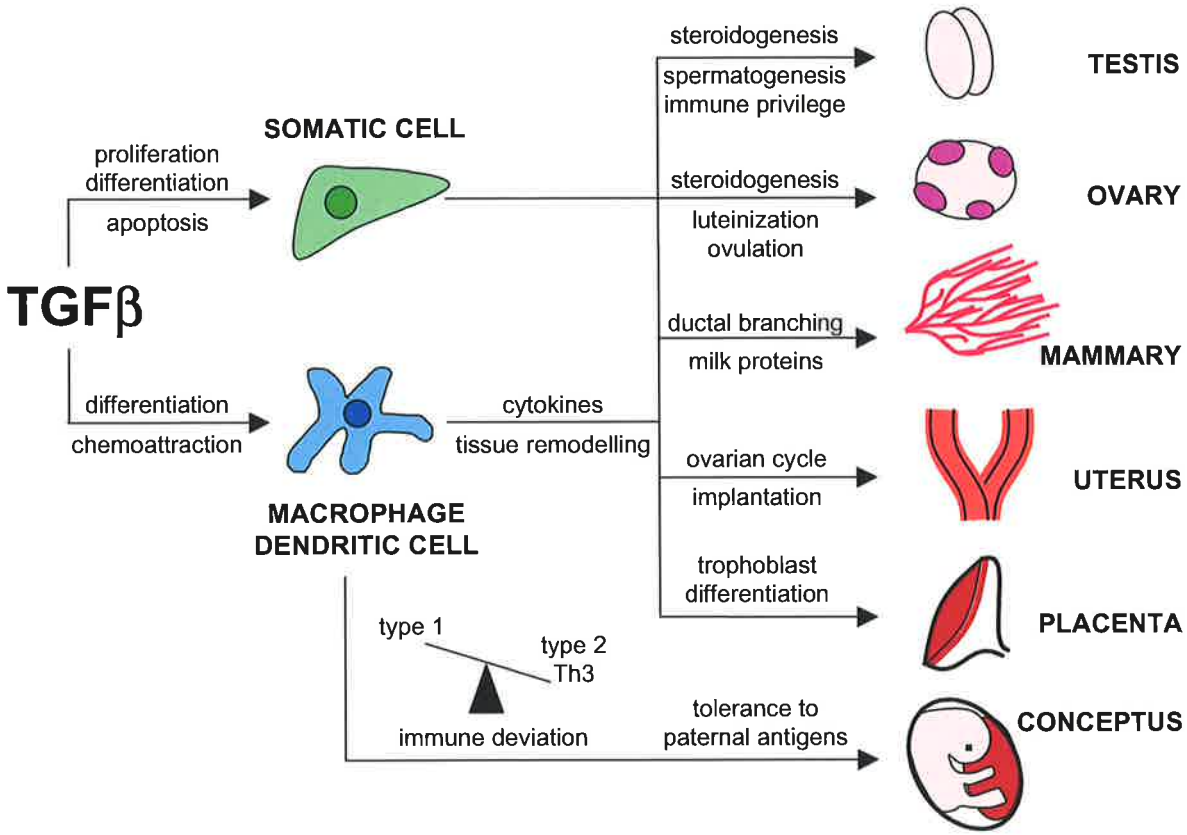

FIGURE 1.2 Diagrammatic representation of experimentally implied and postulated roles of TGFβ in reproductive tissues. TGFβ exerts its effects through direct effects on somatic cells, or indirectly through the agency of infiltrating macrophages and dendritic cells, in the testis, ovary, mammary gland, uterus, placenta and conceptus. TGFβ regulates cytokine release and tissue remodelling processes in macrophages, to influence development and differentiation in reproductive organ development and homeostasis. TGFβ regulates immune-deviating function in dendritic cells to induce and maintain immune tolerance, most notably in the maternal response to fetal antigens, but potentially also to gametes developing in the testes and ovary.

antigens, may be achieved through the agency of TGFβ-driven macrophage populations.

Moreover, expression of TGFβ appears to be attenuated in a number of reproductive pathologies. These include endometriosis, prostate cancer, breast cancer, penile fibrosis, recurrent spontaneous abortion and preeclampsia. However altered expression does not demonstrate causal involvement in disease progression, and a better understanding of the differential significance of each of the TGFβ isoforms in vivo, as well as the endocrine and paracrine messengers which regulate their synthesis and signalling, is required before the roles of TGFβ in reproductive health and disease can be fully unravelled. This will come not only from spatial and temporal analyses of TGFβ expression, but more importantly from studies of the large repertoire of molecules involved in activation of latent TGFβ, formation of TGFβ receptors, sequestration of TGFβ in the extracellular matrix and at the cell surface, as well as those comprising and modulating the TGFβ signalling cascade. In vivo models clearly offer advantages for definitive analyses of TGFβ in reproductive physiology (Table 1.2), through permitting the relative significance of endocrine versus paracrine cytokine, as well as the full gamut of microenvironmental modulating influences, to be evaluated. Powerful tools in this discovery process will be null mutant and transgenic animal models, which have already been used to great effect with mammary tissue. Undoubtedly, sophisticated techniques for tissue-specific regulation of transgene expression used in conjunction with tissue transplantation approaches will provide the most compelling information.

1.9 AIMS

It is widely accepted that TGFβ1 null mutant mice have severely compromised fertility. For those laboratories working with these mice, it is conventional for TGFβ1 deficient progeny to be produced by heterozygous breeding pairs. However, reproductive performance in male or female TGFβ1 deficient mice has never been the subject of formal investigation. Therefore the general aim of this thesis is to examine reproductive function in TGFβ1 null mutant mice to precisely characterise the extent and nature of their infertility. Specifically, the experiments contained herein aim to determine the reproductive lesions leading to infertility caused by TGFβ1 deficiency in mice. In doing so, the relative importance of TGFβ1 in various reproductive tissues and processes will be identified, leading to a greater understanding of the importance of this cytokine in vivo.

Chapter 2 Materials and Methods

2.1 MICE

2.1.1 Animal husbandry

All animal experiments were approved by the University of Adelaide Animal Ethics Committee and conducted in accordance with the National Health and Medical Research Council guidelines (1997) for use of animals for scientific purposes. The colony was maintained in specific pathogen free rooms at the University of Adelaide Laboratory Animal House, in controlled light (12:12 light:dark) and temperature. Food and water were given ad libitum.

2.1.2 Maintenance and production of TGFβ1 null mutant mice

2.1.2.1 Generation of the TGFβ1 null mutation

The mice used to investigate the role of TGFβ1 in reproductive function were homozygous for a null mutation in the *tgfβ1* gene (Fig 2.1A). The null mutation was produced by embryonic stem cell targeting by Shull et al. (1992) at the University of Cincinnati College of Medicine, Ohio, USA. A neomycin resistance gene (*neor*) was inserted into the BamH1 restriction site in exon 6 of the *tgfβ1* gene (Fig 2.1B). The gene was introduced into D3 embryonic stem cells (derived from the 129/Sv strain), and selected for neomycin resistance. The embryonic stem cells were injected into C57Bl/6 blastocysts and implanted into pseudopregnant females.

TGFβ1 null mutant mice are phenotypically normal at birth however develop a wasting syndrome at approximately 3 weeks of age characterised by inflammation and necrosis of many organs including the heart, stomach, liver and lung (Shull et al., 1992). The syndrome causes death within a few days. These observations are consistent with the findings of Kulkarni et al. (1993) who used embryonic stem cell targeting to disrupt exon 1 of the TGFβ1 gene.

Solution recipes found in Appendix

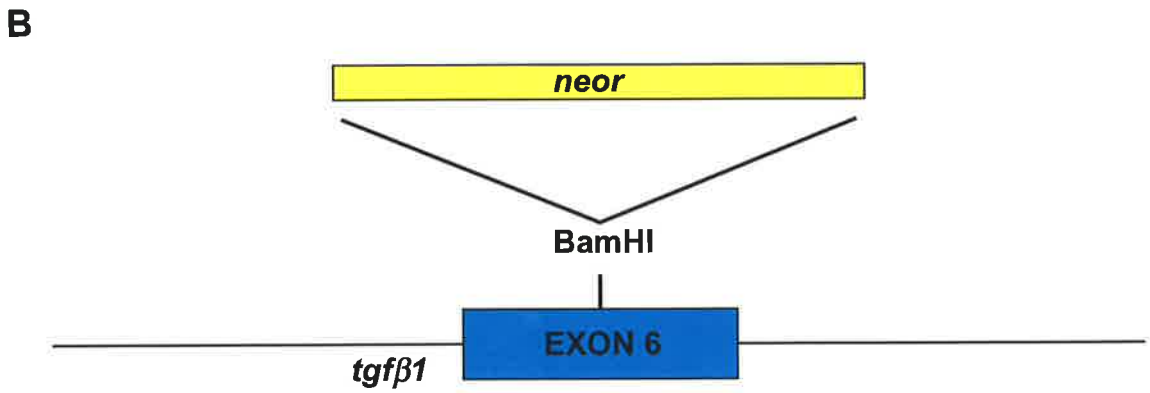

FIGURE 2.1 Embryonic stem cell targeting of the *tgfβ1* gene produces TGFβ1 null mutant mice. (A) Male 10 week old TGFβ1+/+ and TGFβ1-/- mice. (B) Targeted insertion of neomycin resistance gene at the BamHI restriction site in exon 6 of the TGFβ1 gene, leading to TGFβ1 null mutation.

TGFβ1 null mutant mice can survive longer than 3 weeks of age if immunocompromised. Dexamethasone (Letterio et al., 1994) or anti-CD11α (Diebold et al., 1995) treatment, mutation in the beta 2-microglobulin (Kobayashi et al., 1999) or MHC class II (Letterio et al., 1996) genes, or severe combined immunodeficiency (scid) (Diebold et al., 1995) prevent inflammation and increase the life span of TGFβ1 null mutant mice. The scid mutation is caused by a naturally occurring mutation in the catalytic subunit of DNA-dependent protein kinase gene (*pkcs*), and causes defective V(D)J rearrangement, leading to very low numbers of B and T cells in mice (Bosma and Carroll, 1991).

The experiments described herein use TGFβ1 null mutant mice on a scid background, and on a mixed genetic background of CF-1, 129/Sv and C3H. The TGFβ1 heterozygous colony was established at the University of Adelaide specifically for the studies contained in this thesis. Homozygous mutants were generated by heterozygous breeding pairs and detected by DNA analysis.

2.1.2.2 Generation of TGFβ1 null mutant mice on scid background

Mice heterozygous for the TGFβ1 null mutation on a mixed genetic background (CF1/129/C3H) and heterozygous for the *pkcs* gene mutation were imported to the University of Adelaide Laboratory Animal House in 1998, from the University of Cincinnati College of Medicine, Ohio, USA.

The mice were bred to homozygosity of the *pkcs* mutation and heterozygosity for the *tgfβ1* mutation. TGFβ1 null mutant mice (TGFβ1-/-) were produced by heterozygous breeding pairs (TGFβ1+/- x TGFβ1+/-). At the time of weaning (3 weeks), 5 mm of the tail tip of each mouse was cut for genotyping (protocol described later). Mice were toe clipped for identification.

2.1.2.3 TGFβ1 colony database

Each mouse born to TGFβ1 heterozygous breeder pairs was allocated a number in the TGFβ1 colony database. This database recorded the birth date of each mouse, its parents, sex, genotype and toe clip number for identification.

2.1.3 Blood collection

Blood was collected by a nick to the tail. The mice were heated under an infrared lamp for several minutes to increase blood flow to extremities. Up to 0.5 ml of blood could be collected by this method.

Where mice were to be sacrificed, blood was collected by cardiac puncture. The mice were deeply anaesthetised with 0.4-0.5 ml 2% Avertin (2% 2.2.2-tribromomethanol (Aldrich, USA) and 2% 2-methylbutan-2-ol (AJAX, AUS)) and blood was extracted directly from the heart using a 26 g needle. Up to 1 ml of blood could be collected by this method.

Where serum was required, blood was incubated at 4°C for 30 minutes, then centrifuged for 5 min at 10,000 g and stored at -20°C.

2.1.4 Ovarian cycle determination

The stage of the ovarian cycle was determined by analysis of vaginal smears, as described by Snell (1956). Sterile phosphate buffered saline (PBS) (25 µl) was used to lavage the vagina of female mice and the contents smeared on a glass slide and covered with a cover slip. The cellular contents were analysed under a phase contrast microscope to determine the stage of the ovarian cycle.

Proestrous	>50% round epithelial cells
Estrous	>90% cornified epithelial cells
Metestrous	50-90% cornified epithelial cells, rest leukocytes
Diestrous	>50% leukocytes

2.1.5 Analysis of mating behaviour

Adult male mice (approximately 8 weeks old) were housed separately for at least a week prior to observation of mating behaviour. The males were exposed to female adult mice in a Whitten cage for 2 days in order to familiarise them with the female scent. They were then housed separately for another 1 or 2 days before the experiment.

Immature (4 weeks old) female B10.br mice were superovulated with 5 IU PMSG (Folligon; Intervet, Boxmeer, Holland) at 1000 hours on day 1 and 5 IU hCG (Chorulon; Intervet, Boxmeer, Holland) at 1000 hours on day 3. At 2200 hours on day 3 the female was introduced into the male's cage and the first 2 hours of contact was recorded under red light using a 0 lux digital video camera (NV-DS38, Panasonic). In some experiments, the males were given a single intraperitoneal injection of sildenafil citrate (Pfizer, Sandwich, UK) (4 μg/10 g in saline) 30 minutes prior to introduction of the female.

On the following morning the female was sacrificed and the reproductive tract dissected. Superovulation and thus female receptivity was confirmed by detection of oocytes in the ampulla of the oviduct. The presence of a vaginal plug or sperm in the uterine tract flushing indicated whether or not the mice had successfully mated. If the female had not ovulated, the male was tested again the following night with a new female.

A number of male sexual behaviour parameters were quantified in the videos. The analysis was done by a single blinded assessor to remove bias. Starting from the introduction of the female, the percent of time in the first 10 minutes spent in anogenital investigation of the female was recorded as a measure of initial sexual interest. The latency, duration and number of mounts, intromission and ejaculation were recorded. The criteria for these behaviours were as follows (1) mounts; the male stood up with both hind legs on the ground and pulled the female towards him, (2) intromission; the male mounted the female and began pelvic thrusting and (3) ejaculation; the male began intromission and then pulled the female over to one side and the mice remained still and locked together for a considerable period of time. The total number of mounts was given as the combined number of mounts, intromission and ejaculation, and total mounts per minute was determined by the equation

(total number of mounts-1)/(last mount latency –first mount latency)

2.1.6 Testosterone replacement

2.1.6.1 Testosterone implants

Testosterone implants were made from 10 mm silastic tubing (0.062 inch internal diameter (ID), 0.125 inch outside diamter (OD)), plugged with vinyl tubing (1.4 mm ID, 1.9 mm OD) and polyethylene tubing (1 mm ID, 1.5 mm ID). The silastic tubing was sterilised in 70% ethanol, and filled with 150 mg/ml testosterone (Sigma, MO, USA) in oil and melted at each end to seal it. The implant was soaked in sterile PBS overnight.

Implants were given to mice under 2% halothane (Rhone Merieux, Vic, AUS), with 700 ml/min oxygen. All surgical equipment was soaked in 0.05% chlorhexidine (Jurox, NSW, USA) before use. An area on the dorsal rump was shaved, and a small incision through the skin was made. Blunt forceps were used to prise skin from the peritoneal wall to make a small space for the implant, which was inserted under the skin on the rump of the mouse parallel with the spine. The incision was closed firmly with two surgical clips. The mouse was given 100% oxygen until showing signs of conciousness.

2.1.6.2 Adult testosterone supplement

An alternative testosterone replacement therapy was by way of subcutaneous injections. Testosterone propionate (Upjohn, NJ, USA) (200 μg in 100 μl) was given to male TGFβ1 null mutant mice every second day from the age of 5 weeks.

2.1.6.3 Neonatal testosterone supplement

To androgenize the neonatal brain, a single subcutaneous injection of testosterone propionate (100 μg in 20 μl) was given to 1 or 2 day old pups. Together with adult testosterone replacement, this method has been shown to successfully restore mating ability in the hypogonadal male mouse (Livne et al., 1992).

2.1.6.4 Stimulation of steroidogenesis

Adult male mice were given a single intraperitoneal injection of hCG (5 IU/10 g) and blood collected for serum testosterone analysis by cardiac puncture 1 or 4 hours later. The testes were dissected and frozen in liquid nitrogen for analysis of steroidogenic enzyme mRNA expression.

2.2 NUCLEOTIDE ANALYSIS

2.2.1 Genotyping mice

Tail DNA was analysed for the intact and disrupted *tgfβ1* gene by polymerase chain reaction (PCR). The genotype of each mouse used in experiments was confirmed by a second PCR of DNA extracted from ear tissue taken after sacrifice.

2.2.1.1 DNA Extraction

To extract DNA from tail and ear tissue, a salt extraction protocol was employed, using autoclaved reagents and materials. Mouse tails were digested with 0.5 ml digestion buffer containing 17 mM tris, 17 mM EDTA, 170 mM sodium chloride, 0.85% SDS (pH 7.8) with 0.1 mg proteinase K (all from Sigma, MO, USA) at 55°C overnight. The tubes were heated to 95°C for 15 minutes to inactivate the proteinase K followed by addition of 0.25 ml NaCl (6 M) (Sigma, MO, USA), the tubes were then shaken vigorously 200 times. The tubes were incubated on ice for 10 minutes then centrifuged for 5 minutes at 10,000 g. 0.5 ml of supernatant was added to 1 ml of 95% ethanol in water in 1.5 ml eppendorf tubes, mixed and centrifuged for 1 minute at 10,000 g. The supernatant was removed and the pellet air dried for 10-30 minutes. Autoclaved milli-Q (MQ) water (0.4 ml) was added to the tubes and dissolved overnight at 4°C. Before PCR, the tubes were shaken to disperse the DNA.

2.2.1.2 PCR design

Two PCR reactions were used to determine the genotype of the mice in respect to *tgfβ1* mutation (Table 2.1). TGFβ1(fwd) and TGFβ1(rev) span the inserted sequence in exon 6 of the *tgfβ1* gene and produce a 134 bp PCR product (Fig 2.2A). TGFβ1(fwd) and NEOR(rev) detect the neomycin resistance gene inserted into exon 6 of the *tgfβ1* gene, and produce a 545 bp PCR product (Fig 2.2B). A very large PCR product (>1000 bp) produced by amplification of the disrupted *tgfβ1* gene by TGFβ1(fwd) and TGFβ1(rev) primers is sometimes detected (Fig 2.2B).

The primers TGFβ1(fwd) and TGFβ1(rev) and conditions used for amplification were designed by Diebold et al. (1995). The NEOR(rev) primer was designed using PRIMER.DOS software, by analysis of the DNA sequence of *tgfβ1* (Guron et al., 1995) and the neomycin resistance gene found in pMC1neo (Marsh, 1995 unpub.). The sequence, Genbank accession number, nucleotide position and PCR product length of the primers are given in Table 2.1.

The expected bands of 134 bp and 545 bp demonstrate the presence of the intact and disrupted *tgfβ1* gene respectively (Fig 2.2C). The identity of these two products was confirmed by DNA sequencing (protocol described later). The TGFβ1(fwd) TGFβ1(rev) product was found to have 97% sequence homology with murine *tgfβ1* gene (Fig 2.3A). The TGFβ1(fwd) NEOR(rev) product was found to have 97% sequence homology with murine TGFβ1 spliced with pMC1neo cloning vector at the BamHI restriction site (Fig 2.3B).

Gene	Nt position	Primer sequence	Product length	Genbank
tgfβ1 intact	231(fwd)	5'GAGAAGAACTGCTGTGTGCG	134	L42461
	364(rev)	5'GTGTCCAGGCTCCAAATATAGG		
tgfβ1 & *neor*	231(fwd)	5'GAGAAGAACTGCTGTGTGCG	545	L42461 & U43611
	929(rev)	5'CTCGTCCTGCAGTTCATTCA		
pkcs	189422(fwd)	5'GTCAGTCTCATGTTGCCAATG	241 (normal)	AB030754
	190635 (rev)	5'AGTTATAACAGCTGGGTTGGC	211 (impaired)	

TABLE 2.1 PCR primers used to genotype the *tgfβ1* and *pkcs* gene mutations.

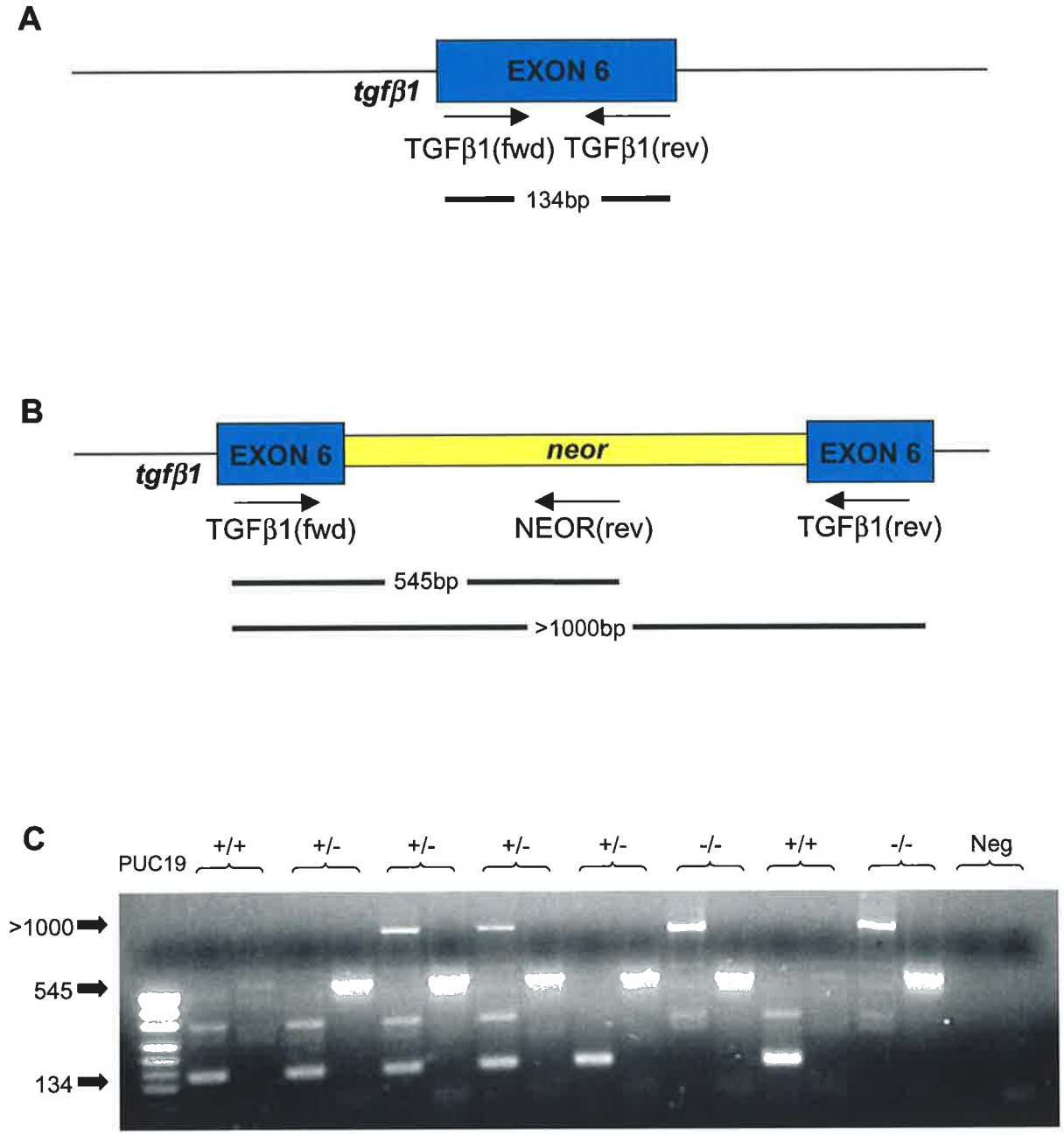

FIGURE 2.2 Genotyping TGFβ1 mutation by PCR. (A) TGFβ1(fwd) and TGFβ1(rev) primers detect intact exon 6 of the *tgfβ1* gene. (B) TGFβ1(fwd), TGFβ1(rev) and NEO(rev) primers detect disrupted exon 6 of the *tgfβ1* gene. (C) PCR products of TGFβ1(fwd) and TGFβ1(rev) (1st lane, then alternate lanes); TGFβ1(fwd) and NEOR(rev) (2nd lane, then alternate lanes) detect wildtype (+/+), heterozygous (+/-) and null (-/-) mice.

A **TGFβ1(fwd) TGFβ1(rev) PCR product sequence**

L42461 **gagaagaact gctgtgtgcg** gcagctgtac attgacttta ggaaggacct gggttggaag tggatccacg

PCR product T GGGTTGGAAG TGNATCCACC

L42461 agcccaaggg ctaccatgcc aacttctgtc tgggaccctg **ccctatatt tggagcctgg acac**

AGCCCAAGGG CTACCATGCC AACTTCTGTC TGGGACCCTG CCCCTATATT TGGAGCCTGG ACAC

B **TGFβ1(fwd) NEOR(rev) PCR product sequence**

BamHI

L42461 **gagaaga actgctgtgt gcg**gcagctg tacattgact ttaggaagga cctgggttgg aagtggatcc

PCR product TGGGTTGG AAGTGNATCC

U43611 tgaattctcg agcagtgtgg ttttgcaaga ggaagcaaaa agcctctcca cccaggcctg gaatgtttcc

TCTAGAGTCG AGCAGTGTGG TTTTCANGA GGAAGCAAAA AACTCTCCA CCCAGGCCTG GAATGTTTCC

U43611 acccaatgtc gagcagtgtg gttttgcaag aggaagcaaa aagcctctcc acccaggcct ggaatgtttc

ACCCAATGTC GAGCAGTGTG GTTTTGCAAG AGGAAGCAAA AAGCCTCTCC ACCCAGGCCT GGAATGTTTC

U43611 cacccaatgt cgagcaaacc ccgcccagcg tcttgtcatt ggcgaattcg aacacgcaga tgcagtcggg

CACCCAATGT CGAGCAAACC CCGCCCAGCG TCTTGTCATT GGCGAATTCG AACACGCAGA TGCAGTCGGG

U43611 gcggcgcggt cccaggtcca cttcgcatat taaggtgacg cgtgtggcct cgaacaccga gcgaccctgc

GCGGCGCGGT CCCAGGTCCA CTTCGCATAT TAAGGTGACG CGTGTGGCCT CGAACACCGA GCGACCCTGC

U43611 agccaatatg ggatcggcca ttgaacaaga tggattgcac gcaggttctc cggccgcttg ggtggagagg

AGCCAATATG GGATCGGCCA TTGAACAAGA TGGATTGCAC GCAGGTTCTC CGGCCGCTTG GGTGGAGAGG

U43611 ctattcggct atgactgggc acaacagaca atcggctgct ctgatgccgc cgtgttccgg ctgtcagcgc

CTATTCGGCT ATGACTGGGC ACAACANACA ATCGGCTGCT CTGATGCCGC CGTGTTCCGG CTGTCANCGC

U43611 aggggcgccc ggttcttttt gtcaagaccg acctgtccg gtgccc**tgaa tgaactgcag gacgag**

ANGGGCGCCC GGTTCTTTTT GTCAANACCG ACCTGTCCG GTNCCCTGAA TNAACTGC

FIGURE 2.3 Nucleotide sequence of PCR products (in capital letters) from mouse DNA amplified with TGFβ1(fwd) primer and TGFβ1(rev) (A) or NEOR(rev) (B) primers. The PCR product sequences were compared to the known nucleotide sequence (in lower case) obtained from Genbank (accession numbers given in left column). Nucleotides that align with the expected sequence are underlined. The forward and reverse primers are depicted in bold. The TGFβ1 and pMC1neo sequence is joined at the BamHI restriction site.

The protocol to detect the *pkcs* mutation was developed by Blunt et al. (1996). PKCS(fwd) and PKCS(rev) primers were used to generate a PCR product (Table 2.1) which could be digested with AluI restriction enzyme. The protocol yields a product of 241 bp for the normal *pkcs* gene and a product of 211 bp for the mutated gene (Fig 2.4).

2.2.1.3 Polymerase chain reaction

PCR reaction mixtures contained 1x PCR buffer, 2.5 mM $MgCl_2$, 0.55 U *Taq* polymerase (all from Biotech, WA, AUS), 200 μM dNTPs (Roche, Germany), 2 μM each primer (Sigma, NSW, AUS) and 2 μl of extracted DNA in a 25 μl reaction mixture. PCR products were stored at -20°C until analysed. The PCR cycle conditions to detect the intact *tgfβ1* gene were one cycle of 95°C for 5 minutes; 30 cycles of 95°C for 20 seconds, 55°C for 50 seconds, 72°C for 1 minute; and one cycle of 72°C for 7 minutes. The PCR conditions to detect the disrupted *tgfβ1* gene were one cycle of 94°C for 5 minutes; 35 cycles of 94°C, 60°C, 72°C for 1 minute each; and one cycle of 72°C for 7 minutes. The PCR cycle conditions to detect the *pkcs* gene were one cycle of 94°C for 5 minutes; 40 cycles of 94°C, 60°C, 72°C for 1 minute each; and one cycle of 72°C for 7 minutes.

2.2.1.4 PKCS restriction digest

PCR products were digested with 2 U AluI restriction enzyme (Geneworks, Adelaide, AUS) for 2 hours at 37°C, and stored at -20°C until analysed.

2.2.1.5 Detection of PCR products

The PCR products were separated by size by gel electrophoresis. TGFβ1 PCR products containing 1x loading buffer were run on a 2% agarose gel (Promega, WI, USA) containing ethidium bromide (Sigma, MO, USA) diluted in TAE buffer for 40 minutes while PKCS PCR products were run on a 3% agarose gel on ice for 2 hours. PUC19 (Geneworks, SA, AUS) was used as a size marker. The gel was photographed using a digital camera (DC 120, Kodak) under UV light to detect bands of PCR products.

