Chapter 5: Immunolocalization of BMP-4 protein is developmentally regulated in mice testes

5. 1. Introduction

The TGF-β superfamily consists of more than 35 proteins including GDF, BMPs, TGF-β, activin, and inhibin (Chang *et al.*, 2002). BMPs were originally identified in demineralized bone extract (Wozney *et al.*, 1988) and have been shown to regulate growth, differentiation, and metabolism of various cell types (Chang *et al.*, 2002). Similar to other members of the TGF-β superfamily, BMPs signal through a heteromeric complex of the type I (BMPR-IA and BMPR-IB) and type II (BMPR-II) serine/ threonine kinase receptors. Formation of this complex allows phosphorylation of type I receptor by type II receptor, which subsequently leads to the activation of smad pathways. Smad 1, smad 5 and smad 8 are responsible for the intracellular signaling of BMPs, while smad 2 and smad 3 propagate TGF β and activin signals (Derynck & Zhang, 2003, Massague, 1998).

In mice, spermatogenesis starts within a day after birth, when the gonocytes resume mitosis and migrate from their central position within seminiferous cord to contact the peripheral basement membrane (Setchell, 1978, Steinberger & Steinberger, 1975). Mice spermatogenesis is divided into two distinct developmental phases: initiation phase (before 6 wk of age) and maintenance phase (after 6 wk of age). The regulation of spermatogenesis is controlled by a complex interplay of endocrine and paracrine/autocrine factors (Itman *et al.*, 2006). Recent studies have shown that several members of the BMP family play an important role in regulation of murine spermatogenesis (Hu *et al.*, 2004, Zhao *et al.*, 1996). BMP8a and BMP8b have been shown to express in spermatogonia and

primary spermatocytes of prepubertal mice, and in stage 6-8 spermatid of adult mice testis (Zhao *et al.*, 1998, Zhao *et al.*, 1996). Similarly, low level of BMP-4 was detected in testis up to 2 wk, but after 2 wk, strong expression was observed in pachytene spermatocytes (Hu *et al.*, 2004). In addition, GDF-9 has been shown to express in pachytene spermatocytes and early round spermatid (Fitzpatrick *et al.*, 1998). However, GDF-9 deficient male mice have shown no defect in fertility, which indicates that GDF-9 is not absolutely required for male fertility (Dong *et al.*, 1996).

The epididymis is a highly convoluted multifunctional male accessory organ. It provides suitable microenvironment for the maturation of spermatozoa, helps in transportation of sperm along the duct, storage and protection of mature spermatozoa until ejaculation (Orgebin-Crist, 1969). Grossly, the adult epididymis is divided into three segments: caput, corpus and cauda. During embryonic and postnatal development, epididymal growth and functions are androgen dependent (Fan & Robaire, 1998). Moreover, castration or ligation of efferent duct with supplementation of androgens leads to thinning and apoptosis of epididymal epithelial cells indicating that some other factors then androgens are also involved in maintenance of epididymal functions (Fan & Robaire, 1998). BMPs knockout mice models have shown severe defects in integrity and functions of epididymis suggesting that BMPs plays a regulatory role in epididymal functions (Hu *et al.*, 2004, Zhao *et al.*, 1998, Zhao *et al.*, 1996). *In situ* hybridization studies have shown the expression of BMP-4, BMP-7 and BMP-8a in epididymis (Chen *et al.*, 1999, Hu *et al.*, 2004, Zhao *et al.*, 1998). BMP-4 is uniformly expressed in epididymis throughout postnatal development. Before 2 wks of age, BMP-4 mRNA expression was higher in epididymis than testis. However, after formation of pachytene spermatocytes (after 2 wks of age) in seminiferous tubules, BMP-4 mRNA expression is higher in testis than epididymis (Hu *et al.*, 2004). Interestingly, expression of BMP-7 mRNA is

developmentally regulated in epididymis (Chen *et al.*, 1999). Before 3 wk of age, BMP-7 mRNA was uniformly detected in epididymal epithelial cells (Chen *et al.*, 1999). At 3 wk of age, BMP-7 expression was more pronounced in the caput segment (Chen *et al.*, 1999). From 4 wk to adulthood, its expression is up regulated and limited to the caput and cauda-vas deferens junction (Chen *et al.*, 1999).

