Chapter 1

The Role of TGF-β Family Members and Their Receptors in Reproduction

1.0 Introduction

Reproduction is regulated by a complex interplay of hypothalamic, pituitary and gonadal endocrine signalling and local gonadal regulation by the autocrine and paracrine action of growth factors. Increasing numbers of growth factors from the transforming growth factor-β (TGF-β) superfamily are being shown to have a significant role in reproduction in both males and females across many different species including humans (Dong et al., 1996; Yi et al., 2001; Hanrahan et al., 2004; Hu et al., 2004; Di Pasquale et al., 2006; Chu et al., 2007).

Over 40 members of the TGF-β superfamily have been identified which include TGF-β, Bone Morphogenetic Protein (BMP), Activin, Glial Cell Derived Neural Cell Factor (GDNF) and Distant Member subfamilies (McDonald & Hendrickson, 1993), encompassing TGF-βs (Burt & Paton, 1992), BMPs, activins, inhibins, growth and differentiation factors (GDFs) (Bottner et al., 2000), GDNF (Eketjall et al., 1999), and anti-Mullerian hormone (AMH) (Burt & Law, 1994). Nearly all TGF-β family members are characterized by 6-9 cystine residues located in the mature domain of the peptide that are folded into a structure known as the cystine knot which is a defining marker of all TGF-β superfamily members (McDonald & Hendrickson, 1993; Vitt et al., 2001). BMPs have particular patterns of 7 cysteine residues in their amino acid sequences, while activin, inhibin and TGF-β contain specific patterns of 9 cysteine residues (Ozkaynak et al., 1990).

TGF-β superfamily members bind type II serine/threonine receptors as homodimers or heterodimers leading to the formation of tetrameric signalling complexes (Kawabata et al., 1995; Rosenzweig et al., 1995; Allendorph et al., 2006), as shown in Figure 1.1 with the exception of GDNF which binds tyrosine

kinase receptors but is still considered to be a TGF-β superfamily member due to the cystine knot in its molecular structure (Eketjall et al., 1999).

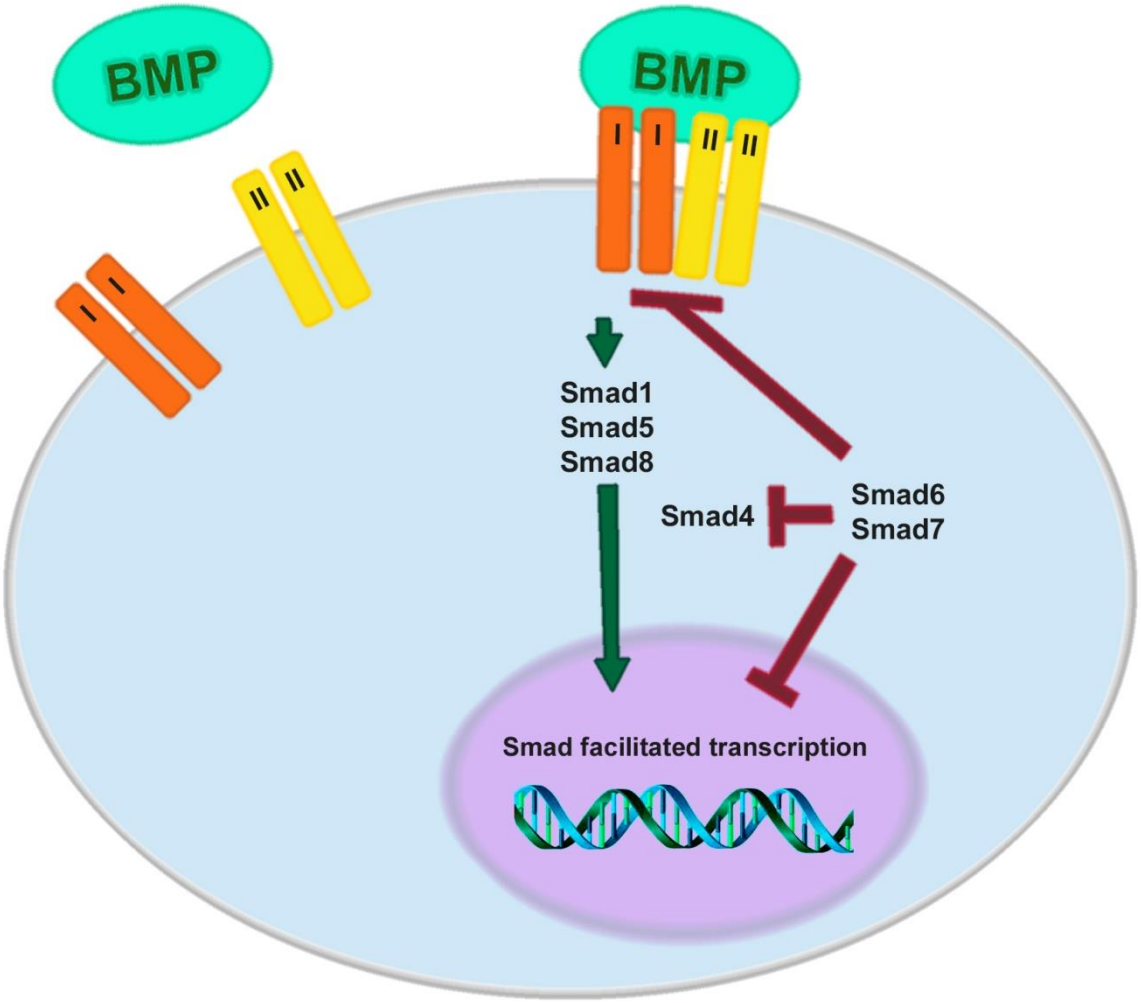


Figure 1.1 Diagram showing tetrameric complex formation of type I and type II serine/threonine receptors when activated by BMPs and consequential Smad signal transducer activities.

BMPs form the largest subgroup of growth factors that belong to the TGF-β superfamily (McDonald & Hendrickson, 1993). BMPs were first purified from bovine bone extracts and identified by their ability to induce the formation of both bone and cartilage at ectopic sites in murine species using both bovine and recombinant human BMP (Wozney et al., 1988; Celeste et al., 1990; Wang et al., 1990). Since

the 1980s researchers using genetic approaches to the identification of BMPs have predicted that these proteins have crucial roles in mammalian development (Lyons et al., 1989b).

BMPs regulate embryonic development, cellular migration and apoptosis, cellular proliferation and differentiation, as well as numerous morphogenic functions such as remodelling of the extracellular matrix in many body systems including the skeletal (Lyons et al., 1989b; Bandyopadhyay et al., 2006), vascular (Erickson & Shimasaki, 2003; Csiszar et al., 2006), nervous (Fu et al., 2006), immune (Kersten et al., 2005; Csiszar et al., 2006) and reproductive systems (Dong et al., 1996; Yi et al., 2001; Hanrahan et al., 2004; Hu et al., 2004; Di Pasquale et al., 2006; Chu et al., 2007).

1.1 Overview of TGF-βs in the Reproductive System

In females reproduction requires the production of a functionally viable oocyte from the ovary and simultaneously a favourable uterine environment to enable a pregnancy. The ovary contains many follicles each usually harbouring a single oocyte at different stages of sequential follicular development (Peters et al., 1975). Follicular development is the process whereby a primordial dormant follicle becomes activated, develops into a primary follicle which progressively develops into an antral ovulatory or atretic follicle (Peters et al., 1975). The hypothalamic – pituitary – ovarian axis forms a significant part of this endocrine process. The secretion of pituitary follicle stimulating hormone (FSH) initiates the growth of secondary and early antral follicles that respond by releasing increasing amounts of estradiol. Estradiol favours luteinizing hormone (LH) release from the pituitary which triggers

ovulation (Rosen & Cedars, 2004), while progesterone secretion from the ovulated follicle is vital for early embryonic life (Sengupta & Ghosh, 2000).

In males the reproductive process requires the production of functionally viable spermatozoa from the testis and fluids from the testis, epididymis, seminal vesicles and prostate which accommodate suitable energy requirements and transport of germ cells (Braunstein, 2004). The testis is mainly comprised of seminiferous tubules and Leydig cells. The seminiferous tubules are formed from tight junctions of Sertoli cells that surround the germ cells and provide an environment required for the transport and development of germ cells from spermatogonia to primary-secondary spermatocytes, spermatids and finally immature spermatozoa. This process also involves pituitary LH and FSH which stimulates Leydig cell testosterone production and Sertoli cell androgen receptor up-regulation respectively (Braunstein, 2004).

Many members of the TGF-β superfamily have been shown to regulate both male and female reproduction and therefore the role of these will be considered in both sexes throughout the general introduction. Furthermore, ligand signalling via BMP receptors, found in many reproductive tissues of mammals and other species, has a vital role in all aspects of development, maturation and function of the reproductive system (Beppu et al., 2000; Wilson et al., 2001; Yi et al., 2001; Ying & Zhao, 2001; Conte & Grumback, 2004). Various naturally occurring gene modifications of BMP-15, GDF-9 and BMPR-IB (ALK-6) in female sheep result in markedly altered reproductive profiles ranging from super-ovulatory to complete infertility (Davis et al., 1992; Mulsant et al., 2001; Wilson et al., 2001; Hanrahan et al., 2004; Chu et al., 2007). Additionally, GDF-9 and BMPR-IB gene knockout in

mice leads to complete infertility due to various defects in ovarian follicular development (Dong et al., 1996; Yi et al., 2001).

In male mice alterations in the genetic expression of BMP-4, BMP-7, BMP-8 and GDF-7 result in either compromised fertility or infertility (Zhao et al., 1998; Settle et al., 2001; Hu et al., 2004). Mouse testis Leydig cell tumors result in severely down-regulated expression of AMHR-II mRNA compared to normal Leydig cells (Hussein et al., 2008) and in human prostate cancer tissue BMP-7 mRNA expression was also significantly reduced compared to healthy tissue (Buijs et al., 2007). This indicates highly important roles for TGF-βs in germ cell development and tissue maintenance in both the female and male reproductive systems.

Gene knockouts for BMPR-IA (ALK-3), BMPR-II, BMP-2 and BMP-4 are less informative in the study of reproduction as they are embryonically fatal (Mishina et al., 1995; Winnier et al., 1995; Zhang & Bradley, 1996; Beppu et al., 2000), however that does not exclude vital functions for these proteins in reproduction, merely that they have multiple roles one of which is essential for embryonic development. Another powerful tool in research utilizes *in vivo* neutralization in which the effects of specific BMPs can be elucidated via their inactivation as demonstrated for BMP-4 in the female mouse reproductive system, illustrating a significant role for this TGF-β superfamily member in early stage follicle development (Tanwar et al., 2008).

In order to understand the physiologic actions of TGF-βs in the reproductive system a detailed understanding of their synthesis and receptor binding properties must be undertaken due to the highly conditional nature of these morphogenic proteins. In addition much of what is understood about BMPs comes from systems other than the reproductive system and therefore these will be briefly investigated to help elucidate what is already known about their function in reproductive tissues.

1.2 Biosynthesis and Structure of BMPs

The endoplasmic reticulum (ER) synthesizes pre-pro-BMP which translocates to the Golgi apparatus for processing by furin pro-protein convertase endoproteases in the trans Golgi network where furin is first observed to be active (Cui et al., 2001; Degnin et al., 2004). Pre-pro-BMP must be cleaved by pro-protein convertases such as furin, also called subtilisin-like pro-protein convertase-1, at S1 and S2 sites of the precursor peptide to produce a mature, stable and bioactive protein (Constam & Robertson, 1999). At pH 7.0 furin efficiently cleaved pro-BMP-4 at the consensus furin S1 site but not its upstream nonconsensus furin S2 site, while enzyme cleavage at S1 and S2 sites were both efficient at pH 6.5 (Degnin et al., 2004). This was due to a conserved P6 histidine which masks the upstream S2 site at neutral pH, while undergoing conformational changes leading to loss of structural integrity at an acidic pH exposing the S2 site for enzyme cleavage (Degnin et al., 2004; Hillger et al., 2005). Cleavage at only the S1 site resulted in rapid degradation by proteosomes and endosomes, while cleavage of the S1 and S2 sites resulted in a much more stable protein conformation, suggesting that different compartments from the trans Golgi network to the post Golgi environment are involved in the secretion of mature, stable and bioactive BMP proteins (Cui et al., 2001; Degnin et al., 2004). Uniquely the S1 consensus furin site must be cleaved first before the noncosensus S2 furin site can be cleaved and this order of pro-hormone processing is necessary for exit of BMP from the ER and for the generation of activated convertases (Cui et al., 2001).

Pro-BMP-2 and digit-removed BMP-2 have been shown to induce ectopic bone formation *in vivo* in rats, however it is not known whether mature BMP-2 cleaved from the pro or digit-removed unit resulted in the osteoinductive activity

(Hillger et al., 2005). Pro-BMP-2 is believed not to be competent to allow for BMP signalling as it interferes structurally with two tryptophan residues that the mature BMP uses to bind the BMP type I receptor (Hillger et al., 2005). Therefore, while there may be variations in different BMPs, mature BMPs are thought to have the major role in cell signalling.

Most BMPs are characterized by seven intermolecular conserved cysteines in their mature segment, of which six are needed for the formation of three disulfide bridges which compose the cystine knot and the seventh cysteine residue is required for dimer formation (Bottner et al., 2000). Exceptions include BMP-15 which lacks the fourth cysteine typically required for intermolecular disulfide bridge formation, as seen in GDF-9 and has therefore also been termed GDF-9B (Otsuka et al., 2000). GDF-9 and BMP-15 are believed to form noncovalent bonds during dimer formation (Dube et al., 1998).

1.3 TGF-β and BMP Signalling

1.3.1 Dimer Formation

Physiologically BMPs have mainly been observed to function as homodimers *in vivo*, while heterodimer units have been created *in vitro* conditions. In cells transduced with AdBMP2/7 in C57BL6 mice and *in vitro* BMP-2/6, BMP-2/7 and *Xenopus* BMP-4/7 in MC3T3-E1 murine osteoblast cells were more potent in increasing osteogenic activity than homodimers of BMP-2, 4 or 7 (Aono et al., 1995; Hazama et al., 1995; Israel et al., 1996; Koh et al., 2008). In ovarian cell lines derived from Chinese hamster and mouse BLK cells BMP-2/7 heterodimers had significantly higher activity in the alkaline phosphatase induction assay (Israel et al., 1996; Koh et al., 2008), and in mouse BLK cells BMP-2/7 enhanced phosphorylation

of BMP recptor activated signal transducers, Smad1, 5 and 8 to a greater extent than BMP-2 or BMP-7 (Koh et al., 2008).

In contrast to these findings, *in vivo* recombinant human BMP-2 was shown to have significantly greater ectopic osteoinductive activity than recombinant *Xenopus* BMP-4/7 in rats (Kusumoto et al., 1997). Animal cap explants using recombinant *Xenopus* BMP-4/7 were shown to potently induce mesoderm formation in vertebrate embryos (Suzuki et al., 1997), while in *Xenopus* embryos *in vivo* BMP-2/7 and BMP-4/7 have not been shown to be required for induction of the mesoderm (Eimon & Harland, 1999). Therefore, whether BMPs signal as homodimers or heterodimers *in vivo* in normal physiologic conditions is debatable and possibly depends on the specific BMP, the cell types through which it signals and the species the BMP originates or is reconsistiuted from.

Recombinant purified *Xenopus* BMP-4, BMP-7 and BMP-4/7 had expected NH2-terminal domains while possessing diverse carbohydrate moieties (Aono et al., 1995). It is possible that glycosylations may have an effect on the circulatory life of BMPs as well as their biological activity similar to the effects of glycosylations on gonadotrophins such as FSH (Creus et al., 2001). Furthermore, whether BMPs originate from *Xenopus*, humans or other species is likely to affect species-specific patterns of glycosylations and receptor binding affinities, which has not yet been investigated, thereby altering cellular responses.

1.3.2 Receptors

Seven type I receptors, five type II receptors and various type III receptors such as endoglin and betaglycan enable the signalling of TGF-β ligands (Shah et al., 2001). The type I receptor is approximately a 55 kDa glycoprotein while the type II

receptor is approximately a 70 kDa glycoprotein (Koenig et al., 1994; Rosenzweig et al., 1995). Type III receptors are proteoglycans that have large molecular weights of approximately 280 kDa (Segarini & Seyedin, 1988; Henis et al., 1992).

To activate the TGF-β family signalling cascade, TGF-β type ligands bind type II serine/threonine receptors as either homodimers or heterodimes thereby causing receptor phosphorylation and activation of the type I receptor leading to the formation of tetrameric signalling complexes (Kawabata et al., 1995; Rosenzweig et al., 1995; Allendorph et al., 2006). While many TGF-β ligands require binding of the type II receptor to initiate signalling, BMP-2 and BMP-4 can bind the type I receptor leading to the recruitment of the type II receptor (Koenig et al., 1994).

Using X-ray crystal diffractions the geometric binding of BMP homodimers to type I and type II receptors was visualized (Allendorph et al., 2006) and type I receptors heterodimerize with type II receptors with no homodimerization occurring among receptor types (Kawabata et al., 1995). Furthermore, BMP receptor binding is enhanced in the presence of both the type I and type II receptors as illustrated in BMP-4 and BMP-7 receptor binding in COS cells using *Caenorhabditis elegans* homolog to mammalian BMPR-II, DAF-4 transfected cells (ten Dijke et al., 1994b). Human BMPR-II also enables signalling via BMP-2, BMP-4 and BMP-7, where BMP-2 and BMP-4 were shown to bind BMPR-II in COS-1 cells. However, the cellular activation of transcription was facilitated by complex formation with the type I receptor (Rosenzweig et al., 1995), highlighting the importance of ligand complex formation with both the type I receptor and type II receptor for signal transduction. Type I receptors bind receptor activated Smad (R-Smad) signal transducers by which TGF-β family members mediate specific transcriptional outcomes (Chen et al., 1998b; Macias-Silva et al., 1998). Different ligands and receptor combinations then

become responsible for a great diversity of nuclear signalling via their particular Smad recruiting properties (Macias-Silva et al., 1998; Aoki et al., 2001).

1.3.3 Binding Epitopes of Type I and Type II receptors

Visualization of receptor-ligand complexes has been performed using X-ray crystal diffractions which identified the distal 'wrist' epitope of BMP-2 to interact with BMPR-IA (Nickel et al., 2001), while the proximal 'knuckle' epitope interacted with the type II receptor (Shah et al., 2001). In BMPR-IA and BMPR-IB the amino acids cysteine 77, phenylalanine 85 or glutamine 86 have exclusive ligand binding properties and are not involved in the structural stability of the receptor, as mutations of these amino acids significantly diminish the ability of receptors binding to BMP-2 (Nickel et al., 2001; Kotzsch et al., 2008).

BMP-2 binds BMPR-IA via its leucine 51 and asparagine 53 residues and substitution of leucine 51 with proline (L51P) eliminates hydrogen bonds between BMP-2 and its type I receptor. L51P mutational BMP-2 did not readily form complexes with either BMPR-IA or BMPR-IB while having no significant effect on its ability to bind BMPR-II, Noggin, Chordin or Gremlin (Keller et al., 2004). In GDF-5 arginine 57 is responsible for strong binding to BMPR-IB and the importance of GDF-5 signalling via BMPR-IB is highlighted by the similarity observed in both GDF-5 and BMPR-IB gene knockout phenotypes (Nickel et al., 2005). GDF-5 will bind BMPR-IA with about 12 times lower affinity than BMPR-IB (Nickel et al., 2005).

1.3.4 Receptor – Ligand Interactions in Surface Plasmon Resonance Studies

Using surface plasmon resonance assays, which measure physical properties of molecular binding using electromagnetic charge, various ligand-receptor binding

possibilities have been explored for TGF-β family members. BMP-2, BMP-7 and activin will bind ActR-II (Allendorph et al., 2006), and with lower affinity BMP-2 will bind ActR-IIB and BMPR-II (Weber et al., 2007). A soluble type I BMP receptor extracellular region generated by the transgenic silkworm expression system bound strongly to BMP-4 as observed by rapid associations and poor dissociations using optical biosensors (Natsume et al., 1997).

Using a similar detection system the extracellular region of BMPR-IA was shown to bind only dimeric recombinant human BMP-2 suggesting that only dimers are involved in cell signalling (Wendler et al., 2005). BMP-2 was shown to bind the extracellular domain of the type I receptors BMPR-IA and BMPR-IB equally even though the receptor amino acid sequence is highly variable (Kotzsch et al., 2008). While these techniques do improve the understanding of ligand receptor interactions, ligand receptor interactions appear to have greater specificity in cell lines and again in actual biological systems *in vivo.* Greater ligand receptor specificity then increases the likelihood of a specific cellular response with a particular ligand receptor combination in the presence of other less specific ligands and receptors as described in the following paragraphs.

1.3.5 Receptor – Ligand Interactions in Cell Lines and Compensation

Many different types of cell lines have been used to study BMP signalling pathways. In MC3T3-E1 osteoblasts BMP-4 binds preferentially to BMPR-IA (ten Dijke et al., 1994b) while in MC3T3-E1 and C2C12 mouse derived osteoblast cell lines BMP-6 binds strongly to BMPR-IB, BMPR-IA, ActR-I (ALK-2) and the orphan receptor ALK-1 (Ebisawa et al., 1999). BMP-7 can bind BMPR-IA though it has a greater affinity for ActR-I in MC3T3-E1, C2C12, human teratocarcinoma cells

(ten Dijke et al., 1994b), and in the embryonic carcinoma cell line P19 where it was also shown to bind ActR-II and ActR-IIB (Macias-Silva et al., 1998). However, there is a possibility that BMPR-II is not expressed in P19 cells, and Ebisawa *et al*. (1999) have hypothesized that the binding of ligands to specific receptors is mediated by the level of receptor expression in cell lines and by the preference of the type II receptor for its type I receptor.

In ROB-C26 osteoprogenitors radio-labeled BMP-2, BMP-6 and GDF-5 bound preferentially to BMPR-IB and BMPR-II in the presence of BMPR-IA (Nishitoh et al., 1996; Ebisawa et al., 1999). In MvLu cells BMP-2 did form complexes with BMPR-IA, however these cells were not shown to express BMPR-IB (Nishitoh et al., 1996). This suggests that in the absence of the preferred receptor BMPs will compensate by binding alternate receptors, however the signalling cascade will be modified and compensation may not be adequate. This phenomenon has been partially elucidated using mouse calvariae derived 2T3 precursor cells, where BMP-2 induces differentiation into osteoblasts via BMPR-IB, while in the presence of indomethacin, a ligand of PPARγ the same cells differentiated into adipocytes via BMPR-IA (Chen et al., 1998a).

BMPR-IA, BMPR-IB and BMPR-II were visualized in 2T3 cells and truncated over-expression of BMPR-IB blocked the ability of BMP-2 to induce bone formation, while truncated BMPR-IA did not, truncated BMPR-IA over-expression even enhanced tissue mineralization without ligand stimulation (Chen et al., 1998a). BMP-7 responded in the same manner as BMP-2 failing to produce calcification in the presence of truncated BMPR-IB over-expression, while being able to in cells over-expressing truncated BMPR-IA (Chen et al., 1998a). In adipocyte forming media truncated BMPR-IB strongly enhanced adipocyte differentiation, while the

over-expression of truncated BMPR-IA prevented adipocyte formation (Chen et al., 1998a). This demonstrates highly specialized receptor dependant roles for BMPs in tissue differentiation and morphology specific to the local cellular environment.

1.3.6 Receptor Autophosphorylation in Transfected Cells

C2C12 cells expressing BMPR-IA and BMPR-II, transfected with BMPR-IB induce specifically the expression of osteocalcin mRNA and alkaline phosphatase activity without BMP-2 receptor binding (Akiyama et al., 1997), which was also observed in 2T3 cells with the transfection of truncated BMPR-IA (Chen et al., 1998a). This illustrates that receptor over-expression can lead to receptor autophosphorylation and possibility induce the non-preferential binding of ligands to receptors that would otherwise not occur.

1.3.7 Receptor Activation Profile for BMP-7 *in vitro* verses *in vivo*

In surface plasmon resonance assays BMP-7 will bind ActR-II (Allendorph et al., 2006). In P19 cells BMP-7 binds ActR-II and ActR-IIB (Macias-Silva et al., 1998) and has a strong affinity for ActR-I in MC3T3-E1, C2C12 and human teratocarcinoma cells (ten Dijke et al., 1994b). BMP-7 also binds ActR-II in COS-1 cells, in Mv1Lu epithelial cells with a 2-3 fold lower affinity than activin A, and in the K562 erythroleukemic cells, where it stimulated differentiation but less effectively than activin A (Yamashita et al., 1995). In rat pituitary cells, however, BMP-7 was unable to stimulate pituitary FSH release via ActR-II or ActR-IB (Yamashita et al., 1995) and in live *Xenopus* embryo culture BMP-7 was unable to induce mesoderm induction at physiological levels via the activin signalling pathway (Yamashita et al.,

1995). While activin receptor binding for BMP-7 has been observed *in vitro*, receptor binding capacity alone was not sufficient for cell signalling *in vivo*.

1.3.8 Smads

Smad substrates serve as signal transducers of the TGF-β family member proteins. Mammalian Smad proteins include Smad1, Smad2, Smad3, Smad4, Smad5, Smad6, Smad7 and Smad8 (Eppert et al., 1996; Hoodless et al., 1996; Lagna et al., 1996; Liu et al., 1996; Macias-Silva et al., 1996; Zhang et al., 1996; Hayashi et al., 1997; Topper et al., 1997; Nishimura et al., 1998; Lee et al., 2001a; Sowa et al., 2004; Kersten et al., 2005). Upon ligand binding, the type II BMP receptor associates with Tribbles-like protein 3 which potentiates R-Smad signalling via the type I receptor (Chan et al., 2007).

TGF-β receptor kinases activated by ligands phosphorylate Smad1, 2, 3, 5 and 8 at their carboxy terminal which have a SSXS motif, as has been demonstrated with Smad1, therefore these Smads are called receptor-regulated Smads (R-Smads) (Kretzschmar et al., 1997). TGF-β, activin and Nodal have been shown to activate Smad2 (Eppert et al., 1996; Macias-Silva et al., 1996) and Smad3 (Zhang et al., 1996; Sowa et al., 2004), while BMPs activate specifically Smad1 (Hoodless et al., 1996; Liu et al., 1996), Smad5 (Nishimura et al., 1998) and Smad8 (Kersten et al., 2005). However, there has been one account that TGF-β can phosphorylate Smad1 (Liu et al., 1998b). Smad4 does not get phosphorylated directly upon ligand binding but instead binds R-Smads that have been receptor activated and co-transports into the cell nucleus (Lagna et al., 1996; Zhang et al., 1996; Nishimura et al., 1998; Lee et al., 2001a), therefore Smad4 is called common mediator Smad (co-Smad) (Hahn et al., 1996). Smad6 and Smad7 bind Smad4 and the type I receptor

and block their signalling, therefore these Smads are referred to as inhibitory Smads (I-Smads) (Hayashi et al., 1997; Topper et al., 1997). Smad6 inhibits mainly BMPs while Smad7 inhibits all TGF-β type proteins by preventing Smad interaction with the DNA binding domains (Zhang et al., 2007). I-Smads also facilitate their inhibitory effects via stimulating Smurfs which are types of HECT E3 ubiquitin ligases which inhibit R-Smads via ubiquitination (Zhang et al., 2000).

All Smads share similarities in their genetic sequences especially in the N-terminal also called the Mad Homology 1 (MH1) domain and the carboxy terminal known as the Mad Homology 2 (MH2) domain. The genetic sequence between MH1 and MH2 is known as the linker region and it is this region that is highly variable among Smads; additionally this region also allows passage though the nucleus via its association with transport proteins (Xu et al., 2007).

Smads enter the cell nucleus through the nuclear pore complex via transport proteins called importins (Xu et al., 2007) or exportins (Kurisaki et al., 2006) based on whether their role is to import or export molecules such as Smads respectively (Xu et al., 2002). In unstimulated cells R-Smads and Smad4 have been shown to constantly shuttle into and out of the nucleus, while TGF-β family member signalling stabilises Smads inside the nucleus by proteins such as Fast1 and reduces their export back into the cytoplasm, thus allowing for a sustained opportunity to alter transcriptional activities (Yingling et al., 1997; Inman et al., 2002; Nicolas et al., 2004; Schmierer & Hill, 2005). Unlike BMPs, TGF-β was unable to activate Fast1 via Smad1 (Chen et al., 1998b), highlighting variables in the transcriptional activation of different TGF-β family members.

There is also evidence that small ubiquitin-related modifier (SUMO) proteins have a role in the regulation and stabilization of Smad signalling across the nuclear

membrane and acetylation via acetyltransferases that act as co-activators of transcription in the nuclear compartment (Chen et al., 1998b).

1.3.9 BMP Signalling and Other Kinase Pathways

In cell culture BMP-2 induces the differentiation of murine derived MC3T3-E1 osteoblast cells via intracellular signalling that involves the inhibition of mitogen activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) and the promotion of alkaline phosphatase activity, however TNF-like weak inducer of apoptosis (TWEAK) signalling via the fibroblast growth factor-inducible 14 (Fn14) activates the MAPK and ERK pathway and blocks BMP-2-stimulated alkaline phosphatase activity which can in turn be blocked by Fn14-Fc chimera (Ando et al., 2006). Furthermore, BMP-2 activity can also be resumed by the addition of PD98057 a MAPK and ERK inhibitor (Ando et al., 2006), it is therefore apparent that the signalling of BMP-2 involves the inhibition of the MAPK/ERK pathways. Anti-p42/44 MAPK siRNAs, chelerythrine, PD 98059 and SB203580 prevented the adhesiveness of monocytes to endothelial cells by preventing p42/44 MAPK phosphorylation by BMP-2 (Csiszar et al., 2006). Chelerythrine treatment blocks protein kinase C (PKC) signalling and was shown to prevent BMP-2 induced reactive oxygen species in epithelium (Csiszar et al., 2006), and in human ovarian granulosa cells the activation of protein kinase A (PKA) and PKC down-regulates BMP-3 transcription (Jaatinen et al., 1996). This provides evidence that in addition to well-known Smad activation, BMP signalling also involves the MARK, ERK, PKA and PKC intracellular pathways and suggests that the morphogenic activities of BMPs can be modulated by interplay with other ligands and signalling pathways.

1.4 BMP Receptors in Reproduction

BMP receptors are found extensively in reproductive tissues of many different species. In sheep and pigs BMPR-IA, BMPR-IB and BMPR-II are found on oocytes, granulosa cells in primary to antral stages of follicular development, the corpus luteum (CL) and the ovarian surface epithelium (Wilson et al., 2001; Souza et al., 2002; Quinn et al., 2004). In Booroola ewes a naturally occurring mutation of BMPR-IB results in a hyper-prolific phenotype with increased ovulation and smaller follicles due to reduced granulosa cell proliferation (Mulsant et al., 2001; Wilson et al., 2001).

Mice with BMPR-IB knockout are infertile due to failure of cumulus granulosa cell expansion, failure of endometrial gland formation leading to a thin uterine lining, and irregular estrus cycles (Yi et al., 2001). While knockout BMPR-IB mice contrast the reproductive profile of mutant BMPR-IB sheep, they both show decreased granulosa cell proliferation; in addition receptor modification verses absence present two very different signalling environments.

In mice BMPR-IA knockout is embryonically lethal (Mishina et al., 1995), as is BMPR-II knockout. BMPR-II knockout yields similar results to BMPR-IA mutation in murine embryos suggesting that BMP signalling via this receptor combination is vital for embryonic development (Beppu et al., 2000). In transgenic AMH-SV40 mice AMHR-II activates Smad1 in granulosa cell tumors (Dutertre et al., 2001), suggesting that it forms complexes with BMP type I receptors that are known to employ Smad1, Smad5 and/or Smad8. BMPR-IB and AMHR-II have been visualized in adult mouse ovary and in fetal and prepubertal rabbit ovary (Gouedard et al., 2000). In rats theca cell derived BMP-4 and BMP-7 bind BMPR-IA, BMPR-IB and BMPR-II receptors present on oocytes and granulosa cells (Shimasaki et al., 1999;

Lee et al., 2001b; Nilsson & Skinner, 2003). In humans BMPR-II was found in a large variety of tissues including the brain, prostate, testis and ovary in both adult and fetal tissues (Rosenzweig et al., 1995), and BMPR-IA, BMPR-II, Smad1, 2, 3, 4 and 5 have been isolated from 6-day granulosa-luteal cell cultures (Jaatinen et al., 2002).

In prepubertal porcines BMPR-IA, BMPR-IB and BMPR-II were strongly expressed in oocytes in all stages of development and in granulosa cells of antral follicles while being moderately expressed in preantral follicles (Quinn et al., 2004). In mature porcines BMP receptor expression was moderate in all oocytes and preantral granulosa cells, strong in antral granulosa cells and very strong in luteinizing granulosa cells of which the expression reduced in the CL (Quinn et al., 2004), displaying developmental regulation in the expression of BMP receptors as well as possible down-regulation by gondotrophins.

While BMP receptors are responsible for much of the outcomes of BMP signalling, BMPs can also affect the signalling of BMP receptors which becomes apparent upon examination of naturally occurring mutations in both receptor and ligands discussed in the next section.

1.4.1 Naturally Occurring Mutant BMP and BMP Receptors

Perhaps one of the most important observations to spur research into TGF-βs and their receptors was the discovery that the hyper-prolific characteristics of various sheep flocks was due to the presence of BMPR-IB and BMP-15 mutations (Galloway et al., 2000; Mulsant et al., 2001; Wilson et al., 2001). In the Australian Booroola ewes a non-conserved substitution in the Fecundity Booroola gene ($FecB^{B}$) at Q249R leads to mutant BMPR-IB signalling that results in a hyper-prolific

phenotype with increased ovulation and litter size (Mulsant et al., 2001; Wilson et al., 2001). Homozygous $FecB^{B}$/ $FecB^{B}$ granulosa cells have reduced proliferative activity compared to non-carrier $FecB^{+}$/ $FecB^{+}$ granulosa cells resulting in reduced follicular size and increased ovulatory follicle numbers in Booroola ovaries (Mulsant et al., 2001; Wilson et al., 2001). The intensity and location of BMPR-IB and BMPR-2 expression between Booroola carriers of $FecB^{B}$ and non-carriers ($FecB^{+}$) was shown to be identical (Wilson et al., 2001), while in granulosa cell culture the inhibitory effects of GDF-5 and BMP-4 on progesterone secretion were significantly less effective in reducing progesterone in $FecB^{B}$/ $FecB^{B}$ mutant Booroola ewes than in $FecB^{+}$/ $FecB^{+}$ ewes (Mulsant et al., 2001), suggesting that the BMP receptor mutation alters downstream signalling components which affect steroidogenesis and cell mitosis. Indeed the mutation of BMPR-IB occurs in the intracellular domain (Wilson et al., 2001).

Homoygous BMPR-IB mutation has also been reported in humans, with a woman carrying an 8 base pair deletion on the BMPR-IB gene (del 359-366) resulting in acromesomelic chondrodysplasia and hypergonadotrophic hypogonadism causing primary amenorrhea, absent ovaries and hypoplastic uterus (Demirhan et al., 2005).

In Romney sheep from New Zealand the Fecundity X Inverdale gene ($FecX^{I}$) situated on chromosome X is responsible for the high prolificacy of this breed (Davis et al., 1991). In Inverdale ewes the $FecX^{I}$ gene was shown to increase ovulation rates in heterozygotes while causing streak ovaries and complete infertility in homozygotes (Davis et al., 1992). The $FecX^{I}$ gene is the Xp11.2-11.4 human ortholog which maps for BMP-15 (Galloway et al., 2000), and BMP-15 transcripts have been detected in ovine oocytes (Bebbere et al., 2008). Intercrossing Inverdale

and Hanna sheep carrying the mutant $FecX^{I}$ and $FecX^{H}$ genes respectively results in identical heterozygote and homozygote profiles, suggesting that the same gene mutation is involved (Davis et al., 2001). Similar reproductive profiles in ewes due to single polymorphisms of BMP-15 were observed in Cambridge sheep ($FecX^{G}$), Belclare sheep ($FecX^{B}$), crossed $FecX^{G}$/$FecX^{B}$ sheep from Ireland (Hanrahan et al., 2004) and in Lacaune sheep carriers of the $FecX^{L}$ gene from France (Bodin et al., 2007).

A point mutation in the GDF-9 gene $FecG^{H}$ was also observed to lead to sterility in homozygous genotypes in Belclare and Cambridge ewes (Hanrahan et al., 2004). Heterozygots for either BMP-15 or GDF-9 had higher ovulation rates and notably the heterozygous combination of BMP-15/GDF-9 increased the ovulation rate further (Hanrahan et al., 2004). However, it is important to note that the specific point mutation on the BMP-15 or GDF-9 gene appeared to have a strong influence on this profile (Hanrahan et al., 2004), suggesting that the amino acid substitutions that constitute these mutations result in modified ligand signalling as opposed to a total lack of function in most cases.

Chinese Small Tailed Han sheep have been identified to carry mutations in both BMPR-IB and BMP-15 genes (Chu et al., 2007). Han sheep heterozygous for the $FecX^{G}$ BMP-15 mutation at Q239Ter (premature stop codon) have increased litter sizes (0.55 times more), while sheep heterozygouse for $FecB^{B}$ BMPR-IB mutation at Q249R have 1.11 times increased litter sizes compared to sheep not carrying the mutation (Chu et al., 2007). Sheep with both BMPR-IB and BMP-15 mutations had even greater litter sizes in excess of twice that of wildtypes (Chu et al., 2007). Immunization against BMP-15 and GDF-9 also has a significant inhibitory effect on ovulation confirming its role in follicular development (McNatty et al., 2007).

In humans two sisters with hypergonadotropic ovarian failure and reduced proliferation of granulosa cells were shown to be heterozygous for an X-linked point mutation of the BMP-15 gene in the pro site of the peptide (Y235C), which was inherited from the unaffected father (Di Pasquale et al., 2004). Furthermore, various gene mutations of BMP-15 have been detected in women with premature ovarian failure (Di Pasquale et al., 2006). In approximately 5 % of Caucasian women with premature ovarian failure BMP-15 gene alterations lead to the amino acid substitution of A180T, R68W and the insertion of 262L of the pro-peptide sequence (Di Pasquale et al., 2006). While the exact cellular mechanism of action is still unclear it is evident that GDF-9 and BMP-15 promote granulosa cell proliferation, regulate steroidogenesis (McNatty et al., 2007) and have a significant role in animal and human reproduction.

1.5 Some Bone Morphogenetic Protein Family Members

The BMP family, also known as the DVR family (Bottner et al., 2000), is amongst a group of morphogenic proteins that have diverse functions in all developmental stages across species. Research on BMPs has elucidated major roles for the growth factors in the formation, development and functioning of almost every body system including the skeletal, vascular, circulatory, nervous, immune and reproductive systems, as well as in organogenesis and tissue orientation during embryogenesis. The amino acid sequences of BMPs are highly conserved across species as can be seen with human BMP-2 and its comparison to BMP-2 and BMP-4 in other species including mammals, monotremes, birds, reptiles and amphibians, Table 1.1

1.5.1 BMP-1

BMP-1 is one of a group of four known metalloproteinases involved in the cleavage of growth hormone, prolactin (Ge et al., 2007) and myostatin (Wolfman et al., 2003), and is not actually a member of the TGF-β superfamily (Wozney et al., 1988). BMP-1 was found to be identical to C-proteinase an enzyme involved in the synthesis of fibrillar collagens from pro-collagen (Li et al., 1996). The genetic homolog to BMP-1, Tolliod in *Drosophila melanogaster* has been found to be necessary for the proteolytic activation of decapentaplegic, a TGF-β family member required for embryonic development and it has been suggested that it may be important for the activation of other transforming growth factors (Finelli et al., 1994).

1.5.2 BMP-2 (BMP-2a)

BMP-2 was first isolated from purified bovine bone extract due to its ability to induce the formation of cartilage and bone *in vivo* (Wozney et al., 1988). BMP-2 is also highly expressed in the hard inner mantle tissue of *Pinctada fucata* saltwater mollusks (Miyashita et al., 2008), indicating a role in hard tissue formation across species. BMP-2 increases the synthesis of proteoglycan and increases the turnover of normal and damaged cartilagenous matrix, while assisting in the repair of damaged cartilage (Davison et al., 2007). In circulation BMP-2 regulates systemic blood iron levels by modulating the hepatic expression of hepcidin, a ferrotransporter antagonist (Truksa et al., 2006; Babitt et al., 2007; Milet et al., 2007). BMP-2 has been shown to regulate the differentiation of adipocytes from precursor mesenchymal C3H10T1/2 mouse cells *in vitro* (Hata et al., 2003). BMP-2 is also expressed in human thymic T cells and $CD34^{+}$, the progenitors of DP, $CD4^{+}$

and $CD8^+$, while not being expressed in the differentiated cells (Cejalvo et al., 2007). This suggests a specific role in early morphogenesis and differentiation.

