The Impact of Exogenous TGFβ1 on Male Reproductive Function

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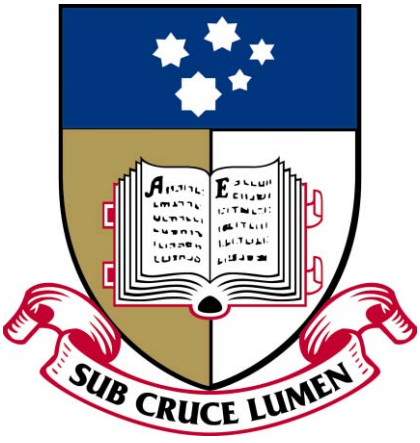

For Paul

None of the work presented in this thesis would have been possible without his generous love, encouragement and support.

For Aaron

His enduring love and patience throughout all of my studies is deeply appreciated.

For my Family and Friends

Their ability to highlight the lighter side of this project has been uplifting.

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ABBREVIATIONS

ºC ..... Degrees celsius
ANOVA ..... Analysis of variance
APES 3-aminopropyltriethoxysilane
Bp ..... Base pair
BrdU ..... Bromodeoxyuridine
BSA ..... Bovine serum albumin
cDNA ..... Complimentary deoxyribonucleic acid
CFSE ..... Carboxyl fluorescein succinimide ester
CSF-1 ..... Colony-stimulating factor-1
CT ..... Cycle threshold
DAB ..... 3,3'-diaminobenzidine
dFUR ..... Fluorodeoxyuridine
DMSO ..... Dimethyl sulphoxide
DNA ..... Deoxyribonucleic acid
dNTPs ..... Deoxynucleotide triphosphates
EDTA ..... Ethylenediaminetetraacetic acid
ELISA ..... Enzyme-linked immunosorbent assay
eNOS ..... Endothelial nitric oxide synthase
FACS ..... Fluorescence activated cell sorter analysis
FSH ..... Follicle stimulating hormone
g ..... Grams
HBSS ..... Hanks buffered salt solution
hCG ..... Human chorionic gonadotropin
HSD3β1 ..... 3β-hydroxysteroid dehydrogenase-1
IFNγ ..... Interferon gamma
Ig ..... Immunoglobulin
iNOS ..... Inducible nitric oxide synthase
IU ..... International unit
KCl ..... Potassium chloride
kDa ..... kilo Dalton
KH2PO4 ..... Potassium di-hydrogen orthphosphate
kg ..... Kilogram
LAP ..... Latency associated peptide
LH ..... Luteinizing hormone
mRNA ..... Messenger ribonucleic acid
mL ..... Millilitre

mg ..... Milligram
Na2HPO4 ..... di-Sodium hydrogen orthophosphate
NaCl ..... Sodium chloride
NaH2PO4 ..... Sodium di-hydrogen phosphate
NCBI ..... National Centre for Biotechnology Information
NCE ..... Non contact erections
NMS ..... Normal mouse serum
nNOS ..... Neuronal nitric oxide synthase
NOS ..... Nitric oxide synthase
PBS ..... Phosphate buffered saline
PCR ..... Polymerase chain reaction
PE ..... Phycoerythrin
PFA ..... Paraformaldehyde
PMN ..... Polymorphonuclear neutrophils
PMSG ..... Pregnant mare serum gonadotropin
PVP ..... Polyvinylpyrrolidone
Rcf ..... Relative centrifugal force
rhLTGFβ1 ..... Recombinant human latent transforming growth factor beta 1
RIA ..... Radioimmunoassay
rpm ..... Revolutions per minute
RPMI ..... Roswell Park Memorial Institute
RT-PCR ..... Reverse transcription - polymerase chain reaction
SCID ..... Severe combined immune deficiency
SDS ..... Sodium dodecyl sulphate
SEM ..... Standard Error of the Mean
TGFβ1-/- ..... Null mice without the TGFβ1 gene
TGFβ1+/- ..... Heterozygote mice with the TGFβ1 gene on one allele
TGFβ1+/+ ..... Wild type mice with the TGFβ1 gene on both alleles
TNFα ..... Tumor Necrosis Factor Alpha
TβRI ..... TGFβ receptor 1
µg ..... Microgram
µL ..... Microlitre

ABSTRACTS AND PUBLICATIONS ARISING FROM THIS THESIS

ABSTRACTS

L. McGrath, R. Robker, SA Robertson. *TGFβ1 is not responsible for erection dysfunction TGFβ1 null mice.* Australian Society for Medical Research. June 2005

L. McGrath, R. Robker, SA Robertson. *Influence of exogenous TGFβ1 on reproductive performance in TGFβ1 null male mice.* Society of Reproductive Biology. July 2005

PUBLICATIONS

L. McGrath, R. Robker, SA Robertson. *Effect of TGFβ1 null mutation on structure and function of the penis in mice* (manuscript in preparation).

L. McGrath, R. Robker, SA Robertson. *Influence of exogenous TGFβ1 replacement on reproductive performance in TGFβ1 null mutant mice* (manuscript in preparation).

ABSTRACT

The TGFβ family of cytokines are potent signalling molecules that regulate tissue development, inflammation and immunity. Previous studies in mice with a null mutation in the *Tgfb1* gene (TGFβ1-/- mice) implicate a key role for TGFβ1 in male reproductive function. These mice show profound infertility due to an inability to copulate successfully, associated with reduced testosterone and sperm production. The focus of this project was to 1) further characterize mechanisms underpinning reproductive deficiency in male TGFβ1-/- mice, 2) identify a reliable physiological marker of TGFβ1 availability in vivo, and 3) to determine whether exogenous TGFβ1 administration influences TGFβ1 availability and restores fertility.

To investigate the causes of unsuccessful copulation by TGFβ1-/- mice, penis morphometry was examined. Penile organ structure, as assessed by scanning electron microscopy, was comparable between genotypes however a superfluous epidermal covering that impeded penile spine protrusion was evident in TGFβ1-/- mice. The epidermal covering was not due to increased epithelial cell proliferation, as measured by Brdu labelling and immunohistology. Behavioural observations of erectile activity showed that TGFβ1-/- mice achieved spontaneous erections albeit at reduced frequency compared to TGFβ1+/+ mice.

The efficacy of exogenous TGFβ1 replacement was evaluated by first identifying measures of in vivo TGFβ1 availability and/or function and selecting an effective route of administration. Serum TGFβ1 and testosterone levels were reliable discriminators of TGFβ1 genotype. Gene expression and phagocytic function of peritoneal macrophages revealed no differences between genotypes. Exogenous sources of TGFβ1 for replacement studies included colostrum, naturally occurring in breast milk and recombinant human latent TGFβ1 (rhLTGFβ1). Colostrum did not increase circulating levels and rhTGFβ1 injection caused only transient elevation of serum levels. Thus mini-osmotic pumps were used to deliver a constant supply of cytokine to TGFβ1-/- mice.

The fertility status of TGFβ1-/- mice receiving exogenous TGFβ1 was investigated. Reproductive behaviour in response to normal receptive female mice was assessed twice during treatment, on day 7 and day 14. Blood, liver and reproductive tissues were collected at sacrifice. Circulating TGFβ1 was increased in TGFβ1 treated TGFβ1-/- mice above TGFβ1-/- control levels, although this did not affect circulating testosterone. Erectile activity and sperm production were unchanged. Videotaping behaviour with estrous females revealed that the TGFβ1+/+ mice successfully mounted and intromitted, unlike the TGFβ1-/- controls. The TGFβ1-/- mice receiving exogenous TGFβ1 displayed moderately enhanced mounting and intromission behaviour although this remained less frequent than in the TGFβ1+/+ controls. Ejaculation behaviour was not observed in any TGFβ1-/- mice regardless of TGFβ1 replacement, compared to TGFβ1+/+ controls where >90% mice displayed ejaculated.

Modest improvement in the copulation activity of the TGFβ1-/- mice receiving exogenous TGFβ1 suggests that systemic TGFβ1 availability can influence reproductive performance in male TGFβ1-/- mice. However since fertility was not restored, locally produced TGFβ1 in the reproductive tract and/or hypothalamic pituitary axis are also implicated in regulating fertility. These findings advance our knowledge of the role of the TGFβ1 cytokine in male reproductive physiology and may have relevance for devising new treatments for infertility and erectile dysfunction in men.

DECLARATION

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

I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

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Leanne Jane McGrath

April 2008

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Chapter 1:
Introduction - The Regulatory Role of TGFβ1 in Male Reproductive Function

1.1 INTRODUCTION

The family of transforming growth factor beta (TGFβ) cytokines are involved in the normal development and homeostasis of many physiological systems as demonstrated by the observation of abnormal TGFβ levels in numerous common diseases including cancer (Akhurst, 1999) (Kong, 1995) (Friedman, 1995), cardiovascular disease (Goumans, 2002) (Wang, 1997) (Scott, 1997), diabetes (Azar, 2000) and allergies (Knight, 2001) (Wohlfahrt, 2003). Additionally, TGFβ and receptor gene polymorphisms, inflammation and disturbances in the cytokine network are also implicated in situations where TGFβ1 is dysregulated (Watanabe, 2002) (Hanada, 2002) (Lawrence, 1996). More recently it has been found that the reproductive tracts of males and females are also sites of TGFβ action (Ingman, 2007) (Ingman, 2002).

Exogenous factors acquired from the environment can also have major effects on the TGFβ system. Drinking alcohol (Chen, 2002) or smoking (Ryder, 2002) (Li, 2003), obtaining an unhealthy body weight (Alessi, 2000), exercise (Heinemeier, 2003) and sun exposure (Dissanayake, 1998) (Quan, 2001) are all lifestyle factors that have been linked to dysregulated TGFβ. Additionally, TGFβ may be absorbed from the diet and consumption of this cytokine may impact on human health (Oz, 2004) (Fell, 2005). Milk is perhaps the most potent potential dietary source of TGFβ (Ginjala, 1998) (Pakkanen, 1998). It is produced by the lactating mammary gland and is thought to influence mammary gland function as well as maturation of the newborn intestinal tract (Goldman, 1996) (Zhang, 1999). As adult fertility can depend on early nutrition and health (Demmelmair, 2006), consumption of TGFβ in early development could have more far reaching reproductive consequences (Figure 1-1).

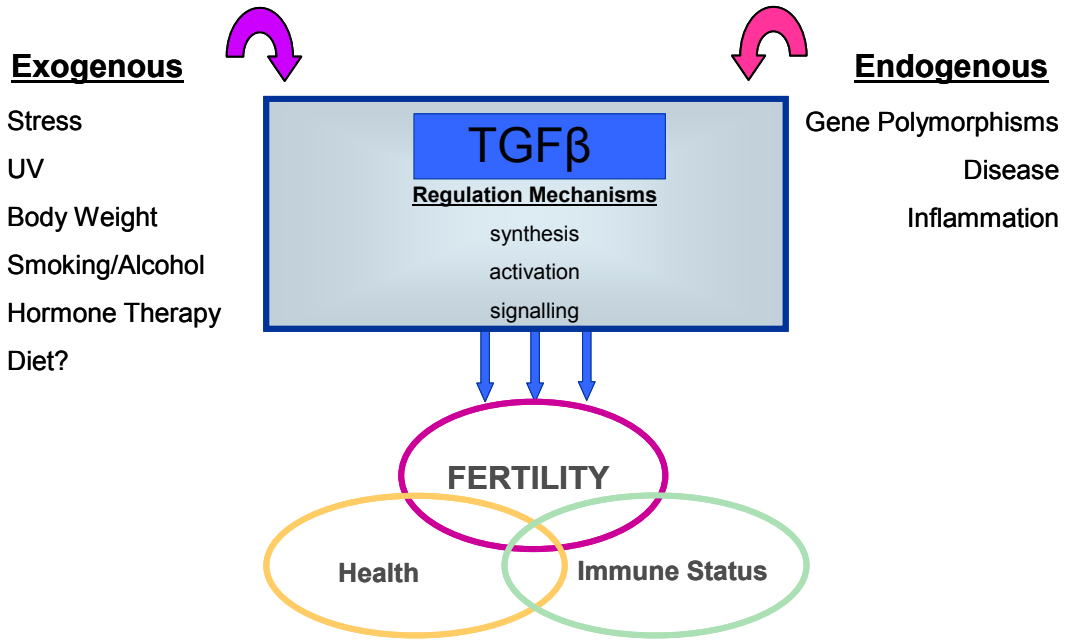


Figure 1-1: Exogenous and endogenous factors that contribute to either circulating or tissue TGFβ bioavailability.

Exogenous factors such as lifestyle and perhaps diet contribute to TGFβ levels, while genetic polymorphisms, disease and TGFβ regulation mechanisms are endogenous factors influencing TGFβ. Delineating the roles of TGFβ on reproductive function is made challenging since both direct effects and indirect effects through health and immune status are likely.

The TGFβs are multifunctional cytokines and TGFβ1 is the most studied isoform, probably due to its critical roles and ubiquity in tissues and blood (Janssens, 2005). The other mammalian isoforms, TGFβ2 and TGFβ3 are shown to have a high sequence homology with TGFβ1 and act on the same cellular receptors (Cox, 1995). However, as these isoforms have differential expression and effects on target cells, they are now known to be essential in their own right (Koch, 2000). Additionally, the conservation of their genetic sequences between species and throughout evolution confirms the biological importance of this family of cytokines (Ottaviani, 2001).

The wide variety of processes that TGFβ participates in makes delineation of its actions challenging. However, animal models have been illuminating in clarifying the independent and overlapping physiological roles of TGFβ, particularly genetically engineered mice with null mutations for the different TGFβ isoforms (Dunker, 2000). TGFβ1 null mutant (TGFβ1-/-) mice deteriorate at 3 weeks of age showing severe lethal inflammation (Shull, 1992) (Kobayashi, 1999). When the TGFβ1-/- mice are treated with anti-inflammatory interventions their lifespan is prolonged into adulthood (Diebold, 1995), providing a means to assess their reproductive capabilities. TGFβ2 and TGFβ3 null mutants die before or immediately following birth with various abnormalities of the heart, lungs or bone (Dunker, 2000). Mice that are heterozygous for TGFβ1 (TGFβ1+/-) present as phenotypically normal, although they do suffer from increased susceptibility to cancer (McKenna, 2001) and gastric lesions (Boivin, 1996). Thus reductions in TGFβ1 levels can cause health problems that are not outwardly obvious, at least initially.

This thesis explores the impact of TGFβ1 deficiency on the male reproductive tract in mice. Penile structure and function were assessed as well as testosterone levels. Levels of TGFβ1 in the blood of TGFβ1 wildtype (TGFβ1+/+) and TGFβ1 heterozygote (TGFβ1+/-) mice were measured to establish their normal ranges. Exogenous sources of TGFβ1 were administered to TGFβ1-/- mice in an attempt to restore blood levels of TGFβ1 to within the normal range. Milk products were tested for their TGFβ1 levels and the product containing the highest amounts of TGFβ1 was fed to mice. A recombinant source of TGFβ1 was also administered to TGFβ1-/- mice to evaluate effects on restoring reproductive behaviour and function.

1.2 BIOCHEMISTRY OF TGFB ACTIVATION AND SIGNALLING

The mechanisms of TGFβ action are varied and context-dependent. TGFβ is kept under tight control by 1) its activation status, 2) differential expression of its receptors and 3) a signalling cascade that is influenced by other cytokines (Wimmel, 2003) (Roberts, 1999). The regulatory mechanisms controlling the actions of this potent cytokine have been extensively reviewed previously (Khalil, 1999).

Synthesis of a precursor form of TGFβ occurs in many cell types. It consists of three parts, the covalently bound binding protein, the non-covalently bound latency associated peptide (LAP) and the active TGFβ form. The binding protein assists in the assembly and secretion of latent TGFβ1 (Miyazono, 1991) and in its targeting to the extracellular matrix (ECM) where it controls ECM synthesis and degradation (Verrecchia, 2002). Once secreted, LAP facilitates shielding of the active TGFβ form, and must be cleaved or conformationally re-arranged to expose the active peptide (Khalil, 1999). Activation in vitro has been demonstrated using heat, acid and urea (Miyazono, 1988) however in vivo activation processes differ and are more numerous depending on tissue type, stage of development and the microenvironment. The main enzymatic activators identified are plasmin (Lyons, 1990) and thrombospondin-1 (TSP-1) (Ribeiro, 1999). Activation can also be achieved in vivo by other enzymes (Horimoto, 1995), reactive oxygen intermediates (Bellocq, 1999) or via sequence specific binding between LAP and the integrin RGD sequence within the extracellular matrix (Annes, 2004) (Murphy-Ullrich, 2000). For further control, once activated, numerous soluble inhibitors can act to prevent the ligand binding to its cellular receptors (Itman, 2006).

For signal transduction, the active forms of TGFβ1 and TGFβ3 bind with high affinity type II TGFβ receptor (TβRII), while TGFβ2 requires the co-receptor TβRIII (also known as betaglycan) to concentrate the ligand at the cellular surface for TβRII binding. These actions in turn phosphorylate the type I receptor (TβRI) for subsequent intra-cellular signalling (Itman, 2006) (Zuniga, 2005) (Blobe, 2001). The type II TGFβ receptors are constitutively expressed on most cell types and bind the TGFβ ligands with high affinity (Wakefield, 1987). However alterations to receptor

concentration can occur following TGFβ binding (Menke, 1999) (Kleeff, 1998) (Liu, 2002) (Wakefield, 1987) (Siegert, 1999) and these appear to be context dependent. The critical importance of these receptors is clearly demonstrated by the death of TβRI null mutant mice during mid-gestation (Larsson, 2001) and the lethal inflammatory disease that develops following TβRII disruption (Leveen, 2002).

Following receptor binding TGFβ signals through the intra-cellular SMAD cascade (Roberts, 1999) (Hanada, 2002). SMAD 2 and SMAD 3 are phosphorylated and form a complex with SMAD 4. Complexed SMADs then translocate to the nucleus to bind to specific DNA elements and operate as transcription factors to regulate responsive genes (Wimmel, 2003). However this process can be antagonised by other cytokines binding to the cell surface. For example, a reciprocal relationship between interferon gamma (IFNγ) and TGFβ occurs where IFNγ stimulation can induce the inhibitory SMAD 6 and SMAD 7, which in turn block the TGFβ signalling SMAD3 (Roberts, 1999) (Itman, 2006) (Strober, 1997), (Figure 1-2).

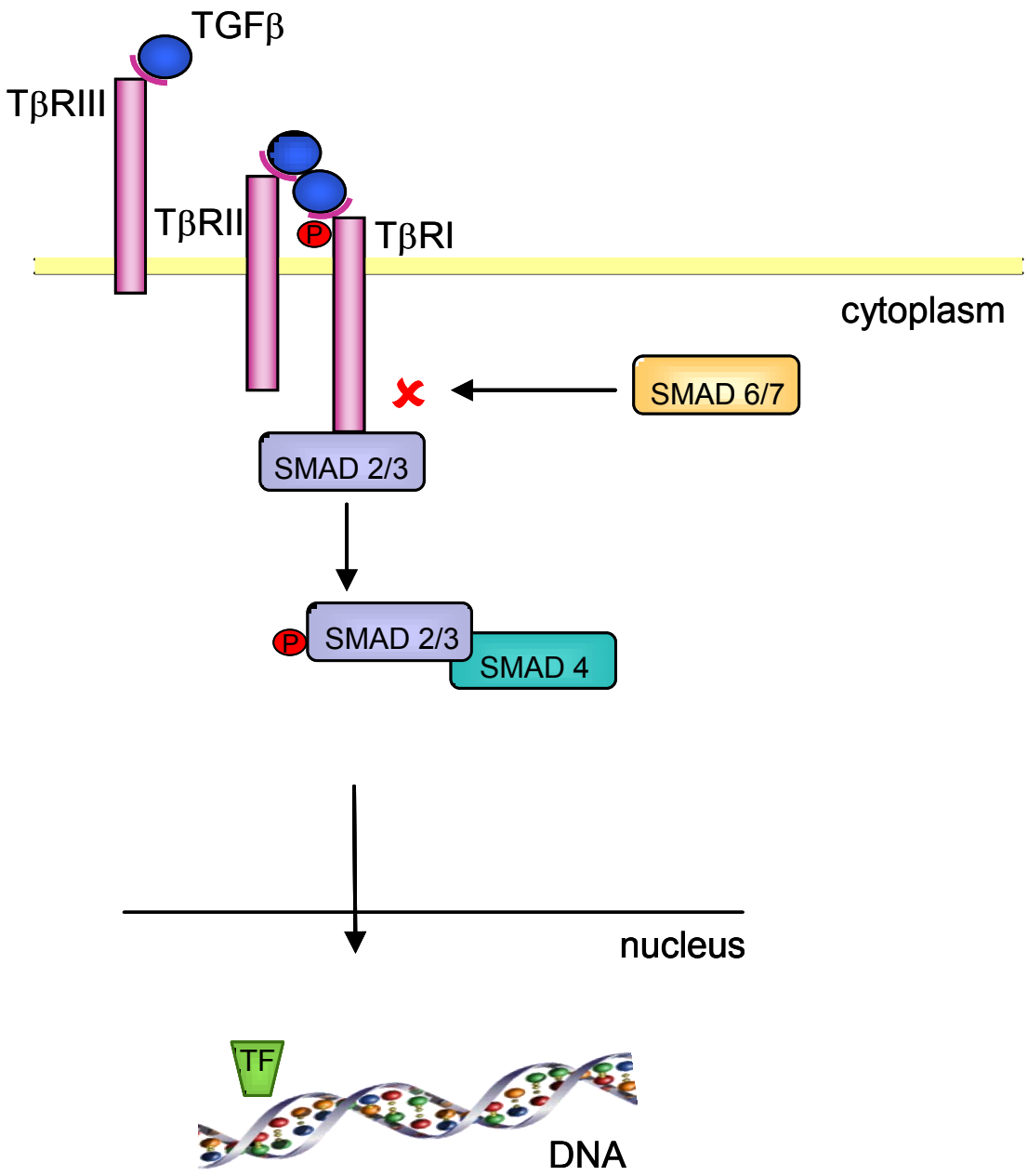


Figure 1-2. TGFβ receptor binding and signalling.

TGFβ binds directly to TβRII or binds to TβRIII which transfers TGFβ to TβRII. TβRII then forms a complex with TβRI to signal to intracellular SMAD complexes which are then translocated to the nucleus to regulate transcription factors (TF). Other antagonistic SMADs may inhibit this signalling pathway.

1.3 TGFB REGULATION OF BIOLOGICAL SYSTEMS

TGFβ regulates many physiological processes in vivo including cellular proliferation, extracellular matrix deposition, apoptosis, chemotaxis, cellular differentiation and hematopoiesis.

TGFβ1 is mainly regarded as an inhibitory cytokine due to its potent ability to suppress cellular proliferation in most cells (Kurokowa, 1987) (Larsson, 2001) (Lawrence, 1996) including those found in tumours (Seoane, 2006) and the immune system (Palmer, 1994). Suppression of proliferation is beneficial as demonstrated by minimising tumour growth (Wimmel, 2003) (Akhurst, 1999), resolving inflammation (Longenecker, 2002) and inhibiting excessive epithelial cell proliferation and shedding which occurs for example during psoriasis (Doi, 2003). In contrast, the proliferation of fibroblasts is up-regulated by TGFβ (Flanders, 2004) (Strutz, 2001). This results in extracellular matrix (ECM) deposition to aid tissue re-modelling and injury repair (Eickelberg, 1999) (Lawrence, 1996) (Grande, 1997), however when this up-regulation is uncontrolled, scarring and fibrosis can occur (Verrecchia, 2002) (Flanders, 2004) (Roche, 1989). The ability of TGFβ1 to induce cellular differentiation through regulating transcription factors suggests a potential for TGFβ to direct the fate of stem cells in various tissues (Moses, 1996). This aspect may be important in the skin where Langerhans cells provide an immune barrier and are completely lacking when TGFβ is absent (Borkowski, 1996) (Strobl, 1998). TGFβ also acts as a chemo-attractant for several cell types (Wahl, 1987) (Janssens, 2005), including those cells of the immune system, depending on the target cell lineage and cellular differentiation state and micro-environment (Ashcroft, 1999) (Wahl, 2000). This property may aid tissue formation and remodelling as TGFβ can play an important part in apoptosis in vitro and in vivo (Schuster, 2002). Hence the normal growth and function of organs is heavily reliant on the appropriate availability of TGFβ occurring in a timely manner.

1.4 REGULATION OF TGFB1 LEVELS AND ACTION

The sources of dysregulated endogenous TGFβ1 levels are multi-factorial as they may be inherited, the product of disease states or influenced by lifestyle. Many polymorphisms within the TGFβ gene are reported while the impact of various disease states and inflammation are linked to altered levels of TGFβ synthesis. Regulation of TGFβ availability may also occur through regulation of binding to its receptor. Finally, exogenous influences such as diet and lifestyle may also be possible contributors to TGFβ regulation.

1.4.1 Endogenous Regulators of TGFβ1

1.4.1.1 GENETIC POLYMORPHISMS

TGFβ bioavailability and signalling can be affected by genetic mutations in the gene itself or its receptors and signalling pathways (Grainger, 1999a) (Janssens, 2003) (Saito, 2001) (Watanabe, 2002). Inherited polymorphisms within the promoter region (G→A at position -800 bp and C→T at position -509 bp) of TGFβ in twins manifest as altered circulating TGFβ levels, with heterozygous individuals showing reduced levels compared to those homozygous for the polymorphism (Grainger, 1999a). Genetic mutations of the LAP region also occur and have led to TGFβ accumulation within the cell, creating excessive SMAD signalling through an internalized receptor complex (Janssens, 2003), (Saito, 2001). A mutation in the TGFβ1 signalling region has been correlated with obesity (Rosmond, 2003) and may partially explain the susceptibility of obese individuals to other diseases involving dysregulated TGFβ1 (Chalmers, 2006). Within the male reproductive system, a TGFβ polymorphism is linked with penile fibrosis (Hauck, 2003). Although these mutations are not regarded as wholly responsible for the symptoms these individuals exhibit, they remain likely contributors.

1.4.1.2 DISEASE AND INFLAMMATION

A previous review has discussed the numerous diseases and pathogens that are shown to cause inflammation and alter TGFβ levels in vivo (Li, 2006). Both Crohn's disease (CD) and ulcerative colitis (UC) are inflammatory conditions of the intestine and both reveal contrasting production of TGFβ in the affected tissues (Hahm, 2001) (Fell, 2000) (di Mola, 1999). These disparate levels are thought to be a consequence of the different polarisation of T-cells that occurs in each disease (Del Zotto, 2003) although this appears to be confined within the intestinal mucosal compartment and is not reflected in the circulation (Sturm, 2000). Some forms of cancer are also shown to impact on circulating levels of TGFβ1, for example, in individuals that experience a recurrence of colon cancer where secretion of TGFβ1 from the tumour appears to be implicated (Friedman, 1995). Additionally, around sites of injury (Grande, 1997), or pathogen invasion (Koch, 2000) (Mills, 2004) elevated levels of TGFβ1 occur to assist in resolving the condition (Li, 2006).

Finally, while chronic elevation of TGFβ appears to pre-dispose some patients to more severe disease states, it is still being elucidated whether dysregulated TGFβ is the cause or consequence (Junker, 2000). However given the ubiquity and context dependent nature of TGFβ, it is likely that the situation differs depending on the individual and the disease (Junker, 2000).

1.4.1.3 OTHER ENDOGENOUS REGULATORS OF TGFB1

TGFβ1 is able to amplify its own production as demonstrated in vitro by the ability of either TGFβ1 or TGFβ2 to up-regulate TGFβ1 gene expression in monocytes (McCartney-Francis, 1990) and epithelial cells (Menke, 1999). Additionally, the availability of the active form of TGFβ is also shown to be influenced by disruptions in the expression of TGFβ activators such as plasmin (Grainger, 1994).

1.4.2 Exogenous Regulators of TGFβ1 Levels and Action

Socio-demographics, the environment and individual lifestyle preferences are known to profoundly influence human health. Diet and lifestyle have been identified as possible sources of exogenous TGFβ1, however more studies are needed to determine how these exogenous sources contribute to TGFβ1 mediated regulation of biological systems and health.

1.4.2.1 LIFESTYLE CONTRIBUTORS TO TGFB1 LEVELS

Stress can cause glucocorticoids and catecholamines to rise. These hormones have been reported to stimulate the production of TGFβ1 (Elenkov, 2002). Obesity, defined by body mass index (BMI), is linked with up-regulated TGFβ1 levels (Alessi, 2000) and interestingly, the underweight condition of anorexia is linked to reduced TGFβ1 levels (Corcos, 2001). Even ultra violet light exposure may alter both TGFβ1 levels and its signalling (Quan, 2001) (Dissanayake, 1998), while smoking alters TGFβ1 levels in both the blood and bronchial airways (Ryder, 2002) (Li, 2003) and excessive alcohol consumption is observed in conjunction with up-regulated TGFβ1 gene expression in the liver (Chen, 2002). Finally, hormone replacement therapy alters TGFβ levels, however this response may also be related to the health status of the individual (Djurovic, 2000) (Figure 1-1).

1.4.2.2 DIETARY CONTRIBUTORS TO TGFB1 LEVELS

The modern human diet contains proteins, hormones and cytokines, particularly derived from animal products. TGFβ1 is present in various foods however the amount of TGFβ which individuals may be consuming has not been adequately determined.

For exogenous TGFβ from the diet to be effective at altering systemic concentrations or endogenous expression, it must first bypass several barriers. In the stomach, foods are exposed to proteases and acidic secretions. TGFβ may be activated by the acidic environment as well as broken down by the gastric proteases (Geibel, 2006)

(Fruton, 2002). Once past the stomach, most foods are further digested to amino acids and simple sugars, then absorbed through the intestinal wall and transported to the blood stream for use or excreted as waste. Intestinal transportation mechanisms have been extensively reviewed previously (Pacha, 2000) where it was suggested that pathways of whole protein absorption might exist. Some proteins may traverse the intestinal wall intact, perhaps through specialised epithelial M-cells that overlay lymphoid follicles in the mucosa (Neutra, 1998). Alternatively, transport may occur through receptor mediated transcytosis, where a protein may bind to its specific receptor prior to being internalised. In support of this, both nerve growth factor (NGF) and epidermal growth factor (EGF) have been shown to traverse the intestinal epithelium this way, however as these proteins are much smaller in size (Lakshmanan, 1989) (Grueters, 1985) compared to TGFβ (Lawrence, 2001) (De Crescenzo, 2006), more research into these transport mechanisms is needed (Figure 1-3).

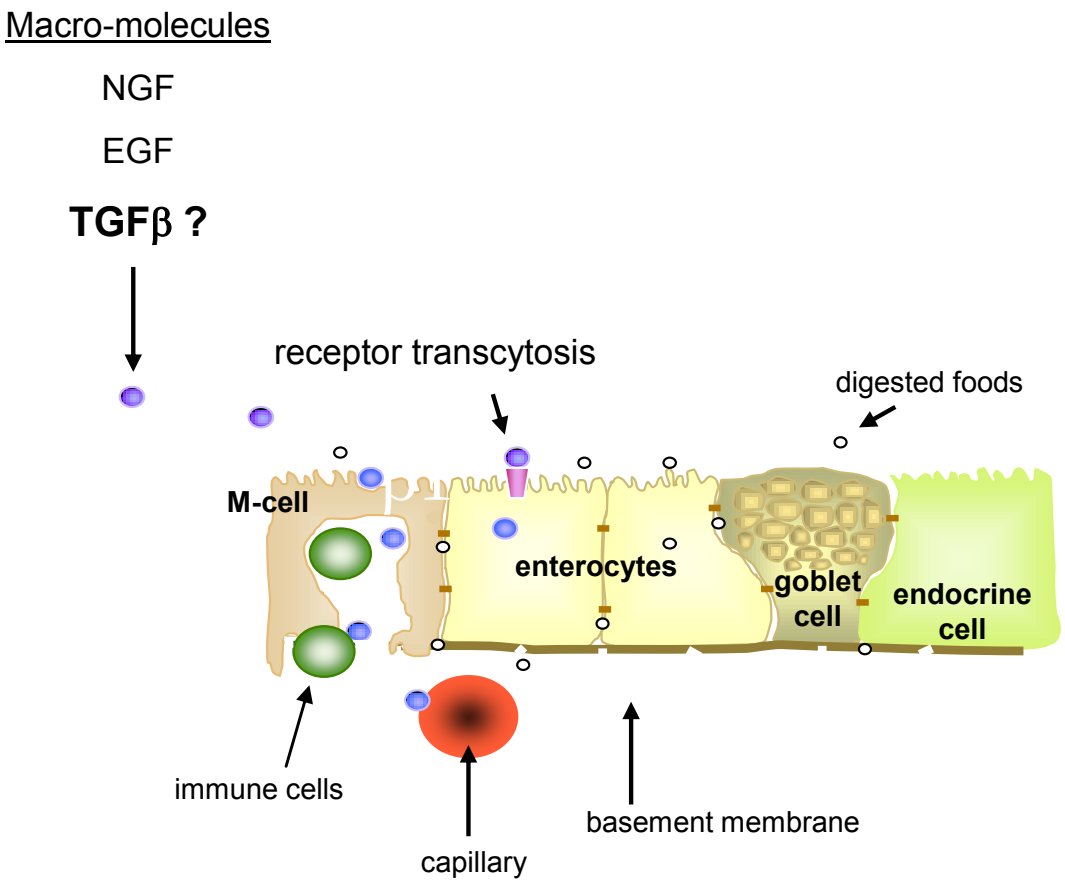


Figure 1-3. Fate of ingested proteins in the gastrointestinal tract.

Various cell types exist in the intestinal tract. NGF and EGF are shown to traverse the intestinal wall intact via receptor mediated transcytosis to enter the blood steam. Another pathway for macro-molecules to traverse the intestinal wall intact may be via specialised M-cells that are associated with the immune system. There is some evidence that TGFβ may be translocated from the lumen to the blood supply via these mechanisms.

1.4.2.2.1 Milk

Many growth factors and cytokines have been detected in milk. Insulin-like growth factor (IGF) is the most abundant, however TGFβ is also found in high levels compared to other growth factors (Ginjala, 1998) (Srivastava, 1996). TGFβ2 is present in milk in greater quantities than TGFβ1 (Szymkowiak, 1995), (McPherson, 2001) and both isoforms are purported to influence the development and immunological function of the mammary gland (Goldman, 1996) as well as to attenuate immune function in the neonatal intestine (Xu, 1996), (Strobel, 2002). Interestingly, no correlation is found between the TGFβ levels in milk and circulating plasma in the mother implying location specific regulation (Hawkes, 2002) and perhaps arguing against a major contribution from the maternal milk to the bloodstream and visa versa.

The composition of milk differs depending on the stage of lactation of the mother. In most species studied, the enriched colostrum fraction appears first and gradually declines over the next 3-4 days to the transitional fraction lasting a few weeks, which then gradually becomes the late or mature milk (Blum, 2008) (Fink, 2007). TGFβ levels are lowest in the late milk fractions (0.8 – 3.5 ng / mL) and considerably less than levels initially present in the colostrum (12 - 43 ng / mL) (Bottcher, 2000) (Ginjala, 1998).

When bovine milk is used for human consumption, it is likely that TGFβ is able to impact on human health due to the high protein sequence homology across species (Van Obberghen-Schilling, 1987) (Nixon, 2000). Indeed, an in vitro experiment suggests that the TGFβ in bovine milk may play a role in the proliferation of human intestinal cells (Purup, 2007), although the number of studies specifically investigating the ability of milk derived bovine TGFβ to impact on human health remains limited. To help understand this, the condition of the TGFβ in cows' milk consumed by humans must be considered as large differences in the concentration and quality of TGFβ between raw and processed milk may exist. Human milk samples reveal almost complete TGFβ2 recovery following pasteurization at 56°C for 30 minutes (McPherson, 2001). This temperature is much lower than those used in another study where TGFβ activation began at around 70°C and temperatures above

90°C led to thermal denaturation (Ansel, 1990). This indicates that while TGFβ might become activated during the normal pasteurisation processes, it is likely to retain biological activity. The pasteurisation standard for milk in Australia is currently set to 78°C for 27 seconds to ensure inactivation of the pathogenic Myobacterium sp. (DairyVale, 2006), however, it has not been determined whether TGFβ survives this process.

1.4.2.2.2 Other dietary sources

Other foods that may contain TGFβ are also derived from animal products such as yogurt, cheeses and meats. Like milk, dairy products undergo much processing and it is not known how this affects TGFβ bioavailability. Fresh meat is another potentially potent source of exogenous TGFβ1, however meat storage and cooking procedures may lead to degradation. Furthermore, dietary components such as salt, oil and food allergens, have been seen to regulate endogenous TGFβ levels (Ying, 1998) (Chandrasekar, 1995) (Perez-Machado, 2003).

1.5 TGFB LEVELS IN BLOOD

Each of the TGFβ isoforms, TGFβ1, TGFβ2 and TGFβ3 are able to act in local autocrine and paracrine fashions. Unlike the other isoforms TGFβ1is found in high levels in the circulatory system, indicating it also functions in an endocrine manner (Wakefield, 1995) (Grainger, 1999b). Both latent and active forms of TGFβ1 are found in the circulation however the latent form is predominant due to its two hour half-life, contrasting with the two minute half-life of the active form (Wakefield, 1990) (Coffey, 1987). Within the circulation TGFβ can bind to soluble receptors such as TβRIII, alpha-2 macroglobulin (A-2m) or beta-2 microglobulin (B-2m) to block cellular receptor binding and subsequent signal transduction (Zhang, 2001) (Kropf, 1997) (Arandjelovic, 2003) (O'Connor-McCourt, 1987).

When the blood is fractionated into serum or plasma, different levels of TGFβ1 are found in each. This is due to the significant amount of TGFβ1 stored in platelets

which is released upon platelet activation during serum preparation (Kropf, 1997). While little work has been done on the fluctuations of TGFβ in blood or tissues over time, one study shows that when the serum of healthy individuals at rest was measured, TGFβ1 levels were constant (at 1155 +/- 30 pg / mL) over 5 consecutive days (Heinemeier, 2003). However, substantial variance amongst TGFβ levels in the plasma of individuals has also been widely reported. In the past, blood was removed and handled without much consideration given to platelet activation, causing erroneous values to be reported. When proper sample handling is practiced by using wide bore needles and an appropriate anti-coagulant, the circulating levels of TGFβ1 are more consistent and show that when disease exists, differences of up to ten-fold occur irrespective of age, gender and perhaps ancestry (Grainger, 2000). Thus, while consistent levels of circulating TGFβ1 may be found within healthy individuals, larger differences occur in diseased individuals.

1.6 TGFB EFFECTS ON PHYSIOLOGICAL FUNCTION AND HEALTH

1.6.1 TGFβ Regulation of Immune Status

Cytokine balance in tissues is an important determinant of protective or pathogenic immune responses. The central role of cytokines is to control the direction, amplitude and duration of immune responses, be they developmentally programmed, constitutive, or antigen-induced. TGFβ plays a major role in these responses and thus has far-reaching consequences in homeostasis, inflammation and autoimmunity (Li, 2006). The extent that TGFβ1 is needed to suppress inflammation may differ depending on which compartment it operates in, with mucosal immunity being more dependent than systemic and organ immunity (Gorelik, 2002). Target cell lineage is also important in determining the responses to TGFβ, as lymphocytes, monocytes and macrophages are differentially modulated by it. These issues have been the subject of several reviews (Banchereau, 1993) (Cavaillon, 1994) (Prud'homme, 2000) (Wahl, 1989) (Wahl, 2000).

Within T-cell lymphocytes, the T helper (Th) type 1 phenotype is responsible for cell mediated immunity (CMI) and is characterised by production of the pro-inflammatory cytokines tumor necrosis factor alpha (TNFα) and interferon γ (IFNγ), while the Th2 phenotype suppresses inflammation and is associated with secretion of IL-10 and humoral antibody responses (Belardelli, 1995). New CD4+ T-cell subsets have emerged, including T regulatory cells and the Th3 subset which are both stimulated by TGFβ (Weiner, 2001). These cells play roles in reducing CMI by secreting TGFβ1 which then exerts bystander effects to produce tolerogenic immune responses, important in protecting autoimmunity (Prud'homme, 2000). The interplay between these cell types and the cytokines they secrete is vital for maintaining a balanced immune response.

TGFβ recruits and activates resting monocytes (Ashcroft, 1999) during the initial stages of inflammation and induces them to secrete TNFα (Bogdan, 1993), which promotes inflammation. However later in inflammation activated macrophages secrete TGFβ to de-activate the macrophage population and suppress inflammation by reducing cytokine production from macrophages (Cavaillon, 1994) (Bogdan, 1993) (Kossmann, 1992) (Assoian, 1987) (Grotendorst, 1989) (Cavaillon, 1995). Despite this, cytokines with opposing actions may not be capable of influencing the same macrophage. This was demonstrated when others found that TGFβ could not suppress nitric oxide (NO) producing or phagocytic macrophages that had been exposed to IFNγ or TNFα (Erwig, 1998). Hence, only un-committed macrophages that are initially exposed to a local or autocrine TGFβ source can produce suppressive actions. However this work conflicts with another study where IFNγ induced nitric oxide (NO) production that occurred in activated mouse peritoneal macrophages, was suppressed by TGFβ acting on nitric oxide synthase (iNOS) mRNA and protein stability (Vodovotz, 1993). It therefore seems likely that macrophage phenotype, activation, location and cytokine concentration play important roles in macrophage regulation (Erwig, 1998).

1.6.2 TGFβ in Male Reproduction

Male sexual characteristics are influenced by events during pre- and peri- pubertal growth and development (Main, 2006) and the levels of testosterone synthesis and

bioactivity (Caretta, 2005) (Itami, 1995). This hormone is produced by the Leydig cells of the testes in response to stimulation by lutenizing hormone (LH) regulated by the hypothalamic pituitary axis (Amory, 2001) (Arzt, 1999). As testosterone also impacts on the hypothalamus to restrict LH production, a negative feedback mechanism occurs (Amory, 2001). Interestingly, macrophages reside in the testes and are found to impact on testosterone production in both humans and rodents (Cohen, 1997) (Hales, 2002). Follicle stimulating hormone (FSH), together with testosterone regulate sperm production (Amory, 2001) (Walker, 2005) (Dohle, 2003). Erections are initiated following central or peripheral neuronal stimulation (Andersson, 2001), where testosterone can trigger dopamine release in the penis which induces nitric oxide (NO) production and a subsequent erection (Hull, 1999) (Steers, 2002) (Hull, 2004). Dopamine is also known to facilitate sexual behaviour and is released following sensory stimulation by both testosterone and NO (Dominguez, 2005) (Putnam, 2005) (Hull, 1997).

The ability of TGFβ to influence erections and behaviour may be related to its effects on dopamine and dopaminergic neurons as indicated by an increased number of neurons following TGFβ1 treatment (Farkas, 2003) and reduced dopamine concentration (70%) following TGFβ2 restriction compared to normal (Andrews, 2006) (Figure 1-4). To further delineate the role of TGFβ1 in reproduction, TGFβ1-/- mice have been informative. While mice with an intact immune system succumb to a lethal autoimmune syndrome in the first few weeks of life (Geiser, 1993), their survival to adulthood and study of their reproductive capability can be achieved by ablating lymphocyte activity (Diebold, 1995) (Bancroft, 1994). A previous study on TGFβ1-/- male mice on a severe compromised immune deficiency (SCID) background revealed profound infertility (Ingman, 2007) comprising of:

1) reduced testosterone production in the testes associated with reduced circulating LH levels and responsive to alleviation by exogenous administration of the LH analog hCG;

2) disruptions in spermatogenesis in some mice, although those with normal spermatogenesis maintained their fertilizing capability, and

3) sexual dysfunction, including altered mounting, intromission and ejaculation activities, despite mice showing interest in females, even when mice were treated to restore testosterone levels.

It was also suggested that the infertility of these mice may in particular be due to erectile dysfunction caused by the absence of penile NOS enzymes in most TGFβ1-/- mice (Ingman, 2002) (Ingman, 2007). Thus, data from the TGFβ1-/- mouse model has revealed that TGFβ1 is involved in male reproduction at multiple levels (Figure 1-4).