The prostate is an exocrine gland associated with the urethra and composed of alveoli and ducts embedded in fibro muscular tissue (Cunha *et al.*, 1987). During ejaculation, secretion of prostate, coagulating gland and seminal vesicle serves as a diluent and vehicle for the transport of sperm (Cunha *et al.*, 1987, Shimasaki *et al.*, 2004). In Polymerase chain reaction (PCR) studies, BMP-4 and BMP-7 mRNA were detected in mouse prostate gland (Thomas *et al.*, 1998). BMP-2, BMP-3, BMP-4 and BMP-6 mRNA are expressed in rat and human prostate and their cancer cell lines (Harris *et al.*, 1994). In humans and rats, BMP-2 and BMP-4 mRNA abundance was significantly higher in normal prostate than prostate cancer derived cell lines (PC-3, LNCaP, PA III cell and PA III tumor) (Harris *et al.*, 1994). In the rat, BMP-6 mRNA and protein levels were similar in both normal and malignant prostate tissues and were androgen independent (Barnes *et al.*, 1995). In humans BMP-6 mRNA and protein expression were higher in prostate cancer as compared with adjacent normal prostate tissue (Barnes *et al.*, 1995). In another study, orchidectomy significantly decreased BMP-7 mRNA whereas treatment with testosterone and dihydrotestosterone significantly increased BMP-7 mRNA expression, indicating that BMP-7 expression in mouse prostate is androgen dependent (Thomas *et al.*, 1998). Recently, Paralkar *et al.* have shown the expression of GDF-15 (aka Prostate derived factor/ PDF) in the epithelium of the normal human prostate. In rat prostate, GDF-15 expression was decreased and increased following orchidectomy and dihydrotestosterone treatment, respectively (Paralkar *et al.*, 1998).

Keeping the above studies in mind, the aims of our current study were (I) to localize the expression of BMP-4 protein in mice testis, epididymis, vas deferens, prostate and seminal vesicles (II) to study the role of BMP-4 in testosterone production by using passive immunization against BMP-4 in prepubertal and adult mice.

5. 2. Materials and Methods

5.2. 1. Tissues and section preparation

For immunolocalization of BMP-4, tissues (testis, epididymis, vas deferens, prostate and seminal vesicle) were collected from male Swiss mice aged 1, 2, 4, 7, 9 and 20 weeks (adult) (Discipline of Physiology, University of New England, NSW, Australia). All the tissues were further processed and sectioned as described in chapter 2.

5.2. 2. Passive immunization against BMP-4

Prepubertal and adult Swiss male mice were divided into four groups (n=5) and given daily subcutaneous injections (100ul) of the following treatments for seven days: the first group was treated with anti BMP-4 (50ug), the second and third groups were treated with eCG (1 IU) with and without anti BMP-4 (50ug), and the fourth group was kept as control and injected with non immune serum (50ug). The mice were killed 12 hours after last injection by CO2 asphyxiation. The experiment was repeated twice and the testes were collected and weighted. Testes were homogenized in four times (V / W) of a homogenization buffer (benzamidine 5 mM, EDTA 5 mM and sodium azide 0.02%). The supernatant was collected after centrifugation for 10 min at 10000 rpm, and then stored at -20 ^{0}C.

5.2. 3. Immunohistochemistry

Method is described in chapter 2

5.2. 4. Testosterone assay

Supernatants collected after homogenization of testes from different treatment groups were measured for testosterone contents. Samples were assayed for testosterone as previously described (McFarlane *et al.*, 1990). Briefly, each sample was extracted in duplicate with a 10 times volume of diethyl ether. The bound tracer was precipitated by using charcoal stripped normal horse serum and 22% polyethylene glycerol (PEG). The tubes were counted in a liquid scintillation counter (model 4430, Packard). The antiserum used in this assay was raised against testosterone-3 CM-BSA (Sirosera PTY, North Ryde, Australia) and 1,2,6,7-^{3}H labeled testosterone (Nycomed Amersham Pty, Buckinghamshire, England). The detection limit for this assay was 40 pg/ml. The within and between assay coefficients were 5 % and 9.5 %, respectively.

5. 3. Results

5.3. 1. BMP-4 immunolocalization in testis, epididymis, vas deferens, prostate and seminal vesicles

In 1 wk old mice, BMP-4 protein was not detected in testis but strong staining for BMP-4 was observed in epithelial cells of epididymis (Fig. 5.1A). In testis, BMP-4 protein was detected in spermatocytes at 2, 4, and 7 wks but was not detected in testis from 9 wk old as well as adult mice (Fig. 5.2B, 5.3C, 5.4D and 5.5E). At 7 wk of age, BMP-4 protein expression was also observed in some interstitial cells of testis (Fig. 5.4D). In epididymis, BMP-4 specific staining was detected in epithelial cell at all the stages of testicular development (Fig. 5.1A and 5.6F). In the vas deferens, BMP-4 protein expression was limited to luminal epithelial cells (Fig. 5.7G). Additionally, BMP-4 specific staining was also observed in epithelial lining of the prostate gland (Fig. 5.8H) but was not detected in seminal vesicles (Fig. not shown). No staining was observed in negative controls (Figure not shown and similar to Fig. 5.5E).