Table 1.1

Comparison of Homo sapiens BMP-2 aa Sequence Across Species

(A) % Sequence aa identity to human BMP-2
(B) % Sequence aa identity to human BMP-4

Species	**Genbank and NCBI accession numbers**	**A**	**B**
Homo sapiens (Human)	BMP2 GB. CAB82007.1; BMP4 NCBI. BAA06410.1	NA	62%
Pan troglodytes (Chimpanzee)	PREDICTED: BMP2 NCBI. XP_514508.2; BMP4 NCBI XP_509954.2	98%	62%
Macaca mulatto (Rhesus monkey)	PREDICTED: BMP2 NCBI. XP_001115987.1; BMP4 NCBI. XP_001084317.1	98%	62%
Sus scrofa (Pig)	PREDICTED: Similar to BMP2 NCBI. XP_001928022.1; BMP4 NCBI. NP_001094501.1	93%	62%
Rattus norvegicus (Norway Rat)	BMP2 NCBI. NP_058874.1; BMP4 NCBI. NP_036959.2	91%	62%
Mus musculus (Mouse)	BMP2 GB. AAB05665.1; BMP4 NCBI. NP_031580.2	91%	62%
Equas caballus (Horse)	PREDICTED: Similar to BMP2 NCBI. XP_001493945.1; BMP4 NCBI. XP_001494992.1	94%	61%
Bos Taurus (Cow)	BMP2 NCBI. NP_001092611.1; BMP4 NCBI. NP_001039342.1	93%	62%
Ovis aries (Sheep)	BMP2 NCBI. CAB82007.1; BMP4 NCBI. NP_001103747.1	93%	62%
Ornithorhynchus anatinus (Platypus)	PREDICTED: Similar to BMP2 precursor NCBI. XP_001514564.1	85%	?
Gallus gallus (Chicken)	BMP2 NCBI. NP_989689.1; BMP4 NCBI. NP_990568.2	80%	62%
Xenopus laevis (African Clawed Frog)	BMP2 NCBI. NP_001095136.1; BMP4NCBI. NP_990568.2	72%	62%
Ambystoma mexicanum (Axolotl)	BMP2 NCBI. ABK34490.1; BMP4 NCBI. ACA34453.1	73%	59%

BMP-2 is found in uterine decidual cells (Lee et al., 2007), in bovine ovarian follicles (Fatehi et al., 2005), and it is expressed in mouse mammary gland (Phippard et al., 1996), the embryonic endoderm (Ying & Zhao, 2001) and in the developing skeletal system and cortex of hair follicles of murine embryos (Lyons et al., 1989b). BMP-2 signalling is necessary to inhibit excessive cellular proliferation in

colon epithelial cells (Kotzsch et al., 2008). In the vascular system BMP-2 is also expressed in endothelial cells and smooth muscle cells and increases the adherence of monocytes to human carotid artery endothelial cells (Csiszar et al., 2006).

BMP-2 has a binding site for NF-kB on its promoter region and can activate it, which is absent in BMP-4 its closest relative (Csiszar et al., 2006). *In vitro* TNFα increased the expression and translation of BMP-2 and was shown to activate NF-kB in endothelial cells in rats (Csiszar et al., 2006). The BMP-2 amino terminus harbours a putative binding site for heparin which can be removed using 'mild trypsin conditions'(Koenig et al., 1994). The heparin binding site on BMP-2 has been shown to modulate receptor binding (Ruppert et al., 1996). Human BMP-2 mRNA constists of 3,150 base pairs (bp) coding a 396 amino acid (aa) (GenBank accession no NM_001200.2).

Mice embryos homozygous for the BMP-2 gene deletion ($bmp2^{ml/}$ $bmp2^{ml}$) die at 7.0 – 10.5 days of gestation, as a result of the pro-amniotic canal not closing causing defects in the amnion, chorion and/or the heart (Zhang & Bradley, 1996). Heterozygous mice for the BMP-2 gene ($bmp2^{ml}$/+) were shown to have a normal phenotype (Zhang & Bradley, 1996). In mice embryos the lack of BMP-2, via the construction of a conditional allele at exon 3, and BMP-4 via the construction of a conditional allele at exon 4, resulted in severely impaired skeletal formation and limb patterning, while the conditional lack of either protein in isolation did not have such pronounced effects suggesting that BMP-2 and BMP-4 are in part functionally interchangeable (Bandyopadhyay et al., 2006). Bmp-2 and Bmp-4 have also been shown to be necessary for pituitary development and patterning, however in order for normal organogenesis to occur, Noggin antagonism of Bmp-2 and Bmp-4 is

required to regulate this process (Davis & Camper, 2007). BMP-2 up-regulates follistatin which inhibits BMP-2 signalling in osteoblastogenesis suggesting a feedback mechanism in rat fetal osteoblast cells (Yukiko et al., 2004).

1.5.3 BMP-3 (Osteogenin)

Homosapiens Pre-pro-BMP-3 mRNA is a 5,734 bp molecule encoding a 472 aa peptide (GenBank accession no NM_001201). In humans BMP-3 is expressed in granulosa cells which respond to human chorionic gonadotrophin (hCG) by down-regulating transcription, indicating that BMP-3 is responsive to endocrine regulation (Jaatinen et al., 1996).The mature region of rat BMP-3 shares 98% homology to human BMP-3, which is expressed in bone, cartilage, lung and ovarian tissue (Takao et al., 1996). BMP-3 interacts with collagen types I, IV and IX found in basement membranes where it is believed to have a role in organogenesis (Paralkar et al., 1990). BMP-3 also binds heparin, which can modulate BMP-3 function (Paralkar et al., 1990).

1.5.4 BMP-3b (GDF-10)

In rats BMP-3b was expressed mainly in bone and brain tissue (Takao et al., 1996), while in mice BMP-3b was also expressed in adipose and uterine tissue and at lower levels in the spleen and liver (Cunningham et al., 1995). The 478 aa constituting BMP-3b is encoded from a 2,674 bp mRNA (GenBank accession no NM_004962.2). BMP-3b is found in murine embryos and murine uterus, while not being expressed in the placenta (Zhao et al., 1999). In mice the expression of BMP-3b increases following conception, peaks in the first week post conception and then decreases until parturition after which levels increase again (Zhao et al., 1999).

BMP-3b is expressed in the stromal cell types not in the uterine epithelium and in normal cycling mice BMP-3b is elevated during early pro-estrus, decreased at estrus and again elevated at di-estrus (Zhao et al., 1999). BMP-3b knockout in mice caused no anatomical or functional abnormalities (Zhao et al., 1999).

1.5.5 BMP-4 (BMP-2b)

Similar to the effects of BMP-2, BMP-4 has been demonstrated to be necessary for blood iron balance as its gene products regulate systemic iron levels by regulating the hepatic expression of hepcidin, a ferrotransporter antagonist (Truksa et al., 2006; Babitt et al., 2007; Milet et al., 2007). In the nervous system BMP-4 is involved in precursor cell migration, morphogenesis and the addition of polysialic acid to neural cell adhesion molecules to increase their adhesion in murines (Fu et al., 2006). BMP-4 was shown to be expressed in human epithelial cells of the thymus cortex and epithelial cell lines thymic T cells and $CD34^+$, the progenitors of DP, $CD4^+$ and $CD8^+$, while not being expressed in the differentiated cells; and in chimeric human–mouse fetal thymic organ culture the addition of BMP-4 strongly reduced the differentiation of $CD34^+$ and inhibited apoptosis and maturation of progenitor T cells (Cejalvo et al., 2007). This suggests that BMP-4 may be a regulatory survival factor.

Like BMP-2, BMP-4 increases the adherence of monocytes to human carotid artery endothelial cells, however in rats unlike BMP-2, BMP-4 was significantly down-regulated by TNFα independent of NF-kB as this effect was not altered by blocking NF-kB with pyrrolydine dithiocarbamate (Csiszar et al., 2006). The 1,957 bp variant 1 of the homosapien BMP-4 mRNA transcript encodes a 408 aa peptide (GenBank accession no NM_001202.3). BMP-4 has been found in the ovaries of

rats (Erickson & Shimasaki, 2003) and mice (Tanwar et al., 2008) and BMP-4 is also expressed in the placenta (Zhao et al., 1998) and in mouse mammary gland (Phippard et al., 1996). In fetal calf serum BMP-4 was shown to form complexes of 100 kda (Kodaira et al., 2006) indicating complex formation with a soluble receptor or binding protein.

1.5.6 BMP-5

Pre-pro-BMP-5 is a 454 aa transcribed from a 2,207 bp mRNA (GenBank accession no NM_021073.2). In culture BMP-5 caused sympathetic nerve cells to develop dendrites via an increase in Smad-1 (Beck et al., 2001). As chimeric BMPR-IA-Fc inhibited the neural dendric growth, BMPR-IA is the likely receptor for BMP-5 in neurons (Beck et al., 2001). Follistatin and noggin also inhibited the effects of BMP-5 on the growth of dendrites in nerve cells *in vitro* (Beck et al., 2001). Rat granulosa cells and oocytes of preantral follicles have been shown to express BMP-5 (Pierre et al., 2005). BMP-5 was demonstrated to have a proliferative effect on granulosa cells while suppressing steroidogenic acute regulatory protein (StAR) expression, basal, FSH-stimulated, and insulin-like growth factor-1-stimulated progesterone production while having no effect on estradiol secretion (Pierre et al., 2005). Follistatin inhibits BMP-5 and was shown to inhibit the suppressive effect of BMP-5 on progesterone production (Pierre et al., 2005).

1.5.7 BMP-6 (Vrg-1)

A 513 aa homosapien BMP-6 peptide is transcribed from a 3,105 bp mRNA (GenBank accession no NM_001718.4). BMP-6 RNA of 3.5 kb length was first isolated from murine tissue and shown to be present at various stages during

embryonic development (Lyons et al., 1989a). In human B cell lymphocytes BMP-6 was shown to inhibit anti-IgM B cell memory via the activation of Smad1, Smad5 and Smad8 (Kersten et al., 2005), indicating a possible function in suppression of the immune response during pregnancy. Furthermore type I receptors ActR-IA and BMPR-IB, and type II receptors BMPR-II and ActR-IIB are found on B cell lymphocytes (Kersten et al., 2005). BMP-6 is important in embryonic development in the mouse being highly expressed in the developing epidermis and skeletal system (Lyons et al., 1989b). In mice embryos BMP-6 null mutation caused mild delays in ossification of the sternum and Bmp5/6 null mutants displayed only mild exacerbation of the sternal defect, suggesting that BMP-2 also expressed in this tissue may compensate for the missing protein (Solloway et al., 1998).

BMP-6 is expressed in murine oocytes (Lyons et al., 1989b) and decreases granulosa cell progesterone production, while having no effect on estradiol secretion in rats (Otsuka et al., 2001a). BMP-6 inhibits FSH-stimulated StAR and P450 side chain cleavage enzyme (P450scc) transcription without effecting P450 aromatase (Otsuka et al., 2001a). BMP-6 had no effect on basal FSH receptor, however it suppressed FSH-stimulated FSH receptor synthesis (Otsuka et al., 2001a). Contrastingly, in chickens BMP-6 increased the expression of inhibin A and progesterone dose dependently and promoted granulosa cell survival *in vitro*. In hierarchy F1 granulosa cells BMP-6 increased LH receptor and highly up-regulated P450scc which progressively decreased by stage F4 granulosa cell development (Al-Musawi et al., 2007). The differences between these two species may reside in the types, distribution and concentration of BMP receptors and BMP binding proteins.

1.5.8 BMP-7 (OP-1)

Human BMP-7 is a 431 aa peptide transcribed from a 4,049 bp mRNA (GenBank accession no NM_001719.2). BMP-7 is implicated in the ossification of chondrocytes (Haaijman et al., 1997) and in human osteoarthrtic cartilage BMP-7 stimulated proteoglycan synthesis in chondrocyte culture (Stove et al., 2006). In rat ovary BMP-7 is secreted by theca cells and up-regulates FSH-stimulated P450 aromatase and estradiol expression, while inhibiting FSH-induced StAR and progesterone production (Lee et al., 2001b). In whole ovary culture BMP-7 stimulated primordial follicle development in mice, indicating that the protein is involved in early follicle recruitment (Lee et al., 2004). BMP-7 is also found in murine prostate (Thomas et al., 1998) and epididymis (Chen et al., 1999).

In mice BMP-7 is expressed in high levels in the kidney and in the uterine endometrium with pregnancy where it rapidly down-regulates and is found in placental trophoblast cells and the fetus (Ozkaynak et al., 1997). Treatment with 17 β-estradiol in non-pregnant mice also down-regulated BMP-7 transcription, while progesterone had no such effect, additionally kidney and ovarian expression of BMP-7 remained unchanged (Ozkaynak et al., 1997), indicating a tissue specific role to BMP-7 modulation. In humans BMP-7 is also expressed in cytotrophoblasts of the placenta and was shown to suppress both progesterone and chorionic gonadotrophin (Martinovic et al., 1996), suggesting that it may be modulated by steroids as well as affecting steroid profiles in reproductive tissues.

1.5.9 BMP-8a (OP-2)

Human BMP8a has a mRNA of 5,642 bp encoding a 402 aa peptide (GenBank accession no NM_181809.3). BMP-8a has been shown to be highly expressed in

uterine decidual cells and it is also expressed in the root of hair follicles and in the testis in postnatal development (Zhao & Hogan, 1996). Bmp-8 DNA spaning both BMP-8a and BMP-8b has been detected in the embryonic skeletal tissue of mice (DiLeone et al., 1997).

1.5.10 BMP-8b (OP-3)

Homosapien BMP8b is a peptide of 402 aa transcribed from a 3,773 bp mRNA (GenBank accession no NM_001720.3). The BMP-8b cDNA sequence has been identified in human hippocampus and murine embryo libraries and is unique among BMPs in that it harbours an additional cysteine residue in its N-terminal domain (Ozkaynak et al., 1992). BMP-8b was strongly expressed in mouse embryos but was not detected postnatally in brain, calvaria, lung, heart or kidney (Ozkaynak et al., 1992), however BMP-8b has been detected in mice in the root of hair follicles and the testis postnatally, as well as being expressed in placental trophoblast cells (Zhao & Hogan, 1996). Homozygous $Bmp8b^{tm1b1h}$ mutant mice exhibited progressive depletion of germ cells due to increased germ cell apoptosis and were rendered infertile (Zhao et al., 1996).

1.5.11 BMP-15 (GDF-9B)

The human BMP-15 precursor is a 392 aa peptide transcribed from 1,179 bp mRNA (NCBI Reference Sequence: NM_005448.1). Also known as GDF-9B, mature mouse BMP-15 is a 125 aa that shares a 70 % similarity to human BMP-15. BMP-15 is specific to the gonads in humans and was shown to be highly expressed in the testis and moderately expressed in the ovaries (Aaltonen et al., 1999), while in mice it was detected strongly in ovaries but not in the testis (Dube et al., 1998). In

humans BMP-15 mRNA is localized in the oocytes of late primary follicles (Aaltonen et al., 1999), and in immature mice BMP-15 is secreted by oocytes in primary to antral follicles (Laitinen et al., 1998). Figure 1.2 illustrates a comparison of the aa carboxy-tail domain of all reviewed BMPs demonstrating highly similar aa sequences within subgroups of the BMP family.

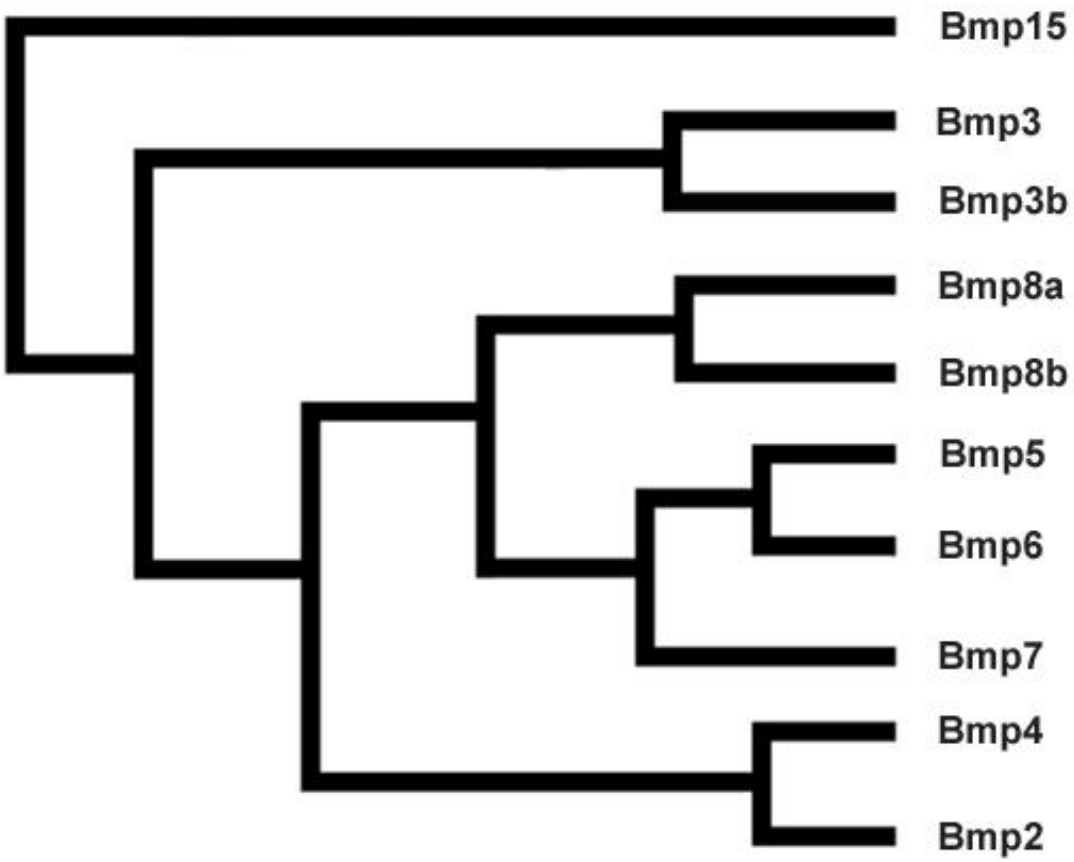


Figure 1.2 Bone morphogenetic protein carboxy-tail domain amino acid sequence map for 10 BMP family members (selective aa map pathways derived from Bottner, Krieglstein & Unsicker, 2000).

1.5.12 GDF-9

GDF-9 mRNA is composed of a 2,020 bp transcript that codes for a 454 aa peptide in humans (GenBank accession no NM_005260.3). In humans GDF-9

mRNA and protien have been detected in the oocytes of primary follicles (Aaltonen et al., 1999). In immature mice GDF-9 is secreted by the oocyte in primary through to antral follicles (Laitinen et al., 1998). GDF-9 is more abundant and is expressed earlier in follicle development than GDF-9B (BMP-15) in humans (Aaltonen et al., 1999). GDF-9 causes the expansion of cumulus cells and increases the adhesiveness of granulosa cells to the oocyte during ovulation which protects the oocyte physically as well as assisting in fertilization (Pangas et al., 2004). GDF-9 secreted from murine oocytes induces gremlin signalling while down-regulating BMP-4 signalling in granulosa cell culture (Pangas et al., 2004). The ovarian follicles of mice with GDF-9 knockout become arrested at the primary follicle stage (Pangas et al., 2004). Collectively this suggests that GDF-9 has a significant role in granulosa cell mitosis and development both directly and possibly indirectly by modulating the effects of other growth factors.

1.6 BMP Agonists and Antagonists

Dragon, a BMP agonist, is a 436 aa glycosylphosphatidylinositol-anchored protein and a member of the repulsive guidance molecules which acts as a co-receptor for BMP-2 and BMP-4 by binding directly to the ligand as well as the extracellular BMP receptor complex (Samad et al., 2005). Dragon enhances signalling by BMP-2 in LLC-PK1 porcine kidney epithelial cells and 10 T1/2 mouse mesenchymal stem cells and binds ActR-I, BMPR-IA, BMPR-IB, ActR-II and ActR-IIB (Samad et al., 2005). Dragon increased BMP-2 and BMP-4 signalling in the reproductive cell lines KGN and Ishikawa cells when the concentrations of BMP ligands were low (Xia et al., 2005). In the reproductive system Dragon is expressed in gonadocytes and in spermatogonia of immature mice, where equine chorionic

gonadotrophin (eCG) was shown to potently up-regulate Dragon in spermatocytes (Xia et al., 2005), which suggests that it may have an endocrine component to its regulation. Supporting this theory Dragon expression in the pituitary was mainly restricted to FSH producing gonadotrophs (Xia et al., 2005). In mature mice Dragon is secreted from spermatocytes and round spermatids (Xia et al., 2005). In female mice Dragon expression was exclusive to the oocytes and especially those from secondary follicles (Xia et al., 2005). Dragon is also expressed in seminal vesicles, epididymis, ovary, uterus and female pituitary (Xia et al., 2005).

Kielin a homolog of chordin-like containing 18 cysteine-abundant domains enhances the signalling of BMP-7 by enhancing ligand binding to the type I receptor in mice kidneys thereby inhibiting renal fibrosis as can be seen with kielin knockout (Lin et al., 2005). In *Xenopus* embryos kielin is expressed in the ectoderm and mesoderm and kielin treatment in *Xenopus* neurula embryos caused dorsalization of marginal ventral explants (Matsui et al., 2000), indicating a function for kielin in tissue patterning similar to BMPs in embryonic development.

Follistatin, chordin, chordin-like, cerberus, noggin, gremlin, FSRP, sclerostin and DAN all belong to a family of BMP antagonists (Sidis et al., 2002; Avsian-Kretchmer & Hsueh, 2004). Follistatin has been demonstrated to antagonize BMP-2, BMP-4, BMP-5, BMP-7, GDF-11 and activin (Beck et al., 2001; Balemans & Hul, 2002). In rats follistatin is present in granulosa cells and is expressed highly in the dominant follicle, in surface interstitial cells where it is only present during the LH surge at pro-estrus and in the CL which is likely to correlate to high progesterone levels, while not being expressed in follicles undergoing atresia (Erickson & Shimasaki, 2003). The expression profile of follistatin indicates that it may block the proliferative effects of BMPs and contribute to follicle rupture necessary for

ovulation, as well as preventing luteolysis thereby promoting the production of progesterone.

Chordin was shown to bind BMP-2 and BMP-4 in the extracellular space via a combination of its four cysteine-rich domains (Larrain et al., 2000). In zebrafish chordino the homolog of mammalian chordin was cleaved by mini-fin the homolog of Tolloid (Blader et al., 1997), indicating that BMP-1 homologs may be important for BMP signalling via their effect on BMP antagonists. Chordin-like antagonizes BMP-4, BMP-5 and BMP-6 (Balemans & Hul, 2002) and has a cysteine-rich domain that functions to bind BMPs and block receptor binding which is conserved among a number of homologs in vertebrates and invertebrates alike (Abreu et al., 2002). In humans Chordin-like has recently been discovered to be up-regulated in response to hypoxia in retinal pericytes (Kane et al., 2008).

Cerberus antagonizes BMP-4, nodal (BMP-16), Wnt and to a lesser degree BMP-2 in embryonic development of *Xenopus* (Piccolo et al., 1999). Noggin antagonizes BMP-2, BMP-4, BMP-7 and GDF-5 (Balemans & Hul, 2002) and was shown to inhibit the actions of Dragon on BMP-2 and BMP-4 *in vitro* (Samad et al., 2005). Noggin knockout in mice causes the pituitary to develop abnormally (Davis & Camper, 2007), highlighting the importance of BMP modulation via its antagonists. In murine granulosa cell culture gremlin was shown to inhibit BMP-4 signalling, while being up-regulated by BMP-4 (Pangas et al., 2004). FSRP antagonizes preferentially activin and to a lesser extent BMP-7 by preventing receptor binding (Sidis et al., 2002). Sclerostin antagonizes BMP-5 and BMP-6 in mice and human osteocytes (Winkler et al., 2003), however there has been a claim that sclerostin does not inhibit BMPs but does inhibit bone formation via other mechanisms (Bezooijen et al., 2004). DAN (Differential Screening-Selected Gene Aberrative in

Neuroblastoma) antagonizes GDF-5 in *Xenopus* embryo (Avsian-Kretchmer & Hsueh, 2004).

Twisted gastrulation (Tsg) is a factor involved in ventralizing activity in *Xenopus* blastomeres and was shown to bind BMP-4 and BMP-2 via its cysteine-rich N-terminal domain also seen in the BMP antagonist chordin (Oelgeschlager et al., 2000). *Xenopus* Tsg was found to bind both BMP-4 and chordin and when Tsg, BMP-4 and chordin where incubated together Tsg was shown to dislodge BMP-4/chordin complexes and bind all BMP-4 (Oelgeschlager et al., 2000). Tsg expression is up-regulated in activated T Cells and Tsg protein potently inhibited the proliferation of reactive $CD4^+$ and their production of cytokines and antibodies (Tzachanis et al., 2007), indicating a possible role in gestational immune suppression.

BAMBI is a pseudoreceptor, similar in structure to the type I receptor and is co-expressed with BMP-4 in embryogenesis of *Xenopus.* BAMBI inhibits the signalling of activin and BMPs via its intracellular domain by binding with the type II receptor and preventing the receptor complexes needed for signal transduction (Onichtchouk et al., 1999). BAMBI mRNA was visualized in ovarian granulosa cells and theca cells of secondary to tertiary follicles in rats (Loveland et al., 2003). BAMBI is expressed in immature rat Sertoli cells, however this expression down-regulates in mature Sertoli cells (Loveland et al., 2003). Initially at birth BAMBI is not expressed in gonocytes but becomes detectable at 5 days, after which it is highly expressed in spermatogonia to spermatocytes then down-regulates and is no longer detectable in elongating spermatids (Loveland et al., 2003). Activin A reduced BAMBI transcription in 24 hour testis fragment culture (Loveland et al., 2003).

1.7 Embryology - Primordial Germ Cells

Autocrine and paracrine growth factor signalling is vital for embryonic development of primordial germ cells (PGCs) and gonadal organogenesis. At embryonic days 7.0-7.25 PGCs are found in the primitive streak mesoderm and the endoderm, and at 8.0 days post coitum (dpc) clusters of PGCs can be seen in the hindgut endoderm of mice (Ginsburg et al., 1990). PGCs migrate across the dorsal mesentery and then lateral into the gonadal ridge where the germ cells become organized into cortical cords in the cortex of the developing gonad under the germinal epithelium (Rosen & Cedars, 2004). Development of an ovary will only happen in the absence of the sex-determining region Y (SRY) with the normal female XX sex chromosome karyotype (Conte & Grumback, 2004). PGCs stain intensely for alkaline phosphatase activity (Ginsburg et al., 1990) and when organized into cortical cords they acquire one surrounding layer of squamous granulosa cells, vital for oocyte survival, after which they are known as primordial follicles (Rosen & Cedars, 2004).

1.7.1 TGF-βs in the Proliferation of Primordial Germ Cells

Brachyury a T box gene is a well defined genetic marker of the primitive streak and its expression is required for the generation of the mesoderm (Wilkinson et al., 1990). Brachyury is expressed transiently in murine embryonic stem cells and when cultured in serum and serum-free^{B4L} media with or without BMP-4, serum-free media without BMP-4 was shown to be unable to induce brachyury and therefore mesoderm formation (Bruce et al., 2007), suggesting that the presence of BMP-4 is vital for mesoderm formation and proliferation of germ cells.

Gastrula mouse 129SvEv 3 x C57BL/6 embryos were shown to express

BMP-2 in the endoderm (embryonic days 6.75 to 7.5), its highest levels occurring simultaneously with the formation of the primitive streak, below the borders of the extraembryonic ectoderm and epiblast (Ying & Zhao, 2001). In normal and heterozygous (Bmp2/+) mouse embryos the PGCs are found on the wall of the hindgut, however in the BMP-2 homozygous mutants the PGCs developed abnormally and were found at the base of the allantois in the posterior streak (Ying & Zhao, 2001). In homozygous mutants for BMP-2 the number of PGCs was significantly reduced as compared to normal and heterozygous mutants, while the heterozygous group also had less PGCs than normal mouse embryos (Ying & Zhao, 2001). PGCs appeared to have normal morphology which suggests that BMP-2 may be important in PGC generation but not necessarily for survival (Ying & Zhao, 2001).

At embryonic days 5.5 to 6.5 high transcript levels of BMP-4 were observed in the proximal region of the extraembryonic ectoderm near the epiblast (Ying & Zhao, 2001). BMP-4 heterozygotes had significantly less PGCs than BMP-2 heterozygotes or wild type mice embryos (Ying & Zhao, 2001), supporting a predominative role for BMP-4 in germ cell proliferation.

At embryonic days 5.5 to 7.5 BMP-8b was expressed in the extraembryonic ectoderm in gastrula mouse embryos (Ying et al., 2000). Homozygous mutations in the BMP-8b gene caused approximately 50 % of embryos to be devoid of PGCs while the other 50 % had severely reduced numbers (Ying et al., 2000). Double gene mutants for BMP-2 and BMP-4 ($Bmp2^{+/-}$;$Bmp4^{+/-}$) had an additive effect showing severely reduced numbers of PGCs and some embryos even lacking them completely (Ying & Zhao, 2001). While PGC numbers were reduced, double heterozyotes for BMP-8b and BMP-2 did not have an additive effect on the inhibition of PGC number (Ying & Zhao, 2001). This suggests that BMP-2 and BMP-4 are in

part functionally interchangeable as the lack of either protein in isolation did not have such pronounced effects and this phenomenon has also been observed in murine embryonic skeletal formation and limb patterning (Bandyopadhyay et al., 2006).

1.7.2 TGF-βs and Male Determination

At embryonic days 7.0 to 7.25 PGCs are found in the primitive streak mesoderm and the endoderm and at 8.0 dpc clusters of PGCs can be seen in the hindgut endoderm of mice (Ginsburg et al., 1990). In XY genotypic males SRY is first expressed at 10.5 to 12.5 days in mice (Conte & Grumback, 2004). SRY encodes an 80 aa peptide domain which binds DNA and regulates transcriptional factors, such as steroidogenic factor-1, which are believed to induce the differentiation of Sertoli and steroidogenic pre-Leydig cells that produce testosterone and stimulate Sertoli cell AMH synthesis (Conte & Grumback, 2004). AMH is essential for the regression of the Mullerian ducts necessary for a normal male phenotype (Conte & Grumback, 2004). In the absence of AMH the Mullerian ducts develop into the upper parts of the female reproductive tract (Behringer et al., 1994), and correspondingly transgenic female mice that over-express AMH lack an internal reproductive track and have ovaries that are largely devoid of oocytes by 2 weeks of age persisting into adulthood (Behringer et al., 1990), illustrating a sex dependant role for AMH in embryonic development.

AMHR-II activates Smad1 in granulosa cell tumors exhibited by transgenic AMH-SV40 mice (Dutertre et al., 2001), suggesting that it forms complexes with BMP type I receptors, which are known to employ Smad1, Smad5 and/or Smad8. BMPR-IB and AMHR-II have been visualized in fetal and prepubertal rabbit testis,

while in AMHR-II transfected CHO-3W cells derived from Chinese hamster ovary, BMPR-IB and AMHR-II have been shown to mediate the effects of AMH via Smad1 (Gouedard et al., 2000). However, in male gonadal cells the role of BMPR-IA in AMH signalling has been demonstrated to predominate. Sertoli cell derived SMAT-1 cells have been shown to express ActR-I, BMPR-IA, BMPR-IB (at a lower level) and AMHR-II, furthermore only BMPR-IA was shown to mediate the actions of AMH while BMPR-IB antagonised this effect (Belville et al., 2005). AMH stimulated signalling by BMPR-IA down-regulated transcription of AMHR-II and P450scc enzyme (Belville et al., 2005). In murines disrupted BMPR-IA expression in the Mullerian duct mesenchyme leads to pseudohermaphrodism similar to mutations of AMHR-II or AMH indicating that AMH exerts its effects on the Mullerian duct regression in male embryos via BMPR-IA and AMHR-II (Jamin et al., 2002). AMH receptor mutant mice develop pseudohermaphroditism identical to the phenotype of both AMH knockout mice and AMH receptor/AMH double mutants indicating that this specific ligand-receptor combination is essential for normal male differentiation, additionally these AMH receptor mutants have seminiferous tubule atrophy and Leydig cell hyperplasia (Mishina et al., 1996).

1.8 BMPs in the Female Reproductive System

It is presently thought that mammalian females are born with a finite number of germ cells (oocytes) which eventually deplete leading to infertility know in humans as menopause. New theories are immerging given the finding that human ovarian surface mesenchymal cells display plasticity and can transform into primitive granulosa cells and germ cells *in vitro* (Bukovsky et al., 2005), presenting a possibility that bipotent ovarian surface epithelium from the tunica albuginea may be

a source of primordial follicles (Bukovsky et al., 2004). Supporting this theory oocyte associated proteins, glycoprotein ZP antigens – heat-solubilized porcine zona protein (Dunbar & Raynor, 1980) and meiotically expressed carbohydrate antigen PS1 have been shown to be present in ovarian surface epithelium of various species including cats, rabbits, cynamolgus monkeys, baboons and humans (Skinner & Dunbar, 1992).

The ovarian surface epithelium is also regulated by TGF-β family signalling and Smad3 knockout in mice resulted in enhanced epithelial growth (Symonds et al., 2003), higher primordial follicle counts, lower primary and antral follicles counts (Tomic et al., 2002), and reduced levels of serum estradiol in mature animals (Symonds et al., 2003). Regardless of which theory is correct, there are several well know endocrine modulators of mature female reproductive function and an emerging role for TGF-βs in the endocrine, paracrine and autocrine signalling of early gonadal development, primordial follicle recruitment and selection of the dominant follicle all taking part in the regulation of the reproductive cycle.

1.8.1 The Hypothalamic – Pituitary – Ovarian Axis

In mammalian females reproductive regulation comprises a complex system of endocrine, paracrine and autocrine signalling in order to achieve a coordinated and functioning reproductive system. The hypothalamic – pituitary – ovarian axis forms a significant part of this endocrine process and is characterised by pulsatile pituitary LH and FSH stimulation of the ovary, and pituitary and hypothalamic modulation by feedback via cyclic ovarian estradiol and progesterone secretion.

Neurons in the hypothalamus secrete gonadotrophin releasing hormone (GnRH) in rhythmic pulses to act on the anterior pituitary to stimulate the release of

FSH and LH from gonadotrophes (Rosen & Cedars, 2004). Slower pulse frequency (90-120 min) is believed to favor the secretion of FSH and pituitary activin (Rosen & Cedars, 2004) which further augments the transcription of the FSH beta subunit (Huang et al., 2001). Developing follicles in the ovary respond to FSH by increasing the production of estradiol, with estradiol acting to increase GnRH pulsatility. Faster pulsatility (60 min) is believed to enhance the secretion of LH and pituitary follistatin (Rosen & Cedars, 2004) which binds activin and reduces its bioavailability. Estradiol then further stimulates pituitary LH release and results in the LH surge which stimulates ovulation to occur (Rosen & Cedars, 2004). Progesterone secretion from the ovulated follicle (CL) then acts to reduce hypothalamic GnRH pulsatility again favoring the secretion of FSH (Rosen & Cedars, 2004), thereby completing a reproductive cycle.

TGF-β family member proteins and their receptors have been found in the pituitary and hypothalamus. BMPR-IA, BMPR-IB and BMPR-II are expressed in the pituitary (however BMPR-IA is more abundant) (Faure et al., 2005). ActR-II and in greater abundance ActR-IIB have been detected in the hypothalamus and a mild expression of both receptors is also found on the anterior pituitary (Cameron et al., 1994). In ewe pituitary cells staining for BMPR-IA and BMPR-II also showed staining for LH, but cells staining for BMPR-IB did not (Faure et al., 2005). Furthermore, BMP-4 and BMP-6 were shown to reduce FSH in 48-hour pituitary culture and activin was able to reverse the effect of BMP-4 (Faure et al., 2005). Conversely, in another study using tissue derived from male and female mice BMP-7 and BMP-6 increased FSH production in oFSHbLuc transgenic mouse gonadotroph derived LβT2 cell culture, while BMP-7 neutralization decreased FSH production in ovine pituitary *in vitro,* however it is unclear whether the ovine pituitary originated from a

male or female animal (Huang et al., 2001) and curiously anti-activin did not significantly reduce FSH production in pituitary culture, which indicates that BMPs may have a predominant role in the regulation of FSH in sheep.

In ovine pituitary cell culture GDF-9, activin A, BMP-2, BMP-4 and BMP-6 were shown to have a suppressive effect on FSH release in Booroola ewes carrying the mutant BMPR-IB gene as compared to wild-type non-carrier ewes (Young et al., 2008), suggesting that mutant BMPR-IB may prematurely favour the secretion of LH and contribute to the hyperprolific phenotype of mutant BMPR-IB carriers.

1.8.2 Follicular Development

Follicular development is the process whereby a dormant primordial follicle becomes activated and develops progressively into a primary, secondary and then into an antral ovulatory or atretic follicle. Follicle atresia can occur at any stage of development resulting in only a few primordial follicles recruited every reproductive cycle to mature into antral follicles (Peters et al., 1975). Ovarian cells communicate with each other by secreting factors that regulate follicular development, steroidogenesis and reproductive function. In recent decades it has been observed that many TGF-βs and their antagonists act in an autocrine and paracrine manner in many ovarian cells to regulate the process of ovarian development (Baarends et al., 1995; Shimasaki et al., 1999; Durlinger et al., 2001; Lee et al., 2001b; Pierre et al., 2005; Davis & Camper, 2007). The current understanding of the initiation of follicular recruitment and dominant follicle selection are still poorly understood but roles for TGF-βs in this area are being identified.

1.8.2.1 TGF-βs Involved in Follicular Development

TGF-β superfamily and BMP receptors are found extensively in the ovaries and other reproductive tissues suggesting that endocrine, paracrine and autocrine BMP signalling has a major role in reproductive functions (Erickson & Shimasaki, 2003). Paracrine and autocrine growth factor signalling between oocytes, granulosa cells and theca cells regulate oocyte growth and granulosa and theca cell proliferation and differentiation from the primordial to antral follicle stages (Dong et al., 1996; Erickson & Shimasaki, 2003; Nilsson & Skinner, 2003; Tanwar et al., 2008). In granulosa cell culture FSH reduced expression of BMPR-IB, BMPR-II and ALK-5, while in combination with estradiol it significantly increased these receptors (Chen et al., 2009), indicating regulation via gonadotrophins and steroids.

1.8.2.2 Primordial to Primary Follicle Transition

Primordial follicles are typically round or ovoid and composed of an oocyte surrounded by one layer of squamous granulosa cells with no zona pellucida proteins between the oocyte and granulosa cells. One known exception is bovine primordial follicles which have been shown to have some cuboidal pre-granulosa cells, however zona pellucida proteins remain absent (van Wezel & Rodgers, 1996). Primary follicles are characterized by the formation of a thick layer of glycoprotein known as the zona pellucida and the transformation of cuboidal granulosa cells from squamous granulosa cells (van Wezel & Rodgers, 1996).

In vivo BMP-4 neutralization caused smaller ovaries containing greater numbers of primordial follicles and proportionally less numbers of primary follicles, which indicates that BMP-4 is an important factor for primordial follicle recruitment in mice (Tanwar et al., 2008), and similarly in ovary culture BMP-4 was shown to

promote the development of primary follicles from primordial follicles, while immunization against BMP-4 resulted in morphologically disorganized ovarian tissue and smaller ovaries associated with increased apoptosis and loss of primordial follicles and oocytes in neonatal rats (Nilsson & Skinner, 2003). BMP-4 was found in theca cell precursor stromal cells of primordial follicles as well as in the follicle basement membrane (Nilsson & Skinner, 2003).

In ovarian organ culture BMP-4 was not shown to effect Kit Ligand (KL) or oocyte derived basic fibroblast growth factor expression (Nilsson & Skinner, 2003) known to enhance granulosa cell, theca cell and ovarian stromal cell proliferation in primordial follicles (Lavranos et al., 1994; Roberts & Ellis, 1999; Nilsson et al., 2001). Leukemia inhibitory factor found in pre-granulosa cells up-regulates the expression of KL, also found on these cells (Parrott & Skinner, 1997) and promotes development of primordial follicles (Nilsson et al., 2002). *In vivo* BMP-7 treatment of rat ovaries promotes the primordial to preantral follicle transition in rats (Lee et al., 2001b) and in immature mouse 4 day ovarian culture (Lee et al., 2004).

In rats BMP-15 mRNA is first detected in low amounts in the oocyte in primary follicles when the squamous granulosa cells undergo morphological change into cuboidal granulosa cells (Erickson & Shimasaki, 2003). In rat oocyte-granulosa cell culture, oocyte derived BMP-15 stimulated KL expression from granulosa cells which then binds c Kit its receptor found on oocytes and down-regulates BMP-15 expression forming a negative feedback loop, additionally as KL enhanced BMP-15 induced granulosa cell mitosis and proliferation in the presence of oocytes, it is suspected that KL is necessary to regulate granulosa cell proliferation by BMP-15 (Otsuka & Shimasaki, 2002).

In mice AMH and the AMHR-II are increasingly synthesized by granulosa cells

from the primary to tertiary stages (Baarends et al., 1995), and AMH knockout in mice causes a three times faster depletion of primordial follicles providing evidence that AMH inhibits follicle transition from primordial to primary stages (Durlinger et al., 1999). AMH knockout mice at 4 months had ovarian weights double that of the control, had more preantral and antral follicles, higher inhibin α subunit levels and lower FSH levels, while at 13 months the inhibin α subunit levels were lower and the FSH levels were higher and accordingly mutant mice contained little or no primordial follicles (Durlinger et al., 1999).

BMPR-IA is weakly expressed in primordial follicle ooctyes and granulosa cells but increases its expression in primary follicles, whereas BMPR-IB mRNA is present in oocytes of both primordial and primary follicles (Erickson & Shimasaki, 2003), indicating that BMPR-IB may have a role in follicle recruitment. BMPR-II is weakly expressed in ooctyes in all stages of development and first appears in granulosa cells at a low level in primary follicles (Erickson & Shimasaki, 2003). BMP receptors are expressed before other known BMPs (Erickson & Shimasaki, 2003) and are believed to facilitate the effects of both primary and secondary follicle derived BMP-2, BMP-4, BMP-6, BMP-7 and AMH (Shimasaki et al., 1999; Nilsson & Skinner, 2003) on primordial follicle granulosa cell development.