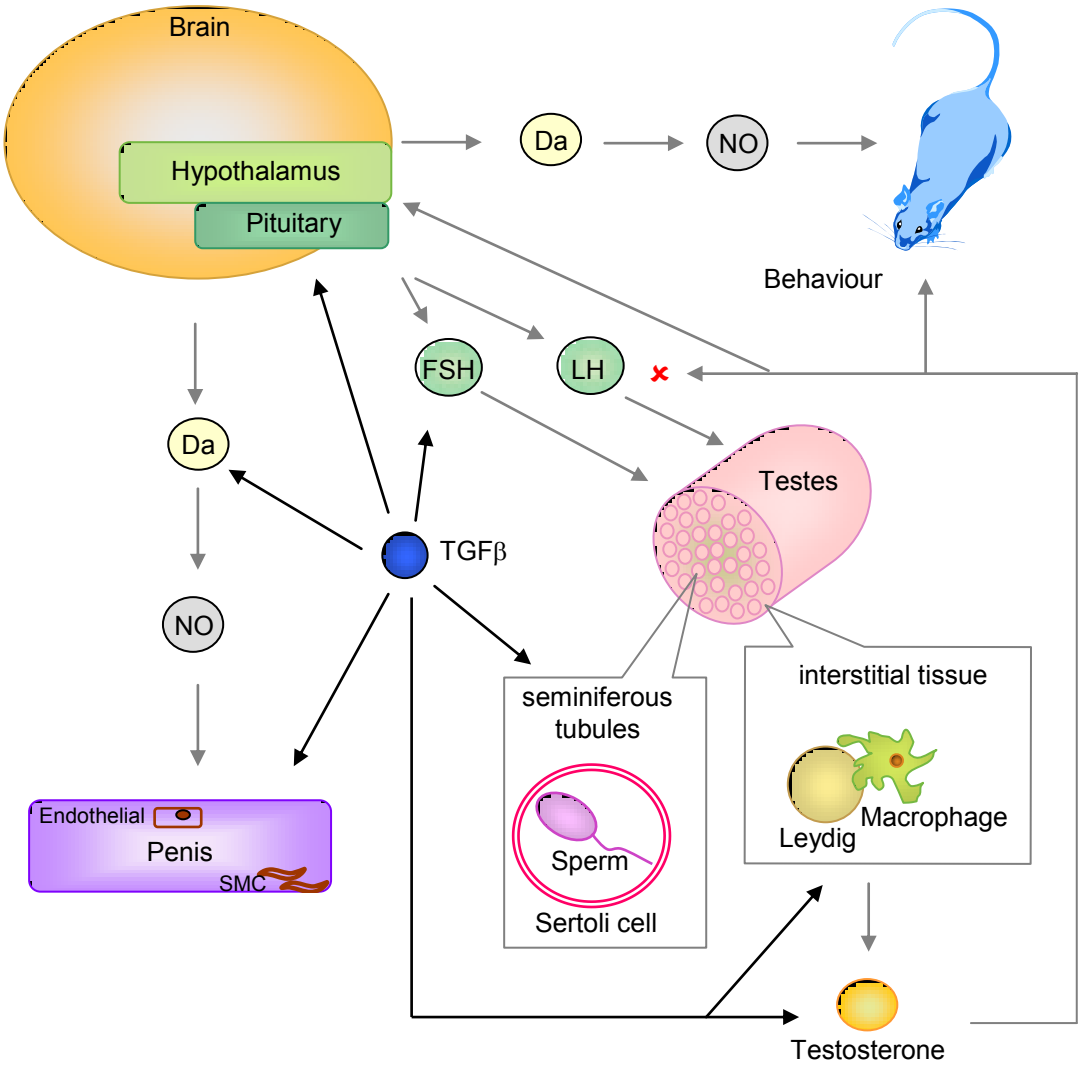


Figure 1-4. Male reproductive parameters affected by TGFβ.

The brain is stimulated by testosterone to produce dopamine (Da) which induces erections and regulates sexual behaviour. TGFβ can influence neuronal cells of the brain as well as regulate dopamine production, by promoting maintenance of dopaminergic neurons. Erections are influenced by TGFβ via its effects on smooth muscle cells (SMC), endothelial cells and nitric oxide (NO). TGFβ influences follicle stimulating hormone (FSH) release and subsequent spermatogenesis in the Sertoli calls residing within the seminferous tubules of the testes. LH released from the pituitary is reduced in TGFβ-/- mice (Ingman, 2007) and the negative feedback loop between LH and testosterone may be affected by TGFβ, via the actions of TGFβ on Leydig cells and the adjacent testicular macrophages in the testes.

1.6.2.1 TGFB1 REGULATION OF TESTOSTERONE PRODUCTION

Testosterone acts on many organs to promote male sexual characteristics as well as secondary sexual traits such as facial hair and behaviour (Zirkin, 1989) (Dohle, 2003) (Traish, 2006) (Itami, 1995). Interestingly, involvement of cytokines and cells of the immune system has increasingly become recognised as participating in regulating these characteristics (Hales, 2002) (Robertson, 2002). Specifically, TGFβ regulates various physiological functions of the testes. This is demonstrated by the findings that differential expression of the TGFβ isoforms occur during development and that gonocyte numbers are altered in the presence of TGFβ, although this may depend on the time of spermatogenesis (Itman, 2006) (Benahmed, 1989) (Mullaney, 1993) (Lui, 2003). Additionally, TGFβ can influence testosterone production via its effects on LH. LH-stimulated testosterone production by fetal testicular cells is inhibited in vitro when TGFβ1 is added to the culture medium (Gautier, 1997) and delivery of an LH analog to TGFβ1-deficient mice results in increased testosterone production (Ingman, 2002). The steroidogenesis cascade is also influenced by TGFβ1 acting on. 3β-hydroxysteroid dehydrogenase (HSD3β1), a key steroidogenic enzyme that converts cholesterol to testosterone (Herrmann, 2002). While this enzyme appears to be both positively and negatively regulated by TGFβ1 in vitro (Fournet, 1996) (Cherradi, 1995), the responses in vivo may be testosterone dependent (Ingman, 2002) (Heggland, 1997). Also, as testicular macrophages produce TGFβ1 and appear to be involved in testosterone regulation (Bryniarski, 2004) (Hales, 2002), TGFβ1 acting through this nexus may play a role in fertility. Importantly, circulating TGFβ1 is shown to have a biphasic effect on testosterone production (Morera, 1988) (Benahmed, 1989), although the mechanism that explains this is not yet understood (Figure 1-4).

1.6.2.2 TGFB1 REGULATION OF SPERM PRODUCTION

Male infertility is often associated with low sperm concentration, motility or morphology, however an impairment in all these variables within an individual is common (Isidori, 2005). Spermatogenesis is regulated by testosterone which diffuses into the seminiferous tubules, the site of sperm production in the testes. Without it, progressive germ cell degeneration can occur, as demonstrated in rodents (Walker,

2005). Indeed, the high testosterone levels in the testes compared to the circulation are required for the production of a normal sperm count [9]. During sperm maturation the Sertoli cells of the testes provide nutritional support and form part of the blood-testes barrier (Fritz, 1994) (Meng, 2005). They are mainly influenced by the release of follicle stimulating hormone (FSH) from the pituitary (Amory, 2001) (Lei, 2004), however testosterone also plays an important role in germ cell maintenance (Walker, 2005) (ten Dijke, 2004) (Figure 1-4).

While seminal fluid contains a large amount of TGFβ1 (Robertson, 2007) developing sperm do not (Avallet, 1997). However TGFβ remains implicated in spermatogenesis through regulating germ cell survival and influencing the blood-testes barrier (Itman, 2006) (Lui, 2003). Somatic cells of the testes express TGFβ1 and its receptors (Fritz, 1994) (Watrin, 1991) (Teerds, 1993) and TGFβ1 is detectable throughout testicular development until adulthood when it declines (Teerds, 1993). Importantly though, it may be the interaction between Sertoli and germ cells that regulates TGFβ1 expression in the testes, as demonstrated by elevated TGFβ1 expression in co-cultures (Avallet, 1997). Over-production of TGFβ1 in the testes may also influence spermatogenesis. When levels of TGFβ1 become excessive, testicular fibrosis may ensue causing a thickening of the seminiferous tubules impairing spermatogenesis (Dobashi, 2002). Genetic absence of TGFβ1 in TGFβ1-/- mice results in modest reduction in sperm production, however this may be partially due to their reduced testicular size (Ingman, 2002) and / or low testosterone levels (Zirkin, 1989).

1.6.2.3 TGFB1 REGULATION OF PENILE ERECTION

Co-ordination between the nervous and vascular systems is crucial for erections to occur [8] and disturbances in either can lead to erectile dysfunction and diminished quality of life, where age is an independent risk factor (McCullough, 2002) (Marumo, 2001) (Nicolosi, 2003) (Thomas, 2002). A cascade of events elicits the erectile response. Neuronal stimulation results in nitric oxide (NO) production from neuronal or endothelial cells causing a subsequent rise in cyclic guanosine monophosphate (cGMP). This triggers smooth muscle relaxation of the penis and vasodilation allowing blood to flow into the organ (Mills, 2002).

The importance of nitric oxide (NO) on erectile function is clearly established (Cartledge, 2001) (Bivalacqua, 2000) and demonstration of the interaction between NO and TGFβ in smooth muscle cells indicates that TGFβ has an effect within the tissues of the penis (Wespes, 2002) (Vodovotz, 1994) (Figure 1-4). Indeed, penile NOS enzymes were undetectable in most TGFβ1-/- male mice previously examined (Ingman, 2002). However, the significance and mechanisms through which disturbances in TGFβ1 affect the erectile response is not clear. Increased plasma TGFβ1 is evident in individuals with erectile dysfunction (Ryu, 2004) and while these elevated levels may be related to vascular health, they highlight the potential of using TGFβ1 measurements to reinforce erectile dysfunction diagnosis. TGFβ1 may also affect the erectile response via its actions on testosterone production, although the effects of testosterone on erectile activity may be context dependent (Traish, 2006) (Wespes, 2002) (Baba, 2000) as rodents appear more responsive to hormonal change than humans (Traish, 2006). TGFβ also impacts on other components of the erectile pathway such as endothelial function (Dunker, 2000) (Saura, 2005) (Bobik, 2006) (Larsson, 2001) smooth muscle cell (SMC) function (Bobik, 2006) (Grainger, 1994) (McCaffrey, 1993) and neuronal innervation (Bottner, 2000) (Ryu, 2004), so many different modes of TGFβ interaction are possible.

Other evidence demonstrates that there is an overlap between erectile function disorders and common diseases where TGFβ1 is dysregulated, such as coronary artery disease, diabetes and atherosclerosis (Fujisawa, 2000) (Francavilla, 2005) (Penson, 2003). As these diseases are related to the vascular system, erectile dysfunction is now suggested to be an early marker of underlying endothelial dysfunction (Francavilla, 2005). Neuronal disorders leading to erectile dysfunction cannot be discounted (De Groot, 1999) (Landtblom, 2006) (Papatsoris, 2006) (Fernandez-Espejo, 2004). In Peyronie's disease, a specific disorder of the penis where higher levels of TGFβ1 occurs, the tissues become fibrotic so that smooth muscle relaxation is impeded and erectile dysfunction occurs (El-Sakka, 1997). Supporting this, in a study of older rats, fibrosis and elevated levels of the TGFβ1 gene occurred more frequently compared to those that were younger (Dahiya, 1999).

Therapies to alleviate erectile dysfunction have focused on rectifying vascular or neuronal deficiencies (Thomas, 2002) (Albrecht-Betancourt, 2004) (Padma-Nathan,

2004). Erectile dysfunction can be restored following sildenafil citrate (Viagra) treatment. This peripherally - acting agent inhibits cyclic guanosine monophosphate (cGMP) breakdown in the penis to allow smooth muscle relaxation, providing adequate NO levels are available (McCullough, 2002). Apomorphine is a potent centrally acting agent that can induce erections (Elabbady, 1995) by acting on the dopamine receptors of the hypothalamus (Padma-Nathan, 2004) to cause NO release (Steers, 2002). As TGFβ1 can strongly influence NO production, the efficacy of these therapies may interact with disturbances in TGFβ1 availability.

1.6.2.4 BEHAVIOUR

Sexual function and behaviour is related to the central and peripheral nervous systems involving the hypothalamus / pituitary axis, which regulates sexual motivation, genital reflexes, and copulation (Romano-Torres, 2007) (Dominguez, 2005) (Hull, 2004). Both dopamine and testosterone are involved in these processes (Hull, 2004) (Klomberg, 2002) (Wespes, 2002) (Livne, 1992) as demonstrated by the following two examples; (1) when male sexual behaviour was assessed following site specific microinjection of dopamine into the brain, sexual behaviour including copulation was facilitated, while a lesion in the same site impaired this behaviour (Dominguez, 2005), and (2) in a study where insufficient testosterone in newborn rodents diminished their reproductive capacity following puberty, neonatal treatment with testosterone allowed intromission and ejaculation behaviours to be displayed (Livne, 1992). Additionally, prior sexual experience can also influence sexual behaviour. Following ad libitum copulation for 4 hours, sexual behaviour was only observed in 63% of mice in the following 4 days, however by day 7 all mice again displayed copulatory activity (Fernandez-Guasti, 2003).

Studies on the impact of TGFβ1 on sexual behaviour are limited, although behavioural studies with TGFβ1-/- mice have clearly shown that the males are sexually motivated as shown by their interest in females. However the reduced number of copulation events they displayed could not be restored following treatment with testosterone or Viagra, indicating that testosterone or cGMP levels alone are not responsible for the infertility of these mice (Ingman, 2002).

1.7 MANIPULATING TGFB LEVELS IN VIVO

While many in vitro studies show the ability of TGFβ to influence a variety of cells, systems and processes that regulate male reproductive tract function, few in vivo studies concerning its influences in male fertility are available.

In vivo delivery of exogenous TGFβ is known to impact on the biochemistry, physiology and function of many different biological systems, although not all attempts have been successful. When radioactive iodine (^{125}I) labelled TGFβ was delivered intravenously to normal rats, most of the label was removed by the liver (83%) and then excreted into bile within 90 minutes, suggesting that the liver may be a target organ or site of metabolism for TGFβ (Coffey, 1987). Despite this quick clearance rate, another study showed hemopoietic changes in normal mice including reduced platelet concentration, following two weeks of subcutaneously delivered TGFβ (Carlino, 1992). In contrast, over-expression of the TGFβ1 gene in the liver resulted in 10-fold higher TGFβ1 levels in the plasma, however hepatic fibrosis and lesions in several organs also occurred (Sanderson, 1995).

An alternative method of delivering exogenous TGFβ in vivo in mice is demonstrated by the finding that when pups suckle on heterozygous (TGFβ1+/-) mothers, maternal TGFβ can be detected in various tissues of the TGFβ1-/- pups resulting in an increased life span (Letterio, 1994). This demonstrates that TGFβ1 in the diet (ie: mother's milk) (1) traversed the gut and (2) influenced immune function and overall health.

Thus, it is hypothesized here that a latent source of human TGFβ1 may be capable of restoring some functional deficiencies in TGFβ1-/- mice. However TGFβ is only functional when present in the active form, this depends on endogenous activation mechanisms in the mouse species being able to activate the human latent form. Activation of exogenous TGFβ1 would be regulated by the endogenous environment and may only occur in certain contexts or tissue locations where TGFβ action is needed. Once activated, TGFβ1 should be able to bind directly to any constitutively expressed TβRII receptor (Wakefield, 1987) and impact on those tissue functions.

1.8 CONCLUSION

TGFβ1 is a multifunctional cytokine implicated in the normal function of many tissues and systems. Its structure and function is evolutionarily conserved between species. Humans experience variations in endogenous TGFβ1 levels for various reasons including disease, genetic makeup, immune status and lifestyle. The reproductive defects of TGFβ1-/- mice acutely impact on reproductive function. While TGFβ1-/- mice are able to mount and occasionally intromit with females, their circulating testosterone levels are significantly reduced and they cannot deposit sperm or produce offspring. Previous attempts to replace testosterone to normal levels have been unsuccessful in alleviating the reproductive deficit, suggesting factors other than testosterone are involved. These other factors could include problems in the structure and function of the penis tissue. While penile weight and internal tissue structures appear normal, erectile capability and external structures such as the spines have not been studied.

Experiments to replace TGFβ1 in TGFβ1-/- mice using an exogenous source of TGFβ1 protein added back to the circulation have not previously been undertaken. This is an important experiment in order to conclusively attribute the reproductive lesions seen in TGFβ1-/- mice to cytokine deficiency and to provide insight into the potential of TGFβ1 as a therapeutic agent.

Demonstration of a role for TGFβ1 in influencing reproductive function would raise the question of whether TGFβ1 supplementation in genetically normal animals could improve reproductive performance. Blood levels of TGFβ1 are varied between and within species and thus may be susceptible to change induced through dietary or systemic delivery of exogenous TGFβ1. Various dietary sources of TGFβ exist, particularly milk products. A more concentrated source would be provided by colostrum and this may have utility in regulating circulatory levels of TGFβ1 in humans. To investigate the levels to which exogenous TGFβ1 would require to be restored in the circulation of TGFβ1-/- mice, quantitation of the levels of TGFβ1 in the blood of both TGFβ1+/+ mice and TGFβ1+/- mice is needed.

In vivo changes to TGFβ1 function might also be identified by assessing TGFβ1 responsive parameters in the highly sensitive immune system, particularly macrophage numbers and function which are associated with TGFβ1 action. Macrophage populations, their functions including phagocyosis and their products such as iNOS and TNFα may be affected by TGFβ1 bioavailability. Exploration of these cells within the TGFβ1 null mutant mouse model could provide helpful information on the impact of perturbing TGFβ1 bioavailability.

The following hypotheses can be formulated from the literature detailed in the preceding sections.

1) TGFβ1 is a key regulator of male reproductive function and null mutation in the TGFβ1 gene influences reproductive competence through altering structure or function of male sex organs or influencing mating ability.

2) TGFβ1 is a key regulator of immune status and null mutation in the TGFβ1 gene alters macrophage populations and function.

3) TGFβ1 can be exogenously replaced by sub-cutaneous or oral administration and increased TGFβ1 bioavailability will influence reproductive and immune parameters in TGFβ1-/- mice.

The aim of the studies presented in this thesis is to investigate the above hypotheses in a mouse model, utilising mice with a null mutation in the TGFβ1 gene.

Chapter 2:
Methods

2.1 MICE

2.1.1 Animal Husbandry

TGFβ1 null mutant mice were bred from heterozygous breeding pairs (TGFβ1+/-) located at the University of Adelaide. The colony was maintained in specific pathogen free rooms with controlled light (12 : 12 light : dark, 7am : 7pm) and temperature. Food and water were given ad libitum.

The TGFβ1 null mutation results in the synthesis of a non-functional TGFβ1 protein. Mice homozygous for a targeted null mutation in the TGFβ1 gene and homozygous for the Prkdc(scid) (severe combined immune deficiency) mutation (TGFβ1-/-) were produced from breeder pairs heterozygous for the TGFβ1 mutation and homozygous for the Prkdc(scid) mutation on a mixed CF1/129/C3H background. They were originally obtained from T Doeschman (University of Cincinnati). Control mice used in these experiments were TGFβ1 replete littermates (TGFβ1+/+) (Ingman, 2007). Some heterozygous (TGFβ1+/-) offspring were also used in some studies, as stated in the relevant chapters. The genetic status of each mouse was determined by PCR analysis of genomic DNA. Their birth date, parents, sex, genotype, ear-punch number as well as an allocated number were recorded in a colony database.

Adult CBA/F1 females obtained from the University of Adelaide, Central Animal House were used in mating experiments to evaluate male reproductive function, while adult C57BL/6 mice from the same supplier were used for harvesting thymocytes.

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.

2.1.2 Genotyping mice

Ear tissue was collected at weaning (3-4 weeks of age) and analysed for the intact and disrupted tgfb1 gene by PCR. The genotype of each mouse used in experiments was confirmed by repeating the PCR on new ear tissue collected at sacrifice.

2.1.2.1 SAMPLE PREPARATION

Ear tissue was digested with 0.35 ml of digestion buffer (Appendix A) containing proteinase K at 55°C for 4 hours. A 5 µL aliquot was taken and diluted with 95 µL of water. The tubes were then heated to 95°C for 15 minutes to inactivate the proteinase K. The mixture was allowed to settle in the tubes and an aliquot of the supernatant was taken for DNA analysis.

2.1.2.2 PCR DESIGN

Two PCR reactions were used to determine the genotype of mice with respect to the TGFβ1 mutation. Primers for the intact gene span a sequence in exon 6 of the TGFβ1 gene and primers for the disrupted gene detect the neomycin resistance gene inserted into exon 6 of the TGFβ1 gene. Primers and products were previously validated by others (Ingman, 2002). The sequence, nucleotide position and PCR product length of the primers are given here (Table 2-1).

Gene	Position	Primer sequence	Product length
Intact TGFβ1	231(fwd)	5'GAGAAGAACTGCTGTGTGCG	134
	364(rev)	5'GTGTCCAGGCTCCAAATATAGG	
Disrupted TGFβ1	231(fwd)	5'GAGAAGAACTGCTGTGTGCG	545
	929(rev)	5'CTCGTCCTGCAGTTCATTCA	

Table 2-1 TGFβ1 genotyping primers.

2.1.2.3 POLYMERASE CHAIN REACTION

PCR reaction mixtures contained 1x PCR buffer, 2.5 mM $MgCl_2$, 0.5 U Taq polymerase (Amersham Pharmacia Biotech AB. Uppsala, Sweden), 16 mM dNTPs (Roche Diagnostics. Mannheim, Germany), 10 µM each primer and 2 µL of ear DNA in a 25 µL reaction mixture. Reactions were overlaid with mineral oil to prevent evaporation. The PCR cycle conditions to detect both products were one cycle of 94°C for 5 minutes; 40 cycles of 94°C for 45 seconds, 61°C for 45 seconds, 72°C for 45 seconds; and one cycle of 72°C for 3 minutes.

2.1.2.4 DETECTION OF PCR PRODUCTS

The genotype of each mouse was determined by visualizing the PCR products after size separation by gel electrophoresis. Samples were mixed with 6 x loading buffer and run on 1.5% agarose gels (Promega Corp, Wisconsin, USA) at 70 volts for 45 minutes (Appendix A). They were then stained for 2 minutes in an ethidium bromide bath containing 0.002% ethidum bromide in water. The gels were photographed using a digital camera (DC 120: Kodak. New York, USA) under UV light (UVP Inc. California, USA) to record the results.

2.2 BLOOD COLLECTION

Prior to bleeding, mice were heated under an infrared lamp for several minutes to increase blood flow to extremities. Blood was collected by making a small nick across the tail vein and collection of blood with a micropipette. Up to 0.2 ml of blood was collected by this method. Where mice were to be sacrificed, blood was collected by cardiac puncture. The mice were deeply anaesthetised with 0.5 mL 2% Avertin (Appendix A) or with 2% halothane in oxygen. Blood was extracted directly from the heart using an 18 G needle and up to 0.8 ml of blood was collected by this method. Mice were then cervically dislocated to ensure death.

2.2.1 Serum preparation

Where serum was required, blood was stored at 4°C overnight to ensure complete coagulation and subsequent TGFβ1 release, then centrifuged at 5,000 rcf for 10 min at 4°C and serum was stored at -70°C.

2.2.2 Plasma Preparation

When required, blood collection methods for plasma samples followed those previously reported to minimize both blood coagulation and platelet activation. Briefly, blood was collected using an 18 gauge needle with 1 x volume of CTAD (Appendix A) in the syringe to prevent blood coagulation and platelet activation. Blood and CTAD were then mixed together by inversion and centrifuged at 5,000 rcf for 10 minutes at 4°C. The supernatant was collected and stored at -70ºC until required.

2.3 SERUM TESTOSTERONE ANALYSIS

Total murine testosterone in serum samples was measured at Repromed Pty Ltd (Adelaide, South Australia) by radioimmunoassay (Diagnostic Systems Laboratories. Texas, USA) according to the manufacturers instructions. The minimum dectectable level of testosterone was 0.4 nM. Samples from mice within the same experiment were collected at the same time of day and processed in the same RIA run.

2.4 TGFB CONCENTRATION ANALYSIS

The concentration of TGFβ protein in samples was analysed in duplicate using commercial ELISA kits. Mouse TGFβ1, human TGFβ2 and human TGFβ3 DuoSets (R&D Systems. Minnesota, USA) were used. The kits are 100% cross reactive with mouse TGFβ1, TGFβ2 and TGFβ3 and all kits are isoform specific with a minimum dectectable level of 16 pg / mL. Analysis was performed according to the

manufacturers' instructions and dilutions were performed to confirm the linear range. The active form of TGFβ in a sample was measured directly while some samples were acid activated in accordance with the manufacturer's instructions to detect total TGFβ. Latent TGFβ was determined by calculating the difference between the active and total values. Serum, plasma, stomach contents, milk and colostrum were assessed in this way. The concentration of TGFβ was determined by comparing sample values with a standard curve of known recombinant TGFβ concentrations. Some samples were assessed on a dry weight basis, this was achieved by measuring the moisture content of the samples (see 2.4.1 below).

2.4.1 Moisture Content

To obtain values of TGFβ1 in the stomach contents of mice on a dry weight basis, the moisture content of the stomach contents was determined by oven drying. A petri dish was oven dried overnight at 105ºC and cooled in a desiccator prior to weighing. A wet sample was added to the dish which was weighed, then returned to the oven for drying overnight. The dried sample was removed from the oven, placed in a desiccator for cooling and re-weighed. The moisture content was determined by difference using the following equation:

$$\begin{aligned} &\textit{Dried dish and dried sample weight} \\ &\quad - \textit{Dry dish weight} \end{aligned} = \textit{Dry matter}$$

$$\textit{Percent moisture \%} = \frac{\textit{Wet sample weight} - \textit{Dry matter}}{\textit{Wet sample weight} \times 100}$$

2.5 PENILE STRUCTURE

To assess penile structure, mice were sacrificed and the penis was promptly inflated with fixative via a needle inserted into the main artery at the base of the penis. The

puncture site was immediately sealed with suture thread prior to excision of the penis. The erect penis samples were then processed and coated with carbon gold using standard techniques to allow analysis by scanning electron microscopy (SEM) (using a PHILIPS XL20 Scanning Electron Microscope (Oregon, USA) (Atkins, 2001). Penile structure and spines were evaluated using Image Analysis Software supplied at the Adelaide Microscopy Centre.

Spines covering the surface of the penile skin were counted in three sections per mouse: the top, mid and base sections each totalled one third of the length of the penis. The lengths of an average of 14 spines (range 8 – 21) were measured in a defined image area with a mean area of 292 nm^2 (range 510 – 119nm^2). The same plane of view was used for each sample as this method allowed the exclusion of spines that might be measured from a distance further away due to curvature of the penis.

A loosely attached superficial material, presumed to be shed epidermal sheets covering the surface of the penis, was evident during SEM assessment of some tissue. Images for epidermal sheet covering were scored using a semi-quantitative scale according to the degree of protrusion of spines evident. Four values in a scale of 0 - 100 percent of spines visible as protruding through the covering were used; 0 – 25%, 25 – 50%, 50 – 75% and 75 – 100% protrusion, to span heavy to negligible covering respectively.

2.6 CELLULAR PROLIFERATION IN TISSUES

2.6.1 Bromodeoxyuridine (BrdU) Incorporation

To detect the rate of cell turnover mice were injected intra-peritoneally with 10 µL / gram of mouse of BrdU (Appendix A). Tissues were retrieved 1.5 hours after injection, fixed overnight in 4% paraformaldehyde (Appendix A) then washed and processed into paraffin blocks (see Section 2.8.1. below).

A commercial BrdU detection kit (BD Pharmingen. California, USA) was used to visualise the BrdU stained cells and procedures were carried out according to the manufacturer's instructions. Tissues were counterstained with haematoxylin to allow visualisation of all cell nuclei (see section 2.8.2.1. below).

Where penile tissues were assessed, both the total epithelial cells and the BrdU positive cells surrounding a spine were counted on the basis of nuclear staining. The number of BrdU stained cells within the population was expressed as a percentage of the total nuclei. This analysis was repeated for the epithelial cells in the tissue between the spines. Results were given as percentages of BrdU stained cells either within or around the spines. Thigh skin tissue was analysed by placing a square grid of 1 x 1 micron squares over the images. The number of squares that overlaid the epithelial areas was deemed to be the total area and the number of BrdU stained cells within that area was recorded. Results were expressed as percentages of BrdU stained cells per area. To analyse the intestinal tract, a cross-section of the tissue was analysed. Total nuclei and BrdU positive cells were counted to calculate the percentage of BrdU positive cells per crypt.

2.6.2 Superfluous Epidermal Covering

Some of the histological samples from the thigh showed sheets of superfluous epidermal material attached to the outer surface of the skin. The area was quantified by placing a square grid of 1 x 1 micron squares over the images. The number of squares that overlaid the epidermal tissue was recorded and compared to the number of squares that overlay the superfluous epidermal covering. The span of the superfluous epidermal covering was quantified and compared to the span of the skin tissue directly adjacent to it. Results were expressed as a percentage of superfluous epidermal covering.

2.7 IMMUNOHISTOCHEMISTRY

2.7.1 Sample Preparation

Tissues including penis, skin, intestine and liver were excised from animals, dissected free of attached fat and fixed in 4% paraformaldehyde (Appendix A) overnight. The tissues were rinsed 2 times in sterile PBS and once in 50% ethanol (BDH: AnalaR. Leicestershire, United Kingdom) followed by storing the samples in 70% ethanol. When embedding the tissues, the penis, skin and intestine samples were placed vertically into paraffin, while the liver was not orientated. Tissue was sectioned at 5 µm with a microtome (HM 325: Microm. Heidelberg, Germany), floated on water heated to 35°C, then placed on APES (Appendix A) coated slides followed by air drying at 37°C overnight.

2.7.2 F4/80 Epitope Analysis

Samples were de-paraffinized through 2 changes in xylene (BDH) for 5 minutes each, then hydrated through 2 minute incubations in 90%, 70% and 50% ethanol and finally 2 minutes in MQ water. Non-specific binding of antibodies was blocked by a 2 minute incubation in 1% w/v of BSA. The BSA was flicked off before application of the primary antibody. In preliminary experiments F4/80 macrophage specific primary antibody was optimised and thereafter used at a concentration of 1:200 and diluted in PBS containing 10% normal mouse serum. This diluent was also used as a negative control in place of the primary antibody. Sections were incubated overnight in a humidified chamber at 4°C. The following morning sections were washed three times in fresh PBS and blocked for non-specific binding as above. Biotinylated rat anti-mouse secondary antibody was diluted to 1:300 (DAKO. Glostrup, Denmark) in PBS. Sections were incubated for 2 hours at room temperature in a humidified chamber before washing three times with fresh PBS. Sections were then incubated with a steptavidin / horseradish peroxidase (DAKO) complex at a 1:400 dilution in PBS for 30 minutes at room temperature. Slides were washed three times and incubated with 3,3'-diaminobenzidine (DAB: Sigma-Aldrich. Missouri, USA) for 10 minutes at room temperature. Samples were counterstained using haematoxylin for approximately 15

seconds, washed in tap water, dehydrated in two 2 minute washes of 100% ethanol and cleared in two 5 minute washes of xylene (BDH) before mounting using Depex (BDH).

2.7.3 Imaging and Analysis

Multiple fields of each tissue section were randomly selected and recorded using an Olympus BH-2 microscope mounted with a Nikon D100 digital camera. Video image analysis software, Video Pro (Leading Edge Software. Adelaide, Australia), was used to analyse the images. The proportion of cells that displayed positive staining was determined by counting the total area of haematoxylin staining and the number of cells stained with DAB (F4/80 positive) within an average of 13 fields (range 7 – 21) per mouse. Results were calculated and expressed as a percentage positivity within the total counterstained area. A single value for each mouse was calculated as the mean of all values for that individual.

2.8 PERITONEAL CELL PHAGOCYTOSIS

2.8.1 Apoptotic Thymocyte Preparation

The thymus tissue of 5 – 8 week old C57BL/6 mice was excised and thymocytes were released into a single cell suspension by mechanical disruption on ice in 1 mL of serum free complete RPMI-1640 medium (cRPMI) containing 2 mM L-glutamine, penicillin (100 IU/ml), and streptomycin (100 µg/ml) (Sigma-Aldrich). Cells were washed by centrifuging at 1200 rpm for 5 minutes, removing the supernatant and replacing with fresh medium. Thymocytes were incubated in cRPMI containing 1 µM of dexamethasone (Sigma-Aldrich) for 4 hours at 37ºC in 5% CO_2 to induce apoptosis. Viability was determined by trypan blue exclusion where 0.4% trypan blue was mixed with an equal volume of thymocytes prior to viewing through a light microscope. The percentage of viable thymocytes that excluded the dye was calculated. Apoptosis was then confirmed via flow cytometry following the manufactures instructions (kit # APOAF. Sigma-Aldrich). Briefly, thymocytes were

resuspended in 500 μL of Binding Buffer in separate plastic tubes at a concentration of 1 x 10^6 cells / mL. This was followed by the addition of 5 μL of Annexin V FITC Conjugate (AV-FITC) and 10 μL of propidium iodide solution (PI) to each tube and incubation in the dark at room temperature for 10 minutes. FACS analysis (FACScan: BD Biosciences. California, USA) was used to count 30,000 events (cells) from each sample. Apoptotic positive thymocytes were positive for AV-FITC staining and negative for staining with PI.

Remaining thymocytes were then washed by centrifuging at 1200 rpm for 5 minutes, removing the supernatant and replacing with cRPMI. To label the cells they were incubated with excess CFSE (Molecular Probes. Oregon, USA) at 37ºC. An unlabelled sample was stored at 4ºC as a control for later flow cytometric analysis. Cells were then washed twice in cRMPI and counted. 100 μL of CFSE-labelled cells were removed and stored at 4ºC as a control for subsequent flow cytometric analysis. The remaining cells were washed by centrifuging at 1200 rpm for 5 minutes, removing the supernatant and replacing with fresh cRPMI to a concentration of 1 - 2 x 10^7 thymocytes per 200 μL.

2.8.2 Phagocytic Capacity

Phagocytosis was evaluated in peritoneal cells by uptake of CFSE labelled apoptotic thymocytes. Mice received 1 - 2 x 10^7 CFSE labelled thymocytes by intra-peritoneal injection and 30 minutes later, were sacrificed and peritoneal cells collected by lavage. The peritoneal cells were washed twice by centrifuging at 1200 rpm for 5 minutes, removing the supernatant and replacing with ice cold HBSS containing 5 mM of EDTA (Appendix A). The peritoneal cells were then counted using a hemocytometer.

2.8.3 Fluorescence Activated Cell Sorter Analysis (FACS)

Before staining, nonspecific binding of antibodies was blocked with anti-FcR antibody 2.4G2 (Fc-Block: BD PharMingen) which was diluted 1:100 in cold FACS wash buffer (Appendix A). Diluted Fc-Block (10 μL) was applied to cells followed by incubation at

room temperature in the dark for 20 minutes. Cells were subsequently washed by centrifuging at 1200 rpm for 5 minutes, removing the supernatant and replacing with FACS wash buffer. Cells were then stained with either 25 µg / mL of PE conjugated F4/80 (eBioscience) or 50 µg / mL of PE conjugated CD11b (BD PharMingen). 50 µg / mL of isotype matched control PE conjugated rat IgG2a (BD PharMingen) was used to ensure the absence of non-specific binding. All antibodies were diluted to the required volume in FACS wash buffer; 10 µL of F4/80 and IgG2a were added per sample while 50 µL of CD11b was used. Antibodies were incubated with cells for 20 min on ice before being washed twice in PBS by centrifuging at 1200 rpm for 5 minutes. Cell pellets were resuspended in 1 ml of FACS fix (Appendix A) and samples were covered in foil and stored at 4ºC until flow cytometric analysis the following day (FACScan: Becton Dickinson. California, USA).

2.8.4 FACS Data Analysis

Data were collected using CellQuest Pro (BD Biosciences) and analysed using Summit software (version 3.1; DakoCytomation. Glostrup, Denmark). Representative scatter plots depict cell populations according to their size (FSC) and complexity (SSC). Ten thousand events were recorded for each sample of peritoneal cells. The positive population was defined by a gate to exclude > 95% of non-specific cells labelled with IgG.(Figure 4-2A) and were then assessed for their F4/80 and CSFE positivity to identify macrophages and phagocytic cells, respectively. The positive population was divided into four gated areas. Two gated areas, capturing cells not labelled with F4/80 were excluded (F4/80- / CFSE- and F4/80- / CFSE+). This left two cell populations, the F4/80+ CFSE+ population (eg: macrophages which had phagocytosed the CFSE labelled apoptotic thymocytes) and the F4/80+ CFSE- population (eg: macrophages which had not phagocytosed thymocytes) (Figure 4-2B). Populations of CD11b+ CFSE+ and CD11b+ CFSE- cells were established using the same approach (Figure 4-2C). The number of phagocytic cells was expressed as a percentage of total F480+ and CD11b+ cells.

2.9 GENE EXPRESSION ANALYSIS OF TISSUES

2.9.1 Primer Design

Peritoneal cells were evaluated for expression of mRNAs encoding the NOS isoforms, nNOS, iNOS and eNOS. The testes were evaluated for mRNAs encoding the steroidogenic enzyme HSD3β1 and the TGFβ receptor 1 (TβRI). The housekeeping genes, 18S and β-actin, were included as reference genes. All mRNAs were quantified using real time – reverse transcription polymerase chain reaction (RT-PCR).

Primers for the NOS, β-actin and 18S genes were designed and validated by others (Ingman, 2002), (Annes, 2004). Primers for HSD3β1 and TβR1 were designed using murine cDNA sequences obtained from the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). Primer sequences were analysed with Primer Express software to ensure they spanned the intron - exon boundaries of the gene. They were then subjected to the NCBI 'BLAST' analysis to confirm their theoretical specificity. Following synthesis (by Sigma Genosys) they were reconstituted to 100 mM with MQ water. Primers were validated by finding the optimum combination of primer and cDNA dilutions that gave a single dissociation peak along with the lowest signal to noise ratio. The PCR products were separated by gel electrophoresis on a 2% agarose gel and product size was confirmed using size markers.

2.9.2 Optimisation of RT-PCR Conditions

Optimal reaction conditions were determined by serially diluting primer sets and control cDNA. Those providing the highest signal and efficiency in the absence of primer dimers and contaminating peaks were found. The dilutions at which reaction linearity was confirmed, by assessing log of the dilution versus cycle threshold (CT) value, were used for subsequent sample analysis (Lin Reg software) (Ramakers,

2003). The primer sequences, nucleotide position and optimal reaction conditions are shown (Table 2-2).

Gene	Position	Primer Sequence	Primer Concentration µM	cDNA dilution
iNOS	883(fwd)	5'CATCAGGTCGGCCATCACT	0.5	10
	968(rev)	5'CGTACCGGATGAGCTGTGAA		
nNOS	2586(fwd)	5'CACCCCAACTCTGTGCAAGAG	0.5	1
	2717(rev)	5'CGGTCCAGTACTTTCAAAGTTGTCT		
eNOS	1467(fwd)	5'CAGGCATCACCAGGAAGAAGA	0.5	10
	1558(rev)	5'CACACGCTTCGCCATCAC		
HSD3β1	235(fwd)	5'GGACAAAGTATTCCGACCAGAAAC	0.5	10
	337(rev)	5'CAGGCACTGGGCATCCA		
TβRI	802(fwd)	5'AGACAACAAAGACAATGGGACATG	0.5	10
	896(rev)	5'CAGTGTATCTATTCAAGTAATCGAAAAGG		
β-actin	24(fwd)	5'CGTGGGCCGCCCTAGGCACCA	0.5	100
	209(rev)	5'ACACGCAGCTCATTGTA		
18S	674(fwd)	5'ACGTCTGCCCTATCAACTTTCG	0.125	1280
	799(rev)	5'CTGCCTTCCTTGGATGTGGTA		

Table 2-2 Primers for real time RT-PCR.

2.9.3 Peritoneal Cell Preparation

Cells were collected after cervical dislocation of mice and lavage of the peritoneal cavity with 5 mL of cold HBSS containing EDTA. After gentle massage of the abdominal wall for one minute, the peritoneal cell population was retrieved and centrifuged for 5 minutes at 1,000 rcf at 4ºC.

2.9.4 RNA Extraction

Tissue samples (approximately 50 – 200 µg) were retrieved from -70ºC storage and homogenised in 500 µL of Trizol (Invitrogen Life Technologies. California, USA) containing 10 µg of carrier glycogen using an Ultra-Turrax (Ika. Staufen, Germany). 200 µL of chloroform was then added and the samples were gently vortexed before placing on ice and centrifuging at 11,000 rpm for 15 minutes at 4°C. The aqueous RNA phase was transferred into a new eppendorf tube and precipitated overnight at -20°C with an equal volume of isopropanol.

2.9.5 DNA Removal

The precipitated samples were washed and re-suspended prior to DNase treatment. Samples were centrifuged at 11,000 rpm for 30 minutes at 4°C and the supernatant poured off, with care not to disturb the pellet. The pellet was washed twice with 500 µL of cold 70% ethanol and centrifuged at 11,000 rpm for 10 minutes at 4°C. The pellet was air dried for approximately 30 minutes before being dissolved in 50 µL of sterile MQ water.

DNase master mix comprising of 25 µL 5x DNA buffer, 1 µL RNase inhibitor and 5 uL DNase I (Roche Diagnostics) was added to the samples and incubated at 37°C for 1 hour. To stop the reaction, 250 uL of water equilibrated phenol:chloroform:isoamyl alcohol at a ratio of 25:24:1 was added, the samples were vortexed then centrifuged at 11,000 rpm for 10 minutes at room temperature. The DNA-free RNA supernatant was collected and precipitated with 2.5 x sample volume of 100% ethanol and 0.1 x

sample volume of 2 M sodium acetate then stored at –20°C until reverse transcription.

2.9.6 Reverse Transcription

Precipitated RNA was washed and re-suspended for quantification. RNA was centrifuged at 11,000 rpm for 20 minutes at 4°C. The supernatant was carefully poured off and the pellet washed twice with 500 µL cold 70% ethanol by centrifuging at 11,000 rpm for 10 minutes at 4°C. The pellet was air dried for approximately 30 minutes before being dissolved in 50 µL of sterile MQ water. The quantity and purity of RNA in the sample was determined by spectrophotometry (DU-50Ò Spectrophotometer: Beckman Instruments Inc. California, USA) at wavelengths of 260 and 280 nm. The quantity of RNA was determined by the following equation:

$$OD260 \times 40 \times dilution \quad = \quad RNA\ \mu g / \mu L$$

To reverse transcribe the RNA, 1 µg was combined with 2 µL of 500 µg / mL random hexamers (Geneworks. Adelaide, South Australia) and incubated for 5 minutes at 65°C, then chilled on ice for a further 5 minutes. Reverse transcription was performed using Superscript III (Invitrogen Life Technologies). A master mix was prepared [8 µL 5 x RT buffer (Invitrogen Life Technologies), 2 µL DTT, 4 µL 10 mM dNTPs (AMRAD Pharmica Biotech: Victoria, Australia), 2 µL Superscript III Enzyme (Invitrogen Life Technologies) per sample] and 16 µL of this was added to each sample, and incubated for 5 minutes at 25°C, followed by 60 minutes at 50°C. The reaction was stopped by heat inactivation for 10 minutes at 70°C. Reverse transcribed samples were chilled on ice and stored at -20°C until real-time RT - PCR application.

2.9.7 Real - Time Reverse Transcription - Polymerase Chain Reaction (RT - PCR)

Quantitative RT-PCR was performed in real-time (using a Geneamp 5700 thermal cycler: Applied Biosystems. California, USA). The reaction mix consisted of (10 µL)

1x SYBR green PCR master mix (Applied Biosystems), 4 µL water, 4 µL cDNA template, 1µL of forward and reverse primers in a 20 µL reaction mixture. Non template controls (NTC), in which cDNA was omitted (and water was substituted) were included as were template controls in which control cDNA was used. Samples were amplified in duplicate using a two step thermo-cycling protocol in 1 cycle of 50ºC for 2 minutes, 95ºC for 10 minutes, 40 cycles of 95ºC for 15 seconds and 60ºC for 1 minute.

The PCR data was retrieved and a background fluorescence baseline was defined at cycles 6–15. A threshold was manually set midway in the exponential phase of the amplification curve and gene expression data normalised to the housekeeping genes was quantified using the 2-ΔΔCT method (Applied Biosystems Bulletin #2). The amount of PCR product in the TGFβ1+/+ control group was multiplied by a constant to give the mean an arbitrary value of 100. This allowed the data for all samples to be expressed relative to the mRNA content of control tissue.

2.10 SURGICAL INSERTION OF MINI-OSMOTIC PUMPS

TGFβ1-/- male mice received 9.6 µg / day rhLTGFβ1 (recombinant human latent TGFβ1, R&D Systems) in a low-endotoxin vehicle of 2% BSA in PBS. TGFβ1+/+ and other TGFβ1-/- male controls received 2% BSA vehicle only (Appendix A). All pumps were primed prior to sub-cutaneous insertion by submersing in sterile saline at 37ºC for 4 hours. Treatment continued for 14 days at a flow rate of 0.22 µL / hr, using 14 day-100 µL mini osmotic pumps (ALZET: Durect Corp. California, USA).

The pumps were inserted using aseptic technique incorporating 70% ethanol. Halothane (Rhone Merieux. Lyon, France) at 2% in combination with 800 mL / min oxygen was used as an anaesthetic. 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 high on the shoulder of the mice. The incision was closed firmly with a 9 mm

wound clip (Becton Dickinson) and mice were given 100% oxygen until showing signs of consciousness.

2.11 BEHAVIOURAL STUDIES

Males were placed individually in clear cages and observed for 1 hour between 13.00 hours and 16.00 hours. Time spent sleeping, genital grooming, and erectile activity were recorded under these conditions.

2.11.1 Sleeping Studies

The amount of time spent asleep and awake were recorded to determine if this influenced the amount of time spent on reproductive behaviours.