5.3. 2. Passive immunization against BMP-4 and testosterone secretion

In prepubertal mice, anti BMP-4 antibody treatment decreased testicular secretion of testosterone compared to controls ($P < 0.05$) (Fig. 5.9). In addition, prepubertal mice treated with anti BMP-4 antibody and eCG had lower concentration of testosterone than eCG treated group ($P < 0.05$) (Fig. 5.9). In adult mice, anti BMP-4 treatment increased the testosterone production compared to control animal ($P < 0.05$) (Fig. 5.10). While, no significant difference was observed in testosterone secretion between anti BMP-4, anti BMP-4 and eCG, and eCG treated animals (Fig.5.10). The mean weight of mice used in

different treatment was not significantly different in different treatment groups (data not shown).

Figure 5. 1(A). BMP-4 protein was localized in epithelium of epididymis, while no expression was observed in testis at 1 wk of age (100 X) (E: epididymis; T: testis).

Figure 5. 2(B). BMP-4 protein was detected in pachytene spermatocytes at 2 wk of age (200 X)

Figure 5. 3(C). Expression of BMP-4 protein was detected in spermatocytes at 4 wk of age (200 X)

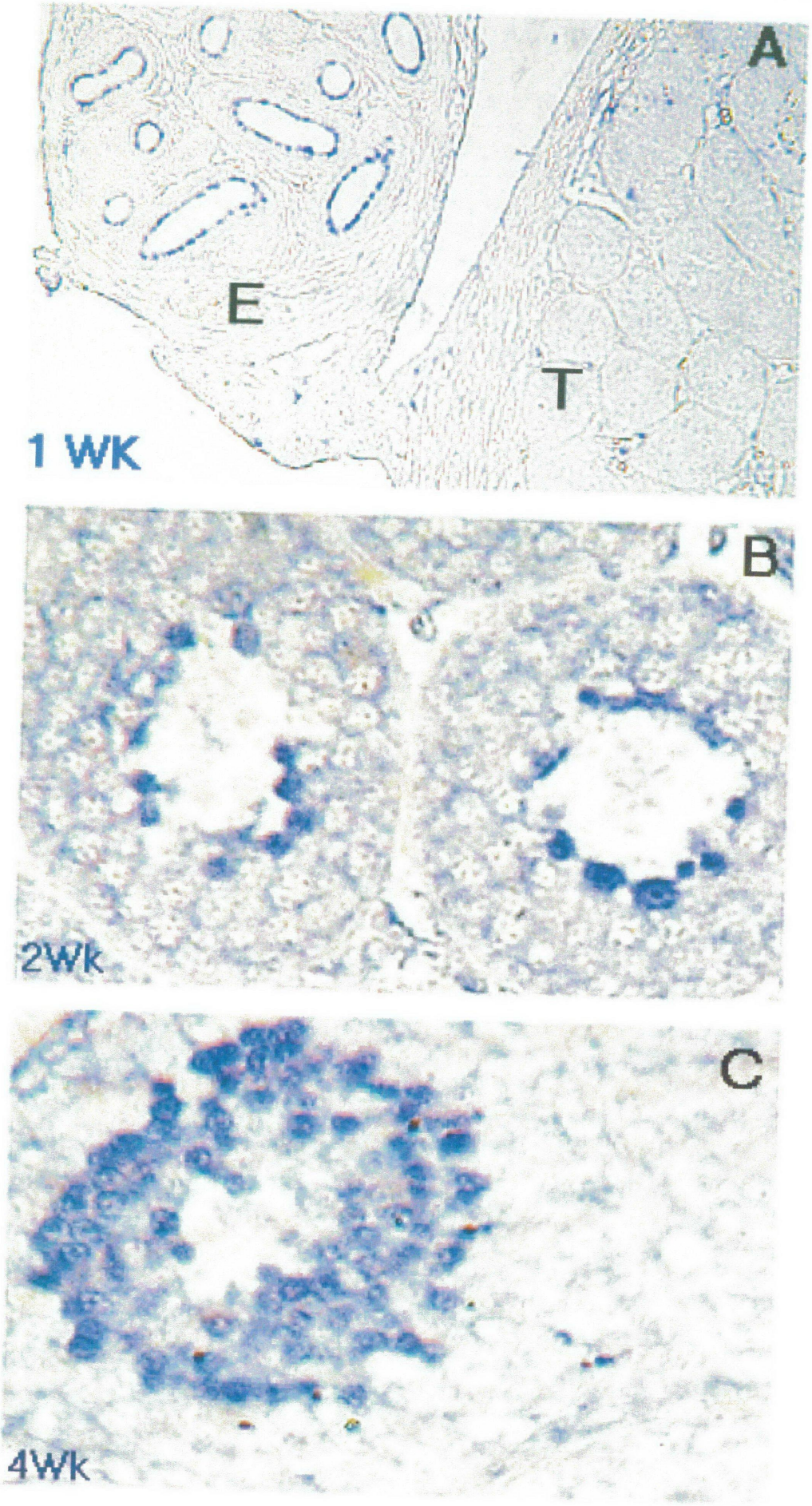
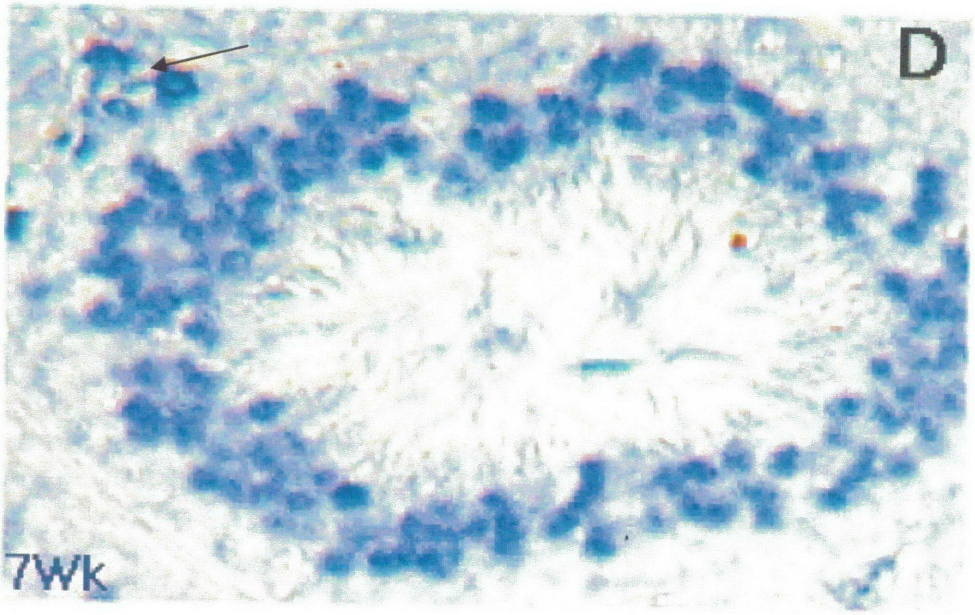