1.8.2.3 Primary to Secondary Follicle Transition

Proliferation of granulosa cells and the acquisition of theca cells occurs at the secondary follicle stage (Rosen & Cedars, 2004). With the acquisition of theca cells follicles become vascularized and gain FSH, androgen and estrogen receptors (Rosen & Cedars, 2004).

As in rat primary follicles, bovine granulosa cells secrete KL while its receptor

c Kit is found on both ooctyes and theca cells (Parrott & Skinner, 1997). In rats KL has been shown to be important for initiating the recruitment and proliferation of theca cells (Parrott & Skinner, 1999) and therefore the progression of the primary to secondary follicle stage. KL may suppress BMP-15 but potentially not its closest relative GDF-9 owing to its higher abundance in human granulosa cells (Aaltonen et al., 1999). BMP-15 is highly expressed in rat secondary follicles when the third layer of granulosa cells had developed (Erickson & Shimasaki, 2003). GDF-9 released from the oocyte enhances primary follicle development (Nilsson & Skinner, 2002) and *in vivo* treatment of recombinant GDF-9 in immature rats enhanced both primary and antral follicle development (Vitt et al., 2000; Vitt & Hsueh, 2001). In mice GDF-9 is also synthesised by oocytes at the primary follicle stage through to ovulation (Dong et al., 1996). The ovarian follicles of GDF-9 knockout mice arrest at the primary stage and these mice are infertile due to a lack of granulosa cell proliferation, illustrating that GDF-9 is required for granulosa cell development (Dong et al., 1996) and therefore the primary to secondary transition.

Oocyte derived GDF-9 down-regulates BMP-4 signalling in granulosa cell culture in mice (Pangas et al., 2004). In rats during the transition of primary to secondary follicle stages, BMP-7 is expressed from the inner layer of theca cells bordering granulosa cells, while BMP-4 was found at its highest expression in the theca externa (Erickson & Shimasaki, 2003). BMP-4 has been shown to stimulate primordial to primary follicle transition in mice (Tanwar et al., 2008) and hence its suppression by GDF-9 may both suppress follicle recruitment and promote selected follicle maturation. BMPR-II and ALK-5 are known receptors for GDF-9 demonstrated *in vitro* using granulosa cells from 25 day old rats (Mazerbourg et al., 2004).

In rats BMP-2 mRNA was detected in granulosa cells and BMP-6 mRNA in oocytes and granulosa cells of secondary follicles onwards (Erickson & Shimasaki, 2003). BMP-6 was shown to inhibit the proliferative effects of FSH in granulosa cells (Otsuka et al., 2001a) suggesting that it decreases sensitivity to FSH. These inhibitory actions may be important to prevent excessive follicle selection as secondary follicles are very sensitive to FSH stimulation. Additionally, BMP-2 up-regulates follistatin an inhibitor of BMP-2 in osteoblasts (Yukiko et al., 2004). Bmp-2 may stimulate high follistatin levels in selected follicles and correspondingly BMP-2 is first expressed in primary granulosa cells whereas follistatin is first expressed in secondary granulosa cells (Erickson & Shimasaki, 2003). BMP-2 and BMP-6 are likely to be negative regulators of follicle selection.

In immature rats at 20 days AMH mRNA was detected mainly in the peripheral areas of the ovary, while the AMHR-II mRNA was localized in the ovarian medulla (Baarends et al., 1995). This indicates that AMH produced by recruited follicles exerts its effects on preantral and early antral follicles which have migrated towards the medulla and accordingly AMH and AMHR-II are expressed in granulosa cells of pre-antral to early antral follicles in mature rats (Baarends et al., 1995). *In vitro* AMH inhibits FSH-stimulated follicle growth in mice by inhibiting granulosa cell proliferation (Durlinger et al., 2001) and in doing so helps control the numbers of follicles destined for ovulation in tandem with other inhibitors of FSH-stimulated follicle growth and steroidogenesis such as BMP-6 and BMP-2.

BMPR-IA and BMPR-IB are abundantly expressed in primary to secondary follicules in oocytes, granulosa cells and theca cells, while BMPR-II is found mainly in granulosa cells and at a low level in ooctyes (Erickson & Shimasaki, 2003)

indicating that classical BMP signalling involving type I and type II BMP receptor formation is most likely to occur in granulosa cells and oocytes.

1.8.2.4 Secondary to Antral Follicle Transition

Tertiary follicles develop an antrum filled with follicular fluid, theca cells form distinct subpopulations, with the theca interna gaining LH receptors and becoming steroidogenic; granulosa cells also become steroidogenic and differentiate into populations of radiata, cumulus oophorus, periantral and membrana granulosa cells (Rosen & Cedars, 2004).

In bovines BMP-2 and BMP-4 were found in the theca interna and some oocytes of antral follicles (Fatehi et al., 2005). In rats BMP-2 is increasingly expressed exclusively in the granulosa cell membrana layer of primary through ovulatory and/or atretic follicles while theca derived BMP-4 drastically down-regulates in atretic follicles (Erickson & Shimasaki, 2003), indicating that BMP-2 and BMP-4 may have opposing roles.

The expression of BMP-6 in membrana granulosa cells is severely down-regulated in the dominant follicle and up-regulated in atretic oocytes, suggesting that BMP-6 inhibits granulosa cell survival (Erickson & Shimasaki, 2003). BMP-2 and BMP-6 are maximally expressed in atretic granulosa cells and according follistatin is highly down-regulated in atretic granulosa cells, while being up-regulated in the dominant follicle (Erickson & Shimasaki, 2003).

In dominant follicles BMP-3b was shown to be strongly expressed in theca interstitial cells (in close proximity to the theca externa cells) and theca externa cells while being almost undetectable in atretic follicles, very similar to the profiles of BMP-4 and BMP-7 for which receptors of these BMPs are found on granulosa cells

(Erickson & Shimasaki, 2003). In small antral follicle culture from rats BMP-7 increased granulosa cell proliferation (Lee et al., 2001b), and in granulosa cell culture it increased estrogen production, while suppressing FSH-induced progesterone secretion (Shimasaki et al., 1999; Lee et al., 2001b). BMP-7 might therefore be implicated in follicle growth and the inhibition of premature selection as well as augmenting estradiol by default and thereby contributing to the LH surge from the pituitary.

BMP-15 is highly expressed in oocytes until ovulation in rats, while follicle atresia causes it to decrease (Erickson & Shimasaki, 2003), which can be expected as BMP-15 enhances granulosa cell proliferation.

AMH has been demonstrated to reduce responsiveness to FSH in preantral and small antral follicles (Baarends et al., 1995; Durlinger et al., 2001). AMH expression is variable among developing follicles and this may have significance in the recruitment of dominant follicles (Baarends et al., 1995). The expression of AMH and AMHR-II decreased to undetectable levels in atretic follicles and it also decreased at estrus in rats (Baarends et al., 1995). This suggests that high FSH levels down-regulate AMH. AMH produced from developing follicles is likely to regulate early antral and antral follicles until ovulation or atresia suppressing dominant follicle development and hence its down-regulation in ovulatory follicles.

Bovine and human AMH inhibited meiosis of rat cumulus cell enclosed and denuded oocytes, however when AMH was homogenously purified it had no significant effect (Takahashi et al., 1986; Ueno et al., 1988). Using oocytes of 25 to 26 day old rats treated with PMSG to induce follicle maturation, immunopurified AMH again was not shown to have a significant effect on meiosis (Tsafriri et al., 1988). In mice meiosis was also not inhibited *in vitro* (Takahashi et al., 1986). It is

possible that glycoslyations transmit the biological activity of AMH being a glycoprotein and that these were modified or eliminated during purification thereby losing their effect presenting the possibility that the activity of other TGF-βs may also have their signalling properties and clearance times altered by glycosylations. Also, other growth factors such as epidermal growth factor (EGF) have been demonstrated to inhibit the inhibitory action of AMH on oocytes thereby allowing meiosis to resume (Ueno et al., 1988) and it is for this purpose that AMH may be down-regulated in late antral follicles.

In rat ovary BMPR-IA was expressed in theca cells of tertiary follicles and its highest levels were detected in the oocytes of secondary to tertiary follicles, while being reduced in dominant follicles and increased in atretic follicles (Erickson & Shimasaki, 2003). If AMH signals via BMPR-IA, as has been demonstrated in amniotes (Jamin et al., 2002) and in Sertoli cell lines (Belville et al., 2005), then it is possible that the effects of AMH will be reduced with the down-regulation of BMPR-IA, furthermore the maintenance of BMPR-IB may facilitate BMP-2-stimulated follistatin synthesis. In the dominant follicle follistatin and BMP-6 have an inverse relationship (Erickson & Shimasaki, 2003), indicating follistatin may have a greater inhibitory effect on BMP-6 then BMP-2.

In dominant follicles BMPR-IB was expressed in theca cells at pro-estrus, and expressed in high levels in granulosa cells as well as oocytes of both dominant and atretic follicles, however following ovulation BMPR-IB was drastically decreased (Erickson & Shimasaki, 2003). This supports an in-expendable role for BMPR-IB, additionally BMPR-IB knockout in mice leads to decreased aromatase synthesis by granulosa cells as cumulus cell expansion fails to develop, which indicates that

signalling via this receptor is required for cell mitosis and steroidogenesis (Yi et al., 2001).

Bovine oocytes and granulosa cells of preantral and antral follicles express BMPR-II (Fatehi et al., 2005). High levels of BMPR-II expression was detected in granulosa cells of dominant and atretic follicles (Erickson & Shimasaki, 2003). BMPR-II is also expressed in all ovine oocytes and granulosa cells of secondary follicles onwards, as well as in the CL, indicating that BMP receptors may serve similar functions across bovines, ovines and murines (Wilson et al., 2001).

FSH is required in females for secondary to tertiary follicle development as demonstrated by FSH deficient mice which have follicles that arrest at the secondary preantral stage (Kumar et al., 1997). Granulosa cells of antral follicles secrete activin which enhances the expression of FSH receptor and stimulates follicular growth (Kumar et al., 1997). Activin A and activin B are homodimers of the βA and βB subunits respectively, whereas activin AB is a heterodimer (Rosen & Cedars, 2004).

Consisting of the same β subunits as activin and different α subunits, inhibin A (α, βA) and inhibin B (α, βB) levels fluctuate throughout the menstrual cycle, of which inhibin B peaks at ovulation while inhibin A levels are elevated in the luteal phase (Rosen & Cedars, 2004), indicating that activins have an autocrine and/or paracrine role while inhibins also have an endocrine role in reproductive regulation. In support of this theory activin βA null mice have less preovulatory follicles, reduced litters and smaller pups (Brown et al., 2000), while activin βB knockout have delayed deliveries, increased litter sizes with pups dieing perinatally, which incidentally also suggests subunit dependant highly specific roles for activin (Vassalli et al., 1994). ActR-IIA knockout mice have reduced FSH secretion and ovarian defects with

follicular arrest at the early secondary stage similar to FSH deficiency (Matzuk et al., 1995). This strongly suggests that FSH and activin have reciprocal roles in antral follicle development. The defects caused by ActR-II knockout were inconsistent with the facial defects occurring in activin knockout suggesting that different ligands have a significant role in ActR-II activation (Matzuk et al., 1995). Activin also appears to have a significant role in the timing of gestation and parturition (O'Connor et al., 1999).

In 4 day preantral follicle culture FSH was shown to have no effect on preantral follicle growth in immature mice while the addition of activin caused an increase by itself and in combination with FSH synergically increased follicle growth (Liu et al., 1998a). In 10 day old rat ovary culture activin A also stimulates preantral follicles to develop onto early antral follicles (Zhao et al., 2001b). In immature mice activin A stimulates antral follicle development, while in mature mice activin A has been shown to inhibit follicle growth (Yokota et al., 1997). In mature mice *in vitro* activin inhibited the effect of FSH on preantral follicles and is believed to lead to dormancy of primary and early preantral follicles (Mizunuma et al., 1999), furthermore in 25 day old rats the administration of activin A caused a reduction in granulosa cell development and resulted in follicular atresia (Woodruff et al., 1990). This indicates a shift in cellular reactions to activin suggesting altered signalling profiles in immature and mature animals which can be anticipated due to the differential developmentally regulated reproductive requirements of immature and mature animals. In immature mice follistatin was shown to inhibit basal follicular growth as well as inhibiting the mitogenic effects of activin and recombinant human growth hormone on follicle development in 4 day preantral follicle culture (Liu et al., 1998a).

TGF-β1 is not shown to effect preantral follicles from immature mice while stimulating preantral development in mature mice, (the opposite being observed for activin A) however, in conjunction with FSH it stimulates both immature and mature preantral mouse follicles *in vitro* (Liu et al., 1999). TGF-β1 and TGFR-II knockout is embryonically lethal (Dickson et al., 1995; Oshima et al., 1996) and TGF-β2 and TGF-β3 knockout are lethal at or shortly after birth (Kaartinen et al., 1995; Sanford et al., 1997).

1.8.3 BMPs in Other Female Reproductive Tissues

High levels of BMP-6 were expressed in the endothelial cells of blood vessels in the ovarian medulla, while BMP-3b, BMP-4, BMP-7 and BMPR-IB were shown to be expressed in rat oviducts (Erickson & Shimasaki, 2003). BMPR-IB was localized in arterial tunica adventia (Erickson & Shimasaki, 2003), and is also expressed in mouse uterus (Yi et al., 2001), suggesting that signalling via this receptor is important in uterine function, and correspondingly several TGF-β ligands have been located in this tissue. BMP-6 is expressed in the uterus of normal cycling mice and the placenta of pregnant mice (Lyons et al., 1989a), BMP-3b is also found in murine uterus (Cunningham et al., 1995), while not being expressed in the placenta (Zhao et al., 1999). In mice the expression of BMP-3b increases following conception, peaks in the first week post conception and then decreases until parturition after which levels increase again (Zhao et al., 1999). BMP-3b is expressed in the stromal cell types not in the uterine epithelium and in normal cycling mice BMP-3b is elevated during early pro-estrus, decreased at estrus and again elevated at di-estrus (Zhao et al., 1999), indicating a possible endocrine component to its regulation.

Many TGF-β family members have been found in the secretory endometrium

of humans including GDF-5, GDF-8, GDF-11, BMP-4 and BMP-7 while BMP-2 and TGF-β were shown to enhance the decidualization process *in vitro* (Stoikos et al., 2008) and similarly in murines BMP-2 is vital for the uterine decidual response (Lee et al., 2007).

1.8.4 Ovarian Steroidogenesis

The main source of ovarian hormones are the developing follicles and the CL (Rosen & Cedars, 2004). At the secondary follicle stage the follicles become overtly responsive to pituitary LH and FSH (Rosen & Cedars, 2004). Theca cells respond to FSH and LH by enhancing the production of low density lipoprotein (LDL) receptors present on the cell surface (Rosen & Cedars, 2004). Upon entering the cell LDL undergo lysosomal degradation resulting in the liberation of free cholesterol (Rosen & Cedars, 2004). Steroidogenic acute regulatory protein (StAR) then translocates the cholesterol into the mitochondria where cytochrome P450scc (CYP11) synthesizes pregnenolone from cholesterol (Rosen & Cedars, 2004). Theca interstitial cells convert pregnenolone into dehydroepiandrosterone via 7α-hydroxylase (CYP17) which in turn is converted into androstenedione via 3β-hydroxysteroid dehydrogenase (3β-HSD). Granulosa cells then convert androstenedione into estrone via aromatase (CYP19) and estrone into estradiol via 17β-hyroxysteroid dehydrogenase (17β-HSD) not found in theca cells (Rosen & Cedars, 2004). After ovulation the follicle develops into the CL which is stimulated by pituitary LH to up-regulate CYP11 and 3β-HSD and thereby progesterone production, progesterone being vital for early embryonic life (Rosen & Cedars, 2004). The enzymatic pathways to steroid sythesis in both the male and female gonad are illustrated in Figure 1.3.

In sheep granulosa cell culture the addition of BMP-2 enhances estradiol production while not affecting granulosa cell proliferation (Souza et al., 2002). In 24 hour human granulosa luteal cell culture, BMP-2 enhances Smad6 expression and inhibin β subunit expression (Jaatinen et al., 2002). In the CL BMP-2 was not expressed during the luteinization process, however it appeared in groups of endothelial cells during luteolysis (Erickson & Shimasaki, 2003), suggesting that BMP-2 may act to down-regulate progesterone production by favoring estradiol synthesis.

In humans BMP-3 is expressed in granulosa cells which respond to hCG by down-regulating synthesis *in vitro* (Jaatinen et al., 1996). Contrastingly in rats BMP-3 is only minimally expressed in theca cells, while murine expressed granulosa cell BMP-2 (Erickson & Shimasaki, 2003) was not shown to be expressed in granulosa cells of humans (Jaatinen et al., 1996). This suggests that BMP-3 is responsive to endocrine regulation, however its reproductive function remains largely unknown and notably there are species differences to be clarified. In rats moderate amounts of BMP-3b were expressed in the theca lutein cells during the luteinizing process but not during luteuolysis (Erickson & Shimasaki, 2003), indicating a possible role in the maintenance of the CL.

HOTT cells (human theca-like tumor cells) express BMPR-IA, BMPR-IB and BMPR-II and respond to BMP-4 treatment by significantly down-regulating forskolin-stimulated CYP17 production, inhibiting androstenedione and stimulating the production of progesterone, similar to the effects of activin in these cells (Dooley et al., 2000). BMP-4 and BMP-6 are highly expressed in theca externa cells of the CL during luteinization and luteolysis in rats (Erickson & Shimasaki, 2003). In rat granulosa cell culture BMP-6 inhibited FSH-stimulated progesterone production

while having no affect on progesterone or estradiol in unstimulated cells (Otsuka et al., 2001a). BMP-6 suppresses the production of androstenedione and progesterone in 6 day porcine granulosa/theca cell culture while stimulating theca cell proliferation (Brankin et al., 2005). This implicates that BMP-4 has a major role in progesterone production, while BMP-6 seems to have an inhibitory function in steroidogenesis, however BMPR-IA is more abundant during luetinization while BMPR-IB is more abundant during lueteolysis (Erickson & Shimasaki, 2003). It is possible that the receptor of preference for BMP-4 is BMPR-IA, while the inhibitory BMP-6 may have a preferential signalling through BMPR-IB as may be the case with inhibitory BMP-2.

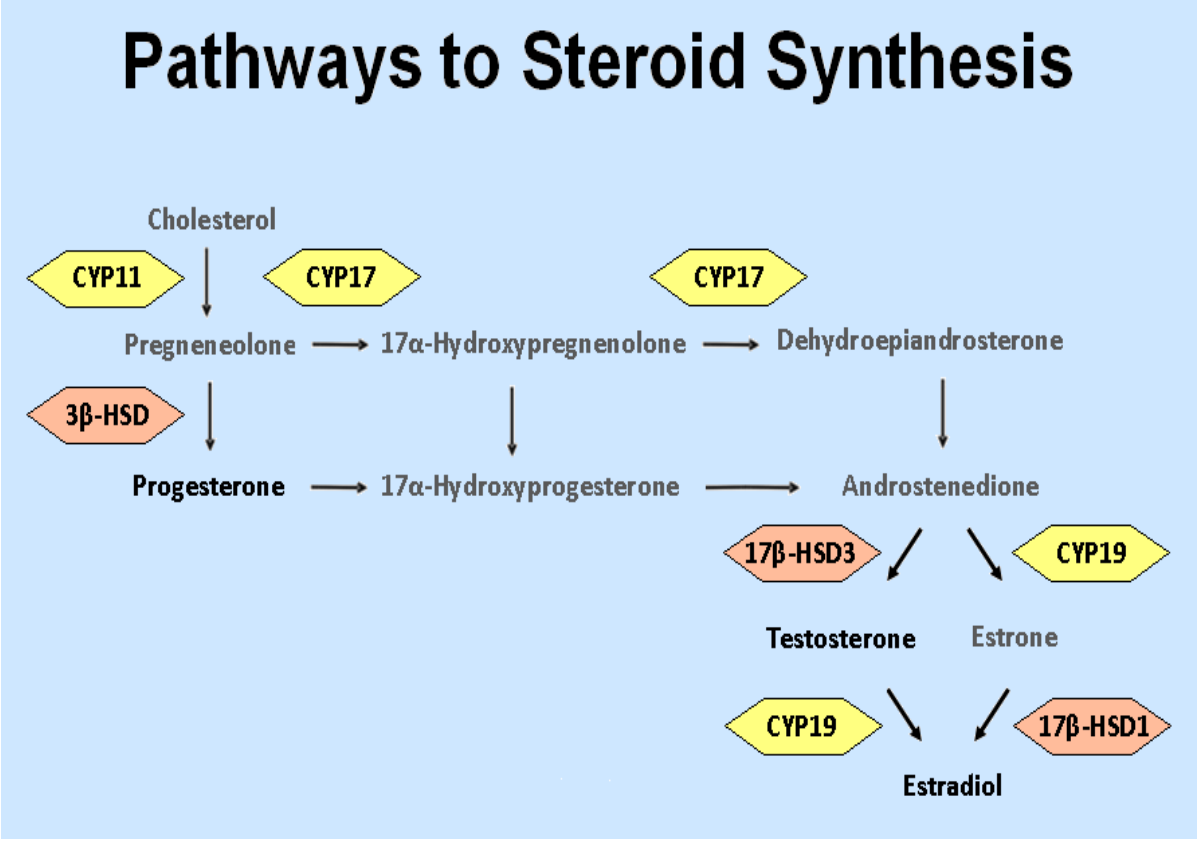


Figure 1.3 Enzymatic pathways to steroid synthesis in the male and female gonad. Cytochrome P450scc (CYP11), 7α-hydroxylase (CYP17) 3β-hydroxysteroid dehydrogenase (3β-HSD), aromatase (CYP19), 17β-hyroxysteroid dehydrogenase (17β-HSD).

In rats BMP-7 increases granulosa proliferation, while suppressing FSH-induced progesterone production by having a suppressive effect on StAR while enhancing P450 aromatase enzyme activity leading to an increase in estradiol production *in vitro* (Lee et al., 2001b). BMP-7 is mildly expressed in the CL and decreases to undetectable levels during luteolysis. The function of BMP-7 may be to suppress progesterone production, while maintaining the CL as a structure.

The oocytes of mice secrete GDF-9 and BMP-15 (Dube et al., 1998; Laitinen et al., 1998) as do human oocytes (Aaltonen et al., 1999; Wu et al., 2007). BMP-15 significantly decreased FSH-stimulated expression of both inhibin and activin subunits (Otsuka et al., 2001b). In human follicular fluid higher levels of BMP-15 were demonstrated to predict higher oocyte quality and blastomere formation which was associated with increased estradiol and decreased FSH (Wu et al., 2007). BMP-15 mRNA is detected in human oocytes of late primary follicles (Aaltonen et al., 1999). In rats BMP-15 has been shown to stimulate the proliferation of granulosa cells independent of FSH, and decrease gransulosa cell FSH-stimulated progesterone production while not affecting estradiol secretion; BMP-15 also decreased FSH-stimulated expression of StAR, P450scc which cleaves cholesterol to yield pregnenolone in theca cells, 3β-HSD which coverts pregnenolone to progesterone in granulosa cells (Otsuka et al., 2000) and expression of the LH receptor in basal and FSH-stimulated states (Otsuka et al., 2001b)

In fetal organ culture of rats, rabbits and sheep AMH decreased granulosa cell aromatase biosynthesis (Vigier et al., 1989), and number of LH receptors in FSH-stimulated immature rat and porcine granulosa cells (Di Clemente et al., 1994), contrasting the role of activin A in immature animals. AMH inhibits cell proliferation and progesterone production in cultured human granulosa/luteal cells after 4 days of

treatment as well as inhibiting the stimulatory effect of EGF on cell proliferation and progesterone synthesis (Kim et al., 1992). Furthermore, treatment with the GnRH-analog leuprolide acetate was shown to reduce AMH 14 times, increase androstenedione 3 times and testosterone by 1.5 times in human granulosa cell culture (Seifer et al., 1993). This indicates that granulosa cell steroidogenesis may be indirectly regulated by hypothalamic feedback via the modulation of AMH.

1.9 BMPs in the Male Reproductive System

In males the reproductive process is typically believed to be simpler than in females however, it is also becoming evident that male reproductive function is tightly regulated by endocrine, paracine and autocrine factors. The main structures involved in the male reproductive system include the testis, epididymis, vas deferens, seminal vesicles, anterior prostate, coagulating gland (dorsolateral prostate) and the urethra as shown in Figure 1.4, which illustrates the reproductive organs of a male mouse. The testis is comprised of seminiferous tubules which are formed from Sertoli cells that surround germ cells and Leydig or interstitial cells that produce testosterone and other hormones necessary for normal male reproductive development (Braunstein, 2004).

In immature mouse testis the expression of BMP activated signalling molecules Smad1, 4, 5, 6, 7 and 8 is extensive while in mature mice this expression becomes increasingly specific to particular cell types illustrating differential developmental regulation (Itman & Loveland, 2008) and this strongly suggests major roles for TGF-βs in male germ cell development, steroidogenesis and reproduction. BMP-6 has been expressed in mature mouse testis (Lyons et al., 1989a) and both BMP-8a and BMP-8b have been detected in spermatogonia, spermatocytes and

spermatids in mice (Zhao & Hogan, 1996; Zhao et al., 1998). Furthermore, in murine male germ cells Smad6 was shown to be variably expressed when stimulated by BMP-2 or BMP-4 dependant on the level of cell differentiation (Itman & Loveland, 2008), indicating that the same hormone may have a different effect in cells based on their level of development and/or that different BMPs signal via different combinations of Smads using the same receptor.

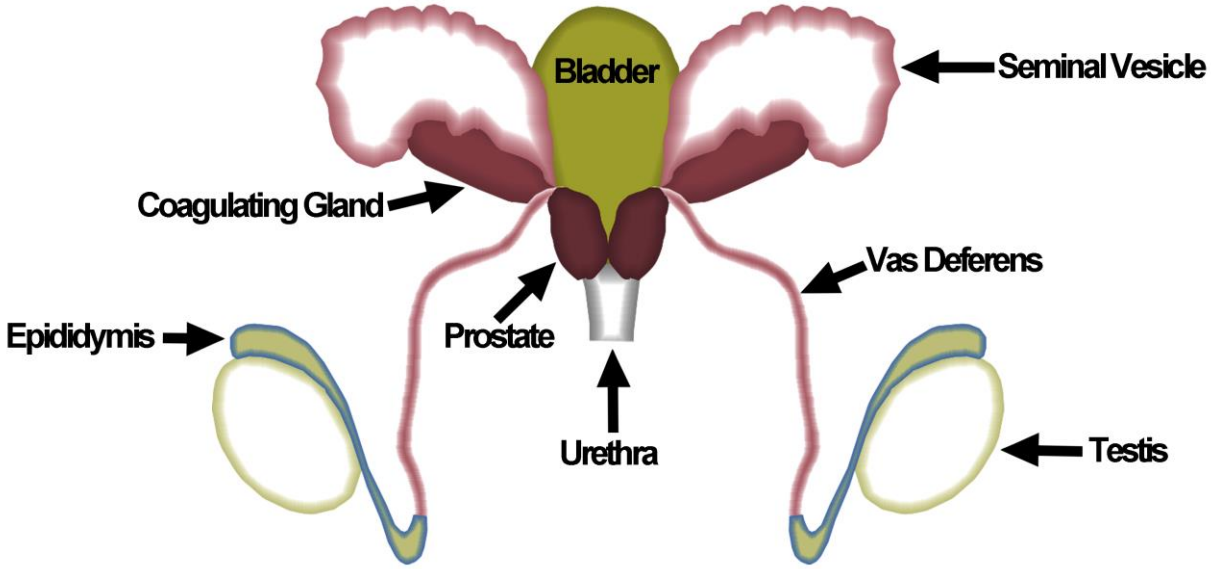


Figure 1.4 The male mouse reporoductive track

BMP receptors are present in male reproductive tissues and BMPR-II has been expressed in adult testis (Rosenzweig et al., 1995), furthermore BMPR-IB and AMHR-II mRNAs have been visualized in fetal and prepubertal rabbit testis (Gouedard et al., 2000). AMH and AMHR-II are found on the immature mouse Sertoli cell line SMAT-1 and AMH can stimulate signalling by Smad1 in ½ hour, Smad4 in 1 hour and the inhibitory Smad6 in 4 hours, similar to the effects of BMP-2 indicating possible regulation by negative feedback, however unlike BMP-2 AMH

was not shown to activate Wnt or NF-kB in SMAT-1 cells (Belville et al., 2005). ActR-IIA was shown to be highly expressed in round spermatids but not in spermatogonia, while ActR-IIB was exclusively expressed in Leydig cells (Cameron et al., 1994) indicating that ActR-IIB probably facilitates the action of activin and other ligands in cellular mitosis and steroidogenic regulation.

Multiple endocrine neoplasia type 1 Leydig cell tumors in mice result in severely down-regulated expression of AMHR-II and Smad1, 5, 3 and 4 (Hussein et al., 2008), indicating that this receptor and its signalling elements are necessary for regulated cell function.

1.9.1 The Hypothalamic – Pituitary – Testicular Axis

In males hypothalamic GnRH is released rhythmically every 1½ - 2 hours to stimulate the release of LH and FSH from the pituitary gonadotrophs. AMH and AMHR-II is found in the pituitary gonadotroph derived LβT2 mouse cell line and in rat pituitary (Bedecarrats et al., 2003). In LβT2 cells AMH has been shown to stimulate FSHβ subunit, FSHβ luciferase promoter and LHβ luciferase promoter genes where it also augmented the effects of GnRH on FSHβ subunit synthesis and synergically increased LHβ luciferase promoter expression (Bedecarrats et al., 2003), indicating that AMH has a role in the synthesis of FSH and LH from the pituitary. LH binds Leydig cell G protein receptors and stimulates testosterone synthesis, while elevated steroid levels then act to inhibit hypothalamic GnRH and pituitary LH release.

FSH is believed to be necessary for the initiation of spermatogenesis and Sertoli cells respond to FSH and/or testosterone by up-regulating androgen-binding proteins on the cell surface which facilitates high testosterone levels required for

germ cell development (Braunstein, 2004). However, transgenic FSH deficient male mice exhibit smaller testis but remain viable (Kumar et al., 1997), indicating that the action of FSH may be carried out in part via the actions of other transforming growth factors and their receptors. *ActR-IIA* is highly expressed in round spermatids in mice (Cameron et al., 1994) and may have a role in pituitary FSH modulation as *ActR-IIA* knockout mice have reduced FSH secretion and small testicular size (Matzuk et al., 1995) similar to FSH mutant mice.

Sertoli cells are also known to secrete inhibin A and inhibin B of which both can inhibit FSH release while having no effect on LH secretion, thereby supporting a high level of steroid production by Leydig cells. In addition inhibin B and FSH levels are found to be reciprocal in serum (Braunstein, 2004) and correspondingly inhibin expression is up-regulated in FSH-stimulated rat testis tissue.

Thereby, through glycoprotein, androgen and growth factor feedback mechanisms between the hypothalamus, the pituitary and the testis steroid production and consequently spermatogenesis is a highly regulated process. The hypothalamic – pituitary – testicular axis and some of its feedback mechanisms are summarized in Figure 1.5.

1.9.2 Spermatogenesis

Seminiferous tubules are formed from the tight junction of Sertoli cells with each other and the basement membrane forming the blood-testis barrier (Braunstein, 2004). The Sertoli cells, also called nurse cells, surround the germ cells, phargocytose residual bodies and damaged germ cells and provide an environment required for the transport and development of germ cells in their sequential progression from spermatogonia, primary-secondary spermatocytes,

round-elongating spermatids to immature spermatozoa (Braunstein, 2004). During this progression germ cells undergo mitosis and meiosis in their transition as illustrated in Figure 1.6.

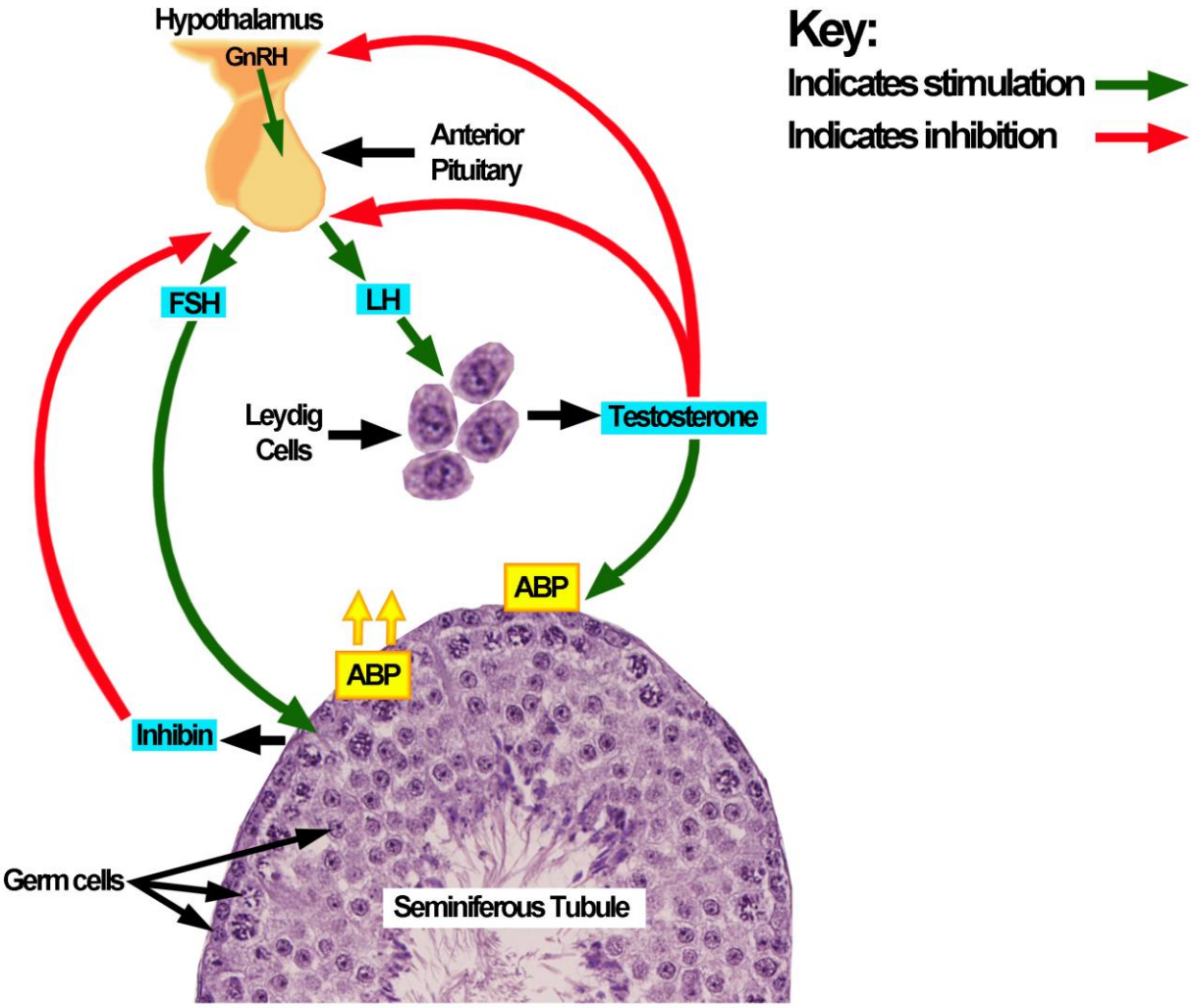


Figure 1.5 Schematic representation of the hypothalamic-pituitary-testicular axis and feedback mechanisms. Green arrows indicate stimulation and red arrows indicate inhibition.

ActR-IIA has been demonstrated to be highly expressed in round spermatids but not in spermatogonia and is likely to contribute to the progression of spermatids to immature spermatozoa (Cameron et al., 1994). In mice before 3 weeks of age low expression of *Bmp8a* and *Bmp8b* was detected in spermatogonia and

spermatocytes (Zhao et al., 1998) and in 3 week old mice *Bmp8a* and *Bmp8b* transcripts were localized in stage 6-8 round spermatids, demonstrating a development shift (Zhao & Hogan, 1996). In the initiation phase of spermatogenesis *Bmp8a* was expressed at a much lower level than *Bmp8b* and correspondingly *Bmp8b* homozygous mutants had greater germ cell degeneration, additionally homozygous Bmp-8b^{tm1b1h} mutant mice exhibited progressive depletion of germ cells due to increased germ cell apoptosis and were rendered infertile (Zhao et al., 1996; Zhao et al., 1998), indicating a function in germ cell survival and maintenance.

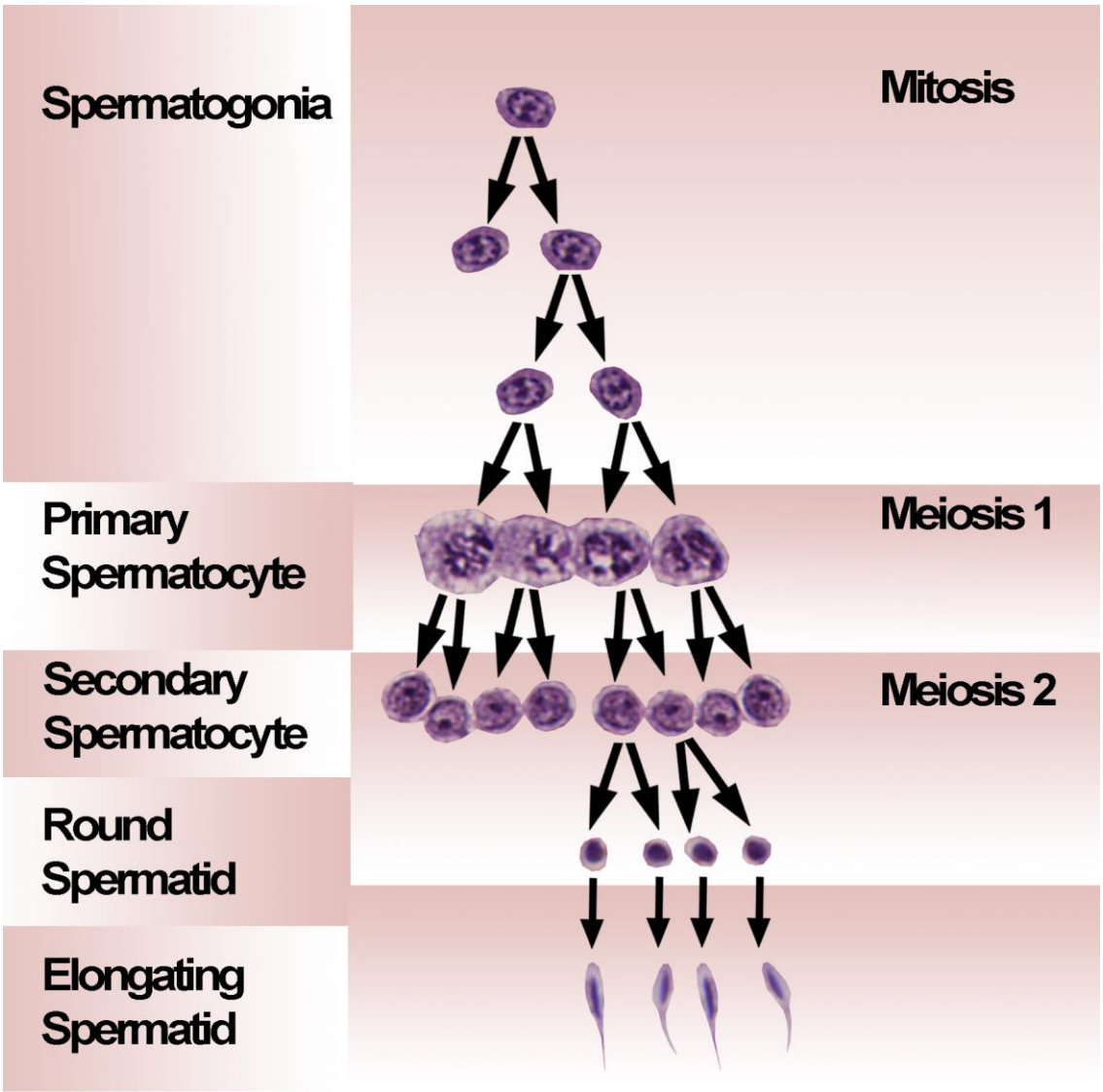


Figure 1.6 Stages of spermatogenesis as germ cells undergo mitosis and meiosis.

1.9.3 Leydig Cell Steroidogenesis

Leydig cells produce the steroids dehydrotestosterone, testosterone and estradiol necessary for germ cell development (Braunstein, 2004) via the steroidogenic enzymes CYP11, CPY17, 3β-HSD, 17β-HSD and CYP19 as shown in Figure 1.3. Leydig cells also produce small amounts of other androgens, estrogens and progestin s such as dehydroepiandrosterone, androstenedione, estrone, pregnenolone, progesterone, 17α-hydroxypregnenolone and 17α-hydroxyprogesterone. A small amount of circulating steroids also comes from the adrenal gland. In circulation albumin binds approximately 38 % of testosterone, while sex hormone-binding globulin binds approximately 60 % reducing the amount of bioactive hormone in circulation while providing high levels of unbound ligand in the testis. Unbound testosterone and dehydrotestosterone transverse the cell membrane and bind the nuclear androgen receptor to effect transcription (Braunstein, 2004).