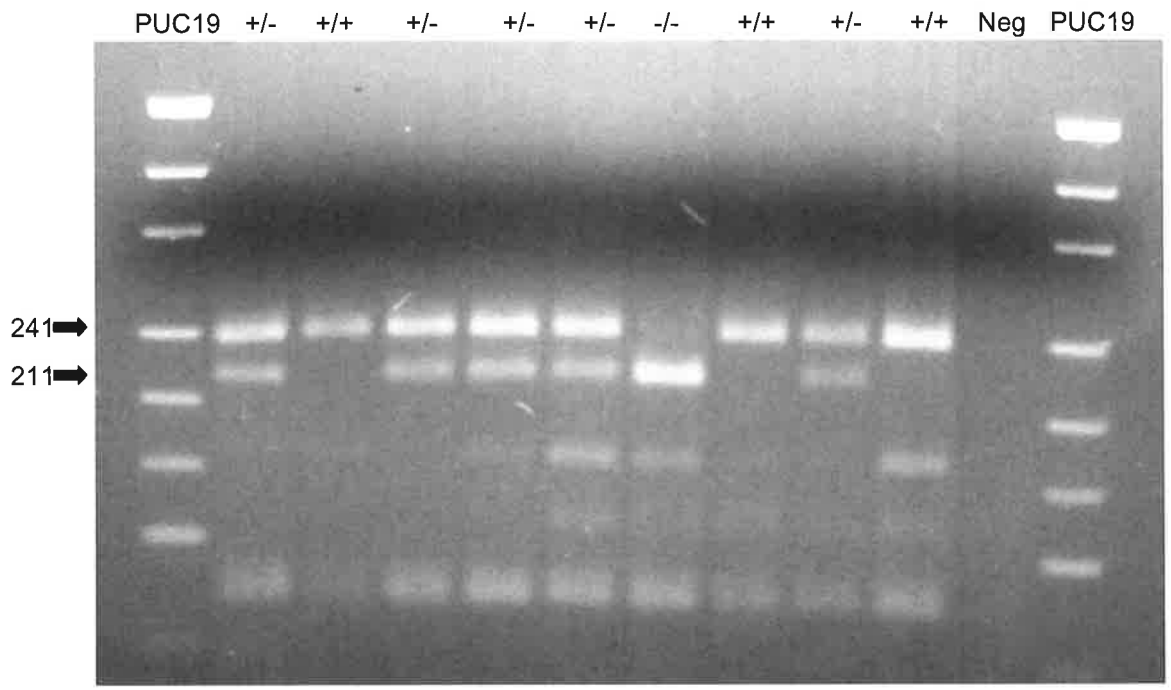

FIGURE 2.4 Genotyping scid mutation by PCR. PCR products amplified by PKCS primers and digested with AluI restriction enzyme, yielding products of 241 bp (normal gene) and 211 bp (mutated gene leading to severe combined immunodeficiency). Each lane represents DNA of single mouse, determined to be wildtype (+/+), heterozygous (+/-) or null mutant (-/-).

2.2.2 Quantitation of mRNA

Quantitative reverse transcription PCR was employed to analyse the quantity of steroidogenic enzyme mRNA in testis and ovarian tissue, and nitric oxide synthase (NOS) mRNA in penis and ovarian tissue. The steroidogenic enzymes analysed were steroidogenic acute regulatory protein (StAR), P450 side chain cleavage (P450scc), 3β-hydroxysteroid dehydrogenase-1 (HSD3β1), P450 17 alpha-hydroxylase/C17-20-lyase (P450c17). The NOS enzymes analysed were neuronal nitric oxide synthase (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS).

2.2.2.1 Primer design

Primers for real time PCR were designed with PrimerExpress 2.0 software using Genbank cDNA sequences. The sequence, Genbank accession number, nucleotide position and PCR product length of the primers are given in Table 2.2.

Exon boundaries for StAR were determined using the complete murine

Gene	Nt position	Primer sequence	Product length	Genbank
star	697 (fwd)	5'CCGGAGCAGAGTGGTGTCAT	158	L36062
	854 (rev)	5'TGCGATAGGACCTGGTTGATG		
p450scc	561 (fwd)	5'ACAGACGCATCAAGCAGCAA	90	AF195119
	650 (rev)	5'CACTGCTGATGGACTCAAAGGA		
hsd3β1	235 (fwd)	5'GGACAAAGTATTCCGACCAGAAAC	103	NM_008293
	337 (rev)	5'CAGGCACTGGGCATCCA		
p450c17	971 (fwd)	5'TGGCTTTCCTGGTGCACAA	81	M64863
	1051 (rev)	5'GTGTTCGACTGAAGCCTACATACTG		
nnos	2586 (fwd)	5'CACCCCAACTCTGTGCAAGAG	132	NM_008712
	2717 (rev)	5'CGGTCCAGTACTTTCAAAGTTGTCT		
inos	883 (fwd)	5'CATCAGGTCGGCCATCACT	86	NM_010927
	968 (rev)	5'CGTACCGGATGAGCTGTGAA		
enos	1467 (fwd)	5'CAGGCATCACCAGGAAGAAGA	92	NM_008713
	1558 (rev)	5'CACACGCTTCGCCATCAC		
β-actin	24 (fwd)	5'CGTGGGCCGCCCTAGGCACCA	186	M12481
	209 (rev)	5'ACACGCAGCTCATTGTA		

TABLE 2.2 PCR primers used to quantify steroidogenic and nitric oxide synthase enzymes.

sequence (King and Lamb, 2001, unpub. Genbank accession number AY032730), for P450scc by comparison with the rat sequence (Oonk et al., 1990) and for HSD3β1 and P450c17 by comparison with the human sequences from Bain et al. (1991) and Kagimoto et al. (1988) respectively. The absence of alternative PCR products and primer dimers in the PCR product was shown by a single dissociation peak and confirmed by visualisation of the PCR product on an agarose gel (Fig 2.5). The PCR products were sequenced (protocol described later) and were found to have >97% homology with the cDNA sequence they were designed to detect (Fig 2.6).

Exon boundaries for nNOS were determined by comparison with rat penile nNOS (Magee et al., 1996), for iNOS by comparison with the complete murine sequence (Coge et al., 2001, unpub. Genbank accession number AF427516), and for eNOS by comparison with the human sequence (Marsden et al., 1993). As with primers designed to detect steroidogenic enzyme mRNA, the absence of alternative PCR products and primer dimers was shown by a single dissociation peak and confirmed by visualisation of the PCR products on an agarose gel (Fig 2.7). The PCR products were sequenced (protocol described later) and were found to have >97% homology with the cDNA sequence they were designed to detect (Fig 2.8).

Primers to detect mRNA for the house keeping genes 18s and β-actin were purchased (QuantumRNA Universal, Ambion, TX, USA) and taken from Jasper (1998) respectively.

2.2.2.2 RNA extraction

Testis, penis and ovarian tissue was dissected, and immediately frozen in liquid nitrogen, the sample was stored at –70°C until extracted.

In an eppendorf tube, 500 µl of RNAzol B (Tel-test, TX, USA) was added to the tissue sample. Testis and ovarian tissue was homogenised with a hand held mixer and penis tissue was homogenised with a sonic homogeniser, and 50 µl of chloroform (Sigma, MO, USA) added. 100 µg of carrier tRNA (Boehringer Mannheim, Germany) was also added to some samples. The sample was incubated

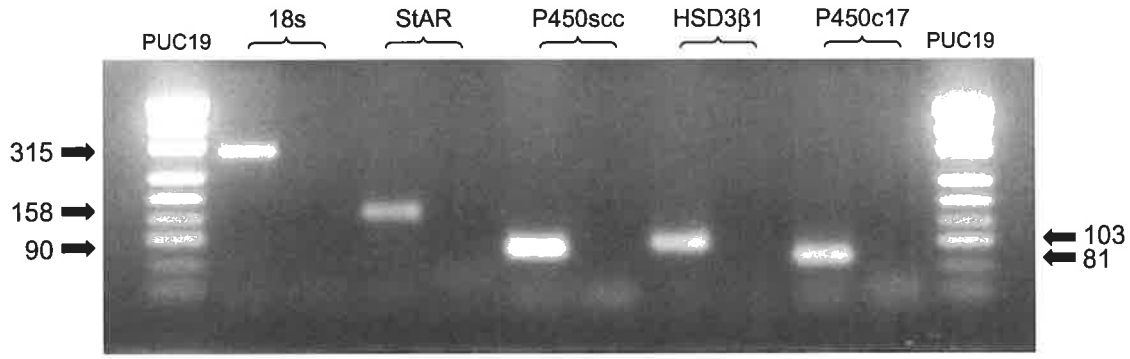

FIGURE 2.5 PCR products amplified from testis cDNA using primers for 18s, StAR, P450scc, HSD3β1 and P450c17. The PCR reactions yield products of 315, 158, 90, 103 and 81 base pairs respectively. Lane 1 and then alternate lanes contain PCR product amplified by specific primers with testis cDNA, lane 2 and alternate lanes contain the same primers without cDNA (non template control).

A **StAR PCR product sequence**

L36062 **ccggagcaga gtggtgtcat** cagagctgaa cacggcccca cctgcatggt gcttcatcca ctggctggaa

PCR product CGGCCCCA CCTGCATGGT GCTTCATCCA CTGGCTGGAA

L36062 gtccctccaa gactaaactc acttggctgc tcagtattga cctgaagggg tggctgccga agacaat**cat**

GTCCCTCCAA GACTAAACTC ACTTGGCTGC TCAGTATTGA CCTGAAGGGG TGGCTGCCGA AGACAATCAT

L36062 **caaccaggtc ctatcgca**

CACCNGGGTC CTATCGCA

B **P450scc PCR product sequence**

AF
195119 **acagacgcat caagcagcaa** aattctggaa atttctcagg ggtcatcagt gatgacctat tccgctttt**c**

PCR product TCTGGNA ATTTCTCAGG GGTCATCAGT GATGACCTAT TCCGCTTTTC

AF
195119 **ctttgagtcc atcagcagtg**

CTTTGAGTCC ATCAGCAGTG

C **HSD3β1 PCR product sequence**

NM_
008293 **ggacaaagta ttccgaccag aaac**caagga ggaattctcc aagctgcaga caaagaccaa ggtgacagtg

PCR product A GGAATTCTCC AAGCTGCAGA CAAAGACCAA GGTGACAGTG

NM_
008293 ttggaaggag acattc**tgga tgcccagtgc ctg**

TTGGAAGGAG ACATTCGGGA TGCCCAGTGC CTG

D **P450c17 PCR product sequence**

M64863 **tggctttcct ggtgcacaa**t cctgaggtga agaggaagat ccaaaaggag attgac**agt atgtaggctt**

PCR product GGAAGAT CCANAAGGCG ATTGCCCAGG ATGTAGGCTT

M64863 **cagtcgaaca c**

CAGTCGAACA

FIGURE 2.6 Nucleotide sequence of PCR products (in capital letters) from testis cDNA amplified with primers for StAR (A), P450scc (B), HSD3β1 (C) and P450c17 (D). The PCR product sequences were compared to the known nucleotide sequence (in lower case) obtained from Genbank (accession numbers given in left column). Nucleotides that align with the expected sequence are underlined. The forward and reverse primers are depicted in bold.

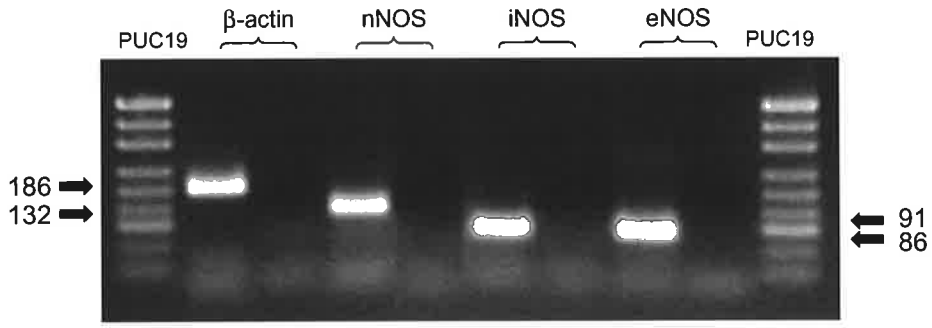

FIGURE 2.7 PCR products amplified from penis cDNA using primers for β–actin, nNOS, iNOS and eNOS. The PCR reactions yield products of 186, 132, 86 and 92 base pairs respectively. Lane 1 and then alternate lanes contain PCR product amplified by specific primers with penis cDNA, lane 2 and alternate lanes contain the same primers without cDNA (non template control).

A nNOS PCR product sequence

NM_
008712 **caccccaact ctgtgcaaga g**gagaggaag agctacaagg tccgattcaa cagcgtctcc tcctattctg

PCR product AG AGCTNCAGGG TCCGATTCNA NAGCGTCTCC TCCTATTCTG

NM_
008712 actcccgcaa gtcatcaggc gatggaccag acctcag**aga caactttgaa agtactggac cg**

ACTCCCGCAA GTCATCAGGC GATGGACCAG ACCTCAGAGA CAACTTNGAA AGTACTGGAC CG

B iNOS PCR product sequence

NM_
010927 **catcaggtcg gccatcact**g tgttccccca gcggagtgac ggcaaacatg acttcaggct ctggaa**ttca**

PCR product CCA GCGGAGTGAN GGCAAACATG ACTTCAGGCT CTGGAACCCA

NM_
010927 **cagctcatcc ggtacg**

CAGCTCATCC GG

C eNOS PCR product sequence

NM_
008713 **caggcatcac caggaagaag a**cctttaagg aagtagccaa tgcagtgaag atctctgcct cactcatggg

PCR product GTAGCCAA TGCAGTGAAG ATCTCTGCCT CACTCATGGG

NM_
008713 cacg**gtgatg gcgaagcgtg tg**

CAGTTNGATG GCGAAGCGTG TG

FIGURE 2.8 Nucleotide sequence of PCR products (in capital letters) from penis cDNA amplified with primers for nNOS (A), iNOS (B) and eNOS (C). The PCR product sequences were compared to the known nucleotide sequence (in lower case) obtained from Genbank (accession numbers given in left column). Nucleotides that align with the expected sequence are underlined. The forward and reverse primers are depicted in bold.

on ice for 15 minutes. Following a 15 minute spin at 11,000 g at 4°C, the top aqueous phase was pipetted off and placed in a new eppendorf tube, together with an equal volume of isopropanol (Sigma, MO, USA). The tube was inverted to mix and incubated overnight at –20°C. The sample was spun at 11,000 g for 30 minutes at 4°C and the supernatant poured off, being careful not to disturb the pellet. The pellet was washed twice with 500 µl cold 75% ethanol, with 10 minute 11,000 g spins in between at 4°C. The pellet was dried on the bench for approximately 30 minutes before being dissolved in 100 µl MQ water.

DNase master mix comprising of 25 µl 5x DNA buffer, 1 µl RNase inhibitor and 5 µl DNase I (both from Boehringer Mannheim, Germany) was added to the sample and incubated at 37°C for 1 hour. 250 µl of water equilibrated phenol:chloroform:isoamyl alcohol at a ratio of 25:24:1 was added to the sample, and after a short vortex to mix, centrifuged at 10,000 g for 10 minutes at room temperature. The top aqueous phase was pipetted off and placed in a new eppendorf tube together with 2.5x sample volume of 100% ethanol and 0.1x sample volume of 2 M sodium acetate (Sigma, MO, USA). The tube was inverted to mix and stored at –20°C until reverse transcribed.

2.2.2.3 Reverse transcription

Samples of extracted RNA were centrifuged at 11,000 g for 20 minutes at 4°C. The supernatant was poured off, being careful not to disturb the pellet. The pellet was washed twice with 500 µl cold 70% ethanol, with 10 minute 11,000 g spins in between at 4°C. The pellet was dried on the bench for approximately 30 minutes before being dissolved in 50 µl MQ water.

The quantity and purity of RNA in the sample was determined with a spectrophotometer (DU-50, Beckman). The sample was sufficiently pure if the ratio of OD_{260}:OD_{280} was >1.7. The quantity of RNA was determined by the following equation:

OD_{260} x 40 x dilution factor = RNA µg/ml

The RNA sample was diluted in MQ water to give a concentration of 1 μg/10 μl. 10 μl was used for reverse transcription, the rest of the sample was reprecipitated in ethanol and sodium acetate as before and stored at –20°C. 1 μl of random hexamer (Geneworks, SA, AUS) (500 μg/ml) was added to the RNA and incubated at 70°C for 10 minutes, then on ice for 5 minutes. A reaction mixture containing 4 μl RT buffer, 2 μl DTT (both from Invitrogen, VIC, AUS) and 2 μl dNTPs (10 mM) was added to the tube and incubated at 43°C for 2 minutes. While still incubating, 1 μl of Superscript II RNase H reverse transcriptase (Invitrogen, Vic, AUS) was added and the sample incubated for a further 1.5 hours at 43°C, then 94°C for 5 minutes. The cDNA sample was made up to 50 μl with MQ water and stored at –20°C until PCR.

2.2.2.4 Polymerase chain reaction

Quantitative PCR was performed in real-time (Geneamp 5700, Applied Biosystems). The PCR reaction mix consisted of 1x SYBR green PCR master mix (Applied Biosystems, Warrington, UK), cDNA template, forward and reverse primers (Sigma, NSW, AUS) in a 20 μl reaction mixture. The PCR cycle conditions were one cycle of 50°C for 2 minutes, one cycle of 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

The concentration of primers was determined in a preliminary experiment with serial dilutions of primer pairs and a control testis or penis cDNA sample, selecting the concentration at which the difference between the threshold cycle (Ct) of the sample (signal) and the Ct of the non template control (noise) was greatest. The StAR, P450scc, HSD3β1 and P450c17 primers were diluted to give a final concentration of 0.05 μM, 1 μM, 0.125 μM, and 1 μM respectively. The β-actin, nNOS, iNOS and eNOS primers were diluted to give a final concentration of 0.5 μM.

The absence of alternative PCR products and primer dimers in the PCR product was shown by a single dissociation peak and confirmed by visualisation of the PCR product on an agarose gel (Fig 2.5 and 2.7). The product was purified and sequenced to verify the identity of the PCR product (Fig 2.6 and 2.8).

To determine the concentration range of cDNA at which Ct increase is linear, serial 1:8 dilutions of testis, ovary and penis cDNA were amplified using each of the primers. It was determined that 4 μl of a 1 in 8 dilution of testis cDNA was required for quantitative analysis of steroidogenic enzymes. 4 μl of a 1 in 500 dilution of ovary cDNA was required for analysis of steroidogenesis enzymes with the exception of P450c17 that required a 1 in 80 dilution. 4 μl of a 1 in 100 dilution of ovary cDNA was required for analysis of iNOS and eNOS enzymes. 4 μl of neat penis cDNA was required for analysis of nNOS enzyme and a 1 in 2 dilution of penis cDNA was required for analysis of iNOS and eNOS enzymes, β-actin was diluted 1 in 100.

The efficiency of each PCR reaction was determined by regression analysis of the fluorescence of the sample at each cycle number. All reactions occurred at a minimum of 70% efficiency.

2.2.2.5 Quantitation of steroidogenesis enzyme mRNA

Each cDNA sample was amplified in triplicate for each primer pair used. The quantity of PCR product amplified by specific primers encoding genes of interest was normalised to the quantity of the housekeeping gene, 18s rRNA for testes and ovary and β-actin mRNA for penis, using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

The amount of PCR product was multiplied by a constant to give an average for the control tissue sample the arbitrary value of 1.

2.2.3 Validation of PCR product sequence

To confirm the identity of PCR products, the products of PCR reactions were sequenced.

To sequence the products from the TGFβ1(fwd), TGFβ1(rev) and NEOR(rev) PCRs, the bands were cut out of the agarose gel and recovered using a SpinBind PCR purification kit (FMC BioGenics, MA, USA) according to the manufacturers instructions. The purified PCR products were amplified with Abi Prism Dye Terminator reaction mixture (Applied Biosystems, Warrington, UK), using the forward

primer (320 ρmol) and 25 cycles of 96°C for 0:30 min, 50°C for 0:15 min and 60°C for 4:00 min. To clean up the sequencing reaction, 2 µl of 3 M sodium acetate was added to 50 µl 95% ethanol. 20 µl of PCR reaction mixture was added and the tube vortexed for 10 seconds and centrifuged for 20 minutes at 4°C at 10,000 g. The supernatant was carefully pipetted off and the pellet washed with 1 ml cold 70% ethanol. The tubes were dried in a speed vac concentrator (RH200-13, Savant).

To sequence PCR products from testis and penis cDNA, the products were purified directly from the reaction mix using a Minelute Reaction Cleanup kit (Qiagen, Germany) according to the manufacturers instructions. The purified PCR products were amplified with Abi Prism Big Dye reaction mixture (Applied Biosystems, Warrington, UK) using the forward primer (3.2 ρmol) and the same cycle protocol as above. To clean up the sequencing reaction, 80 µl of 75% isopropanol was added to the mix and incubated at room temperature for 15 minutes. The tubes were centrifuged at 12,000 g for 20 minutes, the supernatant was aspirated off and 250 ml of 75% isopropanol was added to the tubes and centrifuged as before for 5 minutes. The supernatant was aspirated off and the sample dried on the bench for 20 minutes.

The sequence of the PCR products were analysed at the IMVS, SA, AUS. Homology with all known sequences was determined by BLAST sequence analysis (http://www.ncbi.nlm.nih.gov/BLAST).

2.3 IN VIVO AND IN VITRO EMBRYO DEVELOPMENT

2.3.1 In vitro fertilisation

2.3.1.1 Oocyte collection

3-4 week old female CBA F1 mice (CBA x C57Bl/6) were superovulated, 2 per male. The mice were injected intraperitoneally with 5 IU PMSG at 1530 hours on day 1 followed by 5 IU hCG at 1730 hours on day 3. At 0730 hours on day 4, the mice were sacrificed and the ovulated oocytes collected from the ampulla of the oviduct.

The oocytes were collected into pregassed Gamete-100 media (Vitrolife, Gothenburg, Sweden).

2.3.1.2 Sperm preparation

One hour prior to oocyte collection, male mice were sacrificed and the cauda epididymis dissected and placed into pregassed Gamete-100 media. The cauda were transferred to a fresh dish of 1 ml G-100 and sperm were squeezed out of the cauda using fine forceps. The cauda were removed from the dish and the sperm were incubated for 15 minutes at 37°C in 5% CO_2. Sperm concentration was determined with a haemocytometer. $5x10^5$ sperm were transferred to an IVF dish containing pregassed 0.5 ml rS1 (Vitrolife, Gothenburg, Sweden) and incubated for a further 60 minutes to capacitate.

2.3.1.3 IVF and culture

Oocytes recovered from superovulated females were added to the IVF dish containing sperm (10^6/ml) for 4 hours at 37°C in 5% CO_2. The oocytes were subsequently washed twice in 100 µl drops of rS1 under mineral oil (Sigma, MO, USA) and transferred to 30 µl rS1 droplets under oil. The droplets were cultured as above.

On day 2 of culture, the embryos were transferred to pregassed 30 µl droplets of SQC media (Vitrolife, Gothenburg, Sweden) under mineral oil. The oocytes were scored for cleavage 48 hours post IVF and for blastocyst development 4 and 5 days post IVF.

2.3.2 In vitro culture

Embryos were collected from the ampulla of 27-29 day old superovulated females mated with B10 stud males on day 0.5 post coitum. The embryos were transferred immediately to pregassed 30 µl rS1 droplets. On day 2 of culture, embryos were transferred to pregassed 30 µl SQC droplets.

2.3.3 Assessment of embryos

Embryos flushed from the oviduct and uterus of pregnant mice on day 3.5 pc or on day 2 and 5 of in vitro culture were visually assessed under a dissecting microscope. The embryos were determined to be one-cell, two-cell, four-cell, 8-16 cell, morula or blastocysts.

2.4 TISSUE HISTOLOGY

2.4.1 Tissue preparation

Testis, ovary, seminal vesicle, penis and uterus were dissected and fixed in 10% buffered formalin overnight. The tissues were rinsed four times in PBS over a period of two days. The fixed tissue was stored in 70% ethanol before embedding in paraffin. Serial 5 μm sections of paraffin embedded tissue were cut with a microtome (HM 325, Microm). The sections were floated on water heated to 35°C containing a drop of detergent and placed on APES coated slides. The slides were air dried at 37°C overnight.

Ovaries were dissected from female mice and frozen in tissue-tek OCT compound (Sakura, CA, USA). Sections (7 μm) were cut with a cryostat (Bright, UK) and air dried for 30 minutes. Sections were stored in air tight trays with silica gel at –70°C.

2.4.2 Haematoxylin and eosin staining

Paraffin embedded sections were dewaxed in two 5 minute washes in Safsolv (Labchem, NSW, AUS) followed by two 2 minute washes in absolute ethanol. The sections were hydrated through 2 minute incubations in 90%, 70% and 50% ethanol and finally 2 minutes in MQ water. The sections were stained in haematoxylin (Sigma, MO, USA) for 4 minutes, then dipped for 5-10 seconds in 0.5% ammonia water, 1% hydrochloric acid and 0.5% ammonia water with washes in tap water in between. The sections were counter-stained with eosin Y (Sigma, MO, USA) for 1 minute and washed again in tap water. The sections were dehydrated with 90%

ethanol for 1 minute, absolute ethanol for 1 minute and absolute ethanol again for 2 minutes. Finally the sections were cleared by incubation in Safsolv three times for 5 minutes each and covered with a cover slip using DPX (BDH, Poole, UK).

2.4.3 Analysis of testis pathology

Sections from three separate regions of the testis were analysed for pathology in each mouse. Ten seminiferous tubules from each section were randomly selected. Testis pathology was quantified on the basis of (1) number of germ cell generations in each tubule, (2) percent of tubules containing immature germ cells sloughed off into the lumen of the tubule, and (3) diameter of tubules.

2.4.4 Immunohistochemical analysis of ovaries

Sections of ovary were stained for endothelial cells using the specific endothelial cell antibody, MTS-12 (Godfrey et al., 1988).

2.4.4.1 Immunohistochemical staining

OCT embedded sections were defrosted. The slides were incubated in 96% ethanol at 4°C for 10 minutes and washed 3 times in PBS for 5 minutes each. The slides were coated in 1% bovine serum albumin (BSA) (Fraction V, Sigma, MO, USA) for 2 minutes and incubated with MTS-12 (neat) containing 10% normal mouse serum (NMS) in a humidichamber at 4°C overnight. The slides were washed again as before, coated in 1% BSA and incubated with horse radish peroxidase conjugated anti-rat antibody (DAKO, Denmark) at a 1:100 dilution in PBS with 10% NMS for 2 hours in a humidichamber at 4°C. The slides were washed as before and incubated with 3,3'-diaminobenzidine according to manufacturers instructions (Sigma, MO, USA; prepared following manufacturers instructions) for 10 minutes, producing a brown stain for MTS-12 positive cells. The slides were washed in MQ water and counter-stained with haematoxylin (blue stain). The slides were incubated in two 5 minute washes of 100% ethanol followed by two 5 minute washes in Safsolv and covered with a cover slip using DPX.

2.4.4.2 Quantification of endothelial cells

The amount of MTS-12 positive cells in the corpora lutea of ovaries was quantified by video image analysis, using Video Pro 32 software. The percent of MTS-12 positive cells (brown stain) on each corpus luteum on two separate sections of ovary was determined by quantification of the brown and blue stained areas.

2.4.5 Whole mount preparation and analysis of mammary gland tissue

The entire fat pad containing the inguinal lymph node was dissected from female mice and spread on a glass slide. The slide was incubated in Carnoy's fixative for 2 to 4 hours.

Fixed mammary glands were stained in carmine alum according to protocol described by Robinson (http://mammary.nih.gov/index.html). The slides were washed in 70% ethanol for 15 minutes, transferred to 35% ethanol for 5 minutes then MQ water for 5 minutes. The tissue was stained in carmine alum (2% carmine, 5% aluminium potassium sulphate (both products from Sigma, MO, USA)) overnight. The slides were washed in 70% ethanol for 15 minutes followed by 2 washes in 100% ethanol for 15 minutes each. Finally, the slides were cleared in 2 washes of Safsolv for 15 minutes each and mounted with a cover slip using DPX.

2.5 SERUM HORMONE ANALYSIS

Serum testosterone, androstenedione, progesterone and estradiol were quantified using radioimmunoassay kits (all from Diagnostic Systems Laboratories, TX, USA) at the Queen Elizabeth Hospital. The sensitivity of the assays were 0.2 nM, 0.07 nM, 0.3 nM and 0.02 nM respectively.

2.6 STATISTICAL ANALYSIS

Data were analysed with SPSS 11.0 for Windows software. Nonparametric data were analysed by Mann Whitney-U and Kruskal Wallis-H tests. Parametric data were analysed by independent samples t-test or one-way analysis of variance with post-hoc Tukey's test where appropriate. Significance was inferred at $p<0.05$. In

some cases different superscripts (a, b and c) have been used in figure legends, groups bearing different superscripts differ significantly. In other cases an asterisk identifies a treatment or genotype that differs significantly from the control.

Chapter 3 Impaired steroidogenesis and spermatogenesis in male TGFβ1 null mutant mice

3.1 INTRODUCTION

Transforming growth factor beta 1 (TGFβ1) is a multifunctional cytokine implicated in the regulation of proliferation and differentiation of many cell lineages involved in male reproductive function. Many descriptive and in vitro studies have suggested that TGFβ1 plays crucial roles in the testis during pubertal maturation, in regulation of steroidogenesis and spermatogenesis, and in the development and function of male secondary reproductive organs (Chapter 1).

Androgens have key activities in regulating many physiological processes. Of primary importance, androgens are the principle steroid hormones involved in male sexual function, with roles in spermatogenesis, secondary sex organ function and sexual behaviour. Androgens are synthesised in the testis from cholesterol through a series of enzymatically controlled chemical reactions (Miller, 1988) (Fig 3.1). In vitro culture experiments have indicated that TGFβ1 has the potential to control androgen synthesis in Leydig cells (Avallet et al., 1987; Lin et al., 1987; Morera et al., 1988) through regulation of LH/hCG receptor expression (Avallet et al., 1987; Le Roy et al., 1999), and steroidogenic enzymes including P450 17 α-hydroxylase/C17-20-lyase (P450c17) (Gautier et al., 1997; Le Roy et al., 1999) and 3β-hydroxysteroid dehydrogenase (HSD3β) (Cherradi et al., 1995). Steroidogenic acute regulatory protein (StAR), often considered a rate-limiting enzyme in steroidogenesis through control of cholesterol intramitochondrial transport, is positively and negatively regulated by TGFβ1 in ovarian granulosa (Minegishi et al., 2000) and adrenocortical (Brand et al., 1998) cells respectively.

Within the seminiferous tubules of the testis, TGFβ1 and its receptors are expressed by Sertoli cells and germ cells (Watrin et al., 1991; Mullaney and Skinner, 1993; Teerds and Dorrington, 1993; Le Magueresse Battistoni et al., 1995). The pattern of synthesis of TGFβ1 and the necessity for interaction between germ cells and Sertoli cells that regulate its expression has led several researchers to suggest a role for this cytokine in spermatogenesis (Teerds and Dorrington, 1993; Fritz, 1994; Avallet et al., 1997).