2.11.2 Genital Grooming

A genital grooming event was recorded each time the mice bent over on their hind legs to lick their genitals in the absence of an erection.

2.11.3 Erectile Activity

An erection was counted when the mouse bent over to groom and hold the genitals as the penis emerged. Where erections were recorded for the mice with pump inserts, the tests were performed shortly prior to blood sampling. Some mice were treated with agents immediately prior to evaluation, to attempt erection induction. Sildenafil Citrate (Viagra: Pfizer. New York, USA) was intra-peritoneally injected at various doses, while apomorphine (Sigma-Aldrich) was sub-cutaneously injected into to the shoulder region of other mice at various doses (Appendix A). Occasionally some tests were performed in the presence of ovariectomised females (see 2.11.3.1 below).

2.11.3.1 OVARIECTOMISED FEMALE MICE

Female mice (CBA-F1, 8 - 10 weeks old) had their ovaries removed to allow artificial stimulation into estrous by administration of exogenous steroid hormones. They were then used to induce sexual responses in male erectile studies.

Avertin (15 µL) was injected intra-peritoneally to sedate the female mice. After shaving the dorsal skin and swabbing with 70% ethanol, the skin on the back and the muscle layer beneath was excised to expose the ovaries. The fat pad adjacent to the kidneys was externalised and secured to the exterior surface of the mouse to gain access to the ovaries. The ovaries were gently prised away from the surrounding tissue. The fat pad was returned to its original location and the cavity was closed by suturing. The skin wound was stapled closed and the mice were allowed to recover.

To stimulate estrous, 2.8 µg (in 100 µL) of estradiol benzoate in sesame oil was sub-cutaneously injected two days prior to testing. On the test day 50 µg (in 100 µL) of progesterone in sesame oil was sub-cutaneously injected (Appendix A). Five hours later females were placed in cages with a wire mesh that acted as a physical barrier, separating them from male mice. Behaviour of the male mice was then recorded.

2.11.4 Mating

Males with pump implants were subjected to two rounds of identical mating experiments. These were executed on days 7 and 14 of the treatment protocol where the day of pump insertion = day 1 (Figure 6-1). All mating studies were preceded by a non-contact adaptation period of two days with adult cycling CBA/F1 females to allow males to become accustomed to the female scent. Contact between males and females were excluded during these times by placing the mice in Whitten cages.

For the mating experiments, immature (3 - 4 weeks old) female CBA/F1 mice were treated with exogenous gonadotrophins to induce estrous and receptivity to males. Females were administered 5 IU PMSG (Folligon: Intervet. Boxmeer, The

Netherlands) at 1200 hours on day 5 or 12 and then 5 IU hCG (Chorulon: Intervet) at 1200 hours on days 7 or 14 (Appendix A). Female receptivity was confirmed when uterine tract flushings were positive for cornified epithelial cells (see 2.13. below). The females were introduced into the male's cage 2:1 at 2300 hours on day 7 or 14 under red light. The first 2 hours of contact were recorded from overhead using a video camera (NV-DS38: Panasonic. Yokohama, Japan).

Mating behaviour of the males was quantified from the videos using the following criteria.

1) Mounts: identified by standing with both hind legs on the ground and use of the fore legs to secure the female.

2) Intromissions: identified by the male mounting the female, securing her and thrusting his pelvis for up to 20 seconds.

3) Ejaculations: identified as an intromission event followed by the male rolling over to one side with the female for a few seconds.

The total number of mounts is given as the combined number of mounts, intromissions and ejaculations. The total number of intromissions is given as the combined number of intromissions and ejaculations. Mounting and intromitting are also calculated on a per minute basis, where the total mounts and intromissions respectively, were divided by the interval in minutes between co-caging with females until 1) ejaculation occurred or 2) until 120 minutes passed, whichever was shortest.

$$\frac{\textit{Total Number of Mounts}}{\textit{Minutes Until Ejaculation or 120}} = \textit{Mounts per Minute}$$

This value takes into account the reduced interest of males in further sexual activity following a successful ejaculation. The number of mice displaying ejaculation activity is expressed as a percentage of the total mice assessed. The number of ejaculations

achieved by each mouse within the two hour observation period is expressed as ejaculations per hour.

2.12 UTERINE TRACT FLUSHINGS

On the morning following mating, the females were checked for the presence of a vaginal plug or sperm in the uterine tract flushing, indicating whether or not the mice had successfully mated. Sterile PBS (15 µL) was gently pipetted into the vagina then recovered for microscopic evaluation. The cellular contents of the smear were examined under phase contrast microscope to determine the stage of ovarian cycle and female receptivity as outlined below.

Proestrous >50% round epithelial cells

Estrous >90% cornified epithelial cells

Metestrous 50-90% cornified epithelial cells, rest leukocytes

Diestrous >50% leukocytes

The smears were also examined for the presence of sperm.

2.13 QUANTITATION OF SPERM

Some male mice were sacrificed and both cauda epididymis were placed into HBSS at room temperature. The cauda were dissected and contents were squeezed out with fine forceps into 500 µL of fresh HBSS. The cauda tissue was removed from the dish and the sperm diluted 1:10 or 1:20 in fresh HBSS then placed on a haemocytometer and cover-slipped for sperm counting by phase contrast

microscopy. Samples were counted in duplicate. To calculate the number of sperm per mouse, the following equation was used.

$$\frac{Count}{Haemocytometer\ Depth\ (mm^3)} = Sperm\ per\ mm^3$$

$$\frac{Sperm\ per\ mm^3}{dilution \times initial\ volume} = Sperm\ per\ Organ$$

2.14 SACRIFICE OF MICE RECEIVING TGFB1 VIA MINI-OSMOTIC PUMPS

2.14.1 Sample Collection

On the day of the treatment protocol, the males were anesthetised for cardiac blood collection and were then sacrificed by cervical dislocation for peritoneal cell harvesting and tissue collection. Penis, testes and liver were taken and placed in liquid nitrogen for RNA or protein analysis. Additional samples from those tissues were placed in fixative overnight for subsequent paraffin embedding and immunohistochemical analysis (all methods are described above).

2.15 STATISTICAL ANALYSIS

Where Shapiro-Wilk tests showed a parameter to be not normally distributed, a non-parametric analysis was performed. Where normal distribution was evident, a parametric analysis was performed. Non-parametric analyses were performed using the Mann Whitney U method (2-tailed significance values), while parametric analyses were performed by one-way ANOVA followed by t-test with Bonferroni correction.

Associations between parameters in milk were assessed by Pearson correlation, while the Spearman's rho correlation test was used for testing correlations between the genotype groups.

All data is expressed as the mean ± the standard error of the mean (SEM), unless otherwise stated. Statistical significance was assumed at $p < 0.05$ and all tests were analysed using SPSS version 12 (Apache Software Foundation).

Chapter 3:
The Role of TGFβ1 in Male Reproductive Behaviour and Secondary Sex Characteristics

3.1 INTRODUCTION

Initial investigations in male TGFβ1 null (TGFβ1-/-) mice found them to be infertile due to deficiencies at multiple levels (Ingman, 2002) (Ingman, 2007). The TGFβ1-/- males had lower serum testosterone levels and could not effectively intromit with normal receptive females. As a result, ejaculation and subsequent sperm transfer failed to take place. Histological examination of penis tissue sections stained with haematoxylin and eosin did not detect any morphometric differences within the tissue structure. Investigations into the morphometry of androgen-insensitive mice were found to have undersized penis tissue structures such as the os bone and urethra (Murakami, 1987), suggesting that while low, the testosterone levels in TGFβ1-/- mice are enough to allow normal penile development.

Expression of TGFβ throughout development has been shown for many organs and systems, the penis being no exception. TGFβ1 expression in the penis of rats was found to peak at two months of age and then decrease over the next four months to initial levels (Gelman, 1998). These authors also found evidence that TGFβ functioned to inhibit penile growth in young rats as demonstrated by a significant reduction (38%) in penile shaft weight when exogenous TGFβ1 was delivered directly to the penile corpus cavernosum. To evaluate the effects of deficient endogenous TGFβ, earlier analyses on TGFβ1-/- mice measured the ratio of penis weight to body weight. Those mice showed a slight increase in relative penile weight (mean ± SEM = 13.7 ± 7.7%) compared to the TGFβ1+/+ control mice, although this did not reach significance (Ingman, 2002). Further investigations into the tissue structure of the penis in these mice were not carried out in that study.

The surface of the mouse penis is characteristically covered with keratinised spines which project toward the base of the shaft. In addition, a ‘cup’ structure is evident at the tip of the penis. It is purported that these distal cups are involved in depositing the copulatory plug while the spines aid in anchoring the males to the females during intromission as well as in stimulating the ejaculation response (Breed, 2003). In support of this, penile spines that overlie dermal tactile receptors were found to

influence copulatory behaviour in marmosets (Dixson, 1991). Penile spines are composed primarily of keratin whose production is influenced by TGFβ in other cell types (Freedberg, 2001) (Doi, 2003). In addition, the development of penile spines is proposed to be androgen dependent (Dixson, 1991) (Lester R. Aronson) and thus may be regulated by TGFβ1 indirectly. Penile spine morphometry was therefore analysed in TGFβ1-/- mice, to determine whether spinal alterations may be contributing to intromission failure in these mice.

Erectile behaviour and genital grooming were also investigated in TGFβ1-/- mice. Mice normally and frequently perform oral grooming of the whole body including the genital area. The amount of grooming that specifically occurred in the genital area has been reported for rats but was not found to correlate with testosterone levels (Moore, 1986). As limited information is available on this subject, genital grooming was assessed in the current study to ascertain if this behaviour is associated with erectile behaviour. This could occur since both erectile activity and sexual behaviour are influenced by dopamine and areas of the brain that receive inputs directly from the hypothalamus (Liu, 1997) (Magoul, 1994). To achieve erections, adequate testosterone levels appear to be important as demonstrated by androgen withdrawal and replacement studies in rodents and humans (Baba, 2000) (Caretta, 2005). Thus, deficient serum testosterone concentrations in TGFβ1-/- mice may affect their capacity to achieve an erection and effectively intromit. Additionally, a TGFβ1 deficiency could interfere with brain function, perhaps through its interaction with the neurotransmitter dopamine, which stimulates the erectile response (Hull, 2004) (Sarkar, 2005) (Farkas, 2003).

Biochemically, penile erections occur when intracellular calcium levels fall and local muscles relax (Figure 3-1). Two pathways that reduce calcium are known. Firstly, the nitric oxide / cyclic guanosine monophosphate (NO / cGMP) pathway, which requires that available NO regulated by NOS, binds to guanylate cyclase which stimulates cGMP production causing calcium levels to fall. Secondly, the vasoactive intestinal peptide / cyclic adenosine monophosphate (VIP / cAMP) pathway, which requires that available VIP binds to adenylate cyclase which stimulates cAMP production causing calcium levels to fall (Steers, 2002). Pharmacological agents that reduce intracellular calcium have been widely used in rodents to induce erections (Elabbady,

1995). Sildenafil citrate (Viagra) acts peripherally within the penis to mediate erections, while others such as apomorphine act centrally through the nervous system (Holmes, 2000) (Montorsi, 2003) (Andersson, 2001). Viagra assists erections through inhibiting the breakdown of cGMP in the penile tissues (Carson, 2005). Apomorphine acts by targeting dopamine receptors to cause the release of NO and VIP thereby reducing intracellular calcium levels (Steers, 2002).

Interestingly, earlier work with TGFβ1-/- mice had revealed that intromission behaviour was not improved following Viagra administration (Ingman, 2002). This was attributed to a decreased capacity of the TGFβ1-/- mice to induce mRNA expression of penile endothelial nitric oxide synthase (eNOS), a precursor to NO in the erection pathway. However attainment of erection was not evaluated in that study (Ingman, 2002). To establish the ability of TGFβ1 deficient mice to achieve erections with or without pharmaceutical manipulation of the cGMP and dopamine receptor pathways, they were directly assessed for erectile activity following apomorphine or Viagra treatment.

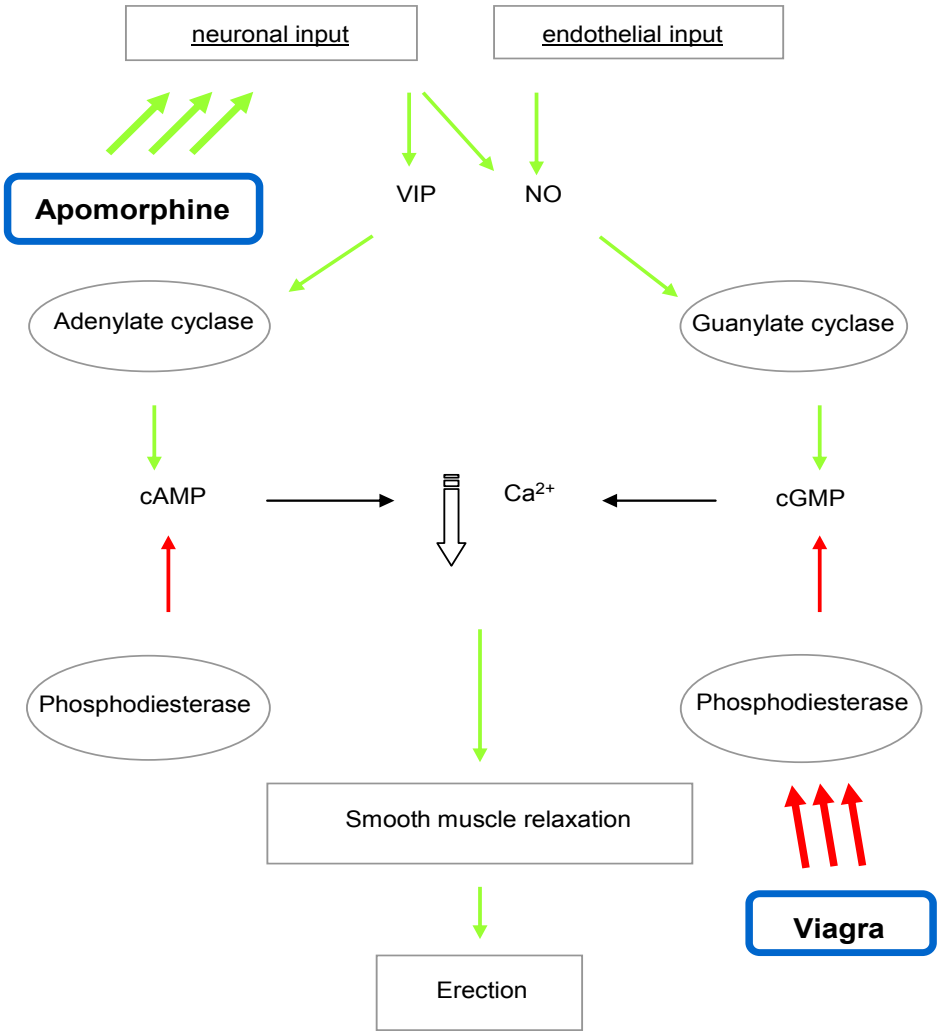


Figure 3-1. Biochemistry of the erectile response.

Nitric oxide (NO) and vasoactive intestinal peptide (VIP) are released from neuronal and endothelial tissues. VIP activates adenylate cyclase which causes a rise in cAMP and a reduction in intracellular calcium leading to smooth muscle relaxation and an erection. NO activates guanylate cyclase which causes a rise in cGMP and a reduction in intracellular calcium leading to smooth muscle relaxation and an erection. Phosphodiesterase inhibits erections by breaking down cAMP and cGMP levels. Red arrows indicate inhibitory actions and green arrows indicate stimulatory actions. Apomorphine acts on neuronal cells to stimulate VIP and NO release, while Viagra inhibits phosphodiesterase activity.

3.2 ANALYSIS OF PENILE STRUCTURE IN TGFB1-/- MICE

To evaluate the cause of reduced intromission behaviour by the TGFβ1-/- mice, the whole penile organ was examined for structural or morphological defects. To perform this, the mice were sacrificed and the penis was promptly inflated with fixative via a needle inserted into the main artery at the base of the penis. The puncture site was immediately sealed with suture thread prior to excision of the penis. The penis samples were then processed and coated with carbon gold to allow analysis by scanning electron microscopy. The structure and penile spines were evaluated using Summit software (Methods 2.5).

3.2.1 Gross Morphology

Scanning electron microscopic images of the penis revealed the gross anatomy of the organs including penile spines. The distal cups were comparable between genotypes although a considerable degree of variation within both genotype groups was observed (Figure 3-2). Despite this, no differences in height were found between the TGFβ1+/+ (mean ± SEM = 3.6 ± 0.4 mm) and TGFβ1-/- (3.2 ± 0.4 mm) genotype groups and similarly no differences in width were found between the TGFβ1+/+ (2.7 ± 0.3 mm) and TGFβ1-/- (2.6 ± 0.2 mm) genotype groups (data not shown).

3.2.2 Spine Density and Spine Length

Penile spines were clearly visible in the scanning electron microscopic images at a higher magnification, allowing quantitative analysis. Measurements of spines were taken from three areas along the length of the penile shaft. The length was divided equally into top, mid and base regions. Spines were counted in a defined image area and their lengths were measured from the base of the spine to the tip (Methods 2.5).

The penile spines in the TGFβ1+/+ mice showed a progressive decrease in spine density from top to base, however the TGFβ1-/- mice did not exhibit the same

progressive decrease (Figure 3-3A). There were no differences in average spine density between genotype groups, when all regions were combined (Figure 3-3B).

When spine lengths were measured in the three regions of penile tissue from TGFβ1+/+ mice, those at the base of the penis were significantly longer than those in their top region. This did not occur in the TGFβ1-/- mice (Figure 3-4A). There were no differences in average spine length between genotype groups, when all regions were combined (Figure 3-4B).

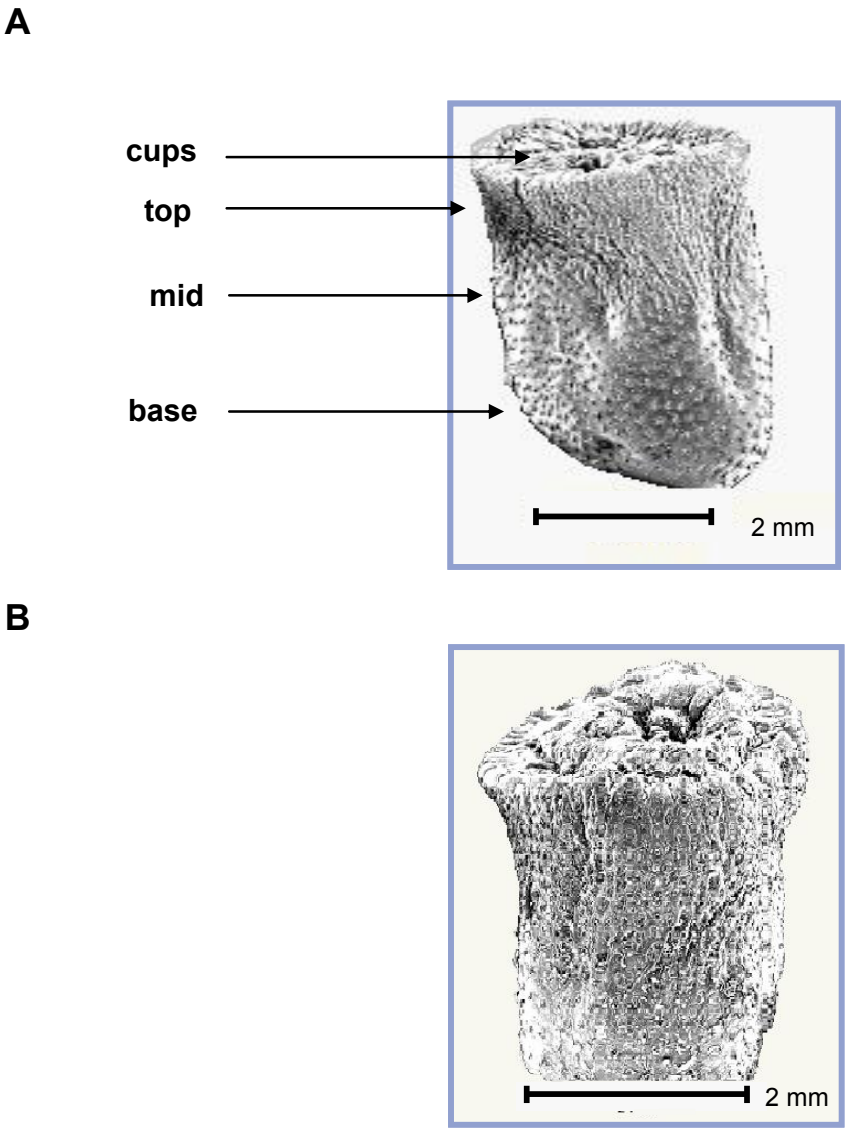


Figure 3-2 Scanning electron microscopy images of the penis in TGFβ1+/+ (A) and TGFβ1-/- (B) male mice.

Distal cups, the top, mid and base regions along the shaft are shown. These images are representative of n=6 TGFβ1+/+ and n=6 TGFβ1-/- males.

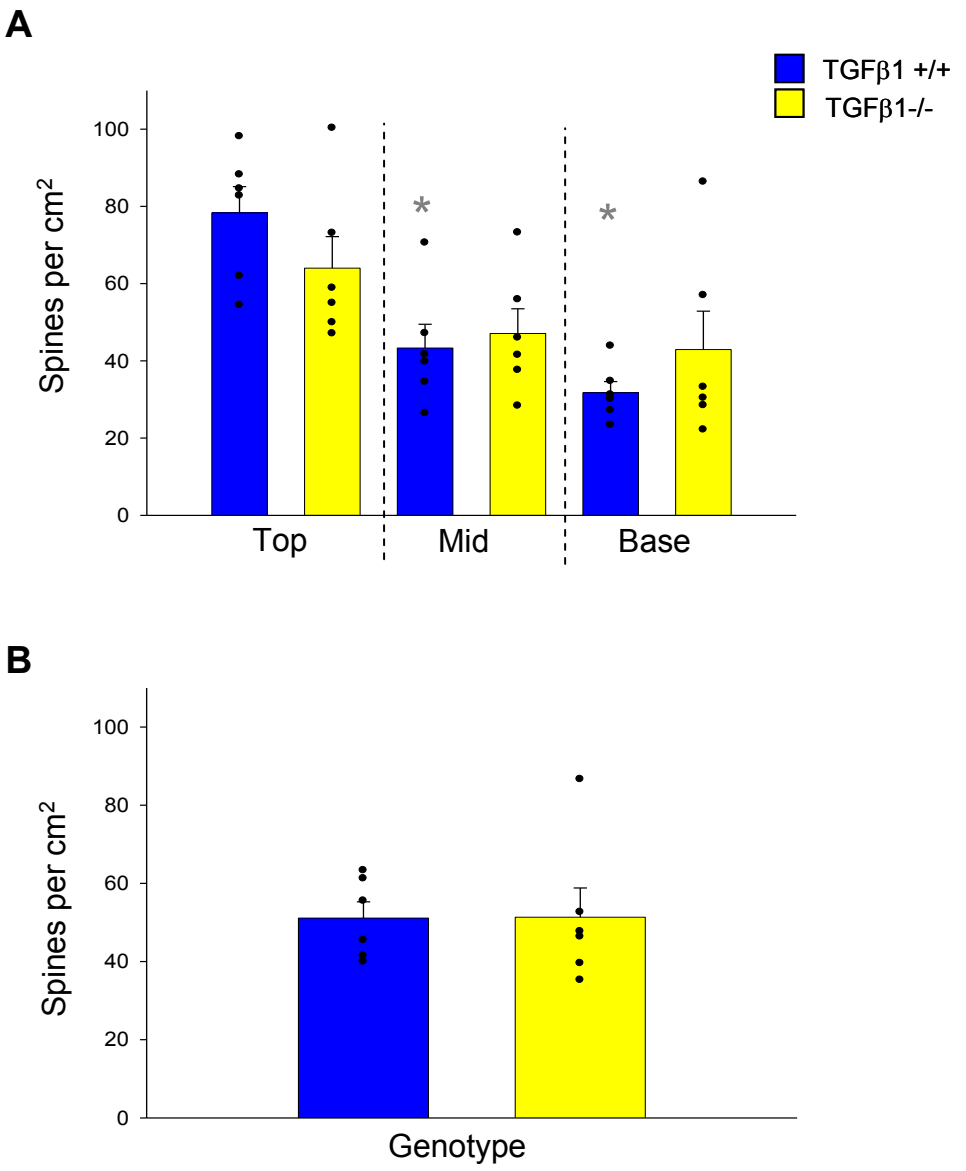


Figure 3-3 Spine density in penile regions (A) and spine density overall (B).

Spines were visualised by scanning electron microscopy and counted in each region of the penis. Bar plots show the mean (± SEM), dot plots show individual sample values. * denotes significant difference from the top region of the TGFβ1+/+ controls ($p<0.05$). Data were analysed using the ANOVA Bonferroni method for (A) and the t-test for (B). n = 6 for each group.

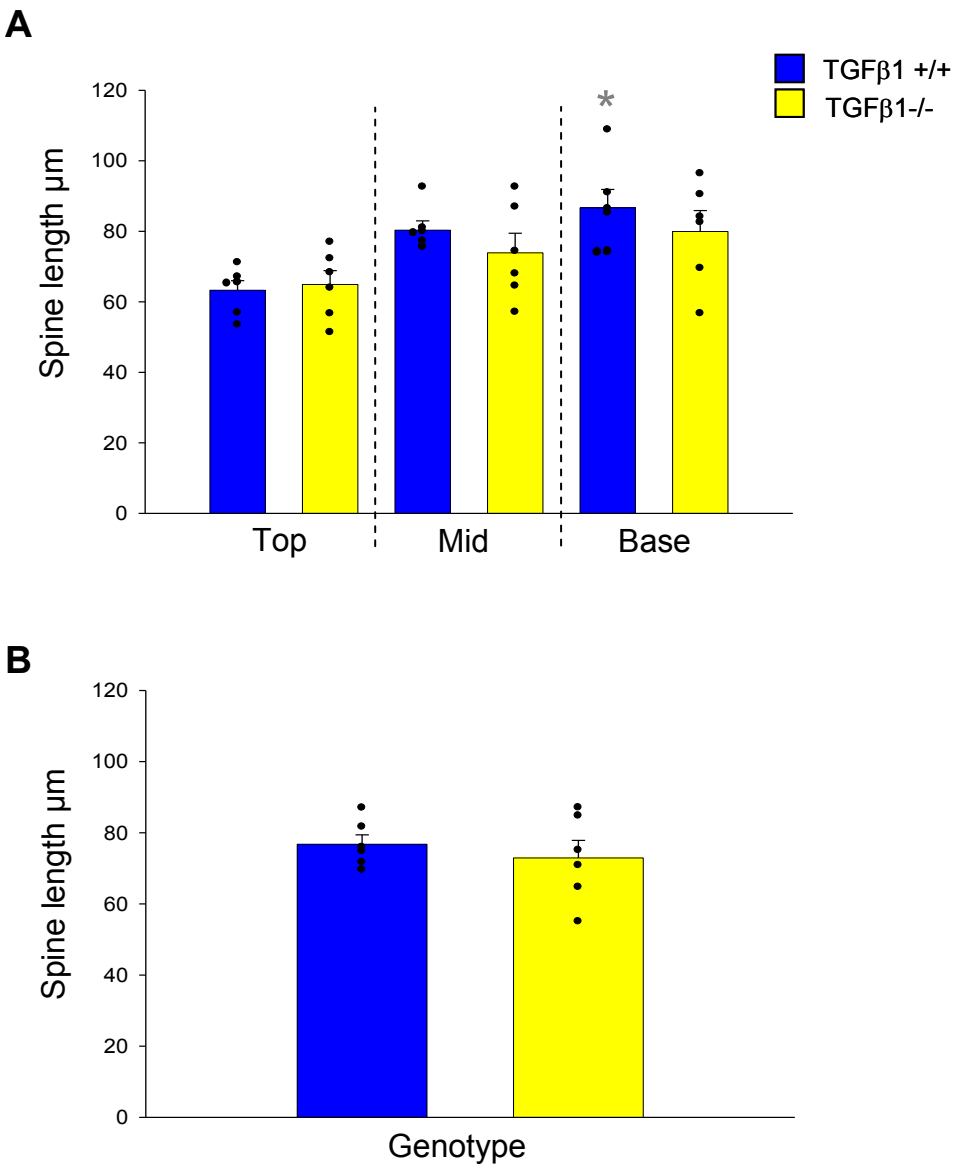


Figure 3-4 Spine lengths per penile region (A) and spine lengths overall (B).

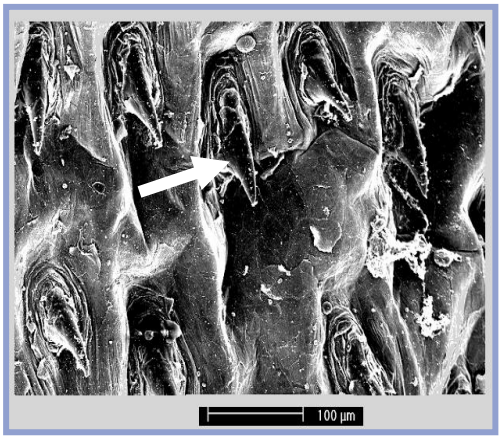
Spines were visualised by scanning electron microscopy and their lengths were measured in each region of the penis. Bar plots show the mean (± SEM), dot plots show individual sample values. * denotes significant difference from the top region of the TGFβ1+/+ controls ($p<0.05$). Data were analysed using the ANOVA Bonferroni method for (A) and the t-test for (B). n = 6 for each group.

3.2.3 Spine Protrusion

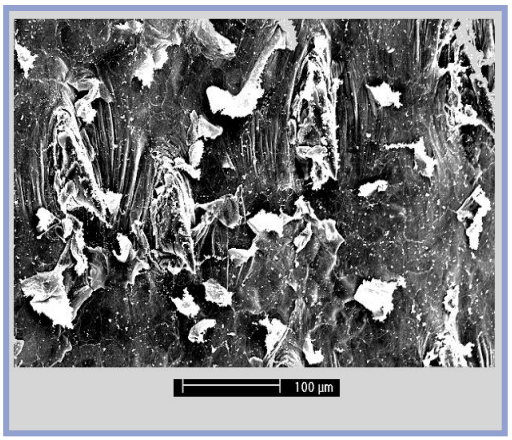
While undertaking the scanning electron microscopy study, it was noted that the exterior penile surface of some TGFβ1-/- mice was covered in material which appeared to obstruct the protrusion of spines (Figure 3-5). The material resembled the keratinized flakes of skin that slough away during normal skin re-generation, although more evidence would be needed to verify this.

Two of six TGFβ1-/- mice had this material completely covering some areas of the exterior surface. To further investigate this observation, the surfaces were arbitrarily measured and compared using 4 ranges in a scale of 0 - 100 percent of spine protrusion through the covering, 0 – 25%, 25 – 50%, 50 – 75% and 75 – 100%. Results revealed that the TGFβ1-/- mice exhibited a significant reduction in spine protrusion compared to the TGFβ1+/+ mice in each penile region (Figure 3-6A) as well as overall (Figure 3-6B). Subsequent experiments sought to determine whether the accumulated surface material was the result of altered epithelial cell proliferation.

A



B



C

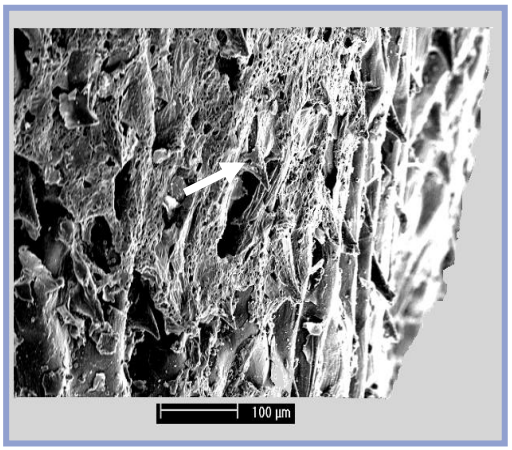


Figure 3-5 Penile spines in TGFβ1+/+ (A) and TGFβ1-/- (B, C) mice.

Spines were visualised by scanning electron microscopy. Representative examples of fully protruded spines (large arrow) and partly protruded spines (small arrow) are shown.

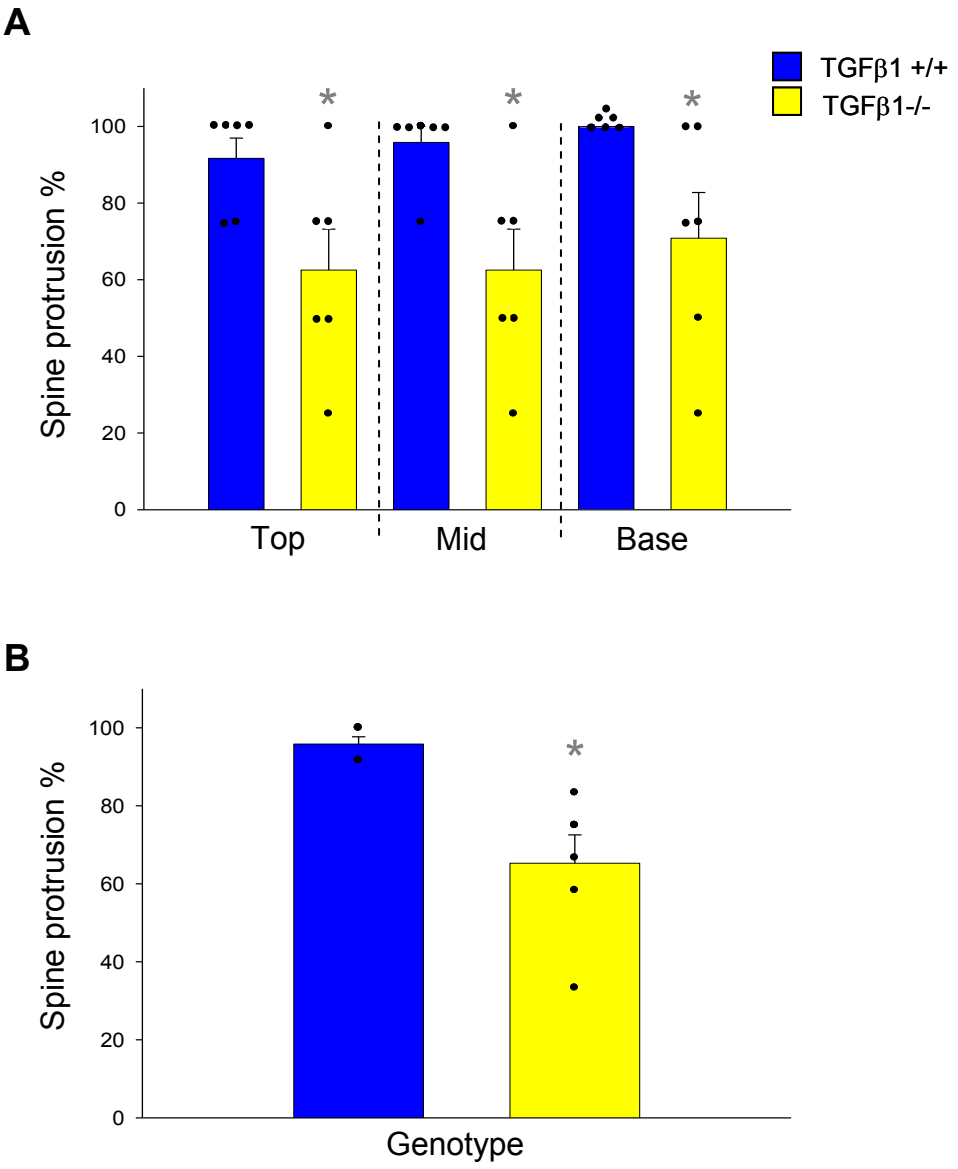


Figure 3-6 Spine protrusion per penile region (A) and spine protrusion overall (B).

Spines protruding through the epidermal covering were visualised by scanning electron microscopy and their protrusion measured in each region of the penis, using a scaled index as described in the methods. Bar plots show the mean (± SEM), dot plots show individual sample values, * denotes significant difference from the TGFβ1+/+ controls ($p<0.05$). Data were analysed using the Mann Whitney U method. n = 6 for each group.

3.3 EPITHELIAL CELL PROLIFERATION

TGFβ plays a major role in suppression of proliferation in various epithelial cell populations (Moses, 1991), therefore it seemed reasonable that endogenous TGFβ1 deficiency might cause increased epithelial cell proliferation to occur. In fact, studies of wound healing in TGFβ1-/- mice have shown an acceleration in wound re-epithelialsation compared to their littermate controls (Koch, 2000). Thus it was hypothesized that the excessive epithelial covering on the penis of TGFβ1-/- mice may be due to excessive epithelial cell proliferation.

To determine if superfluous epithelial proliferation is an underlying cause of the diminished spine protrusion in the TGFβ1-/- mice, proliferation was analysed using the bromo-deoxyuridine (BrdU) method. BrdU was intra-peritoneally injected into mice, and left for 90 minutes to allow incorporation into newly proliferating cells. Tissue sections were then assessed by immunohistological examination to detect BrdU positive cells (Methods 2.6.1 and Methods 2.7).

Since epithelial turnover specifically in penile skin has never been described, skin from the thigh and a region of the intestinal epithelium were also included in these analyses for comparison. The intestinal tract is known as a site of considerable cell turnover due to the rapid epithelial regeneration needed to sustain the highly dynamic digestive system. Newly formed epithelial cells originate in the intestinal crypts and differentiate, then migrate to the villi tip where they are eventually shed (Bullen, 2006).

3.3.1 Epithelial Cell Proliferation in the Penis

A representative image of BrdU staining within the penis skin is shown (Figure 3-7). BrdU positive cells were counted and compared to the total number of cells within two epithelial compartments, 1) adjacent to the spines and 2) between the spines. No differences in the ratio of BrdU positive cells to total epithelial cells were seen between genotype groups, irrespective of their location either adjacent or between

spines (Figure 3-8A). Also, when the two areas were combined, no significant difference was evident between genotype groups (Figure 3-8B).

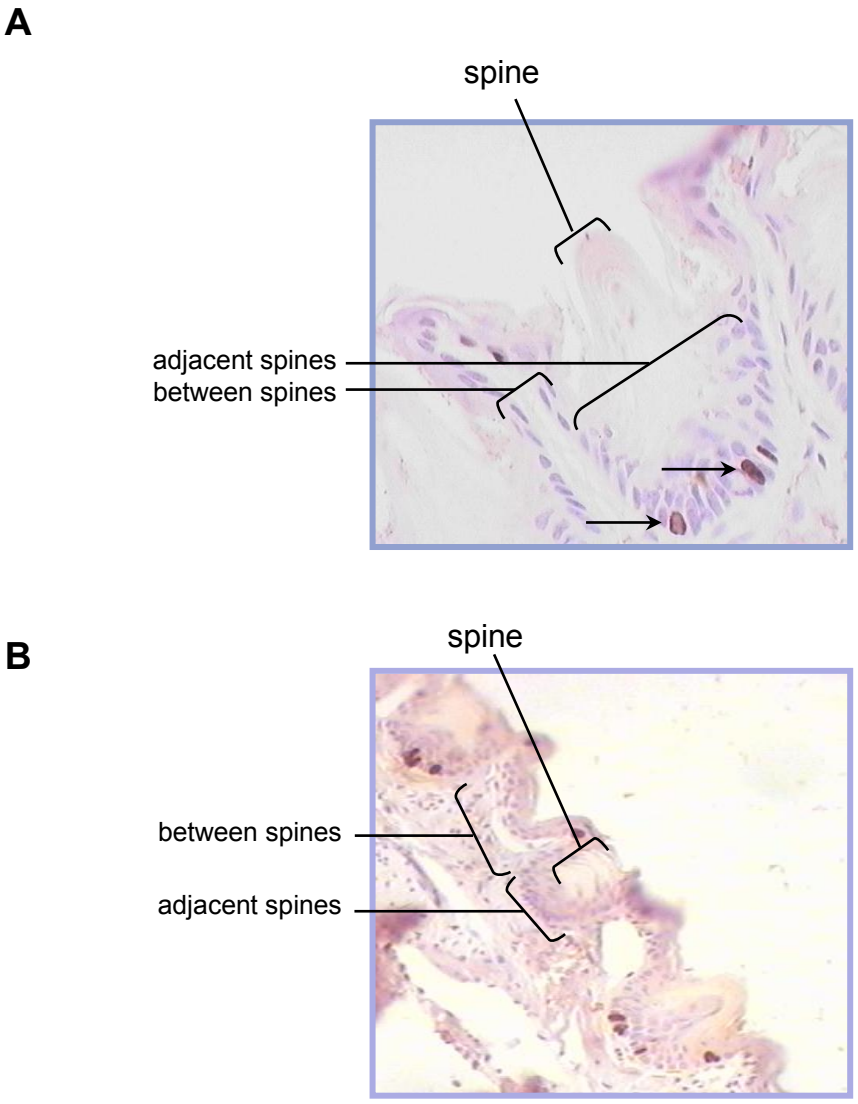


Figure 3-7 Image of proliferating cells in the penis skin.

Penis skin images is representative of all TGFβ1+/+ and TGFβ1-/- samples tested. Magnification x40 (A). Magnification x20 (B). Spines, areas adjacent to and between the spines, and BrdU stained cells are shown (arrows).

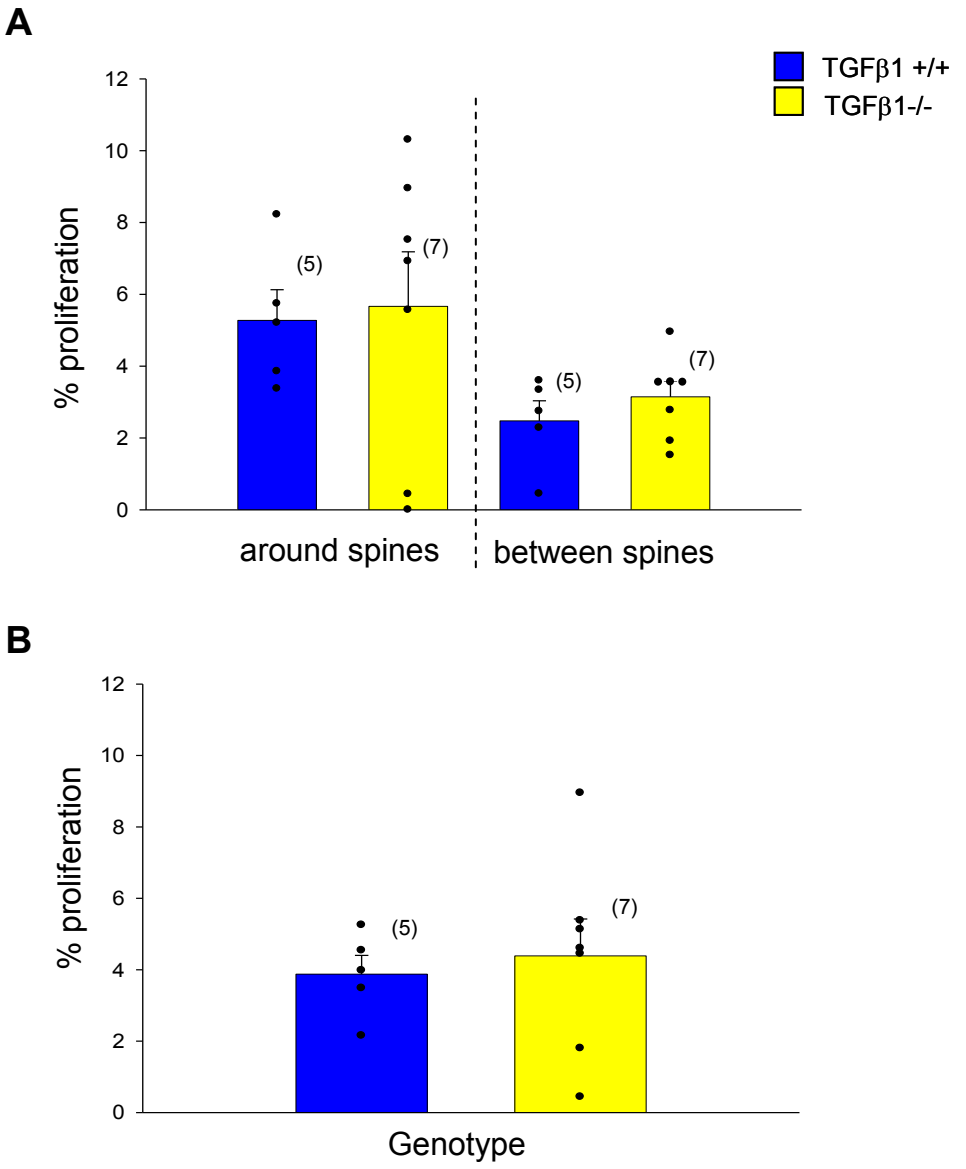


Figure 3-8 Percentage of proliferating cells in each area of the penis (A) and overall (B).

Proliferating cells were stained with BrdU and visualised by immunohistochemistry and compared to the total number of cells. Bar plots show the mean (± SEM), dot plots show individual sample values, no significant differences were found ($p>0.05$). Data were analysed using the ANOVA Bonferroni method for (A) and the t-test for (B). n = 6 for each group.

