Chapter 7: Active immunization against BMP-4 decreases ovulation rate in ewes.

7. 1. Introduction

BMP-4 is a member of the TGF-β superfamily. The TGF-β superfamily consists of over 35 proteins, including TGF-β, activin/inhibin, GDFs, BMPs, AMH, and others, that share common structural motifs. Members of the TGF-β superfamily are involved in multiple cellular activities including proliferation, differentiation, migration, organization and death during pre and postnatal life (Shimasaki *et al.*, 2004).

The mRNA for various BMP ligands including BMP-2, BMP-4, BMP-6 and BMP-7 has been detected in sheep ovary (Fabre *et al.*, 2006, Souza *et al.*, 2003). In sheep ovary, BMP-4 and BMP-7 mRNA are expressed by granulosa and theca cells, whereas BMP-2 mRNA is only expressed by granulosa cells and BMP-6 mRNA by oocytes (Souza *et al.*, 2003). Among the family of BMP receptors, the BMPR-IA, BMPR-IB, and the BMPR-II receptors are expressed mainly in the granulosa cells of primary to late antral follicles in sheep ovary (Souza *et al.*, 2002). The expression of BMPR-IB has also been detected in granulosa cells and oocytes from the primary to the late antral follicle stages, and to a lesser extent, by the theca layer of ovine and bovine antral follicles (Fatehi *et al.*, 2005, Souza *et al.*, 2002, Wilson *et al.*, 2001). Furthermore, the intracellular downstream signaling smad-1 protein has been detected in ovine and bovine granulosa cells (Glister *et al.*, 2004, Pierre *et al.*, 2004), and smad-2 has been detected in bovine granulosa and theca cells (Glister *et al.*, 2004, Glister *et al.*, 2005).

In sheep granulosa cells from small antral follicles, BMP-2, BMP-4, and BMP-6 inhibit both basal and FSH stimulated progesterone production (Fabre *et al.*, 2006). The inhibitory effect of BMP-4 on progesterone secretion is associated with a decrease in expression of StAR, 3β-HSD and P450scc genes at the mRNA and protein levels. The underlying mechanism implies a decrease in FSH-induced cAMP production as well as a decrease in cAMP- induced progesterone production by inhibiting the transcriptional activity of steroidogenic factor-1 (SF-1) (Fabre *et al.*, 2003, Fabre *et al.*, 2006, Pierre *et al.*, 2004). Similar effects, negative on progesterone and positive on estradiol, have been observed for BMP-4, BMP-6 and BMP-7 using bovine granulosa cells taken from antral follicles in basal and insulin-like growth factor-1 (IGF-1)-stimulated conditions (Glister *et al.*, 2004). In addition, BMP-4 inhibits LH-dependent production of progesterone by sheep granulosa cells derived from preovulatory follicles (Fabre *et al.*, 2006). Additionally, BMP-2, BMP-4 and BMP-6 have been shown to decrease LH-induced androstenedione production by ovine theca cells taken from immature follicles (1-3 mm) (Souza *et al.*, 2004). Accordingly, BMP-4, BMP-6 and BMP-7 suppress basal and LH-induced androgen production by cultured bovine theca cells (Glister *et al.*, 2005).

In mice, sheep, and humans, gene deletion and naturally occurring mutations in various components of BMP system have been shown to affect fertility (Demirhan *et al.*, 2005, Elvin *et al.*, 1999a, Fabre *et al.*, 2006, Mulsant *et al.*, 2001, Souza *et al.*, 2001, Wilson *et al.*, 2001). Booroola was the first gene reported to increase ovulation rate (Piper & Bindon, 1982). It is due to the action of a single autosomal gene (FecB), which influences the number of ovulations per estrous cycle. Ewes which are homozygous $FecB^{B}/FecB^{B}$, heterozygous $FecB^{B}/FecB^{+}$ and non-carriers $FecB^{+}/FecB^{+}$ for the Booroola mutation ($FecB^{B}$) can be segregated on the basis of an ovulation rate recording of 5 or more, 3 or 4 and 1 or 2, respectively (Davis & Kelly, 1982, Piper *et al.*, 1985). Two gene