A
E
T
1 WK
B
2Wk
C
4Wk

Figure 5. 4(D). BMP-4 protein was detected in spermatocytes and some interstitial cell (arrow) at 7 wk of age (200 X)

Figure 5. 5(E). No expression of BMP-4 protein was observed in testis of 9 wk old and adult mice (200 X)

Figure 5. 6(F). BMP-4 protein expression in epithelium of epididymis was observed throughout postnatal development (200 X)


D
7Wk


E
9Wk

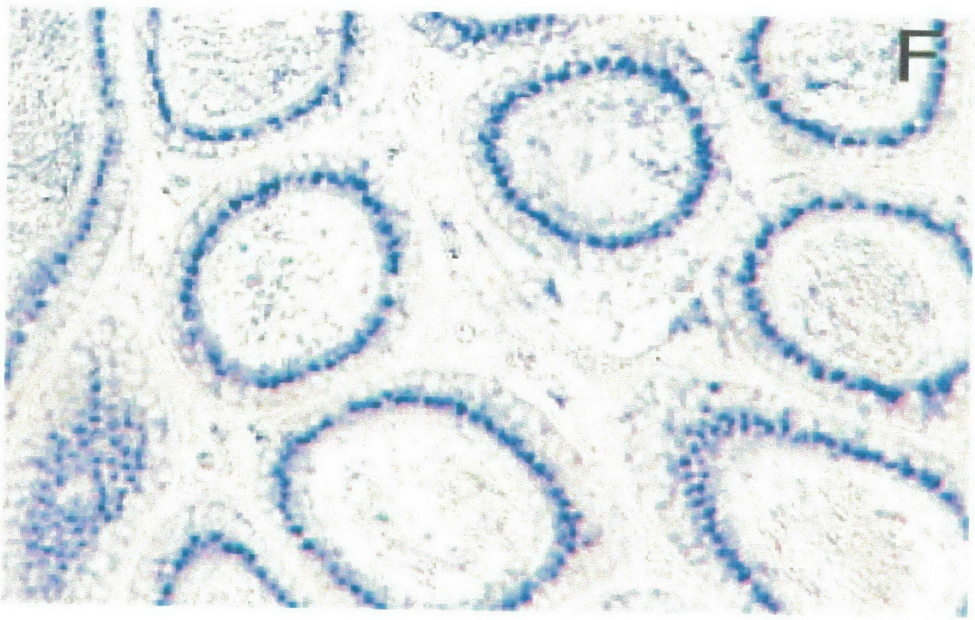
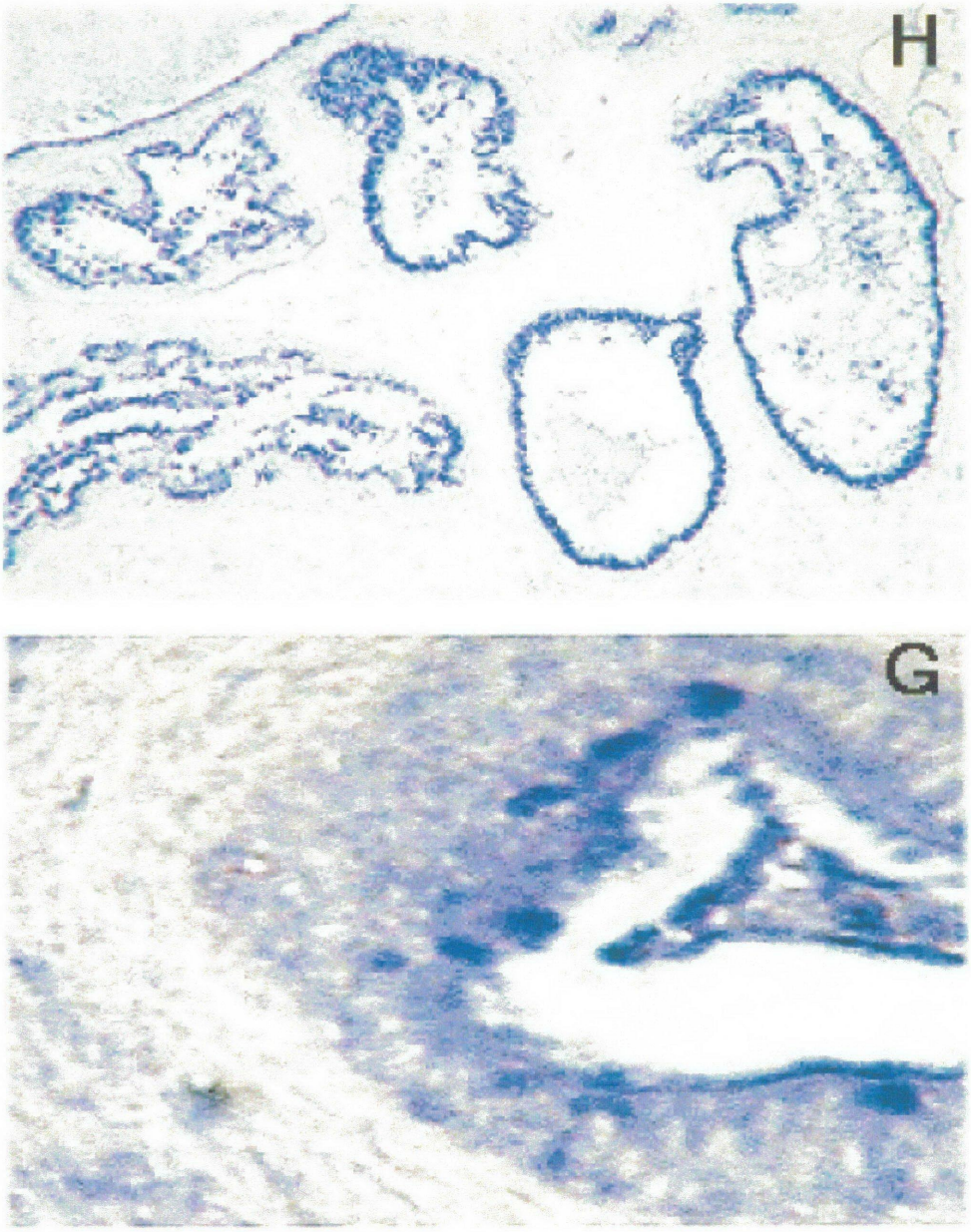

F

Figure 5. 7(G). BMP-4 protein was detected in luminal epithelium of vas deferens (400 X)

Figure 5. 8(H). Expression of BMP-4 protein was observed in epithelium of prostate gland (200 X)