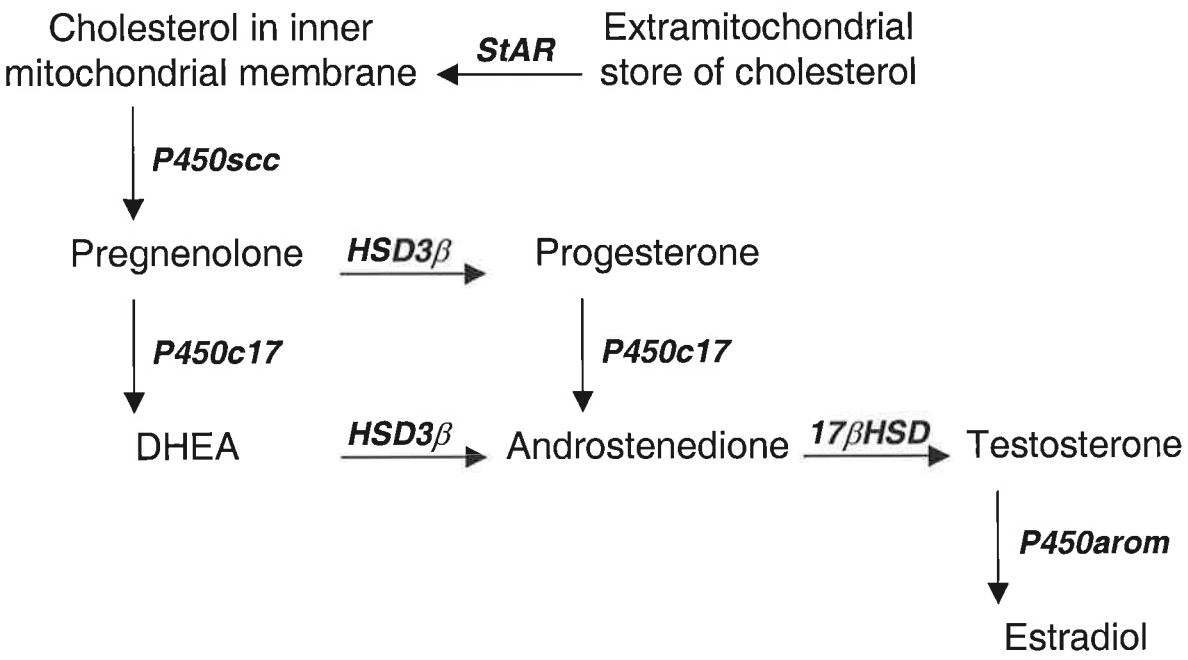

FIGURE 3.1 Principle pathway of sex steroid hormone synthesis in mammals, adapted from Miller, 1987. Cholesterol is transported to the inner mitochondrial membrane by a mechanism involving steroidogenic acute regulatory protein (StAR), and is converted to pregnenolone by P450 side chain cleavage enzyme (P450scc) from which dehydroepiandrosterone (DHEA) or progesterone can be synthesised. These are substrates for androstenedione synthesis by 3β-hydroxysteroid dehydrogenase (HSD3β) or P450 17 alpha-hydroxylase/C17-20-lyase (P450c17) respectively. Androgens are converted to estradiol by P450aromatase (P450arom) enzyme activity.

An opportunity to study the precise physiological significance of TGFβ1 in male reproductive function in vivo is now provided by availability of TGFβ1 null mutant mice (TGFβ1-/-). These mice are on a severe combined immunodeficient (scid) background to prevent death at the age of three weeks due to massive multifocal inflammatory lesions (Diebold et al., 1995).

It is widely accepted that the fertility of TGFβ1 null mutant mice is severely compromised (Kallapur et al., 1999). However, reproductive function in TGFβ1 deficient mice has not previously been the subject of formal investigation. The aim of this chapter is to examine reproductive performance and investigate testicular function in male TGFβ1 null mutant mice.

3.2 EFFECT OF TGFβ1 NULL MUTATION ON MALE FERTILITY

To evaluate the effect of null mutation in the *tgfβ1* gene on male fertility, adult males were housed from 7 to 10 weeks of age with two B10 females, the females were checked daily for plugs or sperm positive vaginal smears. Over a period of three weeks, none of ten (0%) TGFβ1-/- males inseminated either of the females (Fig 3.2). Over a similar period, eleven of twelve (92%) TGFβ1+/±[†] males mated with both females, and pups were delivered by all mated females. Analysis of vaginal smears revealed that all females were cycling normally.

On examination of body weight during pubertal development and adulthood, it was found that male TGFβ1 null mutants were significantly smaller than their TGFβ1 replete littermates (Fig 3.3A). Between the ages of 5 and 10 weeks, these males consistently weighed 20% less than age-matched controls. To evaluate the effect of TGFβ1 null mutation on the weight and morphology of reproductive and other organs, male mice were sacrificed at the age of 10 weeks and male sexual organs as well as other organs were dissected. Relative to body weight, no difference in the wet weight of the testis, seminal vesicle or penis was observed in TGFβ1 mutant

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[†] As wildtype (TGFβ1+/+) and heterozygous (TGFβ1+/-) males did not have any apparent fertility defects, the two genotypes were grouped together in several experiments, this group is deemed TGFβ1+/±.

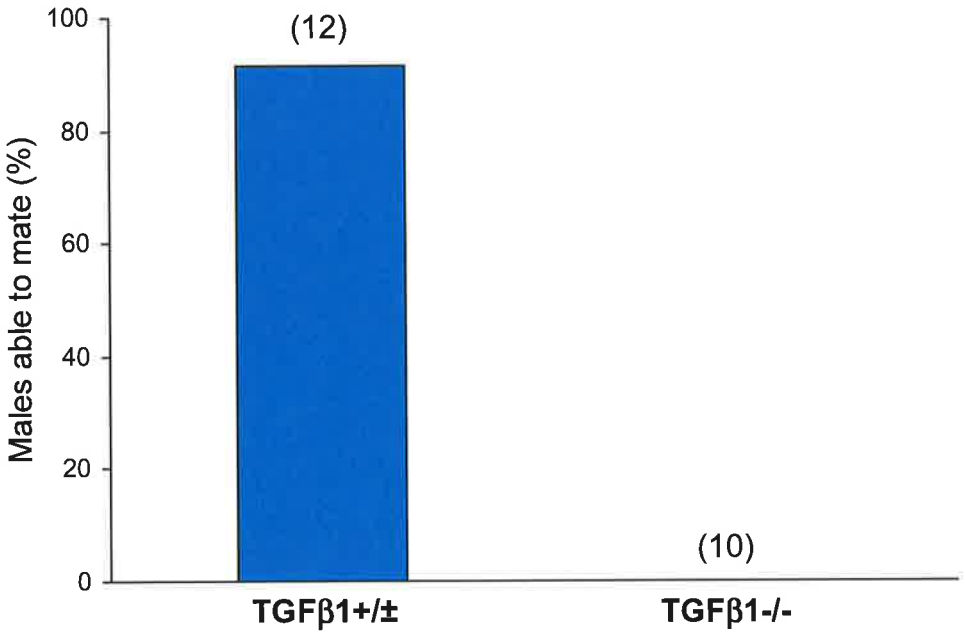


FIGURE 3.2 Mating ability of TGFβ1+/± and TGFβ1-/- males housed with normal adult females. Adult males were housed with two naturally cycling B10 females for a period of 3 weeks. Mating ability was confirmed by the presence of a plug or sperm positive vaginal smear. n is given in parentheses.

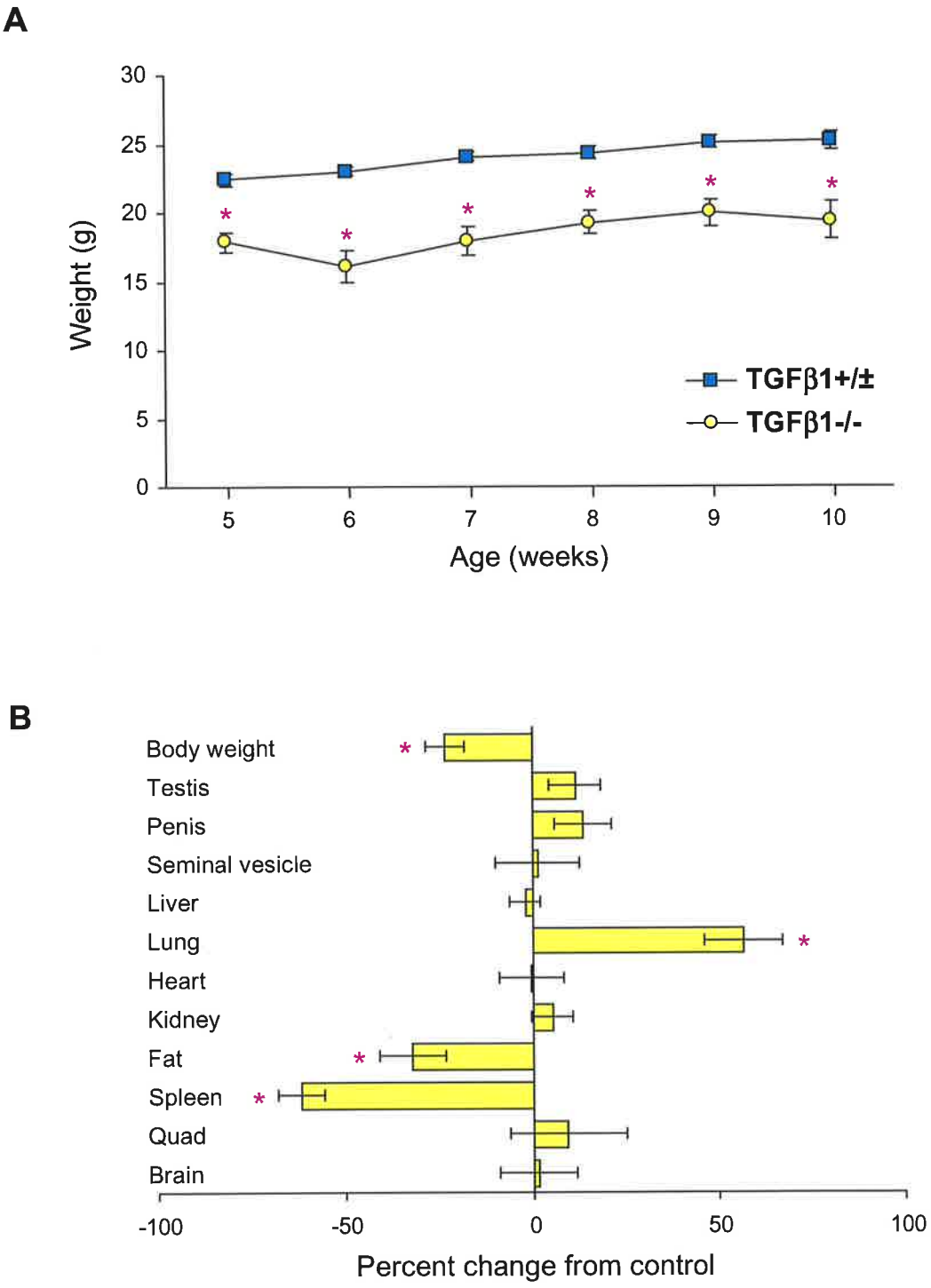


FIGURE 3.3 Body and tissue weights of adult TGFβ1+/± and TGFβ1-/- male mice. (A) Mice were weighed weekly between the ages of 5 and 10 weeks (n=6 per group). Percent change of whole body and organ weights of TGFβ1-/- males from average control. (B) Organ weight was calculated relative to body weight. Data are mean±SEM, and were analysed by independent samples t-test, *denotes significant difference from controls ($p<0.05$).

mice (Fig 3.3B). However, there were differences in the weight of other organs. The lungs of male TGFβ1 null mutant males weighed 50% more than littermate controls relative to body weight. Peritoneal fat and spleen mass were decreased by 40% and 60% respectively. The weight of the liver, heart, kidney, quadreceps and brain were comparable between TGFβ1-/- and TGFβ1+/± littermates relative to body weight.

When the histology of male reproductive tissues was examined, no gross differences were observed in the structure of the seminal vesicle or penis of TGFβ1 null male mice. A similar extent of epithelial branching in the seminal vesicle occurred in TGFβ1 null mutant mice compared to littermate controls (Fig 3.4), and the urethra, corpus cavernosum and epithelium were present and of normal dimensions and appearance in the penis (Fig 3.5).

3.3 EFFECT OF TGFβ1 NULL MUTATION ON SPERMATOGENESIS

To investigate the effect of TGFβ1 null mutation on spermatogenesis, epididymal sperm were quantified in TGFβ1+/± and TGFβ1-/- mice. It was found that a significant proportion of TGFβ1 null mutant males had greatly reduced epididymal sperm numbers (4 of 14 had fewer than 10^7 sperm) compared to TGFβ1+/± males and the other TGFβ1-/- mice analysed (Fig 3.6A). When testis pathology in eight randomly chosen null mice was quantified and compared to control mice, differences in spermatogenesis were observed in some but not all the TGFβ1 null mutants. Low numbers of epididymal sperm were associated with testis pathologies while normal numbers of epididymal sperm in TGFβ1 null mutants was associated with histologically normal testes (Fig 3.7). Similar proportions of males showed evidence of immature germ cells sloughed off into the lumen of the seminiferous tubules irrespective of genotype (Fig 3.6B). One of eight showed a large proportion of tubules containing less than 4 generations of germ cells (Fig 3.6C). A small but significant reduction in mean tubule diameter was detected in the testes of TGFβ1-/- males (Fig 3.6D).

To investigate whether sperm from TGFβ1 null mutants were viable, epididymal sperm were used to fertilise superovulated oocytes from CBA F1 female mice in vitro

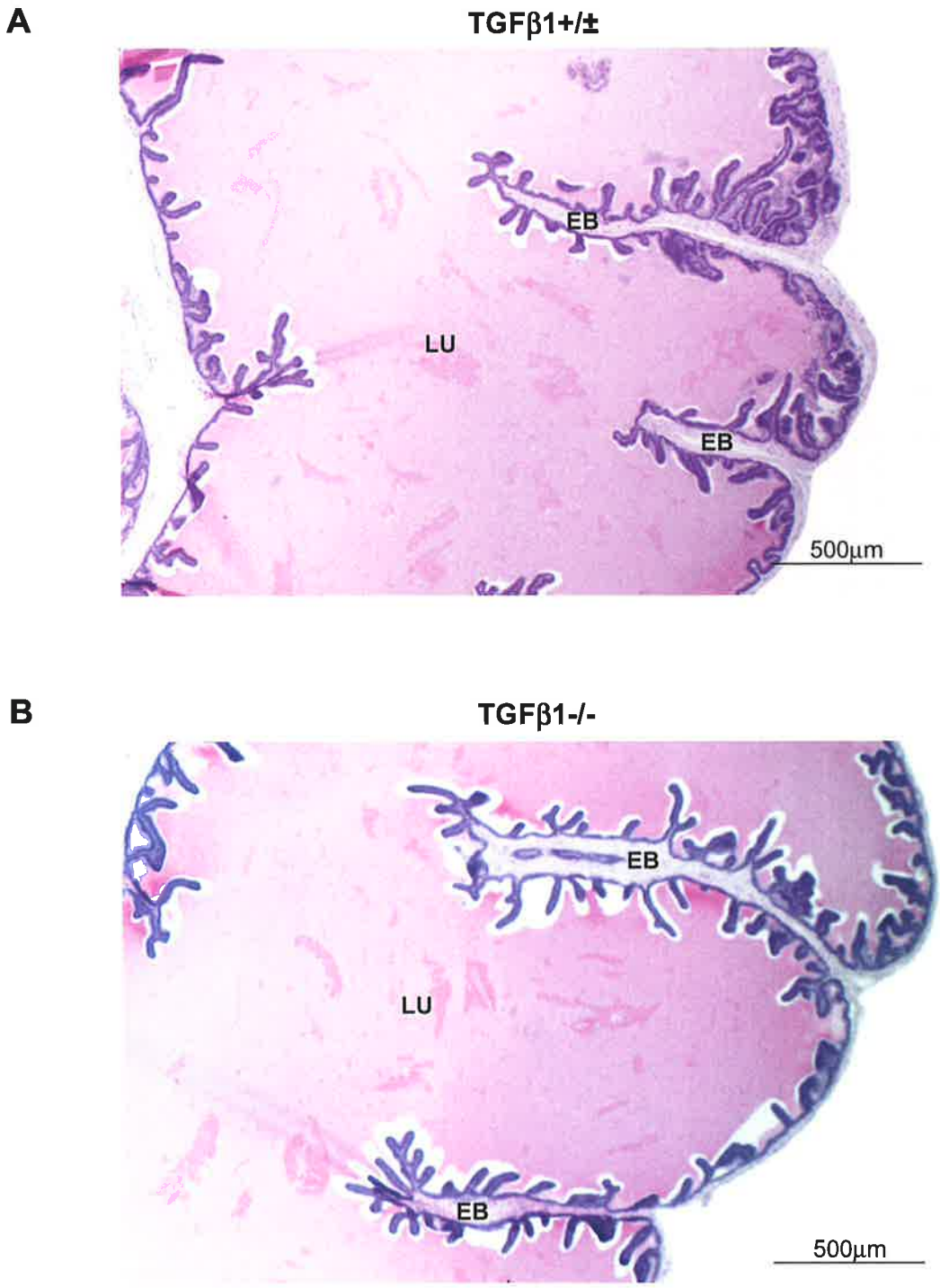


FIGURE 3.4 Representative histology of seminal vesicle of adult TGFβ1+/± (A) and TGFβ1-/- (B) male mice. The seminal vesicles were dissected from 10 week old mice, fixed in 10% buffered formalin and embedded in paraffin. Saggital sections (5µm) were cut and stained with haematoxylin and eosin. The lumen (LU) and epithelial branches (EB) are labelled.

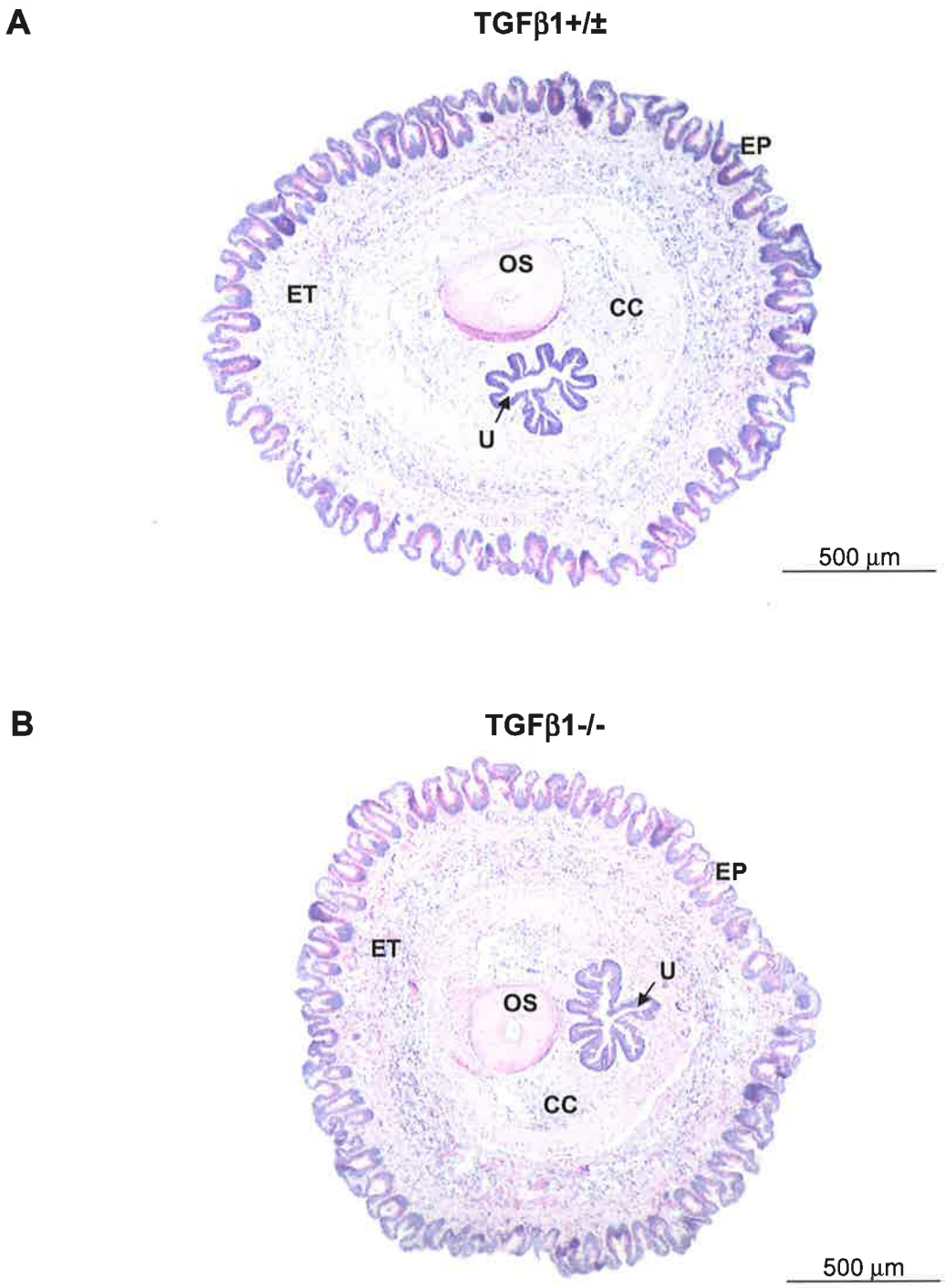


FIGURE 3.5 Representative histology of penis of adult TGFβ1+/± (A) and TGFβ1-/- (B) male mice. The penis was dissected from 10 week old mice, fixed in 10% buffered formalin and embedded in paraffin. Transverse sections (5 µm) were cut and stained with haematoxylin and eosin. The corpus cavernosum (CC), epithelium (EP), erectile tissue (ET), Os penis bone (OS) and urethra (U) are labelled.

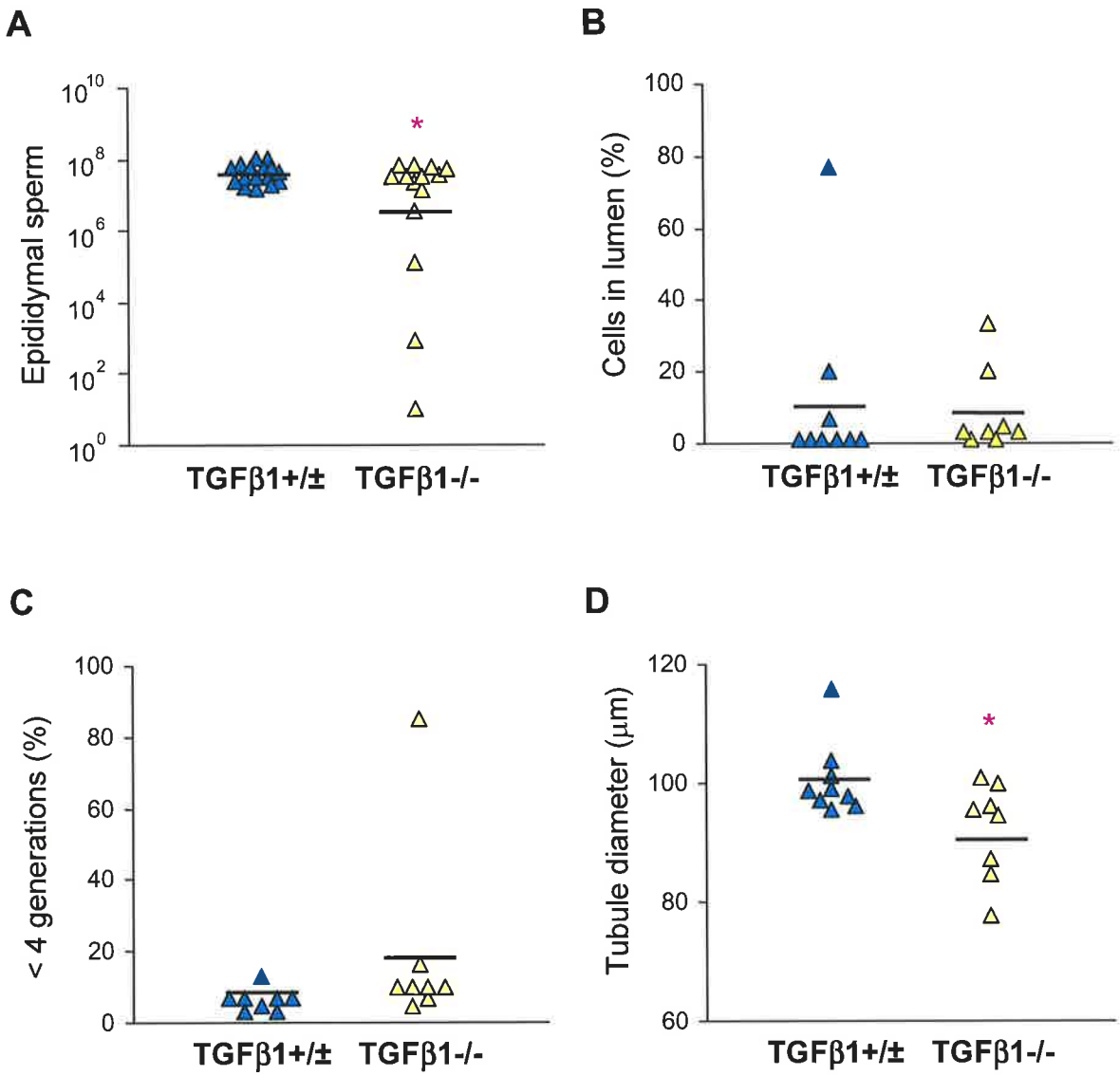


FIGURE 3.6 Quantitation of parameters of testis pathology in 10 week old TGFβ1+/± and TGFβ1-/- mice. Sperm retrieved from the cauda epididymis was counted with a haemocytometer (A). Percent of tubules with cells sloughed off into the lumen (B), less than 4 generations of germ cells (C) and tubule diameter (D) were quantified in 3 separate regions of the testis of each mouse (n=8 per group) by analysis of 10 seminiferous tubules chosen at random. Data from 30 tubules were averaged and represent a single point on each graph, the bar represents the mean. Data were analysed by independent samples t-test, *denotes significant difference from control ($p<0.05$).

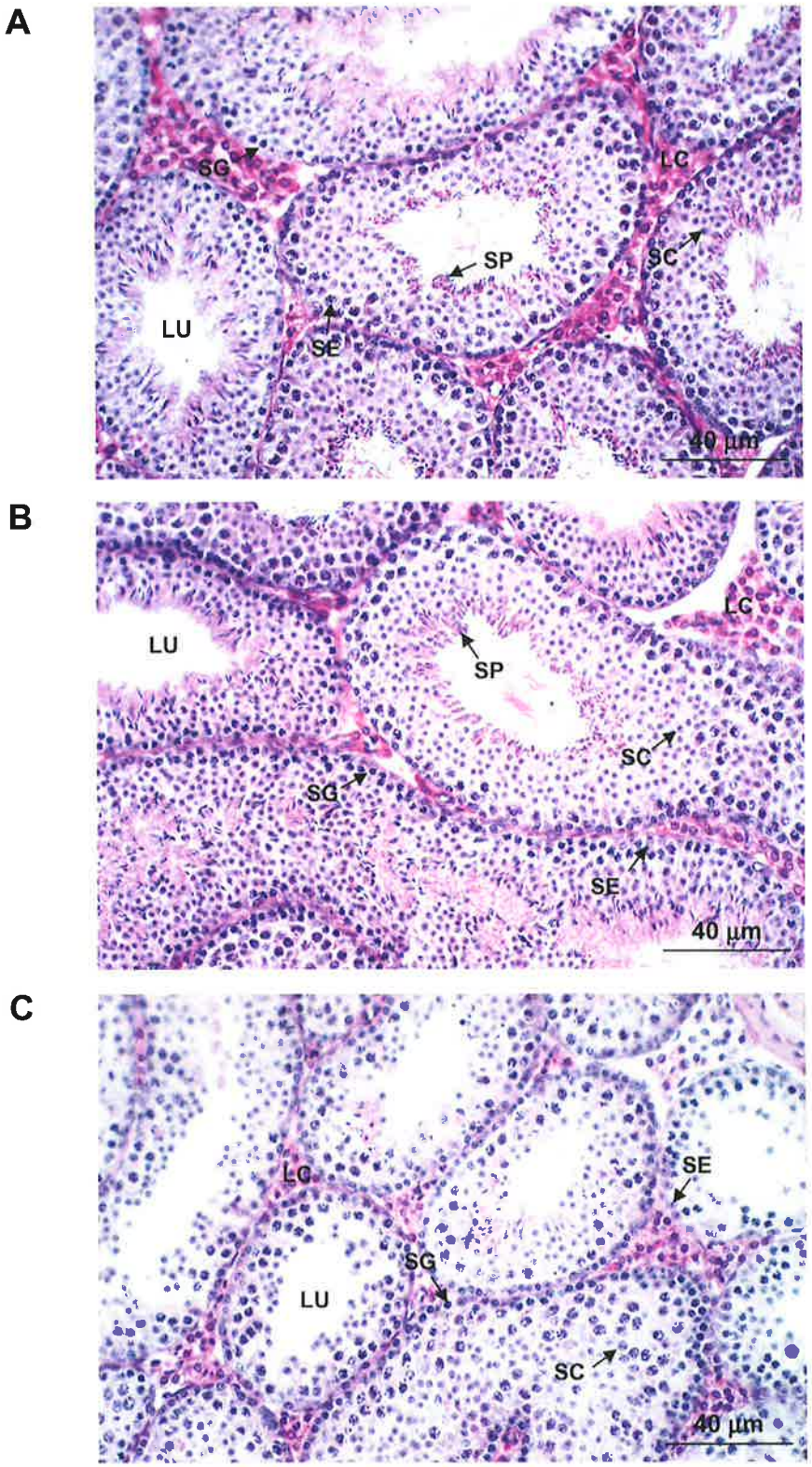


FIGURE 3.7 Histology of testes of TGFβ1+/± (A) and TGFβ1-/- (B and C) male mice. Testes were dissected from 10 week old mice, fixed in 10% buffered formalin and embedded in paraffin. Transverse sections (5 µm) were cut and stained with haematoxylin and eosin. (B) Representative of most TGFβ1-/- mice, (C) pathology in the testis of one TGFβ1-/- male is not representative. Leydig cells (LC), lumen of the seminiferous tubule (LU), Sertoli cells (SE), spermatogonia (SG), developing spermatocytes (SC) and mature spermatozoa (SP) are labelled.

	TGFβ1+/±		TGFβ1-/-	
Expt	Cleave (%)	Blast (%)	Cleave (%)	Blast (%)
1	23/29 (79)	17/23 (74)	46/65 (71)	34/46 (74)
2	47/52 (90)	35/46 (76)	28/38 (74)	17/25 (68)
3	25/37 (68)	19/25 (76)	25/32 (78)	17/25 (68)
TOTAL	**95/118 (81)**	**70/94 (78)**	**99/135 (73)**	**68/96 (71)**

TABLE 3.1 Effect of TGFβ1 null mutation on fertilising ability of sperm. Data are cleavage and blastocyst development rates for oocytes fertilised in vitro with sperm from TGFβ1+/± or TGFβ1-/- males in 3 separate experiments. Percent development is given in parentheses.