specific differences have been consistently associated with FecB mutation. Firstly, follicles mature and ovulate at significantly smaller diameters in FecB carrier ewes than in non-carrier ewes. Secondly, FecB mutation has been associated with higher concentration of FSH during the estrous cycle and higher progesterone concentration during the luteal phase of estrous cycle (McNatty *et al.*, 1989, McNatty *et al.*, 1991, Xia *et al.*, 2003). In 2001, three independent groups published simultaneously the mutation responsible for the hyperprolific phenotype of Booroola ewes (Mulsant *et al.*, 2001, Souza *et al.*, 2001, Wilson *et al.*, 2001). The $FecB^{B}$ allele corresponds to a single mutation in the coding sequence of the bone morphogenetic protein receptor type IB (BMPRIB), also known as activin like kinase receptor-6 (ALK-6), on ovine chromosome 6. The G to A transition at nucleotide position 746 of the cDNA induces a non-conservative substitution of the glutamine with an arginine at position 249 of the protein (Q249R) (Souza *et al.*, 2001). After identification of this FecB mutation, DNA mutation test for FecB has shown the presence of a similar mutation in Indian Garole, Indonesian Javanese, Chinese Hu and small tailed Han Breed (Davis *et al.*, 2002).

The aim of the present study was to examine the role of BMP-4 in sheep reproduction by actively immunizing ewes against BMP-4.

7. 2. Material and Methods

7.2. 1. Animals

The ewes (3 year old heterozygous Booroola merino sheep) used in this study were grazed on open paddocks and run with vasectomized Merino rams.

7.2. 2. Active immunization of ewes against BMP-4

A synthetic peptide equivalent to amino acids 88-102 from mouse BMP-4 (Invitrogen Australia, Mount Waverly Vic 3149 Australia) was conjugated to diphtheria toxoid for the preparation of vaccine for active immunization against BMP-4 in sheep. Ewes were injected (i.m.) with 30 µg of diphtheria toxoid-BMP-4 peptide conjugate (n = 6) or with equivalent amount of diphtheria toxoid (n = 5) in 1ml of Freunds complete adjuvant (FCA) for initial immunization, which was given 4-5 months before onset of the breeding season. The animals were boosted after 4 weeks and after a further 6-8 weeks using half the primary concentration of rbBMP-4 peptide in Freunds incomplete adjuvant (FIA). After final booster injection, blood samples were collected to measure anti BMP-4 antibody titre and then vasectomized rams with marking harnesses were run with the ewes to monitor the estrous cycle. The length of the estrous cycle was calculated as the days between first observed markings of successive cycles by the vasectomized ram. In addition, daily blood samples were collected via the jugular vein for determination of plasma progesterone concentration. Ovulation rate of ewes that displayed estrous behavior was determined by laparoscopy. The person involved in laparoscopy was unaware of individual treatments group. All ewes were subjected to laparoscopy one time in each estrous cycle for three estrous cycles.

7.2. 3. Determination of antibody titer and cross reactivity

Individual plasma samples from actively immunized sheep were tested for reactivity to both rbBMP-4 and rbBMP-2 peptide. To do this we used indirect ELISA technique as described below. Briefly, flat bottomed microtitre plates (Greiner Labortechnik, Austria) were coated with 100 ng/well rbBMP-4 and rbBMP-2 in binding buffer (0.05 M bicarbonate, 0.02% sodium azide, pH 9.6). Excess binding sites were blocked with 200 µl 5 % skim milk in PBS for 1 hr at 37 ^{o} C. The plates were washed 5 times with 0.9% saline and 0.05% Triton-X 100, using a Titertek Microplate washer (Labsystems, Helsinki, Finland). Serial dilutions of antisera in phosphate buffer, pH 8.0, containing 0.5 M sodium chloride, 0.5% Tween-20 and 0.1% BSA (ELISA buffer) were added to the ELISA plate in a volume of 100 µl and incubated overnight at 37 ^{o} C. The plates were washed and biotinylated rabbit anti-sheep IgY (Chemicon, Temecula, CA, USA) at a dilution of 1:10000 in ELISA buffer was added to each well and incubated at 37 ^{o} C for 1 hr. The plates were washed and streptavidin conjugated to alkaline phosphatase at dilution of 1:10000 was incubated for 1 hr at 37 ^{o} C. The plates were developed with p-nitrophenylphosphate disodium salt hexahydrate (NPP) and read at 405 nm using a Titertek Multiskan Plus microplate reader (Labsystems).

7.2. 4. Determination of Progesterone concentration

Samples were assayed for progesterone using a procedure similar to that previously described (McFarlane *et al.*, 1990, Xia *et al.*, 2003). The antiserum was prepared using progesterone-11α-HS-BSA (230#; Bioquest Limited, North Ryde, NSW, Australia). Each sample was extracted in duplicate with a 10 X volume of diethyl ether.