H
G

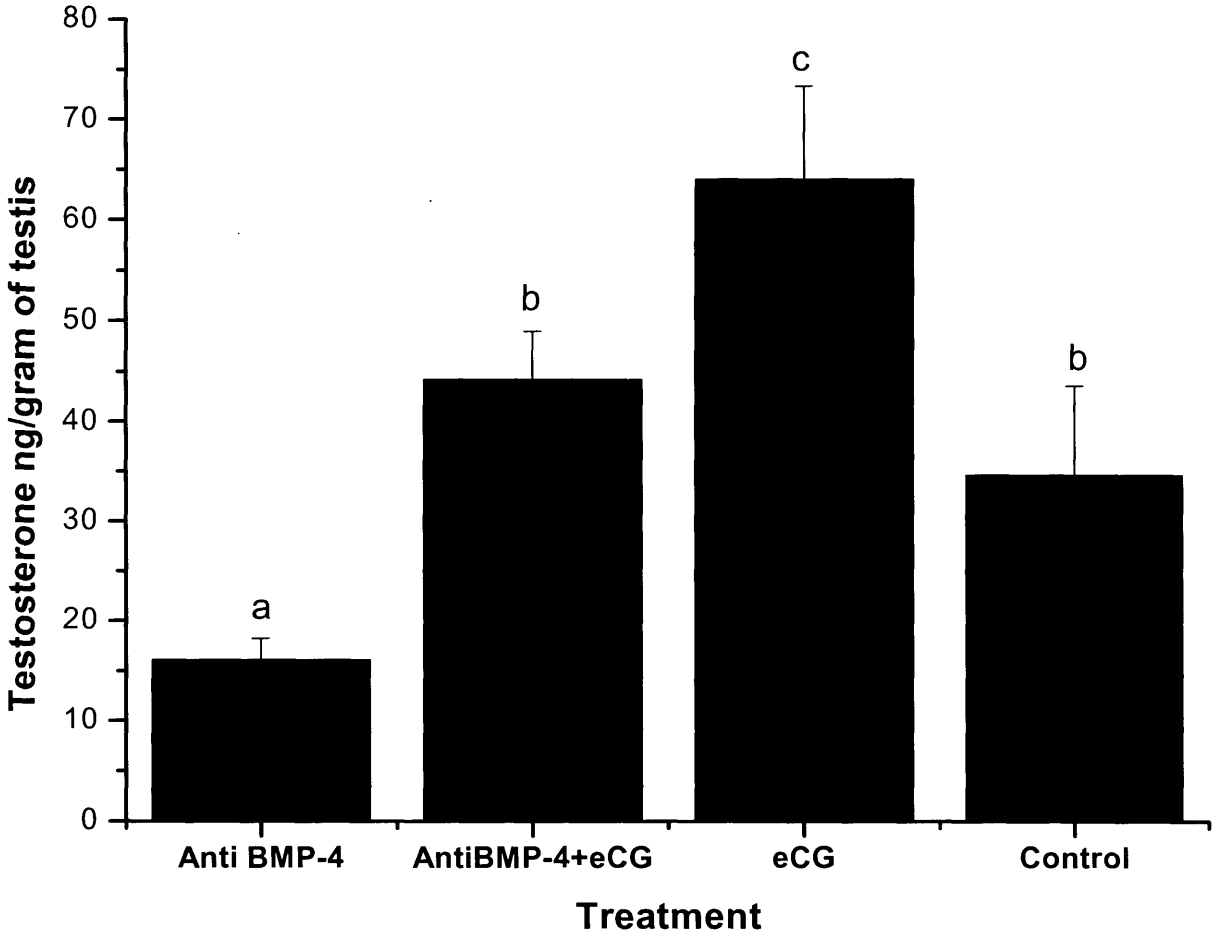


Figure 5. 9. Effect of anti BMP-4, antiBMP-4 & eCG, eCG and non immune serum treatment on testicular testosterone concentration of prepubertal mice. Distinct letters above bars represents statistically significant differences between groups ($P < 0.05$).