There is a growing body of evidence that some TGF-β family members can alter or be altered by androgens such as testosterone (Teixeira et al., 1999). Rat AMHR-II was visualized in Leydig cells and murine derived MA-10 and R2C Leydig cell lines (Teixeira et al., 1999). AMH is believed to signal via its receptors found on Leydig cells which respond by down-regulating the enzymes required for the production of testosterone (Teixeira et al., 1999) and correspondingly in MT-hAMH transgenic male mice over-expressing AMH serum testosterone is reduced (Lyet et al., 1995). AMH knockout in rodents leads to hyperplasia of Leydig cells (Behringer et al., 1994) as does Smad4 over-expresion in transgenic pMIS-Smad4 male mice, in addition to increased testosterone and germ cell apoptosis (Narula et al., 2002),

suggesting that BMP receptor initiated Smad4 activity has a role in the signalling of AMH.

The over-expression of AMH leads to impaired development of the Wolffian ducts and testes that remain undescended (Behringer et al., 1994), and correspondingly transgenic mice (MT-hAMH) over-expressing AMH have reduced levels of CYP17. Similarly, in Leydig cells *in vitro* AMH was shown to significantly down-regulate CYP17 which converts progesterone into androstenedion leading to a marked reduction in testosterone synthesis as well as CYP11, 3β-HSD and LH receptor (Rey et al., 1993; Racine et al., 1998; Teixeira et al., 1999), indicating a direct function in steroid producing cells as well as a role in response modulation to gonadotrophins. In serum AMH and testosterone have been shown to have an inverse relationship (Rey et al., 1993).

1.9.4 TGF-βs in the Prostate

Bmp4 and *Bmp7* were shown to be expressed in prostate tissue in mice (Thomas et al., 1998). *Bmp7* up-regulates its expression in response to testosterone while down-regulation occurs in the absence of testosterone due to orchidectomy indicating that its expression is regulated by testosterone, however *Bmp4* showed no such response suggesting a function independent of androgens in this tissue (Thomas et al., 1998). BMP-6 is detected at similar levels in healthy and cancerous prostate tissue in rats and orchidectomy was not shown to affect the expression of the protein suggesting that BMP-6 regulation is independent from androgens similar to BMP-4 (Barnes et al., 1995). BMP-2, BMP-3, BMP-6 and BMP-4 mRNAs were expressed in both human and rat prostate with BMP-4 predominating in humans (Harris et al., 1994).

In humans BMP-6 is also detected in the prostate and found to be elevated in neoplastic prostate cancer tissue as well as human derived DU145 and PC3 carcinoma cell lines, with higher grade tumors staining stronger for BMP-6 (Barnes et al., 1995). In human prostate *BMP7* was found to be expressed in luminal acinar epithelium, the stromal compartment and the blood vessels, and this expression was significantly reduced in prostate cancer specimens (Buijs et al., 2007). The human prostate cancer cell lines PC-3 and PC-3M-Pro4, noted for their tumorigenic and highly metastic properties were not shown to express detectable *BMP7*, while the less aggressive human prostate cancer cell lines C4-2, C4-2B4 and LNCaP showed a much higher expression of *BMP7* (Buijs et al., 2007). In BALB/c nu/nu mice with human PC-3M-Pro4/luc^{+} cancer cells BMP-7 administration inhibited tumor growth. TGF-β and BMP receptors are found in PC-3M-Pro4 cells and BMP-7 activated type I receptors, while TGF-β in isolation did not; however, together they had a synergic effect on luciferase expression and therefore type I receptor activation (Buijs et al., 2007). BMP-7 may counteract the activation of Smad3/4 nuclear factors by TGF-β (Buijs et al., 2007).

BMPR2 is found in prostate tissue in both adult and fetal human tissues (Rosenzweig et al., 1995). BMP-2 signalling has been shown to inhibit prostate cancer cell growth signalling via Smad1 interacting with the nuclear androgen receptor (Qiu et al., 2007). The signalling of BMP activated Smads, such as Smad1 is necessary for healthy prostate function as indicated by significant down-regulation of BMP receptors and Smads evident in prostate cancer (Qiu et al., 2007). MARK/ERK phosphorylation of the Smad1 linker region is required for Smad1 interaction with the nuclear androgen receptor where it acts as a co-repressor of androgen mediated prostate cancer growth (Qiu et al., 2007).

1.9.5 TGF-βs in Other Male Reproductive Tissues

Bmp4, *Bmp7*, *Bmp8a* and *Bmp8b* are found in murine epididymis (Zhao et al., 1998; Chen et al., 1999; Hu et al., 2004). After 4 weeks of age *Bmp7* was increasingly expressed from the cauda epididymis extending to the vas deferens indicating developmental regulation, while after 6 weeks the more generalized epithelial expression of *Bmp7* restricts to the initial segment of the epididymis (Chen et al., 1999). *Bmp8a* and *Bmp7* are expressed in the cauda epididymis and distal caput epididymis in mice (Zhao et al., 1998). *Bmp8a* synthesized by epithelial cells in the initial segment is believed to promote the survival of the epithelial cells in the cauda and distal caput epididymis and therefore *Bmp8a* is possibly a factor necessary for the maintenance of spermatogenesis (Zhao et al., 1998). C57BL/6 background male mice heterozygous for Bmp-4 presented with degenerated epithelium of the corpus epididymis (Hu et al., 2004). *Bmp8a* and *Bmp8b* homozygous mutants both displayed degeneration of the epithelial cells lining the epididymis (Zhao et al., 1998). Furthermore heterozygous *Bmp7* and homozygous *Bmp8a* double mutant mice show increased exacerbation of *Bmp8a* homozygous epithelial degeneration of the epididymis (Zhao et al., 2001a).

In the vas deferens *Bmp7* was found to be expressed in mice older than 4 weeks of age while not being significantly detected before that age postpartum (Chen et al., 1999).

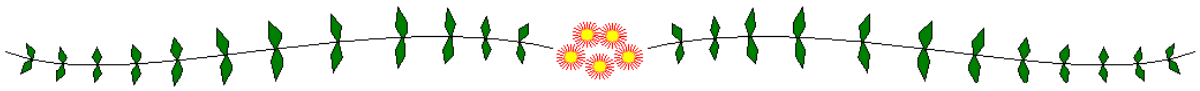
In murine embryos *Gdf7* is first detected in the mesenchymal cells of the seminal vesicles at 17 dpc and postnatally until 9 days of age (Settle et al., 2001), while at 7 days GDF-7 receptors *Bmpr1a* and *Bmpr1b* are found in the seminal vesicle epithelium (Settle et al., 2001). *Gdf7* knockout causes morphological defects due to a lack of mesenchymal to epithelial transition and failure of the seminal

vesicles to produce the seminal vesicle secreted proteins found in wild-types leading to infertility in male mice (Settle et al., 2001). Therefore GDF-7 is vital for the mesenchymal to epithelial transition necessary for normal secretory function.

1.10 Proposed Project

The aim of this research is to elucidate the function of BMPR-IB using passive neutralization of BMPR-IB *in vivo* and *in vitro* placing specific attention to its role in the male reproductive system, given the importance of BMPR-IB in females and the lack of publications available for its function in males. BMP and BMP receptor mRNA expression was also screened in male reproductive tissues to establish the presence and relative quantitation of these genes. We hypothesize that BMPR-IB has important roles in male reproduction based on a limited amount research investigating BMPs and their receptors in this area and the fact that the receptor has crucial roles in females. The significance of any findings could be translated in numerous fields and includes a greater understanding of normal male reproductive physiology, a better understanding of abnormalities present in male infertility and cancer of male reproductive organs as well as the identification of possible future therapeutic targets.

Chapter 2



2.0 Materials and Methods

2.1 Animals

Male Swiss Quackenbush mice (Physiology Animal House, University of New England, NSW, Australia) were housed in sanitary conditions in a light controlled room (12:12) at a constant temperature of 21 °C and had access to a constant supply of standard rodent chow and water. Chickens were also housed at the UNE Physiology Animal House in sanitary conditions at room temperature and had a constant supply of standard poultry pellets and water. Sheep were maintained in open paddocks on pasture. The University of New England Animal Ethics Committee authorized the use of animals needed to conduct this research, which was approved under the Animal Research Act 1985. The use of animals needed to conduct this research was in accordance with the National Health and Medical Research Council: Australian code of practice for the care and use of animals for scientific purposes 7th Edition 2004.

2.2 Antibodies and Reagents

Antibodies were raised in chickens and sheep against synthetic peptides of BMP-2 (C)ISMLYLDENEKVVLK (Invitrogen Mount Waverly Vic), BMP-4 (C)ISMLYLDEYDKVVLK (Invitrogen) and the extra-cellular domain of BMPR-IB (C)NKDLHPTLPPLKDRD (Invitrogen). A cysteine was added to the N terminus of all synthetic peptides to enable conjugation to dipthera toxoid or biotin. Using the NCBI Basic Local Alignment Search Tool (BLAST) the peptide sequence homologies for BMPR-IB, BMP-2 and BMP-4 were examined across species (Table 2.1). BMP-2 and BMP-4 peptides showed 100 % homology in all species examined. BMPR-IB peptide showed 100 % homology to mouse and rat BMPR-IB but was less similar in

other species predominantly due to a single substitution shown boxed in Table 2.2. No similarity was found for BMPR-IA the closest family member receptor to BMPR-IB in mouse using the NCBI BLAST.

Table 2.1 Species comparison of: **BMPR-IB aa** Peptide Sequence (C)NKDLHPTLPPLKDRD
BMP-2 aa Peptide Sequence (C)ISMLYLDENEKVVLK
BMP-4 aa Peptide Sequence (C)ISMLYLDEYDKVVLK

		SEQUENCE IDENTITIES		
Species	**NCBI accession numbers**	**BMPR-IB**	**BMP-2**	**BMP-4**
Mouse	gb AAH65106.1; gb AAB05665.1; gb AAH13459.1	100 %	100 %	100 %
Rat	gb EDL82342.1; gb CAA81088.1; gb AAH78901.1	100 %	100 %	100 %
Human	gb BAA19765.1 ; gb AAF21646.1; gb AAH20546.1	93 %	100 %	100 %
Sheep	NP_001009431.1;AER12105.1;ABW37747.1	93 %	100 %	100 %
Cow	NP_001098798.1;AAI42130.1; AAI05345.1	93 %	100 %	100 %
Chinese hamster	gb EGW11753.1 ; gb EGV93086.1; gb EGW09442.1	93 %	100 %	100 %
Platypus	XP_00151667 stimulated 1.2; XP_001514564.2;	93 %	100 %	NA
Chicken	NP_990463.1;NP_989689.1; CAA53514.1	87%	100 %	100 %

Non-immune Ig was derived from non-immunized chickens and sheep. Sheep anti-chicken antibody was raised in sheep against normal chicken Ig. Equine chorionic gonadotrophin (eCG) (Bioniche Animal Health, Armidale, NSW) was used to atennuate the hypothalamic-pituitary-gonadal axis. Human chorionic gonadotrophin (hCG) (Sigma Pty Ltd) and follicle stimulating hormone (FSH) (Follitropin V Vetrepharm, Canada) were used to examine gonadotrophin specific responses in cells and tissues.

Table 2.2 BMPR-IB Peptide Sequence Comparisons

Species	Start	BMPR-IB protein sequence	End
Mouse	103	NKDLHPTLPPLKDRD	117
Rat	103	NKDLHPTLPPLKDRD	117
Human	103	NKDLHPTLPPLKNRD	117
Sheep	103	NKDLHPTLPPLKNRD	117
Cow	103	NKDLHPTLPPLKNRD	117
Chinese hamster	48	NKDLHPTLPPLKNRD	62
Platypus	63	NKDLHPTLPPLKNRD	77
Chicken	103	NKHLHPTLPPLKNRD	117

2.3 Antibody Purification from Egg Yolk and Plasma

Egg yolks from BMPR-IB, BMP-2 and BMP-4 immunized chickens were diluted 1 in 5 with 0.1 M sodium acetate buffer pH 5.0 and refrigerated over-night. The mixture was centrifuged at 1,910 g for 30 minutes and the precipitate discarded. The supernatant was then adjusted to a pH of 4.5 - 4.8 followed by the addition of caprylic acid 25 ul/ml. This solution was mixed for 30 minutes then centrifuged at 1,910 g for 30 minutes. The supernatant was collected and strained to remove fat while the pellet was discarded. One part 10 x PBS (0.4 M Na_2HPO_4, 0.1 M KH_2PO_4, 1.5 M NaCl, pH 7.4) was added to 9 parts supernatant. Ammonium sulphate was added 0.277 g/ml (to yield 45 % saturation) and mixed for 30 minutes, then centrifuged at 1,910 g for 15 minutes. The supernatant was discarded and the pellet was mixed with approximately 0.5 ml of PBS. Non-immune Ig was prepared identically from the yolks of non-immunized chicken. Ig concentration was determined using a spectrometer at 280 nm and determined using the equation: *280 nm x dilution factor / 14 x initial volume = mg/ml.* Following quantification antibodies were diluted in 50 % glycerol in PBS and stored at -20 °C.

Plasma from non-immunized sheep and sheep immunized against BMPR-IB, BMP-2 and normal chicken Ig was processed as for antibody purification from egg yolk. The complete list of BMPR-IB, BMP-2, BMP-4 and non-immune control antibodies purified from plasma or yolk is shown in Table 2.3.

Table 2.3

Antibody Name	Raised in	Target
JMS#8117	Sheep	BMPR-IB
JMS#8120	Sheep	BMPR-IB
JMS#8124	Sheep	BMPR-IB
JMS#8127	Sheep	BMPR-IB
JMS#8131	Sheep	BMPR-IB
JMS#8113	Sheep	BMP-2
JMS#8114	Sheep	BMP-2
JMS#8115	Sheep	BMP-2
JMS#8121	Sheep	BMP-2
JMS#8133	Sheep	BMP-2
JMS#8111	Sheep	None
JMS#8116	Sheep	None
JMS0375	Sheep	Chicken Ig
JMCK#50	Chicken	BMP-2
JMCK#51	Chicken	BMP-2
JMCK#54	Chicken	BMP-4
JMCK#55	Chicken	BMP-4
JMCK#58	Chicken	BMPR-IB
JMCK#59	Chicken	BMPR-IB
JMCK#1B	Chicken	None

2.4 Antibody Biotinylation

A 2 mg aliquot of antibody and proportional biotinylation binding buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, 0.02 % NaN_3, pH 8.4) were added together to make a total volume of 1 mL in a glass dilution tube. Biotin (NHS-LC-Biotin; Pierce,

Rockford, IL, USA) was equilibrated to room temperature than prepared as a solution in distilled water at 1 µg/uL of which 75 µL was added to the antibody tube. This preparation was placed on ice for 2 hours and stirred occasionally. The antibody plus biotin solution was then centrifuged in a centricon tube (Millipore, Billerica MA USA) at 6,000 rpm at 6 ºC for 30 minutes. Unbound biotin was washed out with 1 mL PBS and centrifuged for 30 minutes. The wash was repeated another 2 times, after which the remaining biotinylated antibody was carefully pipetted into a 2 mL vial and the volume recorded. Biotinylated antibody was brought to a volume of 500 µL using PBS and a further 500 µL ultrapure glycerol (Gibco BRL Life Technologies, Grand Island NY USA) was added to yield a 2 mg/mL biotinylated antibody preparation. Biotinylated Ig was stored at -20ºC.

2.5 Direct ELISA

To measure the binding of purified antibodies to the target antigen 96 well microtitre plates (Greiner) were coated with 100 ng/100 ul peptide (or target Ig) in 0.05 M carbonate binding buffer pH 9.6 and incubated overnight at 37 °C. The plate was then blocked using 200 ul of 5 % skim milk powder in carbonate binding buffer for 1 hour at 37 °C. The plate was washed 5 times with 0.1 M Tris wash buffer pH 8.0. Chicken antibody in high salt ELISA (HSE) buffer (0.5 M NaCl, 0.5 % Tween-20, 0.1 % BSA, pH 8.0) or sheep plasma (1:100) in HSE buffer were added to wells and placed on a plate shaker overnight at room temperature. The plate was washed 5 times with Tris wash buffer and biotinylated antibody (Sheep anti-chicken or donkey anti-sheep) was added at a concentration of 100 ng/well then placed on the plate shaker for 1 hour. The plate was again washed 5 times and incubated with streptavidin alkaline phosphatase (SAP) 1/10,000 in HSE buffer for 45 minutes.

After incubation the plate was washed for a final 5 times. P-nitrophenylphospate disodium salt hexahydrate (NPP) was added to all wells using 100 ul to develop the plate. After developing the plate absorbance was read using a Titertek Multiskan Plus Microplate Reader (Labsystems) at 405 nm. Absorbance values for chicken and sheep antibodies are shown in Figure 2.1 and Figure 2.2 respectively where the controls are a non-immune Ig tested against BMP peptides.

Initially we had only chicken antibodies and while the absorbance of JMCK#58 was higher than JMCK#59 both were significantly higher than the non-immune control (Figure 2.1). As we had substantially more JMCK#59 available for use we tested the antibody binding using a competitive BMPR-IB ELISA which demonstrated a good dose response curve (Figure 2.3). We also tested the bioactivity of both JMCK#58 and JMCK#59 *in vitro* using testicular culture and there was no significant difference between the two antibodies as shown in Figure 2.4.

BMP-2 and BMP-4 antibodies selected for use in experiments were JMCK#51 (anti-BMP-2) and JMCK#54 (anti-BMP-4) based on their higher absorbance values using a direct ELISA (Figure 2.1). BMP-4 antibody binding to BMP-4 peptide was tested using a competitive BMP-4 ELISA which demonstrated a good dose response curve with BMP-4 antibody and no cross reactivity for BMP-2 peptide (Figure 2.5).

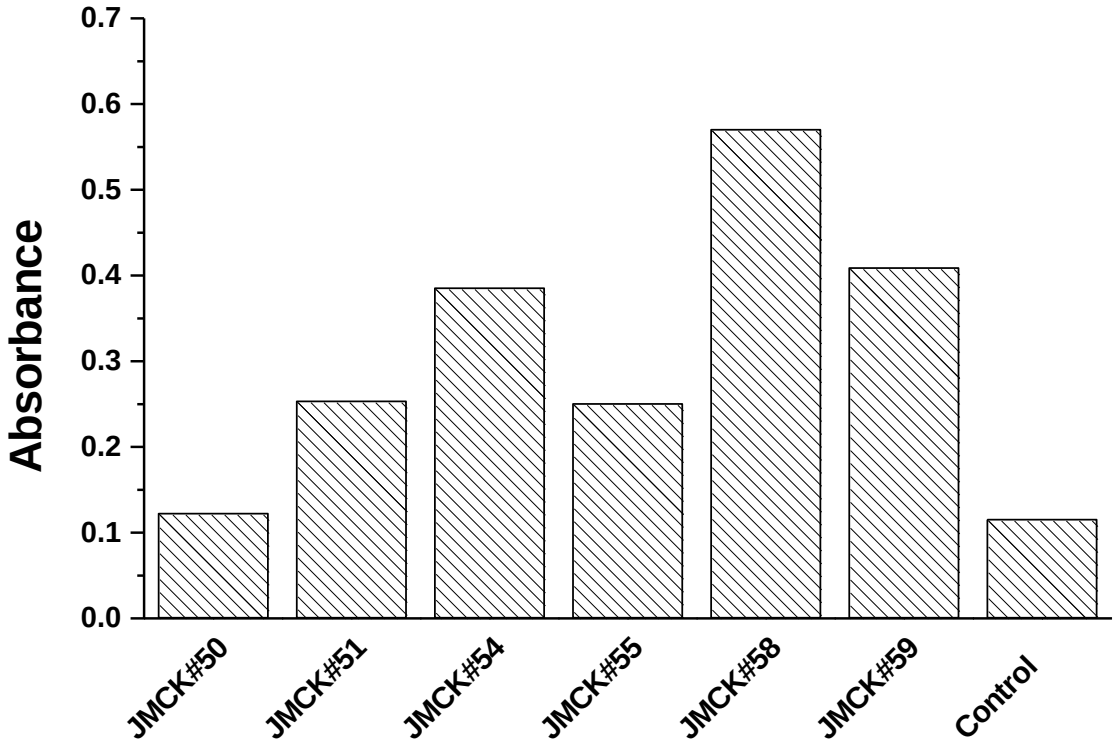


Figure 2.1 This graph shows the absorbance (binding strength) of chicken antibodies against their respective target ligands. JMCK#50 and 51 are BMP-2 antibodies. JMCK#54 and 55 are BMP-4 antibodies. JMCK#58 and 59 are BMPR-IB antibodies. Control (JMCK#1B) was the non-immune control tested against the target ligands.

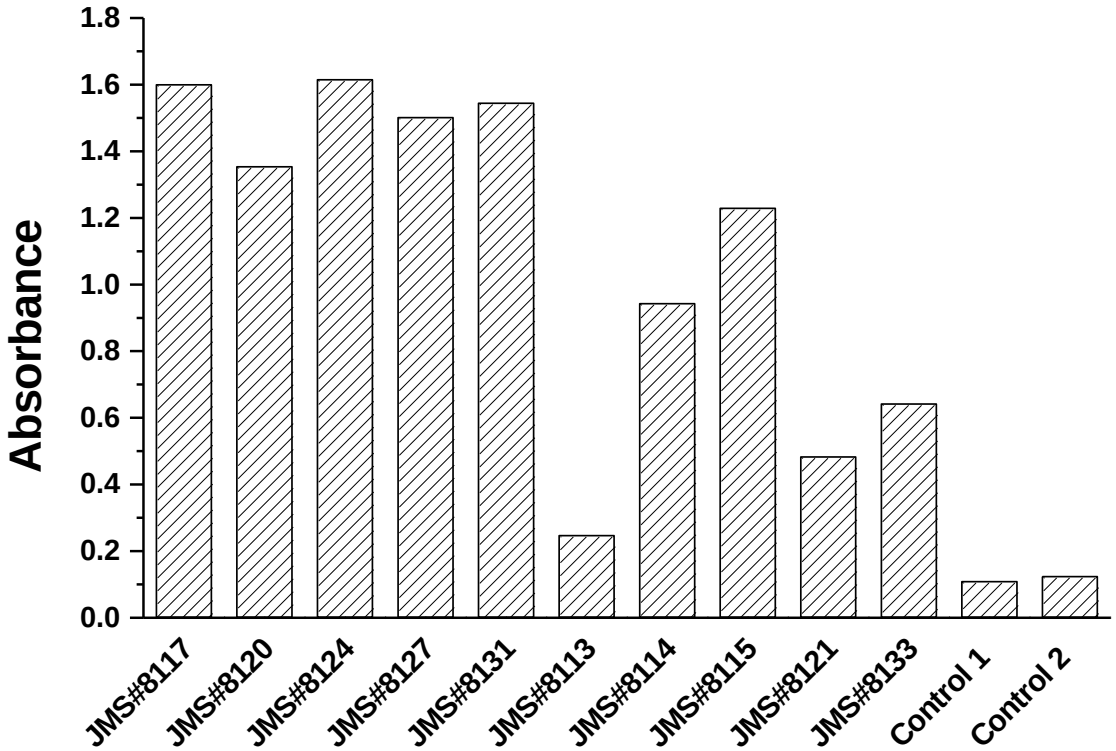


Figure 2.2 This graph shows the absorbance (binding strength) of sheep antibodies against their respective target ligands. JMS#8117, 8120, 8124, 8127 and 8131 are BMPR-IB antibodies. JMS#8113, 8114, 8115, 8121 and 8133 are BMP-2 antibodies. Control 1 (JMS#8111) and control 2 (JMS#8116) are non-immune controls tested against the target ligands.

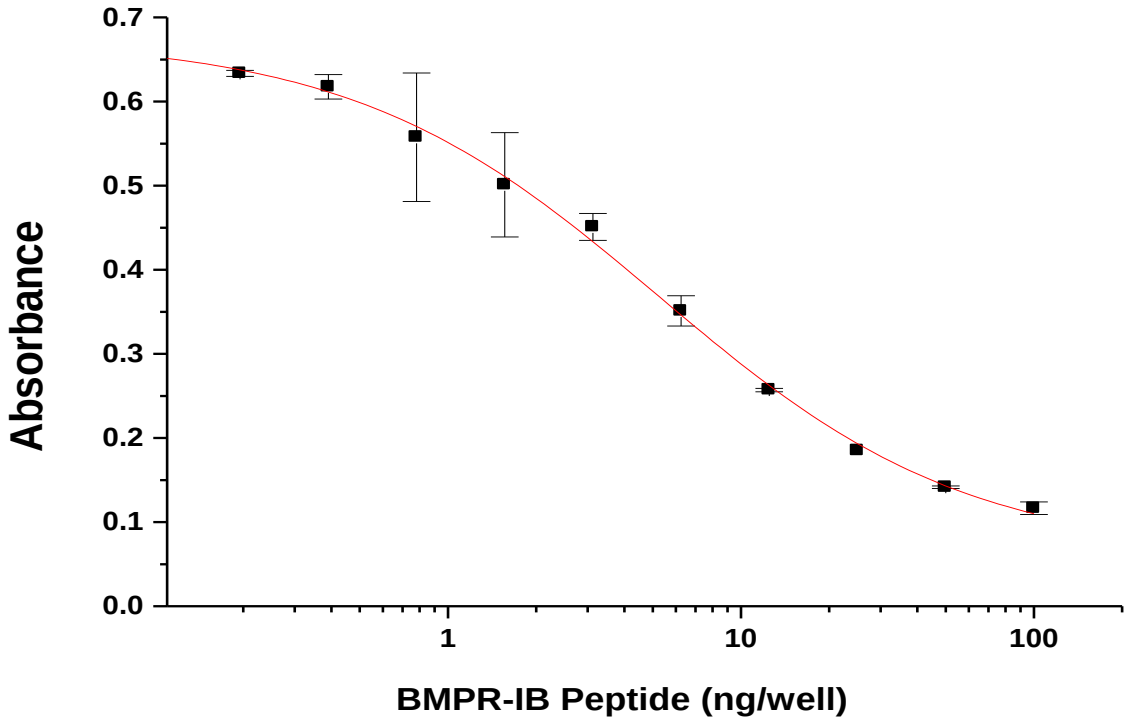


Figure 2.3 A dose response curve using BMPR-IB antibody JMCK#59.

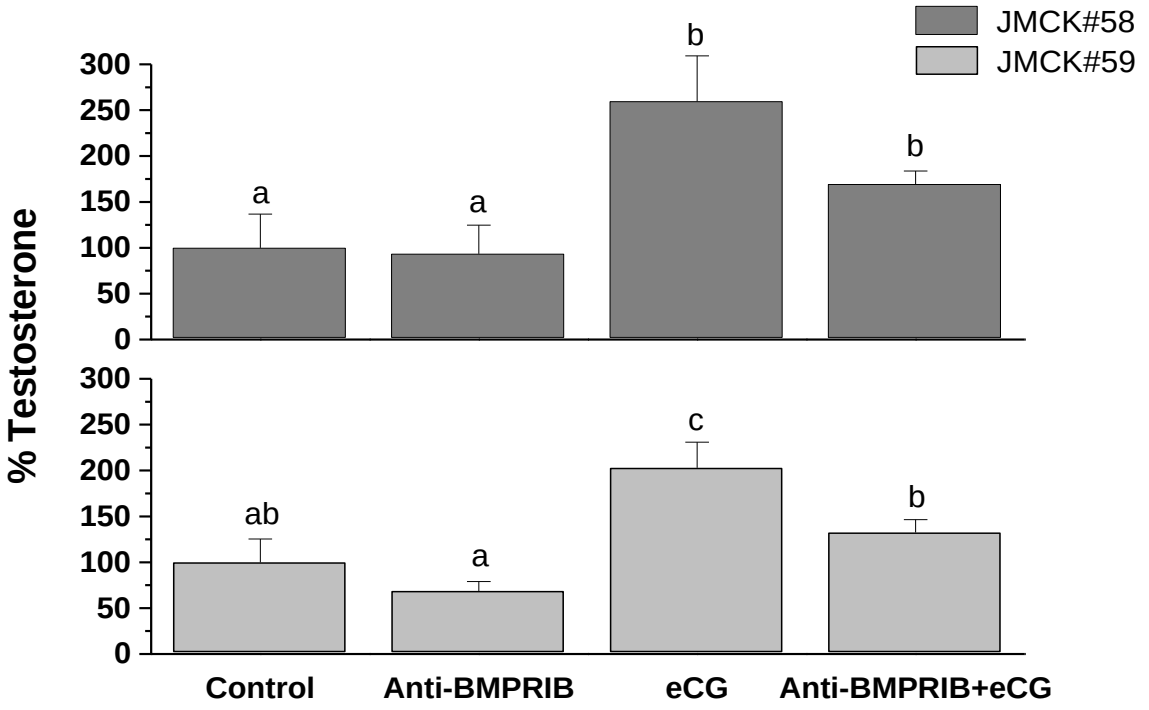


Figure 2.4 *In vitro* comparison of BMPR-IB antibodies JMCK#58 and JMCK#59 on testicular culture testosterone production.

2.6 Competitive ELISA

To measure antibody dose response and test for antibody cross reactivity, 96 well microtitre plates (Greiner) were coated with 30 ng peptide in carbonate binding buffer and incubated overnight at 37 °C. The solution was then discarded and the plate was blocked using 200 ul of 5 % skim milk powder in carbonate binding buffer for 1 hour at 37 °C, after which the plate was washed 5 times with Tris wash buffer.

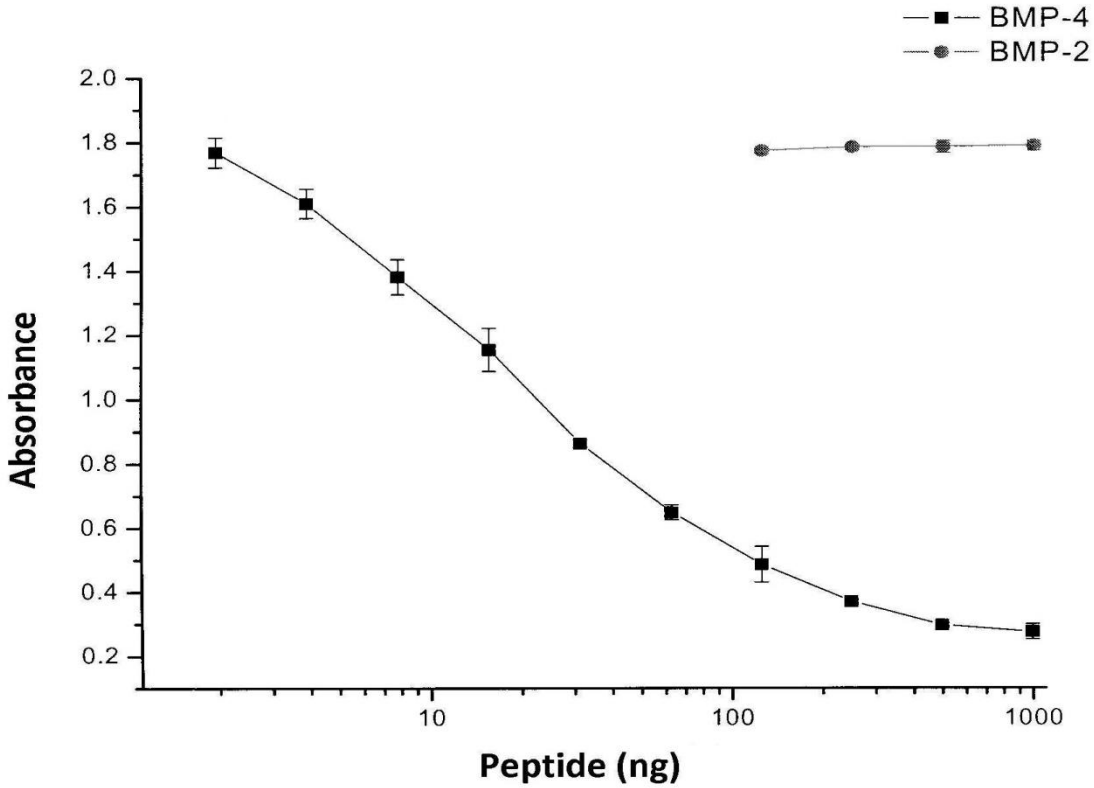


Figure 2.5 This graph demonstrates the competitive binding of BMP-4 antibody with increasing amounts of BMP-4 and BMP-2 peptide.

A serial dilution of peptide was prepared in HSE buffer starting from 100 ng to 0.1 ng. The serial dilutions were added to wells in duplicate and 2 ug antibody was added to each well. The plate was placed on a plate shaker for 10 hours then emptied and washed 5 times with Tris wash buffer. After the wash was discarded 1 ug of biotinylated sheep anti-chicken in HSE buffer was added to wells and placed on a plate shaker overnight at room temperature. The plate was washed 5 times

with Tris wash buffer, the wash discarded and then incubated with SAP 1/10,000 in HSE buffer for 3 hours. After incubation the plate was washed for a final 5 times. NPP was added to all wells using 100 ul to develop the plate. After developing the plate absorbance was read using a Titertek Multiskan Plus Microplate Reader (Labsystems) at 405 nm.

2.7 RNA Extraction

RNAs from tissues were extracted using TRI Reagent (T9424: Sigma-Aldrich Co, St Louis MO). Approximately 50 mg of tissue was stored in RNA later (AM7021: Ambion, Austin TX) overnight at 4 °C. Tissues were briefly dried on tissue paper than homogenized in glass dilution tubes in 1 ml TriReagent (Sigma-Aldrich Co) using a Heilidorfe homogenizer at 3,000 to 4,000 rpm. Homogenates were poured into 1.5 ml Eppendorf tubes (Sarstedt) and incubated for 10 minutes at room temperature. 200 ul of chloroform was added to the tubes, which were then shaken vigorously for 15 seconds, incubated for a further 2 to 3 minutes and centrifuged at 11,000 rpm for 15 minutes at 4 ºC. After centrifugation the homogenate separated into 3 layers – a red protein layer, an interphase containing DNA and an upper aqueous phase containing the RNA. The aqueous phase was harvested taking care not to aspirate any interphase and placed in a sterile 1.5 ml Eppendorf tube and 0.5 ml propane-2-OL added. The tubes were mixed well and incubated for 10 minutes at room temperature then centrifuged at 11,000 rpm for 10 minutes at 4 ºC. The supernatant was removed and the pellet washed in 75 % ethanol and centrifuged at 10,000 rpm for a further 5 minutes. The supernatant was again aspirated and the RNA pellet left to dry on ice for approximately 10-15 minutes. 20-50 ul sterile water was added to the RNA pellet depending on sample size. RNA

was stored at - 80 ºC. RNA integrity was checked on a 1 % RNA agarose gel and quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE).

2.8 RT-PCR

Reverse transcription was performed using 20 ng Oligo(dT)15Primer (DT-1: Fisher Biotec, Subiaco WA) and 2 μg total RNA denatured at 70 °C for 5 minutes then placed on ice. 10 mM dNTP Mix (DM-10M: Fisher Biotec), 1 x RT Buffer, 200 Units M-MLV Reverse Transcriptase (M1705: Promega, Alexandria NSW) and 1 Unit rRNasin(R)RNase Inhibitor (324750: Promega) were added to the reaction mix. Extension was performed at 40 °C for 60 minutes and transcribed cDNA stored at -20 °C until used.

2.9 PCR

Gene specific primers (GeneWorks Pty Ltd, Hindmarsh SA) were designed in house using NCBI Primer-BLAST (NCBI, 2009) or derived from other researchers (Table 2.4). A master mix was prepared containing 10 x PCR buffer, 10 mM dNTP mix (Fisher Biotec), 25 mM $MgCl_2$ (Fisher Biotec), 100 ng each of forward and reverse primer (GeneWorks Pty Ltd) and 1 U DNA Polymerase (Fisher Biotec). The master mix was dispensed into individual PCR tubes to which 80 ng cDNA was added. PCR amplification was performed using the following program:

95 °C for 5 minutes (initial melt)	1 hold
95 °C for 30 seconds (melting) 60 °C for 45 seconds (annealing) 72 °C for 1 minute (extension)	35 cycles
72 °C for 10 minutes (final extention)	1 hold
4 °C for ∞	1 hold

Table 2.4 Primers used for RT-PCR and RT-qPCR

Gene	NCBI reference #	(5'→3')	Sequence	Amplicon length (bp)	Source
β-actin	NM_007393.3	Forward	CGTCGACAACGGCTCCGGCATG	150	In house
		Reverse	TGGGCCTCGTCACCCACATAG		
Cyp19	NM_007810.3	Forward	TGTGTTGACCCTCATGAGACA	190	(Douglas et al., 2006)
		Reverse	CTTGACGGATCGTTCATACTTTC		
Hsd3b1	NM_008293.3	Forward	TGGACAAAGTATTCCGACCAGA	250	(Sarraj et al., 2010)
		Reverse	GGCACACTTGCTTGAACACAG		
Hsd3b6	NM_013821.3	Forward	GGAGGAGATCAGGGTCCTGG	209	(Lin et al., 2009)
		Reverse	CTAGGATGGTCTGCCTGGG		
Hsd17b3	NM_008291.3	Forward	ACAACGTTGGAATGCTCCCCAGC	147	In house
		Reverse	GGCCTTTCCTCCTTGACTCCATGT		
Bmpr1a	NM_009758.4	Forward	AGGTCAAAGCTGTTCGGAGA	178	In house
		Reverse	CTGTACACGGCCCTTTGAAT		
Bmpr1b	NM_007560.3	Forward	TCAATGTCGTGACACTCCCATTCCT	245	In house
		Reverse	TGCTGTACCGAGGTCGGGCT		
Bmpr2	NM_007561.3	Forward	CACCCCCTGACACAACACCACTC	243	In house
		Reverse	GACCCCGTCCAATCAGCTCCAG		
Bmp2	NM_007553.2	Forward	ACCCCCAGCAAGGACGTCGT	197	In house
		Reverse	AAGAAGCGCCGGGCCGTTTT		
Bmp3	NM_173404.3	Forward	AGCAGTGGGTCGAACCTCGGA	199	In house
		Reverse	ACCCCCACCGCTCGCACTAT		
Bmp3b	NM_145741.2	Forward	GGCAACACCGTCCGAAGCTTCC	199	In house
		Reverse	AGGAGGCGGCAGGATGCGTT		
Bmp4	NM_007554.2	Forward	GACTACTGGACACCAGACTAGTCC	180	In house
		Reverse	CTTCCCGGTCTCAGGTATCA		
Bmp5	NM_007555.3	Forward	ATCAGGACCCCTCCAGGATGCC	120	In house
		Reverse	TGATCCAGTCCTGCCATCCCAGATC		
Bmp6	NM_007556.2	Forward	GCAGAGTCGCAACCGGTCCA	153	In house
		Reverse	GGTGCAATGATCCAGTCCTGCC		
Bmp7	NM_007557.2	Forward	CAAGCAGCGCAGCCAGAATCG	161	In house
		Reverse	CAATGATCCAGTCCTGCCAGCCAA		
Bmp8a	NM_007558.2	Forward	TTGGCTGGCTGGACTGGGTCA	209	In house
		Reverse	GCTGTCATAGTACAGCACAGAGGTG		
Bmp8b	NM_007559.4	Forward	GGCTGGCTGGACTCTGTCATTGC	176	In house
		Reverse	AGCTCAGTAGGCACACAGCACAC		
Bmp15	NM_009757.4	Forward	GCCGTCGGCCAACACAGTAAG	202	In house
		Reverse	AGAAGGTAAGTGCTTGGTCCGGCA		

To confirm that primers were specific to target cDNA their product lengths were checked with a 100 bp DNA Ladder using 1.2 % agarose gel electrophoresis. PCRs were optimized for each set of primers using a $MgCl_2$ gradient to enhance the efficiency of amplicon synthesis as demonstrated in Figure 2.6.

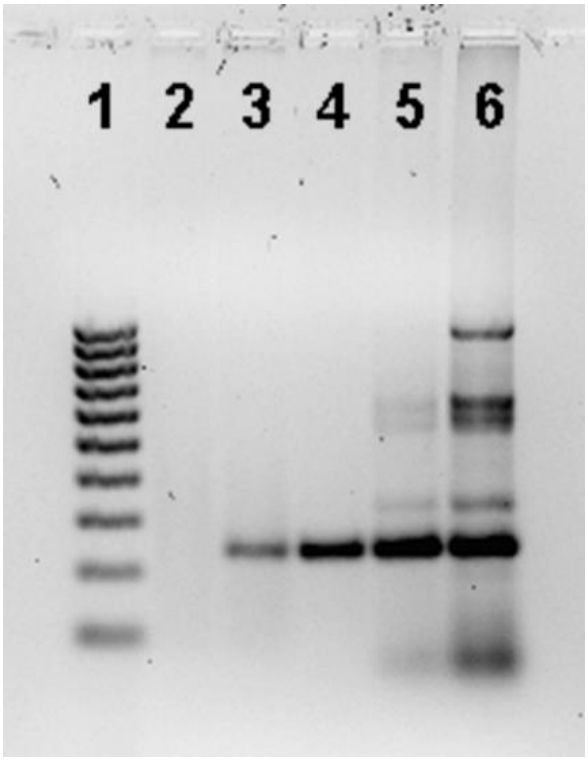


Figure 2.6 This figure shows a *Bmpr1b* PCR optimized using a $MgCl_2$ gradient.

Lane 1 = 100 bp DNA Ladder
Lane 2 = Negative control
Lane 3 = Amplicon using 2.5 mM $MgCl_2$
Lane 4 = Amplicon using 2.8 mM $MgCl_2$
Lane 5 = Amplicon using 3.0 mM $MgCl_2$
Lane 6 = Amplicon using 3.3 mM $MgCl_2$

This *Bmpr1b* PCR was optimized at 2.8 mM $MgCl_2$ resulting in a strong band of the expected size (245 bp) in the absence of non-specific binding.