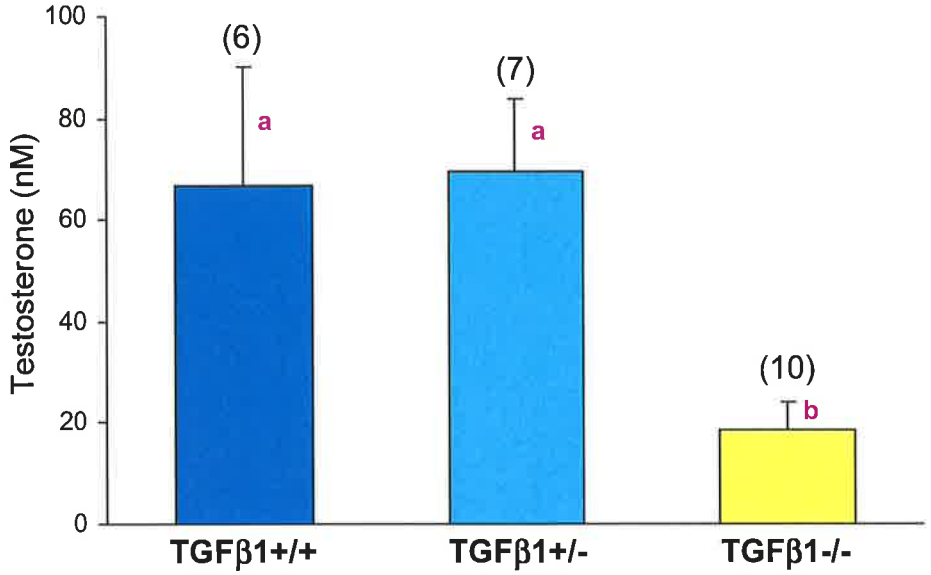
Only males with a minimum of 10^7 sperm recovered from the epididymis were used for IVF. It was found that oocytes cleaved and developed to blastocyst stage at a similar rate when fertilised with sperm from either TGFβ1+/± or TGFβ1-/- males (Table 3.1).

3.4 EFFECT OF TGFβ1 NULL MUTATION ON STEROID SYNTHESIS

To determine the effect of TGFβ1 null mutation on testosterone synthesis, serum testosterone was measured by radioimmunoassay (RIA). Serum testosterone concentration (mean±SEM) in 10 week old wildtype (TGFβ1+/+) and heterozygous (TGFβ1+/-) male mice was 67±23 nM and 70±14 nM respectively (Fig 3.8A). A significant decrease in mean serum testosterone was observed in TGFβ1-/- male mice, with mean±SEM values of 18±6 nM. When TGFβ1+/+ and TGFβ1+/- males were combined together (TGFβ1+/±), serum testosterone in TGFβ1-/- males was found to be reduced by 78% and 72% at the age of 6 weeks and 10 weeks respectively (Fig 3.8B). TGFβ1 null mutation also caused serum androstenedione concentration to be reduced while serum estradiol was unaffected by genotype (Fig 3.9).

To investigate whether testicular steroidogenesis in TGFβ1 null mutant males is responsive to LH induction, TGFβ1+/± and TGFβ1-/- males were given hCG one or four hours before sacrifice. Stimulation with hCG caused increases in serum testosterone to a similar concentration in both TGFβ1+/± and TGFβ1-/- males, a 2-fold and 6-fold increase respectively (Fig 3.10).

A



B

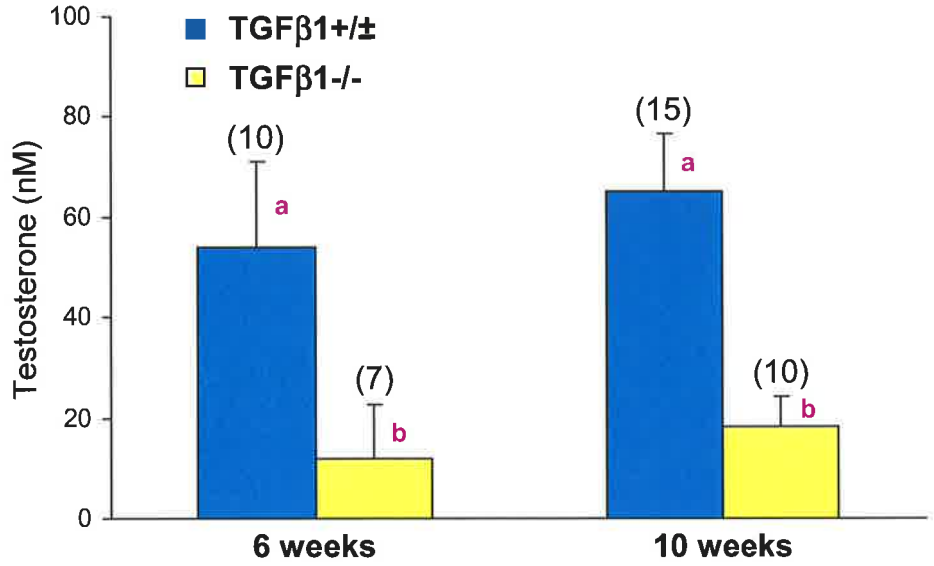


FIGURE 3.8 Serum testosterone concentration in 10 week old TGFβ1+/+, TGFβ1+/- and TGFβ1-/- male mice (A) and 6 and 10 week old TGFβ1+/± and TGFβ1-/- male mice (B). Serum was obtained at sacrifice by cardiac puncture, testosterone concentration was measured by radioimmunoassay. Data are mean+SEM, and were analysed by independent samples t-test, **a** and **b** denote significant difference ($p<0.05$). n is given in parentheses.

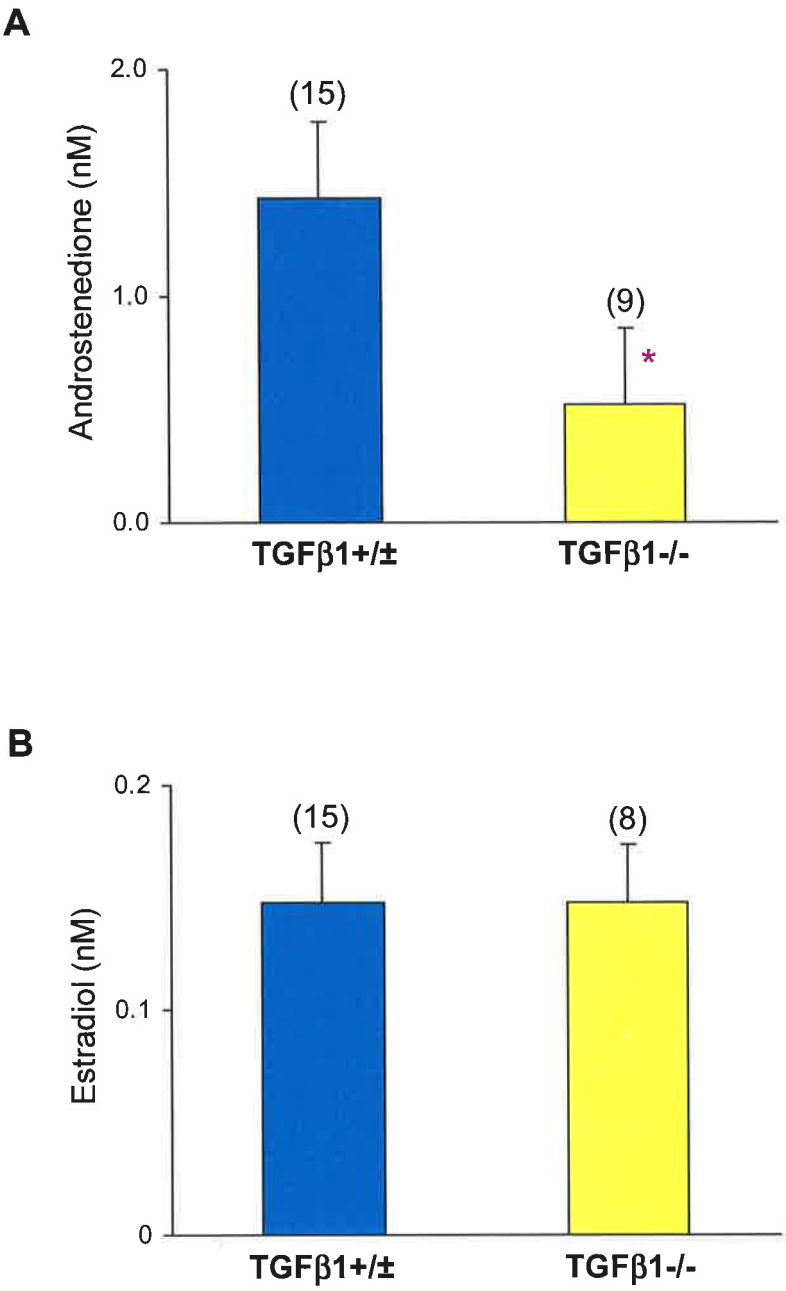


FIGURE 3.9 Serum androstenedione (A) and estradiol (B) concentration in 10 week old TGFβ1+/± and TGFβ1-/- male mice. Serum was obtained at sacrifice by heart bleed, androstenedione and estradiol concentration were measured by radioimmunoassay. Data are mean+SEM, and were analysed by independent samples t-test, *denotes significant difference from controls ($p<0.05$), n is given in parentheses.

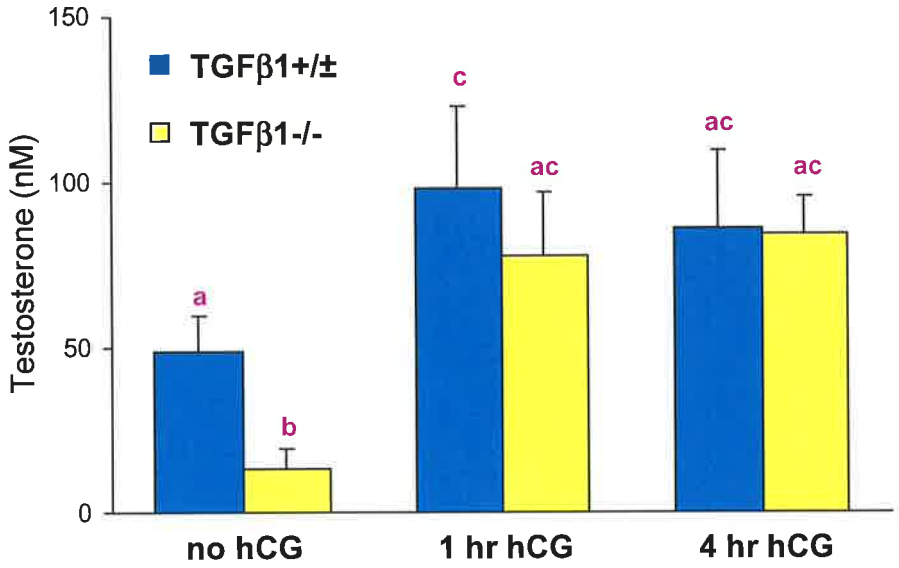


FIGURE 3.10 Serum testosterone concentration in hCG-stimulated 10 week old TGFβ1+/± and TGFβ1-/- male mice. Mice received intraperitoneal injections of hCG (5 U/10 g) and sacrificed one or four hours later, controls (no hCG) were not injected (n=6 per group). Serum was obtained by cardiac puncture, and testosterone content measured by radioimmunoassay. Data are mean+SEM, and were analysed by ANOVA test with Tukey's post hoc, **a**, **b** and **c** denote significant difference ($p<0.05$).

Testicular steroidogenic enzymes were analysed by quantitative reverse transcription PCR using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), normalised to 18s rRNA. The amount of PCR product amplified by specific primers was multiplied by a constant to give an average for the control tissue sample (TGFβ1+/± no stimulation) the arbitrary value of 1. HSD3β1 mRNA levels were 3-fold higher in the testes of unstimulated TGFβ1-/- mice compared to unstimulated TGFβ1+/± mice. StAR, P450scc and P450c17 mRNA expression were not altered by TGFβ1 null mutation (Fig 3.11). Stimulation with hCG did not cause altered expression of StAR, P450scc or P450c17, however HSD3β1 mRNA expression in TGFβ1-/- male was reduced by 5-fold after 4 hours of hCG stimulation.

3.5 DISCUSSION

These experiments clearly demonstrate that genetic deficiency in TGFβ1 causes a profound fertility defect in male mice. TGFβ1 null mutants did not mate with normal adult female mice, suggesting either an inability to perform one or more components of the mating act, or alternatively a lack of sexual interest. These mice were found to have greatly reduced serum testosterone, a steroid necessary for development and maintenance of male reproductive organs, spermatogenesis and induction of sexual behaviour.

3.5.1 General health and reproductive function in TGFβ1 null male mice

From the age of approximately 3 months, TGFβ1 null male mice develop a syndrome of pathologies characterised by dramatic weight loss, and a "wet perineum". The syndrome eventually causes hind limb paralysis and death. The cause of the syndrome is not clear. A similar phenomenon has been observed in the TGFβ1 colony at the University of Cincinnati (GP Boivin, personal communication). We do not believe this syndrome is related to lack of sexual functioning. While TGFβ1 null mutants are smaller than littermate controls throughout adult life, they maintain a steady weight and other overt signs of good health during the course of the mating study. The syndrome becomes apparent at varying ages in TGFβ1 null

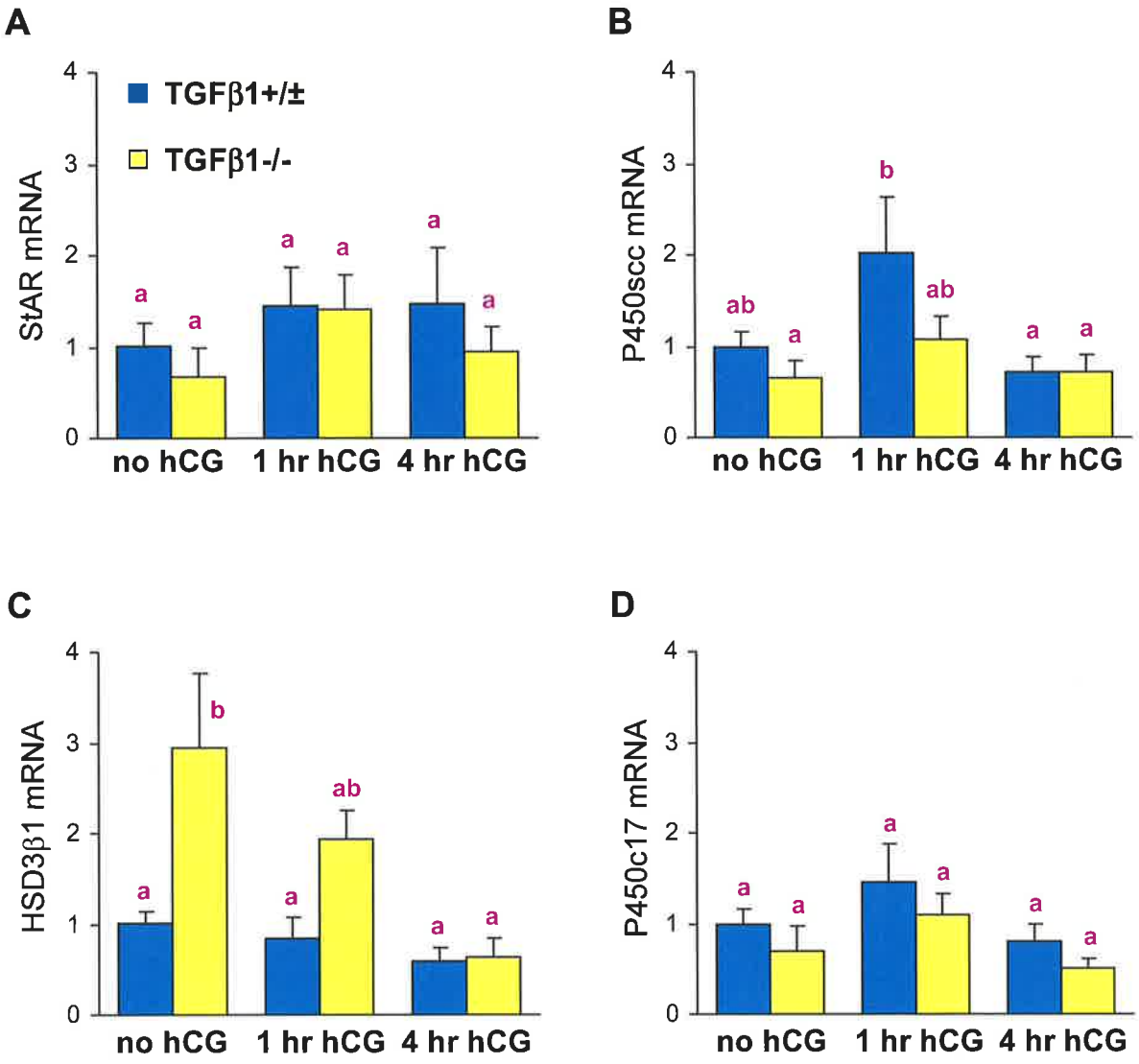


FIGURE 3.11 Expression of mRNAs encoding StAR (A), P450scc (B), HSD3β1 (C) and P450c17 (D) in the testes of hCG-stimulated 10 week old TGFβ1+/± and TGFβ1-/- male mice. Mice were injected ip with hCG (5 U/10 g) and sacrificed one or four hours later, controls (no hCG) were not injected (n=6 per group, except TGFβ1+/± no hCG where n=10). The testes were dissected and frozen in liquid nitrogen. Messenger RNA was extracted and reverse transcribed, then quantitative PCR was performed. Amount of mRNA was normalised to 18s RNA, and expressed in arbitrary units where the average of the control (TGFβ1+/±) is 1. Data are mean+SEM, and were analysed by ANOVA test with Tukey's post hoc, **a** and **b** denote significant difference ($p<0.05$).

mutant males, between 8 and 16 weeks. Males that showed any sign of ill health before the end of experiment at 10 weeks were excluded from the study.

One explanation for the cause of infertility in TGFβ1 null male mice is abnormal secondary sex organ development, particularly in the penis. TGFβ1 has been implicated in penile fibrosis seen in aging men (Moreland et al., 1995) and Peyronie's disease (el Sakka et al., 1997). Furthermore, TGFβ1 is believed to have an inhibitory role in the regulation of penile growth during puberty (Gelman et al., 1998). Aberrent or reduced penile growth might interfere with the ability to penetrate the vagina and/or cervix of female mice. In order to investigate the possibility that a gross structural defect is the cause of infertility, we dissected 10 week old TGFβ1 null males. When organ weight is normalised to body weight, most organs, including the testis, penis and seminal vesicles are of normal weight and dimensions. Furthermore, histological studies failed to reveal any overt structural differences or unusual histology in accessory gland structure in TGFβ1 null mutant males.

However the weight of some organs were altered by TGFβ1 null mutation. The lungs were increased as a result of TGFβ1 deficiency, and fat and spleen weight were decreased. Our data are consistent with the known roles of TGFβ1 in inhibition of lung morphogenesis (Shiratori et al., 1996; Zhou et al., 1996). The vital importance of TGFβ1 in the immune system is already demonstrated by the immune dysregulation observed in immune competent TGFβ1 null mice (Shull et al., 1992; Kulkarni et al., 1993) and it is unsurprising that TGFβ1 null males in the present study exhibit reduced spleen weight. The cause of reduced fat deposits in the absence of TGFβ1 may be due to perturbations in proliferation of adipocyte precursor cells (Butterwith and Goddard, 1991), or by reduced feeding mediated by alterations in leptin production (Gottschling-Zeller et al., 1999).

3.5.2 Spermatogenesis in TGFβ1 null male mice

Analysis of the morphology of the testes revealed differences between TGFβ1 deficient and replete males. The average diameter of seminiferous tubules is reduced in TGFβ1 null males, however as the absolute testis weight is also reduced,

we do not believe this is a finding of functional significance. Two different phenotypes of spermatogenesis emerged in TGFβ1 deficient mice. In approximately 70% of males, spermatogenesis proceeds normally and the sperm are capable of fertilising oocytes from superovulated normal female mice and development of blastocysts occurs at a normal rate. In the remainder, sperm are all but absent from the epididymis. The latter phenotype is accompanied by severely impaired spermatogenesis with a large proportion of seminiferous tubules containing only spermatogonia and spermatocytes with no mature spermatozoa.

The concentration of serum testosterone in TGFβ1 null males approaches the lower end of the threshold level required to maintain spermatogenesis (Zirkin et al., 1989). One genetic modifier of the effect of TGFβ1 deficiency has been described (Bonyadi et al., 1997) and there are likely to be others (Kallapur et al., 1999). The mice used in this study are on a mixed background, so it is unsurprising that the phenotype resulting from TGFβ1 deficiency is stronger in some animals. Presumably when serum testosterone falls below the threshold required to maintain testis function disruption to spermatogenesis occurs. Indeed, TGFβ1 null mice with severely impaired spermatogenesis exhibited very low serum testosterone levels however a statistical association between serum testosterone and impaired spermatogenesis would be difficult to identify given the random cyclical nature of testosterone production.

Alternatively, TGFβ1 deficiency may be directly responsible for compromised spermatogenesis. Male gametes synthesise TGFβ1 with greatest abundance in spermatocytes and early round spermatids (Teerds and Dorrington, 1993) suggesting a role in spermatocyte proliferation and differentiation. The impaired spermatogenesis seen in mutants deficient in mature sperm is consistent with this hypothesis. However the infrequent occurrence of this phenotype implies that any necessity for TGFβ1 in spermatogenesis must be highly dependent on genetic modifiers.

3.5.3 Steroidogenesis in TGFβ1 null male mice

Genetic deficiency in TGFβ1 causes defective steroidogenesis in male mice. Both serum androstenedione and testosterone are inhibited however serum estradiol is not affected by TGFβ1 null mutation. This finding indicates that disturbance in steroidogenesis occurs upstream of androstenedione synthesis while the enzymes which control androstenedione conversion to testosterone (17βHSD) and conversion to estradiol (P450arom) are active. In vitro culture of primary testicular cells from several species demonstrates TGFβ1 inhibition of LH/hCG induced androgen production (Avallet et al., 1987; Lin et al., 1987; Le Roy et al., 1999). These findings are difficult to reconcile with our in vivo analysis of testosterone synthesis in the absence of TGFβ1 where we find a dramatic decrease in serum testosterone. However, at the physiological doses ordinarily used in in vitro studies (1-2 ng/ml) TGFβ1 has also been found to increase LH/hCG stimulated testosterone synthesis by cultured porcine Leydig cells, with inhibition occurring at higher concentrations (Morera et al., 1988).

Two explanations for these discrepancies are suggested. Firstly, the bimodal effect of TGFβ1 on testosterone synthesis in vitro may by a consequence of the impure nature of the cell preparations. Macrophages are capable of both regulation of testicular steroidogenesis (Bergh et al., 1993; Cohen et al., 1996), and responsiveness to TGFβ1, with bimodal effects dependent on differentiation-state and environmental context of the TGFβ1 signal (Bogdan and Nathan, 1993). Testicular macrophages are likely to be active in non-purified primary Leydig cell cultures causing differential effects dependent on different methods employed in the culture system. In addition, the most critical difference between in vitro and in vivo studies of this nature is that we see the net effect of disruption in the intact tissue compared with the less physiologically pertinent observations in specific subpopulations of cells. However, the limitation of in vivo experiments in null mutants is that direct and indirect effects of systemic deficiency in TGFβ1 on testicular function are likely and complicate the analysis. Thus, while TGFβ1 may be important in regulation of testicular function, this cytokine is likely to have an even more crucial role in regulation of endocrine signals that stimulate the testis.

Stimulation of testicular cells by LH/hCG causes testosterone synthesis and induction of some steroidogenic enzymes (Payne and Youngblood, 1995). LH binds with high affinity to specific receptors on Leydig cells resulting in activation of adenylate cyclase and increased production of intracellular cyclic AMP. LH has both acute and chronic effects on testicular steroidogenesis. The acute response involves rapid transport of cholesterol to the inner mitochondrial membrane, and may involve sterol carrier protein$_2$ and cytoskeletal components (Jefcoate, 1992). Chronic stimulation of steroidogenesis also occurs following LH stimulation. In vitro culture studies have indicated that this involves increased expression of the steroidogenic enzymes StAR (Reinhart, 1999) and P450c17 (Anakwe and Payne, 1987).

Steroidogenesis is also positively and negatively regulated by TGFβ1. In vitro addition of TGFβ1 reduces LH/hCG receptor expression (Avallet et al., 1987) and cAMP formation (Avallet et al., 1987; Gautier et al., 1997) and inhibition of TGFβ1 action by an antisense oligonucleotide causes increased LH/hCG receptor expression (Le Roy et al., 1999). TGFβ1 has also been shown to inhibit a number of steroidogenic enzymes. HSD3β mRNA synthesis and protein activity in a mouse adrenal tumour cell line (Cherradi et al., 1995) and P450c17 expression in rat (Gautier et al., 1997) and porcine (Le Roy et al., 1999) Leydig cells and bovine adrenocortical cells (Brand et al., 1998) are both reduced by culture with physiological concentrations of TGFβ1. Expression of StAR is also decreased in primary cultures of porcine Leydig cells (Chuzel et al., 1996) and bovine adrenocortical cells (Brand et al., 1998) treated with TGFβ1, however the same concentration has the opposite effect on FSH induced StAR mRNA expression when rat granulosa cells are pretreated with the cytokine (Minegishi et al., 2000). Other studies have also found upregulation of steroidogenic enzymes by TGFβ1. In long term culture, TGFβ1 increases basal P450scc and HSD3β and LH stimulated P450c17 mRNA expression by ovarian interstitial cells (Fournet et al., 1996).

To analyse induction of steroidogenesis in TGFβ1 null mutant males, we measured serum testosterone and expression of StAR, P450scc, HSD3β1 and P450c17 mRNA in unstimulated mice, and mice stimulated for one or four hours with hCG. Serum testosterone in TGFβ1 null males is elevated one hour following

stimulation to a similar concentration as seen in controls, so the testis is clearly capable of normal testosterone output. This suggests that the major lesion in steroidogenesis occurs upstream of the testis, possibly in LH or GnRH production. However we have also shown that perhaps a less severe consequence of TGFβ1 deficiency occurs in local regulation of HSD3β1 mRNA synthesis. This finding is in agreement with studies on bovine adrenal cells (Le Roy et al., 1996) and the Y1 adrenal tumour cell line (Cherradi et al., 1995) where TGFβ1 negatively regulated HSD3β activity and mRNA expression. Alternatively, overexpression of HSD3β1 mRNA may be the consequence of chronic testosterone deficiency as testosterone negatively regulates expression of this steroidogenic enzyme in Leydig cell cultures (Heggland et al., 1997). Indeed HSD3β1 mRNA expression in TGFβ1 null males fell rapidly upon stimulation with hCG and rising testosterone levels.

3.6 SUMMARY

These experiments have revealed severe fertility defects in TGFβ1 null male mice, accompanied by impaired testosterone synthesis and, in some animals, impaired spermatogenesis. Reduced serum testosterone is likely to be due to impaired endocrine signals that upregulate androgen synthesis. The cause of infertility in TGFβ1 deficient mice with normal sperm production is not yet clear. As the mice do not plug or deposit sperm in the vagina of female mice we suspect a lesion in sexual function and experiments described in the following chapter investigate this.

Chapter 4 Impaired sexual performance in male TGFβ1 null mutant mice

4.1 INTRODUCTION

Sexual behaviour and function in males is dependent on central and peripheral neural signalling, and a process of concerted smooth muscle contraction and relaxation to obtain erection and ejaculation. A diverse range of central signalling molecules are involved in the cascade of events required for sexual intercourse including dopamine, norepinephrine, serotonin and norepinephrine (Andersson, 2000). Peripherally, erection is mediated by production of nitric oxide by penile nitric oxide synthase (NOS) enzyme isoforms, causing cGMP production and consequently relaxation of the corpus cavernosa smooth muscle (Burnett, 1995). However noradrenaline, acetylcholine and dopamine have also been implicated (Andersson, 2001). In addition, sex steroid hormones, including testosterone and estrogen, are believed to have roles in both central and peripheral control of sexual behaviour and function (Harding, 1986). Furthermore, exposure to testosterone during the perinatal period is critical to androgenization of the sexually dimorphic spinal nucleus of the bulbocavernosus, and the ability to copulate (Breedlove and Arnold, 1983).

In the previous chapter it was shown that male mice with a targeted null mutation in the TGFβ1 gene have a fertility defect. The cause of infertility was identified as related to dysregulated sexual behaviour or an inability to perform the mating act as the male mice fail to inseminate adult females or induce psuedopregnancy. It was also found that TGFβ1 null adult males have a 75% decrease in serum androgen content.

In this chapter, investigation into the cause of infertility in male TGFβ1 null mutants is continued. Initially, mating behaviour and penile NOS induction were analysed, and attempts were then made to restore mating ability using a variety of interventions. These interventions included treatment with sildenafil citrate, a pharmacological agent that inhibits breakdown of cGMP via inhibition of phosphodiesterase V (Boolell et al., 1996), thus increasing the effectiveness of NOS in stimulating erectile responses, and replacement of androgens neonatally and during adulthood.

4.2 EFFECT OF TGFβ1 NULL MUTATION ON MALE MATING BEHAVIOUR

To investigate sexual activity in TGFβ1 null males, their behaviour when introduced to a receptive superovulated female was observed. Interaction between the male and female was video taped under red light, for the first two hours of contact, starting at 2200 hr. Male sexual behaviour, including anogenital investigation, mounting, intromission and ejaculation were analysed by examination of the video tapes by a blinded assessor. These behavioural parameters were defined in Chapter 2. Examples of sexual behaviour exhibited by TGFβ1+/±[†] and TGFβ1-/- males can be viewed in Movie 4.1 and 4.2 respectively.

Both TGFβ1+/± and TGFβ1-/- males displayed initial sexual interest in the female by way of anogenital investigation (Table 4.1). Four of six TGFβ1-/- males mounted the female. Two of six TGFβ1-/- males also showed intromission behaviour however these displays were shorter in duration than intromission behaviour displayed by control males. Since it was not possible to view whether insertion of the penis into the vagina did indeed occur, it remains possible that these may be "false" intromissions. All TGFβ1+/± males ejaculated during the test period, and afterwards did not display further sexual interest. In contrast, no TGFβ1-/- males ejaculated during the test period and these mice continued to display mounting behaviour leading to significantly more total mounts over the 2 hour period. However as TGFβ1+/± males displayed the behaviour for a shorter period, the overall number of mounts per minute was comparable. The presence of a plug or sperm positive vaginal smear in the female on the following morning confirmed that ejaculation had occurred and this was evident in all receptive females housed with control males but not in females housed with TGFβ1 null males.