The sensitivity of assay was 25 ng/ml. The inter-assay coefficient of variation was 11%, and the intra-assay coefficient of variation was 4% (method is fully described in appendix).

7. 3. Results

7.3. 1. Antibody titers

Ewes immunized against BMP-4 had measurable antibodies against BMP-4 (Fig. 7.1.). No cross reactivity was observed when plasma (collected after final booster injection) from BMP-4 immunized and control ewes was tested against BMP-2 peptide (Fig. 7.2 and 7.3). Ewes immunized with diphtheria toxiod did not have measurable antibodies to either BMP-2 or BMP-4 (Fig. 7.2 and 3.).

7.3. 2. Effect of active immunization on ovulation rate and follicle number

Ewes immunized against BMP-4 and control maintained regular estrous cycles throughout this study. Active immunization against BMP-4 decreased ovulation rate compared to control animals ($P < 0.05$; Fig. 7.4). The mean ovulation rates of BMP-4 immunized and control ewes were 2 and 3.2, respectively. However, follicle number (diameter $\geq$ 3 mm) observed at laparoscopy was not significantly different in different treatment groups. The mean follicle number of BMP-4 immunized and control ewes were 1.72 and 1.6, respectively ($P > 0.05$; Fig. 7.4).

7.3.3. Effect of active immunization on progesterone concentration

Immunization against BMP-4 affected the secretion of progesterone during luteal phase (day $\geq$ 8 and day < 15) of the estrous cycle. However, concentration of progesterone was similar in follicular phase (day < 8 and day > 15) for both treated and control animals. In luteal phase, progesterone concentration was significantly lower in BMP-4 immunized than control ewes ($P < 0.05$; Fig. 7.5). In follicular phase, concentration of progesterone was not significantly different between the treatment groups ($P > 0.05$; Fig. 7.5).