2.9 QPCR

A qPCR master mix was prepared consisting of 10 nM primer and 2 x Fast EvaGreen qPCR Mix (31020: Biotium, Hayward CA). Using a CAS-1200 automated PCR Setup robot (Corbett Robotics, Eight Mile Plains, QLD) QPCR reactions were set up in duplicate using 16 ng cDNA after which qPCR was performed using a Rotor-Gene R6 6000 Real-time Analyzer (Corbett Life Science, Concorde NSW). Data analysis of qPCR Ct values was performed by the $2^{-\Delta\Delta Ct}$ method using *β-actin* as the reference gene.

2.11 Agarose Gel Electrophoresis

For RNA gels 0.5 g of agarose was boiled in 50 ml of 1 x TAE buffer (40 mM Tris base, 20 mM Acetic acid, 1 mM EDTA) for 2 minutes to yield a 1 % agarose gel after which the solution was allowed to cool slightly. 5 µl of 1/10 Ethidium bromide solution was added to the mixture which was than poured into a prepared gel molt and a comb added. After the gel had solidified the comb and tape around the gel molt were removed and the gel was submurged in 1 x TAE buffer. Samples were loaded into the wells using a 6x DNA loading dye (0.25 % bromophenol blue, 0.25 % xylene cyanol FF, 30 % glycerol in H_2O) and the gel was run at 80 V for 45 minutes. For DNA gels 0.6 g of agarose was boiled in 50 ml of 1 x TAE buffer to yield a 1.2 % agarose gel and the same procedures followed as for RNA gels. DNA was run at 80 V for 75 minutes.

2.12 Tissue Culture

A Thermoline automatic CO_2 incubator (Scientific Equip Pty Ltd, Wetherill Park, NSW) was set to a temperature of 32 ºC at 5.0 % CO_2 in humidified air. Dulbecco's Modified Eagle Medium/F-12 (DMEM) (Gibco Invitrogen Australia Pty Ltd, Mount Waverly, VIC) was prepared according to manufacturer's instructions and enriched with 0.1 % BSA (Sigma Aldrich Pty Ltd, Castle Hill, NSW). Tissue was assayed using 24 well culture plates (Sarstedt Australia Pty Ltd). At 3 weeks and 8 weeks male mice were sacrificed by CO_2 asphyxiation. Without delay testes were extracted, decapsulated and cut into quarters if from 8-week old mice or used whole if from 3-week old mice. Tissues were weighed and approximately 20 mg was placed in each well containing 0.5 ml DMEM following incubation for 15-30 minutes. After pre-incubation the media was removed and 1 ml of treated DMEM preparation

was added to allocated wells. The tissues were incubated for 3 hours, after which the sample tissue culture media was transferred to labeled vials and stored at -20 °C until assayed.

2.13 Leydig Cell Isolation

A Thermoline automatic CO_2 incubator (Scientific Equip) was set to a temperature of 32 ºC at 5.0 % CO_2 in humidified air. DMEM was used as culture media (Gibco Invitrogen) prepared according to manufactures instructions and enriched with 0.1% BSA (Sigma Aldrich Pty Ltd). At 8 weeks male mice were sacrificed by CO_2 asphyxiation and the testes extracted, decapsulated and aspirated with 5 ml of culture media then mechanically dispersed using two syringes joined with a connecting tube gently pushed back and forth 20 times. Freshly dispersed cells were filtered through 10 µm nylon gauze to collect interstitial cells which were washed and loaded onto Percol gradients prepared at 0 %, 20 %, 35 %, 43 %, 68 % and 90 % in Hanks Balanced Salt Solution (8.0 % NaCl, 1.0 % D-glucose, 0.4 % KCl, 0.06 % KH_2PO_4, 0.1 % $MgCl_2$, 0.1 % $MgSO_47H_2O$, 0.09 % $NaHPO_47H_2O$, 0.14 % $CaCl_2$, 0.01 % Phenol Red, pH 7.4) enriched with 0.1 % BSA. The gradients were then centrifuged at 1,230 g at 4 ºC for 30 minutes. After centrifugation 5 bands/zones of visible cells were present. Fraction 4 (43-68 % interface) was harvested for cell culture. Purified cells were counted with a haematocytometer and cell viability was determined using trypan blue exclusion. Fraction 4 contained enriched Leydig cells and always produced a distinct band of 90 % steroidogenic cells as determined by 3β-hydroxysteroid dehydrogenase (3βHSD) staining. Cells were cultured in 96-well polypropylene cell culture plates (Sarstedt Pty Ltd). After 3

hours incubation the culture media was aspirated into labeled vials and stored at -20 ºC until assayed.

2.14 Testicular Interstitial Cell Isolation

A Thermoline automatic CO_2 incubator (Scientific Equip) was set to a temperature of 32 ºC at 5.0 % CO_2 in humidified air. DMEM was used as culture media (Gibco Invitrogen) prepared according to manufactures instructions and enriched with 0.1% BSA (Sigma Aldrich Pty Ltd). Male mice were sacrificed by CO_2 asphyxiation and the testes extracted, decapsulated and aspirated with 5 ml of culture media then mechanically dispersed using two syringes joined with a connecting tube gently pushed back and forth 20 times. Freshly dispersed cells were filtered through 10 µm nylon gauze to collect interstitial cells. The cells were counted with a haematocytometer and cell viability was determined using trypan blue exclusion. Cells were cultured in 96-well polypropylene cell culture plates (Sarstedt Pty Ltd). After 3 hours of incubation the culture media was aspirated into labeled vials and stored at -20 ºC until assayed.

2.15 Steroid Extraction

Steroid extraction was performed on mouse serum samples using 0.7 ml diethyl ether and 50 µl mouse serum in glass dilution tubes that were vortexed for 10 minutes. The tubes were then placed on dry ice until the serum was frozen. The steroid containing ether was decanted into 3.5 ml polypropylene tubes (Sarstedt Australia Pty Ltd) and placed into a thermostate vacuum oven (Townson & Mercer Ltd, Croydon) for 1 hour at 25 ºC to evaporate the ether. Steroids were re-hydrated

using gel buffer (1 % PBS, 0.1 % Gelatine, 0.02 % NaN_3 in H_2O), incubated for 12 hours at 4 °C and assayed using a testosterone radioimmunoassay.

2.16 Testosterone Radioimmunoassay

Testosterone concentrations in serum, homogenates and culture media were determined using a radioimmunoassay. Tritiated tracer at 10,000 cpm per 100 µl and antibody (1:50,000) were added to samples in 3.5 ml polypropylene tubes (Sarstedt Pty Ltd) and incubated for 18 - 24 hours at 4 °C. The antibody bound competitively to the known quantity of radio-labelled testosterone (tracer) and the testosterone in the sample. After incubation 100 µl of 5 mg/ml horse Ig in gel buffer and 1.6 ml of 22 % polyethylene glycol (PEG), were added to each tube and the samples incubated at 4 °C for 30 minutes, before being centrifuged at 4 °C for 30 minutes at 1,230 g to separate the bound from the free testosterone. After centrifuging, the samples were immediately aspirated and 2 ml scintillation fluid (0.01 M PPO, 0.8 mM POPOP, 1.6% Methanol, and 5% Triton X-100 in Toluene) added to each tube to resuspend the pellet containing the bound steroids. Samples were equilibrated for 12 to 18 hours before being read with a scintillation counter (Packard 4000 series β-counter). Steroid levels were measured in ng ml^{-1} and determined by plotting the log/logit transformation of the standard curve, with a range from 100-0 ng ml^{-1} to fit a linear equation in each assay.

2.17 Histology

Freshly isolated tissues were fixed by immersion in Bouin's fixative (75 ml saturated aqueous picric acid, 25 ml formalin 40 %, 5 ml glacial acetic acid). Tissues were left in Bouin's fixative for 6-12 hours after which they were placed in 50 %

ethanol for storage. Using a histokinet tissues were dehydrated and prepared for wax embedding using the following series of solutions: 50 % ethanol - 2 hours, 70 % ethanol - 2 hours, 80 % ethanol - 2 hours, 90 % ethanol - 1 hour, 100 % ethanol I & II - 1 hour, 50 % ethanol with 50 % Xylol - 1 hour, Xylol I - 1 hour, Xylol II - 2 hours, paraffin I - 2 hours, paraffin II - 3 hours. Tissues were then embedded in paraffin wax. Embedded tissues were sectioned using a microtome at 3 µm and placed in a water bath at 42 ºC. Sections were collected on glass slides and placed on a hot plate at 42 ºC for 2-3 hours. Slides were dewaxed and hydrated using a series of solvents in the following order: Xylol (x 2) - 4 minutes, 100 % ethanol (x 2) - 3 minutes, 80 % ethanol - 3 minutes, 50 % ethanol - 3 minutes, distilled water - 3 minutes.

2.18 3β-HSD Histochemistry

Cells or tissues were stained for 3β-HSD using 1 % B-NAD, 0.2 % Nitro blue tetrazolium, 0.1 % BSA in Dulbeccos PBS (NaCl 0.8 %, KCl 0.02 %, KH_2PO_4 0.02 %, Na_2HPO_4 0.012 %, $MgSO_4$ 0.12 %, CaCl 0.0147 %, pH 7.4) combined with 0.06 % pregnenolone in ethanol. The preparation was added to dried cell suspensions or deparaffinised 3 µm testis slices on glass slides and incubated for 1½ hours in a dark place at room temperature. Slides were washed with H_2O and allowed to dry, before microscopic examination. Assessment of the images was assisted by other researchers in the physiology department.

2.19 Trypan Blue Viability Test

Trypan blue staining was carried out using 10 ul 0.5 % trypan blue (0.04 M Na_2HPO_4, 0.01 M KH_2PO_4, 0.15 M NaCl, pH 7.4) in PBS mixed with 10 ul cell

suspension in a 1.5 mL Eppendorf tube. This was gently agitated and loaded onto a haemocytometer slide. Percent cell viability was determined using the formula: *Unstained cell count / Total cell count x 100 = Cell viability*

2.20 Analysis

Data analysis of qPCR Ct values was performed by the $2^{-\Delta\Delta Ct}$ method using *β-actin* as the reference gene. Testicular homogenates were analysed as ng/mg tissue. Tissue culture media testosterone was normalized to ng per 10 mg tissue and Leydig cell testosterone was normalized to ng / 10K cells. Statistical analysis was performed using a general linear model procedure in SAS statistical software (SAS Institute Inc. Cary, NC, USA). The data were evaluated using one-way ANOVA followed by the Student-Newman Keuls *post hoc* test. Values were considered to be significantly different at $P < 0.05$ and presented as mean ± standard error (SE).

Chapter 3

3.0 Postnatal Expression of Bone Morphogenetic Proteins and their Receptors in the Mouse Testis

3.1 Introduction

Bone morphogenetic proteins (BMPs) belong to the decapentaplegic-Vg-related (DVR) family, which forms the largest subgroup of growth factors of the transforming growth factor-β (TGF-β) superfamily (McDonald & Hendrickson, 1993). BMP signalling occurs via heterodimerization of type I and type II serine/threonine receptors (Koenig et al., 1994; Ebisawa et al., 1999), which activate SMAD1, SMAD5 and/or SMAD8 signal transducers (Hoodless et al., 1996; Liu et al., 1996; Nishimura et al., 1998; Aoki et al., 2001; Kersten et al., 2005).

In mice, knockouts for *Bmpr1a* (*Alk-3*) and *Bmpr2* are embryonically fatal (Mishina et al., 1995; Beppu et al., 2000), while *Bmpr1b* (*Alk-6*) deficiency in male mice resulted in compromised fertility which was attributed to defective development of the seminal vesicles (Yi et al., 2001). Expression of mutant *Bmp4* (Hu et al., 2004), *Bmp7* (Zhao et al., 2001a), *Bmp8a* (Zhao et al., 1998) and *Bmp8b* (Zhao et al., 1996) resulted in either compromised fertility or infertility in male mice.

BMP and BMP receptor mRNAs have been detected in murine ovary and shown to fluctuate depending on follicle stage (Yi et al., 2001; Erickson & Shimasaki, 2003; Pierre et al., 2005; Tanwar et al., 2008). Furthermore, *Bmp2*, *Bmp4*, *Bmp5*, *Bmp6*, *Bmp7*, *Bmp8a*, *Bmp8b* and *Bmpr1a* have been shown to be expressed in embryonic mouse testis (Dewulf et al., 1995; Ross et al., 2007) indicating that these genes and their translated products are likely to be important for male germ cell development as well, while studies on the postnatal expression of many BMP genes has been limited. Mouse mRNA expression of *Bmp2* and *Bmp4* was detected in immature testis (Itman & Loveland, 2008), *Bmp7*, *Bmp8a* and

Bmp8b in immature and adult testis (Zhao et al., 1998; Zhao et al., 2001a; Itman & Loveland, 2008), *Bmp5* and *Bmp6* in adult testis (Lyons et al., 1989a; Marker et al., 1997), while *Bmp15* mRNA in adult mouse testis and *Bmp3b (Gdf10)* in silico were reported to be absent (Dube et al., 1998; Katoh & Katoh, 2006).

Inconsistent findings about the expression of BMP receptor mRNAs in the mouse testis or cells derived thereof have also been documented for all three receptors. Pellegrini *et al.* (2003), Dewulf *et al.* (1995) and ten Dijke *et al.* (1994a) reported not finding *Bmpr1b* mRNA in mouse testis using Northern blotting and *in situ* hybridization, while Gouedard *et al.* (2000) reported *Bmpr1b* expression in mouse testis and testicular cell lines MA-10 cells and SMAT-1 cells derived from Leydig cell tumors and immature Sertoli cells respectively using polymerase chain reaction (PCR). In Sertoli cells of immature mice Puglisi *et al.* (2004) detected *Bmpr1a* using the Ribonuclease Protection Assay and *Bmpr2* expression by Northern blot, while on the contrary Pellegrini *et al.* (2003) did not readily detect the same mRNAs in Sertoli cells. In spermatogonia of immature mice Pellegrini *et al.* (2003) identified BMPR-IA protein and *Bmpr1a* and *Bmpr2* transcripts using Northern blot, and while Puglisi *et al.* (2004) also found *Bmpr1a* expressed in spermatogonia, they did not detect *Bmpr2*.

Pellegrini *et al.* (2003) demonstrated that BMP-4 increased proliferation of spermatogonia while its transcripts were expressed by Sertoli cells but not germ cells, indicating BMP-4 had a paracrine function in germ cell signalling. *Bmp4* expression decreased progressively from postnatal day 4 to 17 (Pellegrini et al., 2003). In mice before 3 weeks of age *Bmp8a* and *Bmp8b* mRNAs have been detected in spermatogonia and spermatocytes and at 3 weeks *Bmp8a* and *Bmp8b* mRNAs were localized in stage 6-8 round spermatids, demonstrating a development

shift (Zhao & Hogan, 1996; Zhao et al., 1996). *Bmp8b* homozygous mutants had greater germ cell degeneration than *Bmp8a* mutants, additionally homozygous *Bmp8b*^{tm1b1h} mutant mice exhibited progressive depletion of germ cells due to increased germ cell apoptosis and were rendered infertile (Zhao et al., 1996; Zhao et al., 1998), indicating a function in germ cell survival and maintenance.

The aim of this study was to investigate the developmental gene expression profiles of BMP receptors - *Bmpr1a*, *Bmpr1b* and *Bmpr2*, and BMPs - *Bmp2*, *Bmp3*, *Bmp3b*, *Bmp4*, *Bmp5*, *Bmp6*, *Bmp7*, *Bmp8a*, *Bmp8b* and *Bmp15* in the mouse testis during postnatal development using reverse transcription quantitative PCR (RT-qPCR) analysis of mRNA. Body weights and weights of the testis, epididymis, vas deferens, seminal vesicle, coagulating gland, and prostate were taken as a measure of reproductive development. BMP and BMP receptor genes were also visualized in adult reproductive and steroidogenic tissues using routine PCR to determine their distribution.

3.2 Experimental Design

Male Swiss Quackenbush mice (Physiology Animal House, University of New England, NSW, Australia) were sacrificed by asphyxiation with CO_2 at weekly intervals from 2 to 8 weeks of age, N = 5. Body, testis, epididymis, vas deferens, seminal vesicle, coagulating gland and the prostate were weighed. At 2, 4, 6 and 8 weeks approximately 50 mg testis was collected in RNALater (Ambion, Austin TX), N = 5, and at 8 weeks samples of epididymis, vas deferens, seminal vesicle, prostate, coagulating gland, adrenal gland and adipose tissue were collected in RNALater (Ambion). Collected samples were incubated at 4 °C overnight then stored at -80 °C until RNA extraction. At 2 weeks mice were considered immature, at 4 weeks early pubertal, at 6 weeks late pubertal and at 8 weeks mature. Extracted RNA from testis was assayed in duplicate using RT-qPCR for the following genes: *Bmpr1a*, *Bmpr1b*, *Bmpr2*, *Bmp2*, *Bmp3*, *Bmp3b*, *Bmp4*, *Bmp5*, *Bmp6*, *Bmp7*, *Bmp8a*, *Bmp8b* and *Bmp15*. *β-actin* was used as the reference gene. RT-PCR was used to screen epididymis, vas deferens, seminal vesicle, prostate, coagulating gland, adrenal gland and adipose tissue for BMP receptor and BMP mRNA genes examined in testis.

3.3 Results

3.3.1 Body and Reproductive Organ Weight throughout Postnatal Development of Male Mice

As a measure of growth and reproductive development we measured body, testis, epididymis, vas deferens, seminal vesicle, coagulating gland and prostate weight at weekly intervals from 2 to 8 week old mice (Figures 3.1 – 3.7). Body weight (Figure 3.1), testis weight (Figure 3.2), and seminal vesicle weight (Figure 3.5) increased significantly ($P < 0.03$) on a weekly basis from 2 weeks to 7 weeks at which time point weight plateaued. Prostate weight (Figure 3.7) also increased significantly ($P < 0.03$) on a weekly basis until 6 weeks, was not significantly different at 7 weeks, but was significantly different from 6 week to 8 weeks ($P \leq 0.005$). Epididymis weight (Figure 3.3) and vas deferens weight (Figure 3.4) increased significantly ($P < 0.01$) from 3 weeks to 7 weeks after which weight started to plateau. Coagulating gland weight (Figure 3.6) was only measured from 5 weeks as organs before that age were too small to dissect using the naked eye. Coagulating gland significantly increased its weight from 5 to 6 weeks ($P < 0.03$) and from 7 to 8 weeks ($P < 0.009$).

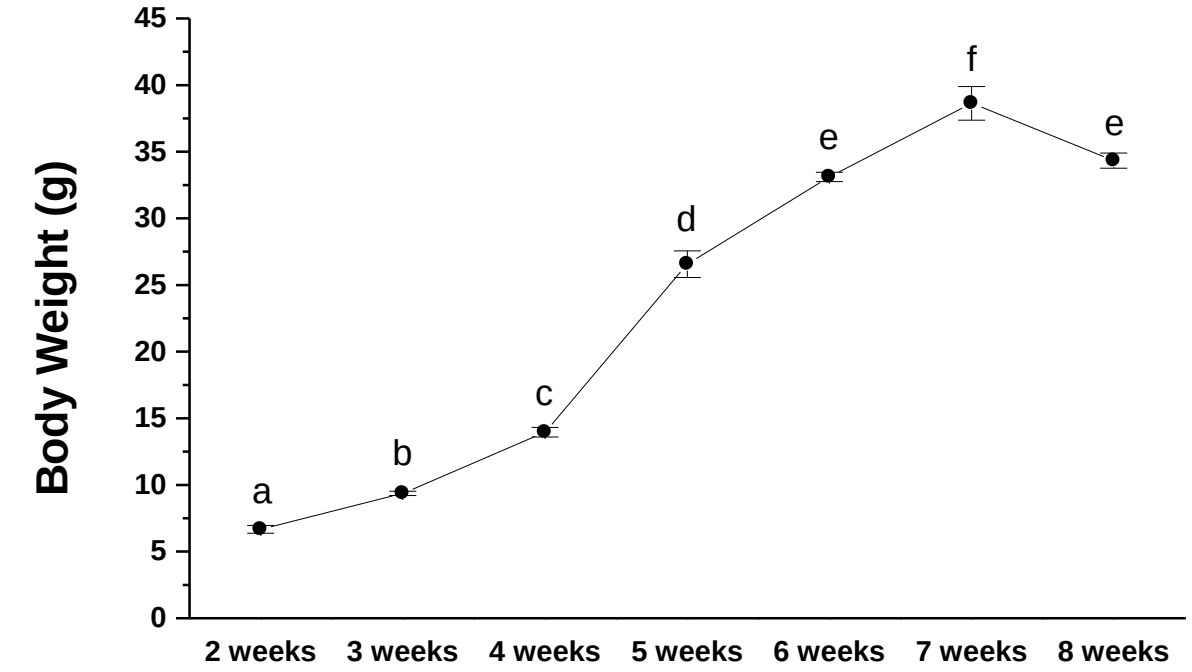


Figure 3.1 Body weight (g) in male mice from 2 to 8 weeks. Results expressed as Mean ± SE. Different letters denote significant differences between means ($P < 0.05$). N = 5.

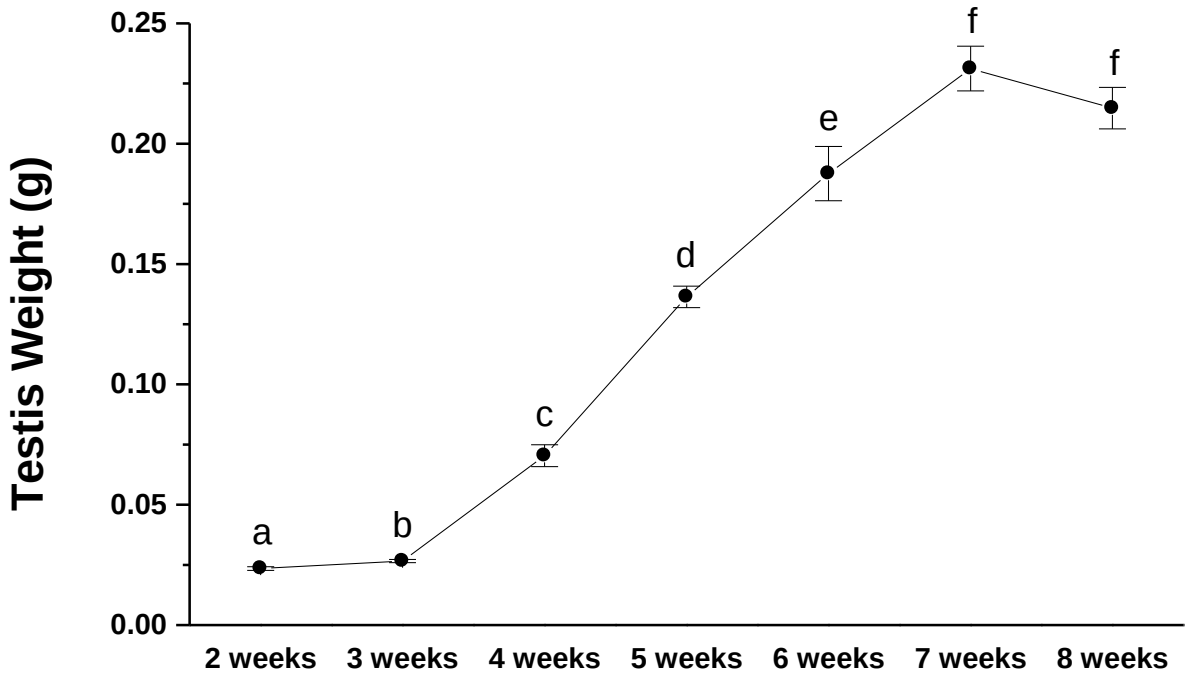


Figure 3.2 Testis weight (g) in male mice from 2 to 8 weeks. Results expressed as Mean ± SE. Different letters denote significant differences between means ($P < 0.05$). N = 5.

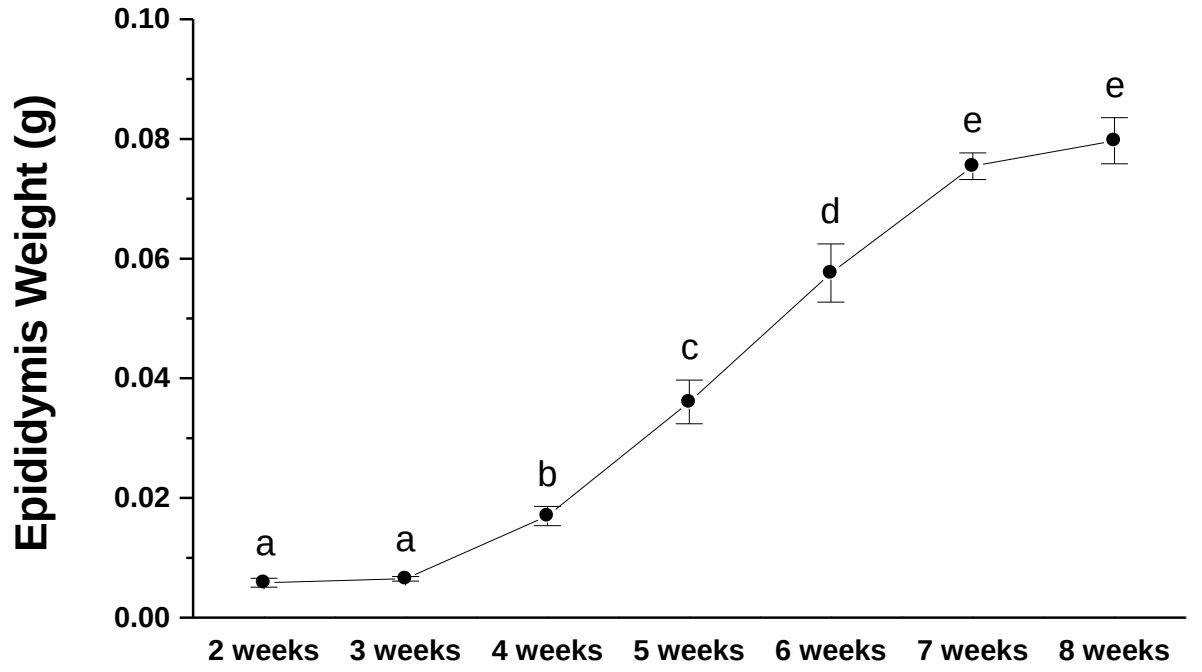


Figure 3.3 Epididymis weight (g) in male mice from 2 to 8 weeks. Results expressed as Mean ± SE. Different letters denote significant differences between means ($P < 0.05$). N = 5.

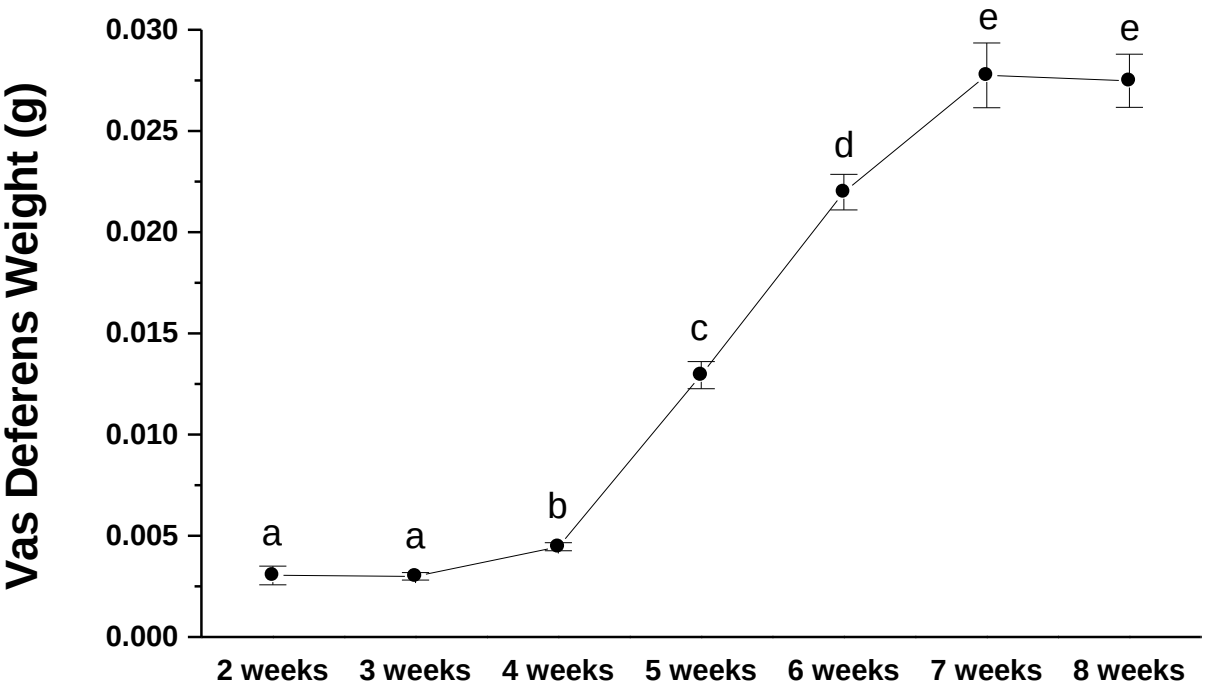


Figure 3.4 Vas deferens weight (g) in male mice from 2 to 8 weeks. Results expressed as Mean ± SE. Different letters denote significant differences between means ($P < 0.05$). N = 5.

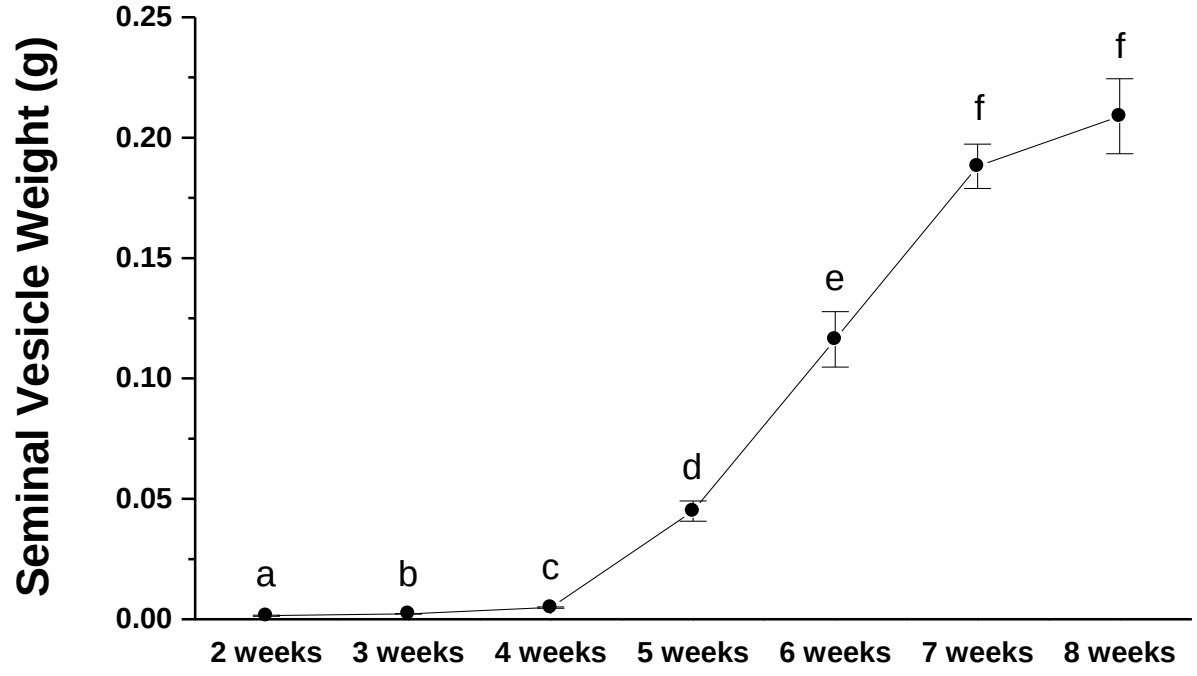


Figure 3.5 Seminal vesicle weight (g) in male mice from 2 to 8 weeks. Results expressed as Mean ± SE. Different letters denote significant differences between means ($P < 0.05$). N = 5.

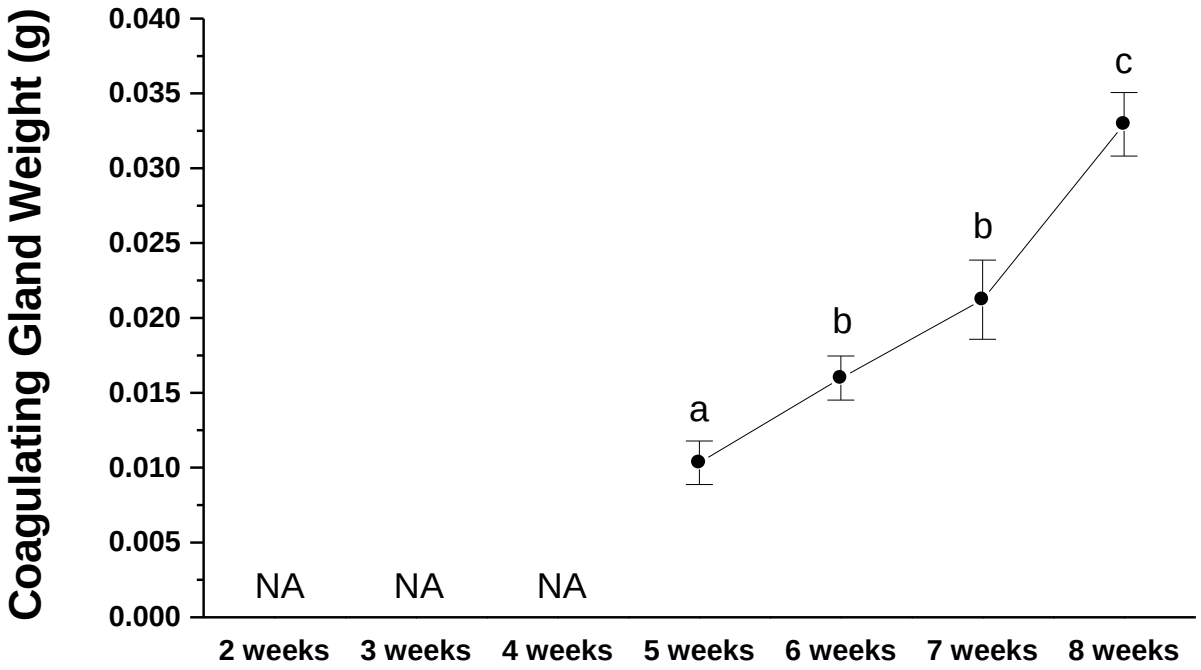


Figure 3.6 Coagulating gland weight (g) in male mice from 2 to 8 weeks. Results expressed as Mean ± SE. Different letters denote significant differences between means ($P < 0.05$). N = 5.

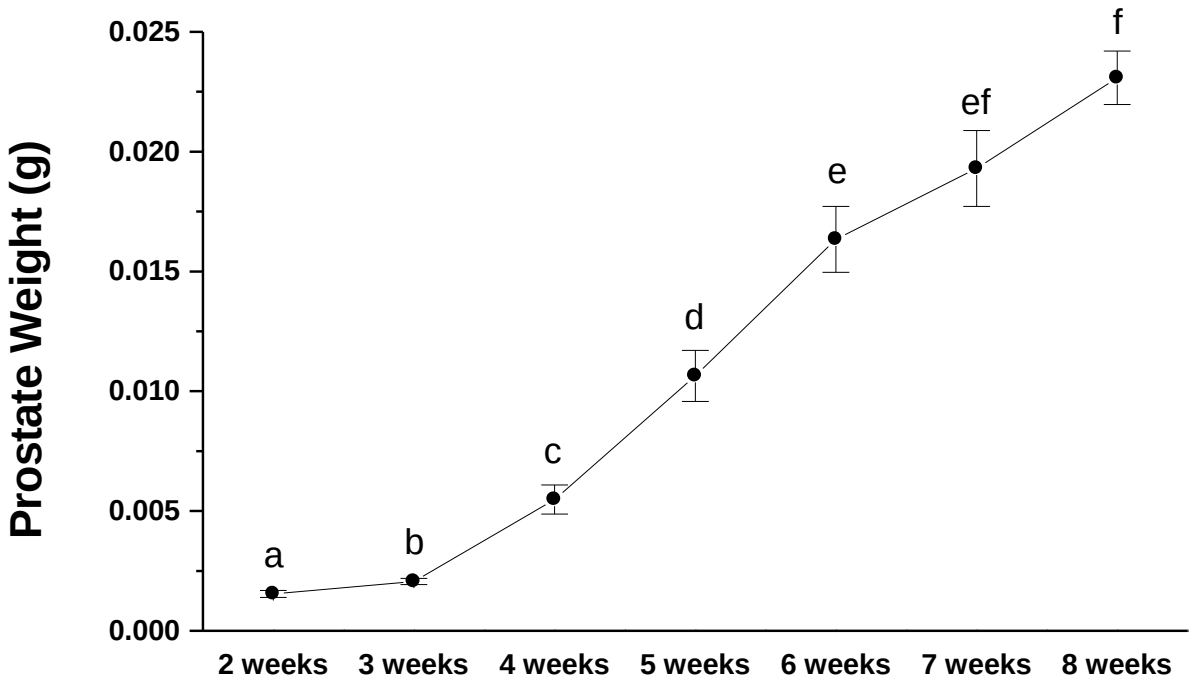


Figure 3.7 Prostate weight (g) in male mice from 2 to 8 weeks. Results expressed as Mean ± SE. Different letters denote significant differences between means ($P < 0.05$). N = 5.

3.3.2 Comparative Analysis of Testicular BMP Receptor Gene Expression During Postnatal Development

The relative changes in *Bmpr1a*, *Bmpr1b* and *Bmpr2* gene expression at the 4 time points examined are shown in Figure 3.8. *Bmpr2* and *Bmpr1a* were the most abundant genes at all ages tested, while *Bmpr1b* was expressed in considerably lower amounts. Overall the pattern of change with age was similar in all three receptors with a marked decline in expression at 4 weeks followed by a slight rise at 8 weeks. At 2 weeks *Bmpr1a* expression was relatively high at 60.3±12 %, while at 4 weeks *Bmpr1a* expression had been reduced 23 fold ($P = 0.0003$) and continued to decline although not significantly to six weeks. By 8 weeks *Bmpr1a* significantly increased by 2 fold ($P < 0.02$) over the expression at 6 weeks. At 2 weeks of age *Bmpr1b* was expressed at significantly lower amounts (1.4±0.4 %) than either

Bmpr1a (60.3±12 %) or *Bmpr2 (295±75 %).* The drop in expression level at 4 and 6 weeks from 2 weeks of age was also much higher (182 and 255 fold) than the decline seen with *Bmpr1a* but similar to that observed with *Bmpr2.* The expression of *Bmpr2* was the highest of the 3 receptors at 2 weeks (296±75 %), and followed a similar expression pattern with age as did Bmpr1b, with a large drop at 4 weeks and remaining low at 6 weeks followed by a slight but significant rise ($P < 0.0001$) at 8 weeks.

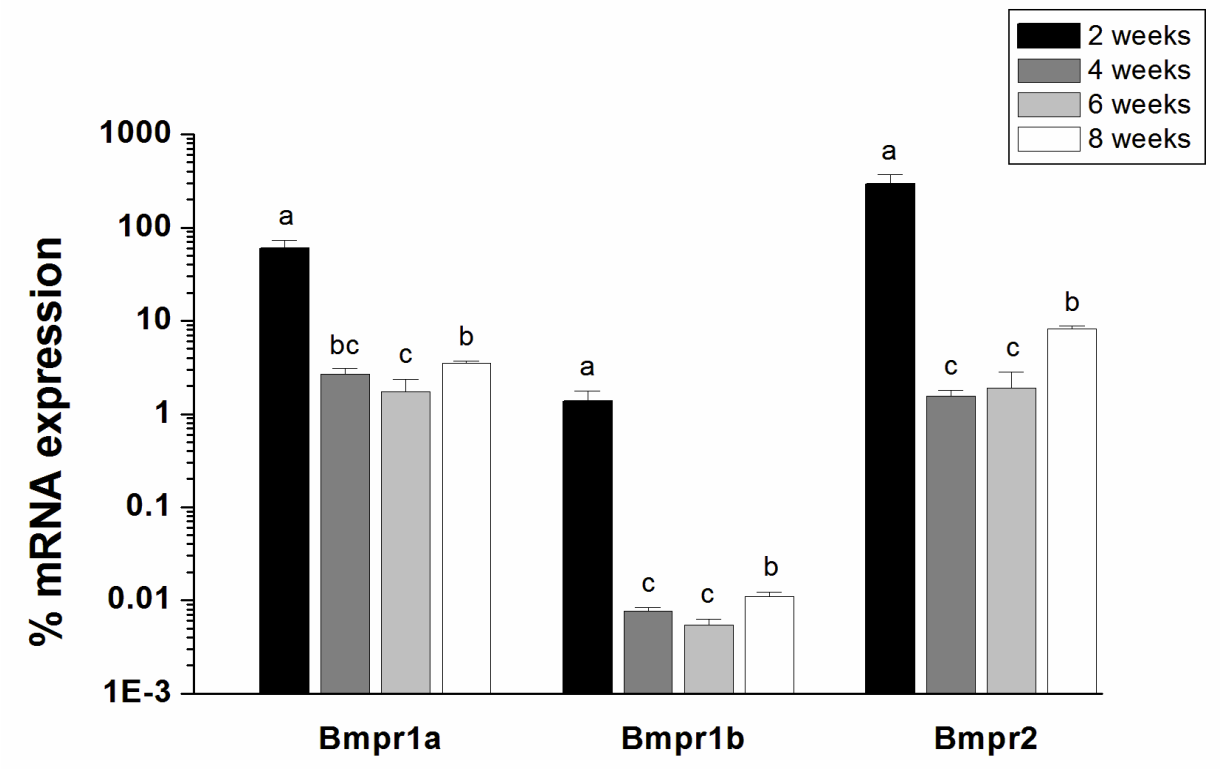


Figure 3.8 *Bmpr1a*, *Bmpr1b* and *Bmpr2* mRNA expression in 2, 4, 6 and 8 week old mouse testis. Results were calculated as a percentage of the housekeeping gene *β-actin* and presented as Mean ± SE. Different letters denote significant differences between means ($P < 0.05$) of individual genes throughout postnatal development. N = 5.