3.3.2 Epithelial Cell Proliferation in Thigh Skin

Cellular proliferation was also analysed in the skin of the inner thigh area as this site is adjacent to the genital area. When the tissue was assessed, BrdU positive cells were scattered throughout the epidermis and around the sebaceous glands associated with hair follicles. In some mice, material resembling superfluous epithelium was observed on the outer surface of the skin (Figure 3-9). The number of BrdU positive cells within the epithelial areas of the epidermis and hair follicles was counted, these were then expressed as a percentage of the total number of cells in those areas. The area of the superfluous epithelium was determined by placing a grid over the image and was expressed as a percentage of the area immediately underlying the epidermis (Methods 2.6.2).

No differences in the number of BrdU positive cells were detected between genotype groups (Figure 3-10A) and despite one TGFβ1-/- mouse showing a large quantity of superfluous material on the exterior surface, no statistically significant differences between the genotype groups were found irrespective of an outlier in the TGFβ1-/- group (Figure 3-10B).

3.3.3 Epithelial Cell Proliferation in the Intestine

The intestinal crypts of TGFβ1-/- mice were assessed for epithelial proliferation (Methods 2.6.1 and 2.7). A representative image of the crypts containing BrdU positive cells is shown (Figure 3-11A).

The number of BrdU positive cells per crypt was counted, however no changes in epithelial proliferation between the genotype groups were found (Figure 3-11B).

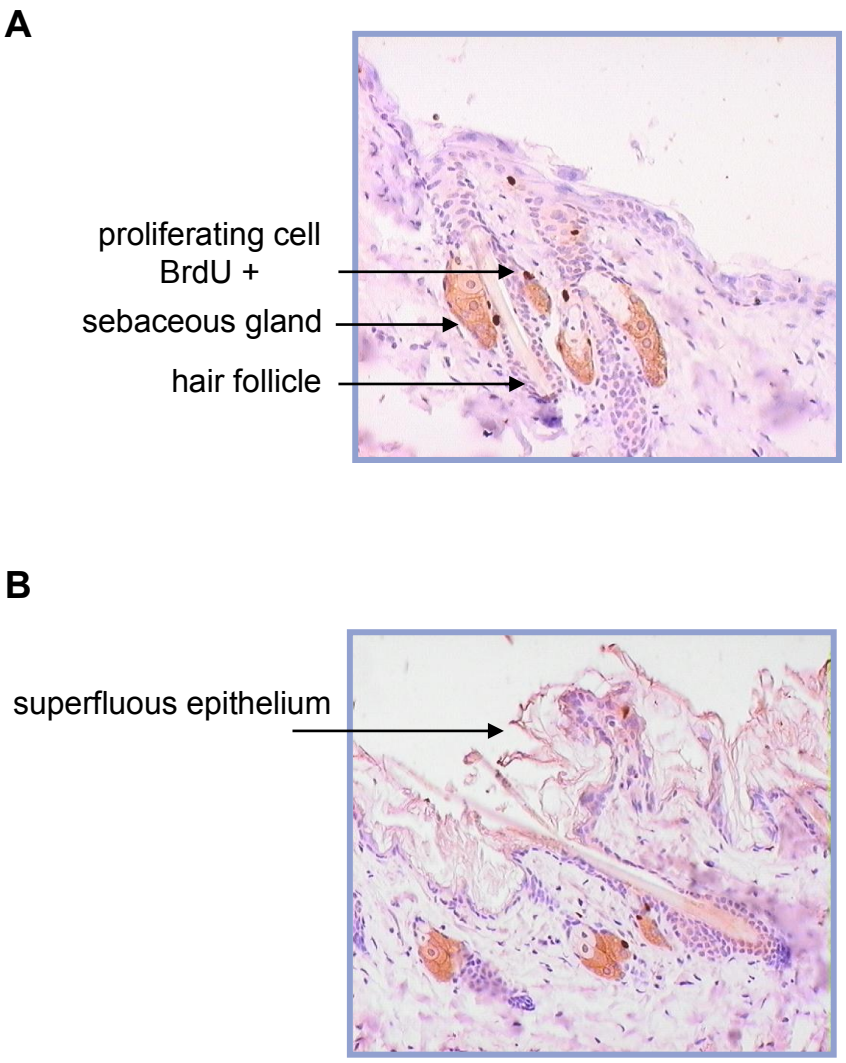


Figure 3-9 Images of BrdU staining in the skin from the thigh region.

Typical image of the thigh region of n=5 TGFβ1+/+ mice (A) and an image of the thigh region in the TGFβ1-/- mouse that was responsible for the outlier where the percentage of superfluous epithelium was measured (B). Arrows indicate the hair follicle, the sebaceous glands, BrdU stained cells and superfluous epithelium.

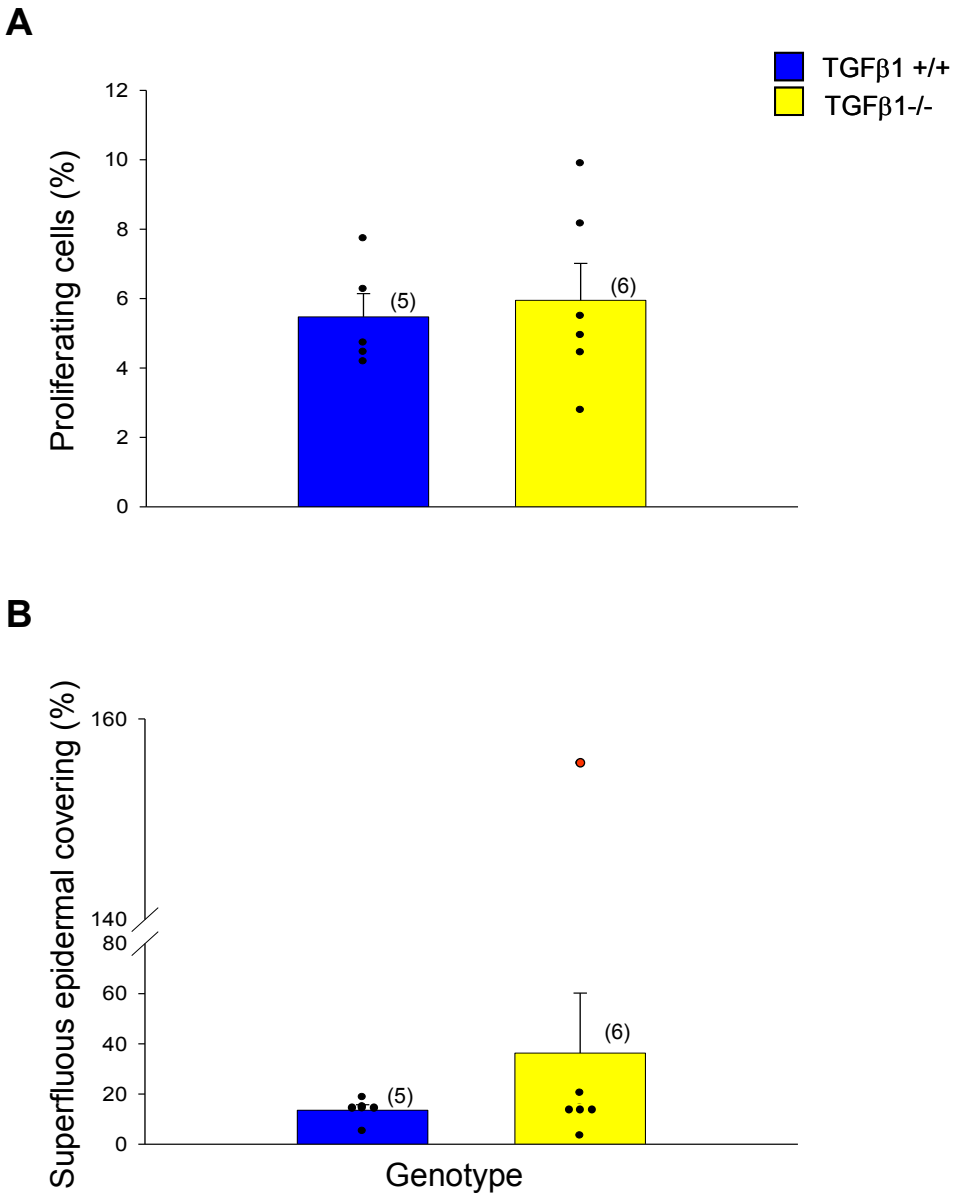


Figure 3-10 Percentage of proliferating cells in thigh skin (A), percentage of superfluous epidermal covering (B).

Proliferating cells were stained with BrdU and visualised by imunohistochemistry then compared to the total number of cells. The area of keratinized epithelium was compared to the surface area of the skin. Bar plots show the mean (± SEM), dot plots show individual sample values (red = outlier), no significant differences were seen ($p>0.05$). Data were analysed using the t-test for (A) and the Mann Whitney U method for (B). n is shown in parentheses.

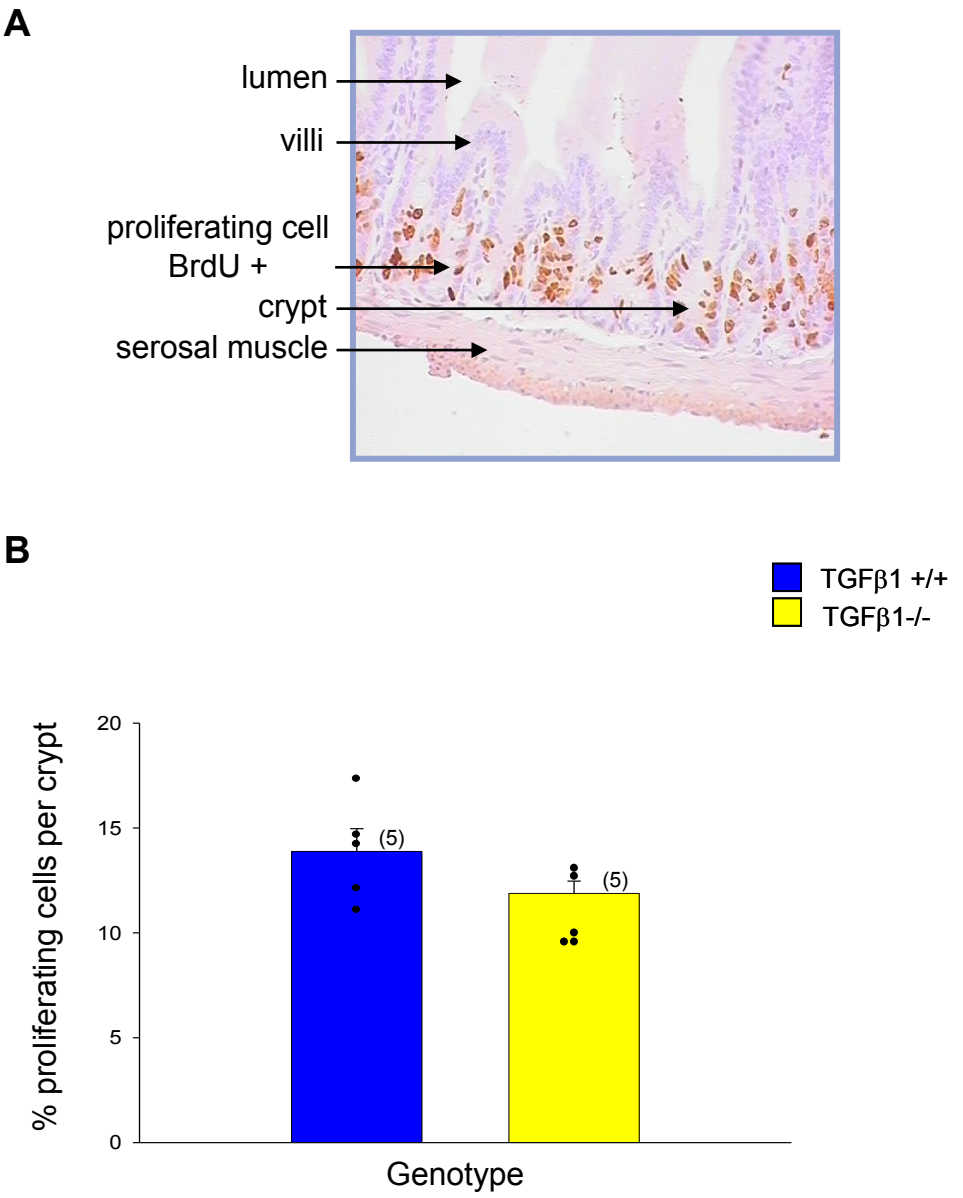


Figure 3-11 Image of proliferation within the intestinal tract (A) and percentage of proliferation per intestinal crypt (B).

Intestinal tract image is representative of all TGFβ1+/+ and TGFβ1-/- samples tested. Arrows indicate the serosal muscle, the crypt, the villi, the lumen and the BrdU stained cells. Cells were stained with BrdU and assessed by immunohistochemistry and are expressed as the percentage of proliferating cells per crypt. Bar plots show the mean (± SEM), dot plots show individual sample values, no significant differences were seen ($p>0.05$). Data were analysed using the Mann Whitney U method. n is shown in parentheses.

3.4 REPRODUCTIVE BEHAVIOURS IN TGFB1-/- MICE

To examine the behaviour of male mice in the absence of female contact, they were placed individually in cages with clear plastic walls and observed for 1 hour between 13.00 hours and 16.00 hours. Genital grooming and erectile activity were recorded and are described here as reproductive behaviours during isolation.

The amount of time spent asleep and awake were also recorded to determine if this influenced the amount of time spent on reproductive behaviours. Per hour, the TGFβ1+/+ mice slept between 0 and 30 minutes (mean ± SEM = 8.0 ± 2.7 minutes, n = 15) and the TGFβ1-/- mice slept between 0 and 40 minutes (9.3 ± 2.9 minutes, n = 22). Genotype groups were not statistically different. As the mice exhibited similar patterns of wakefulness, it was concluded that sleep would not influence time spent on reproductive behaviours.

3.4.1 Genital Grooming

A genital grooming event was recorded each time the mice bent over on their hind legs, in the absence of an erection, to lick the genital area. A similar number of TGFβ1+/+ (88.9%) and TGFβ1-/- (83.3%) mice performed at least one genital grooming act, with the number of grooming acts ranging from zero through to twelve. However a smaller proportion of TGFβ1-/- mice (55.6%) displayed greater than one grooming act compared to the TGFβ1+/+ mice (88.9%) (Figure 3-12A).

A significant difference between genotype groups was also observed when grooming was expressed on a per hour basis. The TGFβ1-/- mice (mean ± SEM = 2.4 ± 0.6 grooming events per hour) were found to perform genital grooming acts less frequently than the TGFβ1+/+ mice (6.4 ± 1.3 grooming events per hour) (Figure 3-12B).

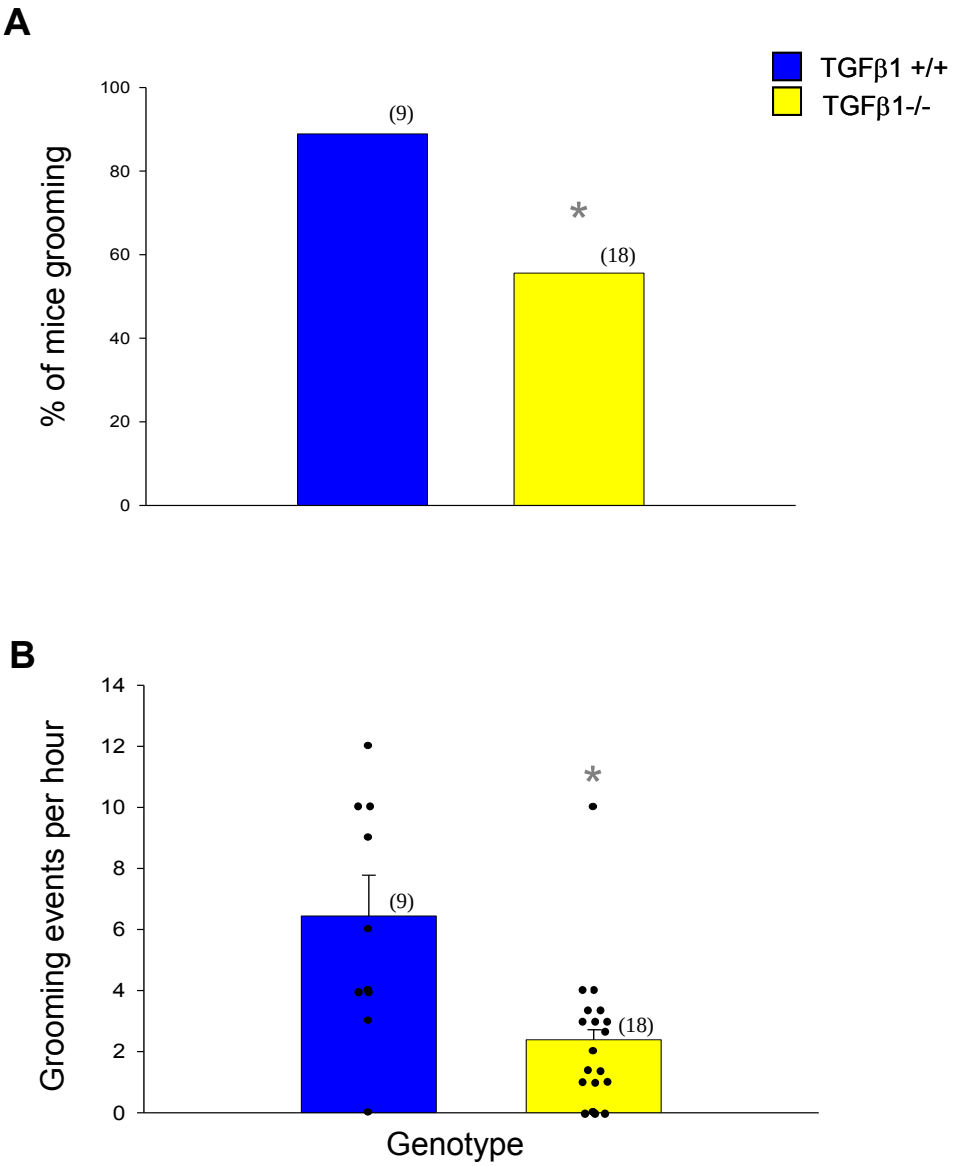


Figure 3-12 Percent of mice grooming genitals (A) and grooming events per hour (B).

Grooming was visualised by placing mice in clear cages and observing for one hour. Both genotypes displayed greater than 1 grooming event during the one hour observation period. Bar plots show the mean (± SEM), dot plots show individual sample values, * denotes significant differences ($p<0.05$). Data were analysed using the Chi square method for (A) and the Mann Whitney U method for (B). n is shown in parentheses.

3.4.2 Erectile Activity

To determine if erectile dysfunction contributes to the infertility of the TGFβ1-/- mice, the number of erections achieved within an hour was counted. Erectile activity was recorded when an erect penis was visible and was distinct from a genital grooming act. Erections persisted for up to 30 seconds (Figure 3-13). Induced erections were assessed following Viagra or apomorphine administration, while spontaneous erections were assessed in the absence of any pharmaceutical intervention. In one experiment, ovariectomised, estrous induced females were caged with the males but the sexes remained physically separated by a wire mesh to allow only the female scent to influence the behaviour of the males (Methods 2.11).

While not directly examined here, it is worth noting that occasionally during animal handling a condition termed 'protruded penis' was observed only in the TGFβ1-/- mice. This condition presented as a reddened and dry penis which remained protruded from the foreskin but in a flaccid, non-erect state. It also appeared painful as evidenced by a reluctance of the animal to move about as well as flinching behaviour when the protruded penis was touched. This condition was noted on perhaps 10 occasions over the course of this study amongst a total of approximately 200 – 300 male TGFβ1-/- mice. Interestingly, a similar condition has been observed in CSF-1 deficient csf^{op}/csf^{op} mice (Ingman, 2007). Sometimes the condition would subside and return to normal, although typically it persisted until sacrifice.

3.4.2.1 SPONTANEOUS ERECTILE ACTIVITY

Initially, mice were observed without any pharmaceutical interventions or presence of females to measure occurrence of spontaneous erections. Both TGFβ1+/+ and TGFβ1-/- genotypes were capable of achieving erections unassisted (Figure 3-14A). The number of TGFβ1-/- mice achieving at least one erection in the one hour observation period (28.6%), was half that of the TGFβ1+/+ mice (58.3%). Also, more TGFβ1+/+ mice achieved greater than 1 erection (4 of 12 mice) within the hour compared to the TGFβ1-/- mice (1 of 21 mice). Analysis of the erection rate per hour showed that compared to TGFβ1+/+ controls (mean ± SEM = 1.2 ± 0.4 erections per

hour), the TGFβ1-/- mice (0.4 ± 0.2 erections per hour) displayed significantly fewer erections per hour (Figure 3-14B).

In addition, the frequency of spontaneous erections in the TGFβ1+/+ mice was negatively correlated with the frequency of genital grooming investigated above ($r = -0.819$, $p = 0.007$). This relationship was not evident in TGFβ1-/- mice.

Figure 3-13 Image of mouse displaying erectile activity.

Mice were placed in clear cages and observed for one hour. An erection was counted when the mouse bent over on his hind legs and held and groomed the penis until the erection subsided.

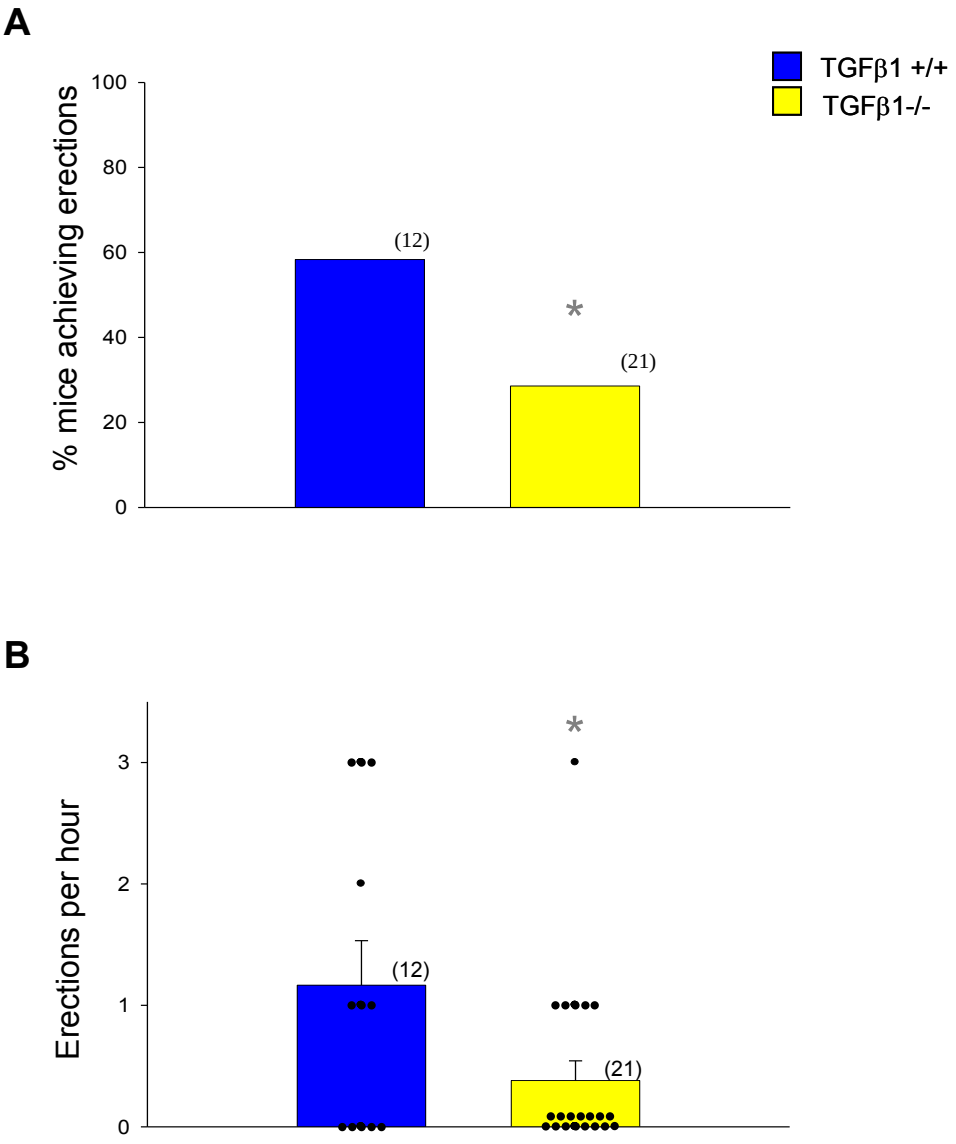


Figure 3-14 Percent of TGFβ1+/+ and TGFβ1-/- mice achieving erections in 1 hour (A) and frequency of erections per hour (B).

Erections were visualised by placing mice in clear cages and observing for one hour. Bar plots show the mean (± SEM), dot plots show individual sample values, * denotes significant difference from the TGFβ1+/+ controls ($p < 0.05$). Data were analysed using the Chi square method for (A) and the Mann Whitney U method for (B). n is shown in parentheses.

3.4.2.2 INDUCED ERECTILE ACTIVITY

3.4.2.2.1 Sildenafil citrate (Viagra) treatment

Next, the effect of pharmacological intervention on erection behaviour was evaluated. The TGFβ1+/+ mice, were used to evaluate the efficacy of administering varying doses of Viagra to induce erections. The effective doses in men range from 0.3 to 1.5 mg / kg and the effective dose in mice is reported to be 1.0 mg / kg (McCullough, 2002). The doses tested in this study approximated those and were 0.1, 1.0 and 1.6 mg / kg. The dose containing 1.0 mg / kg of Viagra was administered in conjunction with exposure to ovulating female mice. Surprisingly, observations following Viagra treatment revealed an average erection rate of less than one per hour for all treatments including those where female exposure occurred. The 0.1 mg / kg dose resulted in one mouse (n = 4) attaining an erection (mean ± SEM = 0.25 + / - 0.25 erections per hour), the 1.6 mg / kg dose resulted in four mice (n = 14) attaining an erection (0.29 + / - 0.13 erections per hour) and erections in the group treated with 1.0 mg dose and female exposure (n = 4) were not detected (Figure 3-15A). Thus there was no significant increase in the number of erections per hour induced by Viagra treatment compared with untreated male mice ($p>0.05$) and further use of this agent in TGFβ1-/- mice was not undertaken.

3.4.2.2.2 Apomorphine treatment

TGFβ1+/+ mice were also used to evaluate the efficacy of administering various doses of apomorphine to induce erections. Doses administered were 2, 10 and 50 μg / kg, which approximated the previously published effective doses of 1.1 and 7.5 μg / kg for mice (Rampin, 2003) and 80 μg / kg for rats (Elabbady, 1995). The lower dose of 2 μg / kg was also administered to a group of mice following prior sexual experience with females. Observations following treatments revealed an average erection rate of one, or less than one, per hour. The 2 μg / kg dose resulted in 0.93 + / - 0.34 erections per hour (n = 14), while the 10 μg / kg dose resulted in 1.0 + / - 0.0 erections per hour (n = 4), erections in the 50 μg / kg dose group (n = 4) were not detected and the group with prior sexual experience that received the 2 μg / kg dose

resulted in 1.0 + / - 0.32 erections per hour (n = 5) (Figure 3-15B). Since there was no significant increase in erection rate induced by apomorphine compared with untreated male mice (p>0.05), further evaluation of this agent in TGFβ1-/- mice was not undertaken.

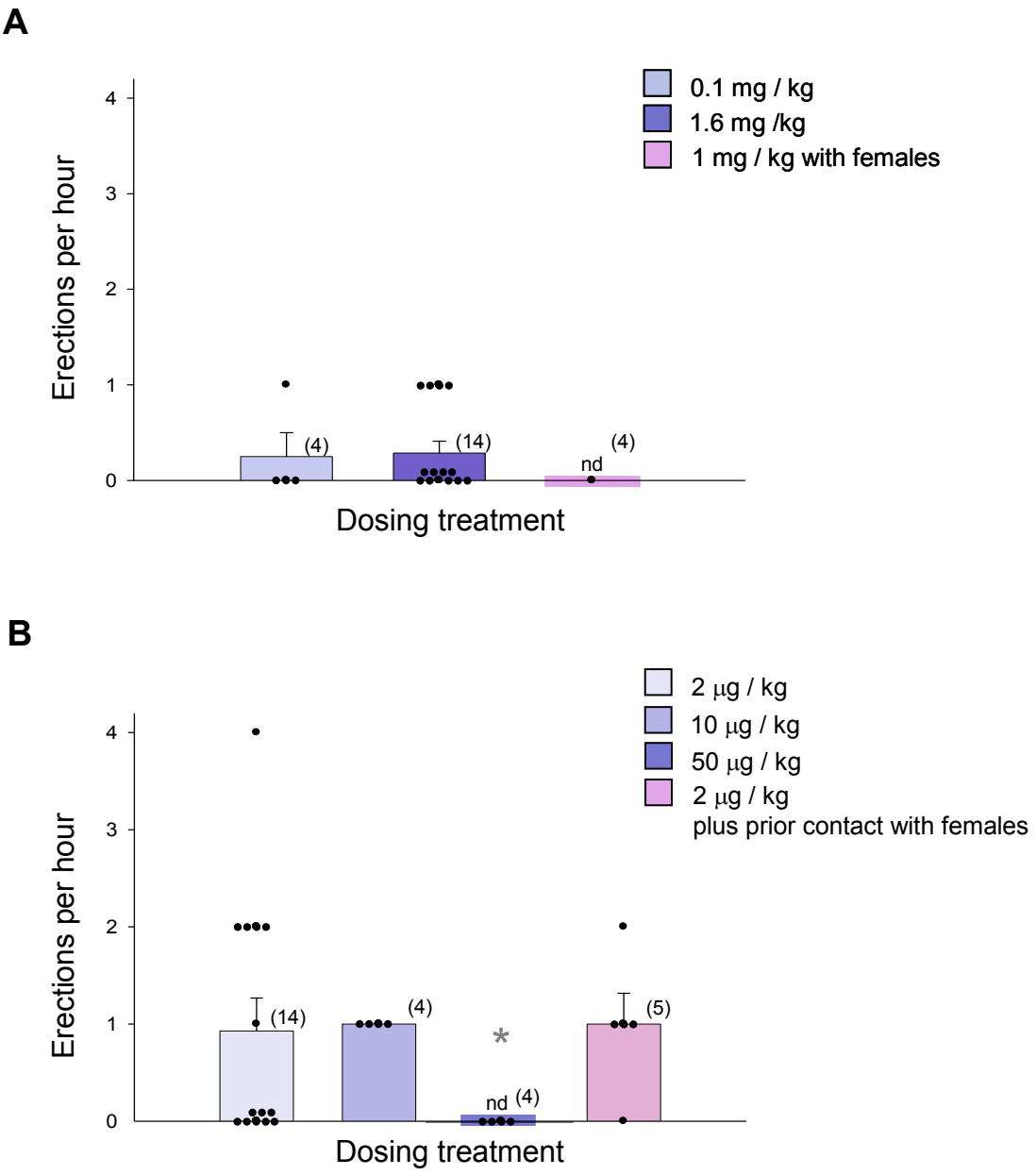


Figure 3-15 Number of erections achieved by TGFβ1+/+ mice, following treatment with Viagra (A) and apomorphine (B).

Erections were assessed by placing mice in clear cages and observing for one hour. Bar plots show the mean (± SEM), point plots show individual sample values. * denotes significant difference (p<0.05). Data were analysed using the Mann Whitney U method. n is shown in parentheses. nd denotes not detected.

3.5 DISCUSSION

The experiments described above using TGFβ1-/- mice were undertaken to analyse whether TGFβ1 is required for normal male reproductive behaviour and development of secondary sex characteristics.

3.5.1 Normal Penile Structure in TGFβ1-/- Mice

Overtly, there was no effect of TGFβ1 deficiency on penile structure suggesting that with normal erectile function the mice should have the capacity to intromit and lodge a copulatory plug effectively. The ability of these mice to produce a normal ejaculate and copulatory plug would be facilitated by the presence of seminal vesicle glands of normal weight and structure (Ingman, 2002).

3.5.2 Altered Spine Distribution and Morphology in TGFβ1-/- Mice

The TGFβ1+/+ mice displayed a different pattern of spine distribution and morphometry than the TGFβ1-/- mice. The TGFβ1+/+ mice were found to have a significant graduated reduction in the density of spines between the top of the shaft to the base region, while the length of those spines significantly increased over the same regions. This distribution pattern may be indicative of different functions of the spines in the different regions. It could be speculated that anchoring to the female might require more spines in the top region, while longer spines in the base region might be associated with the ejaculation response. Thus the TGFβ1-/- mice might experience more difficulty anchoring to the female but if achieved might be able to ejaculate. Overall, spine density and size did not differ between genotype groups.

Studies in cats have found that when the penile spines are completely abrogated, copulatory behaviour is less frequent but still occurs (Lester, 1967). As the reduced testosterone and deficiency in TGFβ1 in these mice has not completely abrogated the formation of spines, normal copulatory behaviour would be expected to be

achievable, irrespective of the marginally changed penile spine distribution. However it remains possible that reduced anchoring function or altered neural sensation in the penis might contribute to failure to intromit and ejaculate.

3.5.3 Normal Epithelial Proliferation in TGFβ1-/- Mice

A clear distinction was seen between genotypes when the outer surface of the penis was evaluated for spine protrusion through what appears to be a keratinized epithelial covering, consistent with a build up of dead skin tissue. It seems possible that the presence of this excess material would further interfere with the anchoring or sensory function of penile spines in the TGFβ1-/- mice. However, this covering was not found to be due to excessive epithelial proliferation, despite several in vitro studies showing the anti-proliferative effects of TGFβ1 on keratinocytes (Moses, 1991) (Pasonen-Seppanen, 2003) (Hashimoto, 2000) (Haber, 2003). However different processes contribute to homeostasis of epithelial surfaces and wound healing and the role of TGFβ1 may be somewhat different where injury or an inflammatory response is involved (Meilin, 1995) since in other studies immuno-deficient TGFβ1-/- mice reveal an increased epithelialisation rate in response to wounding (Koch, 2000). However, as TGFβ1 is reported to act both as a positive and negative regulator of keratinocyte proliferation in vivo (Fowlis, 1996), understanding its precise role remains challenging.

It is possible that the levels of TGFβ2 and TGFβ3 isoforms (Crowe, 2000) (Hibino, 2004) or other keratinocyte modulators (Ansel, 1990), including TβRII (Fowlis, 1996) are providing compensatory functions. Alternatively, a build up of sloughed cells and a failure to shed them is plausible. Penile grooming occurred habitually both in the absence of erections and during every erection observed. As the TGFβ1-/- mice groom less frequently and have fewer erections, they would also have fewer opportunities to physically dislodge superfluous epithelial material on the penis surface. This could also potentially cause irritation, impair function or even be responsible for the protruded penis condition mentioned above. Indeed, disruption to TGFβ1 signalling is implicated in abnormal keratinocyte proliferation, as occurs in psoriasis patients (Doi, 2003).

3.5.4 Reduced Genital Grooming Behaviour in TGFβ1-/- Mice

The proportion of mice performing at least one genital grooming act in the one hour observation period did not differ between genotype groups, however when those displaying two or more acts were assessed, a difference between genotype groups was found. This difference was also reflected when the results were expressed on a per hour basis. The negative correlation found between the genital grooming and erectile activity rates in the TGFβ1+/+ mice (3.4.2 above) suggests that they are less likely to perform genital grooming when they have erections. The loss of this correlation in the TGFβ1-/- mice may be reflective of their reduced capacity to achieve erections. However as site-specific lesions in the brain are seen to affect non-contact erections (NCE) differently from copulatory activities (Liu, 1998), it is not clear whether incidence of erectile activity is directly linked to incidence of genital grooming.

3.5.5 Reduced Erectile Activity in TGFβ1-/- Mice

Previous reports showing increased erectile frequency in normal mice when treated with Viagra or apomorphine, compared to un-treated controls, provided the basis to assess the impact of pharmacological treatments in TGFβ1+/+ mice. Despite repeated efforts, neither pharmacological agent, with or without female influence, induced an increased frequency of erections compared to those achieved spontaneously. The TGFβ1+/+ mice are known to mate successfully and sire multiple litters of offspring (Ingman, 2002), indicating that their erection pathways are operating normally. Care was taken to prepare the drugs freshly each time, at the correct concentrations and in the diluents previously recorded to be stabilising. As testosterone and NO are previously recognised modulators of erections (Thomas, 2002), the effects of apomorphine and Viagra might have been influenced by the absence of an intact immune system in the TGFβ1+/+ mice. Indeed, the absence of lymphocytes in this mouse model could affect the availability of cytokines, which are reported to improve penile adenylate cyclase activity in response to pharmacological inducers (Anderson, 1992). The production of dopamine is also influenced by T-cells

of the immune system (Levite, 2000). Thus the erectile response of these mice to pharmacological inducers may depend on the background strain.

The difference in erectile capacity between the TGFβ1-/- mice compared to the TGFβ1+/+ controls may be due to the effects of TGFβ1 on neuronal innervation of the brain (Farkas, 2003), diminished dopamine production (Hull, 2004) or lesions in the brain (Liu, 1997). Alternatively, a biphasic effect of TGFβ1 on erectile responses may be occurring (Francavilla, 2005) (Penson, 2003). As TGFβ in the blood continuously bathes the vascular system, a constant circulating level of TGFβ1 as well as local production in the penile tissues might be required to regulate endothelial function. In support of this, it was previously found that altered plasma TGFβ1 levels were significantly related to erectile dysfunction and changes to the vascular system (Ryu, 2004).

3.6 SUMMARY

Altered density of penile spines may contribute to the reduced intromission behaviour reported for TGFβ1-/- mice. The reduced erectile frequency and penile grooming that they display may contribute to a build up of superfluous epithelial cells which then impacts on spine protrusion. Thus the TGFβ1 null mutation may influence mating ability through a synergistic effect on altered penile structures, reduced genital grooming and reduced erection frequency. These effects may contribute together with the previously described reduction in testosterone and LH levels to explain the profound infertility and inability of the TGFβ1-/- mice to copulate.

Chapter 4:
Physiological and Biochemical Discriminators of TGFβ1 Levels In Vivo

4.1 INTRODUCTION

In order to evaluate the reproductive consequences of manipulating TGFβ1 levels in vivo, it was important to first develop robust measures of systemic TGFβ1 levels. Physiological and biochemical parameters reported to be responsive to TGFβ1 were investigated in an effort to find discriminators of TGFβ presence and action in vivo. These parameters included plasma TGFβ1 levels as well as secondary parameters such as macrophage gene expression and phagocytosis and testosterone production. The parameters were compared in TGFβ1+/+ and TGFβ1-/- mice to identify a means for assessing the efficacy of exogenous TGFβ1 delivery in subsequent experiments.

As discussed in chapter 1, cells of the immune system are highly sensitive to differences in TGFβ concentrations in vitro and in vivo. In vitro tests show differing effects on the activation or proliferation of lymphocytes and macrophages when exposed to exogenous TGFβ1, depending on the environmental context (Kulkarni, 2002). In vivo, over-expression of TGFβ1 is associated with fibrosis in the liver and lungs of humans (Kelly, 2003) (Tsushima, 1999), while an absence of TGFβ1 in vivo causes immune competent TGFβ1-/- mice to quickly succumb to massive inflammation (Palmer, 1994) (Kulkarni, 1993).

The TGFβ1-/- mice used for this study are devoid of lymphocytes, thus only the macrophages remain as potential indicators of immune change. As many studies report on the various effects of TGFβ1 in macrophages (Bogdan, 1993) (Stoika, 2001) (Vodovotz, 1993) (Ashcroft, 1999), these cells were proposed to serve as a sensitive discriminators of TGFβ1 responses in the mice studied here. Specifically, tumor necrosis factor alpha (TNFα) and inducible nitric oxide synthase (iNOS) are macrophage genes that have been shown to be regulated by TGFβ1 (Bogdan, 1992) (Vodovotz, 1996b).

TNFα allows macrophages to become differentiated into phenotypes that are associated with tissue repair or cytotoxic activities in tissues (Winston, 1999) and

macrophages also produce TNFα upon activation (Brault, 2005). TGFβ1 plays an antagonistic role to TNFα in macrophages by suppressing their activation and inhibiting TNFα mRNA translation (Bogdan, 1993) (Bogdan, 1992), thus TGFβ1 deficiency might allow TNFα production to continue unabated in activated cells. However, TGFβ1 is also reported to induce TNFα in resting monocytes (Bogdan, 1993), providing evidence of an alternate pathway of TNFα production in resting cells. To examine the effect of TGFβ1 null mutation on expression of TNFα mRNA in macrophages, quantitative RT-PCR was used in the peritoneal cell population of mice.

The other macrophage gene of interest in TGFβ1-/- mice is inducible nitric oxide synthase (iNOS). This enzyme produces nitric oxide (NO) a reactive oxygen species synthesised by macrophages during inflammation (Calorini, 2005) (Weinberg, 1995) (Xu, 2002) which is negatively regulated by various agents amongst which TGFβ1 appears to be the most potent (Vodovotz, 1996b). As iNOS can be generated from cultured macrophages ex vivo without stimulation (Vodovotz, 1996b), resting peritoneal cells were assessed to determine if differences occur between genotype groups.

To assess macrophage function, phagocytosis of apoptotic cells was investigated. In vivo, even under homeostatic conditions, macrophages continually remove the cellular debris of apoptotic cells (Savill, 1993) (Iyoda, 2004). TGFβ1 is found to inhibit this phagocytic capacity in vitro (Stoika, 2001) (Li, 2006) and when phagocytosis is studied in inflamed tissues, inflammation is resolved in response to TGFβ1 (Maderna, 2003). This suggests a regulatory role of TGFβ1. To determine whether macrophage phagocytic function is altered in TGFβ1-/- mice, a standard phagocytosis assay using flow cytometric analysis of peritoneal macrophages was used.

Macrophages also reside in the liver (Kupffer cells), a primary site of TGFβ1 processing (Coffey, 1987). When excessive TGFβ1 production occurs in the liver, fibrosis is also found (Sanderson, 1995) (Eickelberg, 1999) (Schuppan, 2003). When TGFβ1 is administered to mice, the hepatic and Kupffer cell populations alter to

regulate liver regeneration (Schackert, 1990). Thus macrophage density in the liver was measured to evaluate its value as a discriminator of TGFβ1 levels in vivo.

A biochemical measure of TGFβ1 action in vivo might be testosterone, as in vivo assessments of TGFβ1-/- mice have previously found reduced circulating testosterone levels (Ingman, 2002), while diabetic rats reveal a negative correlation between TGFβ1 and testosterone (Salama, 2001). TGFβ1 is also found to affect testosterone levels in vitro as demonstrated by its ability to inhibit basal and luteinizing hormone (LH)-induced testosterone secretion (Gautier, 1997). The impact of TGFβ1 on testosterone levels may however occur indirectly via the interactions between TGFβ1 and testicular macrophages, as these cells are influenced by TGFβ1 (Ashcroft, 1999) and are reported to play an important role in hormone production (Cohen, 1997). Thus, testosterone levels were assessed to confirm the impact of TGFβ1 deficiency on testosterone production in this study.

Circulating TGFβ1 concentrations are variable between individuals, perhaps partly under genetic control (Grainger, 1999a). In the blood TGFβ1 is found freely available in the plasma as well as stored within platelets. Plasma TGFβ1 may be derived from the tissues, as a correlation is found between circulating TGFβ1 and tissues that produce varying amounts of TGFβ1 (Kong, 1995) (Tsushima, 1999). Collection of plasma results in low levels of TGFβ1 due to the addition of anti-coagulants, while serum collection results in high levels, due to platelet activation during the coagulation process (Coupes, 2001). However, studies where circulating TGFβ1 is measured are often confounded by a high degree of variability in plasma measurements, due to differences in sample preparation methods, such as unintentional platelet activation during blood collection (Coupes, 2001). To overcome this, methods that minimise platelet activation were implemented here and used to compare serum and plasma levels of TGFβ1. This was done to determine the levels of TGFβ1 in wild type mice and to evaluate whether delivery of exogenous TGFβ1 could result in detectable TGFβ1 in TGFβ1-/- mice.

The experiments described below analyse whether TGFβ1 is required for normal macrophage gene expression, function or density and whether testosterone and TGFβ1 levels can be used as discriminators of TGFβ1 in vivo. Peritoneal cells were

assessed using real time RT-PCR, flow cytometry and immunohistochemistry. Testosterone was measured using commercial RIA, while TGFβ1 was measured using commercial ELISA.

4.2 PERITONEAL CELL GENE EXPRESSION

To determine the expression of TNFα mRNA and iNOS mRNA in resting macrophages, peritoneal cells were collected from mice. Total RNA was extracted and assessed for mRNA expression relative to the β-actin housekeeping gene.

4.2.1 Tumor Necrosis Factor Alpha

The expression of TNFα mRNA in peritoneal cells of TGFβ1-/- mice (mean ± SEM = 32.6 ± 19.1) was not different compared to the TGFβ1+/+ mice (100.4 ± 64.5), despite the higher mean in the TGFβ1+/+ group. Exclusion of a single high outlier in the TGFβ1+/+ group did not affect the statistical significance of the data (Figure 4-1A).