4.3 EFFECT OF TGFβ1 NULL MUTATION ON PENILE NOS EXPRESSION

To analyse the effect of TGFβ1 null mutation on the molecular mechanisms in the

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[†] As wildtype (TGFβ1+/+) and heterozygous (TGFβ1+/-) males did not have any apparent fertility defects, the two genotypes were grouped together in several experiments, this group is deemed TGFβ1+/±.

	TGFβ1+/±	TGFβ1-/-
% time anogenital investigation	27 (18-44)	37 (15-48)
MOUNTS	5/5	4/6
Latency (min)	7 (4-48)	10 (2-44)
Number	2 (0-13)	45 (0-68)
Duration (sec)	3 (3-6)	3 (3-3)
INTROMISSIONS	5/5	2/6
Latency (min)	51 (4-53)	11, 97
Number	9 (3-13)	0 (0-6)*
Duration (sec)	12 (11-18)	5, 6*
EJACULATION	5/5	0/6
Latency (min)	63 (32-85)	NA
Duration (sec)	33 (23-44)	NA
TOTAL MOUNTS	11 (10-27)	48 (0-71)*
Mounts per minute	0.39 (0.19-0.59)	0.47 (0.39-0.75)

TABLE 4.1 Effect of TGFβ1 null mutation on mating behaviour by male mice. Virgin adult male mice were housed individually for a period of one week, then housed adjacent to adult female mice for 2 days. Males were then housed with an immature superovulated B10 female and behaviour was video taped under red light. Percent of time during the first 10 minutes after introduction of the female spent in anogenital investigation was recorded. The latency (ie time taken before behaviour is displayed), number and duration of mounts, intromissions, ejaculation, and the number of mice displaying the behaviour, were recorded over the 120 minute period. The proportion of mice in each group that displayed the behaviour was also recorded. The total number of mounts (including mounts, intromissions and ejaculation) was calculated and the time from the first to the last mount was used to determine the number of mounts per minute. Data was not available (NA) when the behaviour was not observed in any mice in that group. Data are median (range) and were analysed by Kruskal Wallis-H test followed by Mann Whitney-U test, *$p<0.05$.

penis that mediate erection, mRNA for NOS enzymes were quantified in penis tissue, in unstimulated and stimulated 10 week old males. Unstimulated animals were caged alone from the age of 7 weeks and sacrificed at the age of 10 weeks. Two NOS induction protocols were investigated. The first was stimulation for four hours with hCG, which is expected to mimic the LH surge male mice experience when a female is encountered (Maruniak and Bronson, 1976; Bronson and Desjardins, 1982). The second stimulation protocol involved subcutaneous injections every second day with 200 μg testosterone propionate (TP) from the age of 5 weeks, with penis dissection occurring 24 hours after the last injection. Messenger RNA was extracted and reverse transcribed, and quantitative PCR performed to detect neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) mRNA expression.

Many penis cDNA preparations showed expression of NOS genes below the detectable limit of the quantitative PCR assay. In these samples, the amount of NOS PCR product as detected by fluorescence of the sample did not increase to cycle threshold (Ct) after 40 cycles, despite having normal levels of the housekeeping genes β-actin and 18s. These samples were assigned a Ct value of 40, and the amount of PCR product relative to the housekeeper gene was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The values were multiplied by a constant to give an arbitrary mRNA quantity so that the average of the control is 1 arbitrary unit (in this case the control was TGFβ1+/± + hCG as this was the only group that consistently displayed detectable levels of NOS enzyme mRNA). These "negative" values varied between samples as the amount of housekeeper gene varied. The highest "negative" value for each primer pair was determined to be the "detection limit" of the PCR assay and all values below this limit were assigned this value. When NOS enzyme mRNA expression was normalised to β-actin mRNA expression the detection limit for the assays were 0.04, 0.006 and 0.0003 arbitrary units for nNOS, iNOS and eNOS respectively.

Both unstimulated TGFβ1+/± and unstimulated TGFβ1-/- males exhibited very low levels of nNOS, iNOS and eNOS mRNA expression (Fig 4.1). In seven of eight unstimulated TGFβ1+/± males, nNOS expression was below the detectable limit of the quantitative PCR assay, and six of eight had below the detectable limit of iNOS and eNOS. No unstimulated TGFβ1-/- males had detectable mRNA for NOS isoforms. When stimulated for 4 hours with hCG, which was shown in the previous chapter to induce testosterone synthesis in both TGFβ1+/± and TGFβ1-/- males, four of six TGFβ1+/± males were induced to express mRNA for nNOS isoforms and five of six expressed iNOS and eNOS. In contrast, NOS enzyme mRNA was expressed was induced in only one of six hCG-stimulated TGFβ1-/- males, and in one of five TP-treated TGFβ1-/- males. Expression of penile nNOS and eNOS mRNA was significantly increased by hCG treatment of TGFβ1+/± males, compared to unstimulated TGFβ1+/± males and there was no statistical difference in expression of these enzymes when TGFβ1-/- males were treated with hCG (data were analysed by ANOVA with Tukey's post hoc, $p<0.05$). Regression analysis revealed no

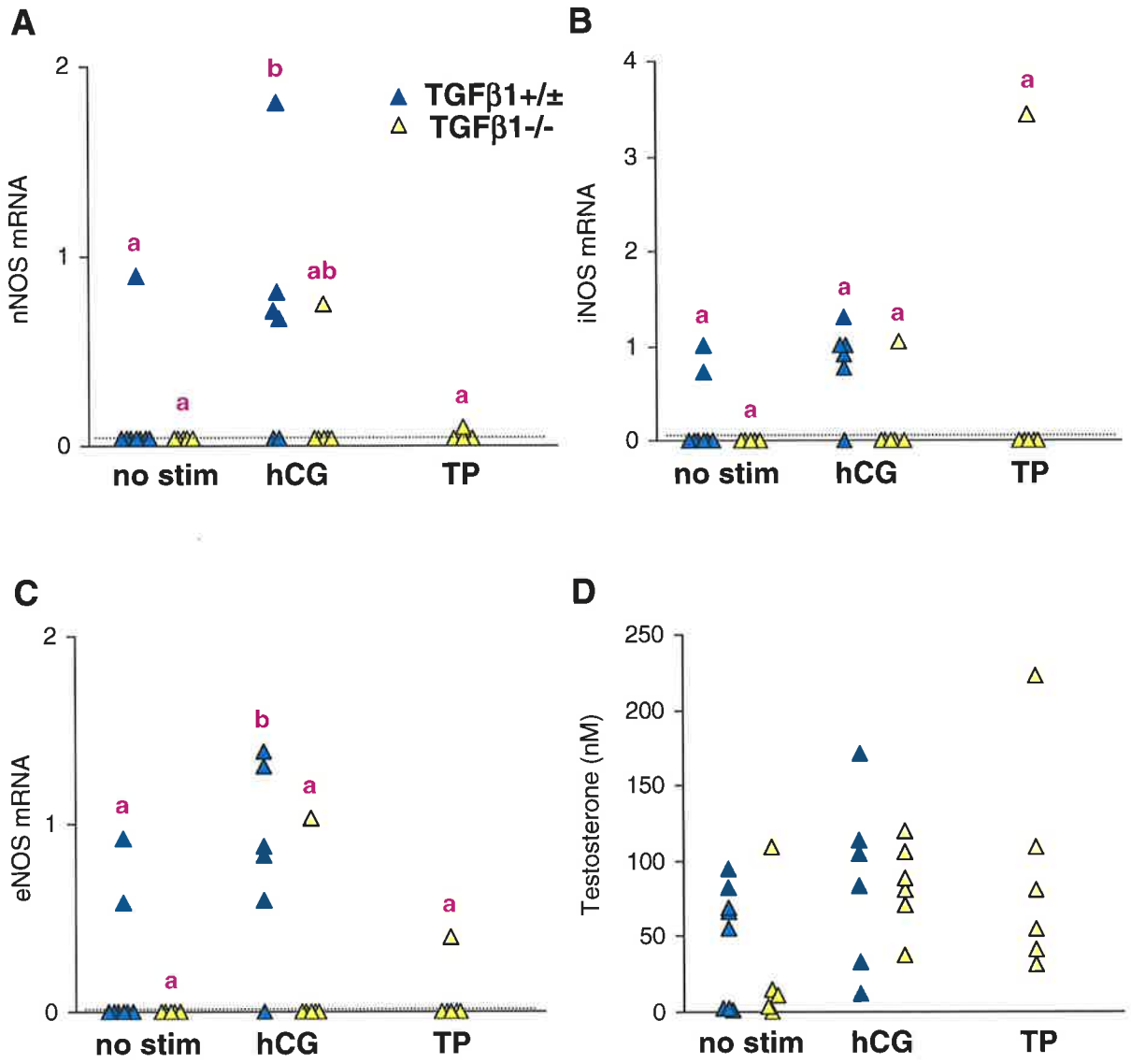


FIGURE 4.1 Expression of mRNAs encoding nNOS (A), iNOS (B) and eNOS (C) induced by hCG and testosterone in penis of TGFβ1+/± and TGFβ1-/- male mice. Males were housed alone, and untreated, or injected with hCG (5 U/10 g) 4 hours prior to sacrifice or injected subcutaneously with testosterone propionate every second day from the age of 5 weeks, and sacrificed 24 hours after the last injection. The penis was dissected and frozen in liquid nitrogen. Messenger RNA was extracted and reverse transcribed, then quantitative PCR was performed. Amount of mRNA was normalised to β-actin expression, and is given in arbitrary units where the average of the control (TGFβ1+/± + hCG) is 1, values that fell below the detectable threshold for the assay (dotted line) were assigned the threshold value. (D) Serum testosterone was collected at sacrifice and assayed for testosterone by radioimmunoassay. Each point represents mRNA quantity for single mouse, data were analysed by ANOVA with Tukey's post hoc, **a** and **b** denote significant difference ($p<0.05$).

significant relationship between serum testosterone concentration and NOS enzyme mRNA expression.

4.4 EFFECT OF SILDENAFIL CITRATE TREATMENT ON MATING ABILITY OF TGFβ1 NULL MUTANT MALES

To enhance the nitric oxide - relaxation pathway and attempt to improve erectile function in TGFβ1 null males, mice were injected with sildenafil citrate (4 μg/10 g) 30 minutes prior to introduction of a receptive superovulated female. The dose of sildenafil citrate used was double that required to enhance intracavernous pressure by electrical stimulation in anaesthetised mice (Mizusawa et al., 2001). Sildenafil citrate did not restore mating ability to TGFβ1-/- males. This drug did not affect the ability of control males to mate (Table 4.2).

	TGFβ1+/± (sildenafil citrate)	**TGFβ1-/- (sildenafil citrate)**
% time anogenital investigation	24 (23-32)	41 (31-48)
MOUNTS	3/3	3/5
Latency (min)	10 (3-30)	10 (2-22)
Number	3 (1-3)	2 (0-62)
Duration (sec)	4 (3-4)	3 (2-4)
INTROMISSIONS	3/3	0/5
Latency (min)	43 (18-59)	NA
Number	22 (1-27)	0
Duration (sec)	11 (11-15)	NA
EJACULATION	3/3	0/5
Latency (min)	76 (46-90)	NA
Duration (sec)	38 (32-43)	NA
TOTAL MOUNTS	26 (5-29)	2 (0-51)
Mounts per minute	0.44 (0.12-0.44)	0.05 (0.02-0.45)

TABLE 4.2 Effect of sildenafil citrate treatment on mating behaviour by TGFβ1+/± and TGFβ1-/- male mice. Adult male mice were housed as described in Table 4.1 and injected (ip) with sildenafil citrate (4 μg/10 g) 30 minutes prior to introduction of an immature superovulated B10 female. Data was not available (NA) when the behaviour was not observed. Data are median (range) and were analysed by Kruskal Wallis-H test followed by Mann Whitney-U test.

4.5 EFFECT OF TESTOSTERONE REPLACEMENT ON MATING ABILITY OF TGFβ1 NULL MUTANT MALES

To determine whether testosterone depletion is the cause of infertility in TGFβ1 null mutant males, the effect of exogenous testosterone administration in the neonate and during adulthood was examined. Testosterone was delivered to adult mice in silastic implants (containing 150 mg/ml testosterone) from 5 weeks of age, or given subcutaneously (TP 200 μg) every other day starting at 5 weeks of age. In a third group of mice, neonatal testosterone propionate was given as a single subcutaneous injection (100 μg) on day 1 or 2 after birth. To maintain serum testosterone concentration in adulthood in this group, TP was given subcutaneously from 5 weeks of age, as described above.

Each of these three different testosterone treatments were found to result in

	TGFβ1+/± neonatal T adult T sc	**TGFβ1-/- adult T sc**	**TGFβ1-/- neonatal T adult T sc**
% time anogenital investigation	18 (15-19)	22 (6-32)	13 (8-21)
MOUNTS	4/4	3/5	4/5
Latency (min)	44 (10-116)	5 (3-19)	6 (5-24)
Number	2 (1-8)	18 (13-20)	6 (0-29)
Duration (sec)	3 (3-3)	2 (2-2)	3 (2-3)
INTROMISSIONS	3/4	0/5	0/5
Latency (min)	59 (10-116)	NA	NA
Number	5 (1-13)	0	0
Duration (sec)	12 (8-13)	NA	NA
EJACULATION	4/4	0/5	0/5
Latency (min)	120 (48-120)	NA	NA
Duration (sec)	41	NA	NA
TOTAL MOUNTS	8 (3-15)	13 (0-20)	6 (0-29)
Mounts per minute	0.22 (0.08-1.12)	0.23 (0.18-0.28)	0.43 (0.16-0.71)

TABLE 4.3 Effect of testosterone replacement on mating behaviour by TGFβ1+/± and TGFβ1-/- male mice. Male mice received subcutaneous injections of testosterone propionate neonatally (100 μg) and/or every second day during adulthood (200 μg). Adult male mice were housed with an immature superovulated B10 female as described in Table 4.1. Data was not available (NA) when the behaviour was not observed. When ejaculation was known to occur however was not observed, the ejaculation latency for that male was assigned a value of 120 minutes. Data was not available (NA) when the behaviour was not observed in any mice in that group. Data are median (range) and were analysed by Kruskal Wallis-H test followed by Mann Whitney-U test.

TGFβ1+/±	TGFβ1-/-	TGFβ1+/± neonatal T adult T sc	TGFβ1-/- adult T sc	TGFβ1-/- neonatal T adult T sc	TGFβ1-/- adult T implant
92% (12)	0% (10)	100% (5)	0% (5)	0% (5)	0% (5)

TABLE 4.4 Effect of testosterone replacement on mating ability by TGFβ1+/± and TGFβ1-/- male mice. Data are percent of TGFβ1+/±, TGFβ1-/- or testosterone supplemented males that inseminated adult female mice during a three week period. Testosterone supplements were given in the form of testosterone in a silastic implant at 5 weeks of age (adult T implant), subcutaneous testosterone propionate given every other day starting from 5 weeks of age (adult T sc) or subcutaneous testosterone propionate given on day 1 or 2 after birth, followed by subcutaneous testosterone propionate given every other day starting from 5 weeks of age (neonatal T + adult T sc). Vaginal smears indicated that the females were cycling normally, with no extended diestrus indicative of pseudopregnancy. n is given in parentheses.

normal levels of serum testosterone in TGFβ1 null males at 10 weeks of age (Fig 4.2), but all failed to alleviate the infertility phenotype as assessed by mating behaviour when the males were housed with immature superovulated B10 females (Table 4.3). Interestingly, ejaculation behaviour in three of four TGFβ1+/± males given neonatal TP followed by subcutaneous TP injections did not occur in the 2 hour observation period, however these males did mate with the females, confirmed by the presence of a plug or sperm positive vaginal smear the following morning. These males were assigned an ejaculation latency of 120 minutes. This increase in ejaculation latency in TGFβ1+/± males, when compared to TGFβ1+/± males with no intervention tended towards significance (median (range); 63 (35-85) minutes for control TGFβ1+/± males compared to 120 (48-120) minutes for testosterone-treated TGFβ1+/± males, data were analysed by Mann Whitney-U test, p=0.08). Testosterone-treated TGFβ1+/± males mated with adult cycling B10 females when housed together for a period of 3 weeks (Table 4.4). In contrast, no testosterone treatment was effective in restoration of mating ability of TGFβ1-/- males housed with adult females. As was observed in adult females housed with untreated TGFβ1-/- males in Chapter 3, daily vaginal smears indicated that the females housed with testosterone-treated TGFβ1-/- males cycled normally and evidence of pseudopregnancy was not observed.

The abundance of sperm recovered from the epididymis of adult testosterone-treated (subcutaneous TP) TGFβ1-/- males was similar to that recovered from untreated TGFβ1+/± males (Table 4.5).

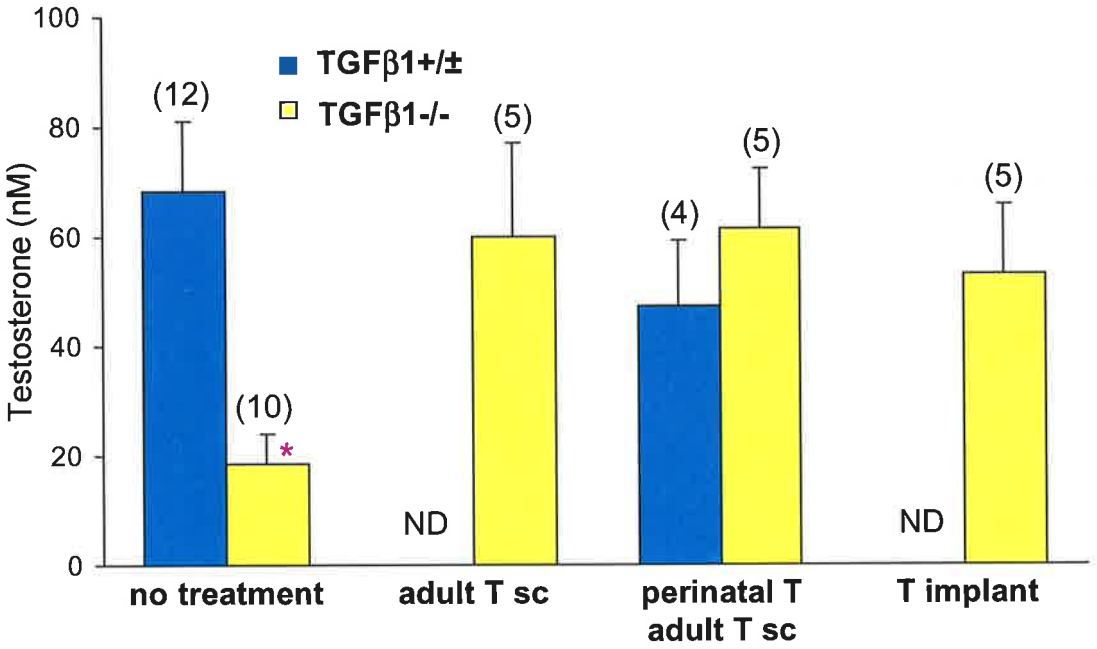


FIGURE 4.2 Serum testosterone concentration in testosterone supplemented TGFβ1+/± and TGFβ1-/- male mice. Testosterone supplements were given in the form of testosterone in a silastic implant at 5 weeks of age (adult T implant), subcutaneous testosterone propionate injected every other day starting from 5 weeks of age (adult T sc) or subcutaneous testosterone propionate given on day 1 or 2 after birth, followed by subcutaneous testosterone propionate given every other day starting from 5 weeks of age (perinatal T + adult T sc). Serum was obtained at sacrifice by heart bleed when the mice were 10 weeks of age, 24 hours after last testosterone injection. Testosterone concentration were measured by radioimmunoassay. Data are mean+SEM, and were analysed by independent samples t-test, *denotes significant difference from control, no treatment ($p<0.05$). ND=not done, n is given in parentheses.

TGFβ1+/±	TGFβ1-/-	TGFβ1-/- adult T sc
$4.8x10^7 \pm 7.4x10^6$	$2.9 x10^7 \pm 6.4x10^6$ *	$4.0x10^7 \pm 7.4x10^6$

TABLE 4.5 Effect of testosterone replacement on epididymal sperm number in TGFβ1-/- male mice. Male mice received subcutaneous injections of testosterone propionate every second day from the age of 5 weeks (200 μg). Sperm were recovered from the cauda epididymis at sacrifice at the age of 10 weeks. Data are mean±SEM, and were analysed by independent samples t-test, *denotes significant difference from control ($p<0.05$).

4.6 DISCUSSION

In this chapter, infertility in TGFβ1 null mutant male mice is shown to be caused by a lesion in sexual function. The males show all the outward signs of sexual interest including anogenital investigatory behaviour and mounting of females. In some cases, TGFβ1 null males displayed intromission behaviour, which was shorter in duration than TGFβ1 replete littermates, however ejaculation was never observed. In addition, TGFβ1 null males do not achieve sufficient penetration of the cervix to induce pseudopregnancy in normal cycling adult females. Analysis of mRNA in the penis revealed that hCG does not induce expression of NOS enzyme isoforms in TGFβ1 null males.

These observations led us to consider two hypotheses for the cause of impaired sexual performance in TGFβ1 null mutant males (1) androgen insufficiency during the crucial stages of development required for adult sexual function; during the perinatal period and/or adulthood and (2) erectile failure in penile tissue caused by dysregulated nitric oxide synthesis. The first possibility was investigated by attempts to restore sexual function by androgen replacement. To explore the second possibility, the therapeutic function of sildenafil citrate in TGFβ1 null animals was examined.

4.6.1 The effect of androgen replacement on sexual function in TGFβ1 null male mice

Androgens are of critical importance in the induction of sexual behaviour and ability to perform the mating act (James and Nyby, 2002). Androgens act centrally, stimulating the brain to respond to a sexual stimulus, and peripherally, facilitating erection via induction of NOS enzymes (Marin et al., 1999). Consistent with this,

administration of testosterone has improved sexual function in several testosterone deficient mouse models (Livne et al., 1992; Cohen et al., 1996; Rao and Lei, 2002).

Two methods of androgen replacement during adulthood were utilised in an attempt to restore fertility in TGFβ1 null males. Initially, testosterone implants were given. While the implants increased serum testosterone levels, it is possible that their use compromised mating ability in the mice. Wound healing is impaired by TGFβ1 deficiency (Crowe et al., 2000) and it was observed that the incision made to insert the implant did not heal properly and may have caused stress or poor health in the TGFβ1 null males. In the second approach, the males were given injections of testosterone propionate every second day. These injections improved serum testosterone concentration but, like the implants, failed to improve mating ability. However androgen replacement did appear to improve the percent of TGFβ1 null males with normal sperm numbers, suggesting that low testosterone is responsible for impaired spermatogenesis described in Chapter 3.

The findings of the behavioural study, where TGFβ1 null mutant males displayed intromission behaviour without any indication of penetration, is consistent with a deficiency in neonatal androgenization. The hypogonadal mouse is also androgen deficient (Cattanach et al., 1977), and testosterone administration during adulthood leads to similar behaviour as that seen in the TGFβ1 null mutant – mounting and intromission but not ejaculation (Livne et al., 1992). This phenotype in the hypogonadal mouse can be rescued by neonatal androgenization (Livne et al., 1992). Following the same protocol described for neonatal androgenization of the hypogonadal mouse, we could not restore sexual function in TGFβ1 null males.

However, the neonatal androgenization protocol may be flawed. It was surprising that neonatal androgenization together with testosterone administration during adulthood tended to increase the ejaculation latency of TGFβ1 replete males. The significance of neonatal androgenization has been better studied in the rat and only one protocol for neonatal androgenization in mice was available in the literature (Livne et al., 1992). Neonatal androgenization followed by testosterone replacement during adulhood caused three of eight hypogonadal males to mate with receptive females overnight, however ejaculation was not actually observed during the two

hour surveillance period. In another study using the same protocol, fertility was restored in three of five LH receptor null male mice however mating behaviour was not observed (Rao and Lei, 2002). A control group of neonatally androgenized normal males were not used in either of these studies. While it is not certain that a delay in ejaculation latency in TGFβ1 replete males observed in the present study was caused by neonatal androgenization or adult testosterone administration, these findings taken together suggest that the neonatal androgenization protocol described by Livne et al. (1992) is suboptimal in its ability to mimic androgenization required for normal sexual function in mice. Androgens are elevated as early as day 14 of gestation in male mice and decline in the first couple of days after birth (Pointis et al., 1980). Neonatal androgenization of normal male mice may disrupt the natural development of sexual dimorphic nuclei that control copulation.

4.6.2 Induction of penile NOS enzymes in TGFβ1 null male mice

Formation of nitric oxide is catalysed by the three isoforms of NOS (Griffith and Stuehr, 1995) and is regulated at the level of NOS gene transcription, mRNA and protein stability, and by availability of the NOS substrate L-arginine (Mori and Gotoh, 2000; Fulton et al., 2001). TGFβ1 has been shown in vitro to suppress nitric oxide synthesis by each of these mechanisms (Vodovotz, 1997) and increased expression of iNOS occurs in immunocompetent TGFβ1 null mutant mice (Vodovotz et al., 1996). In addition, NOS activity in the penis is positively regulated by androgens (Seo et al., 1999). Excessive production of nitric oxide may impair stimulation of neurons or smooth muscle relaxation/contraction involved in penile erection (Rajasekaran et al., 2001), while diminished nitric oxide will be insufficient to cause the increased level of cGMP required to induce erection (Hedlund et al., 2000).

We postulated that dysregulated NOS enzyme synthesis might be responsible for the inability of TGFβ1 null males to mate. Induction of NOS enzymes in response to hCG stimulation occurred in TGFβ1 replete males but failed to occur in five of six hCG-stimulated TGFβ1 null males. Without NOS enzyme activity, nitric oxide will be diminished, in which case the male will not achieve an erection. This observation is consistent with the reduced number of TGFβ1 null males that demonstrate

intromission behaviour and accounts for the shortened intromission times observed in those that did intromit.

As the NOS induction mechanism is impaired in TGFβ1 null males, sildenafil citrate was administered to increase smooth muscle relaxation. Sildenafil citrate assists the nitric oxide - erection pathway by reducing the breakdown of intermediate cGMP. Therefore the mechanism of action of this pharmacological agent requires synthesis of cGMP via NOS and nitric oxide. As NOS enzyme mRNA was for the most part undetectable in TGFβ1 null males by the highly sensitive quantitative PCR assay, it was perhaps unsurprising that sildenafil citrate was ineffective. To determine whether impaired nitric oxide synthesis is the primary cause of sexual dysfunction in TGFβ1 null males, measurement of erectile responses following replacement of penile nitric oxide levels together with further attempts to improve mating ability by stimulation of this pathway would be of great value.

A surge in LH occurs when a male mouse is introduced to a new mouse (Bronson et al., 1973; Bronson and Desjardins, 1982). This surge is part of the stimulus for aggression towards other males, and sexual behaviour towards receptive females, and causes an acute increase in serum testosterone (Mendis-Handagama, 1997). As pituitary hormone synthesis is likely to be impaired in TGFβ1 null males (Chapter 3), it is uncertain whether the LH surge occurs when a TGFβ1 null male is introduced to a new female. Therefore exogenous stimulation of the pathway was preferable to female-induced stimulation in order to determine whether the penile mechanism of erection via nitric oxide synthesis was intact in TGFβ1 null males. Exogenous hCG was used to stimulate the LH receptor in mice similarly to endogenous LH (Pierce and Parsons, 1981).

LH receptors are not isolated to steroidogenic tissues (Rao, 2001) and have been found in seminal vesicles (Tao et al., 1998) and may be present in the penis. The effect of LH/hCG on penile NOS expression has not been previously described. The findings reported here raise further questions on the relative roles of testosterone, hCG and TGFβ1 in this mechanism.

It might be postulated that the sexual dysfunction phenotype in TGFβ1 null mice is the consequence of impaired hypothalamo-pituitary function, particularly LH secretion, as was postulated for the reduced serum testosterone phenotype observed in Chapter 3. However mating dysfunction and infertility caused by LH receptor null mutation can be reversed by treatment with neonatal androgenization followed by adult testosterone replacement (Rao and Lei, 2002). Therefore, the principle role of LH in mating behaviour is via testosterone production.

We did not find a relationship between serum testosterone concentration and NOS enzyme mRNA expression although previous studies have shown that testosterone is a crucial hormone in the regulation of NOS enzyme expression, whereby castrated rats do not produce NOS in response to electrical stimulus unless administered exogenous testosterone (Marin et al., 1999; Seo et al., 1999). It is uncertain whether testosterone acutely or chronically stimulates NOS expression in the normal male, and differences in the regulation of NOS between rats and mice may occur. The data presented here suggest that a stimulus threshold is required for acute induction of penile NOS enzymes. In the present study, the two TGFβ1 null males that did have detectable levels of penile NOS enzyme mRNA also had the highest serum testosterone levels. It is possible that NOS enzymes may be regulated by androgens in TGFβ1 null males, but a higher concentration of testosterone is required, perhaps due to reduced sensitivity to androgens. In this situation, sildenafil citrate together with testosterone administration might assist in facilitating sexual function. Further research on the mechanism of acute NOS induction and dose responses to sildenafil citrate may lead to successful mating and restoration of fertility in these mice.

4.7 SUMMARY

These data demonstrate that TGFβ1 null mutant males show normal sexual interest and behaviour but fail to intromit successfully or inseminate females. Sexual dysfunction is clearly the primary cause of infertility in the TGFβ1 null male and is not restored by testosterone treatment. The underlying cause may be related to lack of induction of NOS enzymes leading to impotence.

Chapter 5 Ovarian dysfunction in female TGFβ1 null mutant mice

5.1 INTRODUCTION

The female reproductive system is unusual in comparison with other organs and tissues in that its development and maturation occurs largely after birth. The great extent of tissue differentiation and remodelling involved in the onset of sexual maturity, ovarian cycles, implantation and lactation require regulation by hormones and growth factors. The ovary is a site of a great deal of change during development and in the reproductive phase of life. These changes impinge on many other systems and processes via sex steroid production.

Evidence for a role for TGFβ1 in processes involved in female reproductive function comes largely from in vitro culture systems, where this cytokine has been shown to be capable of influencing oocyte maturation, ovulation, implantation, early embryogenesis and mammary gland development (Chapter 1). TGFβ1 is believed to modulate the effects of pituitary hormones on the ovary, influencing steroid hormone production (Adashi et al., 1989) and DNA synthesis (Roy, 1993), and regulating oocyte maturation. However the role of TGFβ1 in these processes in vivo remains undefined.

The TGFβ1 null mutant mouse offers a unique opportunity to investigate the role of TGFβ1 in female reproductive function in vivo. Here we demonstrate that TGFβ1 is indeed crucial to reproductive success, primarily through its effects on ovarian function.