3.3.3 Comparative Analysis of BMP Gene Expression in Mouse Testis during Postnatal Development

The relative changes in *Bmp2*, *Bmp3*, *Bmp3b*, *Bmp4* and *Bmp5* gene

expression at the 4 time points examined are shown in Figure 3.9 panel A, while relative changes in the expression of *Bmp6*, *Bmp7*, *Bmp8a*, *Bmp8b* and *Bmp15* at the same time points examined are shown in Figure 3.9 panel B. The majority of genes were expressed at a significantly higher level at 2 weeks than 4 weeks with the exception of *Bmp5.* From 4 to 8 weeks approximately 50 % of genes tested increased their expression while the other 50 % reduced their expression or remained unchanged. *Bmp15* was the least expressed gene at 2 weeks. Genes that significantly increased their expression from 4 to 8 weeks were *Bmp3*, *Bmp5*, *Bmp7*, *Bmp8a* and *Bmp8b*. Genes that did not change significantly from 4 to 8 weeks were *Bmp2*, *Bmp4*, *Bmp6* and *Bmp15*, while *Bmp3b* was the only gene to significantly decrease its expression by 8 weeks. At 4 weeks *Bmp3b* was reduced by 12 fold ($P \leq 0.04$), *Bmp4* by 22 fold ($P \leq 0.0007$), *Bmp7* by 5 fold ($P = 0.0008$), *Bmp8a* by 5 fold ($P \leq 0.02$) and Bmp8b by 9 fold ($P = 0.0463$), while *Bmp2* was reduced 8 fold to less than 0.1 % ($P \leq 0.0001$), and *Bmp3* was reduced 65 fold to less than 0.01 % were $P = 0.009$. *Bmp3* expression declined the most radically of all the BMP mRNAs becoming the least expressed gene. By 6 weeks *Bmp5*, *Bmp8a* and *Bmp8b* significantly increased their expression were $P \leq 0.0001$, $P = 0.003$ and $P \leq 0.004$ respectively, and *Bmp3b* and *Bmp7* expression reduced significantly were $P \leq 0.03$ and $P = 0.02$ respectively from 4 weeks. Expression of *Bmp2*, *Bmp3*, *Bmp4*, *Bmp6* and *Bmp15* did not alter and *Bmp3* remained the least expressed gene. At 8 weeks expression of *Bmp7* and *Bmp8a* increased by 4 fold ($P \leq 0.005$) and 1 fold ($P = 0.04$) respectively. Expression of *Bmp2*, *Bmp3*, *Bmp3b*, *Bmp4*, *Bmp5*, *Bmp6*, *Bmp8b* and *Bmp15* did not change, however *Bmp15* was the least expressed gene at 8 weeks, being expressed at only 0.004 % of the housekeeping gene.

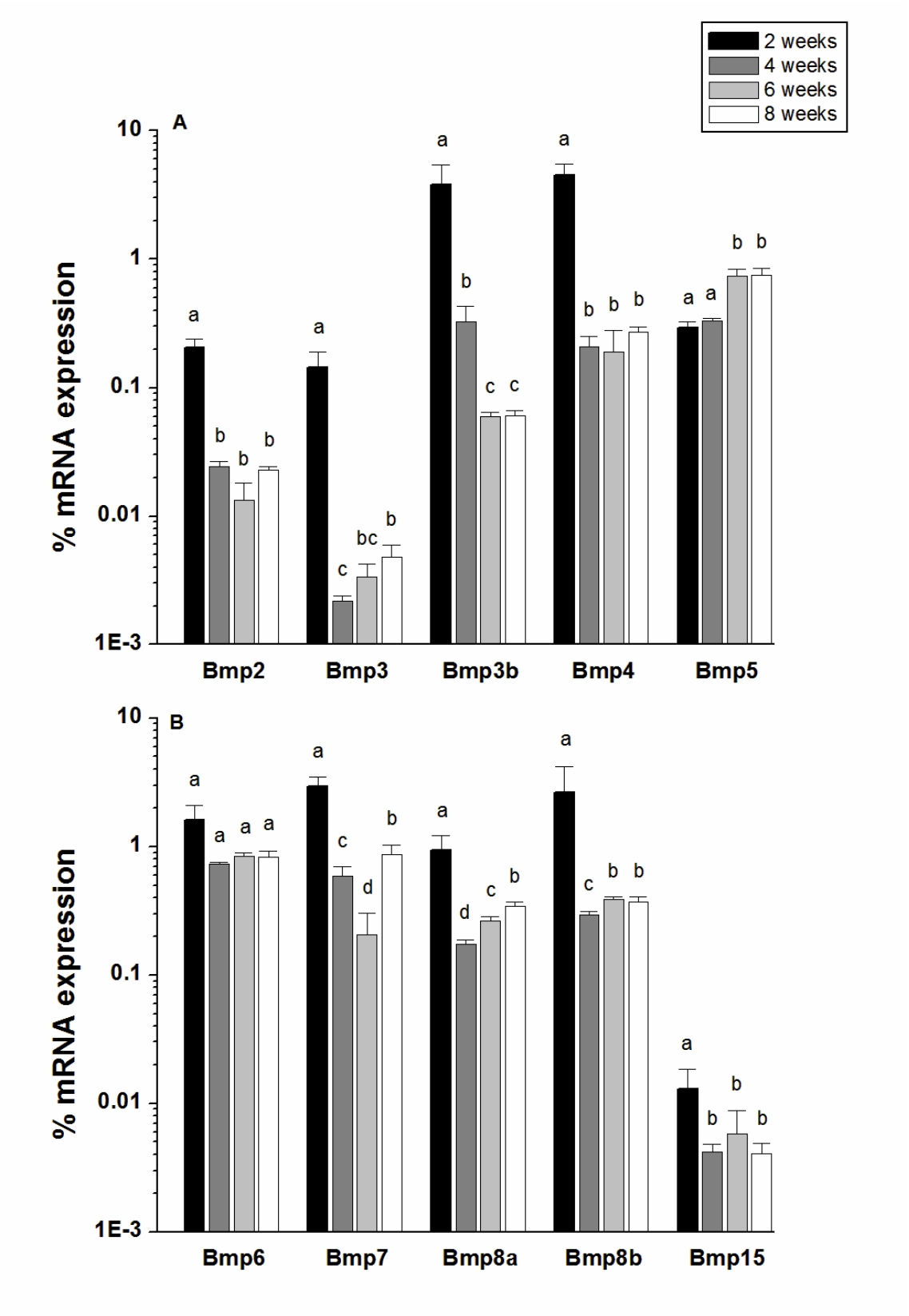


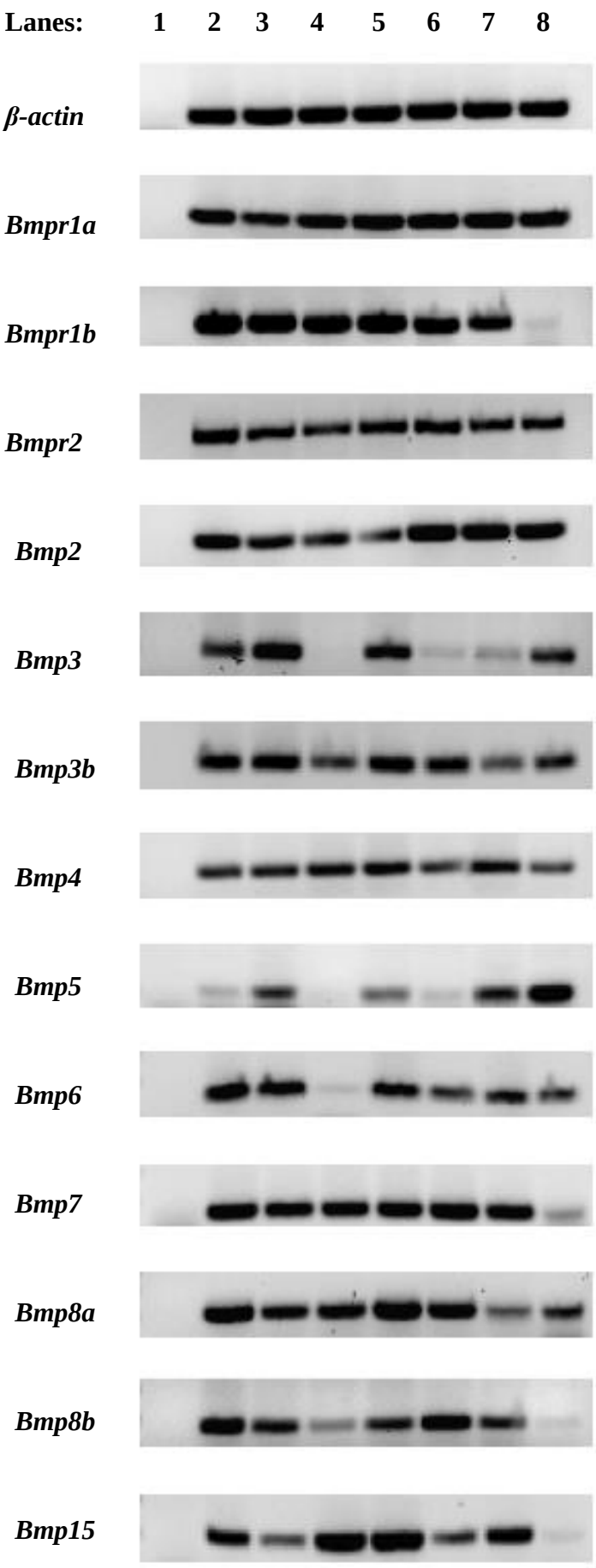
Figure 3.9 (Panel A) *Bmp2*, *Bmp3*, *Bmp3b*, *Bmp4* and *Bmp5,* **(Panel B)** *Bmp6*, *Bmp7*, *Bmp8a*, *Bmp8b* and *Bmp15* mRNA levels in 2, 4, 6 and 8 week old mouse testis. Results were calculated as a percentage of the housekeeping gene *β-actin* and presented as Mean ± SE. Different letters denote significant differences between means ($P < 0.05$) of individual genes throughout postnatal development. N = 5.

3.3.4 Distribution of BMP Receptor and BMP Genes in Adult Male Reproductive and Steroidogenic Tissues

The distribution of *Bmpr1a*, *Bmpr1b* and *Bmpr2*, and BMPs - *Bmp2*, *Bmp3*, *Bmp3b*, *Bmp4*, *Bmp5*, *Bmp6*, *Bmp7*, *Bmp8a*, *Bmp8b* and *Bmp15* mRNA in epididymis, vas deferens, seminal vesicle, prostate, coagulating gland, adrenal gland and visceral adipose tissue of adult mice is shown in Figure 3.10. *Bmp5* expression was low in epididymis while all other genes showed a high level of expression. In vas deferens *Bmp5* and *Bmp15* expression was reduced compared with the relative high levels of expression of all other genes. *Bmp3* and *Bmp5* were not readily visible in seminal vesicles and *Bmp6* and *Bmp8b* expression was low while other genes were highly expressed. *Bmp5* followed by *Bmp2* were the least expressed genes in dorso-lateral prostate with other genes being highly expressed while in coagulating gland (anterior prostate) *Bmp5* and *Bmp3* expression were reduced. In coagulating gland *Bmp4*, *Bmp6* and *Bmp15* were only moderately expressed compared to the higher expression of other genes. In the adrenal gland the expression of *Bmp3b* was moderate while *Bmp3* and *Bmp8a* were the least expressed genes. Adipose tissue showed reduced expression of *Bmpr1b*, *Bmp7*, *Bmp8b* and *Bmp15* however, *Bmpr1a* and *Bmpr2* were readily expressed along with *Bmp2* and *Bmp5* being amonst the highest expressed genes.

Figure 3.10 BMP receptor and BMP mRNA gene expression in adult male reproductive and steroidogenic tissues using RT-PCR.

Lane 1 = Negative control
2 = Epididymis
3 = Vas deferens
4 = Seminal vesicle
5 = Prostate
6 = Coagulating gland
7 = Adrenal gland
8 = Adipose tissue



3.4 Discussion

In our study testis, epididymis, seminal vesicles and prostate weights ranged inbetween the values previously reported by Sterger et al. (1998) and Cagen et al. (1999) while body weight was similar. Body weight and growth of the majority of reproductive organs plateaued by 7-8 weeks indicating that adulthood had been reached. However, prostate, coagulating gland and seminal vesicle weights were still increasing and in future studies this experiment should probably be continued until 10 weeks. BMP receptor and BMP gene expression was also extensive in all male reproductive and steroidogenic tissues tested, similar to findings by Erickson & Shimasaki (2003) who reported widespread expression of BMP receptor and BMP genes in the reproductive tissues of female rats. This suggests that these genes are important in both males and females. The developmental studies carried out demonstrate that BMP and BMP receptor mRNAs are expressed in the mouse testis. Our novel discoveries include finding postnatal expression of *Bmpr1b*, *Bmpr2*, *Bmp3*, *Bmp3b*, *Bmp4*, *Bmp5*, *Bmp6* and *Bmp15* in the testis at all developmental stages.

Bmpr1b mRNA has been reported to be absent in mouse testis by numerous researchers (ten Dijke et al., 1994a; Dewulf et al., 1995; Pellegrini et al., 2003) with one exception (Gouedard et al., 2000), and it likely to have been overlooked because its expression is low both relative to the housekeeping gene and to *Bmpr1a* and *Bmpr2* as determined in this study using highly sensitive RT-qPCR. This is supported in part by Belville *et al.* (2005) who found that *Bmpr1b* was expressed at a significantly lower level than *Bmpr1a* in SMAT-1 cells. We found *Bmpr1a* and *Bmpr2* mRNA in relatively high levels at all developmental stages, however BMP receptor expression was significantly higher in immature animals than in adult

animals. Similarly, Puglisi *et al*. (2004) reported that *Bmpr1a* and *Bmpr2* expression in the testis decrease significantly with age, however they were unable to detect *Bmpr2* mRNA by 30 days of age (~ 4 weeks) or older. Given that *Bmpr2* was one of the most abundant genes tested in our study the reason for this discrepancy is unclear as it would seem unlikely to be mouse strain specific.

Bmp2, *Bmp4* and *Bmp7* mRNAs were present at all ages screened but had their highest expression in immature testis. Itman and Loveland (2008) reported *Bmp2*, *Bmp4* and *Bmp7* mRNA in 5-day old mouse testis, and demonstrated that BMP-2 and BMP-4 treatment stimulated signalling of SMAD 1, 5 and 8 in Sertoli cell and spermatogonial cultures. Furthermore, BMP-2 had a role in the proliferation of spermatogonia in concert with FSH but not alone, and BMP-7 had a role in the proliferation of Sertoli cells in the presence of FSH (Puglisi et al., 2004), while BMP-4 increased proliferation of spermatogonia independent of gonadotrophins (Pellegrini et al., 2003). This demonstrates a great diversity of actions by these closely related BMPs and demonstrates how they can have vastly different functions under different conditions including the presence or absence of gonadotrophin stimulation.

Gene expression of *Bmp7* was highest in immature testis, lowest during early puberty and then significantly increased during late puberty and more so in adult testis. This suggests upon translation BMP-7 may have a role in the initiation of germ cell proliferation and maintenance of late stage spermatogenesis. Our findings are in agreement with Zhao *et al.* (2001) who found abundant expression of *Bmp7* in spermatogonia of immature mice, while in adult mice *Bmp7* mRNA was found mainly in spermatids and suggested to have a supporting role for maintenance of spermatogenesis. Similar to Zhao, *et al.* (1998) we found *Bmp8a* was significantly

less expressed than *Bmp8b* in pubertal testis but not in adult testis. This supports the suggestions that BMP-8a is important in late stage spermatogenesis with mRNA being identified in round spermatids (Zhao et al., 1998), while BMP-8b was shown to be necessary for both initiation and maintenance of spermatogenesis being expressed in spermatogonia and spermatids of pubertal mice and at high levels in round spermatids of adult mice (Zhao & Hogan, 1996).

In humans *BMP3B* (*GDF10*) mRNA has been detected in the testis (Hino et al., 1996) and by using *in-silico* expression analysis Katoh and Katoh (2006) identified *BMP3B* in human testis but reported the gene absent in mouse testis. We found low-level expression of *Bmp3* and significantly higher expression of *Bmp3b* in all age groups. *Bmp3b* expression was highest in immature mice, reduced significantly during early puberty and late puberty and remained unchanged in adult testis. Based on its expression patterns we hypothesize *Bmp3b* may have a role in germ cell proliferation while factors released from spermatids and/or spermatozoa present at 6 weeks of age (Seok et al., 2004) may down regulate its expression.

Bmp5 mRNA has been detected in adult mouse spermatogonia (Marker et al., 1997) and of interest *Bmp5* was the only gene we tested that had a lower expression at 2 weeks than all other age groups and increased significantly between 4 and 6 weeks which suggests a possible role in late stage spermatogenesis. *Bmp6* has been reported to be expressed in mature mouse testis (Lyons et al., 1989a), and in our study *Bmp6* expression stayed relatively high compared with other BMP genes indicating potential for a role in testicular functioning. We also detected *Bmp15* mRNA in mouse testis, which was previously not detected using Northern blotting (Dube et al., 1998). Compared to other BMP genes *Bmp15* was expressed at a low level throughout development.

Given that altered BMPR-IB signalling by putative BMPs is implicated in reproductive cancers including testicular cancer (Miyazaki et al., 2004; Bokobza et al., 2009; Fustino et al., 2011; Neumann et al., 2011), which is one of the most common cancers in young Caucasian men with a high incidence rate in developed countries (Rosen et al., 2011), a mouse model profiling the postnatal expression of BMP and BMP receptor genes in the testis may be of clinical interest.

This study establishes an extensive BMP system in mouse testis throughout postnatal development at the mRNA level, as well as widespread BMP and BMP receptor expression in male reproductive and steroidogenic tissues. Studies of protein expression will be needed to confirm the translation and abundance of the BMP and BMP receptor proteins not already detected. Given the predominance of BMP and BMP receptor genes reported in our study, and based on available research findings, it is likely that many of these genes have vital roles in germ and somatic cell proliferation, cellular homeostasis and steroid production. As *Bmpr1b* was expressed in mouse testis and other reproductive tissues at all ages tested we next examined the effects of BMPR-IB immunization on the reproductive characteristics of pubertal and adult mice.

Chapter 4

4.0 The Effects of *in vivo* BMPR-IB Immunization on Reproductive Characteristics of Pubertal Male Mice

4.1 Introduction

In males steroidogenesis, spermatogenesis and fertility are regulated by endocrine hormones from the pituitary and increasingly identified local growth factors from the transforming growth factor-β (TGF-β) superfamily. Pituitary gonadotrophins luteinizing hormone (LH) and follicle stimulating hormone (FSH) are amongst the most well known endocrine factors to be involved in both the initiation and maintenance of reproductive competence. LH has been shown to be required for the up-regulation of testosterone production by the testis (Bilinska & Szoltys, 1981), while FSH in combination with testosterone induces Sertoli cell differentiation and male germ cell development (Schlatt et al., 1995; Tesarik et al., 2002). Furthermore, increased androgen production facilitates maturation and secretory activity of the coagulating gland, prostate and seminal vesicles via steroid receptors found in these tissues (Yamashita, 2004). Seminal vesicles are extremely sensitive to androgen-mediated vascularization, growth and secretory activity (Tajana et al., 1984; Kashiwagi et al., 2005) and can be used as an indirect but specific measure of testosterone production.

A growing body of evidence also suggests major developmental roles for TGF-β superfamily members and their receptors in the modulation of testosterone synthesis (Teixeira et al., 1999), germ cell maturation (Zhao et al., 1998), integrity of reproductive tissues (Zhao et al., 1998; Hu et al., 2004) and epithelial secretory function (Settle et al., 2001), all of which are vital for normal reproductive capacity. The largest subgroup of growth factors belonging to the TGF-β superfamily are the

decapentaplegic-Vg-related (DVR) family also known as the BMP family (McDonald & Hendrickson, 1993), which signal as dimers via heterodimerization of type I and type II serine/threonine receptors (Koenig et al., 1994; Ebisawa et al., 1999).

BMP receptors have been localized in reproductive tissues and cells. *Bmpr1b* mRNA was expressed in mouse testis and testicular cells types SMAT-1 and MA-10 cells derived from immature Sertoli cells and Leydig cell tumors respectively (Gouedard et al., 2000) while in female mice *Bmpr1b* was detected in the ocyte and granulosa cells within the ovary (Yi et al., 2001). In female mice *Bmpr1b* deficiency led to irregular oestrous cycles, defective cumulus cell expansion attributed to decreased aromatase production by granulosa cells and the absence of endometrial glands. In male mice *Bmpr1b* deficiency resulted in compromised fertility which was attributed to defective development of the seminal vesicles (Yi et al., 2001) and correspondingly *Bmpr1a* and *Bmpr1b* mRNA are expressed in seminal vesicle epithelium of immature mice (Settle et al., 2001). In SMAT-1 cells AMH signalling via BMPR-IA suppressed P450 side chain cleavage enzyme (*P450scc*) transcription, while BMPR-IB antagonised this effect (Belville et al., 2005), suggesting that type I BMP receptors are likely to modulate steroid production by altering steroidogenic enzyme mRNA expression.

In the testis steroidogenic enzymes are crucial for regulated steroid synthesis. P450scc is responsible for converting cholesterol into pregnenolone (Rosen & Cedars, 2004), while 17 α-hydroxylase P450 (Cyp17A) metabolises the steroid precursor pregnenolone into dehydroepiandrosterone (Payne & Hales, 2004). 3β-hydroxysteroid dehydrogenase (3β-HSD) is required for converting pregnenolone and dehydroepiandrosterone into androstenedione while 17β-HSD converts androstenedione into testosterone (Payne & Hales, 2004). Aromatase

converts androstenedione and testosterone into estrone and estradiol respectively (Payne & Hales, 2004). Together these enzymes allow for highly regulated steroid production. Steroidogenic enzymes 3β-HSD, 17β-HSD, P450scc, 17 α-hydroxylase P450 and aromatase have been reported in Leydig, Sertoli and germ cells in numerous species (Bilaspuri & Guraya, 1984; Tsubota et al., 1993; Prisco et al., 2008). Furthermore, in spotted ray 3β-HSD and 17β-HSD have been characterized in Leydig cells during meiosis and in Sertoli cells before meiosis and post spermiation (Prisco et al., 2008), suggesting that the distribution and intensity of 3β-HSD expression in Leydig, Sertoli and germ cells plays a role in the regulation of steroid production required for developing germ cells in spotted ray. It could be possible that this phenomenon is also important in other species however this remains to be tested.

In this study we investigated the function of BMPR-IB in growth, reproductive development, serum and intra-testicular testosterone and mRNA expression of key testicular steroidogenic enzymes (*Cyp19*, *Hsd3b1*, *Hsd3b6* and *Hsd17b3*) in pubertal male mice using *in vivo* immune neutralization of BMPR-IB.

4.2 Experimental Design

Male Swiss Quackenbush mice (Physiology Animal House, University of New England, NSW, Australia) aged 3 weeks were directed into 4 groups (A, B, C, D) containing five animals each and there was no significant difference in body weight (Table 4.1). Treatments for each group were administered via subcutaneous injection using 100 µl PBS as the carrier. Group A received 50 µg of non-immune Ig as negative controls, group B received 50 µg anti-BMPR-IB, group C received 2 IU eCG as positive controls and group D received a combination of 2 IU eCG and 50 µg anti-BMPR-IB. The treatments were administered every day for 6 days at 10:00 am. On the 7th day the mice were sacrificed by asphyxiation with CO_2 and the testis and seminal vesicles were removed and weighed. In chapter 3 the mean testis weight of 3 week old mice was ~25mg, while in chapter 4 the mean testis weight was ~100mg owing to natural variations in the growth rate of mouse pups based on litter size – small litter sizes means pups grow much faster resulting in greater weight while large litter sizes means pups grow much slower resulting in significantly smaller weights, however most pups catch up by 8 weeks (indicated in Figure 3.2 and Figure 5.1). Testis samples for 3β-HSD histochemistry were collected in bouins fixative for histology as described in the general materials and methods in Chapter 2. Testis samples were also stored at -20 °C for homogenization and approximately 50 mg testis was placed in RNALater (Ambion, Austin TX) and incubated at 4 °C overnight then stored at -80 °C until RNA extraction followed by RT-qPCR using *β-actin* as the reference gene. Blood samples were taken via cardiac puncture and the serum collected and stored at -20 °C until steroid extraction. Steroid extracts and homogenates were assayed using a testosterone radioimmunoassay. The experiments were repeated twice.

4.3 Results

4.3.1 Body Weight of Pubertal Mice Immunized Against BMPR-IB

BMPR-IB immunization had no significant effect on weight gain in basal or eCG-stimulated conditions and over the period of 7 days mice in all treatment groups gained on average 7.7 grams (Table 4.1).

Table 4.1

Body Weight (g) of Pubertal Mice						
	Start of Experiment		*End of Experiment*		*Weight Gain*	
Treatment	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**
Control	11.15	0.39	18.58	0.62	7.43	0.56
Anti-BMPRIB	11.09	0.37	18.45	0.64	7.36	0.67
eCG	11.47	0.44	20.09	0.60	8.62	0.53
Anti-BMPRIB+eCG	11.32	0.39	18.78	0.67	7.46	0.55

4.3.2 Seminal Vesicle and Testis Weight in BMPR-IB immunized Pubertal Mice

In pubertal mice treatment with equine chorionic gonadotrophin (eCG) significantly increased seminal vesicle weight by 2.9 fold ($P < 0.0001$) and BMPR-IB neutralization decreased eCG-mediated seminal vesicle weight gain by 1.4 fold were $P \leq 0.002$ (Figure 4.1 panel A). In basal conditions the BMPR-IB immunized group seminal vesicle weight was similar to the control. BMPR-IB immunization had no effect on testis weight in either basal or eCG-stimulated conditions (Figure 4.1 panel B).

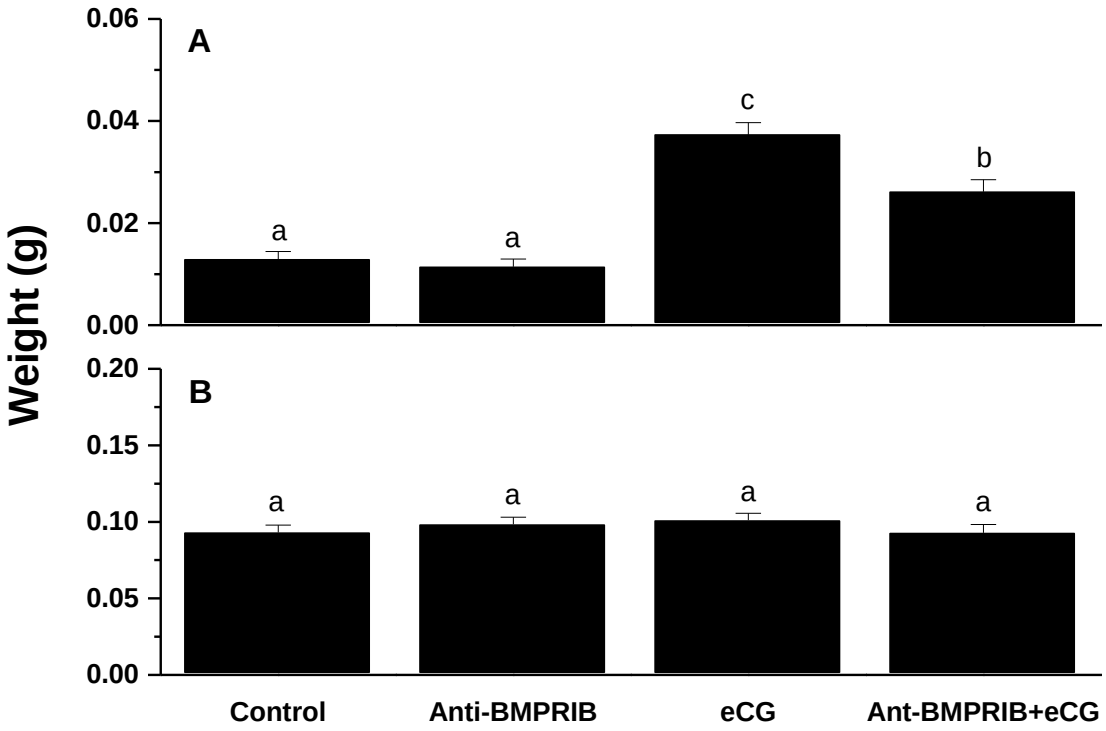


Figure 4.1 (Panel A) Seminal vesicle weight (g) and (Panel B) testis weight (g) in control, BMPR-IB immunized, eCG and BMPR-IB immunized plus eCG treatment groups of pubertal male mice after 6 days of treatment. Results expressed as Mean ± SE. Different letters denote significant differences between means ($P < 0.05$). N=15.

4.3.3 Serum and Intra-testicular Testosterone in BMPR-IB Immunized Pubertal Male Mice

In eCG treated pubertal mice, serum testosterone was elevated 1.5 fold compared to the control ($P < 0.004$) as shown in Figure 4.2. BMPR-IB immunization appeared to reduce basal testosterone levels however this was not statistically significant and while BMPR-IB immunization in combination with eCG did not significantly alter eCG-stimulated testosterone production, it was also not statistically different from the control ($P < 0.06$) indicating a mild reduction in testosterone (Figure 4.2).

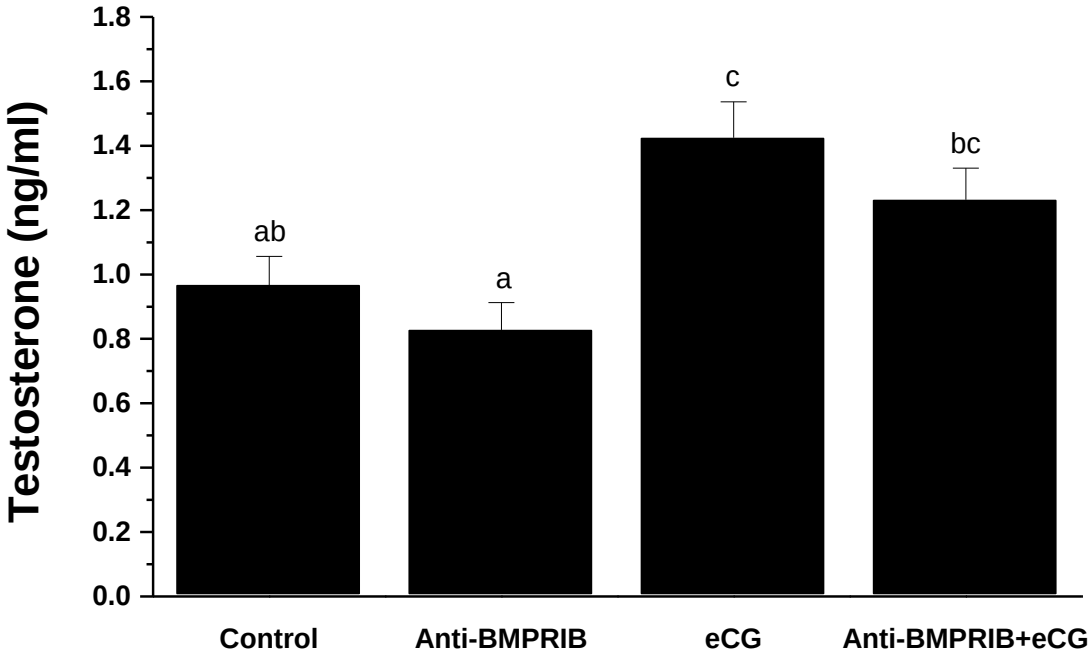


Figure 4.2 Serum testosterone (ng/ml) in control, BMPR-IB immunized, eCG and BMPR-IB immunized plus eCG treatment groups of pubertal male mice after 6 days of treatment. Results expressed as Mean ± SE. Different letters denote significant differences between means ($P < 0.05$). N = 15.

Testosterone levels from testis homogenate had a similar pattern to serum testosterone (Figure 4.3). Equine CG increased testosterone by 3.8 fold from the control ($P < 0.0001$), and while BMPR-IB immunization had no significant effect on basal or eCG-mediated testosterone production, BMPR-IB immunization appeared to reduce eCG-stimulated testosterone.

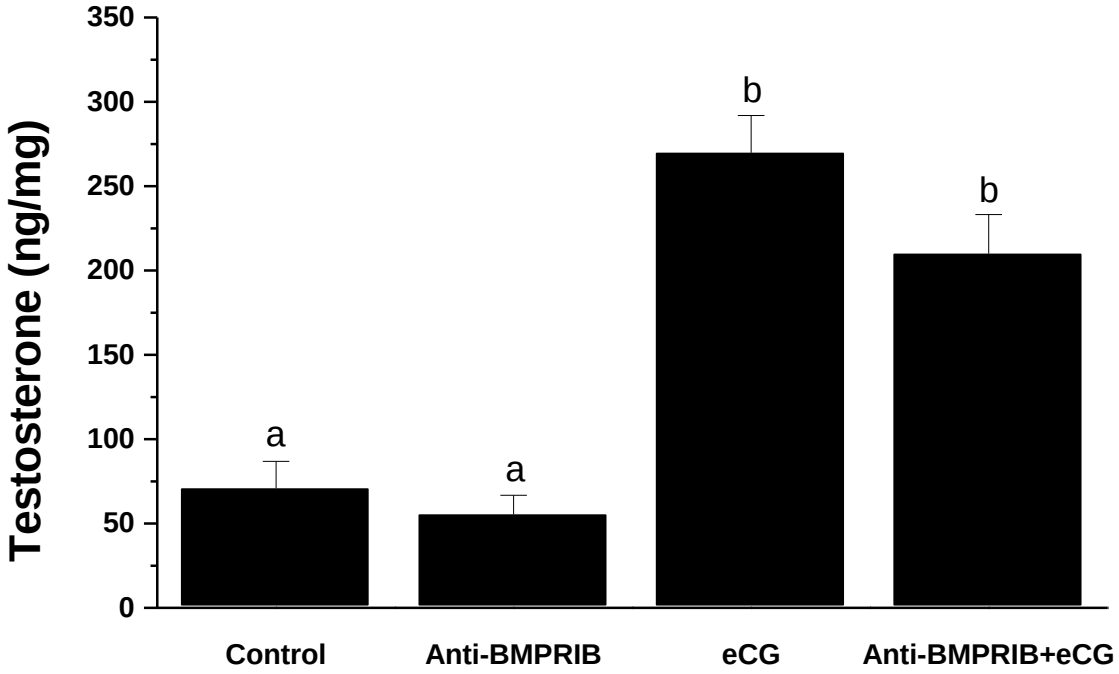


Figure 4.3 Intra-testicular testosterone (ng/mg) in control, BMPR-IB immunized, eCG and BMPR-IB immunized plus eCG treatment groups of pubertal male mice after 6 days of treatment. Results expressed as Mean ± SE. Different letters denote significant differences between means ($P < 0.05$). N = 15.

4.3.4 3β-Hydroxysteroid Dehydrogenase Staining in BMPR-IB Immunized Pubertal Mouse Testis

3β-HSD staining in control, BMPR-IB immunized, eCG treated and BMPR-IB immunized plus eCG treated groups of pubertal mouse testis are shown in Figure 4.4 panels A, B, C and D respectively. Staining for 3β-HSD was detected in spermatogonia and spermatocytes in control testis while BMPR-IB immunization markedly reduced spermatogonial and spermatocyte 3β-HSD staining. Equine CG treatment up-regulated Leydig cell 3β-HSD staining and down-regulated enzyme staining in spermatogonia and spermatocytes, while immunization against BMPR-IB notably reduced eCG-stimulated Leydig cell 3β-HSD staining.

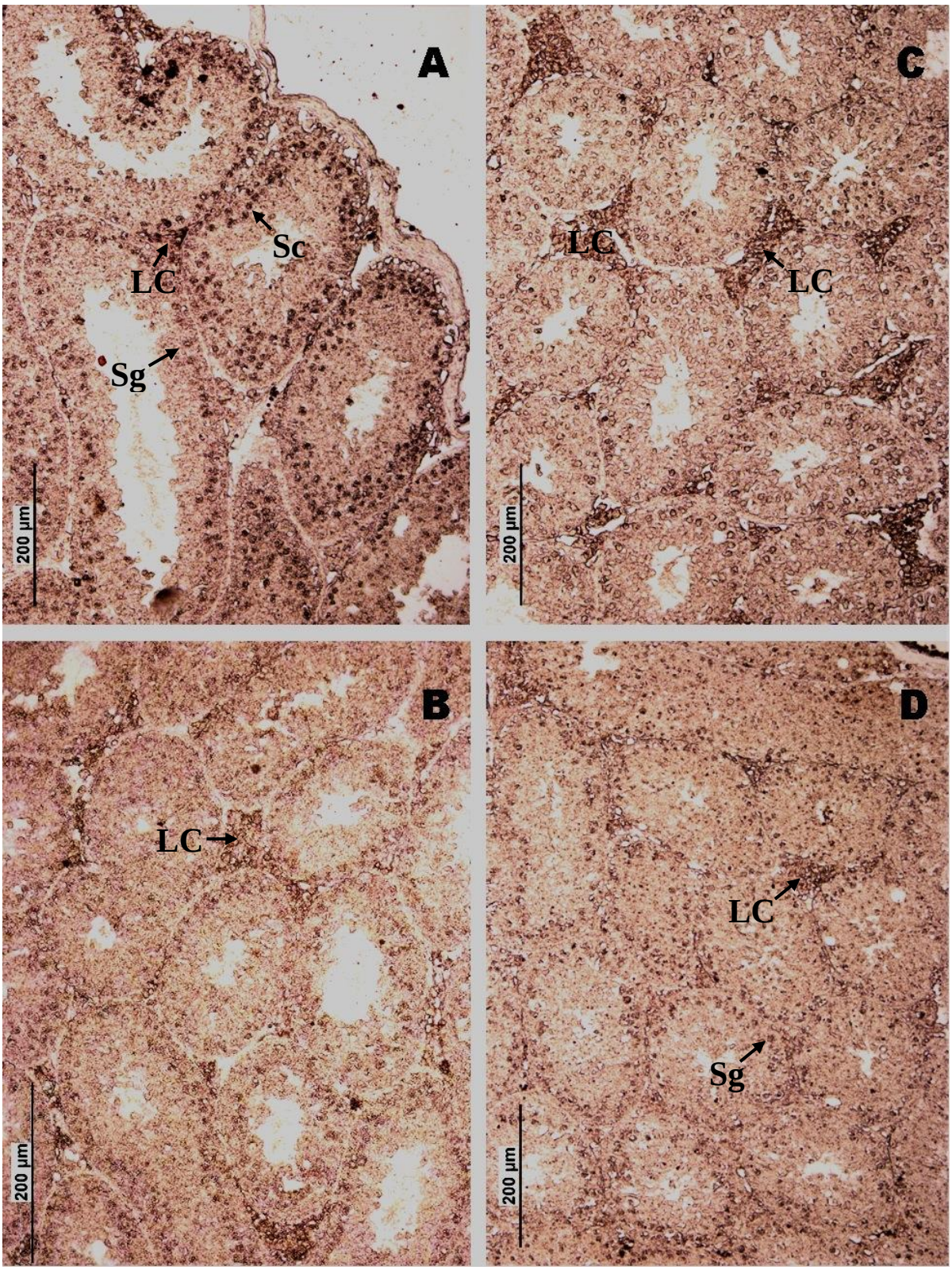


Figure 4.4 3β-HSD staining in (A) Control, (B) BMPR-IB immunized, (C) eCG treated and (D) BMPR-IB immunized plus eCG treated groups of pubertal mouse testis at 20x magnification. Spermatogonium (Sg), spermatocyte (Sc), Leydig cells (LC).

4.3.5 The mRNA Expression of *Cyp19, Hsd3b1*, *Hsd3b6* and *Hsd17b3* in Pubertal Mouse Testis Immunized Against BMPR-IB

Testicular expression of *Cyp19*, *Hsd3b1*, *Hsd3b6* and *Hsd17b3* mRNA in control, BMPR-IB immunized, eCG treated and BMPR-IB immunized plus eCG treated groups of pubertal mice is demonstrated in Figure 4.5. Treatment with eCG significantly increased *Hsd3b1* by 1.9 fold (P = 0.0004) and decreased *Hsd3b6* by 1.8 fold (P < 0.02). BMPR-IB neutralization had no significant effect on *Cyp19*, *Hsd3b1* or *Hsd17b3* expression in basal or stimulated states while it mildly increased *Hsd3b6* in eCG-stimulated testis.