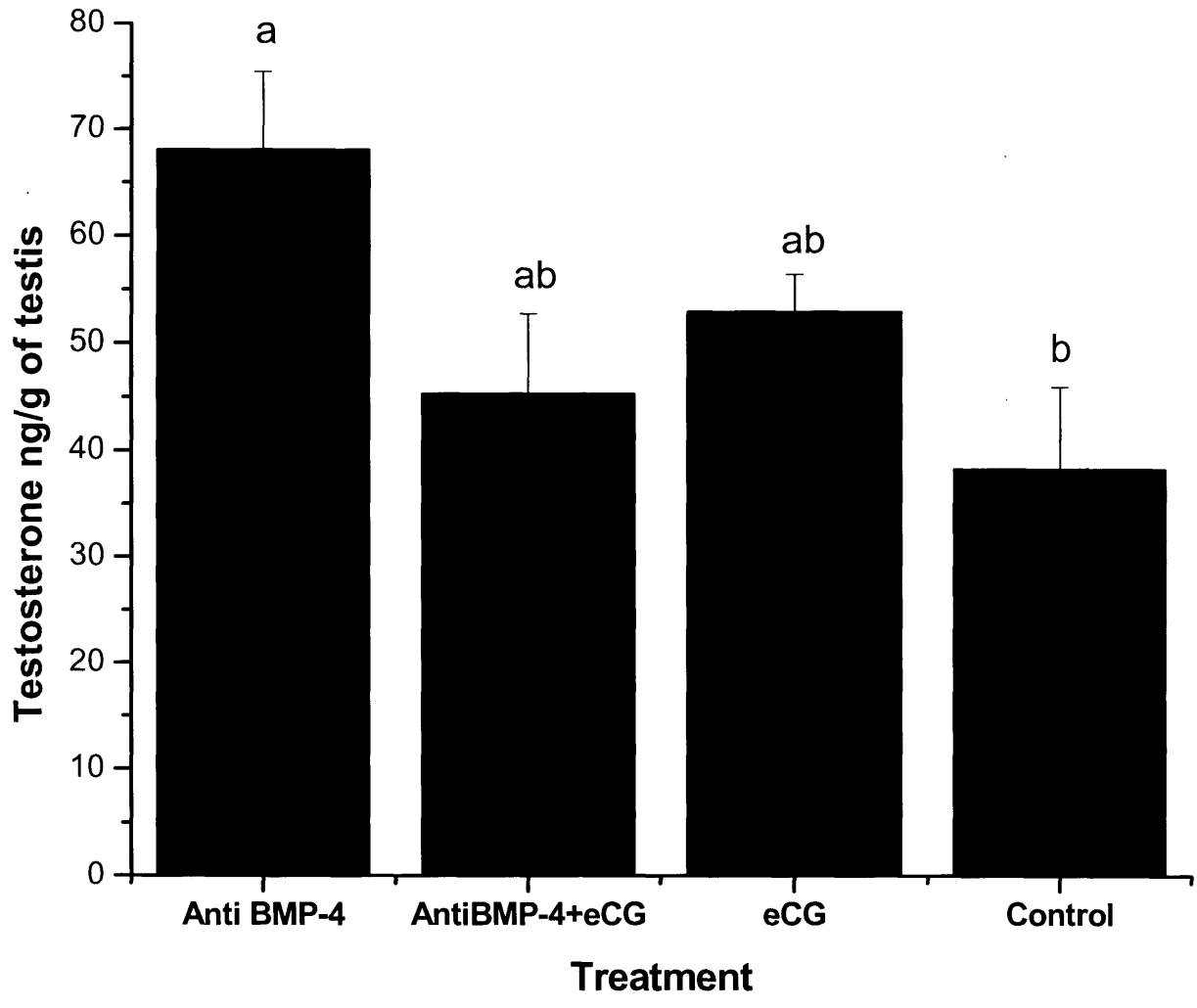


Figure 5.10. Effect of anti BMP-4, antiBMP-4 & eCG, eCG and non immune serum treatment on testicular testosterone concentration of adult mice. Distinct letters above bars represents statistically significant differences between groups ($P < 0.05$).

5. 4. Discussion

Our studies have shown that BMP-4 protein expression is developmentally regulated in mice testes and also present in epididymis, vas deferens and prostate. Expression of BMP-4 protein in testis was first observed in spermatocytes at 2 wk of age. Interestingly, at this stage of testicular development pachytene spermatocytes first appeared in seminiferous tubules (Steinberger & Steinberger, 1975). At 4 and 7 wk of age, BMP-4 protein was detected in most of the round spermatids, while no expression of BMP-4 was observed in 9 wk and adult testis. Our studies have suggested that BMP-4 plays a major role in initial stages of spermatogenesis. *In situ* hybridization studies have shown similar pattern of expression of BMP-4 mRNA except BMP-4 mRNA can also be detected in adult testis (Hu *et al.*, 2004). Furthermore, gene profiling studies have shown presence of BMP-4 mRNA during testicular development (Schultz *et al.*, 2003, Shima *et al.*, 2004). In addition, expression of BMPR-IA and BMPR-II in testis starts decreasing at 30 day postnatal (Puglisi *et al.*, 2004), further supporting the role of this protein in initial stage of spermatogenesis.

During initiation of spermatogenesis, somatic and germ cell events are controlled by gonadotrophins and local gonadal factors. Various members of the TGF-β superfamily such as activin, inhibin, follistatin, BMP-2 and BMP-7 have been shown to regulate gonadotrophin functions in testis (De Kretser & McFarlane, 1996, Itman *et al.*, 2006, Puglisi *et al.*, 2004). BMP-4 has been shown to affect FSH production in pituitary cell cultures (Faure *et al.*, 2005) and LH induced androgen production in thecal cells of ovary (Glister *et al.*, 2005). Moreover, *in vitro* treatment with BMP-2 and FSH increases proliferation of spermatogonia, whereas treatment with BMP-7 and FSH increases Sertoli cell number (Puglisi *et al.*, 2004). In our studies, we found that passive immunization

against BMP-4 in prepubertal male mice leads to a decrease in testosterone production. However, anti BMP-4 treatment in adult mice stimulated testosterone secretion. In addition, anti BMP-4 treatment with eCG in prepubertal animal's decreases testosterone secretion in comparison to eCG treated animals, while no difference was observed in adult animals. These findings indicate that BMP-4 stimulates and down regulates testosterone production in prepubertal and adult mice, respectively.