5.2 EFFECT OF TGFβ1 NULL MUTATION ON ESTROUS CYCLICITY AND OVULATION

To investigate the effect of TGFβ1 null mutation on estrous cyclicity, cycles were tracked for a period of 28 days by histological analysis of vaginal smears in adult females. Cycles were severely perturbed in null mutant mice, with deficiency in TGFβ1 causing a 2-fold increase in estrous cycle length compared to TGFβ1 replete females (Table 5.1). However the proportion of the cycle spent in each phase was unaffected by genotype (Fig 5.1).

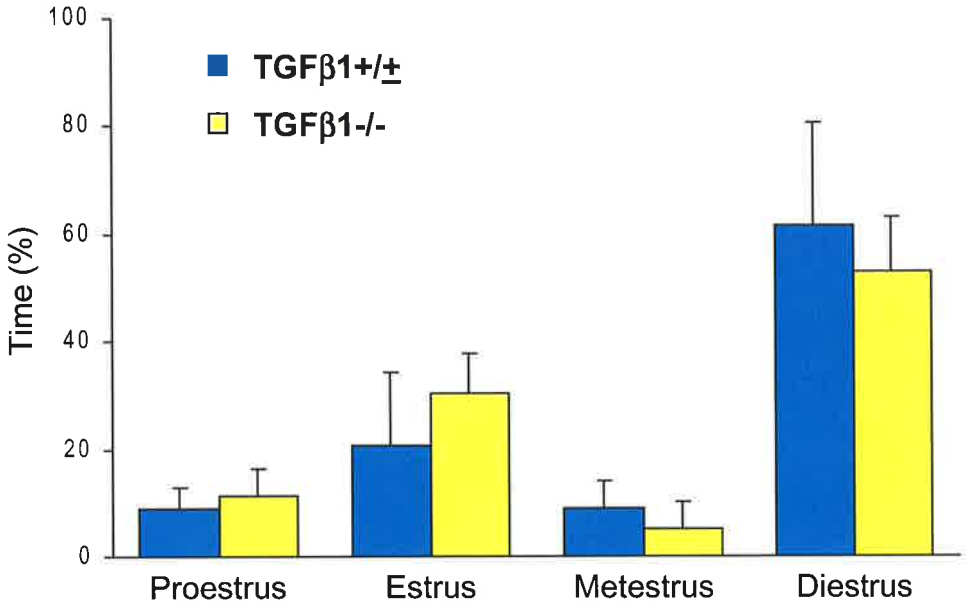


FIGURE 5.1 Percent time spent in each phase of the estrous cycle in adult TGFβ1+/± and TGFβ1-/- female mice. Daily vaginal smears were analysed by phase contrast microscopy in female mice (n=6 per group) over 28 days. The percent of viable epithelial cells, cornified epithelial cells and leukocytes in the smears distinguished mice in proestrus, estrus, metestrus and diestrus. Data are mean+SEM, and were analysed by independent samples t-test.

	TGFβ1+/±	TGFβ1-/-
Cycle length (days)	5.5±0.4	9.4±0.9*
Cycles (number)	5.3±0.4	2.8±0.4*
Ovulations (number)	10.6±0.3	6.3±1.6*
Ovarian weight (%)	0.030±0.017	0.017±0.004*

TABLE 5.1 Ovarian cycles, ovulation and ovarian weight in adult TGFβ1+/± and TGFβ1-/- females. Estrous cycles were followed for 28 days (females were approximately 6 to 10 weeks of age) by histological analysis of vaginal smears. Estrus was defined as when >90% of cells in vaginal smear were cornified epithelial cells. A single complete cycle was defined as the first day of estrus through to the first day of the next estrus (n=6 per group). Female mice mated with stud B10 males were sacrificed on day 3.5 pc. The number of corpora lutea were taken as indicative of number of ovulations and ovarian weight is given as percent of body weight (n=10 TGFβ1+/±; n=6 TGFβ1-/-). Data are mean±SEM, and were analysed by independent samples t-test, *p<0.05.

Ovarian function was further analysed in naturally mated day 3.5 post coitum (pc) females. When the ovaries of day 3.5 pc mice were compared, there was found to be a 40% reduction in the number of ovulations in TGFβ1-/- females and the ovarian weight, when compared as a percentage of body weight, was halved (Table 5.1). The ovaries of day 3.5 pc TGFβ1-/- mice were similar to TGFβ1+/±[†] mice in the histology of the corpora lutea (Fig 5.2). Ovarian follicles in various stages of development were observed in ovaries from both genotypes.

5.3 EFFECT OF TGFβ1 NULL MUTATION ON FERTILITY

To determine the effect of TGFβ1 null mutation on female fertility, TGFβ1+/± and TGFβ1-/- adult females were housed with normal stud B10 males and checked each day for the presence of plugs and subsequent litters. Only 14 of 34 (41%) TGFβ1-/- females mated during a 28 day period, compared with 35 of 35 (100%) TGFβ1+/± females (Fig 5.3A). Of the TGFβ1 null females that did mate, the mean±SEM interval between caging with males and detection of the plug was 12.3±2.5 days, compared to 5.2±1.0 days for TGFβ1+/± females (data were analysed by independent samples t-test, p<0.05). When TGFβ1 null mutant females mated with B10 stud males, pregnancy outcome was dramatically compromised,

[†] As wildtype (TGFβ1+/+) and heterozygous (TGFβ1+/-) females did not have any apparent fertility defects, the two genotypes were grouped together in several experiments, this group is deemed TGFβ1+/±.

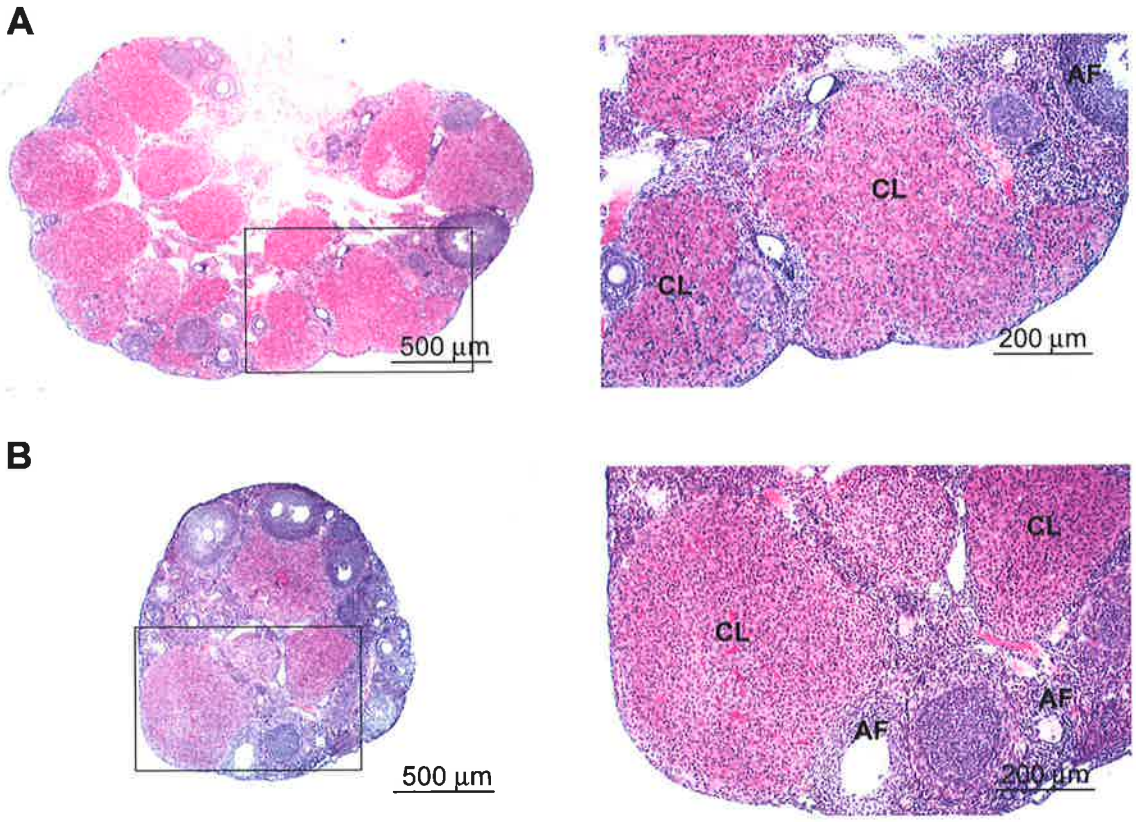


FIGURE 5.2 Representative histology of ovaries on day 3.5 post coitum from TGFβ1+/+ (A) and TGFβ1-/- (B) mice. Female mice were mated with normal stud B10 males and sacrificed on day 3.5 pc. Dissected ovaries were fixed in 10% buffered formalin and embedded in paraffin. Sections (5 µm) were cut and stained with haematoxylin and eosin. Corpora lutea (CL) and antral follicles (AF) are labelled.

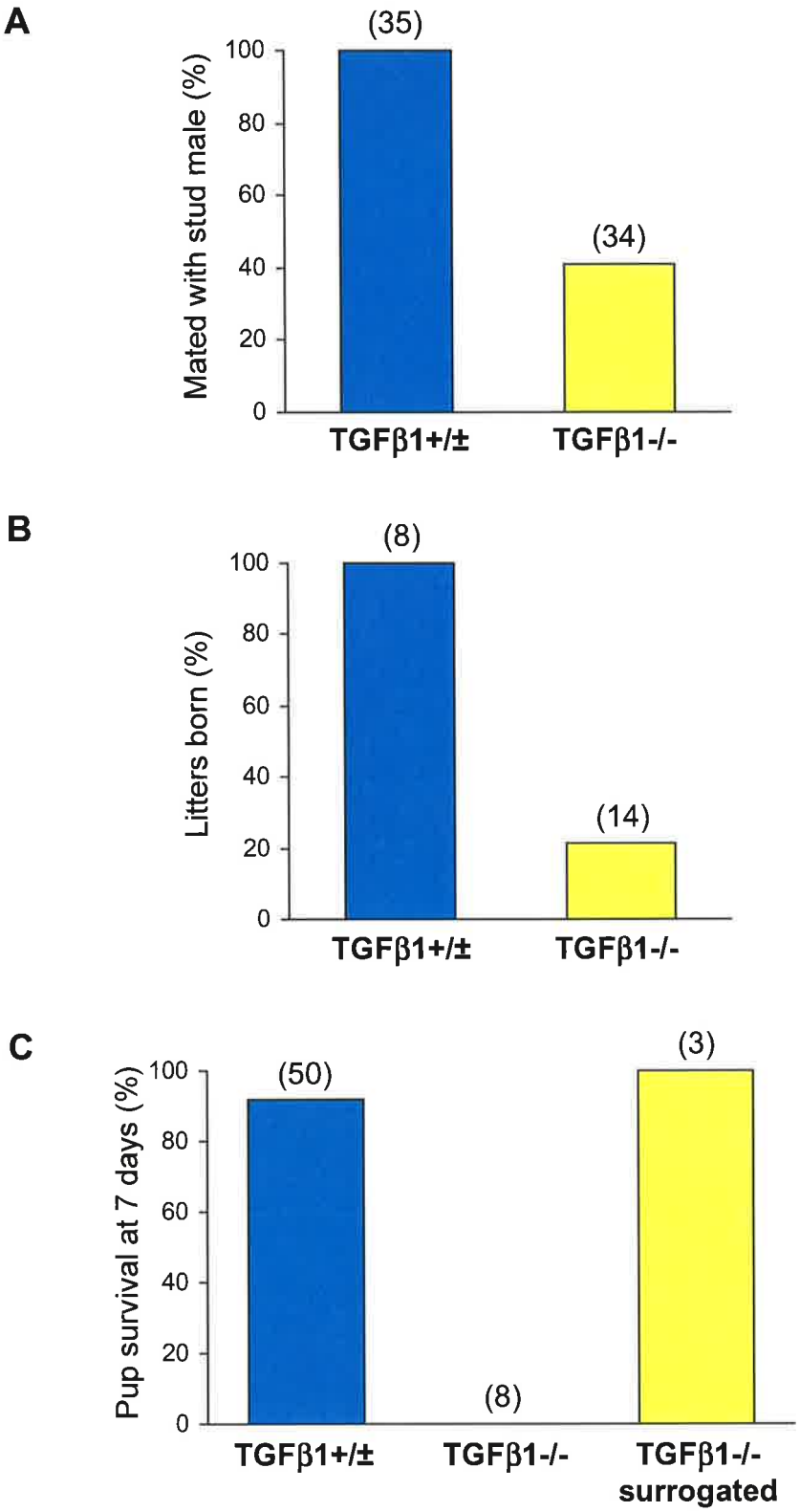


FIGURE 5.3 Fertility of TGFβ1+/± and TGFβ1-/- females. (A) Female mice were caged with normal stud B10 males and checked daily for plugs as evidence of a mating event. (B) After mating, the female was monitored for birth of pups, indicative of pregnancy success. (C) Survival of pups born to TGFβ1+/± and TGFβ1-/- mothers was monitored for the first 7 days after birth. n is given in parentheses.

with the frequency of live litters born reduced by 80% (Fig 5.3B). The females that did not give birth to pups did not show visible signs of pregnancy. Survival of pups born to TGFβ1 null mothers was also severely reduced (Fig 5.3C). Pups born to two TGFβ1 null mothers failed to thrive and did not survive to seven days of age. A third litter of three pups was fostered immediately after birth to a normal lactating female. These pups survived to adulthood.

5.4 EFFECT OF TGFβ1 NULL MUTATION ON UTERINE MORPHOLOGY

To investigate the effect of TGFβ1 null mutation on uterine morphology, sections of fixed, paraffin embedded uteri from day 3.5 naturally mated females were stained with haematoxylin and eosin (Fig 5.4). The uteri from TGFβ1-/- females were morphologically similar to uteri dissected from TGFβ1+/± females in terms of luminal diameter, presence and relative proportion of endometrial and myometrial layers and uterine glands.

5.5 EFFECT OF TGFβ1 NULL MUTATION ON PREIMPLANTATION EMBRYO DEVELOPMENT

To further investigate pregnancy failure caused by TGFβ1 null mutation, females were sacrificed on day 3.5 of pregnancy and embryos were flushed from the reproductive tract. A comparable percentage of oocytes flushed from the reproductive tract of TGFβ1+/± and TGFβ1-/- females had undergone the first cellular division. However few embryos from TGFβ1-/- females were developed to the blastocyst stage, the majority being delayed in the morula stage of development (Table 5.2 and Fig 5.5). Radioimmunoassay of serum from these mice revealed a 75% reduction in mean progesterone concentration in TGFβ1 null mutant females when compared to TGFβ1 replete controls (Fig 5.6A). Progesterone output per corpus luteum was also significantly reduced (Fig 5.6B). Serum estradiol concentration was not altered by genotype (Fig 5.7).

To further investigate the reasons for failure of preimplantation embryo development, a series of experiments were devised in an attempt to rescue

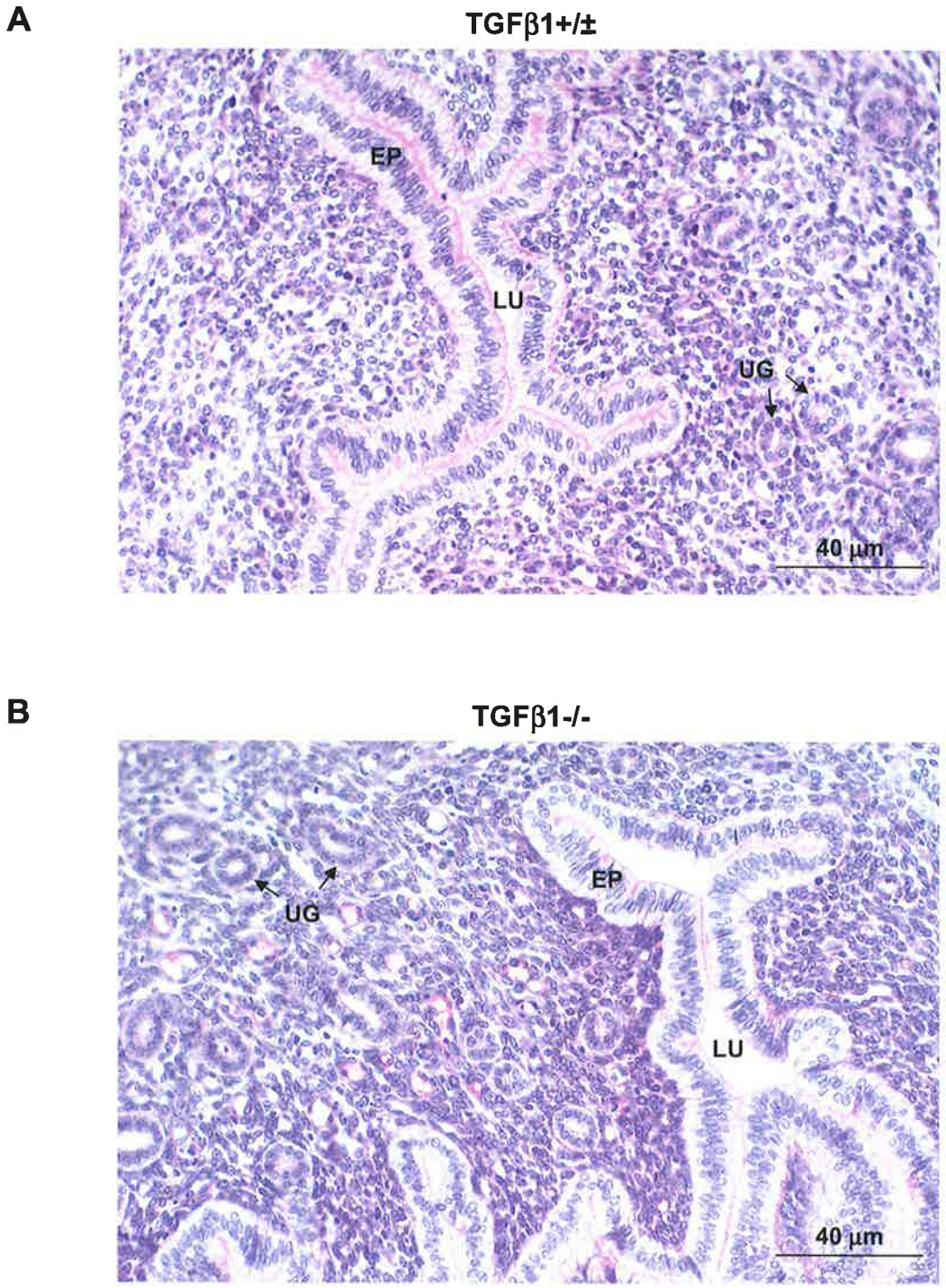
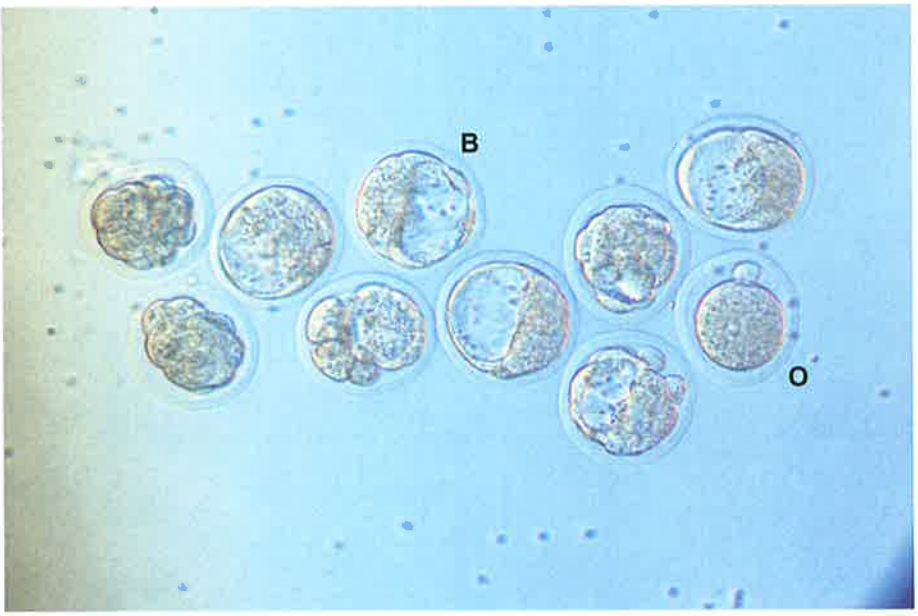


FIGURE 5.4 Representative histology of endometrium on day 3.5 post coitum from TGFβ1+/+ (A) and TGFβ1-/- (B) mice. Female mice were mated with normal stud B10 males and sacrificed on day 3.5 pc. The dissected uteri were fixed in 10% buffered formalin and embedded in paraffin. Sections (5 μm) were cut and stained with haematoxylin and eosin. The luminal epithelium (EP), uterine lumen (LU) and uterine glands (UG) are labelled.

A

TGFβ1+/±



B

TGFβ1-/-

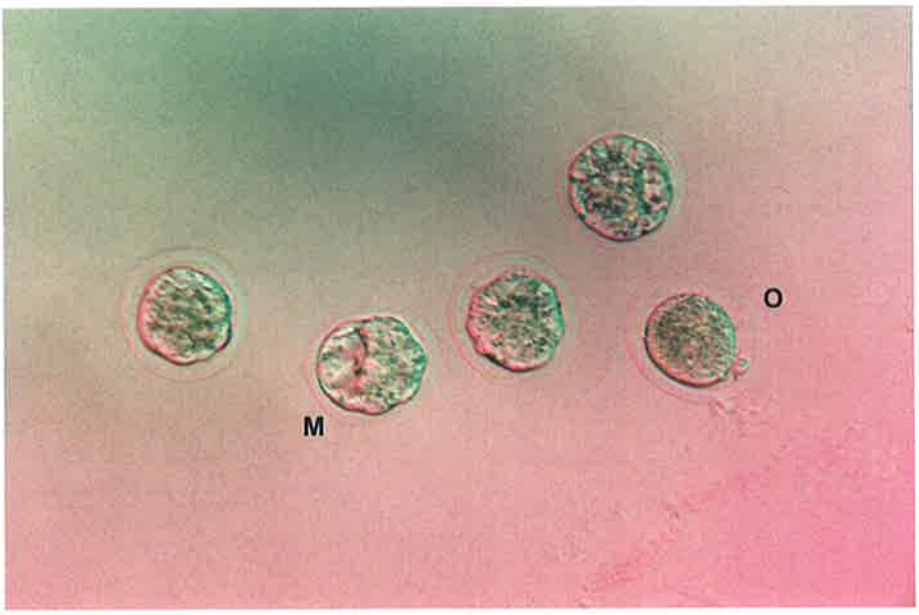


FIGURE 5.5 Embryos flushed on day 3.5 post coitum from TGFβ1+/± (A) and TGFβ1-/- (B) female mice. Embryos were flushed from the uterus and oviduct. These photomicrographs are representative of number and developmental stage of embryos flushed from naturally mated adult females (see Table 5.1 and 5.2). Examples of one-cell (O), morula (M) and blastocysts (B) are labelled.

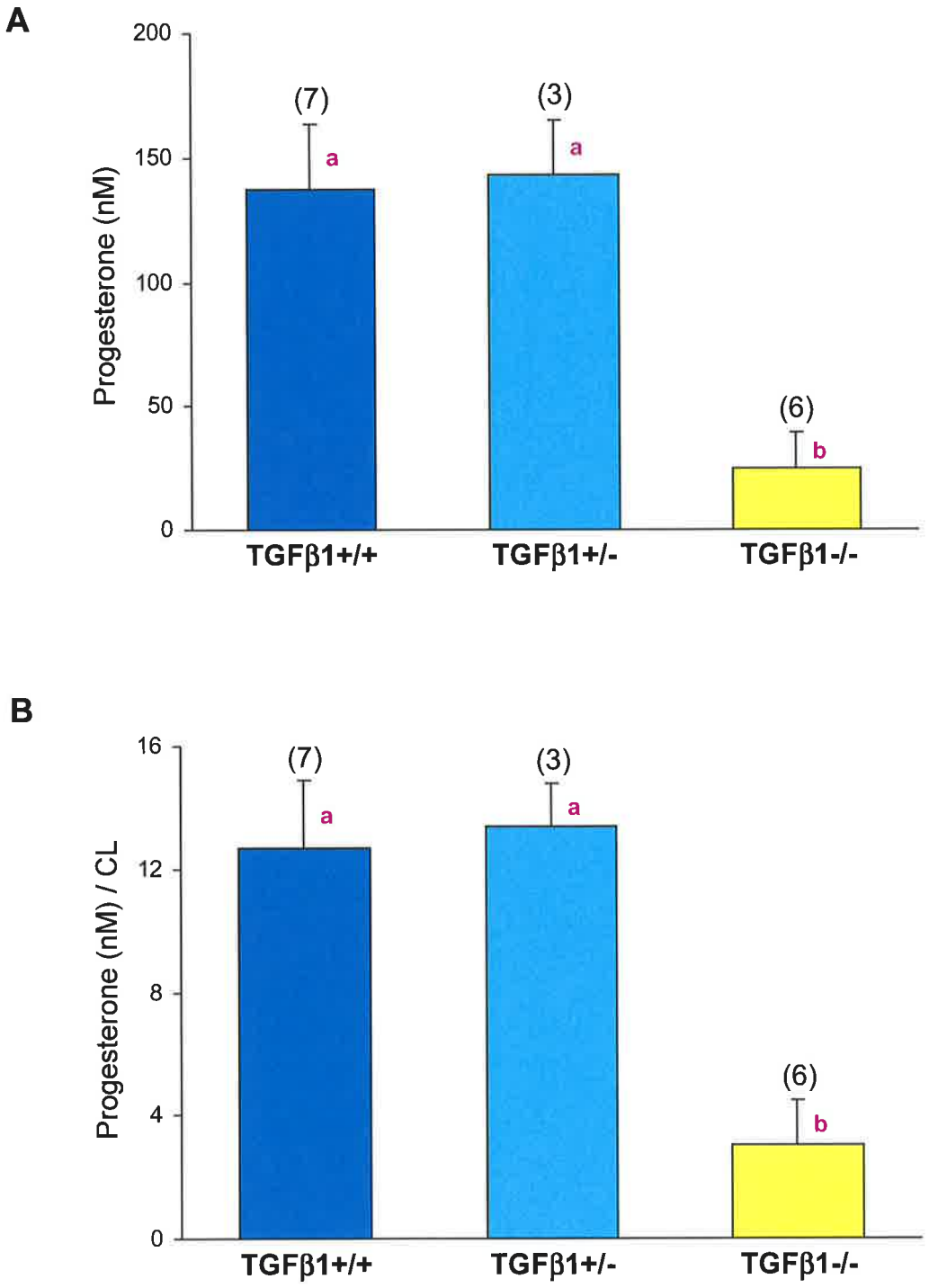


FIGURE 5.6 Serum progesterone concentration on day 3.5 post coitum in TGFβ1+/+, TGFβ1+/– and TGFβ1-/- female mice. (A) Serum was obtained from adult mated females at sacrifice on day 3.5 pc by cardiac puncture, progesterone concentration was measured by radioimmunoassay. (B) Serum progesterone was divided by the number of corpora lutea (CL) to calculate progesterone output per CL. Data are mean+SEM, and were analysed by independent samples t-test, **a** and **b** denote significant difference ($p<0.05$). n is given in parentheses.

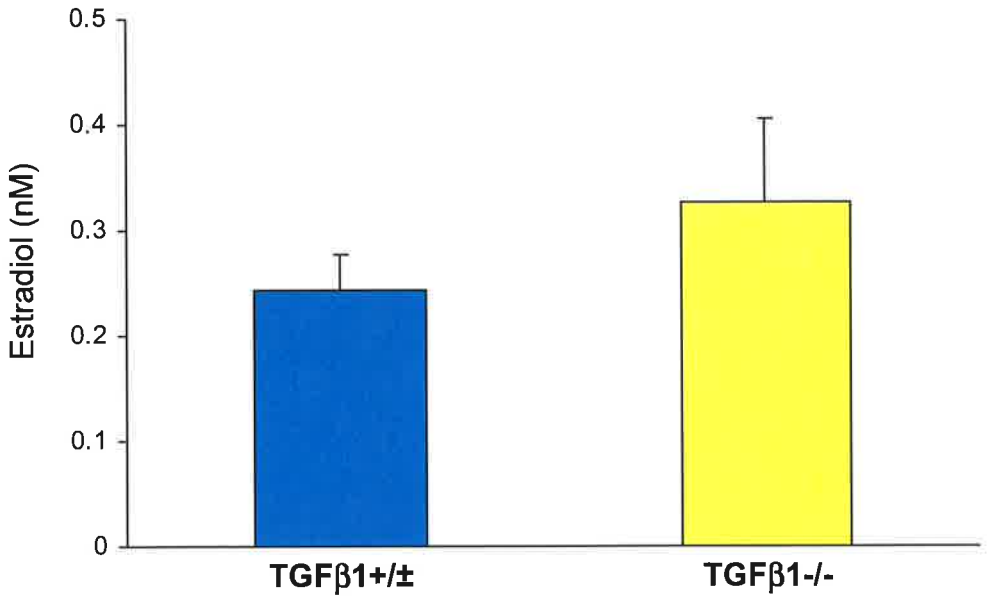


FIGURE 5.7 Serum estradiol concentration on day 3.5 post coitum in TGFβ+/± and TGFβ1-/- female mice. Serum was obtained from adult mated females at sacrifice on day 3.5 pc by cardiac puncture, estradiol concentration was measured by radioimmunoassay. (n=6 per group). Data are mean+SEM, and were analysed by independent samples t-test. n is given in parentheses.