4.2.2 Inducible Nitric Oxide Synthase

The expression of iNOS mRNA in the TGFβ1-/- mice (mean ± SEM = 210.8 ± 197.6) was not different compared to the TGFβ1+/+ mice (100.3 ± 80.2), despite the higher mean value in the TGFβ1-/- group. Exclusion of a single high outlier in the TGFβ1-/- group did not affect the statistical significance of the data (Figure 4-1B). Expression of genes encoding the two other NOS isoforms, eNOS mRNA and nNOS mRNA was assessed but they were not detected in theseperitoneal macrophage preparations (data not shown).

4.3 PERITONEAL CELL PHAGOCYTOSIS

CFSE labelled (CFSE+) apoptotic thymocytes were administered by intra-peritoneal injection and incubated in the peritoneal cavity of conscious mice for 30 minutes. Peritoneal cells were then collected by lavage. The cells were incubated with antibody reactive with F4/80 to detect macrophages (F4/80+ cells) and antibody against CD11b to detect both macrophages and polymorphonuclear neutrophils (PMN) (CD11b+ cells) (McFarland, 1992). Phagocytic cells were defined as those that had internalized the fluorescent CFSE+ cells. Isotype matched control rat immunoglobulin (Ig) G was used to determine non-specific binding. Cells were assessed by flow cytometry and the digital output was analysed. The cell populations were shown as dot-plots according to their size (forward scatter (FSC)) and complexity or density (side scatter (SSC)). The F4/80 or CD11b positive population was defined by a gate to exclude > 95% of presumably dead cells non-specifically labelled with anti-IgG (Figure 4-2A). Dual-labelling for CFSE and F4/80 (or CFSE and CD11b) was used to quanify the phagocytic capacity of the macrophages (Figure 4-2B, Figure 4-2C) (Methods 2.8).

Approximately 42% of the F4/80+ cells were phagocytic and no difference in the proportion of phagocytic versus non-phagocytic F4/80+ cells was found between genotype groups. The total percentage of F4/80+ cells within the entire gated macrophage population was also similar between genotype groups (Figure 4-3A). Within the CD11b population, approximately 40% were phagocytic. Again no differences were detected in phagocytic capacity between the genotype groups, or in the total percentage of CD11b+ cells within the entire gated macrophage population (Figure 4-3B).

4.4 MACROPHAGE DENSITY IN THE LIVER

As TGFβ1 is known to affect macrophages of the liver (Kulkarni, 2002) (Kelly, 2003) (Tsushima, 1999), the density of cells expressing the mouse macrophage-specific antigen F4/80 was quantified in tissue sections of liver by immunohistochemistry and

video image analysis to determine whether endogenous TGFβ1 deficiency alters the abundance of liver macrophages (Figure 4-4A, Figure 4-4B).

No difference between the density of F4/80 macrophages in the TGFβ1+/+ mice (mean ± SEM = 3.9 ± 1.3%) and TGFβ1-/- mice (4.9 ± 0.7%) was found (Figure 4-4C).

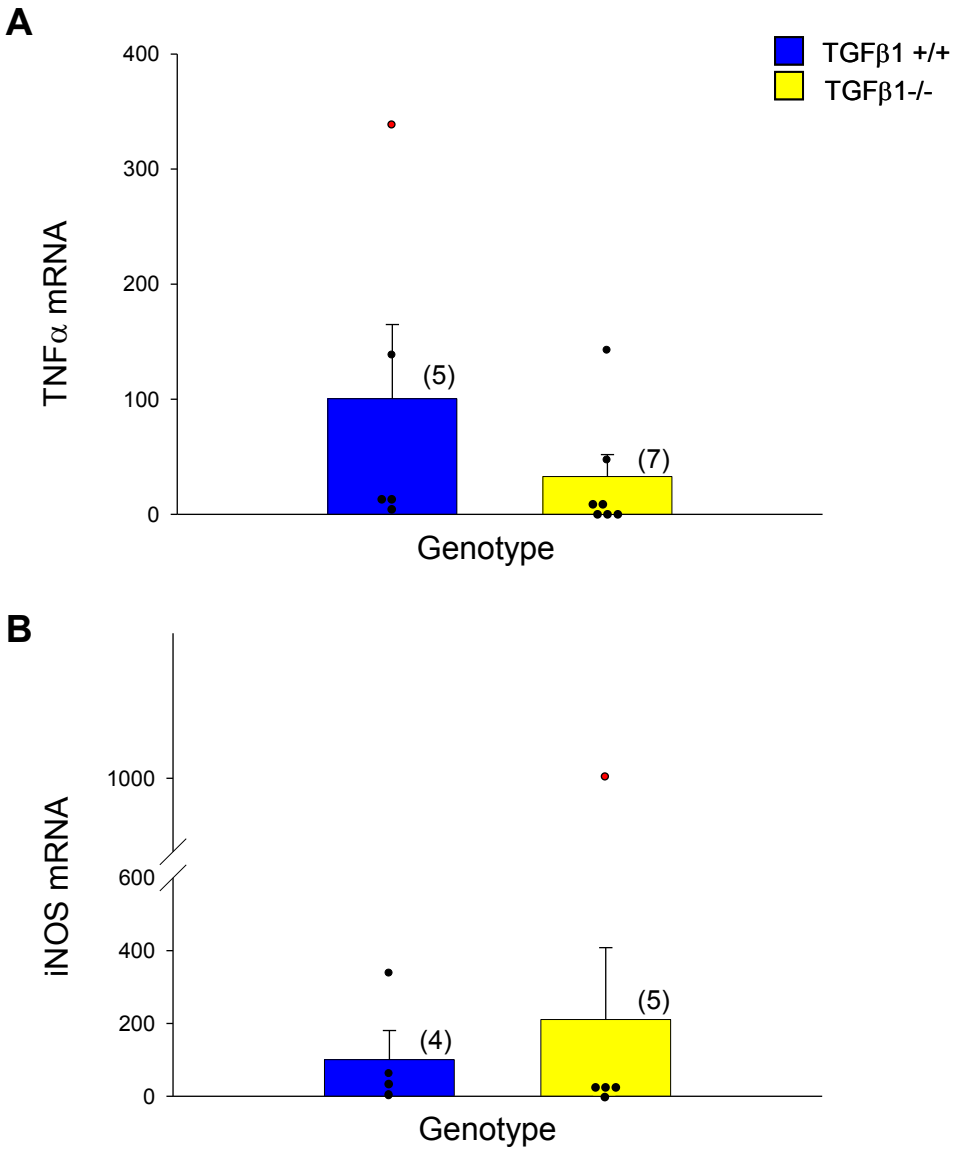


Figure 4-1 TNFα mRNA expression (A) and iNOS mRNA expression (B) in unstimulated peritoneal cells.

Real Time RT-PCR was used to quantify mRNA and values were normalised to β-actin mRNA content. Bar plots show the mean (± SEM), dot plots show individual sample values (red = outlier). No significant differences between genotype groups was found, irrespective of inclusion of outliers ($p>0.05$). Data were analysed using the Mann Whitney U method. n is shown in parentheses

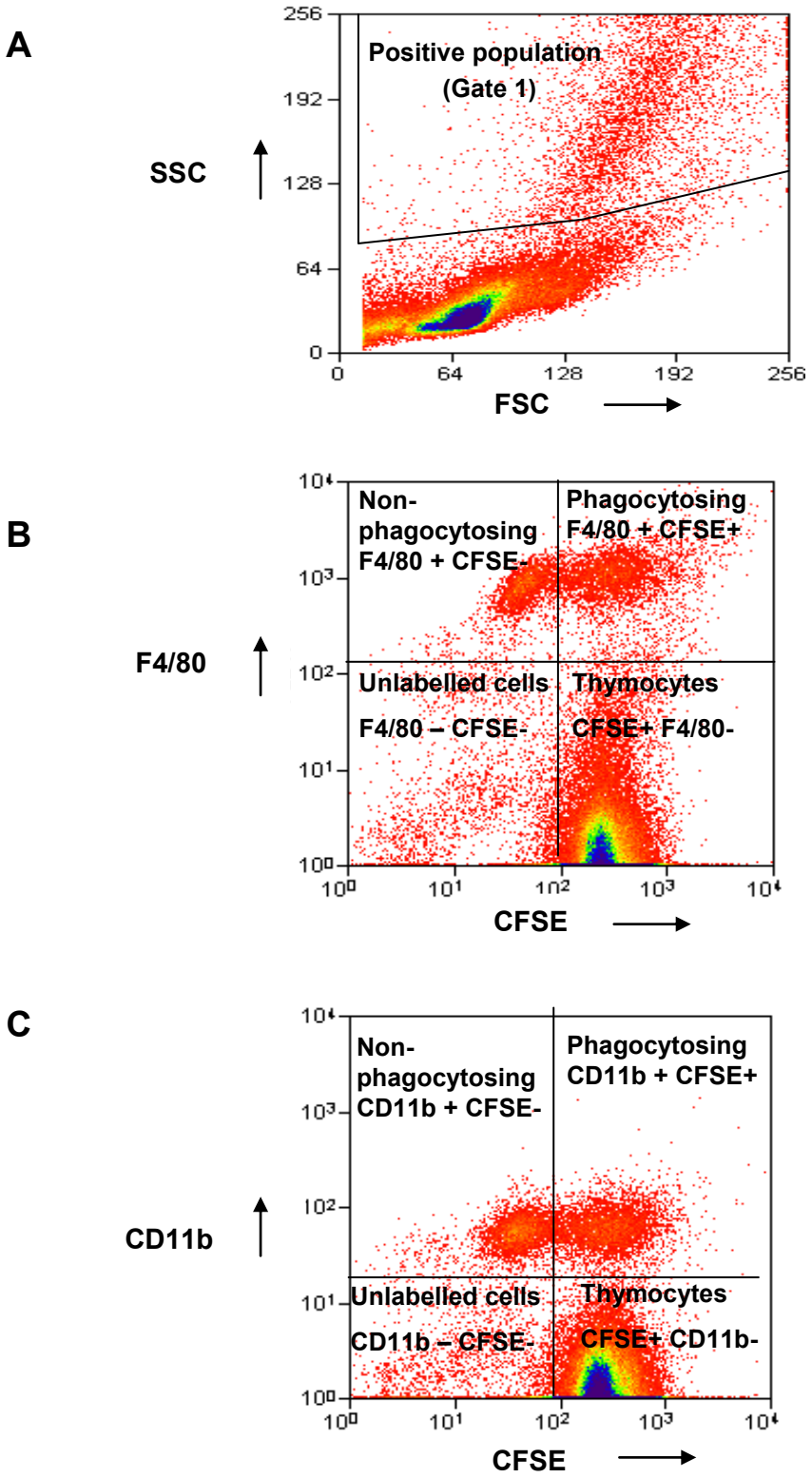


Figure 4-2 Flow cytometric assay of phagocytosis of CFSE-labelled apoptotic thymocytes by peritoneal macrophages.

Example of forward (FSC) and side (SSC) scatter plot of peritoneal cells, analysis was confined to cells lying within Gate 1 (A). Analysis of uptake of CFSE+ cells by F4/80+ cells (B) and analysis of uptake of CFSE+ cells by CD11b+ cells (C). Peritoneal cells were assessed by flow cytometry. F4/80+ quadrants and CD11b+ quadrants are shown.

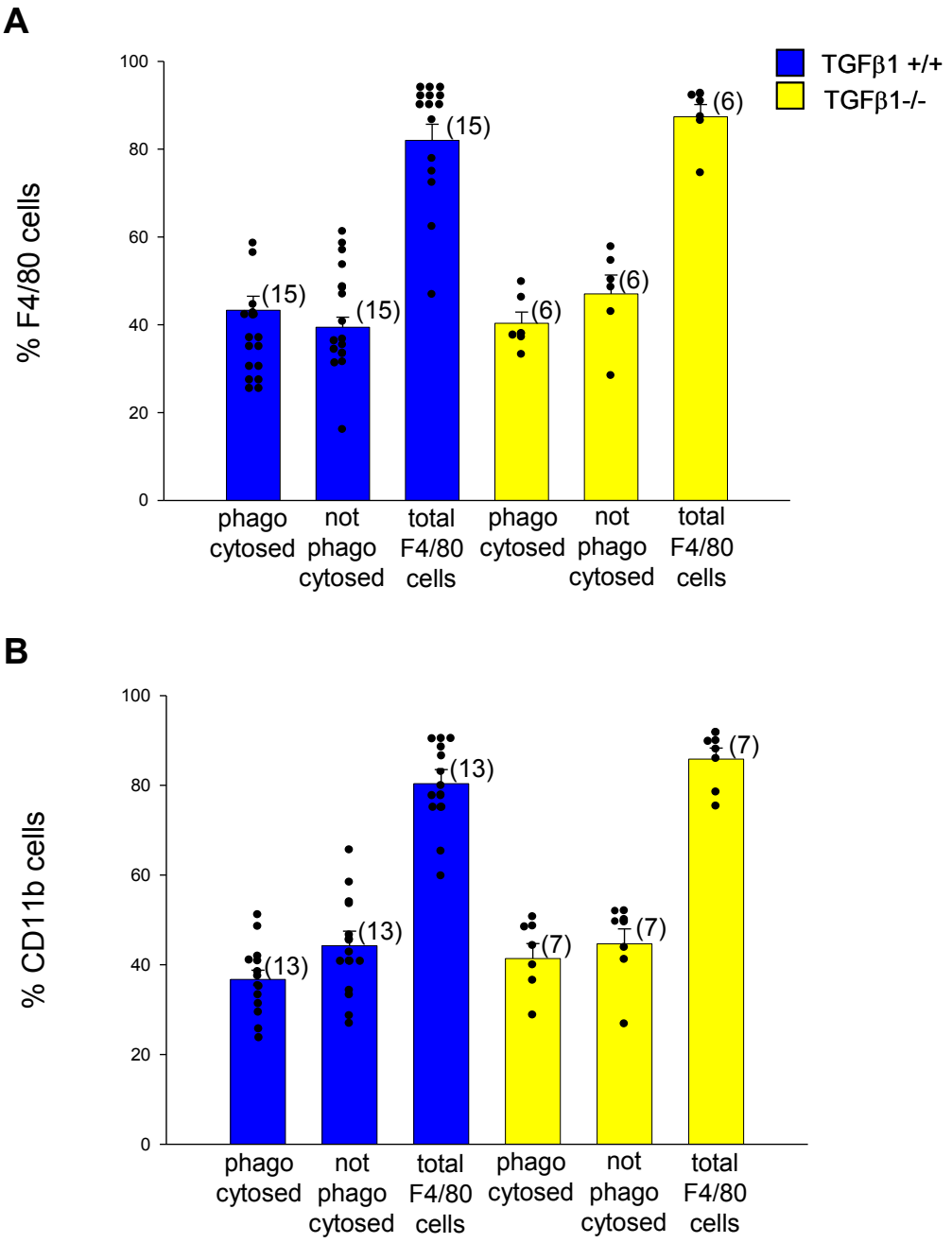


Figure 4-3 Phagocytic activity of F4/80+ cells (A) and CD11b+ cells (B).

Bar plots show the mean (± SEM), dot plots show individual sample values. No significant difference between genotype groups was was found ($p>0.05$). Data were analysed using the Mann Whitney U method. n is shown in parentheses.

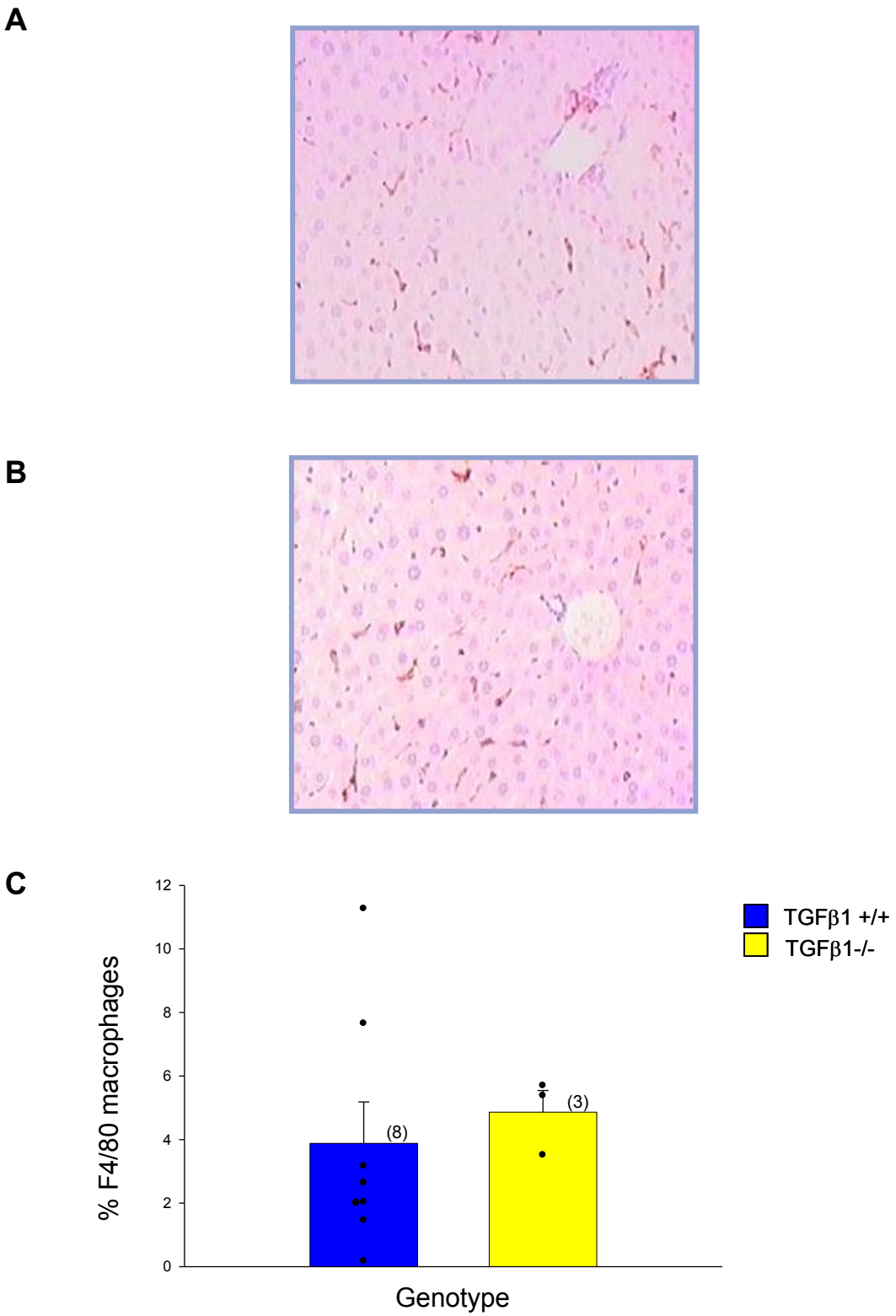


Figure 4-4 F4/80 liver macrophages in TGFβ1+/+ mice (A) and TGFβ1-/- mice (B); Percent F4/80 macrophages (C).

Macrophages were labelled by immunohistochemistry using antibody to F4/80 and counterstaining with haematoxylin. F4/80 cells were quantified by image analysis. Bar plots show the mean (± SEM), dot plots show individual sample values. Significant differences between genotype groups was not reached ($p>0.05$). Data were analysed using the Mann Whitney U method. n is shown in parentheses.

4.5 SERUM TESTOSTERONE

Mice were housed individually for five days without any prior exposure to females or female bedding. Blood was collected from the tail vein to obtain serum which was measured by radioimmunoassay (RIA) for testosterone content.

Serum of the TGFβ1-/- mice showed a significantly reduced testosterone content (mean ± SEM = 2.7 ± 1.1 nmoles / L) compared with the TGFβ1+/+ mice (8.2 ± 4.9 nmoles / L) (Figure 4-5). The level of statistical significance did not alter when the outliers were removed from analysis (both p <0.5).

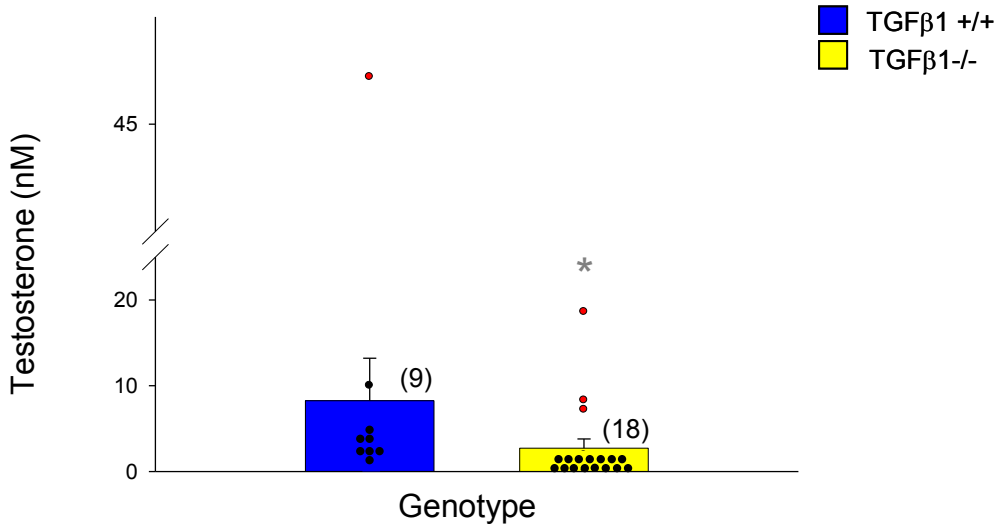


Figure 4-5 Total serum testosterone content in TGFβ1+/+ and TGFβ1-/-mice.

RIA was used to detect total testosterone. Bar plots show the mean (± SEM), dot plots show individual sample values (red = outlier). * denotes statistically significant difference from TGFβ1+/+ controls (p<0.05). Data were analysed using the Mann Whitney U method. n is shown in parentheses.

4.6 CIRCULATING TGFB1 CONCENTRATIONS

4.6.1 Serum

Blood was collected from mice by cardiac puncture, then left to clot at 4°C overnight to ensure complete activation of platelets and the release of all stored TGFβ1 into the serum. Samples were then acid-activated to measure total TGFβ1 by ELISA (Methods 2.2 and Methods 2.4).

The serum from TGFβ1+/+ mice (mean ± SEM = 54.3 ± 4.2 ng / mL) contained twice as much TGFβ1 than the serum of TGFβ1+/- mice (23.7 ± 2.0 ng / mL), while no TGFβ1 was detected in the serum of TGFβ1-/- mice (Figure 4-6).

4.6.2 Plasma

Cardiac blood was collected mid-flow from mice using a wide-bore needle, and immediately mixed in the syringe with CTAD, an inhibitor of platelet activation. Samples were then centrifuged and the supernatant was acid-activated and analysed by ELISA (Methods 2.2 and 2.4).

The TGFβ1+/+ mice had twice as much plasma TGFβ1 (10.2 ± 1.9 ng / mL) as the TGFβ1+/- mice (4.6 ± 0.6 ng / mL), (Figure 4-6). TGFβ1 was not detectable in the serum of TGFβ1-/- mice.

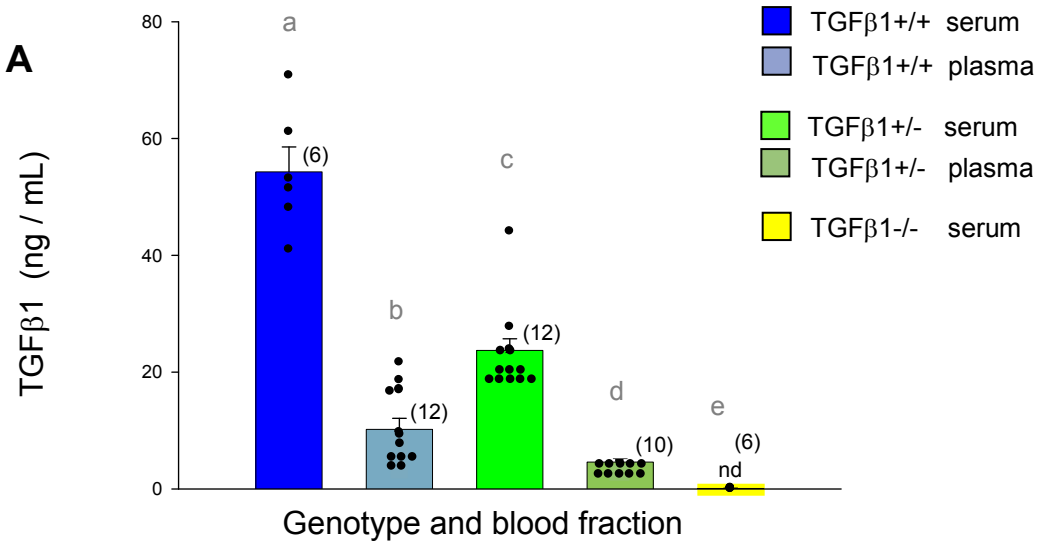


Figure 4-6 Serum and plasma TGFβ1 concentrations.

ELISA was used to detect the concentrations of TGFβ1. Bar plots show the mean (± SEM), dot plots show individual sample values, different superscripts denote a statistically significant difference between groups ($p<0.05$). Data were analysed using the Mann Whitney U method. n is shown in parentheses. nd denotes not detected.

4.7 DISCUSSION

4.7.1 Peritoneal macrophage gene expression

Lymphocytes and macrophages play a key role in driving the lethal inflammatory reactions observed in immune competent TGFβ1-/- mice. In the immune deficient TGFβ1-/- mice used herein, we were unable to detect any differences between genotype groups in the parameters we measured. We conclude that these aspects of macrophage phenotype remain unaltered in the absence of TGFβ1.

TNFα mRNA was produced in resting peritoneal cells providing evidence that constitutive TNFα production occurrs in the absence of macrophage activation (Brault, 2005), (Bogdan, 1993) and is independent of TGFβ1 regulation. To suppress inflammation, TGFβ1 interferes with TNFα translation in activated macrophages (McDonald, 1999) (Yoon, 1998). It is unclear whether the effects of TGFβ1 are as evident when macrophages are not activated. TNFα suppression may not occur until TNFα levels rise above a threshold level. Also, as IL-10 is a more potent suppressor of TNFα production in macrophages compared with TGFβ1 (Bogdan, 1993), the complete absence of TGFβ1 here may not be sufficient to alter TNFα expression in vivo due to regulation by IL-10. Furthermore the other TGFβ isoforms, TGFβ2 and TGFβ3 are likely to play a compensatory role (Yamagami, 2004).

No difference in iNOS mRNA expression was found between genotype groups. This is surprising since TGFβ is a potent suppressor of nitric oxide (NO). While others have found comparable iNOS protein expression in peritoneal macrophages between TGFβ1 genotypes (Vodovotz, 1996b) it is also known that for example, in a study extending over 4 weeks, TGFβ1-/- mice were found to express more iNOS in the heart and kidney than TGFβ1+/+ littermates, with differences peaking at 2 - 3 weeks of age (Vodovotz, 1996a). Thus, either the adult age of the mice, or the tissues analysed in the studies herein may be responsible for the lack of genotype differences in iNOS gene expression. In another study using peritoneal macrophages ex vivo from TGFβ1-/- mice, no differences were found compared to TGFβ1+/+

controls when NO_2^- (a reaction product of NO) production was assessed (Vodovotz, 1996b). Additioinally, as lymphocytes are the main cellular source of IFNγ, the level of peritoneal macrophage activation by this cytokine (Taylor-Robinson, 1997) may be constrained in the TGFβ1-/- (SCID) mice used in these experiments.

4.7.2 Peritoneal Cell Phagocytosis

Failure to detect any differences in phagocytosis between genotype groups implies that TGFβ is not an important regulator of macrophage phagocytosis under the experimental conditions examined. However the method employed here or the physiological state of the macrophages or apoptotic cells could have influenced the outcome, since phagocytic activity can alter depending on the microenvironment or status of the phagocytic cell, or the cell being engulfed (Maderna, 2003) (Krysko, 2006).

Activated peritoneal macrophages are typically used for phagocytosis studies (Misawa, 2001) (May, 2005) however this method was not employed here as it may interfere with reproductive function. This was important as a key objective of these experiments was to define an indicator of TGFβ bioactivity in vivo that could eventually be measured in the same mice in which reproductive parameters would also be evaluated. Additionally, phagocytosis was observed after only a single time point of 30 minutes in this study. Rapid uptake of apoptotic cells by phagocytosis has been shown in vitro (Licht, 1999). However, a 30 minute time period may not have been sufficient in vivo, where the time taken for an initial inflammatory reaction to occur and subsequently be subject to suppression by TGFβ1 may be longer (Uchimura, 1997) (Kurosaka, 2003) (Misawa, 2001). Alternatively, the differentiation state of the macrophage or the status of the apoptotic cell may affect the ability of the macrophage to phagocytose apoptotic cells. For example, differences in phagocytic responses, such as reduced cytokine production, were found when apoptotic cells are in a state of early apoptosis versus late apoptosis (Kurosaka, 2003). The apoptotic thymocytes used in this study could be considered early apoptosis, so the role of TGFβ during removal of these cells by phagocytes may have been less important. Additionally, phagocytic function of the peritonel cell population may

remain intact due to the presence of other regulatory mechanisms (Niedergang, 2005).

4.7.3 Serum Testosterone

Basal serum testosterone concentrations were different between genotype groups. The lower levels found in the TGFβ1-/- mice are similar to those reported previously (Ingman, 2002) (Ingman, 2007) and may at least in part account for their reduced erectile activity shown in Chapter 3.

As a previous investigation of circulating testosterone levels in these mice was conducted in the presence of bedding from females' cages (Ingman, 2002), it may have reflected a failure to respond to female pheromones, since the female scent of females alters testosterone levels in other mammals (Zhang, 2001) (Swann, 1997). Here, however, the difference in testosterone levels depending on genotype were found in the absence of any female influence, thus basal testosterone levels are affected by TGFβ1 deficiency. Although this relationship is not thought to be linear as heterozygotes with half the TGFβ1 gene expression are able to produce normal levels of testosterone that are not different to the TGFβ1+/+ controls (Ingman, 2007).

It is recognised that TGFβ1 added to LH-induced Leydig cell cultures can reduce testosterone production (Fauser, 1988) (Gautier, 1997), a converse finding compared with the absence of TGFβ1 in vivo resulting in reduced testosterone levels. These contradictory findings are difficult to reconcile, thus two possible reasons are provided. As severely reduced testosterone levels are found in mice deficient in colony stimulating factor - 1 (CSF-1) which are devoid of macrophages (Cohen, 1997), interactions between testicular function and immune modulators may be important. Thus the effect of TGFβ1 on circulating testosterone levels here might be mediated locally in the testis through altered macrophage presence or function. Alternatively, as TGFβ1 is seen to influence Leydig cell testosterone production in a biphasic manner by impacting on the steriodogenic cascade (Morera, 1988) (Benahmed, 1989), low or absent TGFβ1 in vivo may be restricting the availability of precursors to steroidogenesis. In addition, as the TGFβ1-/- mice have low serum LH

and are capable of up-regulating their testosterone production following administration of the luteinizing hormone (LH) analogue, human chorionic gonadotrophin (hCG) (Ingman, 2007), reduced LH is implicated as responsible for their perturbed serum testosterone levels.

4.7.4 Circulating TGFβ1 Concentrations

Differences in serum and plasma TGFβ1 levels found between genotype groups were in proportion to the allelic status of the TGFβ1 gene as a 50% reduction of TGFβ1 was found in mice with only one copy of the intact functional gene. Humans with a non-functioning allele have also produced abnormal circulating TGFβ1 levels (Grainger, 1999a). Also, there is an inverse correlation between incidence of some diseases, such as malaria, and TGFβ serum levels (Wenisch, 1995). When plasma concentrations in both genotype groups were tested, they were found to be one fifth (1/5th) of that detected in the serum, revealing the impact of coagulation and platelet activation on TGFβ1 biovailability. While plasma TGFβ1 is thought to be derived from most tissues, it is not known if this TGFβ1 has a physiological role or is simply an irrelevant by-product of metabolic tissue processes. However, freely available TGFβ1 in the plasma could boost processes in distant tissues or provide an immediate source for regulation of sudden inflammatory events.

4.8 SUMMARY

Previously recognised TGFβ1-dependent macrophage pathways were not found to be different in TGFβ1-/- mice. Thus the peritoneal cell population cannot be used here to detect changes in TGFβ1 levels in vivo. Testosterone production was sensitive to the TGFβ1 deficiency, and may be a useful parameter for assessment of exogenous TGFβ1 action. However as expected, the circulatory system was the most informative discriminator of the presence of TGFβ1 in vivo in these mice as TGFβ1 levels in the serum and plasma were directly related to the genotype status of the mice. Using serum to measure in vivo TGFβ1 or testosterone levels has the

added advantage that serum is easily accessible and can be informative without the need for any additional intervention.

Chapter 5:
Sources and Routes of Administration for the Administration of Exogenous TGFβ1

5.1 INTRODUCTION

The experiments described in the previous chapter sought to identify biological parameters that would act as direct or indirect measures of TGFβ1 bioavailability in vivo. In this chapter, natural and recombinant sources of TGFβ1 and routes of administration were investigated for their potential usefulness in protocols for TGFβ1 replacement in TGFβ1 null mutant mice.

Circulating TGFβ1 in the blood is largely under genetic control as demonstrated by observations of altered plasma levels in individuals with polymorphisms within the TGFβ1 gene (Grainger, 1999a) (Nagpal, 2005). However, studies show that TGFβ1 levels in blood as well as tissues are also correlated with age (Okamoto, 2005b) and various disease states (Junker, 2000) (Wenisch, 1995) (Steiner, 1994) where disease severity may play a role in those correlations (Flisiak, 2002). These types of studies typically analyse TGFβ1 levels in the circulation prior to and following an intervention. In contrast, longitudinal investigations of TGFβ1 levels over time are limited, although one study in humans at rest, did show similar TGFβ1 levels in blood samples taken over a 5 day period (Heinemeier, 2003). Hence, day-to-day homeostatic adjustments do not appear to affect circulating TGFβ1 levels. To determine if similar regulation occurs in mice and to establish a baseline, TGFβ1 levels were examined in the TGFβ1+/+ mice. Two blood samples were taken from mice over different time frames, from 0 - 90 minutes and at two times one week apart.

In the absence of polymorphisms or disease, the diet may also influence TGFβ1 levels independently of age, either indirectly by promoting endogenous production or directly by adding to the circulatory pool. Maternal milk is a natural dietary source of TGFβ, due to secretion from mammary epithelial cells (Heid, 2005). Concentrations of TGFβ in milk are relatively high and vary widely depending on stage of lactation and species (Xu, 1999) (Ginjala, 1998) (Pakkanen, 1998) (Hawkes, 2002). In humans, colostrum is produced during the initial stages of lactation and is thought to be the richest source of nutrients for the newborn (Playford, 1999) while mature milk

is gradually produced after a few weeks. Both the TGFβ1 and TGFβ2 isoforms have been detected by ELISA in human and bovine milk at the levels summarised below (Table 5-1).

	TGFβ1 (ng / mL)			TGFβ2 (ng / mL)		
Colostrum	12-43	bovine	(Ginjala, 1998)	150-1150	bovine	(Pakkanen, 1998)
	0.07-0.2*	human	(Kalliomaki, 1999)	1.4-5.4*	human	(Kalliomaki, 1999)
Milk	0.8-3.5	bovine	(Ginjala, 1998)	12-71	bovine	(Pakkanen, 1998)
	0.2-3.5	human	(Hawkes, 2002)	0.01-14	human	(Hawkes, 2002)

Table 5-1 TGFβ concentrations in human and bovine colostrum and milk.

* While numerous studies show TGFβ levels in human colostrum and milk samples (Bottcher, 2000) (Kalliomaki, 1999), generally these were not evaluated within one month of sample collection. Samples left frozen for more than one month at -20ºC begin to acidify which lowers the amount of TGFβ detectable (Ogundele, 2002).

As TGFβ is highly homologous across species (Yamaguchi, 1992) it is possible that consumption of bovine TGFβ in milk can impact on TGFβ1 responsive processes in humans. However, the amount of TGFβ in our supermarket milk or in colostrum products, such as the high protein formulations marketed for athletes (Buckley, 2002) (Playford, 1999), has not been formally assessed and reported. Thus cow's milk for human consumption, before and following pasteurisation and a commercially-available bovine colostrum sample, were assessed for their TGFβ content.

Following consumption, TGFβ bioavailability becomes a subject for investigation. Some evidence exists showing the alleviation of intestinal disorders following consumption of TGFβ-rich diets (Fell, 2000) and in support of this, abrogation of TGFβ1 signalling in the intestine of rodents has contributed to intestinal bowel disease (IBD) (Hahm, 2001). Also, when immature TGFβ1-/- mice suckled on TGFβ1 replete mothers, maternal TGFβ was detected in various organs (Letterio, 1994). This demonstrates that TGFβ was transferred through the intestinal wall in this

model, although intestinal closure can not be assumed in suckling rodents (Teichberg, 1990) (Pacha, 2000). For proteins to traverse the intestinal wall intact to influence health, several barriers must be bypassed, such as gastric acidity and proteases (Miranda, 1983) (Yvon, 1993) (Yoneda, 2001), which act to degrade proteins into their constituent amino acids for intestinal uptake (van Goudoever, 2006). However, TGFβ is acid stable and would be expected to survive the low pH of the gastrointestinal tract, indeed it might be activated as a result of the acid environment.

Mechanisms that allow whole proteins to traverse the intestinal epithelium, such as specialised epithelial M-cells or receptor-mediated transcytosis, are also thought to exist (Pacha, 2000). Insulin-like growth factor (IGF) may pass through via M-cells (Philipps, 2002), while nerve growth factor (NGF) and epidermal growth factor (EGF) are likely to be transferred via receptors (Figure 1-3) (Pacha, 2000). However, evaluation of whether TGFβ can cross the intestinal wall through any of these mechanisms has not been undertaken. To assess the possibility of dietary TGFβ entering the circulation, experiments were undertaken to evaluate uptake of TGFβ in a bovine colostrum product was fed to adult TGFβ1-/- mice.

Recombinant TGFβ1 was also investigated for replacement experiments. It was determined that the latent form would be used, since the active form of TGFβ1 is labile with a short half life in the circulation of less than three minutes. This is likely due to hepatic degradation as well as rapid depletion by peripheral tissues (Wakefield, 1990), (Coffey, 1987). Latent TGFβ1 persists longer in the circulation as degradation of exogenous protein was seen to occur only after 90 minutes (Wakefield, 1990). Also, as active TGFβ binds available receptors without restriction and latent TGFβ is subject to regulation by activation mechanisms, the latent form may be a more physiologically relevant method of delivering exogenous TGFβ1. Thus, the ability of TGFβ1-/- mice to take up and retain recombinant latent TGFβ1 in the circulation was assessed here following intra-peritoneal injection. Recombinant human latent TGFβ1 was chosen for use in these studies for several reasons. 1) The human form of TGFβ1 retains over 90% protein sequence homology with the murine form and has comparable bioactivity in both species (NP_035707, 2007) (NP_000651, 2007). 2) Previous experiments had shown that exogenous latent

TGFβ can prolong the life of TGFβ1-/- suckling mice (Letterio, 1994). 3) Human latent TGFβ1 is commercially available and understanding its activity would be relevant to potential clinical studies in humans.

5.2 VARIATION IN SERUM TGFB1 LEVELS OVER TIME

Adult male TGFβ1+/+ mice were subjected to three bleeds over 90 minutes (0, 30 and 90 minutes) or two bleeds one week apart (day 0 and day 7). The mice being assessed over one week were allowed access to females during the test period. Blood was primarily taken from the tail vein, although cardiac puncture was used for the final 90 minute bleed.

The initial tail bleed of mice tested over 90 minutes revealed total TGFβ1 serum levels (mean ± SEM = 78.3 ± 8.7 ng / mL) that were significantly higher than subsequent bleeds at 30 minutes (58.0 ± 8.4 ng / mL) and at 90 minutes (45.6 ± 9.0 ng / mL). The difference between the second and third bleeds was not significant. While most mice demonstrated reduced TGFβ1 levels in the subsequent bleeds, some mice revealed higher TGFβ1 levels (Figure 5-1A).

When blood samples were assessed one week apart, the initial tail bleed contained significantly more total serum TGFβ1 (78.2 ± 6.8 ng / mL) than the bleed taken one week later (52.3 ± 3.8 ng / mL) (Figure 5-1B).

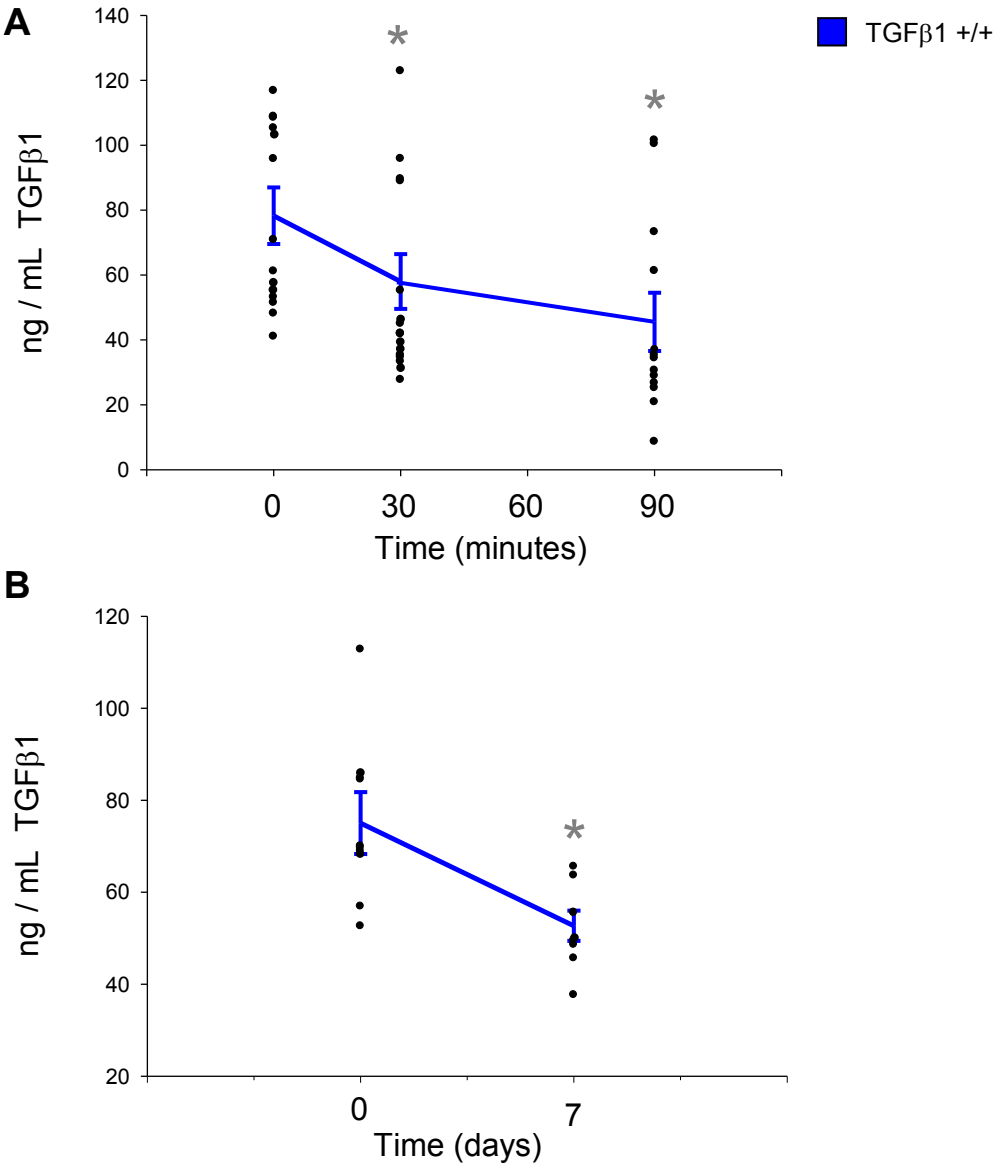


Figure 5-1 Circulating TGFβ1 in TGFβ1+/+ mice over (A) 90 minutes and (B) one week.

TGFβ1 was measured by ELISA. Line plots show the mean (±SEM), point plots show values for individual mice. * denotes significant difference from first bleed value ($p<0.05$). Data were analysed using the Mann Whitney U method (A) and by t-test (B). n = 13 (A) and n = 7 (B).

5.3 TGFB IN COLOSTRUM AND MILK

Milk and colostrum were investigated as sources of TGFβ that might be used for oral administration of TGFβ.

5.3.1 TGFβ in Bovine Milk

To assess the stability of TGFβ in milk following factory processing, transport and storage, samples of 1) raw, 2) freshly-pasteurised (78ºC / 27 seconds) and 3) commercially available milks purchased from the supermarket were evaluated for their TGFβ concentrations. Samples were collected from a local milk processing factory and each raw and pasteurised sample was verified to have originated from the same batch. Full cream and low fat varieties of commercially available milks were purchased from local supermarkets. Care was taken to ensure that the brands selected were produced by the same manufacturer of the raw and pasteurised samples, although it was impractical to match the batches with those collected at the factory. Some full cream samples from a second manufacturer were also collected. All samples were frozen at -20ºC for less than four weeks and were analysed simultaneously. Commercial ELISA was used to measure the three mammalian isoforms of active and latent TGFβ in the samples (Methods 2.4).