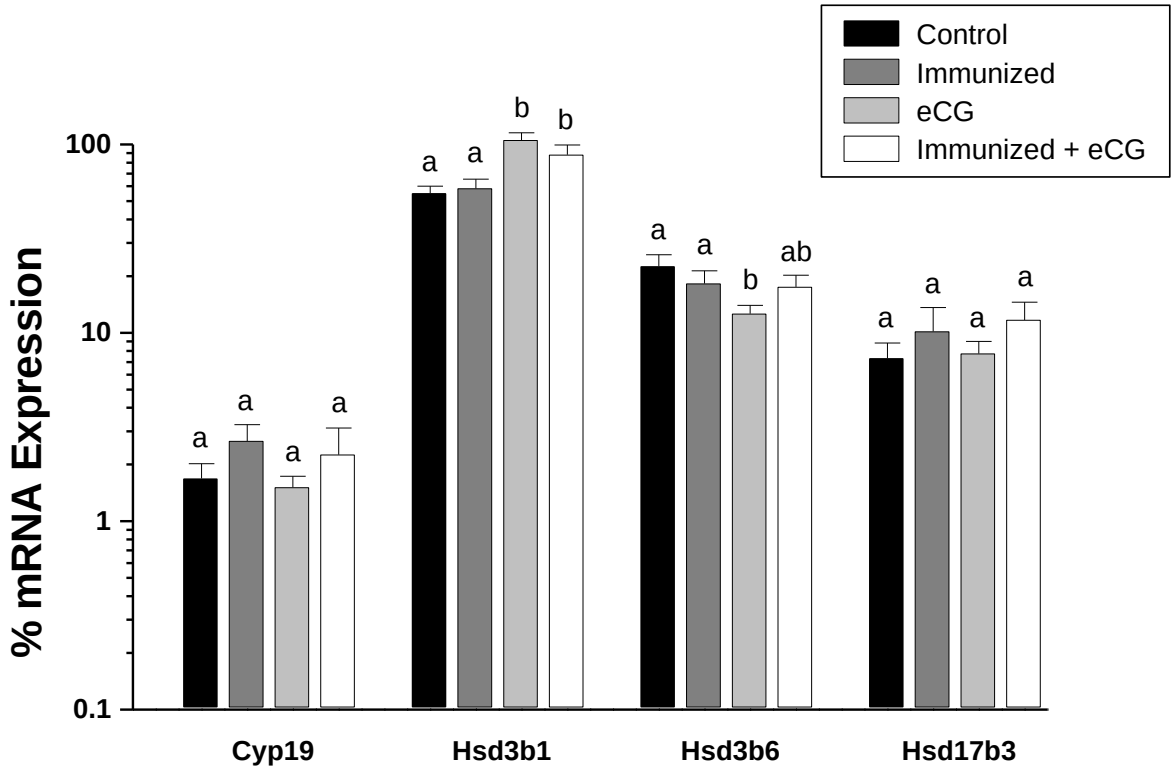


Figure 4.5 *Cyp19, Hsd3b1, Hsd3b6* and *Hsd17b3* mRNA expression in control, BMPR-IB immunized, eCG and BMPR-IB immunized plus eCG treated pubertal mouse testis after 6 days of treatment. Results were calculated as a percentage of the housekeeping gene *β-actin* and presented as Mean ± SE. Different letters denote significant differences between means (P < 0.05) of individual genes. N = 5.

4.4 Discussion

Using BMPR-IB immunization, our study demonstrates that the type I BMP receptor has a role in gonadotrophin stimulated seminal vesicle growth and a minor role in testosterone production of pubertal mice.

While serum testosterone has been known to fluctuate throughout the day (Lucas & Eleftheriou, 1980), mean values of basal testosterone in pubertal mice have previously been recorded at 1 ng/ml (Grasso et al., 1997), which was consistent with our findings. All mice in our study responded to eCG by up-regulating testosterone production as eCG possesses unique LH and FSH activity (Moore & Ward, 1980). Seminal vesicles of eCG-stimulated mice were significantly heavier than the control and correspondingly seminal vesicles have been demonstrated to be extremely sensitive to androgen dependent vascularization, growth and secretory activity (Tajana et al., 1984; Kashiwagi et al., 2005).

In vivo anti-BMPR-IB caused a significant decrease in eCG-stimulated seminal vesicle weight gain of pubertal mice, but did not significantly affect eCG-stimulated steroidogenic enzyme expression or intra-testicular testosterone while it appeared to reduce 3β-HSD staining and mildly reduce serum testosterone. *Bmpr1b* mRNA has been shown to be expressed in the seminal vesicle epithelium of immature mice (Settle et al., 2001) and *Bmpr1b* deficiency in male mice caused defective growth of seminal vesicles leading to undersized seminal vesicles in adults (Yi et al., 2001). This corresponds with our findings that signalling through BMPR-IB is important in seminal vesicle growth in pubertal mice. The fact that BMPR-IB immunization reduced seminal vesicle weight significantly without having a marked effect on eCG-mediated testosterone production may suggest the sensitivity of seminal vesicles to

small changes in testosterone but it is more likely that BMPR-IB signalling is downstream of testosterone stimulation.

Testosterone was mildy reduced in BMPR-IB immunized pubertal mice indicating a minor role in the regulation of androgen production. BMPR-IB had been detected in SMAT-1 cells derived from immature Sertoli cells where BMPR-IB antagonized the action of AMH signalling via BMPR-IA and mediated the up-regulation of steroidogenic enzyme *P450scc* mRNA (Gouedard et al., 2000; Belville et al., 2005). This suggests that BMPR-IB may enhance testosterone production by modulating steroidogenic enzymes, however we did not detect definitive changes in steroidogenic enzyme mRNA expression. 3β-HSD activity was detected in germ cells and somatic cell types using 3β-HSD staining and BMPR-IB immunization may have subtle effects on enzyme distribution, which could explain why net testicular steroidogenic enzyme expression did not yield any conclusions. In female mice 3β-HSD has been detected in ovarian interstitium and granulosa cells (Hart et al., 1966) while 3β-HSD activity has also been reported in mouse oocytes (Niimura & Kawakami, 2003) indicating that the presence of 3β-HSD in somatic and germ cells within the gonads is not exclusive to males.

3β-HSD staining in pubertal mouse testis suggests that BMPR-IB may be implicated in the up-regulation of spermatogonial and spermatocyte 3β-HSD under basal conditions while indicating only modest activity in Leydig cells, while in gonadotrophin-stimulated conditions, where eCG was used to bypass the hypothalamic-pituitary-gonadal axis BMPR-IB is implicated in the stimulation of Leydig cell 3β-HSD. Putative ligands of BMPR-IB, BMP-2 and BMP-7 have been shown to enhance FSH-stimulated proliferation of Sertoli cells and spermatogonia in immature mouse testis (Puglisi et al., 2004), furthermore, Sertoli cell derived

SMAT-1 cells express BMPR-IB (Belville et al., 2005), and blocking this signalling pathway may decrease the stimulatory actions of gonadotrophins on Leydig cell 3β-HSD seen in the pubertal mice in our study. Low intensity 3β-HSD staining (low serum and intra-testicular testosterone) occurred in controls and high intensity activity (elevated serum and intra-testicular testosterone) was observed in eCG-stimulated testis. Accordingly, in humans hCG is known to stimulate 3β-HSD activity in the testis (Labrie et al., 1994). While BMPR-IB immunization had no pronounced effect on steroidogenic enzymes *Cyp19*, *Hsd3b1*, *Hsd3b6 or Hsd17b3*, eCG significantly increased *Hsd3b1*, decreased *Hsd3b6* and had no effect on *Hsd17b3*. This pattern of expression is very different to what is seen in adults with Baker et al. (1997) and Baker et al. (2003) demonstrating that *Hsd3b6* and *Hsd17b3* expression increased with gonadotrophin treatment, while *Hsd3b1* expression decreased mildly.

In conclusion, BMPR-IB has a role in promoting eCG-mediated seminal vesicle growth and a minor role in the augmentation of testosterone production in pubertal male mice.

Chapter 5

5.0 The Effects of *in vivo* BMPR-IB Immunization on Reproductive Characteristics of Adult Male Mice

5.1 Introduction

In males LH and FSH from the pituitary and growth factors including transforming growth factor-β (TGF-β) superfamily members regulate steroidogenesis, spermatogenesis and fertility. TGF-β superfamily ligands which include BMPs and their receptors have been identified to have important roles in fertility via their effects on testosterone production, germ cell maturation, epithelial secretory function and integrity of reproductive tissues (Zhao et al., 1998; Teixeira et al., 1999; Settle et al., 2001; Hu et al., 2004).

BMPR-IB is expressed in mouse, rabbit and sheep testis (Gouedard et al., 2000; Wilson et al., 2001). *BMPRIB* mRNA has also been found in human testicular cancer with germ cell origins (Fustino et al 2011) while a *Bmpr1b* mutation in zebrafish testis was directly linked to germ cell tumors (Neumann et al. 2011). In adult humans BMPR-IA, BMPR-IB and BMPR-II have been detected in the prostate (Kim et al., 2004) and we have shown high levels of these receptor mRNAs in mouse prostate as well (Chapter 3). This suggests that signalling via these receptors is likely to have important functions in reproductive physiology.

In male mice *Bmpr1b* deficiency resulted in compromised fertility attributed to defects in seminal vesicle development (Yi et al., 2001), however whether *Bmpr1b* mutants had altered testicular function has not been reported. Seminal vesicles express *Bmpr1b* mRNA in immature mice (Settle et al., 2001) and we showed that *Bmpr1b* is expressed in adult seminal vesicle as well.

Steroidogenic enzymes P450scc, 3β-HSD, 17β-HSD, 17 α-hydroxylase P450 and aromatase have been reported in both steroidogenic and non-steroidogenic cells of the testis in a variety of species (Bilaspuri & Guraya, 1984; Tsubota et al., 1993; Prisco et al., 2008) and are vital for regulated steroid production. In SMAT-1 cells BMPR-IA activation suppressed *P450scc* mRNA (Belville et al., 2005), while in ovarian cells BMP-2, BMP-4 and BMP-6 have been shown to enhance estradiol or suppress androstenedione (Dooley et al., 2000; Souza et al., 2002; Brankin et al., 2005) indicating that BMPs via their receptors can influence steroidogenic enzyme activity and thereby steroid production.

AMH and AMHR-II found on SMAT-1 cells stimulated signalling by Smad1 and Smad4 similar to the effects of BMP-2 (Gouedard et al., 2000), indicating receptor complex formation with BMPR-IA or BMPR-IB, which can stimulate the same combination of Smads. In AMHR-II transfected CHO-3W cells derived from Chinese hamster ovary, AMHR-II has been immuno-precipitated with BMPR-IB (Gouedard et al., 2000), while disrupted BMPR-IA expression in embryonic murine Mullerian duct mesenchyme leads to pseudohermaphrodism similar to mutations of AMHR-II or AMH indicating receptor complex formation between BMPR-IA and AMHR-II (Jamin et al., 2002). This suggests differential roles for BMP receptors based on cell type and collectively that upon ligand activation type I BMP receptors are likely to modulate steroid production in the reproductive system via their effect on steroidogenic enzymes.

In this study we investigated the function of BMPR-IB in weight regulation, reproductive development, serum and intra-testicular testosterone and testicular expression of *Hsd3b1*, *Hsd3b6*, *Hsd17b3* and *Cyp19* in adult mice using *in vivo* immune neutralization of BMPR-IB.

5.2.1 Experimental Design

Male Swiss Quackenbush mice (Physiology Animal House, University of New England, NSW, Australia) aged 8 weeks were placed into 4 groups (A, B, C, D) containing five animals each and there was no significant difference in body weight (Table 5.1). Treatments for each group were administered via subcutaneous injection using 100 µl PBS as the carrier. Group A received 50 µg of non-immune Ig as negative controls, group B received 50 µg anti-BMPR-IB, group C received 2 IU eCG as positive controls and group D received a combination of 2 IU eCG and 50 µg anti-BMPR-IB. The treatments were administered every day for 6 days at 10:00 am. On the 7th day the mice were sacrificed by asphyxiation with CO_2 and the testis and seminal vesicles were removed and weighed. Testis samples for 3β-HSD histochemistry were collected in bouins fixative for histology as described in the general materials and methods in Chapter 2. Testis samples were also stored at -20 °C for homogenization and approximately 50 mg testis was placed in RNALater (Ambion, Austin TX) and incubated at 4 °C overnight then stored at -80 °C until RNA extraction followed by RT-qPCR using *β-actin* as the reference gene. Blood samples were taken via cardiac puncture and the serum was collected and stored at -20 °C until steroid extraction. Steroid extracts and homogenates were assayed using a testosterone radioimmunoassay. The experiments were repeated twice.

5.3 Results

5.3.1 Body Weight of Adult Mice Immunized Against BMPR-IB

BMPR-IB immunization had no effect on body weight in basal or eCG-stimulated conditions in adult male mice (Table 5.1). Variable amounts of weight gain were observed in individual animals attributed to fat deposition.

Table 5.1

Body Weight (g) of Adult Mice						
	Start of Experiment		*End of Experiment*		*Weight Gain*	
Treatment	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**
Control	36.46	0.53	37.51	0.56	1.05	0.47
Anti-BMPRIB	36.13	0.52	37.05	0.55	0.91	0.55
eCG	36.63	0.75	36.71	0.93	0.08	0.98
Anti-BMPRIB+eCG	36.06	0.76	37.55	0.74	1.50	0.64

5.3.2 Seminal Vesicle and Testis Weight in BMPR-IB Immunized Adult Mice

In adult mice group seminal vesicle weight was significantly elevated in the equine chorionic gonadotrophin (eCG) stimulated cohort by 1.3 fold relative to the control ($P \leq 0.0001$), however anti-BMPR-IB did not significantly alter basal or eCG-stimulated seminal vesicle weight (Figure 5.1 panel A). BMPR-IB immunization had no effect on testis weight in basal or eCG-stimulated conditions in adult mice (Figure 5.1 panel B).

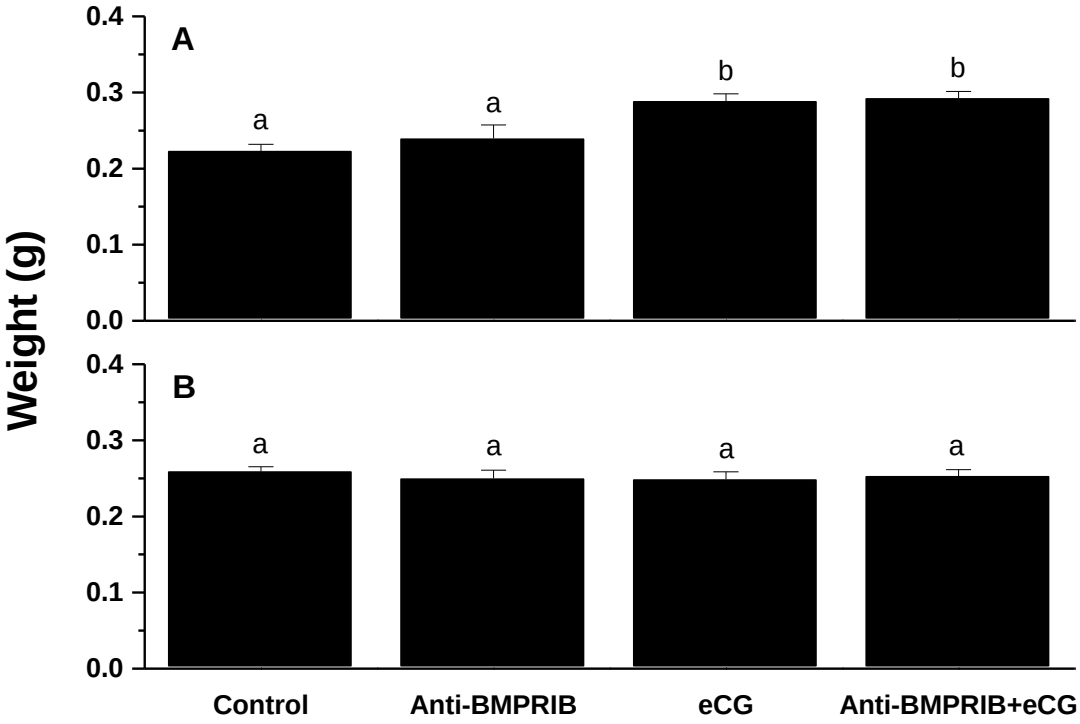


Figure 5.1 (Panel A) Seminal vesicle weight (g) and (Panel B) testis weight (g) in control, BMPR-IB immunized, eCG and BMPR-IB immunized plus eCG treatment groups of mature male mice after 6 days of treatment. Results expressed as Mean ± SE. Different letters denote significant differences between means ($P \leq 0.05$). N=15.

5.3.3 Serum and Intra-testicular Testosterone in BMPR-IB Immunized Adult Male Mice

In adult male mice serum testosterone concentration in the anti-BMPR-IB treatment group was significantly increased by 4.3 fold relative to the control ($P < 0.04$) as shown in Figure 5.2. Testosterone was also increased in the eCG treated cohort by 5.5 fold ($P < 0.0001$), while immunization did not alter eCG-induced testosterone production. Intra-testicular testosterone had a similar pattern to that of serum testosterone (Figure 5.3), where BMPR-IB immunization significantly increased basal intra-testicular testosterone content by 3.1 fold ($P < 0.04$), and treatment with eCG increased testosterone 5.2 fold ($P < 0.0001$), while immunization had no effect on eCG-stimulated testosterone.

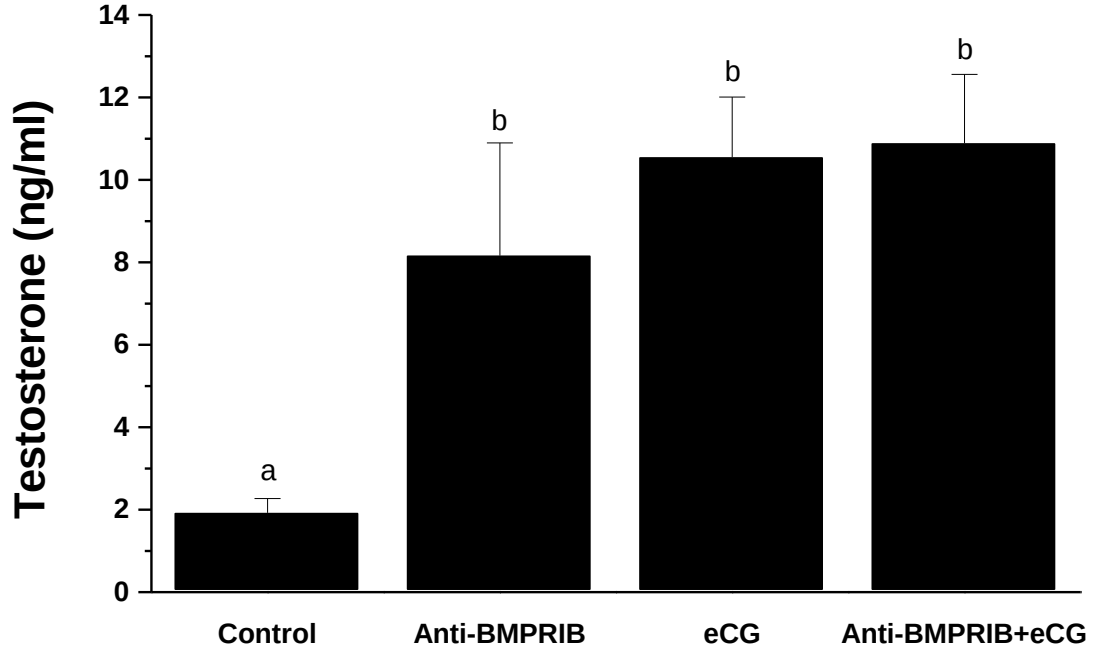


Figure 5.2 Serum testosterone (ng/ml) in control, BMPR-IB immunized, eCG and BMPR-IB immunized plus eCG treatment groups of mature male mice after 6 days of treatment. Results expressed as Mean ± SE. Different letters denote significant differences between means ($P < 0.05$). N = 15.

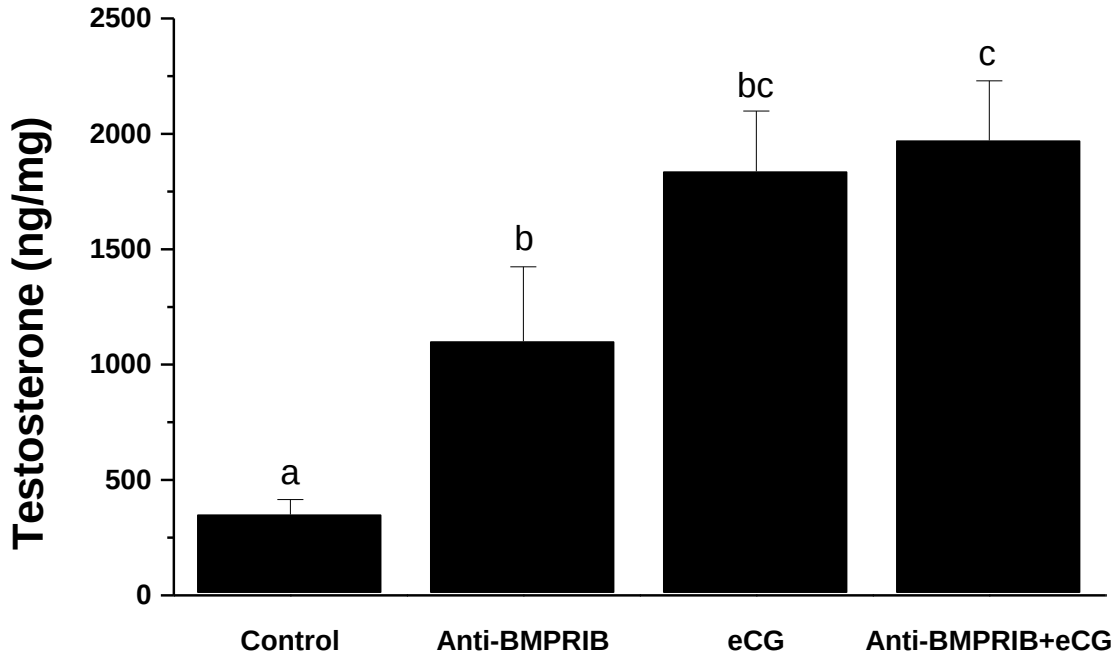


Figure 5.3 Intra-testicular testosterone (ng/mg) in control, BMPR-IB immunized, eCG and BMPR-IB immunized plus eCG treatment groups of mature male mice after 6 days of treatment. Results expressed as Mean ± SE. Different letters denote significant differences between means ($P < 0.05$). N = 15

5.3.4 3β-Hydroxysteroid Dehydrogenase Staining in BMPR-IB Immunized Adult Mouse Testis

Staining for 3β-HSD in control, BMPR-IB immunized, eCG treated and BMPR-IB immunized plus eCG treated groups of adult mouse testis are visualized in Figure 5.4 panels A, B, C and D respectively. In mature mouse testis 3β-HSD staining localized the enzyme in Leydig cells, spermatogonia, elongating spermatids and spermatozoa, and BMPR-IB immunization reduced germ cell 3β-HSD staining to non-detectable levels, while strongly enhancing Leydig cell 3β-HSD. Equine CG treatment also enhanced Leydig cell 3β-HSD staining, while reducing enzyme staining in spermtogonia and maintaining it in elongating spermatids and immature spermatozoa. BMPR-IB immunization had no effect on eCG-stimulated 3β-HSD staining.

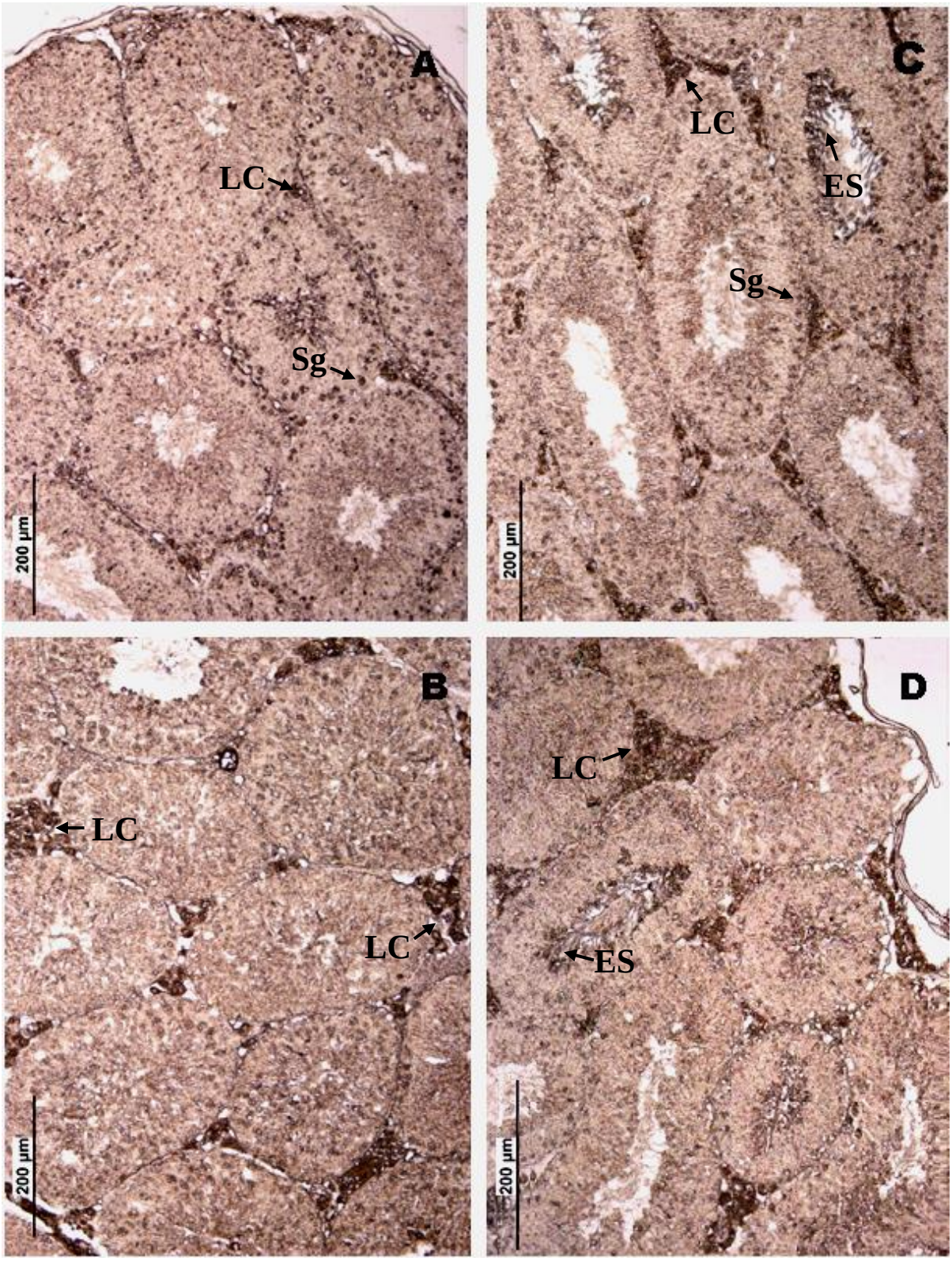


Figure 5.4 3β-HSD staining in (A) Control, (B) BMPR-IB immunized, (C) eCG treated, and (D) BMPR-IB immunized plus eCG treated groups of mature mice at 20x magnification. Spermatogonium (Sg), elongating spermatids (ES), Leydig cells (LC).

5.3.5 The mRNA Expression of *Cyp19*, *Hsd3b1*, *Hsd3b6* and *Hsd17b3* in Adult Mouse Testis Immunized Against BMPR-IB

In adult testis in basal conditions BMPR-IB immunization significantly reduced *Cyp19* expression by 1.4 fold (P ≤ 0.005), increased *Hsd3b1* expression by 1.5 fold (P ≤ 0.006) and reduced *Hsd3b6* expression by 2 fold (P ≤ 0.03), while having no effect on *Hsd17b3* (Figure 5.5). Treatment with eCG significantly decreased the expression of *Cyp19* by 1.6 fold (P < 0.02), Hsd3b1 by 2 fold (P ≤ 0.01), while it increased the expression of *Hsd3b6* by 2.5 fold (P < 0.0009) and *Hsd17b3* by 1.9 fold (P ≤ 0.02). BMPR-IB immunization in eCG-stimulated conditions caused a mild increase in *Cyp19* and *Hsd3b1* expression but had no effect on *Hsd3b6* or *Hsd17b3*.

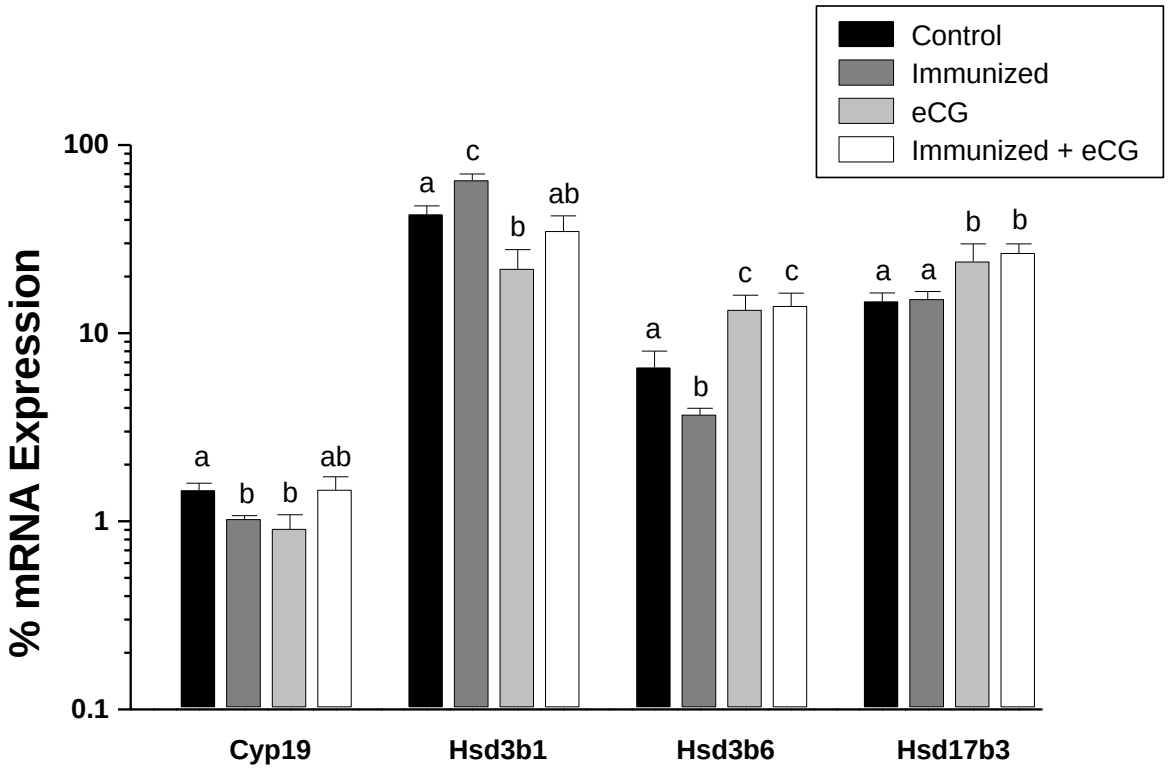


Figure 5.5 *Cyp19, Hsd3b1, Hsd3b6 and Hsd17b3* mRNA expression in control, BMPR-IB immunized, eCG and BMPR-IB immunized plus eCG treated adult mouse testis. Results were calculated as a percentage of the housekeeping gene *β-actin* and presented as Mean ± SE. Different letters denote significant differences between means (P < 0.05) of individual genes. N = 5.

5.4 Discussion

This study demonstrates that the BMPR-IB receptor has a significant role in testosterone production in mature male mice. In adult mice basal testosterone levels were recorded around 2 ng/ml, which was consistent with basal testosterone values recorded by other researchers (Wilson & LeBlanc, 2000; Capkova et al., 2006; Alvarez et al., 2008).

Immunization against BMPR-IB caused no obvious anatomical or histological differences in the testis of adult mice. This is similar to the observation that *Bmpr1b* deficiency in female mice also presented with no obvious histological differences regarding ovarian follicular development (Yi et al., 2001). In adult mice BMPR-IB signalling by TGF-β superfamily ligands may be implicated in the inhibition of testosterone production under basal conditions but not gonadotrophin-stimulated conditions as immunized mice had significantly increased production of testosterone only in basal conditions. BMPs which may facilitate a reduction in testosterone indirectly, derived from studies of ovarian cells, include BMP-2 shown to enhance estradiol production in sheep granulosa cell culture (Souza et al., 2002), BMP-4 shown to inhibit androstenedione in human theca-like tumor cells (Dooley et al., 2000) and BMP-6 demonstrated to suppress androstenedione in 6 day porcine granulosa/theca cell culture (Brankin et al., 2005). As estradiol is synthesized from testosterone and its precursor androstenedione, signalling by these BMPs could result in decreased testosterone.

3β-HSD staining was detected in testicular interstitial cells and germ cells particularly elongating spermatids, which is similar to findings by Bilaspuri & Guraya (1984) who also identified 3β-HSD in goat testicular interstitial cells and seminiferous tubules particularly in spermatozoa and elongated spermatids. Staining

for 3β-HSD suggests that in unstimulated conditions BMPR-IB is implicated in the detection of spermatogonial 3β-HSD, however, it also seems to have strong inhibitory effects on Leydig cell 3β-HSD as evidenced by intense staining for Leydig cell 3β-HSD in immunized animals. Furthermore, the intensity of 3β-HSD staining correlated to testosterone levels and there appeared to be an inverse pattern of 3β-HSD staining between Leydig cells and spermatogonia in immunized and non-immunized mature mice, indicating that Leydig cells may influence germ cell expression of 3β-HSD and vice versa. In support of these observations 3β-HSD and 17β-HSD have been characterized in Leydig cells during meiosis and in Sertoli cells before meiosis and post spermiation in spotted ray (Prisco et al., 2008). Furthermore, spermatogonia have also been shown to have steroidogenic enzymes and additionally the ultra-structural components of steroid synthesizing cells (Prisco et al., 2008), evidence that cell communication between Leydig, Sertoli and germ cells may play a role in normal reproductive physiology.

The effect of BMPR-IB neutralization on the testicular expression of *Hsd3b1* and *Hsd3b6* was opposite to the effects of eCG. In adult testis BMPR-IB immunization significantly increased *Hsd3b1* expression and significantly decreased *Hsd3b6* expression indicating that androgen production was regulated via the modulation of specific isoforms of HSD. By deduction we demonstrate that signalling through BMPR-IB functions to suppress basal and eCG-stimulated *Hsd3b1* expression, while being necessary for uninhibited expression of *Hsd3b6* only under basal conditions. Immunization also reduced *Cyp19*, which upon translation is known as aromatase and is required for converting testosterone to estradiol (Rosen & Cedars, 2004). We provide evidence that BMPR-IB is required for uninhibited *Cyp19* expression in basal conditions and inhibited expression of *Cyp19*

in eCG-stimulated conditions. BMPR-IB seems to have a role in aromatase production in males and females as *Bmpr1b* mutant female mice also have reduced *Cyp19* expression within the ovary (Yi et al., 2001).

As the regulation of *Hsd3b6* and *Hsd17b3* are gonadotrophin dependent (Baker et al., 1997; Baker et al., 2003) also observed in our study, BMPR-IB is likely to mediate the action of BMPs or other TGF-β superfamily members locally within the testis to decrease testosterone production and is unlikely to enhance the production of the pituitary gonadotrophins, because if LH secretion had been stimulated *Hsd3b6* expression would have been up-regulated which was not the case. We found that gonadotrophin treatment significantly down-regulated *Hsd3b1* while Baker *et al.* (2003) found *Hsd3b1* to be gonadotrophin independent. Baker *et al.* (2003) used multiple treatments of gonadotrophin in GnRH-null mice which reduced *Hsd3b1* expression but this did not reach significance. The fact that we used normal mice and treated them over a longer time period is likely to have contributed to this difference. The decrease in *Cyp19* expression and increase in *Hsd3b1* expression correlates well to the increased testosterone production observed in immunized male mice.

In conclusion, BMPR-IB functions to suppress testosterone production via its affect on *Cyp19*, *Hsd3b1* and *Hsd3b6* mRNA expression in adult male mice *in vivo*.

Chapter 6

6.0 Effects of anti-BMPR-IB on Testosterone Production in Testicular Culture and Leydig Cell Culture of Male Mice

6.1 Introduction

Studies on transforming growth factor-β (TGF-β) superfamily members have shown that these proteins and their receptors are involved in the regulation of steroid production in the testis, while little is known about BMPR-IB specifically. Anti-Mullerian hormone receptor II (AMHR-II) has been found in rat Leydig cells and in mouse Leydig cell derivatives R2C cells and MA-10 cells (Teixeira et al., 1999) where BMPR-IB and Smad1 have been co-localized (Gouedard et al., 2000). Furthermore, AMHR-II activated Smad1 in granulosa cell tumours exhibited by transgenic AMH-SV40 mice (Dutertre et al., 2001) suggesting that it forms complexes with BMP type I receptors, which are known to employ SMAD1, SMAD5 and/or SMAD8 (Hoodless et al., 1996; Liu et al., 1996; Nishimura et al., 1998; Aoki et al., 2001; Kersten et al., 2005).

Bmpr1a, *Bmpr1b*, *Amhr2* and Smad1 mRNAs were also expressed in the Sertoli cell line SMAT-1 (Gouedard et al., 2000; Belville et al., 2005) where AMH signalling via BMPR-IA down-regulated *Amhr2* and *P450scc*, the later essential for steroid production, while BMPR-IB antagonized the actions of BMPR-IA signalling (Belville et al., 2005). Collectively, this indicates that BMP receptor signalling components are present on steroidogenic and non-steroidogenic cells of the testis, and that they are likely to have key roles in the modulation of androgen production.

In previous chapters we have demonstrated that BMPR-IB is involved in the modulation of testosterone *in vivo* and have shown that BMP mRNA expression is present in the testis. Based on the actions of BMPs in ovarian steroidogenic enzyme expression and steroid production, roles for BMPs in male steroidogenesis can be

hypothesized. BMPs which may by indirect means result in decreased testosterone based on the enhancement of estradiol or inhibition of androstenedione in unstimulated conditions include BMP-2, BMP-4 and BMP-6 (Dooley et al., 2000; Souza et al., 2002; Brankin et al., 2005), while in FSH-stimulated conditions BMP-7, BMP-15 and AMH seem to have very similar functions (Lee et al., 2001b).

In this study we wanted to determine whether BMPR-IB had an autocrine or paracrine function in the regulation of male testosterone production *in vitro.* To determine which cell/tissue types BMPR-IB signals to modulate testosterone production, testicular tissue from pubertal and mature mice, and Leydig cells from mature male mice were cultured for 3 hours in normal and gonadotrophin treated media in the presence or absence of anti-BMPR-IB and testosterone secretion analyzed. Additionally, anti-BMPR-IB treated and/or gonadotrophin treated and non-treated seminiferous tubule pre-conditioned media and Leydig cell pre-conditioned media were also cultured with Leydig cells to investigate the contribution of Leydig cells and seminiferous tubules to anti-BMPR-IB reactivity in Leydig cell testosterone production. Lastly the functions of BMP-2 and BMP-4 were investigated in testicular interstitial cells using antibodies targeted against BMP-2 and BMP-4 to determine if they were likely ligands for BMPR-IB.

6.2 Experimental Design

Testicular tissue was obtained from mice aged 3 and 8 weeks for two separate experiments. The tissues were weighed and 20 mg was placed in each well of a 24-well polypropylene tissue culture plate (Sarstedt Pty Ltd) in one of 4 groups consisting of the control, anti-BMPR-IB, eCG and anti-BMPR-IB plus eCG in 1 ml of DMEM:F12. Anti-BMPR-IB was used at a concentration of 50 µg/ml and eCG at 0.1 IU/ml. Each well consisted of the following: 50 ul treatment (or PBS for controls), 1,000 ul cuture media DMEM:F12, and 20 mg testicular tissue. The tissue was cultured for 3 hours, after which the culture media was collected and assayed for testosterone using a radioimmunoassay. Experiments were repeated four times. N = 5.

Three separate Leydig cell experiments in (1) Unconditioned, (2) Leydig cell conditioned and (3) Seminiferous tubule conditioned media were undertaken. In all Leydig cell cultures DMEM:F12 was used as cell culture media, BMPR-IB antibodies were used at a concentration of 50 µg/ml, hCG was used at 1 IU/ml and FSH was used at 0.1 µg/ml. Each well consisted of the following: 5 ul treatment (or PBS for controls), 100 ul cuture media DMEM:F12 and 50 ul cell stock (1,000 Cells in 50 ul DMEM:F12).

Experiment 1 - Leydig cells were purified from 8 week old mouse testis and 50 µl cell stock (1,000 cells/well) cultured in 100 µl DMEM:F12 in treatment groups of control, anti-BMPRIB, hCG and anti-BMPRIB plus hCG in 96-well polypropylene cell culture plates (Sarstedt Pty Ltd). After 3 hours incubation the culture media was harvested and assayed for testosterone using a radioimmunoassay. The experiment was repeated four times. (N = 5).

Experiment 2 - Leydig cell conditioned media was harvested and pooled from Leydig cell culture in four respective groups (control, anti-BMPRIB, hCG and anti-BMPR-IB plus hCG). The Leydig cell conditioned media was then cultured for 3 hours with a fresh batch of Leydig cells using 50 µl cell stock (1,000 cells/well) in 100 µl Leydig cell conditioned media on 96-well polypropylene culture plates (Sarstedt Pty Ltd). After incubation the media from each group was harvested and stored at -20 °C. Leydig cell conditioned media testosterone was determined using a radioimmunoassay. Leydig cell conditioned medias were assayed before experimentation and the testosterone subtracted from the culture media. The experiment was repeated four times. (N = 5).