	TGFβ1+/±	TGFβ1-/-
Efficiency of embryo retrieval (%)	82±5	87±8
Fertilised (%)	86±6	89±5
Degenerating (%)	8±7	11±11
Pre-morula (%)	3±2	22±16
Morula (%)	16±6	51±20*
Blastocyst (%)	74±7	16±12*

TABLE 5.2 Preimplantation embryo development in adult TGFβ1+/± and TGFβ1-/- females. Embryos were recovered from the reproductive tract of females on day 3.5 pc and visually classified as fertilised, degenerating, pre-morula, morula or blastocyst. The percent of embryos in each developmental stage was determined for each mouse (n=10 TGFβ1+/±; n=6 TGFβ1-/-). Efficiency of embryo retrieval is the number of embryos retrieved as a percent of the expected number of oocytes ovulated as determined by the number of corpora lutea. Degenerating, pre-morula, morula or blastocyst are given as a percent of total fertilised. Data are mean±SEM, and were analysed by independent samples t-test, *p<0.05.

development of fertilised oocytes from TGFβ1-/- mice. Initially mice were treated with gonadotrophins to elicit superovulation. It was discovered that 70% of immature TGFβ1 null female mice were responsive to exogenously administered gonadotrophins as reflected by successful induction of ovulation and the number of oocytes retrieved were comparable to oocytes retrieved from TGFβ1+/± females. Two methods were employed to investigate embryo development in a defined culture media, using TGFβ1+/+ (either CBA F1 or B10) males as a source of sperm in order to produce TGFβ1 replete (TGFβ1+/-) embryos. Both in vitro fertilisation followed by in vitro culture, and in vitro culture from day 0.5 pc after natural mating, resulted in a similar rate of cleavage of oocytes from TGFβ1+/± and TGFβ1-/- females (Table 5.3). However, TGFβ1+/- embryos from oocytes obtained from

	TGFβ1+/±		TGFβ1-/-	
Responsive to gonadotrophins (%)	10/10 (100)		7/10 (70)	
Oocytes retrieved	9±1		7±1	
	Cleaved (%)	Blastocyst (%)	Cleaved (%)	Blastocyst (%)
In vitro fertilisation/ in vitro culture	12/25 (48)	6/12 (50)	5/10 (50)	0/5 (0)
In vivo fertilisation/ in vitro culture	36/49 (73)	20/36 (56)	12/18 (67)	0/12 (0)
TOTAL	48/74 (65)	26/48 (54)	17/28 (61)	0/17 (0)

TABLE 5.3 Cleavage and blastocyst development rates for embryos recovered from 27-29 day old superovulated TGFβ1+/± and TGFβ1-/- females, subjected to in vitro culture for 4 days following in vitro or in vivo fertilisation. Standard superovulation protocol was used (5 IU PMSG followed by 5 IU hCG 48 hours later).

TGFβ1-/- females failed to develop to blastocyst stage and most frequently did not survive past 2-cell stage.

5.6 EFFECT OF TGFβ1 NULL MUTATION ON OVARIAN STEROIDOGENESIS

To further investigate the cause of reduced serum progesterone concentration in TGFβ1 null females, steroidogenic activity in ovaries was explored. In an initial attempt to investigate the effect of TGFβ1 null mutation on corpus luteum structure and function, vascularization of the corpora lutea on day 3.5 pc was analysed by immunohistochemistry using an endothelial cell specific antibody (Fig 5.8). Video image analysis revealed that the density of endothelial cells in corpora lutea was unaffected by genotype.

To investigate the effect of TGFβ1 null mutation on expression of ovarian steroidogenic enzymes, immature TGFβ1+/± and TGFβ1-/- females were hyperstimulated with gonadotrophins and quantitative RT-PCR was used to quantify expression of mRNAs encoding steroidogenic enzymes including steroidogenic acute regulatory protein (StAR), P450 side chain cleavage (P450scc), 3β-hydroxysteroid dehydrogenase-1 (HSD3β1) and P450 17 α-hydroxylase/C-17-29-lyase (P450c17) (see Figure 3.1 for roles of these enzymes in steroidogenesis). The quantity of PCR product amplified by specific primers was normalised to 18s rRNA using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). This value was multiplied by a constant to give an average for the control tissue sample (TGFβ1+/±) the arbitrary value of 1. There was a 50% decrease in HSD3β1 mRNA expression and a 4-fold increase in P450c17 mRNA in TGFβ1-/- mice compared to TGFβ1+/± mice (Fig 5.9). StAR and P450scc were similarly expressed in TGFβ1-/- and TGFβ1+/± ovaries.

Nitric oxide synthase (NOS), particularly the iNOS and eNOS isoforms, are important mediators of steroidogenic activity and ovarian function (Wehrenberg et al., 1998; Nakamura et al., 1999; Jablonka-Shariff and Olson, 2000; Hefler and Gregg, 2002), and production of NOS enzymes is impaired in the penis of TGFβ1 null males on a scid background (Chapter 4), and systemically in immunocompetent TGFβ1 null mice (Vodovotz et al., 1996). Quantitative PCR to detect iNOS and

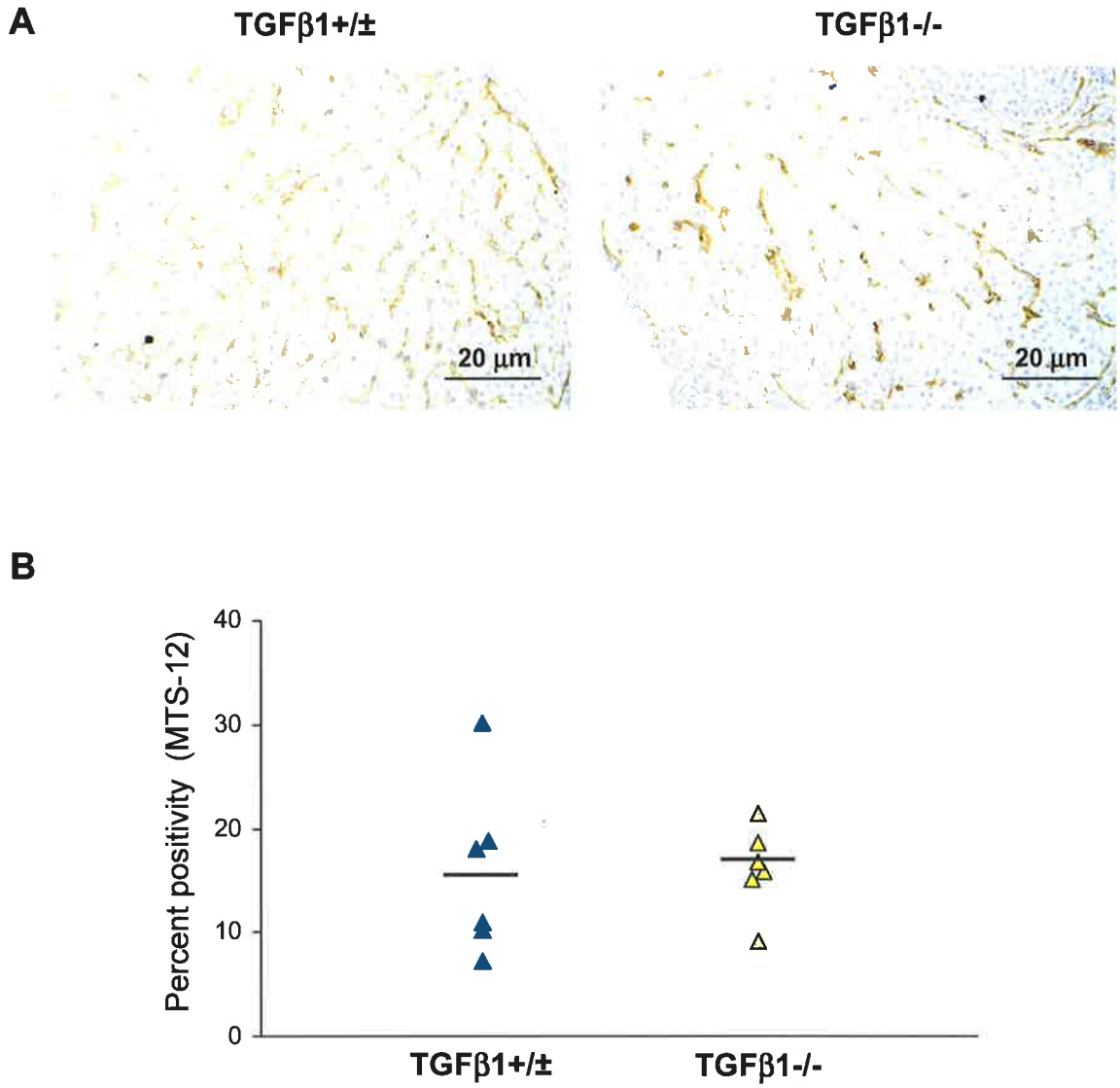


FIGURE 5.8 Endothelial cells in corpora lutea from day 3.5 post coitum TGFβ1+/± and TGFβ1-/- females. (A) Sections (7 µm) of fresh frozen ovaries were fixed in ethanol and labelled with the endothelial cell specific monoclonal antibody MTS-12, using an indirect immunoperoxidase technique. (B) Percent of MTS-12 positive cells in the corpora lutea were quantified by video image analysis of all corpora lutea visible in two individual sections of ovary from each mouse (n=6 per group). Symbols represent mean values of between 2 and 7 corpora lutea from each ovary, bar represents the mean, data were analysed by independent samples t-test.

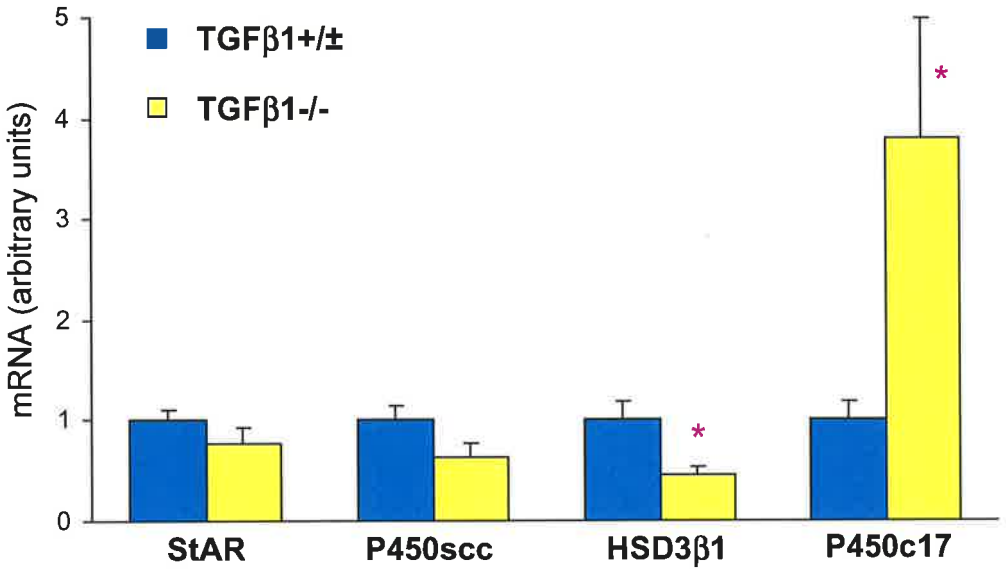


FIGURE 5.9 Expression of mRNAs encoding ovarian steroidogenic enzymes in immature superovulated TGFβ1+/± and TGFβ1-/- female mice. Ovaries were dissected 14 hours post-hCG and frozen in liquid nitrogen. Messenger RNA was extracted and reverse transcribed, then quantitative PCR was performed. Amount of mRNA was normalised to 18s rRNA, and expressed in arbitrary units where the average of the control (TGFβ1+/±) is 1. Data are mean+SEM, and were analysed by independent samples t-test, *denotes significant difference from control ($p<0.05$).

eNOS mRNA expression was performed on ovary cDNA from immature superovulated females 14 hours post-hCG, and normalised to 18s rRNA. No differences between expression of these enzymes in TGFβ1+/± and TGFβ1-/- females were identified (Fig 5.10).

5.7 EFFECT OF TGFβ1 NULL MUTATION ON MAMMARY GLAND DEVELOPMENT

To investigate mammary gland morphogenesis in TGFβ1 null mutant mice, inguinal mammary glands from 3 month old virgin TGFβ1+/+ and TGFβ1-/- females (n=6 per group) were dissected and stained as whole mounts with carmine alum. Mammary duct branching was found to be considerably impaired in virgin TGFβ1 null females, with fewer secondary branch points evident in glands of TGFβ1-/- females compared with TGFβ1+/+ females (Fig 5.11). In mammary glands from two of six TGFβ1-/- females the ducts had not extended to the edge of the fat pad.

5.8 DISCUSSION

These data clearly indicate a critical role for TGFβ1 in the reproductive function of female mice. We have demonstrated three distinct lesions in female fertility caused by TGFβ1 null mutation. Firstly, the number of TGFβ1 null females able to mate with normal males is reduced. Secondly, of the females that do mate with males, production of live born litters occurs rarely. Thirdly, two null females that did give birth did not nurture their young and survival of pups in a third litter was achieved by surrogating them to a normal lactating female.

The reduced frequency of mating when TGFβ1 null females were housed with normal males is likely to reflect perturbations in the ovarian cycle. The length of the cycle is doubled by TGFβ1 deficiency and the increased time between co-caging and detection of a plug is consistent with this. However, half the TGFβ1 null females housed with males for a four week period did not mate at all. This may be due to defective cycles in these animals whereby ovulation does not occur at all or because the signals that indicate female receptivity are impaired. The mechanistic basis of

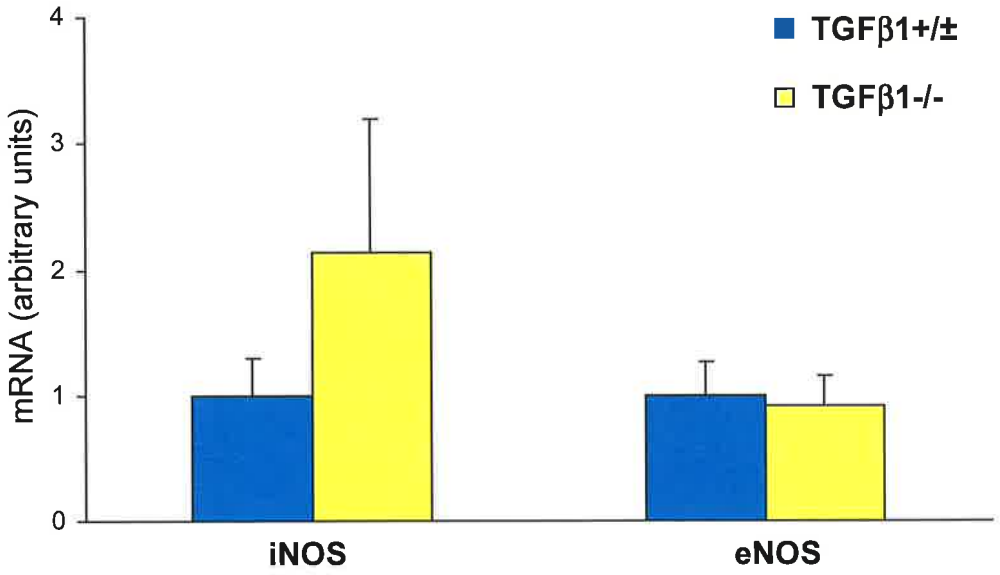


FIGURE 5.10 Expression of mRNAs encoding ovarian nitric oxide synthase enzymes in immature superovulated TGFβ1+/± and TGFβ1-/- female mice. Ovaries were dissected 14 hours post-hCG and frozen in liquid nitrogen. Messenger RNA was extracted and reverse transcribed, then quantitative PCR was performed. Amount of mRNA was normalised to 18s rRNA, and expressed in arbitrary units where the average of the control (TGFβ1+/±) is 1. Data are mean+SEM, and were analysed by independent samples t-test.

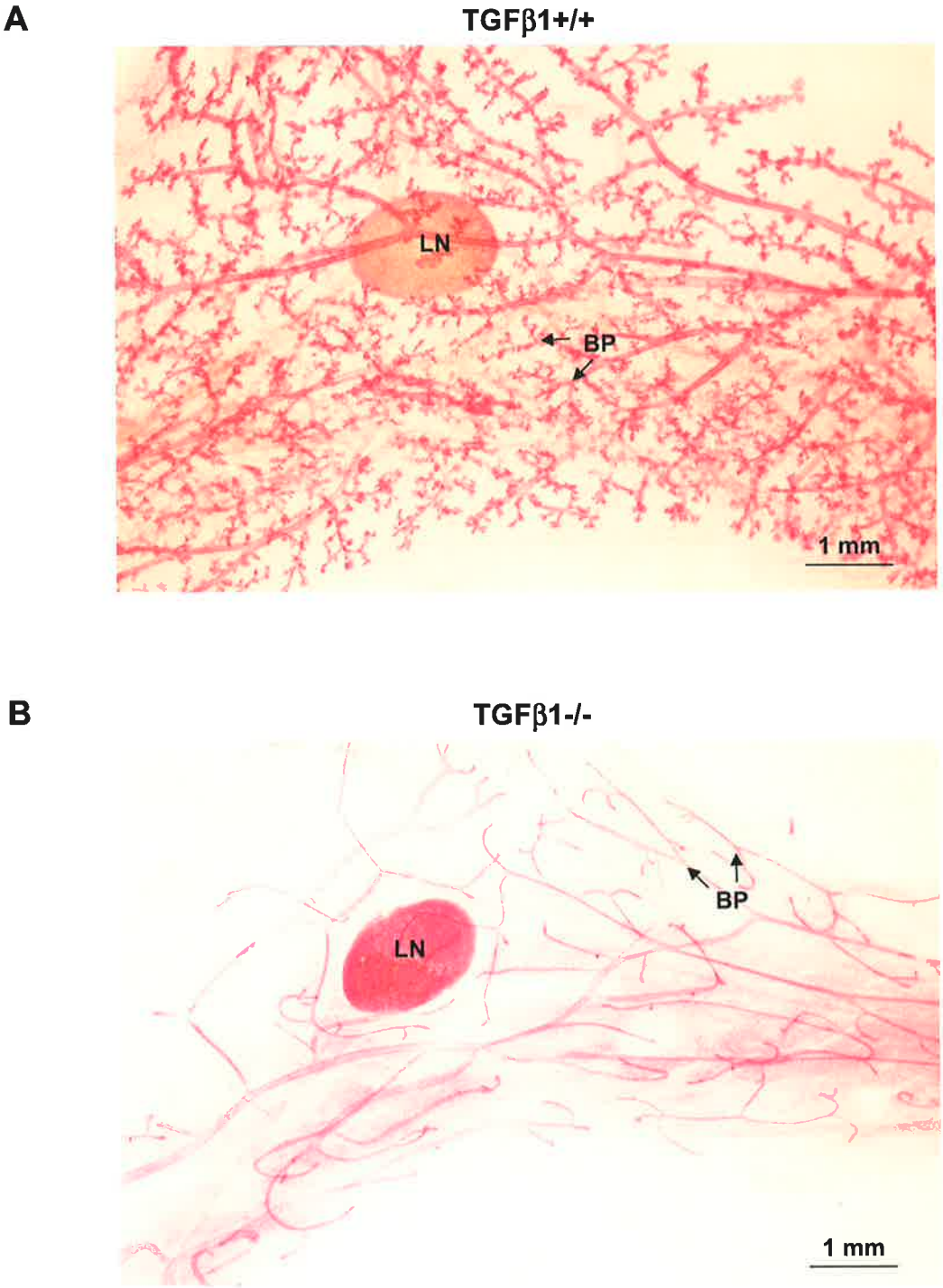


FIGURE 5.11 Adult virgin mammary gland tissue from TGFβ1+/+ (A) and TGFβ1-/- (B) female mice. The mammary gland was dissected from 12 week old female mice from around the inguinal lymph node (LN) region, spread on a glass slide and fixed in Carnoy's solution. The gland was stained with carmine alum. Branch points (BP) are labelled.

defective ovarian function caused by TGFβ1 deficiency is unclear. TGFβ1 is implicated in a diverse array of roles in reproductive function and unravelling the relative importance of each will be a challenging task. It is reasonable to hypothesise that TGFβ1 deficiency causes (1) perturbations in the hypothalamo-pituitary-ovarian axis which would have downstream effects on ovarian steroidogenesis, oocyte maturation and ovulation, (2) impaired cell-to-cell communication within the ovary necessary for appropriate differentiation and/or proliferation of somatic cells required for folliculogenesis and luteogenesis, or (3) an altered number or phenotype of ovarian leukocyte populations, also required for folliculogenesis and luteogenesis.

5.8.1 Ovarian function and hormone synthesis in TGFβ1 null female mice

Analysis of steroidogenic enzymes in the hyperstimulated ovary suggests that somatic cell function is impaired in the absence of TGFβ1. Following PMSG and hCG administration, HSD3β1 and P450c17 mRNA expression in TGFβ1 null ovaries were decreased and increased respectively, when compared to TGFβ1 replete littermates. HSD3β1 catalyses the conversion of pregnenolone to progesterone, and P450c17 catalyses the conversion of progesterone to androstenedione. The decreased and increased relative expression of these enzymes in hyperstimulated immature TGFβ1 null mutants is consistent with the observation of reduced progesterone production in naturally mated TGFβ1 null females on day 3.5 pc. Therefore TGFβ1 deficiency in the ovary appears to cause impaired communication in the paracrine or autocrine signals that regulate progesterone synthesis.

Whether the reproductive phenotypes of TGFb1 null females is a direct effect of lack of local TGFβ1 in the ovary, or the consequence of dysregulated gonadotrophin synthesis, cannot be determined on the basis of the existing data. Some of the effects of TGFβ1 deficiency we have noted may stem from dysregulated hormone synthesis in the hypothalamus or pituitary. Altered GnRH, LH or prolactin levels would be expected to have consequences for ovarian cyclicity, ovulation and corpus luteum development and maintenance, and lactation in the post-partum mammary gland. Serum testosterone is reduced in TGFβ1 null mutant males due to perturbations in hormone regulation upstream from the testis as hCG administration

caused increased testosterone production (Chapter 3). Therefore the possibility exists that a similar impairment in female TGFβ1 null mutants is responsible for much of the phenotype described in this chapter. Intriguingly, there are some parallels between the reproductive phenotype exhibited by TGFβ1 null mutants and the phenotype exhibited by the colony-stimulating factor-1 (CSF-1) null mutant (Pollard, 1997). It has recently been reported that perturbed signalling within the hypothalamus of these mice is the primary cause of ovarian dysfunction (Cohen et al., 2002). Further experiments employing ovarian transplant technology will elucidate the relative importance of local and systemic TGFβ1 deficiency.

Macrophages are highly responsive to TGFβ1 (Bogdan and Nathan, 1993) and have specific roles in ovarian function (Fukumatsu et al., 1992; Matsuyama and Takahashi, 1995; Van der Hoek et al., 2000). Ovarian macrophages are involved in tissue remodelling required during the ovarian cycle and they produce an array of cytokines that may have roles in promoting follicle and oocyte development. Depletion of macrophages in the ovary causes reduced ovulation (Van der Hoek et al., 2000), suggesting macrophages promote follicle rupture. Interestingly, administration of TGFβ1 to the bursa of the mouse ovary causes a similar inhibition of ovulation (Juneja et al., 1996). This finding contrasts with our finding of fewer ovulations in the absence of TGFβ1 however these results can be reconciled when the functions of ovarian macrophages are considered. TGFβ1 has bimodal effects on macrophages dependent on concentration and context (Bogdan and Nathan, 1993), whereby TGFβ1 both activates and suppresses macrophage number and activation in peripheral tissues. Administration of TGFβ1 to the bursa might result in unphysiological concentrations of TGFβ1 in the ovary and may alter macrophage number and/or function. Similarly the absence of TGFβ1 would also have effects on macrophages present in the ovary and at the location of follicle rupture. Therefore we postulate that the cause of reduced ovulation in the case of too little or too much TGFβ1 is mediated by macrophages.

Ovarian macrophages also have important roles in development of the corpus luteum, through remodelling required for vasculogenesis and through production of growth factors (Matsuyama and Takahashi, 1995). Interestingly, yolk sac

vasculogenesis is impaired in some TGFβ1 null fetuses, leading to embryo lethality (Dickson et al., 1995) and it was hypothesised that disrupted vasculogenesis of the corpus luteum may be responsible for impaired progesterone synthesis during early pregnancy. By immunohistochemical analysis of ovary sections from day 3.5 pregnant females we did not find reduced numbers of endothelial cells in the corpora lutea of TGFβ1 null mutant females. However the number of endothelial cells in TGFβ1 replete ovaries varied from 10 to 30 percent of the total cellular area of the corpora lutea and may not be a reliable measure of functional vasculogenesis. The organisation of endothelial cells within the corpus luteum also varied between mice. Therefore it remains a possibility that vasculogenesis is impaired in TGFβ1 null ovaries although endothelial cells have been found in normal quantities. Furthermore, it is possible that endothelial function may be perturbed, although ovarian eNOS mRNA expression was not altered by TGFβ1 null mutation.

5.8.2 Impaired preimplantation embryo development and TGFβ1 null mutation

Pregnancy failure was found in 80% of adult TGFβ1 null females mated with normal stud males, due to impaired preimplantation embryo development. In addition, fertilised oocytes collected from immature superovulated TGFβ1 null females do not develop in vitro past the 2-cell stage. This developmental failure might have been expected to be caused by reduced oocyte competence, or poor embryo development due to reduced embryonic or maternal tract sources of TGFβ1.

5.8.2.1 Maternal reproductive tract TGFβ1 deficiency

TGFβ1 has been suggested to be involved in the regulation of early embryo development (Chapter 1) and we considered the possibility that a deficiency in the oviduct and uterus of TGFβ1 null mice may be the cause of delayed preimplantation embryo development. However the results reported herein suggest this hypothesis to be incorrect. Early embryo developmental failure occurs irrespective of whether embryos develop in tracts of null mutant females, with strategies including IVF and in vitro culture of early embryos failing to rescue embryonic arrest. This finding does not show that maternal sources of TGFβ1 in the reproductive tract are not

necessary, but rather that TGFβ1 deficiency in the maternal tract does not account for the observed impairment to embryo development.

5.8.2.2 Embryonic TGFβ1 deficiency

TGFβ1 mRNA transcription is activated in the developing embryo as early as the 2-cell stage and its level increases during the early stages of cell division (Paria et al., 1992). Embryo lethality has also been reported in TGFβ1 null mutant mice, where a reduction in TGFβ1-/- and to a lesser extent TGFβ1+/- offspring are observed, according to the Mendelian predicted ratio of wildtype, heterozygous and homozygous mutants produced by heterozygous breeder pairs (Kallapur et al., 1999). This suggests that normal levels of embryonic TGFβ1 improve survival of early embryos. Therefore it could be postulated that preimplantation embryos produced by TGFβ1-/- females mated with wildtype TGFβ1+/+ males will have a reduced chance of survival due to embryonic deficiency in TGFβ1, particularly if the *tgfβ1* gene is maternally imprinted.

However, it is unlikely that embryonic TGFβ1 deficiency is responsible for developmental failure in embryos from TGFβ1-/- females mated with TGFβ1+/+ males described in this chapter (summarised in Figure 5.12). Previously it has been shown through IVF that sperm from TGFβ1-/- males fertilise TGFβ1+/+ oocytes, and that the cleaved embryos (all TGFβ1+/-) develop to blastocysts at a similar rate to those oocytes fertilised by TGFβ1+/+ sperm (Chapter 3). Therefore when the null mutation is paternally inherited, TGFβ1+/- embryos develop to blastocyst stage normally. This is in stark contrast to results reported here, where we find that when the defective gene is maternally inherited, TGFβ1+/- embryos are developmentally impaired. A possible interpretation of the data is that maternally imprinted expression of the *tgfβ1* gene in the developing embryo is required. However, whether the null gene was inherited maternally versus paternally was found not to influence the expected frequency of the null gene in offspring (Kallapur et al., 1999). Taken together, these findings indicate that TGFβ1 null mutation in the oocyte and sperm contribute equally to reduced number of heterozygous preimplantation embryos produced by heterozygous breeder pairs described by Kallapur et al. (1999).

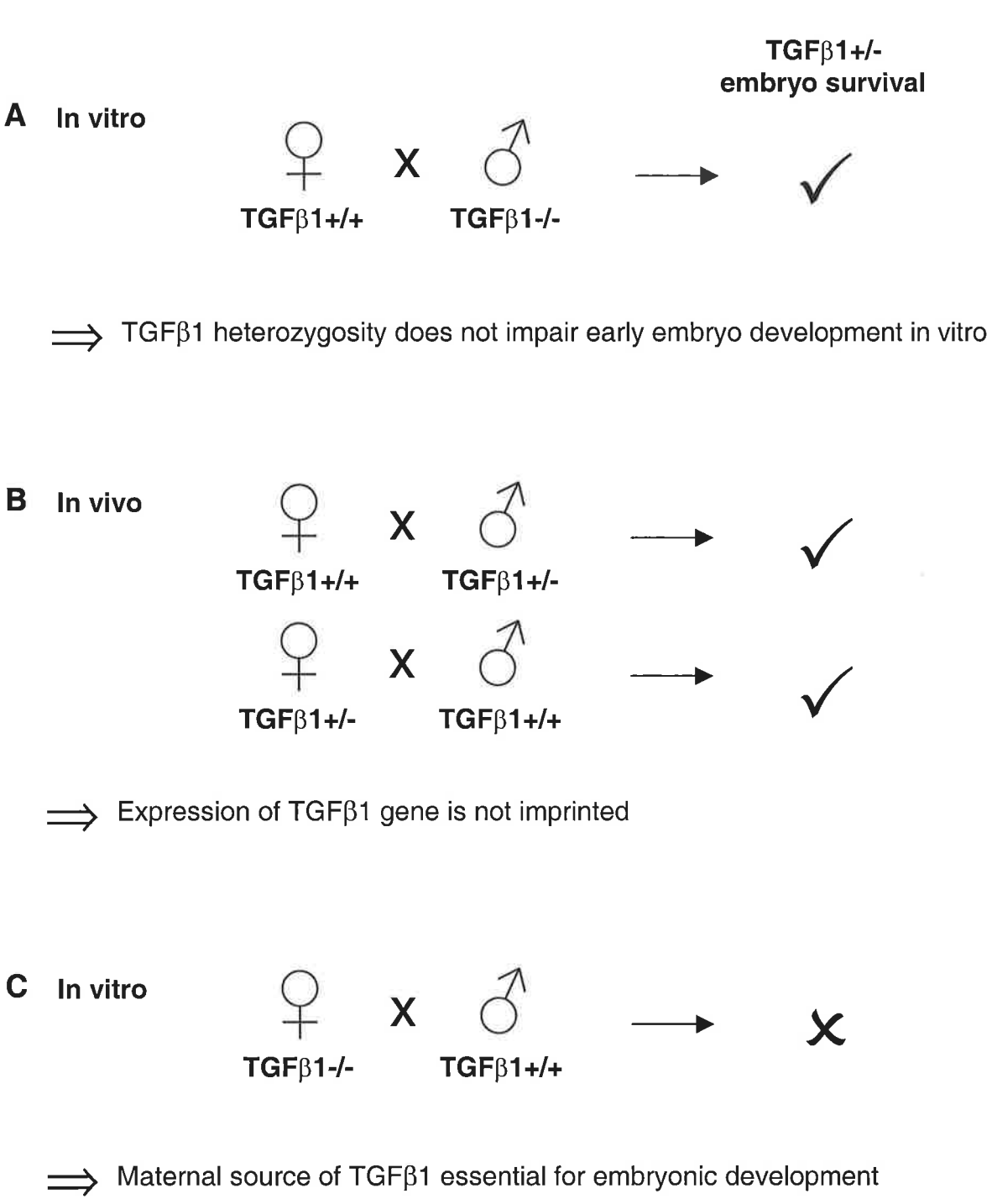


FIGURE 5.12 Embryonic deficiency in TGFβ1 versus maternal deficiency: effects on embryo development. Evidence suggests that maternal sources of TGFβ1 are critical to TGFβ1 heterozygous embryo development. (A) In vitro fertilisation study (Chapter 3), (B) in vivo fertilisation and development (Kallapur et al., 1999), (C) in vitro fertilisation study (this chapter).