No significant differences were found between the low fat or full cream varieties for any isoform or activation status ($p > 0.05$), therefore the results were combined. Total TGFβ1 in raw milk samples (mean ± SEM = 7.6 ± 0.3 ng / mL) was significantly less than total TGFβ2 (43.4 ± 2.2 ng / mL) and pasteurisation diminished these levels by 55% and 85% respectively ($p < 0.05$) (Figure 5-2A). Levels of TGFβ1 and TGFβ2 in commercially purchased milks were the same as levels in freshly pasteurized milk. TGFβ3 was not detected in any samples. Additional testing on another manufacturer's milk samples did not detect any significant differences in TGFβ1 or TGFβ2 levels between manufacturers (data not shown). When comparing proportions of the two isoforms, TGFβ2 made up 85% of the total TGFβ in raw bovine milk.

Pasteurisation was associated with an increase in the ratio of active to latent TGFβ. TGFβ1 (6.2 ± 0.2 ng / mL) and TGFβ2 (43.4 ± 2.2 ng / mL) were predominantly present in the latent form in raw milk samples, however this altered following pasteurisation so that both latent TGFβ1 (1.9 ± 0.1 ng / mL) and latent TGFβ2 (1.3 ± 0.3 ng / mL) substantially decreased. In contrast, raw milk samples contained very little active TGFβ1 (0.7 ± 0.03 ng / mL) or active TGFβ2 (0.1 ± 0.02 ng / mL). Following pasteurisation, the levels of active TGFβ1 (1.5 ± 0.07 ng / mL) and active TGFβ2 (5.3 ± 0.4 ng / mL) rose significantly. Again, the levels of latent and active TGFβ1 and TGFβ2 found in commercially available milks were similar to those found following pasteurisation (Figure 5-2B).

Total TGFβ1 and total TGFβ2 were also found to correlate in the raw ($r = 0.938$, $p = 0.006$) (Figure 5-3A) and pasteurised milks ($r = 0.934$, $p = 0.006$) (Figure 5-3B). Active levels of the two TGFβ isoforms in the pasteurised milks was also found to correlate ($r = 0.847$, $p = 0.033$) (Figure 5-3C). Active TGFβ in the raw milk samples could not be tested for correlations due to the very small amounts detected.

5.3.2 TGFβ in Bovine Colostrum

A single sample of a commercially available bovine colostrum powder (Intact, Numico Australia) was evaluated. The published moisture content of the product was 5% (Numico, 2006), allowing the TGFβ concentrations to be reported here on a 'dry weight basis'.

Total TGFβ2 (844.7 ng / g) was much higher than total TGFβ1 (44.3 ng /g) and made up 95% of the TGFβ evaluated. It was predominately in the latent form (Figure 5-4).

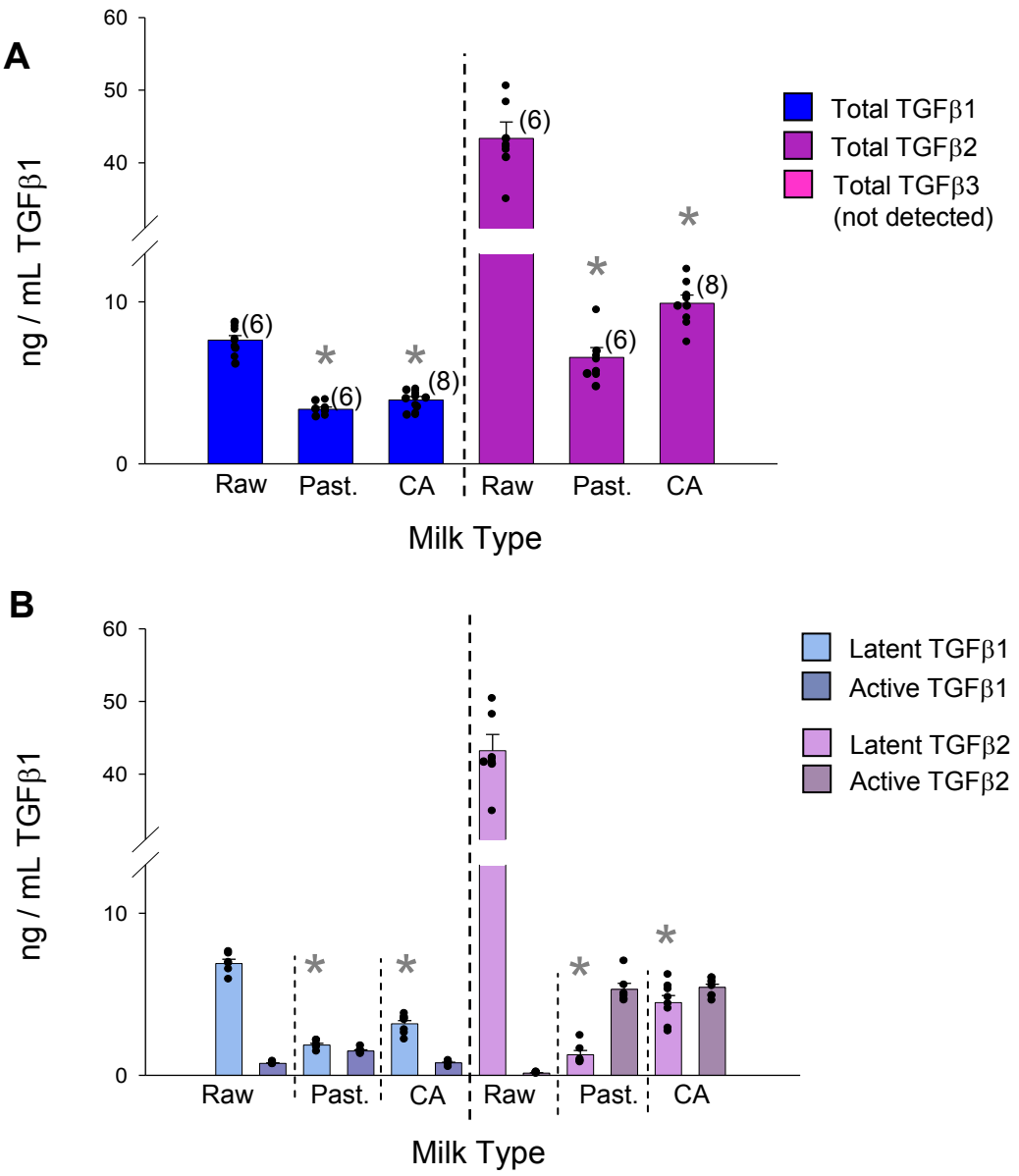


Figure 5-2 TGFβ isoforms in milk. Total TGFβ1 and TGFβ2 (A) and latent and active TGFβ1 and TGFβ2 ((B).

TGFβ was measured by isoform specific ELISA in raw, pasteurised (past.) and commercially available (CA) milks. Bar plots show the mean (± SEM), dot plots show individual sample values. * denotes significant differences from the raw milk samples, within isoforms ($p<0.05$). Data were analysed using the Mann Whitney U method. n is shown in parentheses in (A).

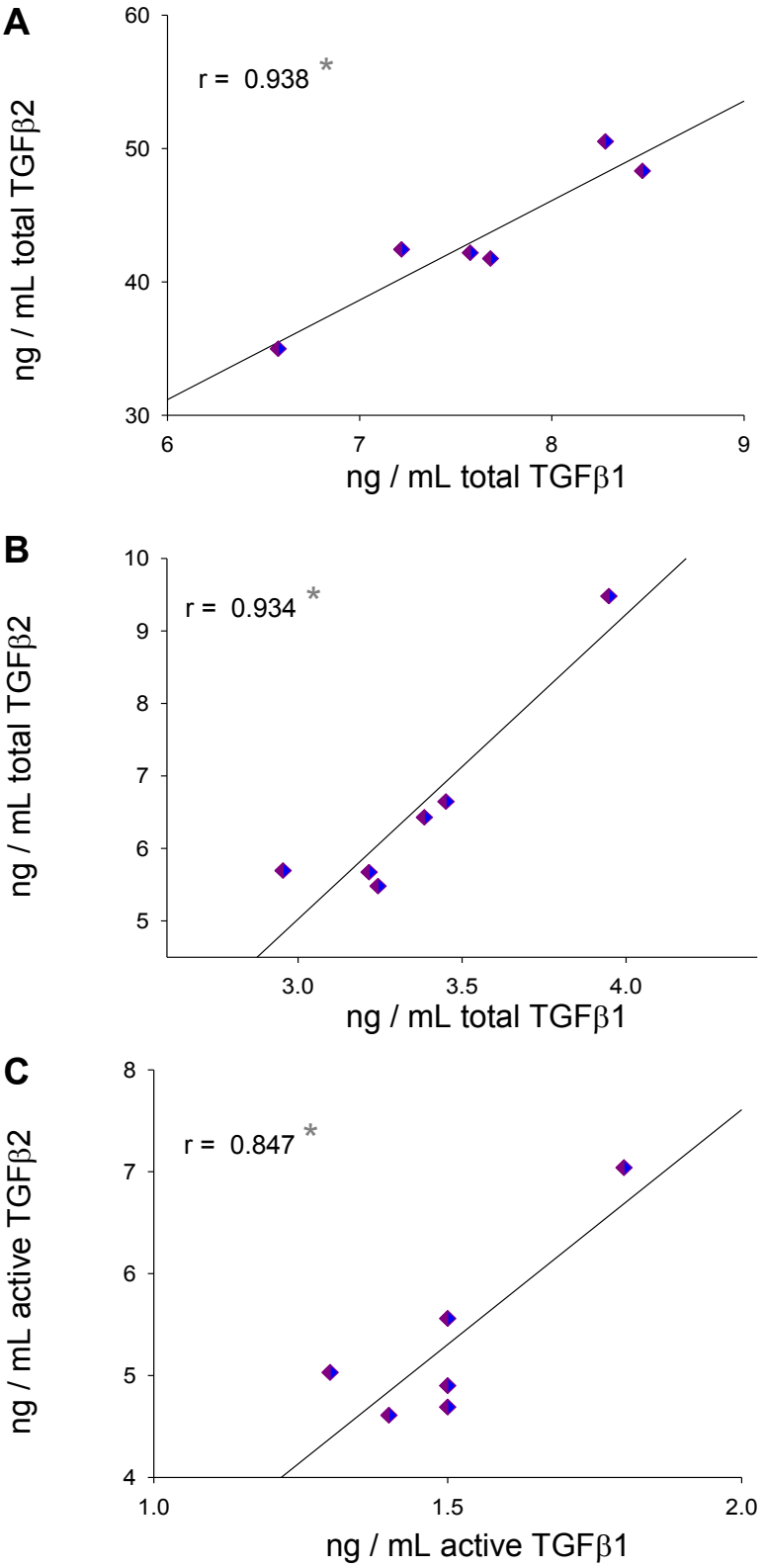


Figure 5-3 Correlations between TGFβ1 and TGFβ2 in milks.

Total TGFβ in raw milk (A), total TGFβ in pasteurised milk (B) and active TGFβ in pasteurised milk (C). TGFβ was measured by isoform specific ELISA. Line plots show linearity, data points show individual samples. * denotes a significant correlation ($p<0.05$) by Pearson's correlation. n = 6.

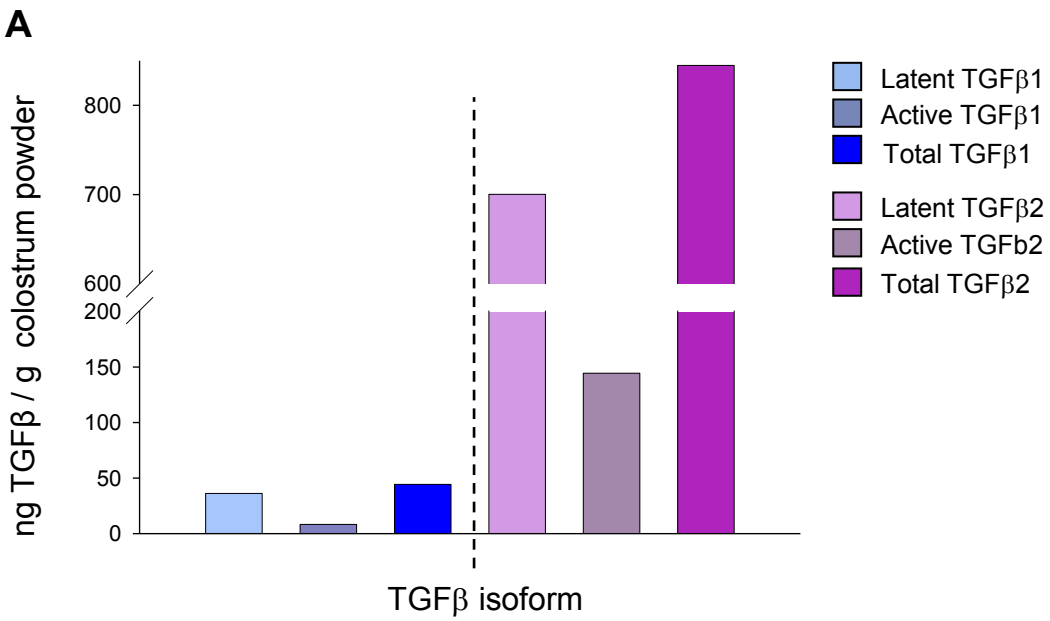


Figure 5-4 TGFβ in a commercially available colostrum powder.

TGFβ1 and TGFβ2 were measured by isoform specific ELISA.

5.4 TGFB1 REPLACEMENT IN TGFB1-/- MICE

5.4.1 Colostrum Consumption by Mice

5.4.1.1 TGFB IN THE STOMACH CONTENTS

A pilot study was conducted to evaluate the feasibility and efficacy of orally administering TGFβ. Adult TGFβ1-/- mice were fed powdered colostrum identified as a rich source of TGFβ1 and TGFβ2, for 2 days in place of normal chow. The only other dietary component provided was water. Mice were then sacrificed and the stomach contents were analysed to verify that TGFβ had been ingested.

The moisture content of the stomach contents was determined by oven drying (Methods 2.4.1). In TGFβ1-/- mice, on a dry weight basis, more TGFβ2 (mean ± SEM = 37.3 ± 7.4 ng / g) was detected than TGFβ1 (14.5 ± 0.5 ng / g), although none of the TGFβ detected was activated (Figure 5-5A). The proportions of TGFβ1 and TGFβ2 detected in the stomach contents were altered compared to the colostrum sample, so that TGFβ2 made up 72% of the TGFβ detected in the stomach content preparation compared to 95 % of the TGFβ detected in the colostrum powder. TGFβ was not evaluated in the stomach contents of TGFβ1+/+ mice without colostrum feeding as none was not expected to be present (Ishizaka, 1998).

5.4.1.2 TGFB IN THE SERUM

Despite the presence of TGFβ1 and TGFβ2 in the stomach contents of TGFβ1-/- mice fed colostrum, when serum collected at sacrifice was assessed, no TGFβ1 or TGFβ2 was detected. The serum of TGFβ1+/+ controls showed the expected levels of TGFβ1 (Figure 5-5B).

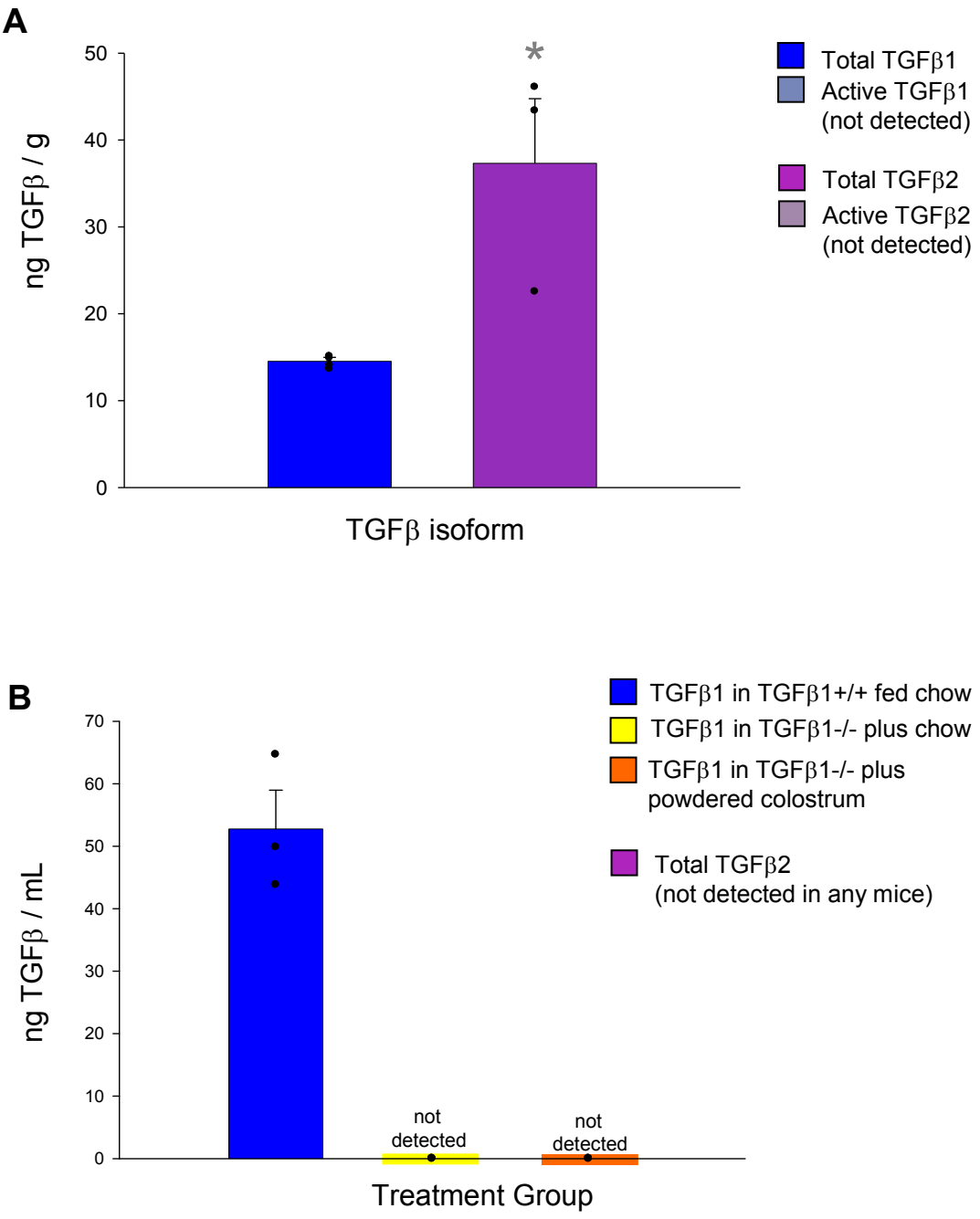


Figure 5-5 Effect of colostrum feeding in TGFβ1-/- mice.

TGFβ in the stomach contents of TGFβ1-/- mice (A) and in the serum of TGFβ1+/+ and TGFβ1-/- mice (B) following feeding with colostrum for two days. TGFβ was measured by isoform specific ELISA. Bar plots show the mean (± SEM), dot plots show individual sample values. * denotes significant difference from TGFβ1 isoform. Data were analysed using the t-test. n = 3 per group.

5.4.2 Recombinant Human Latent TGFβ1 Injection in Mice

Since orally administered TGFβ1-containing colostrum appeared not to be a viable source for TGFβ1 uptake into the circulation, a recombinant source of latent TGFβ1 was investigated. A small pilot study was undertaken where latent human TGFβ1 was injected intra-peritoneally to determine whether TGFβ1 could enter the circulation via this route. Two TGFβ1-/- mice were injected with 635 ng rhLTGFβ1 (equivalent to 31.8 ng / mL), while one TGFβ1+/+ mouse and one TGFβ1-/- mouse were injected with phosphate buffered saline (PBS) as controls. The dose of TGFβ1 was chosen to approximate the mean serum content of TGFβ1+/+ and TGFβ1+/- mice (approximately 39 ng / mL, see Chapter 4.6). Blood was recovered over 2.5 hours (at 30, 90 & 150 minutes after administration).

The maximum serum TGFβ1 level recorded for one TGFβ1-/- mouse receiving exogenous TGFβ1 was 21.7 ng / mL at 90 minutes with a slight decline over the next hour to 17.6 ng / mL at 150 minutes. The second TGFβ1-/- mouse receiving exogenous TGFβ1 showed a maximum of only 8.0 ng / mL at 90 minutes, dropping to 3.4 ng / mL at 150 minutes. Active TGFβ1 was not detected in these samples (Figure 5-6).

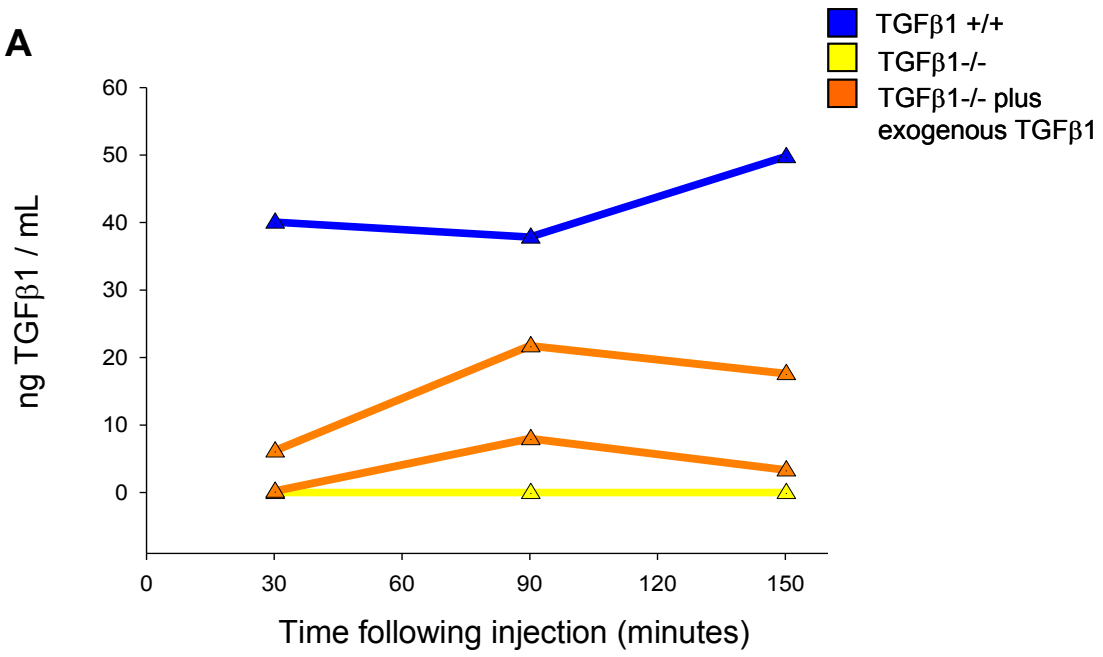


Figure 5-6 Exogenous TGFβ1 in the serum of TGFβ1-/- mice over time.

Two TGFβ1-/- mice injected sub-cutaneously with 635 ng of rhLTGFβ1 and two PBS injected controls. TGFβ1 was measured by ELISA. Line plots show measurements from individual mice over time.

5.5 DISCUSSION

5.5.1 Altered Serum TGFβ1 Levels over Time

Changes in TGFβ1 concentrations in serum were detected in TGFβ1+/+ mice over a 90 minute and over a one week time span. Blood was recovered always at a similar time of day to avoid circadian influences. Bleed location did not appear to influence the TGFβ1 concentration detected, since the tail blood values at 30 minutes were not significantly different from the blood recovered from the heart at 90 minutes. When assessing the TGFβ1 levels in the samples that were taken one week apart, the exposure to females may have influenced the outcome, perhaps due to the effect of TGFβ1 on testosterone production (Avallet, 1994). Alternatively, stress caused by the experimental procedure of extracting blood may also be responsible for differences, as TGFβ1 levels can change in response to stress hormones such as glucocorticoids and catecholamines (Elenkov, 2002). In support of this, various stressors are seen to contribute to altered TGFβ1 levels. For example, in vitro exposure to hypoxic conditions or exercise performed by healthy human individuals was shown to increase the TGFβ1 concentration (Heinemeier, 2003). Thus, stress in various forms may alter circulating TGFβ1 levels.

5.5.2 Reduced TGFβ in Milk Following Pasteurisation

The TGFβ1 and TGFβ2 isoforms were similar in proportions and quantity in the bovine milk samples as described elsewhere (Jin, 1991) (Ginjala, 1998) (Pakkanen, 1998). Both isoforms were significantly depleted following pasterurisation, however TGFβ2 appeared more susceptible to degradation, presumably due to differences in secondary or tertiary protein structure. Betaglycan, a soluble receptor in milk (Zhang, 2001), is reported to preferentially bind to TGFβ2 (Velasco-Loyden, 2004), providing a mechanism that under some circumstances might preferentially sequester and protect TGFβ2 compared with TGFβ1.

In raw milk, TGFβ was predominantly in its latent form, whereas pasteurisation not only reduced total TGFβ but also activated it. TGFβ in the breast milk of many species is predominately in the latent form (Xu, 1999) (Pakkanen, 1998) (Srivastava, 1996) implying that TGFβ entering the digestive tract is not naturally in the active form. Active TGFβ entering the intestine would presumably be able to bind to receptors on gut epithelial cells without prior regulation by the activation process, perhaps causing aberrant TGFβ signalling. Thus consumption of pasteurised milk could result in different exposures of the gut to active and latent TGFβ than un-pasteurised milk.

Low levels of TGFβ were found in the pasteurised and commercially available milks and the slight differences between the milks could be due to the different batches they were likely derived from. These results also show that sample packaging, transport and shelf storage time of around one week at 4ºC in the supermarket, had little effect on TGFβ stability. As TGFβ-rich diets are capable of repairing some intestinal disorders (Oz, 2004), the reduction of TGFβ in our modern milk supply might be a contributing factor in allergies and intolerances that some consumers of cows' milk experience (Kalliomaki, 1999) (Saarinen, 1999). It is interesting to speculate that intestinal disturbances might be reduced if enough latent TGFβ remained in milk or elsewhere in the diet to impose its tolerogenic properties.

5.5.3 High Levels of TGFβ in Colostrum

Both TGFβ1 and TGFβ2 were high in a colostrum based protein drink and the concentration process that extracts water during manufacture is likely the main contributor to this. As with raw milk, the latent form in the powdered colostrum was much higher than the active form. The higher proportion of TGFβ2 (95%) in this sample compared to the milk samples may represent the mammary gland differentially regulating relative amounts of TGFβ1 and TGFβ2 synthesised in early versus late lactation.

5.5.4 TGFβ is Found in the Stomachs of Colostrum Fed Mice

TGFβ1-/- mice clearly ingested the powdered colostrum as both TGFβ1 and TGFβ2 isoforms were detected in the stomach contents. Despite this, the amounts detected by ELISA were lower than those present in intact colostrum powder (by 68% and 96% respectively) and this may be due to dilution after ingestion or coagulation of the milk proteins within the gastric environment (Berendsen, 1980). When comparing the different isoforms, the proportion of TGFβ2 (72%) in the stomach contents was somewhat reduced compared to the colostrum powder (95%). This suggests that TGFβ2 may be more susceptible to proteolytic or acid-mediated breakdown than TGFβ1.

Surprisingly, no active TGFβ1 or TGFβ2 was detected in the stomach contents of mice fed colostrum powder. Protease inhibitors in milk and the milk milieu of the protein can minimize TGFβ activation (Playford, 1993) (Ishizaka, 1998), however it is more likely that active TGFβ in the stomach is relatively labile and difficult to detect.

5.5.5 TGFβ is Not Found in The Serum of Colostrum Fed Mice

Following two days of consumption, neither TGFβ isoform was detected in the blood implying that TGFβ delivered orally in the diet does not substantially affect systemic levels of TGFβ. As these mice were adults, they should possess a mature intestinal tract (Teichberg, 1990), where the digestive barriers are also fully formed and M-cell or receptor mediated transport mechanisms might be required for TGFβ uptake. This result contrasts with a systemic effect of orally administered TGFβ reported in a mouse model of food allergy (Okamoto, 2005a). However it is not known if the effects in this TGFβ replete model were due to TGFβ entering the circulation or acting at the gut epithelial surface or via other indirect immune modulating pathways.

5.5.6 Recombinant Human Latent TGFβ1 Injection in Mice

TGFβ1 was found to enter the circulation after intra-peritoneal injection of recombinant latent cytokine. Levels were highest at 90 minutes, however its stability or ability to be taken up into the circulation may vary between individual mice. Active TGFβ was not detected. While it may have become associated with alpha 2-macroglobulin (alpha 2M) or beta 2- macroglobulin (beta 2M), this would not have precluded it from being detected by ELISA (Feige, 1996) (Hegele, 2002). Alternatively, any exogenous TGFβ1 converted to the active form may have been immediately taken up into tissues, activated and then utilised.

5.6 SUMMARY

Both native and recombinant sources of TGFβ were evaluated as candidate sources for oral and intraperitoneal delivery into TGFβ1-/- mice respectively. TGFβ1 and TGFβ2 were present in milk but were activated and substantially reduced in concentration following pasteurisation. High levels of TGFβ were found in a commercially available colostrum powder. When the colostrum powder was fed to TGFβ1-/- mice, TGFβ1 was detected in the stomach contents but not in the serum, showing that oral ingestion of TGFβ does not contribute to detectable circulating TGFβ1 levels in the TGFβ1-/- mice. Intra-peritoneal administration of exogenous TGFβ1 showed that while TGFβ1 reached the circulation, it began to decline after 90 minutes thus was also of limited utility as a method for systemically delivering a constant supply of exogenous TGFβ1.

Chapter 6:
Reproductive Impact of Exogenous TGFβ1 Administration

6.1 INTRODUCTION

In chapter 4, the most effective method of detecting TGFβ1 bioavailability in vivo was found to be measurement of circulating levels of TGFβ1 in serum. However in chapter 5, neither oral ingestion of TGFβ nor a single intra-peritoneal injection of TGFβ1 was found to be suitable for delivering a constant level of cytokine. To resolve this, sub-cutaneously implanted mini-osmotic pumps were next utilised here to provide a continuous infusion of exogenous TGFβ1 to the circulation of TGFβ1-/- mice. Serum levels of TGFβ1 were assessed to confirm delivery to the circulation, while the biochemical and physiological consequences in tissues and on reproductive behaviour were assessed to elucidate those parameters that may be responsive to exogenous TGFβ1 administration.

The most evident reproductive lesion in male TGFβ1-/- mice was reduced testosterone, consistent with other studies showing TGFβ1 regulation of male steroidogenesis (Salama, 2001) (Gautier, 1997). The steroidogenic enzyme 3β-hydroxysteroid dehydrogenase (HSD3β1) was previously found to be elevated in TGFβ1-/- mice (Ingman, 2002) potentially due to a disrupted negative-feedback loop with testosterone (Heggland, 1997) (Fournet, 1996) (Cherradi, 1995) (Herrmann, 2002). Therefore it was of interest to evaluate whether an exogenous source of TGFβ1 applied in vivo may subsequently impact on testosterone production or HSD3β1 expression.

For exogenous TGFβ1 to induce such changes in the testes, it needs to bind to TβRII and signal through TβRI (Kolodziejczyk, 1996). As TβRII in epithelial kidney cells from TGFβ1-/- mice are significantly over-expressed compared to their TGFβ1+/+ littermates (Sarkar, 2005), it seems reasonable that adequate levels of TβRII would be available for exogenous TGFβ1 to bind with. Additionally, as TβRI is under-expressed in kidney cells from TGFβ1-/- mice and is capable of up-regulation in the presence of exogenous TGFβ1 (Sarkar, 2005), the TGFβ1 treatment provided here may influence TβRI expression levels in the testes of the TGFβ1-/- mice.

Another parameter that may be affected by exogenous TGFβ1 is sperm production. TGFβ1 is found to be influential in spermatogenesis (Teerds, 1993) (Fritz, 1994) (Avallet, 1997) and is purported to play a direct role in germ cell proliferation and differentiation in vitro (Haagmans, 2003). Furthermore, some TGFβ1-/- mice are seen to suffer a severe disruption to epididymal sperm numbers (Ingman, 2002). Thus, sperm production in vivo was also examined here following exogenous TGFβ1 treatment.

The ability of the TGFβ1-/- mice to achieve erections, as described in chapter 3, implies that their erectile pathways are functional however their reduced erection rate indicates that some components of the pathway are compromised. Since a relationship between circulating levels of TGFβ1 and erectile activity is also seen in humans (Ryu, 2004), the delivery of exogenous TGFβ1 to the circulation of TGFβ1-/- mice may restore erectile frequency. TGFβ1 may also affect their behavioural activities as demonstrated by their reduced capacity to perform genital grooming (Chapter 3). Additionally, previous behavioural studies in the presence of females revealed that the TGFβ1-/- males could not effectively mount, intromit or ejaculate (Ingman, 2002). Thus, the ability of exogenous TGFβ1 to influence genital grooming as well as sexual behaviour towards receptive females was determined here.

The experiments described below analyse whether subcutaneous delivery of exogenous TGFβ1 can alter circulating levels of TGFβ1 in TGFβ1-/- male mice and subsequently affect reproductive parameters. TGFβ1 was measured by commercial ELISA, while testosterone was measured using RIA. HSD3β1 and TβRI gene expression was assessed in the testes using real time RT-PCR and sperm was counted in the cauda epididymis. Genital grooming and erectile activities were observed directly, while mounting, intromission and ejaculation behaviours with females were video taped for later analysis.

6.2 SYSTEMIC DELIVERY OF EXOGNEOUS TGFB1

6.2.1 Administration of TGFβ1

TGFβ1-/- mice were given human recombinant latent TGFβ1 with a 2% BSA carrier, sub-cutaneously via mini osmotic pumps, for 14 days at 27 ng per gram of body weight per hour. This rate was estimated to supply TGFβ1 to a level similar to that found in the serum of fertile TGFβ1+/- mice (mean ± SEM = 23.7 ± 2.0 ng / mL) (Figure 4-5) assuming the exogenous TGFβ1 was turned over every hour. Control mice were TGFβ1+/+ mice and TGFβ1-/- mice with pumps containing PBS and 2% BSA carrier. The experimental protocol is depicted below (Figure 6-1). Males were subjected to two rounds of identical mating experiments, on days 7 and 14 following pump insertion. All mating studies were preceded by a non-contact adaptation period of three nights (days 2 - 4 and days 9 - 11) with adult female mice in Whitten cages. Blood samples were taken on day 7 and at post-mortem on day 14 to determine circulating TGFβ1 and testosterone levels. Tissue samples were taken at post-mortem on day 14 to determine macrophage density in the liver as well as HSD3β1 mRNA and TβR1 mRNA in the testes. Sperm in the cauda epididymis was counted. Responses for the TGFβ1 treated TGFβ1-/- mice were compared to both TGFβ1+/+ and TGFβ1-/- age-matched controls with pumps containing BSA carrier only. Body weights were recorded throughout the trial as a general measure of overall health. Additionally, to further examine responses, each treatment group was individually assessed using the Spearman's rho correlation test to determine if any of the investigated parameters might be causally related to each other.

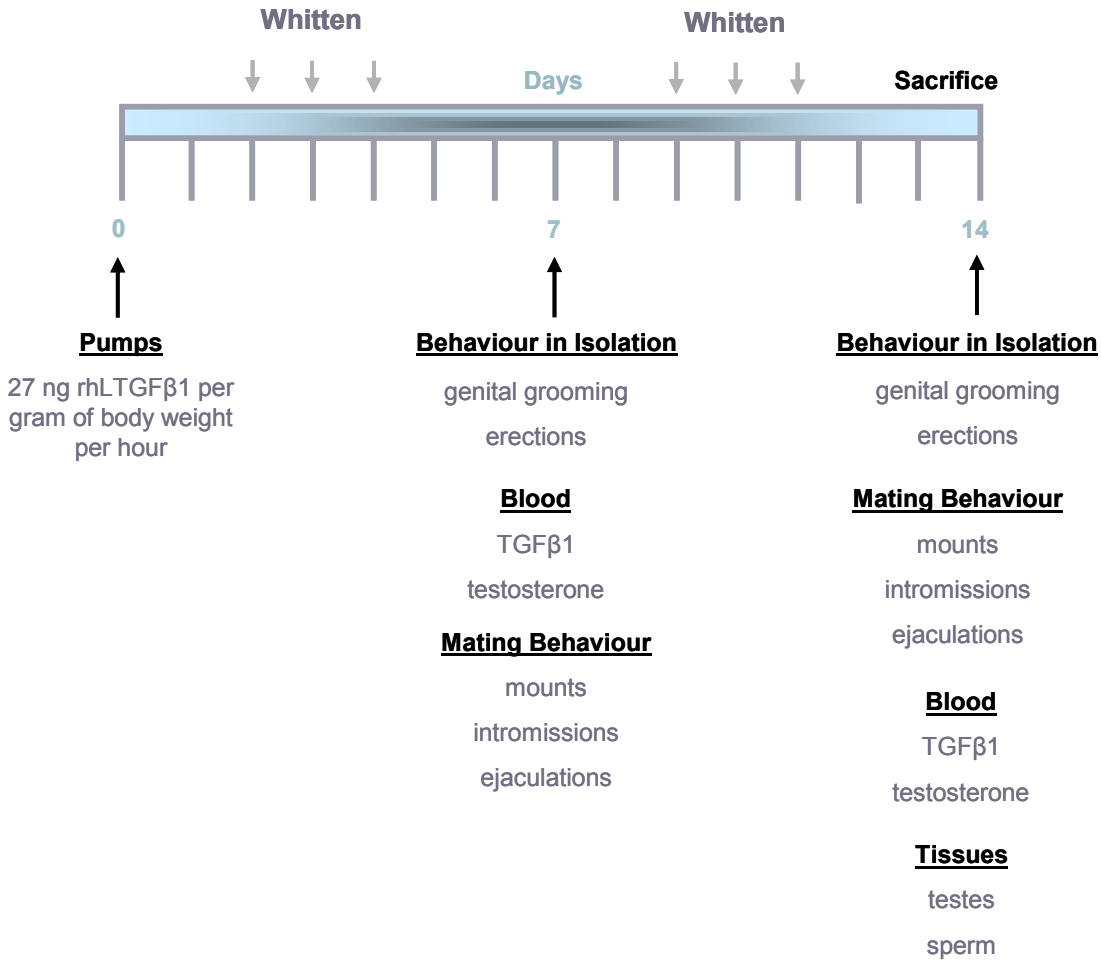


Figure 6-1 Timeline of exogenous TGFβ1 replacement for assessment of TGFβ1 delivery and reproductive parameters.

6-8 week old male mice were housed individually for at least 5 days prior to pump insertion. On day 0 mini-osmotic pumps containing recombinant latent TGFβ1 were implanted. Males were exposed to female mice in Whitten cages prior to nocturnal mating experiments. On day 7 a blood sample was collected and behaviour was assessed, on day 14 behaviour was assessed then blood and tissues were collected upon sacrifice.

Initial body weights differed (34.6%) between the TGFβ1+/+ controls (mean ± SEM = 21.7 ± 0.7 grams) and the TGFβ1-/- controls (14.2 ± 0.5 grams) ($p < 0.05$). By completion of treatment there was no significant difference between the weights of TGFβ1-/- mice used as controls and the TGFβ1-/- mice that received exogenous TGFβ1 (15.4 ± 0.8 grams). Differences were not evident when the intermediate (day 7) and final (day 14) body weights were analysed separately (data not shown). Interestingly, it was noted that the difference in body weights between the genotypes used here was significantly greater than the difference reported earlier using TGFβ1-/- mice of a similar age, from the same colony where the TGFβ1-/- mice weighed approximately 20% more at the same age (mean ± SEM = 19.4 ± 1.3 grams) (n = 7) (Ingman, 2002).

6.2.2 Serum Levels of TGFβ1

Blood was obtained at 7 and 14 days following mini-osmotic pump insertion. TGFβ1 was then measured in the serum by ELISA (Methods 2.4).

The TGFβ1-/- mice receiving exogenous cytokine had detectable TGFβ1 in their blood on day 7 (mean ± SEM = 0.8 ± 0.2 ng / mL) and higher levels on day 14 (2.0 ± 0.5 ng / mL). These levels were higher than the background levels found in the TGFβ1-/- controls on both days (0.1 ± 0.01 ng / mL) but were significantly lower than those found in the TGFβ1+/+ controls at day 7 (75.0 ± 6.7 ng / mL) and at day 14 (52.7 ± 3.3 ng / mL). With regard to the TGFβ1 treated TGFβ1-/- mice (n = 10), they were deemed not to have achieved successful TGFβ1 replacement if the serum value obtained on day 14 was similar to the background values obtained for the TGFβ1-/- controls (0.1 ng / mL). Two of ten mice with TGFβ1 implants fell into this category on day 14. As they were ‘not replaced’ (NR), the results of data sets in this chapter exclude these two mice (n = 8). With exclusion of these two values the mean TGFβ1 serum concentration of the remaining mice at day 14 was (2.5 ± 0.4 ng / mL). All TGFβ1 treated TGFβ1-/- mice on day 7 (n= 10) are included regardless of their serum TGFβ1 levels on day 7 as treatment was deemed to be ongoing. All TGFβ1+/+ controls were included (n = 13) for analysis. Significant differences were not found when comparing the TGFβ1-/- mice across the two test days, irrespective of TGFβ1

treatment ($p > 0.05$). However, a significant difference was found between the two test days when the TGFβ1+/+ controls were assessed ($p < 0.05$) (Figure 6-2).

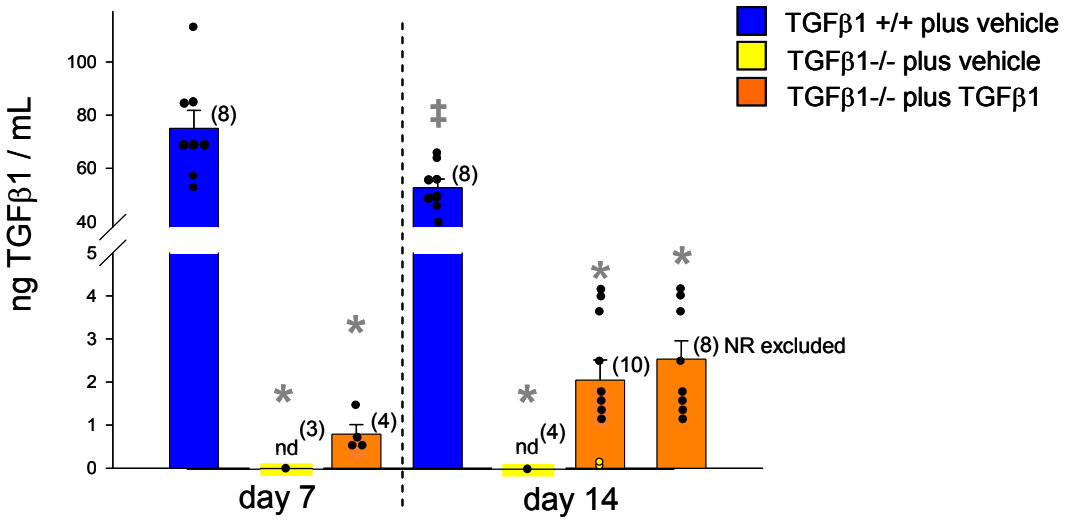


Figure 6-2 Serum levels in TGFβ1-/- mice treated with exogenous TGFβ1.

Levels of TGFβ1 were measured by ELISA in serum recovered on day 7 and day 14 of the treatment protocol. Bar plots show the mean (± SEM), dot plots show individual sample values. Data are shown with and without not replaced (NR) mice (defined as not showing TGFβ1 levels above background) (yellow = NR mice). * denotes significant difference from TGFβ1+/+ controls and ‡ denotes significant difference between days within groups. Data were analysed using ANOVA followed by the Bonferroni t-test. n is shown in parentheses. nd denotes not detected.

6.3 REPRODUCTIVE EFFECTS OF TGFB1 REPLACEMENT

6.3.1 Total Testosterone in Serum

Serum collected at days 7 and 14 was measured for total testosterone by radioimmunoassay (RIA) (Methods 2.3).

Total testosterone in the TGFβ1-/- control group (mean ± SEM = 3.8 ± 2.9 nM) was significantly reduced compared to the TGFβ1+/+ control group (8.9 ± 5.7 nM) on day 7. The TGFβ1-/- mice treated with exogenous TGFβ1 revealed a higher mean testosterone level (13.3 ± 8.8 nM) and this value was not significantly different to the TGFβ1+/+ controls ($p = 0.08$). Measurements on day 14 showed again that the TGFβ1-/- (2.2 ± 1.2 nM) controls were significantly different to TGFβ1+/+ controls (10.7 ± 3.7 nM), and mice receiving exogenous TGFβ1 showed similar testosterone levels (5.7 ± 4.0 nM) to the TGFβ1+/+ controls ($p = 0.07$) (Figure 6-3). Despite these results it is worth noting that neither day showed a statistical difference between the TGFβ1-/- control group and the TGFβ1 treated TGFβ1-/- mice. No significant differences in testosterone levels were found when comparing the same treatment groups across the two test days ($p > 0.05$).