In mammals, fetal gonads have potential to develop into an ovary or a testis depending upon gender specific signals (Bowles *et al.*, 2006). The primordial germ cells form future gametes and they arise within the proximal epiblast of mouse embryo in response to extra embryonic signals (Ying *et al.*, 2001). In mice embryos, BMP-4 and BMP-8b are expressed in extra embryonic ectoderm at 5.5-7.5 dpc, while BMP-2 is expressed in visceral endoderm at 5-7.5 dpc (Lawson *et al.*, 1999, Ying *et al.*, 2001, Ying *et al.*, 2000). BMP-4 and BMP-8b gene deletion during embryonic development in mice leads to failure of development of primordial germ cells and defective allantois development (Ying *et al.*, 2001). Furthermore, BMP-2 gene knockout mice embryos also have reduced number of primordial germ cells (Lawson *et al.*, 1999, Ying & Zhao, 2001). *In vitro* co-culture studies have shown that extra embryonic ectoderm derived BMP-4 and BMP-8b proteins act synergistically to induce primordial germ cell formation from the epiblast (Ying *et al.*, 2001). The presence of BMPR-IA and BMPR-II in migratory primordial germ cell (Pellegrini *et al.*, 2003), with defective PGCs development in smad-1 and smad-5 knockout mice embryos (Chang & Matzuk, 2001, Hayashi *et al.*, 2002), further signify the importance of BMPs in primordial germ cell formation.

TGF-β has been shown to down regulate kit receptor expression in hematopioetic cell (Sansilvestri *et al.*, 1995), while it up regulates kit receptor expression in T-leukemia

cells (Tomeczkowski *et al.*, 1998) and in neural crest derived cell line (NCC-melb4) (Kawakami *et al.*, 2002). In the ovary, GDF-9 (Elvin *et al.*, 1999b) and BMP-15 (Otsuka & Shimasaki, 2002a) have been shown to inhibit and stimulates KL (Kit ligand) expression in mice granulosa cells. While, *in vitro* treatment with BMP-4 of neonatal rat ovary has no effect on KL expression (Nilsson & Skinner, 2003). The KL/ kit system plays an essential role in spermatogenesis as kit mutated male mice showed impaired spermatogenesis and leydig cell hyperplasia (Kissel *et al.*, 2000). In undifferentiated spermatogonia cell culture from day 4 mouse testis, BMP-4 treatment induces germ cell differentiation by inducing Kit ligand sensitivity in Kit negative spermatogonia (Pellegrini *et al.*, 2003).

In mice, BMP-8b gene deletion leads to germ cell deficiency and infertility (Zhao *et al.*, 1996). During early puberty, BMP-8b deficient males showed a marked reduction in proliferation and differentiation of the germ cells (Zhao *et al.*, 1996). While, BMP-8b deficient adult mice showed a significant increase in spermatocytes apoptosis leading to germ cell depletion and infertility (Zhao *et al.*, 1996), suggesting a role of BMP-8b in initiation and maintenance of spermatogenesis. In contrast to the BMP-8b knockout mice, BMP-8a gene knockout mice showed no germ cell defect during initiation of spermatogenesis (Zhao *et al.*, 1998). However, 47% of BMP-8a-deficient adult mice showed germ cell degeneration and severely compromised spermiogenesis, indicating a role of BMP-8a in maintenance of spermatogenesis, but not in initiation of spermatogenesis (Zhao *et al.*, 1998). Additionally, BMP-8a deficient mice show degeneration of epididymal epithelium, which further leads to granuloma formation and sterility (Zhao *et al.*, 1998). BMP-4 knockout mice were unable to deliver information regarding role of BMP-4 in spermatogenesis as they die at birth (Lawson *et al.*, 1999). On

other hand, BMP-4 deficient heterozygous male mice survive to adulthood and showed defect in spermatogenesis due to degeneration of germ cells, reduced sperm number and decline in sperm motility (Hu *et al.*, 2004). Furthermore, BMP-4 knockout heterozygous male mice also had extensive degeneration of epididymal epithelium (Hu *et al.*, 2004). In another study, BMP-4 deficient heterozygous mice have shown significant increase in size of ventral prostate due to increase in overall length of ducts of prostate (Almahbobi *et al.*, 2005). Overall these studies, with our findings, indicate that BMP-4 plays an important role in regulation of male reproductive functions.