Experiment 3 - Seminiferous tubule conditioned media was created by dissecting 3 x 1 cm random segments of seminiferous tubles cultured in 5 ml DMEM in glass Petri dishes in control, anti-BMPR-IB, FSH and anti-BMPR-IB plus FSH treatment groups for 6 hours. After incubation the media from each group was collected and stored at -20 °C. For seminiferous tubule conditioned Leydig cell culture 100 µl seminiferous tubule conditioned media and 50 µl cell stock (1,000 cells/well) was used for each well in one of four groups (control, anti-BMPR-IB, FSH and anti-BMPR-IB plus FSH) and incubated for 3 hours. Testosterone was determined using a radioimmunoassay. Seminiferous tubule conditioned medias were assayed before experimentation and the testosterone from residual Leydig cells subtracted from the culture media. The experiment was repeated four times. (N = 5).

Testicular interstitial cell culture was performed using interstitial cells harvested from 3 and 8 week old mice and 50 µl cell stock (20,000 cells/well) cultured in 100 µl DMEM in treatment groups of control, anti-BMP-2, anti-BMP-4,

eCG, anti-BMP-2 plus eCG, and anti-BMP-4 plus eCG using 96-well polypropylene culture plates (Sarstedt Pty Ltd) for 3 hours. All antibodies were used at a concentration of 50 µg/ml and eCG was used at a concentration of 0.1 iu/ml. Each well consisted of the following: 5 ul treatment (or PBS for controls), 100 ul cuture media DMEM:F12 and 50 ul Cell stock (20,000 Cells in 50 ul DMEM:F12). After incubation the media from each group was collected and stored at -20 °C until being assayed for testosterone using a radioimmunoassay.

6.3 Results

6.3.1 Effect of anti-BMPR-IB on Testosterone Secretion by Testicular Tissue in Pubertal and Adult Mice

In tissue culture of pubertal testis stimulation by eCG resulted in a 2.7 fold increase in testosterone production ($P < 0.0001$) however, anti-BMPR-IB had no effect on either basal or eCG-mediated testosterone secretion (Figure 6.1). In adult testicular culture treatment with anti-BMPR-IB significantly decreased testosterone production by 1.5 fold relative to the control ($P < 0.05$) as shown in Figure 6.2. Stimulation by eCG increased testosterone by 1.9 fold ($P < 0.0003$), while anti-BMPR-IB significantly reduced the eCG-mediated increase in testosterone 1.5 fold ($P < 0.02$) (Figure 6.2).

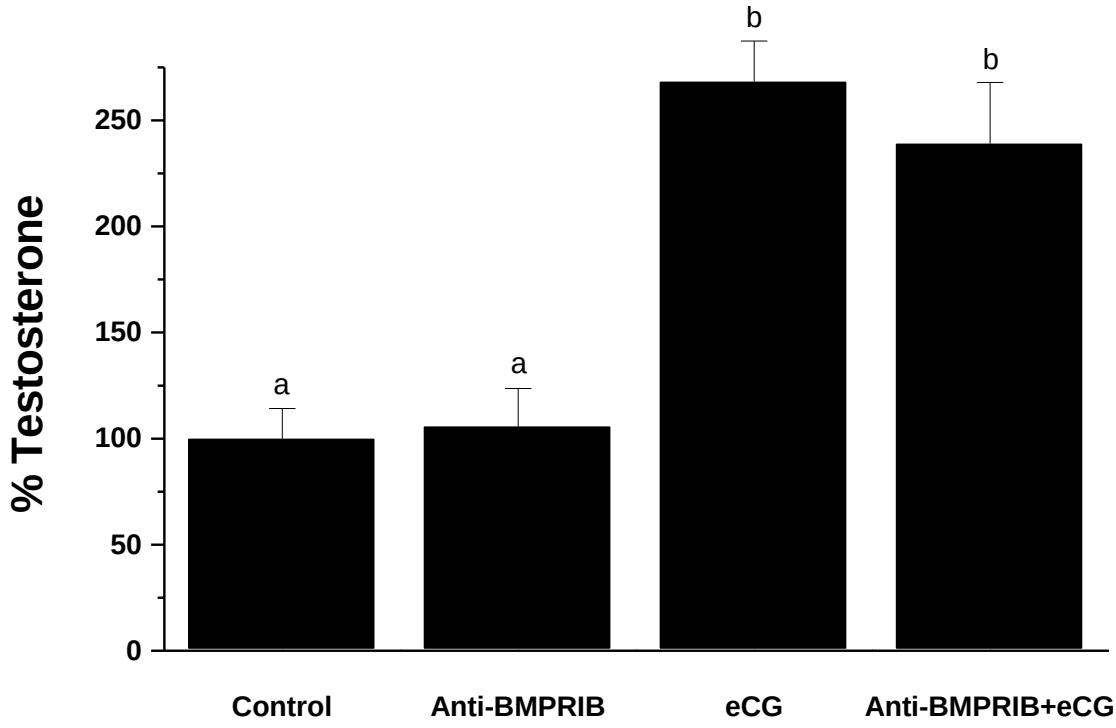


Figure 6.1 Percent testosterone in pubertal testis culture media after treatment with anti-BMPR-IB, eCG and anti-BMPR-IB plus eCG in comparison to no treatment (control). Results expressed as Mean ± SE. Different letters denote significant differences between means ($P < 0.05$). N = 20.

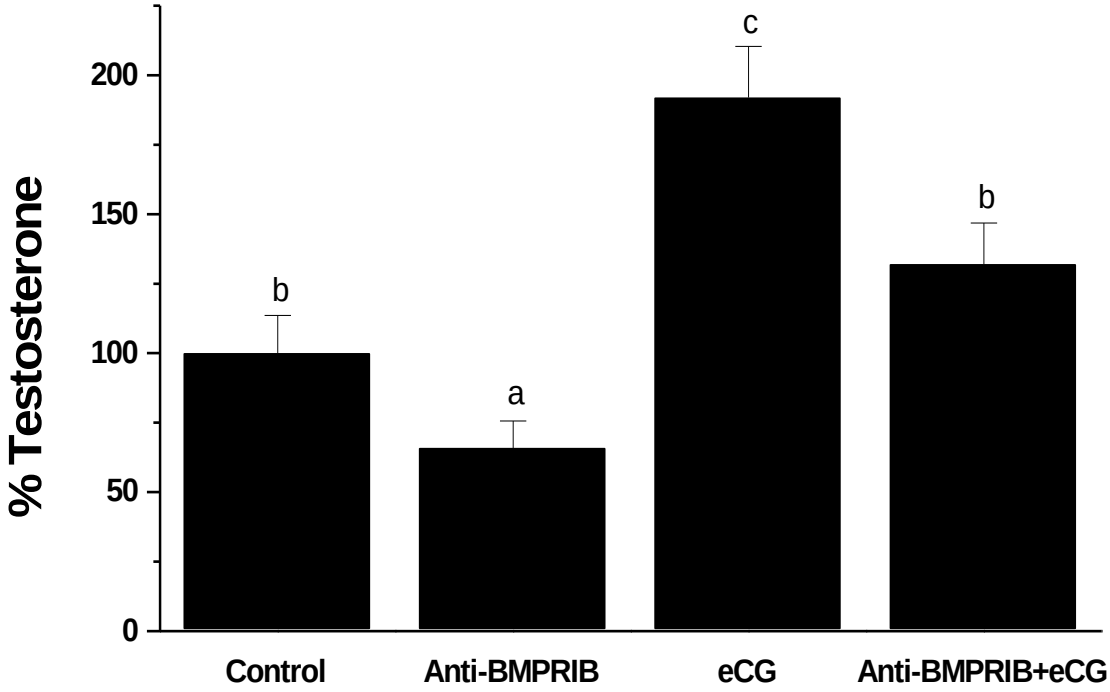


Figure 6.2 Percent testosterone in mature testis culture after treatment with anti-BMPR-IB, eCG and anti-BMPR-IB plus eCG in comparison to no treatment (control). Results expressed as Mean ± SE. Different letters denote significant differences between means ($P < 0.05$). N = 20.

6.3.2 Effect of Anti-BMPR-IB on Testosterone Secretion by Leydig Cells

In purified Leydig cells from adult mice anti-BMPR-IB significantly increased testosterone 1.4 fold from the control ($P < 0.0001$) (Figure 6.3). Treatment of Leydig cells with hCG significantly increased testosterone production by 45 fold ($P < 0.0001$), while anti-BMPR-IB had no effect on hCG-induced testosterone production.

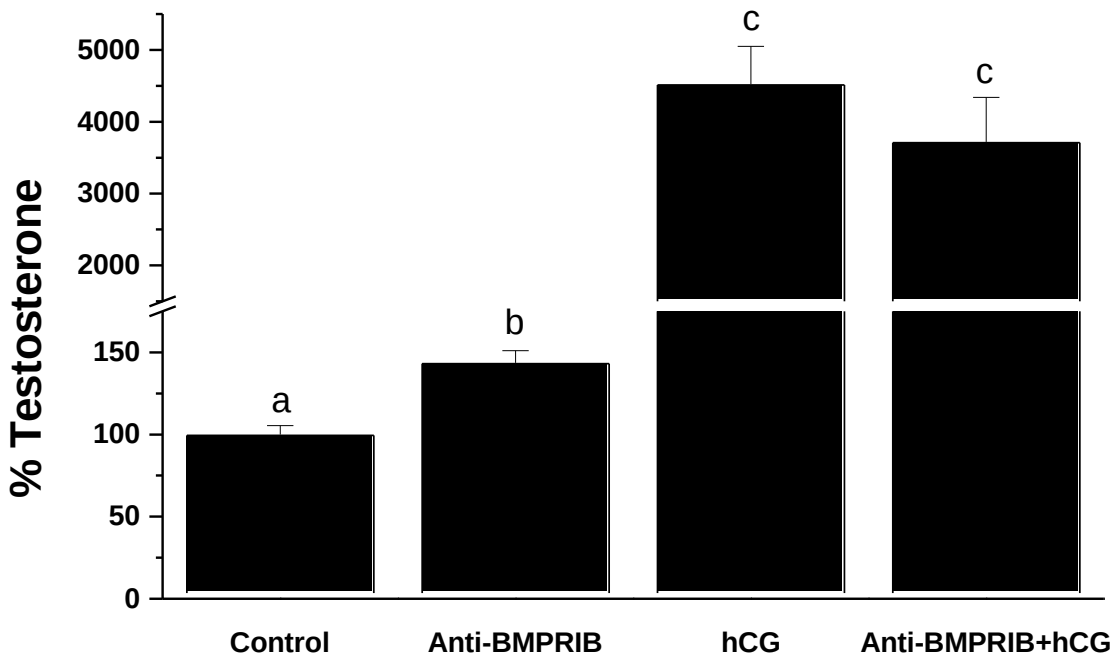


Figure 6.3 Percent testosterone in adult Leydig cell culture media after treatment with anti-BMPR-IB, hCG and anti-BMPR-IB plus hCG in comparison to no treatment (control). Results expressed as Mean ± SE. Different letters denote significant differences between means ($P < 0.05$). N = 20.

6.3.3 Testosterone Secretion by Leydig Cells in Leydig Cell Conditioned Media

In Leydig cells incubated with Leydig cell conditioned media anti-BMPR-IB significantly increased testosterone production by 1.6 fold ($P < 0.0005$) (Figure 6.4). Leydig cells cultured in Leydig cell hCG-treated Leydig cell conditioned media had a powerful inhibitory effect on testosterone production to below recordable levels, suggesting increased conversion of testosterone into other steroids by aromatase, whereas anti-BMPR-IB significantly inhibited the decrease seen in the hCG treated cohort ($P < 0.0005$) (Figure 6.4).

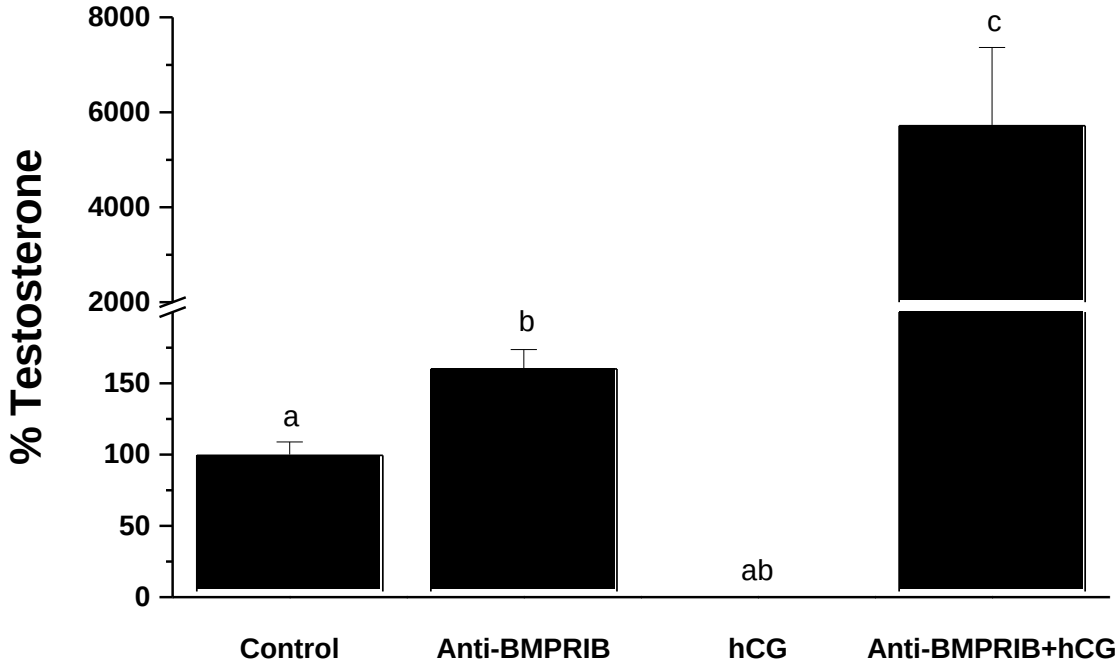


Figure 6.4 Percent testosterone in adult Leydig cell culture media after treatment in Leydig cell conditioned media previously treated with anti-BMPR-IB, hCG and anti-BMPR-IB plus hCG in comparison to no treatment (control). Results expressed as Mean ± SE. Different letters denote significant differences between means ($P < 0.05$). N = 20.

6.3.4 Testosterone Secretion by Leydig Cells in Seminiferous Tubule Conditioned Media

In Leydig cells cultured with seminiferous tubule conditioned media anti-BMPR-IB decreased testosterone by 1.3 fold in basal conditions (Figure 6.5). FSH induced factors significantly increased Leydig cell testosterone production by 3.7 fold ($P < 0.0001$), and while anti-BMPR-IB appeared to reduce FSH-modulated testosterone production the effect was not significant.

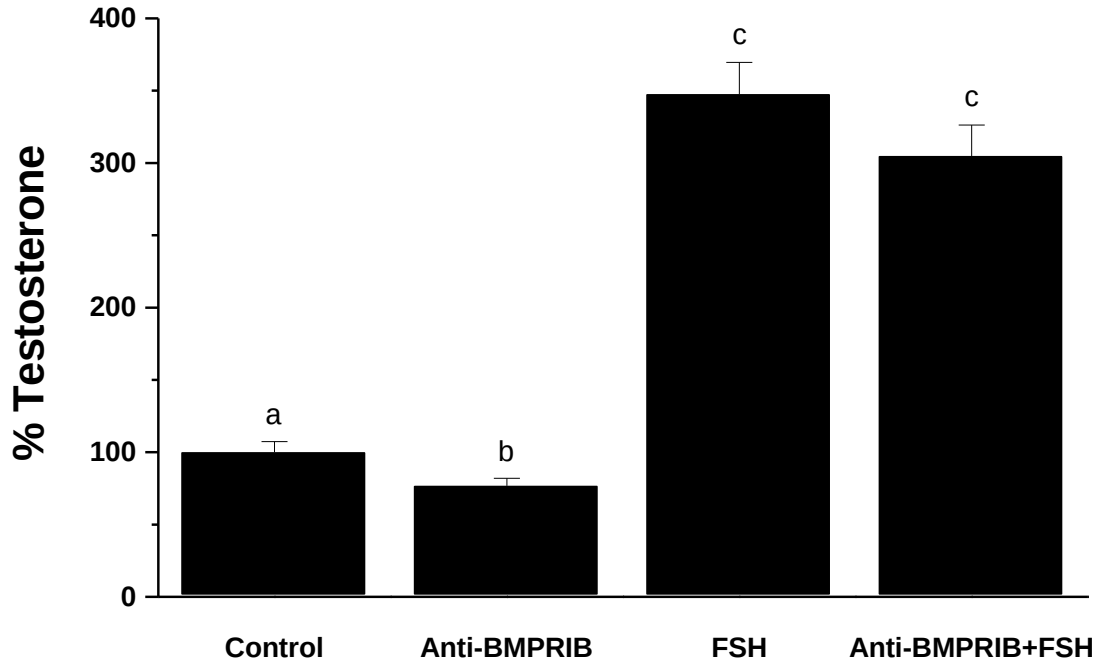


Figure 6.5 Percent testosterone in adult Leydig cell culture media after treatment with seminiferous tubule conditioned media previously treated with anti-BMPR-IB, FSH and anti-BMPR-IB plus FSH in comparison to no treatment (control). Results expressed as Mean ± SE. Different letters denote significant differences between means ($P < 0.05$). N = 20.

6.3.5 Comparison of the Effect of Anti-BMPR-IB on Leydig Cell Testosterone Secretion in Normal, Leydig Cell Conditioned and Seminiferous Tubule Conditioned Media

Relative comparison of anti-BMPR-IB treatment on Leydig cell testosterone in normal (unconditioned), Leydig cell conditioned and seminiferous tubule conditioned media is examined in Figure 6.6. In unstimulated conditions Leydig cells cultured in normal and Leydig cell conditioned media produced equivalent testosterone, however the hCG in the Leydig cell conditioned treated media cohort reduced the peak in testosterone production seen in the normal Leydig cells treated with hCG to undetectable. Testosterone production in the seminiferous tubule conditioned media control was 2.4 fold increased relative to the normal media control, $P < 0.0001$

(Figure 6.6). As expected FSH treated seminiferous tubule conditioned media resulted in significantly less Leydig cell testosterone production than hCG treated Leydig cells in normal media, $P < 0.0001$ (Figure 6.6).

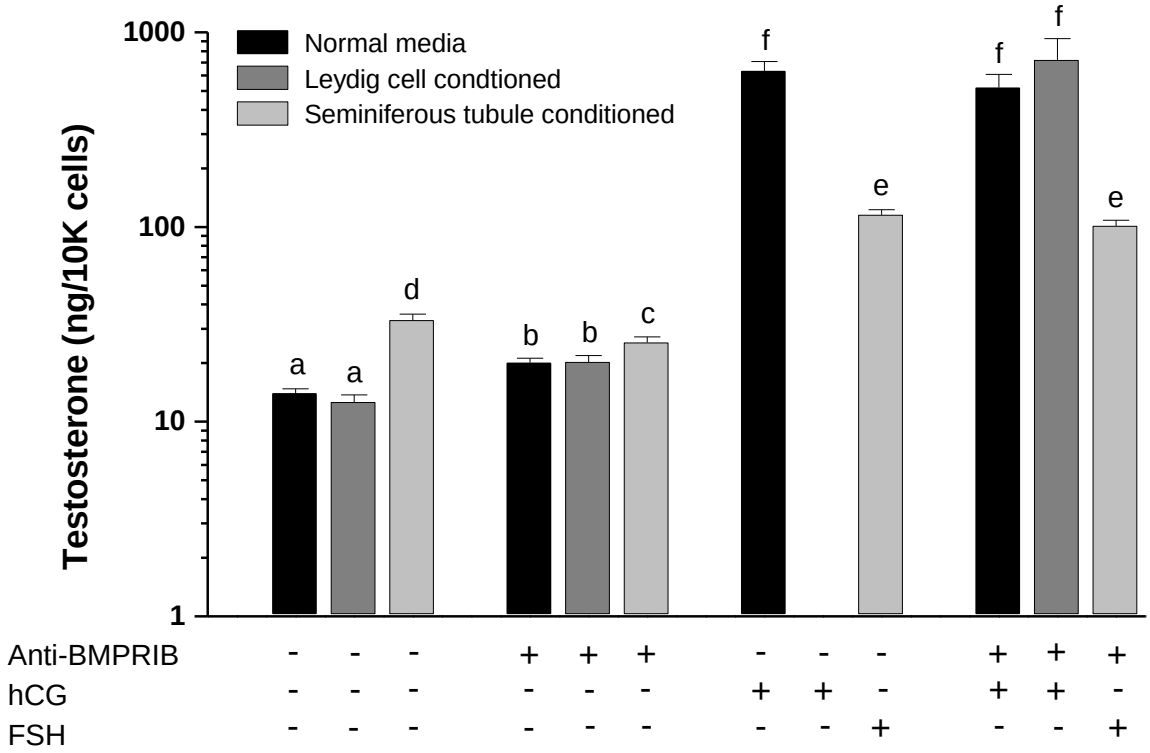


Figure 6.6 Leydig cell culture media testosterone in ng/10K cells in control, anti-BMPR-IB, gonadotrophin and anti-BMPR-IB plus gonadotrophin treatment groups in normal (unconditioned) media, Leydig cell conditioned media and seminiferous tubule conditioned media. Results expressed as Mean ± SE. Different letters denote significant differences between means ($P < 0.05$). N = 20.

6.3.6 The Effects of anti-BMP-2 and anti-BMP-4 on Pubertal and Adult Mouse Testicular Interstitial Cell Testosterone Production

The effects of anti-BMP-2 and anti-BMP-4 on pubertal testicular interstitial cell testosterone production are shown in Figure 6.7. Anti-BMP-4 significantly decreased testosterone production to 69.0±9.0 % ($P < 0.02$), while anti-BMP-2 resulted in testosterone production that was intermediate of the control (100±7.2 %) and BMP-4 immunized cohort at 83.0±7.9 %. Treatment of cells with eCG significantly increased testosterone production to 4,492±488 % ($P < 0.0001$), while anti-BMP-4 and anti-BMP-2 had no effect on eCG-induced testosterone production at 4,517±472 % and 5,175±674 % respectively.

Testosterone production in response to anti-BMP-2 and anti-BMP-4 in adult interstitial cells is shown in Figure 6.8. Anti-BMP-4 significantly decreased testosterone production to 54.0±13 % ($P < 0.02$), while anti-BMP-2 resulted in testosterone not being significantly different from the control (100±11.8 %) or anti-BMP-4 treated cohort at 66.7±11 % indicating a mild reduction. Treatment of cells with eCG significantly increased testosterone production to 4,707±374 % ($P < 0.0001$), while anti-BMP-4 significantly reduced eCG-induced testosterone production to 3,218±348 % ($P < 0.02$). Testosterone in response to anti-BMP-2 (4,064±373 %) in eCG-stimulated conditions was not significantly different from the eCG treated or anti-BMP-4 plus eCG treated cohort indicating a mild reduction.

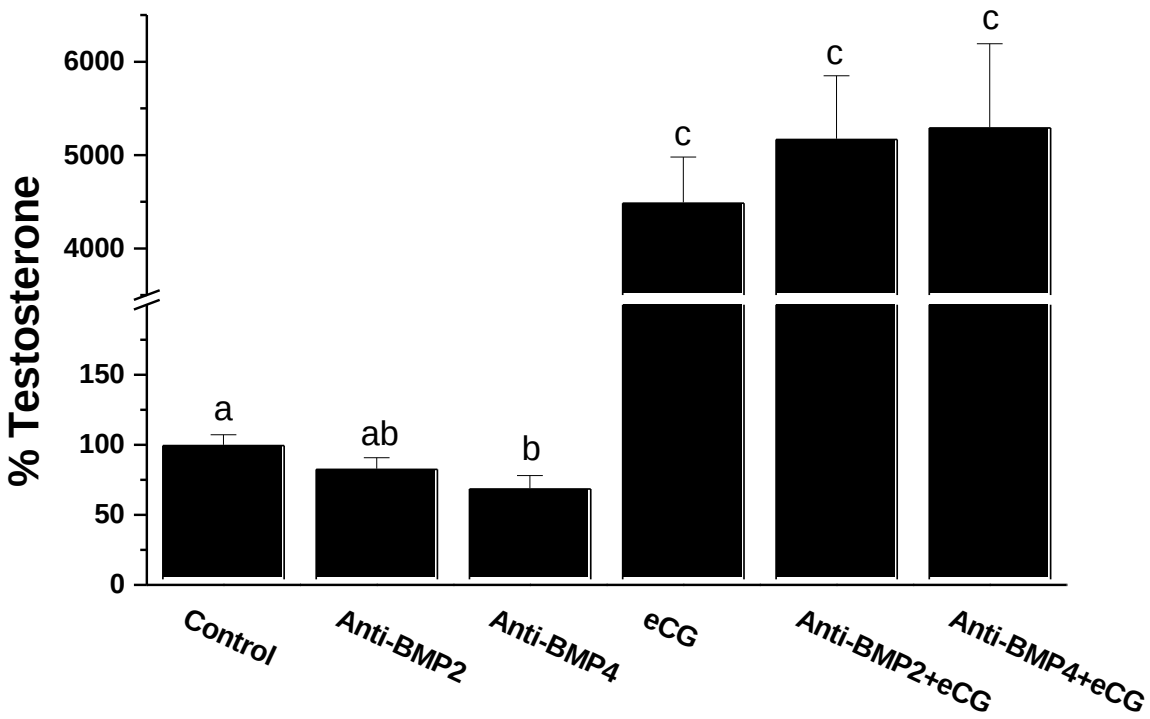


Figure 6.7 Testosterone as percent of the control in pubertal interstitial cell culture media in treatment groups of anti-BMP-2, anti-BMP-4, eCG, eCG plus anti-BMP-2, and eCG plus anti-BMP-4 in comparison to no treatment (control). Results expressed as Mean ± SE. Different letters denote significant differences between means ($P < 0.05$). $N \geq 12$.

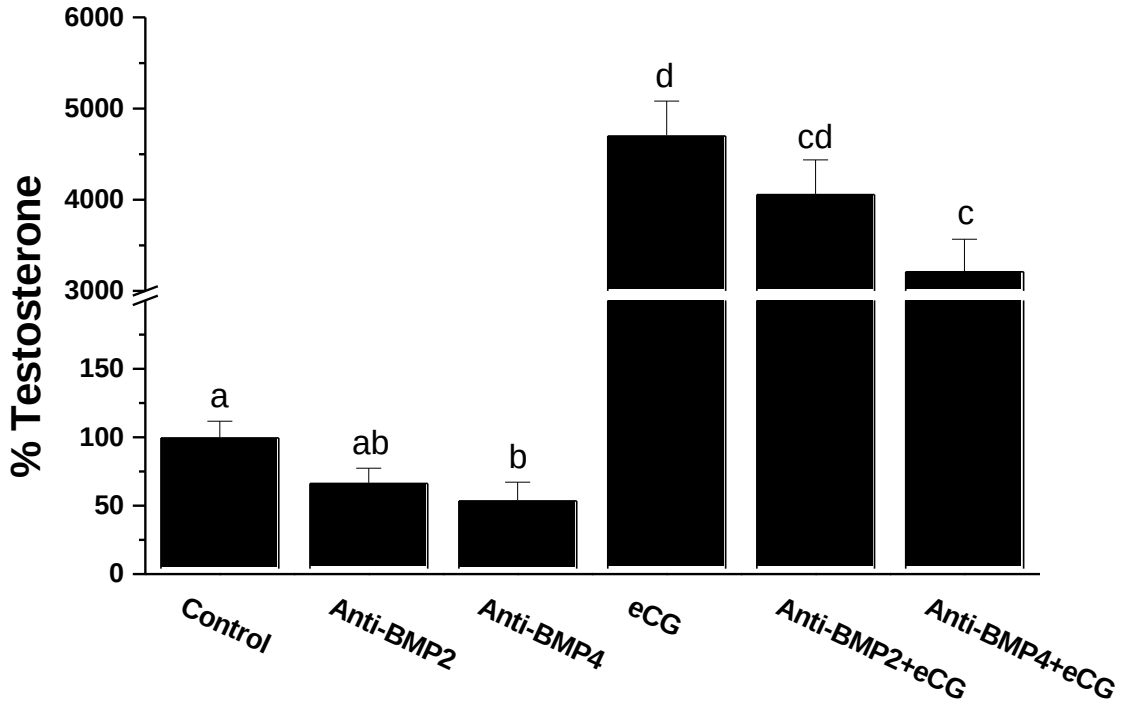


Figure 6.8. Testosterone as percent of the control in mature interstitial cell culture media in treatment groups of anti-BMP-2, anti-BMP-4, eCG, eCG plus anti-BMP-2, and eCG plus anti-BMP-4 in comparison to no treatment (control). Results expressed as Mean ± SE. Different letters denote significant differences between means ($P < 0.05$). $N \geq 14$.

6.4 Discussion

Using *in vitro* tissue and cell culture from male mice, we provide evidence that BMPR-IB has a role in altering steroidogenesis via its action on both steroidogenic and non-steroidogenic cells contained within the testis. In testicular culture eCG significantly increased testosterone production indicating that tissues were viable and responsive. In pubertal testis anti-BMPR-IB had no significant effect on basal testosterone or eCG-stimulated testosterone production, while in adult testis anti-BMPR-IB reduced both basal and eCG-mediated testosterone secretion by 37 % and 41 % respectively. Supporting this finding AMH signalling via BMPR-IA mediated a reduction in *P450scc* mRNA expression, while BMPR-IB antagonized this effect in Sertoli cell derived SMAT-1 cells indicating a role for BMPR-IB in the up-regulation of enzymes required for testosterone production in males (Belville *et al.,* 2005).

BMPR-IB is found in murine ovary (Yi et al., 2001) for which potential ligands BMP-6, BMP-7 and BMP-15 have been shown to decrease gransulosa cell FSH-stimulated progesterone production and/or modulate steroidogenic enzymes such as StAR, 3β-HSD and aromatase (Otsuka et al., 2001a; Lee et al., 2001b; Otsuka et al., 2000). As BMPR-IB and a wide range of BMP mRNAs are present in the testis BMPR-IB is likely to be facilitating the action of BMPs in mouse testis also. While tissue culture demonstrated a role for BMPR-IB in the up-regulation of testosterone in adult testis tissue, the question of which cell types BMPR-IB signals to modulate testosterone production remained, either directly via the Leydig cells or indirectly via the Sertoli cells and/or germ cells. In order to answer this question Leydig cell culture was performed and anti-BMPR-IB significantly increased basal testosterone by approximately 50 %, while having no significant effect on hCG-mediated

testosterone production. As blocking endogenous BMPR-IB decreased testosterone in mature testis culture but increased testosterone in Leydig cell culture we next investigated the effects of Leydig cell conditioned, and seminiferous tubule conditioned media formerly treated with or without anti-BMPR-IB in control and gonadotrophin primed media groups on freshly extracted untreated Leydig cells. Leydig cell conditioned media produced the same testosterone levels as the unconditioned Leydig cells in control and anti-BMPR-IB treated groups showing significantly increased testosterone in the anti-BMPR-IB treated group. However, the media from Leydig cells previously treated with hCG inhibited the synthesis of testosterone to below recordable levels indicating that testosterone was being converted into other steroids by steroidogenic enzymes. Human CG has been shown to acutely increase aromatase expression in Leydig cells, in a process known as aromatization (Valladares & Payne, 1979). Aromatase converts testosterone to estradiol (Rosen & Cedars, 2004) and may have contributed to the reduction in testosterone while aromatase is not know to exit Leydig cells or have presence in media and is unlikely to be having a direct effect. In zebra finch brain aromatase enhanced Bmp2 expression as demonstrated using the aromatase blocker Fadrozole (Walters et al. 2008) and this study provides some evidence that aromatase may increase growth factors which inhibit testosterone in mouse testis as anti-BMPR-IB in the hCG treated Leydig cell conditioned media group inhibited the inhibition of testosterone production. BMPR-IB may indirectly augment aromatase activity, and supporting this theory BMPR-IB immunization significantly decreased aromatase expression in adult mouse testis. It also suggests that BMPR-IB shown to be important for aromatase production in females (Yi et al., 2001) may also be important for aromatase production in males.

In culture media previously conditioned with seminiferous tubules anti-BMPR-IB significantly decreased testosterone production, indicating that the decrease in testosterone seen in mature testicular culture is likely to be mediated by BMPR-IB receptors on cells contained within seminiferous tubules, where BMPR-IB acts to indirectly increase testosterone. Ligand signalling by BMPR-IB may increase steroidogenic enzymes in Sertoli and/or germ cells, while acting to decrease testosterone in Leydig cells by a similar mechanism. In support of these observations, *in vivo* BMPR-IB immunization acted to increase 3β-HSD staining in Leydig cells and reduce enzyme staining within the seminiferous tubules of adult mice. Seminiferous tubule primed media has been shown to contain both inhibitory and stimulatory steroidogenic factors based on the concentration of the media used (McFarlane et al., 1994). Similarly Hedger & Eddy (1990) have also demonstrated that Leydig cells release autocrine factors that alter testosterone secretion. This supports our findings and explains how BMPR-IB immunization can impact testosterone production differentially based on cell type and provides further evidence of the cellular complexity that governs regulated steroid production and germ cell development.

We also investigated the function of BMP-2 and BMP-4 as possible ligands of BMPR-IB in interstitial cell preparations. In both pubertal and adult unstimulated interstitial cells anti-BMP-2 caused a mild decrease in testosterone while anti-BMP-4 caused a significant reduction. Anti-BMP-2 and anti-BMP-4 caused a similar decrease in eCG-stimulated testosterone production in adults but not in pubertal mice. Both BMPs seem to augment testosterone production which could explain one mechanism of how BMP-4 expressed by Sertoli cells increased proliferation of spermatogonia (Pellegrini *et al.,* 2003) and how BMP-2 in concert with FSH also

enhanced spermatogonial proliferation (Puglisi et al., 2004). On the basis of these findings BMPR-IB would seem unlikely to be facilitating the actions of BMP-2 and BMP-4, however performing the same experiment using purified Leydig cells in place of interstitial cells would be required for confirmation as testicular interstitial cell preparations contain Sertoli cells and germ cells.

Collectively, we provide evidence that the testicular functions for BMPR-IB are developmentally regulated, that the receptor has autocrine and paracrine roles in the modulation of steroidogenic enzymes and consequential testosterone production in adult mice.

Chapter 7

7.0 General Discussion

Bone morphogenetic proteins (BMPs) belong to the decapentaplegic-Vg-related (DVR) family, which forms the largest subgroup of growth factors belonging to the transforming growth factor-β (TGF-β) superfamily (McDonald & Hendrickson, 1993). BMP signalling occurs via heterodimerization of type I and type II serine/threonine receptors (Koenig et al., 1994; Ebisawa et al., 1999). BMP receptor mRNAs - *Bmpr1a*, *Bmpr1b* and *Bmpr2*, and BMP RNAs - *Bmp2*, *Bmp3*, *Bmp3b*, *Bmp4*, *Bmp5*, *Bmp6*, *Bmp7*, *Bmp8a*, *Bmp8b* and *Bmp15* are expressed in mouse testis during postnatal development. BMP and BMP receptor mRNAs are also expressed in numerous other male reproductive and steroidogenic tissues, indicating the presence of an extensive BMP system, which is likely to regulate aspects of organ growth, steroid production and germ cell development.

BMPR-IB developmentally regulated seminal vesicle growth partially independent of testosterone in pubertal mice, as *in vivo* immunization caused a significant decrease in eCG-stimulated seminal vesicle weight gain of pubertal mice, but did not markedly affect eCG-stimulated steroidogenic enzyme expression, or serum and intra-testicular testosterone. In support of this finding *Bmpr1b* mRNA was found to be expressed in seminal vesicle epithelium of immature mice (Settle et al., 2001) and we also found *Bmpr1b* mRNA in adult seminal vesicle, where immunization had no effect. This suggests that BMPR-IB is important in seminal vesicle growth during postnatal development but not organ maintenance once adulthood has been reached.

BMPR-IB immunization has reveled that BMPR-IB has a developmental role in steroid production as immunization had no marked effect on testosterone in pubertal mice while significantly increasing basal testosterone production in adult male mice.

BMPR-IB has differential roles in Leydig cells and seminiferous tubules in adult mice as demonstrated by decreased testosterone in anti-BMPR-IB treated mature testis culture and seminiferous tubule pre-conditioned Leydig cell culture, and increased basal testosterone in normal and Leydig cell pre-conditioned Leydig cell culture. This suggests that in seminiferous tubules BMPR-IB acted to augment testosterone synthesis while in Leydig cells it acted to inhibit synthesis. In support of these observations BMPR-IB immunization *in vivo* acted to increase 3β-HSD staining in Leydig cells and reduce enzyme activity within the seminiferous tubular compartment of adult mice. Additionally, BMPR-IB has been detected in SMAT-1 cells and MA-10 cells (Gouedard et al., 2000; Belville et al., 2005), while in SMAT-1 cells BMPR-IB was shown to up-regulate *P450scc* mRNA (Belville et al., 2005). This supports our findings and explains how anti-BMPR-IB can impact testosterone production differentially based on cell type.

BMPR-IB modulated isoforms of hydroxysteroid dehydrogenase (HSD) at the mRNA level as shown in adult testis where BMPR-IB immunization significantly increased *Hsd3b1*, decreased *Hsd3b6* and had no effect on *Hsd17b3* expression. As the up-regulation of *Hsd3b6* and *Hsd17b3* are gonadotrophin dependent (Baker et al., 1997; Baker et al., 2003) and immunization did not increase the expression of these HSD isoforms, BMPR-IB is likely to be acting locally within the testis to decrease testosterone production *in vivo*. BMPR-IB immunization plus eCG treatment resulted in *Hsd3b1* expression that was intermediate of the treatments in isolation, which was not the case for *Hsd3b6* expression suggesting that the pathway to *Hsd3b1* expression are distinctly different in both basal and eCG-stimulated conditions, whereas the effects of BMPR-IB immunization on *Hsd3b6* expression are blocked by the eCG-stimulated steroidogenic pathway.

We provide evidence that BMPR-IB augments aromatase activity in mouse testis, as factors in media from Leydig cells previously treated with hCG inhibited the synthesis of Leydig cell testosterone production to below recordable levels indicating that testosterone was being converted into other steroids by steroidogenic enzymes, while anti-BMPR-IB in this media group obliterated the secondary response. This indicates that BMPR-IB signalling is likely to stimulate enzymatic conversion of testosterone into other steroids. In the process of aromatization hCG has been shown to acutely increase aromatase (*Cyp19*) expression in Leydig cells, (Valladares & Payne, 1979) which is likely to have contributed to the drastic reduction in testosterone seen in the hCG pre-conditioned media group, which suggests that BMPR-IB acts to augment aromatase activity. Supporting this theory BMPR-IB immunization significantly decreased *Cyp19* mRNA expression in adult mouse testis. However, aromatase is not known to exit Leydig cells or have presence in media and is more likely to be stimulating the release of growth factors which consequentaly stimulate aromatase activity. In zebra finch brain aromatase enhanced *Bmp2* expression as demonstrated using the aromatase blocker Fadrozole (Walters et al. 2008) and this study provides some evidence that aromatase may also increase growth factors which inhibit testosterone in mouse testis as anti-BMPR-IB in the hCG pre-conditioned media group inhibited the inhibition of testosterone production. The observation that BMPR-IB is involved in aromatase activity offers an interesting concept that BMPR-IB already shown to be important for aromatase production in females (Yi et al., 2001) may also be important for aromatase production in males.

When comparing the role of BMPR-IB in the male mouse reproductive system with its role in females based on studies using BMPR-IB immunization and *Bmpr1b*

mutant mice, many similarities in function become apparent. *Bmpr1b* mRNA is expressed in mouse testis, Sertoli cell derived SMAT-1 cells and Leydig cell derived MA-10 cells (Gouedard et al., 2000; Belville et al., 2005) while in female mice *Bmpr1b* is expressed in granulosa cells and oocytes of antral follicles (Yi et al., 2001). *Bmpr1b* mutant males were subfertile (with testicular causes not reported) and *Bmpr1b* mutant females were infertile *in vivo* while their oocytes were capable of vertilization *in vitro* (Yi et al., 2001). BMPR-IB immunization caused no obvious histological differences in the testis in our study and female mice with *Bmpr1b* deficiency also showed no obvious histological differences regarding ovarian follicular development (Yi et al., 2001). BMPR-IB immunization resulted in decreased aromatase expression in the testis while Yi et al. (2001) reported that *Bmpr1b* mutant female mice experience failure of granulosa cell proliferation due to defective aromatase production. The gonads of both male and female *Bmpr1b* mutant mice appear normal, however *Bmpr1b* deficiency caused obvious defects in other reproductive organs. In *Bmpr1b* mutant males seminal vesicle growth was defective (Yi et al., 2001) and we demonstrate that BMPR-IB immunization significantly reduced eCG-stimulated seminal vesicle growth in pubertal mice. In females *Bmpr1b* deficiency led to defective/absent endometrial gland formation of the uterus (Yi et al., 2001). BMPR-IB immunization decreased aromatase and increased testosterone in adult male mice and as aromatase is decreased in females, androgens are likely to increase however this remains to be tested. Regulation of *Hsd3b1* and *Hsd3b6* expression in the testis in response to BMPR-IB immunization is opposite to regulation by eCG indicating that BMPR-IB does not enhance pituitary LH or FSH and has a local role within the testis. In support of

autocrine and/or paracrine roles for BMPR-IB, *Bmpr1b* mRNA was not detected in mouse pituitary (Yi et al., 2001).

We demonstrate widespread expression of BMP and BMP receptor RNAs in male reproductive and steroidogenic tissues, developmental functions for BMPR-IB *in vivo* and *in vitro*, and comparable functions for BMPR-IB between male and female mice with disrupted receptor signalling. In conclusion BMPR-IB has significant roles in seminal vesicle growth, the modulation of steroidogenic enzymes and consequently testosterone production in male mice.

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