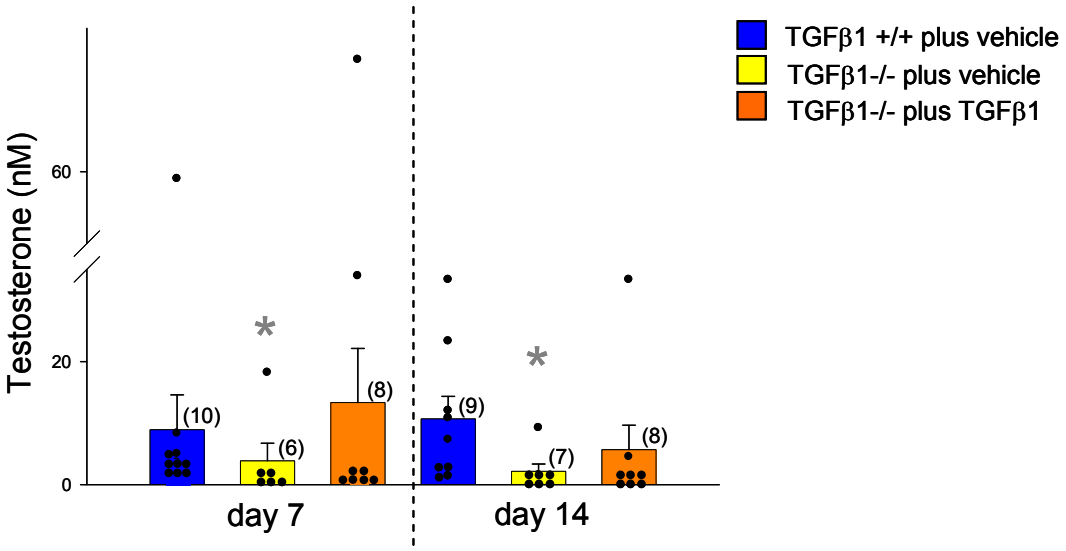


Figure 6-3 Effect of TGFβ1 replacement on total serum testosterone.

Serum levels of testosterone were measured by RIA on day 7 and day 14 of the treatment protocol. Bar plots show the mean (± SEM), dot plots show individual sample values. * denotes significant difference from the TGFβ1+/+ controls ($p < 0.05$). Data were analysed using the Mann Whitney U method. n is shown in parentheses.

6.3.2 3β-hydroxysteroid dehydrogenase (HSD3β1) mRNA in Testes

The testes were excised at post mortem on day 14 of TGFβ1 replacement. HSD3β1 mRNA expression was assessed using real time RT-PCR and normalised to the β-actin housekeeping gene. The mean of the TGFβ1+/+ control group was assigned an arbitrary value of 100 (Methods 2.9).

There were no significant differences in HSD3β1 mRNA between the TGFβ1+/+ controls (mean ± SEM = 100.3 ± 42.0), the TGFβ1-/- controls (45.8 ± 11.9) or the TGFβ1-/- mice receiving exogenous TGFβ1 (46.7 ± 9.3), irrespective of an outlier included in the analysis of in the TGFβ1+/+ control group (Figure 6-4A).

6.3.3 Transforming Growth Factor β Receptor 1 (TβRI) mRNA in Testes

TβRI mRNA expression was assessed in the testes using real time RT-PCR and normalized to the 18S housekeeping gene. The mean of the TGFβ1+/+ control group was assigned an arbitrary value of 100 (Methods 2.9).

There were no significant differences in TβRI mRNA between the TGFβ1+/+ controls (mean ± SEM = 100.4 ± 12.6), the TGFβ1-/- controls (108.7 ± 44.1) or the TGFβ1-/- mice receiving exogenous TGFβ1 (76.0 ± 22.4), irrespective of an outlier included in the analysis of the TGFβ1-/- control group (Figure 6-4B).

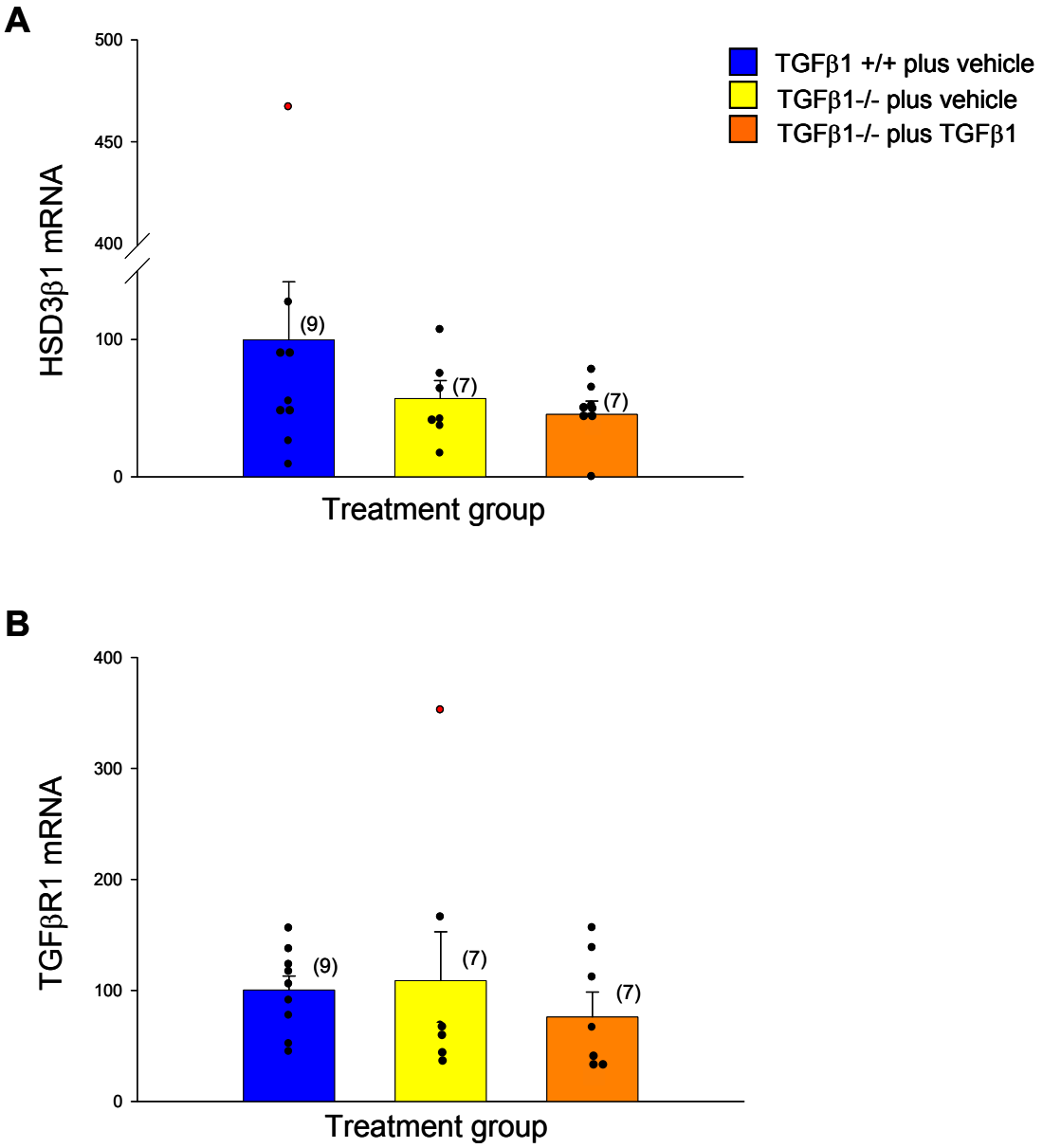


Figure 6-4 Effect of TGFβ1 replacement on HSD3β1 (A) and TGFβRI (B) mRNA expression in the testes.

mRNA levels were quantified by RT-PCR in tissues recovered on day 14 of the treatment protocol. Bar plots show the mean (± SEM), dot plots show individual sample values (red = outlier). No significant differences between treatment groups were found irrespective of outliers ($p>0.05$). Data were analysed using the Mann Whitney U method. n is shown in parentheses.

6.3.4 Sperm Count

Sperm was extruded from both cauda at post mortem on day 14 and diluted in Hanks Balanced Salt Solution (HBSS) and an aliquot was counted to determine the total number of epididymal sperm per mouse (Methods 2.13).

Analysis did not reveal any significant differences in sperm counts between the TGFβ1+/+ controls (mean ± SEM = 16.0 ± 5.0 million), the TGFβ1-/- controls (10.0 ± 4.8 million) or the TGFβ1-/- mice receiving exogenous TGFβ1 (13.0 ± 4.6 million) (Figure 6-5).

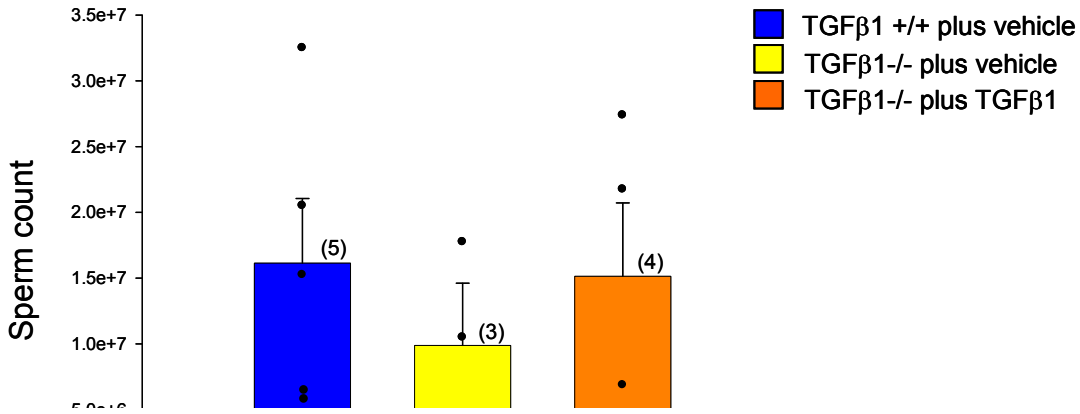


Figure 6-5 Effect of TGFβ1 replacement on sperm count.

Sperm was extruded from the cauda epididymis of each mouse. An aliquot was counted by light microscopy on day 14 of the treatment protocol. Bar plots show the mean (± SEM), dot plots show individual sample values. No significant differences were found ($p>0.05$). Data were analysed using the ANOVA Bonferroni method. n is shown in parentheses.

6.4 EFFECTS OF TGFB1 REPLACEMENT ON BEHAVIOUR DURING ISOLATION

To examine the behaviour of male mice in the absence of female contact, they were placed individually in clear-walled, plastic cages and observed for 1 hour between 13.00 hours and 16.00 hours on day 7 and 14 following pump insertion. Genital grooming and erectile activity were recorded (Methods 2.11).

6.4.1 Wakefulness During Observation Period

The amount of time spent asleep and awake were also recorded to determine if this influenced the amount of time spent on reproductive behaviours. Data collected on day 7 showed that per hour, the TGFβ1+/+ controls slept between 0 and 25 minutes (mean ± SEM = 6.6 ± 2.2 minutes, n = 12) and the TGFβ1-/- controls slept between 0 and 40 minutes (11.7 ± 6.9 minutes, n = 6). The TGFβ1-/- mice receiving exogenous TGFβ1 slept between 0 and 30 minutes (10.8 ± 4.5 minutes, n = 6). On day 14 the TGFβ1+/+ controls slept between 0 and 30 minutes per hour (11.5 ± 3.4 minutes, n = 10) and the TGFβ1-/- controls slept between 0 and 10 minutes (5.0 ± 2.2 minutes, n = 6). The TGFβ1-/- mice receiving exogenous TGFβ1 slept between 0 and 25 minutes (10.0 ± 4.1 minutes, n = 6). No statistically significant differences between groups were found on any day. As the mice exhibited similar patterns of wakefulness, it was concluded that sleep would not influence time spent on reproductive behaviours.

6.4.2 Genital Grooming

A genital grooming event was recorded each time the mice bent over on their hind legs, in the absence of an erection, to lick their genital area. Assessment of genital grooming was carried out for a 1 hour test period as described in Chapter 3, where the number of mice displaying greater than one genital grooming act per hour was identified as a useful parameter.

On day 7, a similar number of TGFβ1+/+ controls (88.9%), TGFβ1-/- controls (85.7%) and TGFβ1-/- mice receiving exogenous TGFβ1 (71.4%) performed greater than one genital grooming act, with the number of grooming acts ranging from zero to fourteen. Day 14 results were comparable, where a similar number of TGFβ1+/+ (87.5%) and TGFβ1-/- controls (71.4%) performed genital grooming behaviour. The percentage of TGFβ1 treated TGFβ1-/- mice was lower (50.0%) but this value was not statistically different to the control groups. The number of grooming acts at day 14 ranged from zero to ten (Figure 6-6A). No significant differences were found when the same treatment groups were compared across the two test days ($p > 0.05$).

The number of genital grooming acts performed at day 7 by the TGFβ1-/- controls (mean ± SEM = 5.3 ± 1.8 grooming events per hour), the TGFβ1+/+ controls (7.7 ± 1.5 grooming events per hour) and the TGFβ1-/- mice receiving exogenous TGFβ1 (4.5 ± 1.7 grooming events per hour) were similar. At day 14, genital grooming rates were again similar for the TGFβ1-/- controls (4.7 ± 1.4 grooming events per hour), the TGFβ1+/+ controls (5.3 ± 1.1 grooming events per hour) and the TGFβ1-/- mice receiving exogenous TGFβ1 (3.0 ± 1.1 grooming events per hour) (Figure 6-6B). No significant differences were found when the same treatment groups were compared across the two test days ($p > 0.05$).

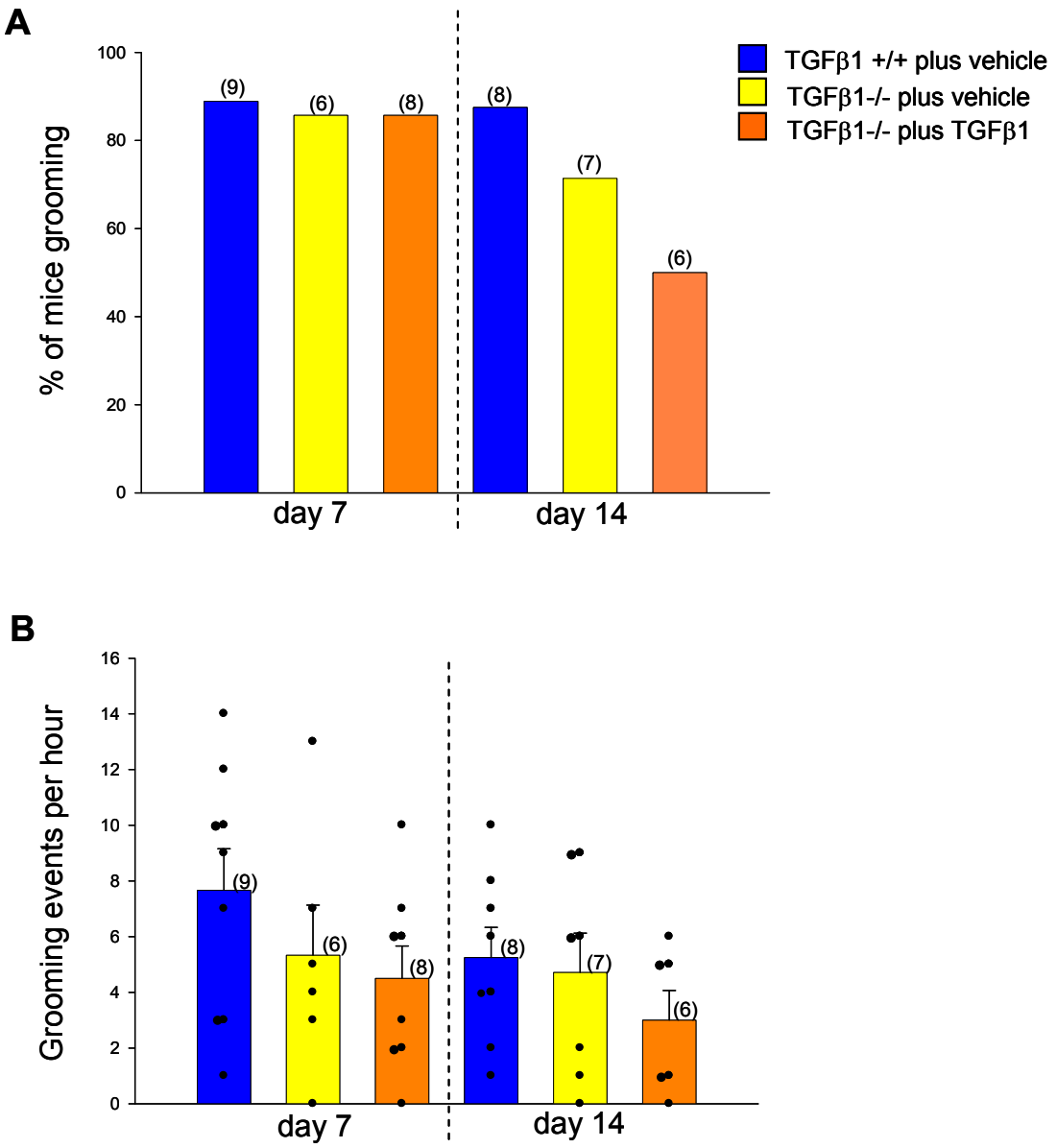


Figure 6-6 Effect of TGFβ1 replacement on the percent of mice grooming genitals (A) and grooming frequency per hour (B).

Grooming was visualised by placing mice in clear cages and observing for one hour on day 7 and day 14 of the treatment protocol. Bar plots show the mean (± SEM), dot plots show individual sample values. No significant differences were found ($p>0.05$). Data were analysed using the Chi-Square test (A) and Mann Whitney U method (B). n is shown in parentheses.

6.4.3 Erectile Activity

Erectile activity was recorded when an erect penis was visible and was distinct from a genital grooming act. Erections persisted for up to 30 seconds (Figure 3-13).

At day 7 (Figure 6-7A), significantly reduced numbers of TGFβ1-/- controls (28.6%) had one or more erections in the 1 hour test period compared to the TGFβ1+/+ controls (81.8%). The proportion of TGFβ1-/- mice receiving exogenous TGFβ1 displaying erectile behaviour (42.9%) was no longer different to the TGFβ1+/+ controls. Day 14 (Figure 6-7A) observations also revealed that the percentage of TGFβ1-/- controls achieving erections (14.3%) was significantly reduced compared to TGFβ1+/+ controls (60.0%), however this time the mice receiving exogenous TGFβ1 did not display any erections (0%) (Figure 6-7A). No significant differences were found when the same control treatment groups were compared across the two test days ($p > 0.05$). However, a significant reduction in incidence of erections was seen at day 14 compared with day 7 in the TGFβ1-/- mice receiving exogenous TGFβ1 ($p < 0.05$).

An erection rate was calculated following the observation that some mice achieved greater than one erection during the 1 hour test period. Analysis of the erection rate per hour on day 7 (Figure 6-7B), showed the TGFβ1-/- controls (mean ± SEM = 0.3 ± 0.2 erections per hour) had a significantly reduced erection rate compared to the TGFβ1+/+ controls (1.1 ± 0.2 erections per hour) and that replacement of TGFβ1 had no effect on this (0.4 ± 0.2 erections per hour). Day 14 observations (Figure 6-7B) also revealed that the rate of erections achieved by the TGFβ1-/- controls (0.1 ± 0.1 erections per hour) was significantly reduced compared to the TGFβ1+/+ controls (0.9 ± 0.3 erections per hour) and again treatment with TGFβ1 did not increase this (0 erections per hour) (Figure 6-7B). No significant differences were found when the same treatment groups were compared across the two test days ($p > 0.05$).

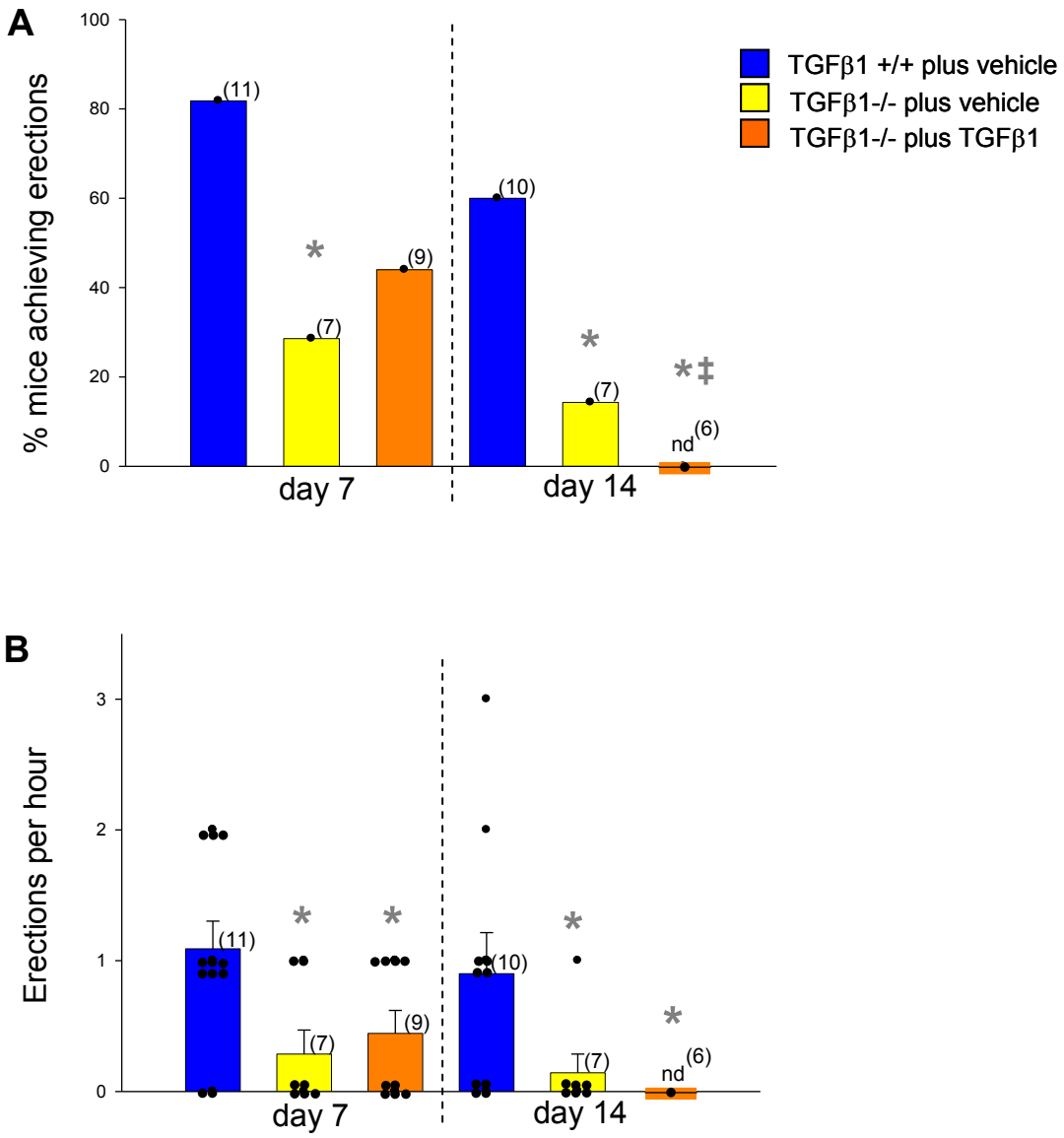


Figure 6-7 Effect of TGFβ1 replacement on the percent of mice achieving erections in 1 hour (A) and frequency of erections per hour (B).

Erections were visualised by placing mice in clear cages and observing for one hour on day 7 and day 14 of the treatment protocol. Bar plots show the mean (± SEM), dot plots show individual sample values. * denotes significant difference from the TGFβ1+/+ controls ($p<0.05$), ‡ denotes significant difference within groups over time and † denotes significant differeance from TGFβ1-/- controls. Data were analysed using the Chi square test (A) and Mann Whitney U method (B). n is shown in parentheses. nd denotes not detected

6.5 EFFECTS OF TGFB1 REPLACEMENT ON BEHAVIOUR DURING CONTACT WITH FEMALES

The reproductive behaviours of males when caged with females were assessed twice during the two week trial. For these mating experiments, two 4 week old, estrous induced, female mice were introduced into each male's cage at 23:00 hours on day 7 and 14. The first 2 hours of contact was recorded from overhead using a video camera under red light. Mating behaviour of the males was quantified from the videos using the following criteria given in Methods 2.11.4.

6.5.1 Mounting

The total number of mounts includes all mounts irrespective of whether they resulted in intromission or ejaculation. Mounting rate was also calculated on a per minute basis, where the total mounts were divided by the number of minutes elapsed until ejaculation occurred or 120 minutes, whichever was first. This value takes into account the reduced interest in pursuing the female mice following a successful ejaculation. Also, the time taken for the males to initiate a mounting act, up to a maximum of 120 minutes, was quantified and is expressed here as mount latency.

Observations on day 7 of the total number of mounts performed by the TGFβ1-/- controls (mean ± SEM = 3.8 ± 3.6 mounts) was significantly reduced compared to the TGFβ1+/+ controls (26.0 ± 4.2 mounts) and replacement with TGFβ1 (9.3 ± 3.5 mounts) had no statistically significant effect. Results were similar at day 14, where the TGFβ1-/- controls (4.1 ± 3.3 mounts) and the TGFβ1-/- mice treated with exogenous TGFβ1 (7.1 ± 3.9 mounts) remained lower than the TGFβ1+/+ controls (21.0 ± 3.3 mounts) ($p < 0.05$) (Figure 6-8A). No significant differences were found when the same treatment groups were compared across the two test days ($p > 0.05$).

The results were similar when the mounts were analysed on a per minute basis. Observations of the number of mounts per minute on day 7 revealed the TGFβ1-/-

controls (mean ± SEM = 0.03 ± 0.03 mounts per minute) mounted females at a significantly lower rate than the TGFβ1+/+ controls (0.34 ± 0.06 mounts per minute) and that treatment with exogenous TGFβ1 (0.08 ± 0.03 mounts per minute) did not improve this. Results were similar at day 14 when the TGFβ1-/- controls (0.04 ± 0.03 mounts per minute) and those treated with exogenous TGFβ1 (0.06 ± 0.03 mounts per minute) mounted at a reduced rate compared to the TGFβ1+/+ controls (0.56 ± 0.11 mounts per minute) (Figure 6-8B). No significant differences were found when the same treatment groups were compared across the two test days ($p > 0.05$).

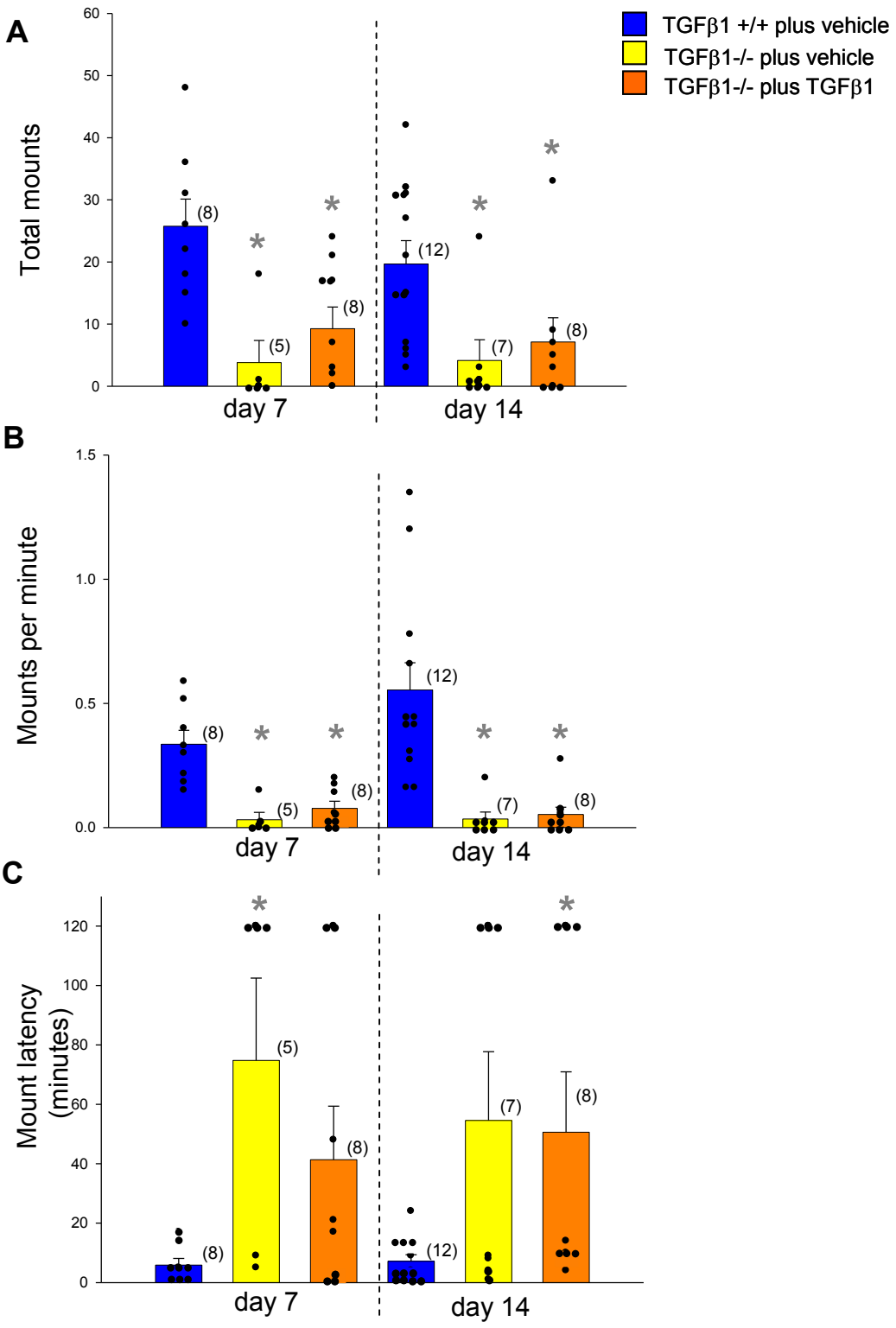


Figure 6-8 Effect of TGFβ1 replacement on total mounts (A), mount rate (B) and mount latency (C).

Mice were placed in clear cages and video recorded for two hours on day 7 and day 14 of the treatment protocol. Bar plots show the mean (± SEM), dot plots show individual sample values. * denotes significant difference from TGFβ1+/+ controls ($p<0.05$). Data were analysed using the Mann Whitney U method. n is shown in parentheses.

Observations on day 7 of the mount latency displayed by the TGFβ1-/- controls (mean ± SEM = 74.8 ± 27.7 minutes) revealed that they mounted the females significantly later in the test period than the TGFβ1+/+ controls (5.9 ± 2.3 minutes). However, treatment with exogenous TGFβ1 (41.4 ± 18.0 minutes) significantly reduced this, so that mount latency was restored to a duration similar to that experienced by the TGFβ1+/+ controls. At day 14, mount latency displayed by the TGFβ1-/- controls (54.6 ± 23.2 minutes) was not different to the TGFβ1+/+ controls (7.2 ± 2.2 minutes), while the TGFβ1-/- mice treated with exogenous TGFβ1 (50.6 ± 20.3 minutes) were different (Figure 6-8C). Significant differences were not found when the same treatment groups were compared across the two test days ($p > 0.05$).

6.5.2 Intromissions

The total number of intromissions includes both those that led to ejaculation and those that did not. Intromission rate was also calculated on a per minute basis, where the total intromissions were divided by the number of minutes elapsed until ejaculation occurred or 120 minutes, whichever was first. This value takes into account the reduced interest in pursuing the female following a successful ejaculation. Also, the time taken for the males to initiate an intromission act, up to a maximum of 120 minutes, was quantified and is expressed here as intromission latency.

On day 7, the total number of intromissions performed by the TGFβ1-/- controls (0 intromissions), was significantly reduced compared to the TGFβ1+/+ controls (mean ± SEM = 12.1 ± 3.2 intromissions). Replacement with exogenous TGFβ1 (1.3 ± 0.6 intromissions) did not affect this. At day 14, the results between the controls were similar to those obtained at day 7, where the TGFβ1-/- controls (0 intromissions) were significantly reduced compared to the TGFβ1+/+ controls (10.8 ± 2.3 intromissions) and TGFβ1 treatment (0 intromissions) had no effect (Figure 6-9A). No significant differences were found when comparing the same control treatment groups across the two test days ($p >0.05$). However, a significant difference was found between the two test days when the mice receiving exogenous TGFβ1 were assessed ($p < 0.05$).

The results were similar when the intromissions were analysed on a per minute basis. Observations of the number of intromissions per minute on day 7 revealed that the TGFβ1+/+ controls (0.18 ± 0.05 intromissions per minute) intromitted with females significantly more than the TGFβ1-/- controls (0 intromissions per minute) and treatment with exogenous TGFβ1 altered this (mean ± SEM = 0.01 ± 0.006 intromissions per minute), so that the number of intromissions per minute was increased compared to the TGFβ1-/- controls, however significance was not reached ($p = 0.07$). At day 14, neither the TGFβ1-/- controls (0 intromissions per minute), nor the TGFβ1 treated mice (0 intromissions per minute) intromitted with the females. The TGFβ1+/+ controls (0.33 ± 0.08 intromissions per minute) continued to display intromission behaviour (Figure 6-9B). No significant differences were found when the same control treatment groups were compared across the two test days ($p > 0.05$). However, a significant difference was found between the two test days when the mice receiving exogenous TGFβ1 were assessed ($p < 0.05$).

Due to the absence of intromission behaviour at day 7, the TGFβ1-/- controls were deemed to have an intromission latency of 120 minutes, the maximum value obtainable for this study. This was significantly longer than that obtained for the TGFβ1+/+ controls (mean ± SEM = 12.6 ± 4.5 minutes). Treatment with exogenous TGFβ1 (88.4 ± 13.3 minutes) altered this, so that intromission latency was shorter in duration compared to the TGFβ1-/- controls, however significance was not reached ($p = 0.07$). At day 14, as no TGFβ1-/- mice displayed intromission behaviour, irrespective of TGFβ1 treatment, their latency was also deemed to be 120 minutes. These latency values were both considerably longer than that found for the TGFβ1+/+ controls (12.3 ± 2.5 minutes) ($p < 0.05$) (Figure 6-9C). No significant differences were found when comparing the same control treatment groups across the two test days. However, a significant difference was found between the two test days when the mice receiving exogenous TGFβ1 were assessed ($p < 0.05$).

When individual TGFβ1 treated TGFβ1-/- mice were assessed, those with higher serum TGFβ1 levels did not necessarily show enhanced intromission or mounting activities and neither did those with high testosterone levels. However when both high TGFβ1 and high testosterone levels occurred in one animal, the total number of mounts improved as did the time taken to initiate mounting and intromission

behaviours. This was demonstrated in two of the TGFβ1 treated TGFβ1-/- mice and is compared to 2 control groups comprising (1) two TGFβ1 treated TGFβ1-/- mice with high TGFβ1 and low testosterone and (2) two control TGFβ1-/- mice showing the highest levels of testosterone (Figure 6-10).

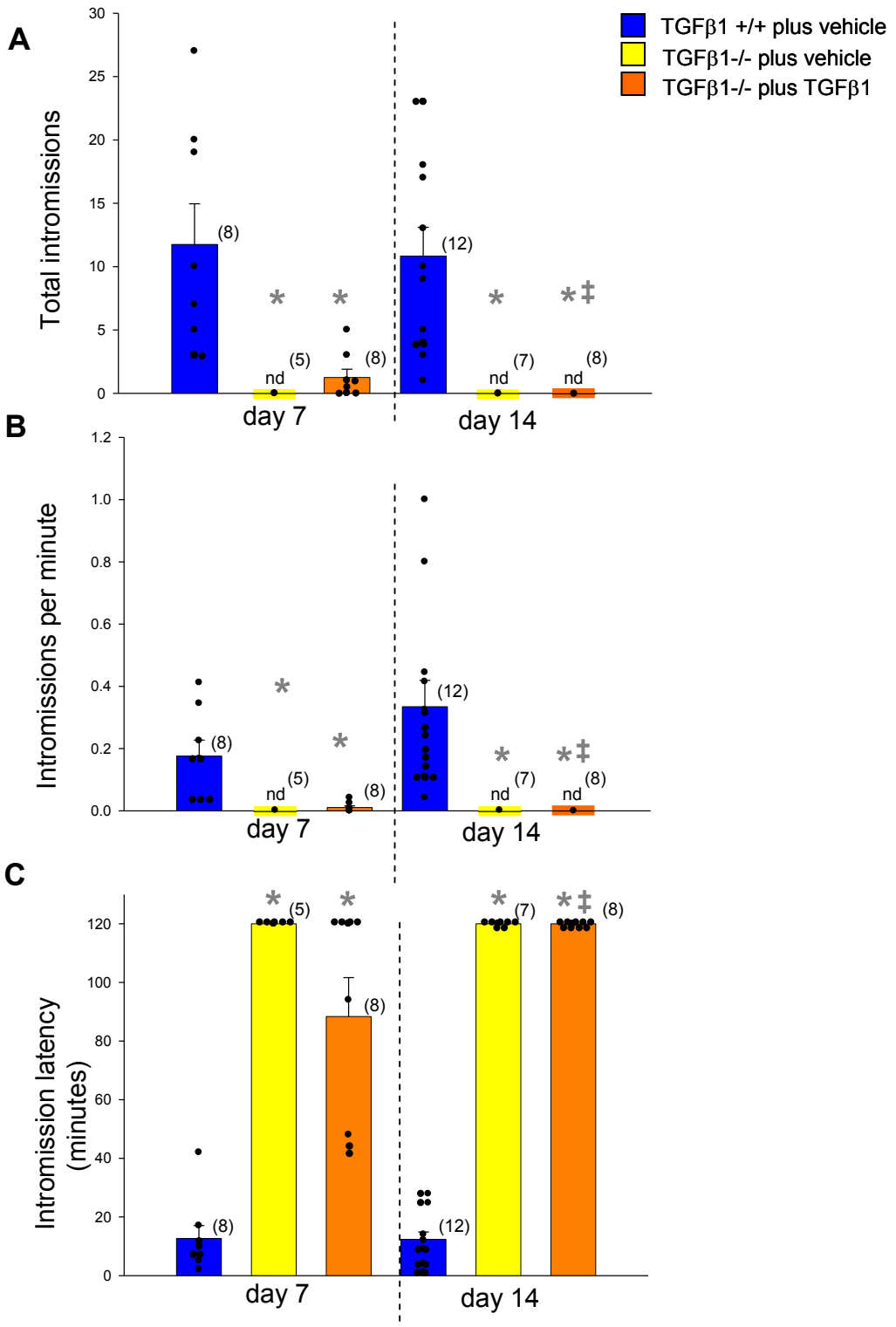


Figure 6-9 Effect of TGFβ1 replacement on total intromissions (A), intromission rate (B) and intromission latency (C).

Mice were videoed for two hours on day 7 and day 14 of the treatment protocol. Bar plots show the mean (± SEM), dot plots show sample values. * denotes significant difference from TGFβ1+/+ controls ($p<0.05$), † denotes significant difference from TGFβ1-/- controls and ‡ denotes significant difference within groups over time. Data were analysed using the Mann Whitney U method. n is shown in parentheses.

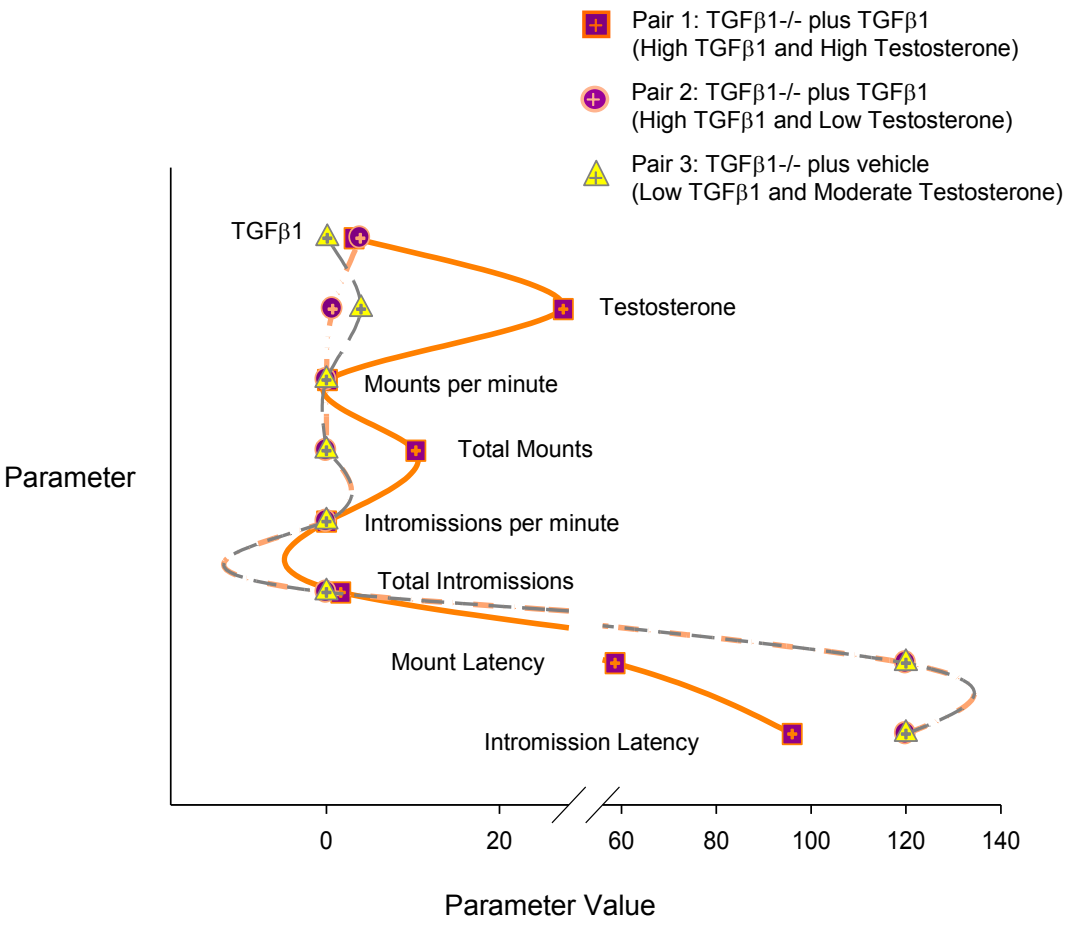


Figure 6-10 Combined TGFβ1 and testosterone levels compared to copulation activity levels.

Each curve is the averaged result of both test days for two mice. Pair 1 was selected for having the highest levels of both TGFβ1 and testosterone in the TGFβ1 treated TGFβ1-/- group. Pair 2 was selected as a high TGFβ1/low testosterone control group. Pair 3 was selected as a low TGFβ1 control group (two with the highest levels of testosterone were chosen).

6.5.3 Ejaculations

The number of mice achieving ejaculations and the total number of ejaculations achieved within the two hour observation period were recorded. Also, the time taken for the males to perform an ejaculation act, up to a maximum of 120 minutes, was quantified and is expressed here as ejaculation latency.

Day 7 observations revealed that none of the TGFβ1-/- mice (0.0%) displayed ejaculation behaviour even following TGFβ1 treatment (0.0%) and that these values were significantly lower than that obtained for the TGFβ1+/+ controls (37.5%). Results were similar at day 14, where none of the TGFβ1-/- mice (0.0%) displayed ejaculation behaviour, irrespective of TGFβ1 treatment (0.0%), again significantly lower than the proportion of ejaculation success in the TGFβ1+/+ controls (91.7%) (Figure 6-11A). Additionally, the TGFβ1+/+ controls displayed more ejaculation events on day 14 than on day 7 ($p < 0.05$).

Due to the absence of ejaculation events for the TGFβ1-/- mice, irrespective of exogenous TGFβ1 treatment, the ejaculation rates were assigned a zero value for these two groups for both days. Using this value, they were found to have a reduced number of ejaculations per hour than the TGFβ1+/+ controls (mean ± SEM = 0.4 ± 0.2 ejaculations per hour) on day 7, however due to the low number of ejaculations displayed by the TGFβ1+/+ controls, this was not significant. On day 14 the TGFβ1-/- mice, irrespective of exogenous TGFβ1 treatment, were again found to have a significantly reduced ejaculation rate compared to the TGFβ1+/+ controls (1.4 ± 0.2 ejaculations per hour). Further analysis revealed that the TGFβ1+/+ controls ejaculated at a significantly higher rate on day 7 than on day 14 ($p > 0.05$) (Figure 6-11B). Interestingly, more than one ejaculation event was observed for half of the male TGFβ1+/+ mice over the 2 hour assessment period on day 14 (6 of 12 mice, data not shown).