Therefore neither the TGFβ1- oocyte is itself compromised when developed in a TGFβ1+/- ovary, nor is the maternally derived copy of the *tgfβ1* gene more important for early embryo development than the paternally derived copy.

We conclude that the failure in early embryo development from oocytes from naturally ovulated or superovulated TGFβ1 null females results from a lack of maternal TGFβ1 available to the oocyte, resulting in reduced developmental competence. TGFβ1 mRNA and protein have been detected in ovarian somatic cells and in the follicular fluid (Ghiglieri et al., 1995). Furthermore, TGFβ1 has been shown to influence granulosa and cumulus cell function (Adashi et al., 1989; Salustri et al., 1990; Roy, 1993) and its level in follicular fluid has been correlated with pregnancy success following IVF in humans (Fried and Wramsby, 1998). Therefore we hypothesise that maternal TGFβ1 is essential for optimal oocyte development via autocrine or paracrine effects.

5.8.2.3 Oocyte development in TGFβ1 null females

Oocyte development is a complex series of stages concerning both nuclear and cytoplasmic maturation. It is cytoplasmic maturation that is often suggested to be impaired in otherwise healthy oocytes, causing embryo developmental failure after fertilisation. Cytoplasmic maturation remains somewhat of a "black box" and quantification is usually left to outcome of number of blastocysts and other developmental stages in the embryo. We suggest that without maternal sources of TGFβ1, cytoplasmic maturation and thus developmental competence of the fertilised ovum is impaired.

Interestingly, embryos produced from oocytes from superovulated TGFβ1 null mutants and cultured in vitro did not progress at all past the cleavage stage of development. The difference in development between naturally mated adult mice and superovulated immature mice may be due to the amount of time spent in the female reproductive tract. A preliminary study was designed to test this hypothesis. An immature superovulated TGFβ1 null female was mated with a stud B10 male and the embryos were allowed to develop in the maternal reproductive tract until day 3.5 post coitum. Of eight embryos flushed from the reproductive tract, two were morula

and two were eight cell. Eight embryos were also flushed from an immature superovulated TGFβ1+/+ female which had been mated the same night, of these, three were blastocysts. This finding, while preliminary, lends support to the hypothesis that the TGFβ1 null reproductive tract is a better environment for embryo development than in vitro culture.

Further experiments are required to confirm the hypothesis that impaired preimplantation embryo development in TGFβ1 null females is due to dysfunctional oocyte development in the ovary. Ovarian transplants will provide important evidence as to the relative effects of systemic versus local TGFβ1 deficiency, as well as the roles of the hypothalamus and pituitary in ovarian dysfunction. Embryo transfers between TGFβ1 replete and TGFβ1 null females will provide compelling information on the effects of maternal tract versus ovarian TGFβ1 deficiency.

5.8.3 Post-partum survival of pups born to TGFβ1 null females

While only three TGFβ1 null females gave birth to pups over the course of this study, we found that the pups appeared normal at birth and if surrogated to a normal lactating female, they survived to adulthood and remained healthy. This suggests that pups born to TGFβ1 null mothers do not have a high rate of congenital defects and that pup survival is compromised by an inability of the mother to nurture her young. The pups failed to thrive and we believe this was due to defective lactation. Indeed analysis of adult virgin mammary gland revealed greatly reduced ductal branching which would be expected to lead to reduced milk production capacity post-partum. However more research is necessary to investigate this, as in some cases a hypoplastic ductal tree has been shown capable of alveoli formation during pregnancy and normal lactation (Pierce et al., 1993).

The finding of reduced ductal branching in the TGFβ1 null female is in direct contrast with a study of TGFβ1 heterozygous females which have less than 10% the normal mammary concentration of TGFβ1 (Ewan et al., 2002). The finding of increased ductal development during puberty and pregnancy in these latter experiments support transgenic studies that indicate TGFβ1 is an inhibitor of growth

in the mammary gland (Jhappan et al., 1993; Pierce et al., 1993; Gorska et al., 1998; Joseph et al., 1999). Taken together, these studies suggest that reduced ductal branching in TGFβ1 null mutants may not be due directly to TGFβ1 deficiency in the mammary gland. Given the severe perturbations in ovarian function in these mice, it is possible that the observed mammary gland phenotype is due to dysregulated production of ovarian steroid hormones. Mammary gland transplants between TGFβ1 replete and TGFβ1 null mutant females at various stages of development would assist in evaluating this hypothesis.

5.9 SUMMARY

Several fertility defects in TGFβ1 null mutant females have been observed and investigated. Primarily, the cause of reproductive failure is believed to stem from ovarian dysfunction in TGFβ1 null mutant mice. The ovary is smaller in weight, ovulates fewer oocytes less frequently and produces less progesterone in early pregnancy. Perturbed ovarian cycles and steroid hormone production may also lead to lactation defects in the post-partum animal. However perhaps the most important finding is that oocytes ovulated from the ovaries of TGFβ1 null mutant mice are developmentally incompetent, subsequently leading to impaired development of preimplantation embryos.

Chapter 6 General Discussion

6.1 INTRODUCTION

These studies have identified TGFβ1 as a vital component of the reproductive system, with genetic deficiency causing severe fertility defects in male and female mice. In this chapter, the reproductive phenotypes of male and female TGFβ1 null mutant mice are discussed, and parallels between the two are drawn. Future research to improve our understanding of the function of TGFβ1 in reproductive tissues and the prospects for novel therapies is highlighted.

6.2 PERTURBATION OF REPRODUCTIVE FUNCTION IN MALE TGFβ1 NULL MUTANT MICE

TGFβ1 is indeed critical for reproductive function in male mice. Two lesions have been uncovered in the TGFβ1 null mutant male (1) reduced androgen production leading to disruptions in spermatogenesis in approximately 30% of mice, and (2) sexual dysfunction (Fig 6.1).

6.2.1 Impaired steroidogenesis in male TGFβ1 null mutant mice

Studies investigating steroidogenesis in TGFβ1 null mutant males revealed that the testis is responsive to gonadotrophic stimulation, suggesting that the cause of reduced serum androgens is upstream of testis function, either in the hypothalamus or the anterior pituitary. To confirm this, serum gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels need to be measured in these animals. Due to the random cyclical nature of secretion, it is estimated that 50 serum samples from each genotype would be required, making this measurement difficult in TGFβ1 null males produced in such low numbers by our heterozygous breeding colony.

Neither the hypothalamus nor the anterior pituitary has been studied in TGFβ1 null mutant mice. The phenotype does however have some similarities to another mouse model of infertility – the colony-stimulating factor-1 (CSF-1) null mutant.

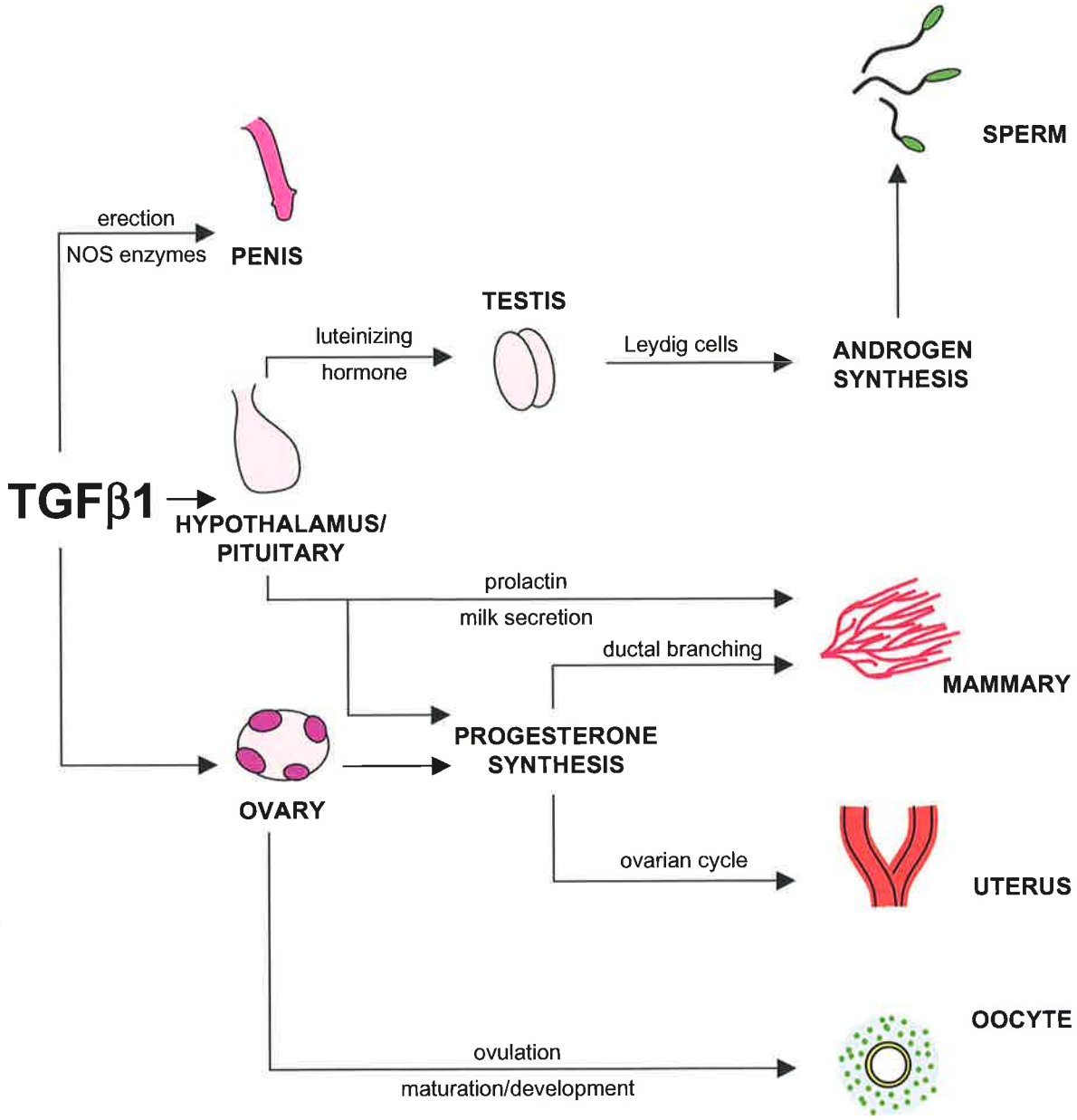


FIGURE 6.1 Diagrammatic representation of postulated roles of TGFβ1 in reproductive tissues, from analysis of the TGFβ1 null mutant mouse. TGFβ1 has critical roles in both male and female reproductive function, exerting effects indirectly through control of pituitary hormone secretion, causing reduced steoridogenesis, spermatogenesis and possibly mammary gland development, and directly on the ovary, causing impaired progesterone synthesis and oocyte maturation. TGFβ1 also directly or indirectly affects penile erection.

Males of this genotype have greatly reduced circulating LH levels, resulting in low serum testosterone concentration, however the pituitary of CSF-1 null mutants is capable of normal production of LH in response to a GnRH agonist (Cohen et al., 1997). Interestingly, negative feedback to the hypothalamus/pituitary is perturbed, testosterone treated animals exhibit increased serum LH, and castrated males have low LH (Cohen et al., 1997). From analysis of CSF-1 null mutant females, the lesion in the male is suggested to be caused by impaired development of the hypothalamus during the first 2 weeks of life (Cohen et al., 2002). Similar impaired hypothalamus function may be the cause of reduced testosterone production in TGFβ1 null male mice.

6.2.2 Impaired mating ability in male TGFβ1 null mutant mice

The second lesion in TGFβ1 null mutant male reproductive function is sexual dysfunction. Behavioural studies have clearly shown that the males are sexually interested in females and display sexual behaviour including mounting and attempted intromission. However penile NOS enzymes are undetectable in 80% of TGFβ1 null males, and therefore relaxation of the corpus cavernosa smooth muscle and the erectile response is unlikely to occur. It is unclear whether TGFβ1 deficiency causes inhibition of NOS induction via a central or peripheral mechanism.

Male copulation is the culmination of a complex series of events, involving both the central and peripheral nervous system, and concerted relaxation and contraction of smooth muscle. Organisation of these neural structures begins before birth with androgenization of the sexually dimorphic spinal nucleus of the bulbocavernosus (SNB) (Breedlove and Arnold, 1983). The perineal muscles innervated by the SNB motor neurons control some penile reflexes (Sachs, 1982). Male mice that are not androgenized by day 2 after birth display mounting and intromission behaviour but cannot copulate (Livne et al., 1992).

Perinatal and adult testosterone replacement were ineffective in restoring mating ability in TGFβ1 null males however the hypothesis that the SNB – penile reflex pathway is impaired in TGFβ1 null males remains valid. TGFβ1 has many

direct and indirect roles in central nervous system development and function, including regulation of glial cell function and neuronal cell differentiation and survival (Bottner et al., 2000). TGFβ1 replacement during critical periods of postnatal development and/or adulthood may restore NOS enzyme induction via improved central nervous system function. While the suckling TGFβ1 null mutant pup receives TGFβ1 from maternal milk, ingestion of which can be transferred to several organs (Letterio et al., 1994), maternal transfer of TGFβ1 has been shown incapable of reversing the embryo lethality observed in some TGFβ1 null offspring (Kallapur et al., 1999; Chagraoui et al., 2001) and therefore cannot be assumed to be a complete substitute for endogenous production.

The alternative hypothesis, that impaired NOS expression may be the result of a direct requirement for TGFβ1 in the penis is also a possibility. All three isoforms of NOS are inhibited in TGFβ1 null males, suggesting a common mechanism in the role of TGFβ1 in NOS induction, more likely to act at the cellular level. However, the literature suggests that the primary role of TGFβ1 is in inhibition of NOS enzymes. Indeed, TGFβ1 deficiency did not cause dysregulated NOS expression in immature superovulated ovaries (Chapter 5). Investigation of the effect of TGFβ1 deficiency in specific organs such as the penis using tissue specific knockouts will provide useful information on the central versus peripheral effects of TGFβ1 deficiency.

6.2.3 Other aspects of the health male TGFβ1 null mutant mice

While TGFβ1 null mutant male mice on a scid background appear healthy in early adulthood, they eventually develop a syndrome characterised by weight loss, a wet perinium presumably caused by incontinence, and hind limb paralysis. The onset of the syndrome usually occurs at around 3 months of age however it does occur as early as 8 weeks in some animals and at 4 months of age in others. Animals that developed the syndrome early were excluded from the experiments reported here as they had been euthanised before the age of 10 weeks. Female TGFβ1 null mutant mice did not appear to suffer the same symptoms as male mice

however, like the males, they also tended to lose weight around 3 months of age and perhaps suffered a less severe form of the syndrome.

The syndrome was found to be associated with a bladder obstruction in one male mouse studied however it is not known whether this is a cause or an effect of the syndrome. Among the possibilities for the cause of the syndrome are kidney dysfunction leading to a buildup of crystalised salts, epithelial hyperplasia at the bladder outlet or blockage by a neurological impairment.

6.3 PERTURBATION OF REPRODUCTIVE FUNCTION IN FEMALE TGFβ1 NULL MUTANT MICE

The principle cause of infertility in TGFβ1 null mutant female mice is ovarian dysfunction (Fig 6.1). There is strong evidence that TGFβ1 deficiency perturbs ovarian function to interfere with important aspects of female fertility, including preimplantation embryo development and steroidogenesis (discussed in Chapter 5).

What is unclear however is to what extent local TGFβ1 deficiency in the ovary itself is responsible for infertility, and whether perturbations in the hypothalamus or pituitary may also account for, or contribute to, impaired progesterone production, mammary gland development and ovarian cycle perturbation. Ovarian transplants between TGFβ1 deficient and competent mice, together with further research into the function of the hypothalamus and pituitary in TGFβ1 null mutant animals will shed light on these questions.

6.4 IMPAIRED NEUROLOGICAL FUNCTION IN TGFβ1 NULL MUTANT MICE

The possibility exists that the phenotypes exhibited by male and female TGFβ1 null mutant mice originate from a common lesion. Parallels between male and female TGFβ1 null reproductive phenotypes are consistent with a lesion in the hypothalamo-pituitary-ovarian axis, presumably in secretion of LH.

CSF-1 and TGFβ1 are key regulators of both macrophages in reproductive function, and the macrophage related microglia in brain function (Bottner et al., 2000; Cohen et al., 2002). In view of the known effects of CSF-1 on central nervous system function, a defect in hypothalamic function in TGFβ1 null mice is a reasonable postulate. In addition, impaired development of central regulators of penile erection, discussed above, may be the cause of impotence in male mice. The role of microglia has primarily been described as phagocytic however there is also evidence that they are crucial to appropriate development of particular neural pathways (Rezaie and Male, 1999). Further analysis of the central nervous system, in particular the presence and phenotype of microglia, in TGFβ1 null mutants would be informative in unravelling the effect of TGFβ1 deficiency on male and female reproductive function.

6.5 INTERACTION BETWEEN TGFβ1 AND OTHER GENES

6.5.1 Embryo lethality

In many aspects of their physiology, mice deficient in TGFβ1 display great variations in phenotype. Defective yolk sac vasculogenesis and haematopoiesis at day 10.5 post coitum (pc) are the cause of death in half of the TGFβ1 deficient embryos (TGFβ1-/-) and 25% of TGFβ1 heterozygous embryos (TGFβ1+/-) produced by heterozygous breeder pairs on a mixed background of NIH/Ola x C57Bl/6J x Sv129 (Dickson et al., 1995), giving rise to a severe deviation in Mendelian frequency of the TGFβ1 mutated gene. This variation in phenotype has been linked to a major modifier gene of unknown role on chromosome 5 (Bonyadi et al., 1997). However embryo lethality in a predominantly CF-1 strain occurs earlier than day 10.5 pc, at the preimplantation stage on day 2.5 pc (Kallapur et al., 1999), and this is likely to account for the deviation in Mendalian frequency reported by the same group in 1994 (Shull and Doetschman, 1994).

Between 1998 and 2002, 1400 progeny were produced by TGFβ1 heterozygous breeding pairs at the University of Adelaide Central Animal House. Of

these, 18% were homozygous for the TGFβ1 mutation, 50% were heterozygous and 32% were wildtype. This represents a deviation of the predicted Mendelian ratio of 1:2:1 (+/+:+/-:-/-) to 1:1.57:0.56. This result is similar to other reports of 1:1.6:0.5 (Dickson et al., 1995) and 1:1.5:0.4 (Shull and Doetschman, 1994).

The deviation in the expected Mendelian frequency observed in the TGFβ1 colony at the University of Adelaide is likely to be caused by impaired preimplantation embryo development as the colony was derived from the same mice generated at the University of Cincinnati and reported by Shull and Doetschman (1994) and Kallapur et al. (1999).

6.5.2 Strain variation and reproductive function

We have found that spermatogenesis pathologies and expression of penile NOS enzymes in males, and pregnancy success in females vary greatly between animals. This is most likely to be the result of genetic variation. It is expected that many modifier genes will be found to be involved in determining the physiological effect of TGFβ1 deficiency. Variance amongst these genes presumably exists within our TGFβ1 population as the colony is on a mixed genetic background (C3H/Sv129/CF-1). In addition, it is possible that the severe infertility reported in this thesis reflects gene interactions specific to this particular colony rather than the absolute deficiency in TGFβ1 itself.

In order to begin to investigate these possibilities, the TGFβ1 mutation was backcrossed onto a pure genetic background. The C57Bl/6 strain was chosen as a suitable background strain to study as this strain expresses the H-2^b antigen which was foreseen as useful for mating studies involving T cell transgenic B10.br females carrying T cell receptors reactive with class I MHC antigens of the H-2^b haplotype.

6.5.2.1 TGFβ1 and the C57Bl/6 background strain

It has previously been reported that the F2 generation of TGFβ1 heterozygous mice backcrossed onto the C57Bl/6 background are unable to produce TGFβ1 deficient offspring, indicating an interaction between TGFβ1 deficiency and an as yet uncharacterised C57Bl/6 gene(s) (Kallapur et al., 1999). In preliminary experiments TGFβ1 heterozygous scid homozygous mice were backcrossed with C57Bl/6 immune competent mice, selecting for TGFβ1 heterozygosity and scid homozygosity in the F2 progeny. It was found that F2 generation mice were able to produce TGFβ1 null homozygous offspring suggesting either (1) the gene(s) responsible for 100% embryo lethality was not transferred into the genome of mice bred at this facility, or (2) the adaptive compartment of the immune system in either the mother or the fetus is involved in the embryo lethality reported by Kallapur et al. (1999), and mice on a scid background are therefore not susceptible.

Interestingly, one TGFβ1 deficient male that was produced by F2 breeders was able to mate and impregnate two B10.br females, suggesting that interaction with other genetic influences was indeed contributing to the phenotype of TGFβ1 deficient mice (discussed later). Therefore we continued the process of backcrossing the TGFβ1 mutation onto the C57Bl/6 strain.

C57Bl/6 mice homozygous for the scid mutation were imported from the Jackson Laboratory, USA in 2000. The mice were mated with TGFβ1+/- scid mice and the progeny selected for heterozygosity in the TGFβ1 mutation. Eight backcrosses are required to transfer the mice onto the new genetic background. At the time of writing, the final backcross has been completed, producing the F8 generation. However the fertility of TGFβ1 null animals produced from this new colony has not yet been explored.

6.5.2.2 Male infertility linked to genetic interaction

Over the course of these experiments, we have had little success in mating TGFβ1 null mutant males. Males used in all experiments reported in Chapters 3 and 4 of this thesis failed to mate at all. However, one TGFβ1 null male was produced that did successfully mate. This male was the progeny of F2 mice used in backcrossing onto the C57Bl/6 genetic background. The male was caged with two B10 females and left unchecked for a period of approximately 4 weeks. This male sired offspring with both females. The male survived longer than the expected 12 weeks however subsequent matings with new females did not occur. The male eventually succumbed to wet perinium syndrome.

The reason why this TGFβ1 null male alone was able to mate is not known, however a number of points must be made. Firstly, over the course of this research there have been many TGFβ1 null mutant male mice housed with normal females and left unchecked which have not sired litters, indicating that the ability of this male to mate was not related to the cage being left alone. Secondly, the introduction of C57Bl/6 genes into the genome of this mouse may be responsible for its ability to mate, although two other TGFβ1 null males produced by this same breeder pair did not do so. If this is the case, it suggests that the mating inability of TGFβ1 null males may be the consequence of interaction with other as yet undefined genes. Interestingly, this male was identified by flow cytometry as expressing high levels of H-2K^b antigen on the surface of its white blood cells. This antigen is found on cells from C57Bl/6 mice and not on cells from the general TGFβ1 colony. The two other TGFβ1 null males from the same breeder pair that did not mate did not express high levels of H-2K^b antigen.

The progeny produced from this TGFβ1-/- male were normal at birth when compared to 3 litters sired by a TGFβ1+/- H2-K^b high littermate. At the age of 5 weeks however, the average weights of progeny sired by the TGFβ1 null male was reduced, by 1.3 g for males and 1.7 g for females (a 6% and 9% decrease in body weight respectively). This difference was also seen at 6 weeks of age. However the

significance of these findings cannot be considered unless further TGFβ1-/- matings occur.

6.6 FUTURE RESEARCH

The experiments reported here clearly demonstrate that genetic TGFβ1 deficiency causes infertility in male and female mice, a finding that has the potential to lead to new therapeutics to treat infertility in humans. However it is not yet known whether (1) TGFβ1 deficiency is a cause of infertility in humans or (2) fertility can be restored by TGFβ1 treatment.

6.6.1 TGFβ1 deficiency as a cause of infertility in humans

Infertility affects 15% of Australian couples trying to conceive (AIHW National Perinatal Statistics Unit, 2001). Of the couples that go on to seek assisted conception, the cause of infertility is unknown in approximately 20% of cases.

TGFβ1 action in specific human tissues may be perturbed for a range of reasons, and may arise from a variety of causes including dysregulated isoform expression, activation, abundance of soluble and membrane bound binding proteins, expression of TGFβ receptors and intracellular Smad signalling circuitry, as discussed in Chapter 1. The cause of perturbation may be genetic, and due to polymorphisms (Andreotti et al., 2002) or dependent on interaction with modifier genes, or caused by lifestyle factors such as diet or stress. Indeed, the concentration of TGFβ1 in seminal plasma of men varies by two-fold between individuals (Sharkey and Robertson, unpublished observations).

If deficiency in TGFβ1 is a cause of female infertility, it might be expected to be characterised by poor preimplantation embryo quality, resulting from perturbation in ovarian function. This hypothesis is supported by the finding that TGFβ1 protein levels in the ovarian follicle correlate with pregnancy success after IVF (Fried and Wramsby, 1998). However in the general population, ovarian TGFβ1 would be

difficult to quantify. A simpler approach would be to measure TGFβ1 in blood, however this may not yield useful data as it is not known whether ovarian concentration of TGFβ1 is reflected in circulating levels.

It is also reasonable to conclude from this study that links may exist between TGFβ1 levels and erectile function in men. Further research into the mechanism by which male TGFβ1 null mice are unable to mate could lead to a new understanding of impotence in men.

6.6.2 Restored fertility by exogenous TGFβ1 treatment

These considerations beg the question of whether exogenous delivery of TGFβ1 might be employed to improve reproductive function. Experiments to restore fertility in TGFβ1 null mutant mice by treatment with exogenous TGFβ1 have been cost prohibitive for these studies as more information on how many doses or most importantly when to administer the dose was required. However we are now at a stage where TGFβ1 treatment can be considered.

For restoration of male reproductive function, TGFβ1 treatment in the first 2 weeks of life compared to treatment for 2 weeks in adulthood would reveal important information on whether TGFβ1 deficiency during development or during adulthood is most important for hypothalamo-pituitary function and mating ability. To restore female reproductive function, TGFβ1 must be delivered to the ovary and possibly also the hypothalamus and pituitary, similar to the male.

It is not known whether exogenous TGFβ1 is delivered to these specific tissues and which route of delivery is most effective. Orally administered TGFβ1 quickly dissipates to the lung, heart and liver (Letterio et al., 1994) however it is not known whether it specifically accumulates in reproductive tissues or the central nervous system. Indeed, it may not be able to function in these tissues as endogenous TGFβ1 might. For example, TGFβ1 delivered to the ovary may not accumulate in the follicle in the same quantity as TGFβ1 produced in the follicle by granulosa cells.

These questions must be taken into consideration when designing experiments to replace TGFβ1 in TGFβ1 null mutant animals.

An alternative strategy to replace TGFβ1 is to target expression of a transgene expressing high levels of the protein. The transgene could be controlled by a tissue specific and/or an inducible promoter. This would enable endogenous production of TGFβ1 at the site of interest or temporal production during a particular phase of development, and be of great benefit to unravelling the relative roles of TGFβ1 in the reproductive processes found to be impaired by TGFβ1 deficiency.

Whether treatment with exogenous TGFβ1 might be usefully exploited as a therapeutic strategy in infertility is an exciting prospect. Both men and women could be potential candidates for such an approach. Perhaps the only effective way to evaluate the therapeutic value of exogenous TGFβ1 in human fertility is a clinical trial, dependent on the outcomes of replacement of TGFβ1 experiments in TGFβ1 deficient mice.

6.7 CONCLUSION

Analysis of reproductive function in the TGFβ1 null mutant mouse has revealed many critical roles of this cytokine in the fertility of male and female animals. This work has exposed the relative importance of this cytokine in reproductive processes initially suggested by descriptive and in vitro studies reported by others, including crucial roles in ovarian function and steroidogenesis. In addition, this research has highlighted other aspects of reproductive function regulated by TGFβ1 that were unexpected, including male sexual performance and the hypothalamo-pituitary-gonadal axis. Further research on the effect of TGFβ1 deficiency and replacement in mice and humans could lead to the development of novel therapeutics for some forms of infertility.

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APPENDIX

All products from Sigma, USA, unless otherwise stated

Tail digestion buffer

0.268 g TRIZMA base (pH 7.5)
0.633 g Ethylenediaminetetraacetic acid (EDTA) sodium salt (AJAX, AUS)
1 g Sodium chloride (BDH, AUS)
0.85 g Sodium dodecyl sulfate (SDS)
100 ml MQ water
Adjust pH to 7.8, autoclave and store at room temperature

Agarose gel

2 g Aragose (Promega, USA)
100 ml 1x TAE buffer

TAE buffer (50x)

100 ml 0.5M EDTA
57.1 ml Glacial acetic acid (AJAX, AUS)
242 g TRIZMA base
Make up to 1L with MQ water, autoclave and store at room temperature

0.5M EDTA

18.61 g EDTA sodium salt
80 ml MQ water
2 g Sodium hydroxide pellets
Stir and adjust pH to 8
Adjust volume to 100 ml

Loading buffer (6x)

0.05 g Bromophenol blue
0.05 g Xylene cyanol FF
6 ml Glycerol

Adjust volume to 20 ml with sterile MQ water, filter (0.22 μm) into 1 ml aliquots

DNA 5x buffer

1.25 ml 2M Tris-HCL
1 ml 500 mM $MgCl_2$
7.75 ml MQ water
Adjust pH to 7.2, autoclave and store in 1 ml aliquots

Avertin

1 g 2.2.2-Tribromomethanol (Aldrich, USA) dissolved in
1 ml 2-Methylbutan-2-ol (AJAX, AUS)
Make up to 50 ml with MQ water
Shake vigorously or heat to dissolve
Aliquot and store at -20C

PBS (10x)

4 g Potassium chloride (BDH, AUS)
4 g Potassium dihydrogen orthophosphate (BDH, AUS)
160 g Sodium chloride
23 g di-Sodium hydrogen orthophosphate, anhydrous (UNIVAR, AUS)
2 L MQ water

Dulbecco's PBS

100 ml 10x PBS
0.133 g Calcium chloride dihydrate (AJAX, AUS)
0.1 g Magnesium chloride (AJAX, AUS)
900 ml MQ water
Adjust to pH 7.4 and osmolality 280

APES Coating slides

Incubate slides in 1% hydrochloric acid for 1 minute
Wash in MQ water

Immerse slide briefly in 2% 3-aminopropyltriethoxysilane (APES) diluted in absolute ethanol

Wash slides in absolute ethanol

Wash slides under running tap water

Wash slides in MQ water

Dry slides overnight in fume hood

Carnoy's fixative

6 parts ethanol

3 parts chloroform

1 part glacial acetic acid

Carmine Alum

1 g carmine

2.5 g aluminium potassium sulphate

500 ml MQ water

Boil for 20 minutes and filter, add a crystal of thymol