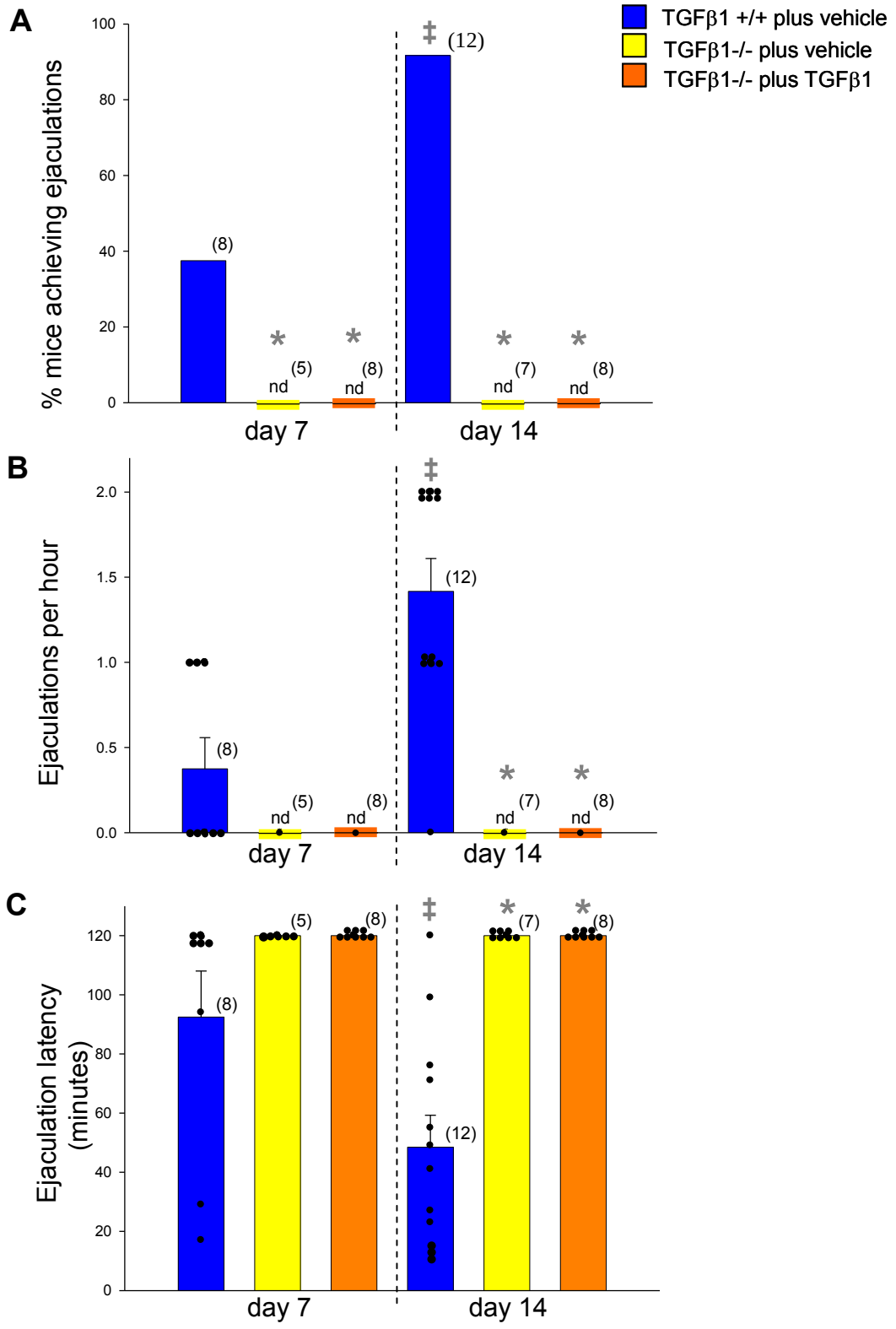


Figure 6-11 Effect of TGFβ1 replacement on mice achieving ejaculations (A), total ejaculations (B) and ejaculation latency (C).

Mice were placed in clear cages and video recorded for two hours on day 7 and day 14 of the treatment protocol. Bar plots are mean +/- SEM, dot plots show individual sample values. * denotes significant difference from TGFβ1+/+ controls ($p<0.05$), ‡ denotes significant difference within groups over time using the Mann Whitney U method. n is shown in parentheses. nd denotes not detected.

Due to the absence of ejaculation events for the TGFβ1-/- mice, irrespective of exogenous TGFβ1 treatment, ejaculation latency was deemed to be 120 minutes for both groups for both days. On day 7, the TGFβ1-/- controls, irrespective of TGFβ1 treatment, were different from the TGFβ1+/+ controls (mean ± SEM = 92.5 ± 15.5 minutes), however this did not reach significance. On day 14 the TGFβ1+/+ controls ejaculated significantly sooner (48.5 ± 10.8 minutes) than the TGFβ1-/- mice irrespective of TGFβ1 treatment. Also, the TGFβ1+/+ controls ejaculated sooner on day 14 than on day 7 ($p < 0.05$) (Figure 6-11C).

6.6 DISCUSSION

6.6.1 Exogenous TGFβ1 was Delivered to the Circulation

Sub-cutaneously implanted mini-osmotic pumps were utilised to provide a continuous infusion of TGFβ1 to the circulation of TGFβ1-/- mice. While the resulting levels of TGFβ1 were found to be significantly lower (< 5 ng / mL) than the amount anticipated (27 ng / mL) by the dose administered, they were higher than the background values obtained for the TGFβ1-/- controls. Importantly the amount of TGFβ1 in the circulation of cytokine replaced mice was comparable to the lowest values for plasma for TGFβ1 seen in TGFβ1+/- heterozygous mice (Figure 4-6). On this basis it was considered that successful TGFβ1 replacement was achieved, albeit at the lowest threshold of what might be considered physiologically normal.

Osmotic function of the pumps was confirmed following comparisons of the initial and final volumes (data not shown), thus three other explanations for low serum levels are provided here. (1) The TGFβ1 may have entered the tissues, been activated and subsequently utilised. A similar explanation has been proposed in previous rodent studies where exogenous TGFβ1 was found to restrict penile growth rate (Gelman, 1998), inhibit intestinal cellular proliferation (Potten, 1995) and reduce numbers of circulating platelets (Carlino, 1992). (2) The exogenous TGFβ1 may have been activated in vivo and subsequently sequestered by a binding protein such as alpha 2-macroglobulin (alpha 2-M) (Arandjelovic, 2003), although the circulating latent TGFβ1

sequestered to binding proteins should still have been detectable using the acid activation method employed here (O'Connor-McCourt, 1987). (3) Alternatively, exogenous TGFβ1 may have been metabolised by the liver (Coffey, 1987). This scenario is consistent with the observation of significantly increased numbers of F4/80+ macrophages in the livers of TGFβ1-/- mice receiving exogenous TGFβ1 (9.6 ± 3.0%, n = 6) compared to the control mice investigated in chapter 4 ($p < 0.05$) [TGFβ1-/- controls (4.9 ± 0.7%), n = 3 and TGFβ1+/+ mice (3.9 ± 1.3%, n = 8)]. This effect could be explained by the ability of TGFβ1 to be chemotactic for Kupffer cells in vitro and ex vivo (Kossmann, 1992).

6.6.2 TGFβ1 and Testicular Function

6.6.2.1 EFFECT OF EXOGENOUS TGFB1 ON SERUM TESTOSTERONE LEVELS

In vitro studies show that exogenous TGFβ1 can alter testosterone levels in cultured Leydig cells in a biphasic manner (Benahmed, 1989) (Morera, 1988), thus exogenous TGFβ1 may influence production from the testes in vivo. In this study, some variation in testosterone levels within genotype groups made assessments of statistical significance difficult. Some of the samples were assessed twice on different occasions to confirm the result, however there was no disparity between assay results (data not shown). Thus the variation within groups may have been due to the fluctuating nature of testosterone production in males (Bartke, 1975). As the day 14 measurements were taken following two rounds of mating experiments, the combined effect of exogenous TGFβ1 and exposure to females must be considered. Previous studies found that circulating testosterone levels could be altered by female influences (Amstislavskaya, 2004). The studies conducted herein did not demonstrate this as testosterone levels in the TGFβ1+/+ controls, were not significantly different between days 7 and 14, nor did they differ from the levels found in TGFβ1+/+ mice that had not been exposed to females (Chapter 4). Thus, in this model female exposure did not appear to affect testosterone levels.

6.6.2.2 EXOGENOUS TGFB1 DID NOT ALTER HSD3B1 OR TBRI GENE EXPRESSION

The similarity of HSD3β1 mRNA levels in the testes between each genotype and treatment group was not expected given the previously reported effects of TGFβ1 on expression of this enzyme (Benahmed, 1989). As androgen levels may also affect this enzyme (Heggland, 1997), testosterone levels were compared to HSD3β1 gene expression. A large reduction in mean testosterone levels (72%) was previously seen in TGFβ1-/- mice in conjunction with altered HSD3β1 expression (Ingman, 2002) (Ingman, 2007). However in the current study a similar difference in testosterone between TGFβ1 genotypes (79%) did not alter HSD3β1 expression and neither did TGFβ1 treatment of the TGFβ1-/- mice where mean testosterone levels were relatively increased. Despite TGFβ being reported to regulate HSD3β expression (Cherradi, 1995), a prior in vitro study has shown that TGFβ1 induced testosterone suppression likely occurs prior to the steroid synthesis steps involving this enzyme (Avallet, 1987). Thus the absence of any change in HSD3β1 expression here may be consistent with this.

When the TβRI gene was analysed in the testicular tissue, no change in mRNA expression was found, indicating that deficiency in endogenous TGFβ1 does not alter receptor expression in the testes in this model. The ability of endogenous TGFβ2 (Deng, 1999) (Rodriguez, 1995) or TGFβ3 (Xia, 2005) to compensate for TGFβ1 deficiency was not examined. Since the cytokines use the same receptor and elicit an overlapping range of responses, an up-regulation in their expression to compensate for the absence of TGFβ1 might be expected.

6.6.2.3 SPERM PRODUCTION IS UNAFFECTED BY TGFB1 AVAILABILITY

Previous investigations on spermatogenesis found that most TGFβ1-/- mice produced a normal sperm count and sperm were able to fertilize oocytes in vitro (Ingman, 2002) (Ingman, 2007). However some of those TGFβ1-/- mice exhibited a severe disruption in epididymal sperm numbers, which may have been related to low testosterone levels (Zirkin, 1989). However no change in sperm production was found here between genotypes irrespective of TGFβ1 treatment, although these

results would be more compelling if a larger number of samples had been available for assessment.

6.6.3 TGFβ1 and Reproductive Behaviours

6.6.3.1 GENITAL GROOMING IS NOT ALTERED BY EXOGENOUS TGFB1

In the experiments reported in this chapter no significant difference was seen between genotype groups in the rate of genital grooming performed and there was no correlation between the erectile activity and genital grooming rates. This conflicts with the result found earlier (chapter 3). No obvious changes in activity levels which might be attributed to the surgical intervention were noted. Also, as the TGFβ1+/+ control group displayed a similar incidence of genital grooming as that noted previously (chapter 3), the inclusion of females in this assessment was not likely to have influenced the results. The difference in results for the TGFβ1-/- mice may be more reflective of the number of mice assessed, with fewer mice in the later experiment (n = 6) compared with the earlier experiment (n = 18) (Chapter 3).

6.6.3.2 ERECTILE ACTIVITY IN RESPONSE TO TGFB1

While the proportion of TGFβ1 treated TGFβ1-/- mice displaying erectile activity was not different to the TGFβ1+/+ controls, in contrast to untreated TGFβ1-/- controls, it appeared to be transient and did not reflect the erection rate. The reduced number of erections in the TGFβ1 treated TGFβ1-/- group at day 14 compared with the day 7 response might be attributed to natural fluctuations in the behaviour of mice, as the TGFβ1-/- controls also showed a similarly lower response on the second test day.

Both low and high (Francavilla, 2005) circulating levels of TGFβ1 are associated with erectile dysfunction, thus a biphasic effect of TGFβ in penile function might operate. A threshold level of both testosterone and TGFβ1 are likely to be required for a normal erectile response (Traish, 2006) (Armagan, 2006). However, the possibility that sustained reduction in testosterone and / or absence of TGFβ1 in these mice during growth and development might indirectly affect erectile function cannot be

excluded. These mice may have underlying health problems that affect their ability to achieve erections. This is consistent with studies in men showing that erectile dysfunction may be an early indicator of underlying vascular diseases (Francavilla, 2005).

6.6.3.3 MODEST CHANGE IN MOUNT LATENCY AND INTROMISSION BEHAVIOUR IN RESPONSE TO TGFB1

The total number of mounts and the mount rate displayed by TGFβ1-/- mice did not improve after exogenous TGFβ1 treatment, despite the elevated serum TGFβ1 content. However, their mount latency was comparable to the TGFβ1+/+ controls at day 7, implying that this measure of their libido was enhanced, although wide variances in mouse behaviour may also be influencing the data. As an exogenous TGFβ1 source is shown not to cross the blood-brain barrier (Kastin, 2003), it could be that TGFβ1 is acting in reproductive tissues possibly in conjunction with testosterone to affect mount behaviour. This is consistent with observations that two of the TGFβ1 treated TGFβ1-/- mice had higher serum TGFβ1 levels coinciding with higher testosterone levels. They mounted more often and commenced mounting and intromission behaviours earlier, implying that the combination of both TGFβ1 and testosterone might contribute to restoring mating behaviour.

Intromission behaviour displayed by the TGFβ1-/- mice receiving exogenous TGFβ1 showed some enhancement on day 7 compared to the TGFβ1-/- controls however significance was not reached. Wide variances in mouse behaviour may be influencing the data. No correlations were found between serum levels of either TGFβ1 or testosterone and the intromission behaviour. Also worth noting is that mice with the highest levels of testosterone, in any group, were not found to be the most vigorous responders during mating tests.

Body weight may be a factor in the ability of males to clasp the female in position for mating. The observation that the TGFβ1-/- mice used in this study weigh 20% less than the age matched TGFβ1-/- mice used in a previous study (Ingman, 2002) suggest their overall condition has deteriorated and more advanced underlying health and developmental problems are occurring.

Body weight is suggested to be associated with locomotor abilities (Klomberg, 2002), and it did correlate with intromission rates in the TGFβ1 treated TGFβ1-/- mice ($r = 0.954$, $p = 0.001$). However, no significant changes in body weight occurred between test days, so this does not explain the difference in response between the two test days. Perhaps other underlying health problems in the TGFβ1-/- mice contribute to their sexual dysfunction, as it is now established that cardiac disorders in men are associated with erectile dysfunction (Jackson, 2006) (Billups, 2005) (Russell, 2004).

Strong correlations were evident between intromission and mount rates in both the TGFβ1+/+ control group ($r = 0.792$, $p = 0.002$) and the TGFβ1 treated TGFβ1-/- mice ($r = 0.855$, $p = 0.003$). This indicates that their ability to perform intromission is related to their level of interest and capacity to attempt copulation.

6.6.3.4 EJACULATION BEHAVIOUR IS NOT ALTERED BY EXOGENOUS TGFB1

Of the three groups only the TGFβ1+/+ controls could effectively clasp, mount and intromit with the females until ejaculation. Scores for ejaculation parameters were improved on day 14 compared to day 7, presumably due to the experience mice achieved at day 7 (Coquelin, 1982). This interpretation is supported by (1) the absence of change in serum testosterone levels over the same period, (2) the absence of any correlation between testosterone levels and ejaculation behaviour and (3) as with intromission behaviour, the observation that the highest testosterone levels did not always coincide with the highest ejaculation events for individual mice. This is consistent with previous suggestions that hormonal factors can be overridden by social experiences (Doty, 1986). The TGFβ1+/+ controls ejaculated sooner when they mounted and intromitted with the females more frequently, as correlations between ejaculation latency and mounting ($r = 0.864$, $p = 0.000$) and intromission ($r = 0.620$, $p = 0.032$) rates were found.

The ability of TGFβ1 treated TGFβ1-/- mice to copulate remained severely compromised after TGFβ1 replacement. In GnRH-deficient hypogonadal (hpg) mutant mice which are not able to copulate despite displaying mounting and intromission behaviour, androgen deficiency in early life was identified as a central control factor in the phenotype (Livne, 1992). Therefore it is possible that TGFβ1-/-

mice may have irreversible reproductive impairment due to testosterone deficiency during growth and development. However, previous studies show that TGFβ1-/- mice supplemented with testosterone in early life or as adults show no improvement in copulation behaviour (Ingman, 2002). Thus, it may be that the combination of both TGFβ1 and androgens during growth may be important for the development of sexual function.

These studies were limited by our ability to fully replace TGFβ1 to levels present normally in TGFβ1+/+ wild type mice. Better TGFβ1 replacement might be expected to be achieved by a longer study period or use of higher concentrations of TGFβ1 in pumps. However the high cost of recombinant TGFβ1 was prohibitive in undertaking these studies. If higher TGFβ1 levels could have been achieved, it might have been expected that greater improvements in a wider range of reproductive performance parameters would have been observed.

6.7 SUMMARY

Exogenous TGFβ1 was delivered to the circulation of TGFβ1-/- mice to produce low serum levels of TGFβ1. As testosterone is necessary for development and maintenance of male reproductive organs, spermatogenesis and sexual behaviour, significant improvements in these areas were anticipated. However, testosterone, HSD3β1, and TβRI in the testes, sperm production and genital grooming were not altered after TGFβ1 replacement.

TGFβ1 replacement did lead to modest improvements in mating behaviour, although as this did not translate into ejaculation events, the fertility of the TGFβ1-/- mice was not restored. The combination of testosterone and TGFβ1 may play a role as implied by the finding that the most improved behavioural responses were from those mice that showed the highest levels of both testosterone and TGFβ1. However, erectile and copulating function is likely to require the integrated actions of several physiological systems and the general health of the mice did not improve with TGFβ1 replacement. Therefore the absence of TGFβ1 throughout growth and development

might be causal and not able to be restored by TGFβ1 treatment in adulthood. However it must be noted that firm conclusions on the efficacy of TGFβ1 replacement cannot be drawn until further studies are undertaken with a longer treatment protocol and / or higher doses of TGFβ1 to achieve higher levels in serum and tissues.

Chapter 7:
Discussion

7.1 INTRODUCTION

Studies in TGFβ1 deficient mice clearly demonstrate that the bioavailability of TGFβ1 is paramount in coordinating homeostasis and health (Palmer, 1994) (Dunker, 2000). The life of these mice is extended by ablating their lymphocytes and therefore their capacity to raise antigen-specific immune responses (Diebold, 1995), thereby allowing the role of TGFβ1 in fertility to be studied. This model has been informative in understanding the biological role of TGFβ1 in male reproductioin. However there are several limitations which must be taken into account in interpreting this information, as well as caveats on the extent to which any animal model can be extrapolated to the human situation. As TGFβ1 levels are variable within individuals and a complete lack of TGFβ1 in humans cannot be studied, it will be essential to undertake further studies on the role of TGFβ1 in humans before the full significance of reproductive function of TGFβ1 can be understood.

7.2 THE TGFB1-/- MOUSE MODEL

The phenotypic lesions of the TGFβ1-/- mouse in the immune response and inflammatory system are increasingly well-documented (Kulkarni, 1993) (Vodovotz, 1996a) (McLennan, 2000). However the full range of biological functions of this molecule is still being discovered, as recent work has also determined that TGFβ1-/- mice may have under-developed neuronal (Tesseur, 2006) (Flanders, 1998) and vascular structures (Bobik, 2006) (Bertolino, 2005). It is important to appreciate that each of these systems interact and this may extend indirect effects on fundamental components of their health and fertility. The effects of the genetic background and immune status of these TGFβ1-/- mice are described below.

7.2.1 Genetic Background

It is increasingly becoming recognised that the strain of a genetically modified model can influence the outcome of a research study (Funkat, 2004), (Bilkei-Gorzo, 2004), (McLennan, 2004), (Tang, 2003). This is likely to be an important factor in accounting for the variation observed between mice in this study, since the mice are on a mixed genetic background (CF-1, 129/Sv, C3H). Differences in embryo lethality are seen to occur between TGFβ1-/- mice on the CF-1 and a 129/Sv backgrounds (Ingman, 2002) (Bonyadi, 1997) and complete embryo lethality occurs when the mice are crossed onto a full C57BL/6 background (Ingman, 2007). The effect of genetic background is attributed to a range of genetic modifiers which if active in humans, indicate that disease outcomes or treatment strategies for TGFβ associated diseases could be affected by the genetic background of an individual.

7.2.2 Immuno-compromised Background

Research outcomes using the TGFβ1-/- mouse model may also be influenced by the status of their immune system. The SCID mutation effectively deletes all T-cells and B-cells from the mouse but should not affect other leukocytes. The TGFβ1-/- mutation is expected to independently influence the actions of a wide range of leukocytes, particularly macrophages. Although dendritic cells may be more refractory to TGFβ1 deficiency as the Langerhans cell populations in the skin of TGFβ1-/- mice (Borkowski, 1996) appear to be normal. Also depending on immune status, some intracellular structures within the liver of TGFβ1-/- mice are altered (Williams, 1996). Interestingly, one line of TGFβ1-/- mice raised on the immuno-compromised nude (Whn-/-) background may have improved fertility (McLennan, 2000), raising the question of whether the SCID background used in this study may have affected the research outcomes. Thus the effects observed in these mice may be specific to this colony and comparisons to data from other related mouse models should be made carefully.

7.3 STUDY OUTCOMES

This study investigated the effects of TGFβ1 deficiency and exogenous TGFβ1 administration on macrophage, epithelial and reproductive parameters in vivo. The choice of parameter and the techniques used for analysis were in some instances guided by previous studies (Ingman, 2002). Others were chosen for being likely to be affected by exogenous TGFβ1 and required development and validation as they had not previously been used in this laboratory.

7.3.1 Health Status of the TGFβ1 / SCID mice

TGFβ1 regulates tissue morphogenesis and homeostasis (Schuster, 2002) which affects several physiological systems and therefore adult health. The most common general measure of health in animal trials is body weight. In the previous study using this colony of mice, the difference in body weight between the TGFβ1+/+ and TGFβ1-/- genotype groups was lower (20.8 %) (Ingman, 2002) compared to the difference found at a comparable age in the current study (34.6 %). This suggests that the general health of the colony has worsened over time. This may have affected their capacity to conduct normal physical activity (Tou, 2002). Although the parameters of their waking activity were not assessed here, they were not found to sleep any more than their TGFβ1+/+ littermates and otherwise appeared overtly normal in their outward signs of health and physical activity, compared to control mice.

This study found that exogenous TGFβ1 delivered sub-cutaneously reaches the circulation, although serum content was lower than anticipated suggesting a short half-life and rapid removal by the liver. The reason for this may involve the macrophages of the liver as exogenous TGFβ1 delivered systemically resulted in a significant increase in these cells. This reveals that the Kupffer cells in TGFβ1-/- mice are capable of responding to the exogenous TGFβ1 as seen previously in vitro and ex vivo (Kossmann, 1992).

Health is also affected by cells of the immune system and the absence of lymphocytes in these mice may influence the role of TGFβ1 in vivo. There is some

evidence that the abundance and / or phenotype of macrophages is affected by TGFβ1 deficiency (Palmer, 1994) (Kulkarni, 1993) (Vodovotz, 1996b) and this would be expected to impact many tissue systems. However we were unable to identify differences in the relative densities of macrophages in the liver or the peritoneal cavity due to TGFβ1 genotype. In addition, peritoneal macrophage phagocytosis as well as iNOS and TNFα gene expression were not altered in the TGFβ1 deficient group. This was unexpected in view of the previously recognised interaction between the products of these genes and TGFβ1. IFNγ may responsible for the contrasting result as it is a known lymphocyte derived cytokine that activates macrophages to produce various cytokines and to phagocytose. To facilitate better evaluation of the effects of manipulating TGFβ1 levels in vivo, our understanding of macrophage regulation and function will need to be increased. The identification and access to appropriate probes for distinguishing altered macrophage phenotype and function is required. This work has begun to contribute by showing that basal iNOS and TNFα mRNA expression occurs in the absence of TGFβ1 and lymphocyte- derived cytokines.

Another area that was investigated herein was epithelial proliferation. Despite superfluous epithelial material being found on the outer penile surface and thigh skin, no changes in cellular proliferation were found in penile or thigh skin or intestinal epithelial tissues. As TGFβ1 is a potent inhibitor of epithelial proliferation in other in vitro and some in vivo studies (Doi, 2003) (Kurokowa, 1987) (Moses, 1994), it must be concluded that at least in the mouse model used here, TGFβ1 is not an essential regulator of epithelial cell proliferation. However, is difficult to exclude the possibility of an effect of the genetic background of the mouse model. Interestingly, increased epithelialisation was seen in another immuno-compromised TGFβ1-/- mouse model following wounding (Koch, 2000). Thus, components of the epithelial proliferation that occurs in tissue regeneration following wounding versus tissue homeostasis may be differentially regulated by TGFβ1. Furthermore, the interaction between TGFβ1 and female hormones has been seen to affect epithelial proliferation (Ewan, 2002) and this may also be true for male hormones such as testosterone.

7.3.2 Fertility Status of the Male TGFβ1 / SCID Mice

The infertility of the TGFβ1-/- mice reported previously (Ingman, 2002) (Ingman, 2007) was confirmed here. Testosterone levels were low in the absence of endogenous TGFβ1 and statistical evaluations did not produce any correlations with TGFβ1 levels. This study also found that endogenous TGFβ1 did not affect HSD3β1 gene expression and neither did exogenous TGFβ1. Thus another component of the pathways regulating testosterone production must be responsible for the alterations to testosterone levels observed in the TGFβ1-/- mice. TGFβ1 regulation of the hypothalamic – pituitary axis and specifically LH production appears to be central to this, since TGFβ1-/- mice have reduced circulating LH in both males and females (Ingman, 2007).

Expression of TβRI mRNA was postulated to potentially alter between genotype groups or following exogenous TGFβ1 delivery. However this did not occur, indicating that TGFβ1 receptors in testicular tissue are not regulated in response to TGFβ1 bioavailability. It could be suggested that exogenous TGFβ1 did not reach the testes, however as it was detected in the circulation, the testes would have been expected to receive this supply.

When the penis of TGFβ1-/- mice was assessed previously, the internal tissue structures appeared normal (Ingman, 2002) and erections could be achieved following electrical stimulation (Ingman, 2007). The current study found the gross morphology of the penis of the TGFβ1-/- mice was normal in overall size and spine structure, however the outer skin layer of the penis was covered in superfluous epithelial material that may impede spine protrusion and reduce the ability to copulate with females. Observations of the TGFβ1+/+ mice for erectile frequency revealed that they did not respond to the pharmacological inducers, apomorphine and sildenafil citrate (Viagra) as anticipated. Further analysis of spontaneous erections revealed that both the TGFβ1+/+ and TGFβ1-/- genotype groups could achieve erections without any pharmacological assistance, however fewer TGFβ1-/- mice achieved erections. Subsequent administration of exogenous TGFβ1 did not alter this. In addition, concurrent observations of their genital grooming behaviour

revealed that the TGFβ1-/- mice groomed less than their TGFβ1+/+ littermates and exogenous TGFβ1 had no effect.

This study confirmed that the ability of the TGFβ1-/- mice to copulate was severely restricted. Following delivery of exogenous TGFβ1, the TGFβ1-/- mice did display some improvement in mounting and intromission behaviour with females seven days after TGFβ1 treatment was initiated. No greater effect was seen after two weeks of TGFβ1 replacement and testosterone implants in a previous study did not restore mating behaviour either (Ingman, 2002). Therefore it seems likely that effects of TGFβ1 exerted in early development, via testosterone or independently, are necessary for mating competence in the adult mouse. In addition, the absence of any major improvement after TGFβ1 replacement may partly reflect the health status of the mice. While their body weight was not significantly reduced at day 14 compared to day 7, underlying problems associated with TGFβ1 deficiency cannot be discounted. It also seems possible that greater levels of circulating TGFβ1 might have been achieved at the earlier time point.

Finally, the combination of threshold levels of both TGFβ1 and testosterone may help to restore fertility in the TGFβ1-/- mice, irrespective of their levels initially. This is suggested as the best improvements in this study of mating behaviour were displayed by the two TGFβ1-/- mice with the highest TGFβ1 and testosterone levels. This also supports the conclusion that if greater circulating concentrations could have been achieved in the replacement experiments, more impressive effects on reproductive performance might have resulted.

7.3.3 Altering Systemic TGFβ1 Levels In Vivo

TGFβ1 is a circulating cytokine that constantly bathes the tissues via the vascular system. It seems reasonable that circulating levels of this cytokine should be tightly controlled particularly since many vascular diseases are associated with dys-regulated TGFβ1 levels (Bobik, 2006) (Li, 2003) (Scott, 1997) (Azar, 2000). This study sought to manipulate levels of TGFβ1 in the circulation of TGFβ1-/- mice. The latent form was used as this was thought to mimic the physiological situation due to

its dominance in all biological fluids and the fact it is regulated in context-dependant manners throughout the body. Latent TGFβ may circulate until it is endogenously activated at sites where it is needed (Ribeiro, 1999) (Blakytny, 2004) (Lyons, 1990) (Geiser, 1993). In contrast, active TGFβ is degraded more quickly (Philip, 1991), binds to sequestering ligands (Arandjelovic, 2003) (O'Connor-McCourt, 1987) and signals indiscriminately through a repertoire of receptors (Wahl, 1987).

Using this model was illuminating in revealing how exogenous TGFβ1 acts in vivo. Most striking was the discrepancy between the results obtained from replacing TGFβ1 in a TGFβ1 deficient system compared with the expected results based on experimental systems replete for TGFβ1. This highlights the likely complexity of the pathways influenced by TGFβ1 and the probable existence of redundant mechanisms for protecting against changes to TGFβ1 levels in vivo. It is also worth considering that tissue location (Sarkar, 2005), age (Gautier, 1997) and extent of TGFβ1 perturbation may play a role in resilience to TGFβ1 fluctuations in vivo (Benahmed, 1989). These data indicate that any clinical studies to investigate perturbation of TGFβ1 concentrations within humans should be designed with care.

7.3.3.1 SOURCES OF EXOGENOUS TGFB1

These studies were initiated with a view to the eventual possibility of utilising pharmaceutical or neutraceutical approaches to deliver exogenous TGFβ1 to humans as a therapeutic for reproductive and / or immunological conditions attributable to TGFβ1 deficiency. TGFβ1 could potentially be obtained through in vivo gene technology, recombinant protein production or naturally occurring sources such as milk. In vivo generation of the protein using gene technology could be useful as high levels of the TGFβ1 protein can be produced and expressed in the desired tissue (Longenecker, 2002) (Moritani, 2005). However regulating the concentration of the resultant protein is difficult and this technology is not readily transferable to humans (Romano, 2006). Recombinant forms of the TGFβ protein are physiologically identical to the natural sources and can be manufactured in both the active and latent forms (Systems, 2007). However their production is expensive and the latent forms must currently be formulated in a glycerol / BSA carrier that helps stabilize the protein. Finally, natural sources of both TGFβ1 and TGFβ2 can be concentrated or

purified from milk (Jin, 1991) or whey (Ansel, 1990). This study confirmed the presence of high levels of TGFβ in milk and colostrum and shows that it occurs mainly in the latent form. Notably, it was also shown that pasteurisation of raw milk destroys significant amounts of latent TGFβ and also increases the activation status of that which remains. Despite this degradation, commercially available milk still contains some TGFβ that may impact on human health after ingestion, since unlike many cytokines, there is evidence that TGFβ can survive the acid environment of the stomach (Ishizaka, 1998) and may be taken up into the blood stream across the gut epithelium (Pacha, 2000). Also, there is high homology in amino acid sequence between bovine and human TGFβ to result in efficient cross-species bioactivity of the cytokine (Van Obberghen-Schilling, 1987) (Nixon, 2000) (Fell, 2005).

7.3.3.2 METHODS OF EXOGENOUS TGFB1 DELIVERY

In this study the intra-peritoneal route for exogenous TGFβ1 delivery was investigated using a single injection of recombinant TGFβ1 protein. It was found that while TGFβ1 reached the circulation, it began to disappear after 90 minutes. To find a method where exogenous TGFβ levels could be sustained, a commercially available colostrum powder was orally delivered to TGFβ1-/- mice. TGFβ was subsequently detectable in the stomach contents but could not be detected in the serum. Thus either the dose or uptake of oral TGFβ appears to have been insufficient to result in systemic bio-availability. Even though the effect on the intestinal or immune system was not investigated here, previous studies in humans with intestinal bowel disorders and allergies (Fell, 2000) (Kalliomaki, 1999) suggest that some benefits are gained by long term consumption of TGFβ.

Greater success for delivering TGFβ to the circulation was achieved when recombinant latent TGFβ was delivered sub-cutaneously using mini-osmotic pumps. This technology was able to deliver a constant supply of a known amount of latent TGFβ1 and results showed that TGFβ1 was detectable in the circulation, albeit at lower than predicted levels. As the pumps were found to be almost empty following treatment, it is likely that they did release the TGFβ1. Thus the cytokine must have either been degraded or taken up by the tissues and utilised. Future studies are

needed to investigate whether higher initial doses of rhLTGFβ1 in pumps might achieve a higher level of circulating cytokine.

7.4 FUTURE RESEARCH

A greater understanding of the mechanisms and consequences of TGFβ1 regulation may lead to effective therapeutic strategies in the future. Clinical trials will ultimately determine the usefulness of exogenous TGFβ1 treatment however much research in animal and culture models is still needed before applying this technology to devising new therapies for treating reproductive disorders or infertility in humans.

This study delivered exogenous TGFβ1 for two weeks in adult mice. While it would be interesting to monitor this treatment in younger developing mice it is currently not surgically practical due to their small size. Delivering a more concentrated dose to these adults may have generated a different response due to the biphasic effect of TGFβ1 on several systems including testosterone production (Morera, 1988) (Saunier, 2006) (Thompson, 2006) (Henckaerts, 2004). While the TGFβ1 content of tissues from these mice was not measured due to cytokine disruption during tissue extraction procedures, it would be interesting to determine the level of TGFβ1 in a range of target tissues.

Future studies could employ the use of radioactive or otherwise tagged TGFβ1 to show the precise location and extent of TGFβ1 accumulation in the tissues. Alternatively, genetic strategies such as the CRE/LOX system could be used to achieve TGFβ1 expression in specific tissues. This would avoid the global effect of systemic TGFβ1 action and focus on the biological system under investigation. With regard to fertility, such investigations could initially include evaluating effects on macrophages and neuronal and vascular cells in reproductive tissues. This would allow investigation of the mechanism of TGFβ1 on testosterone production and the genetic modifiers of TGFβ1 action. Knowledge in these areas could reveal important features on the mechanisms by which TGFβ1 regulates reproductive events.

Finally, it would be interesting to further investigate the possible benefits of TGFβ1 ingested in milk. As milk consumption by some individuals evokes lactose intolerance or allergies (Ansel, 1990) (Pakkanen, 1998), or perhaps even testicular and prostatic cancers in men (Ganmaa, 2002), a better understanding of the therapeutic properties of milk, such as those reported for breast milk (Bottcher, 2003) (Kalliomaki, 1999) (Saarinen, 2000) might be useful. Alternative processing methods that maintain high TGFβ levels in milk without compromising exposure to hazardous bacteria, may result in the production of new value-added foods. Additionally, other foods such as yoghurt and meats could be investigated as potential sources of TGFβ1 with neutraceutical value. Despite the disappointing results of no elevation of TGFβ1 in the circulation following colostrum consumption this was only a preliminary investigation and further studies of specific tissues linked to the intestinal tract could reveal important roles for TGFβ in the diet.

7.5 CONCLUSION

Analysis of the male TGFβ1-/- mouse has revealed that body weight, penile function, testosterone production and sexual behaviour are significantly disrupted in the complete absence of TGFβ1. Individually these are unlikely to be the cause of their complete infertility however these deficiencies combined with possible under-developed neuronal or vascular structures are likely to amount for the profound effects of TGFβ deficiency on reproductive function and fertility.

A range of potential sources of TGFβ for manipulation of endogenous TGFβ levels revealed benefits and pitfalls associated with each. The analysis revealed that commercial processing significantly diminished the TGFβ content of milk. While commercially available colostrum contains high levels of TGFβ, two days of consumption did not impact on systemic TGFβ levels. Continuous sub-cutaneous infusion of recombinant latent TGFβ1 was successful in elevating TGFβ1 levels in the circulation, however sufficiently high levels in circulation were not achieved possibly due to degradation by macrophages in the liver.

Despite this, an effect of exogenous TGFβ1 treatment was shown as copulatory activity was modestly and temporarily improved. Ultimately however, as no ejaculation events were displayed by any TGFβ1-/- mice, their fertility was not restored and further studies are required to devise better strategies for alleviating the effects of TGFβ deficiency. These are likely to require (a) higher doses of TGFβ to achieve higher circulatory levels, (b) a longer treatment protocol, (c) targeted delivery to specific tissues and (d) replacement of TGFβ1 throughout the growth and development of the individual to ensure adequate responsiveness of the reproductive tissues to TGFβ in adulthood.

APPENDIX A

All chemicals were of analytical grade and obtained from Sigma-Aldrich (Missouri, USA) unless otherwise indicated. All buffers and solutions were prepared with 18.2 mΩ MilliQ water (Millipore. Massachusetts, USA).

APES (3-AMINOPROPYLTRIETHOXYSILANE) COATED SLIDES

To prepare slides, wash them in detergent for 30 minutes, then place under running tap water for 30 minutes, then in distilled water for 2 x 5 minutes. Place slides in 95% ethanol 2 x 5 minutes, then air dry for 10 minutes. To coat slides, immerse them in a solution of 2% APES in 5% acetone for 5 seconds. Shake off the excess liquid, rinse in distilled water for 2 x 30 seconds, then dry overnight at 42ºC prior to storing at room temperature.

APOMORPHINE (1µG / KG DOSE)

Combine 2 µL of 0.1 µg / µL Apomorphine hydrochloride with 1998 µL of 0.1% ascorbic acid. Sterile filter through 0.2 µM. Make up fresh each time. Inject 200 µL per 20 g mouse.

Ascorbic acid (0.1%)

Combine 10 mg of ascorbic acid into 10 mL of PBS (makes 1% ascorbic acid), then combine 1 mL of 1% ascorbic acid with 9 mL of PBS.

Apomorphine hydrochloride (0.1 µg / µL)

Combine 5 mg of apomorphine hydrochloride with 1.67 mL of 1% ascorbic acid (makes 3% apomorphine hydrochloride), then combine 50 µL of 3% apomorphine hydrochloride with 1450 µL of 0.1% ascorbic acid (makes 0.1 µg / µL of apomorphine hydrochloride).

AVERTIN

Combine 1 mL of 2methyl-2-butanol (tert-amyl alcohol) with 1 g of 2,2,2-tribromomethanol (AJAX. California, USA). Make up to 50 mL with water and shake vigorously in hot water to dissolve. Aliquot and freeze at -20ºC.

BROMODEOXYURIDINE (BRDU) / FLURODEOXYURIDINE(DFUR)

Dissolve 100 mg of BrdU in 10 mL of saline. Prepare dFUR by mixing 10 mg with 1 mL of water. Combine 1 mL of BrdU with 100 µL of dFUR just prior to use.

CITRIC ACID, THEOPHILINE, ADENOSINE, DIPRIDAMOLE (CTAD)

Combine 270 mg of theophiline, 99.9 mg of adenosine 9.9 mg of dipridamole, 2.1 mg of citric acid (BDH. Leicestershire, United Kingdom) and 3.6g of HEPES. Make up to 100 mL with water. Adjust to pH 5 with 10M NaOH.

PARAFORMALDEHYDE (PFA)(4%)/POLYVINYLPYRROLIDONE (PVP)(2.5%) FOR IMMUNOHISTOCHEMISTRY

Heat 600 mL of water until steaming (~60ºC), add 25 g PVP-40, slowly stir in 40 g PFA, add 1M NaOH drop wise until the solution clears, and stir until dissolved (can take 30-60 mins), add 4.0 g Na2HPO4 (BDH), add 6.5g. Prepare in a fume hood.

SILDENAFIL CITRATE (VIAGRA) (1 MG / KG DOSE)

Dissolve 1 x 25 mg tablet into 2.5 mL of saline (makes 10 mg / mL). Take 20 µL and add to 1980 µL of saline (makes 0.1 µg / g). Inject 200 µL in to a 20 g mouse.

SOLUTIONS FOR PCR GENOTYPING

Digestion Buffer

Combine 2.5 mL 1M Tris, 3.75 mL 5M NaCl, 12.5 mL 0.5M EDTA and 25 mL 10% SDS. Make up to 1 litre. Combine 1mL with 5 uL of Proteinase K (20 mg / mL) just prior to use.

Agarose gels (1.5%)

Add 15.0 g agarose (Promega Corp, Wisconsin, USA) to 100 mL of TE buffer. Dissolve in a microwave, pour into a tray with a well comb fitted, leave to set at room temperature.

STOCK SOLUTIONS

EDTA (0.5 M)

Dissolve 46.5 g of NaCl in a total volume of 250 mL of water then adjust pH to 7.8 with 10 M NaOH.

FACS Wash Buffer

Dissolve 1 g of BSA and 40 mg of Azide in a total volume of 100 mL of PBS.

FACS Fix

Dissolve 1.0 g glucose and 1.25 mL of formalin with 45 mL of PBS.

Loading Buffer

Combine 0.4 % bromophenol blue, 0.4 % xylene cyanol FF, 0.3 % glycerol.

NaOH (10M)

Dissolve 40 g of NaOH into a total volume of 1 litre of water.

Phosphate Buffered Saline(PBS)

Combine 0.2 g KCL, 0.2 g KH2PO4, 8.0 g NaCl, and 1.15 g Na2HPO4 (anhydrous). Make up to 1.0 litre of water. Adjust pH to 7.4.

Proteinase K (20 mg / mL)

Add 20.0 mg of Proteinase K to 1 mL of saline. Store aliquots at –20ºC until use.

Saline

Dissolve 90 mg of NaCl in a total volume of 100 mL of water. Sterile filter through 0.2µM.

Sodium dodecyl sulphate (SDS) (10%)

Dissolve 50.0 g of SDS in a total volume of 500 mL of water.

Sodium Chloride (5 M)

Dissolve 29.2 g of NaCl in a total volume of 100 mL of water.

Tris EDTA (TE) Buffer

Dissolve 6.1 g Tris Base, 0.37 g ethylenediaminetetraacetic acid (EDTA) in a total volume of 1 litre of water, Adjust pH to 8.0 using concentrated hydrochloric acid (HCl).

Trisma base (1 M)

Dissolve 12.1 g of trisma base in a total volume of 100 mL of water.

SOLUTIONS FOR ESTROUS INDUCTION OF FEMALES

Ovariectomized Females

Estradiol benzoate and progesterone were sub-cutaneously injected, once each to ovariectomized females two days and five hours respectively, prior to using them in experiments with male mice.

ESTRADIOL BENZOATE

Dissolve 7 mg of estradiol benzoate in a total volume of 1 mL of 100% ethanol. Take a 40 µL aliquot and combine with 1 mL of sesame oil.

PROGESTERONE

Dissolve 5 mg of progesterone in a total volume of 1 mL of 100% ethanol. Take a 100 µL aliquot and combine with 900 µL of sesame oil.

Immature Females

Pregnant mare serum gonadotropin and human chorionic gonadotropin were sub-cutaneously injected, once each to immature females two days and twelve hours respectively, prior to using them in experiments with male mice.

HCG

Add 30mL of sterile saline to 1500 IU of chorulon chorioic gonadotrophin.

PSMG

Add 20mL of sterile saline to 1000 IU of folligon serum gonadotrophin.

REAGENTS FOR MINI-OSMOTIC PUMPS

All reagents were purchased new from the Sigma-Aldrich cell culture tested range, they were prepared in a sterile cabinet and used aseptically to minimize endotoxin contamination.

rhLTGFβ1 Aliquots

rhLTGFβ1 (cat# 299-LT: R&D Systems. Minnesota, USA) was supplied as a 0.2 µM filtered solution (1.05 mL) in PBS and 25% glycerol containing rh Latent TGFβ1 at a concentration of 0.952 mg / mL and 50 µg of bovine serum albumin (BSA) per 1 µg of cytokine.

74.3 µL aliquots containing 70.7 µg of cytokine were stored at -70ºC.

PBS / Glycerol

Combine 750 µL of PBS with 250 µL of glycerol. Sterile filter through 0.2 µM.

TGFβ1 containing pump

Combine a 74.3 µL (70.7 µg) aliquot of rhLTGFβ1 with 102.5 µL of PBS / Glycerol. Use this to fill a 100 µL, 14 day, Alzet mini-osmotic pump (model # 1002). Pumps were weighed before and after filling to confirm they were full, then placed in sterile saline at 37ºC for 4 hours for priming.

BSA containing pump

Add 20 mg of low endotoxin BSA into 1 mL of PBS / Glycerol. Sterile filter through 0.2 µM. Use this to fill a 100 µL, 14 day, Alzet mini-osmotic pump (model # 1002). Pumps were weighed before and after filling to confirm they were full, then placed in sterile saline at 37ºC for 4 hours for priming.

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