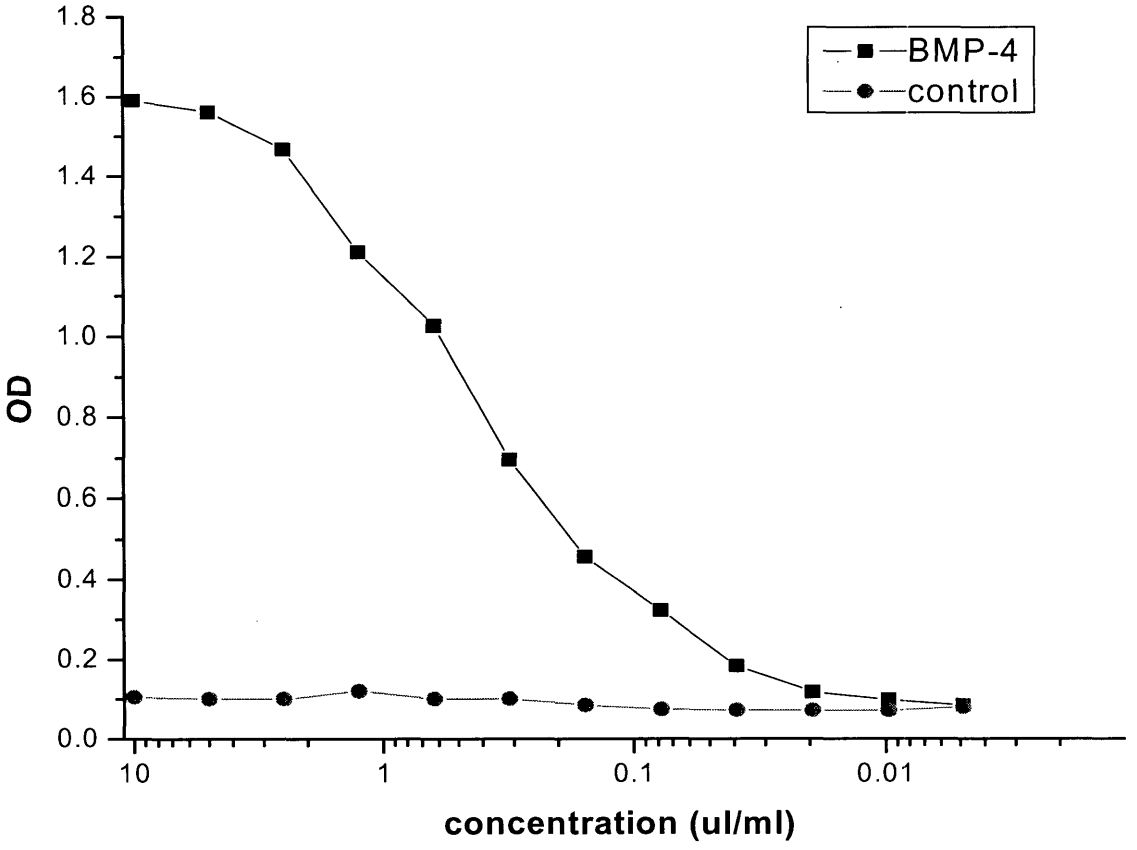

Figure 7. 1. This graph shows the binding of plasma collected from BMP-4 immunized and control ewes to 100 ng of rbBMP-4 protein. Plasma from BMP-4 immunized ewes showed significant binding with BMP-4 peptide while no binding to BMP-4 peptide was observed with plasma collected from control ewes.

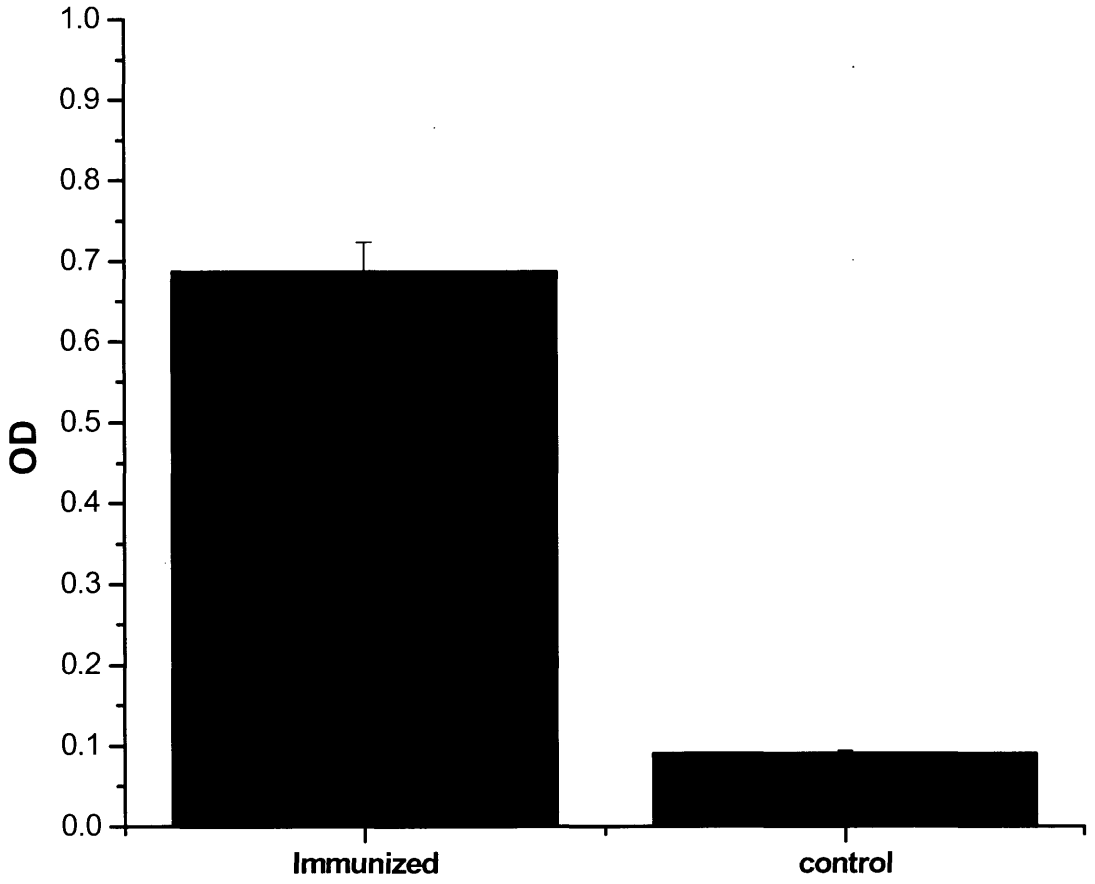

Figure 7. 2. Plasma from different treatment groups was tested for reactivity with BMP-4 peptide.

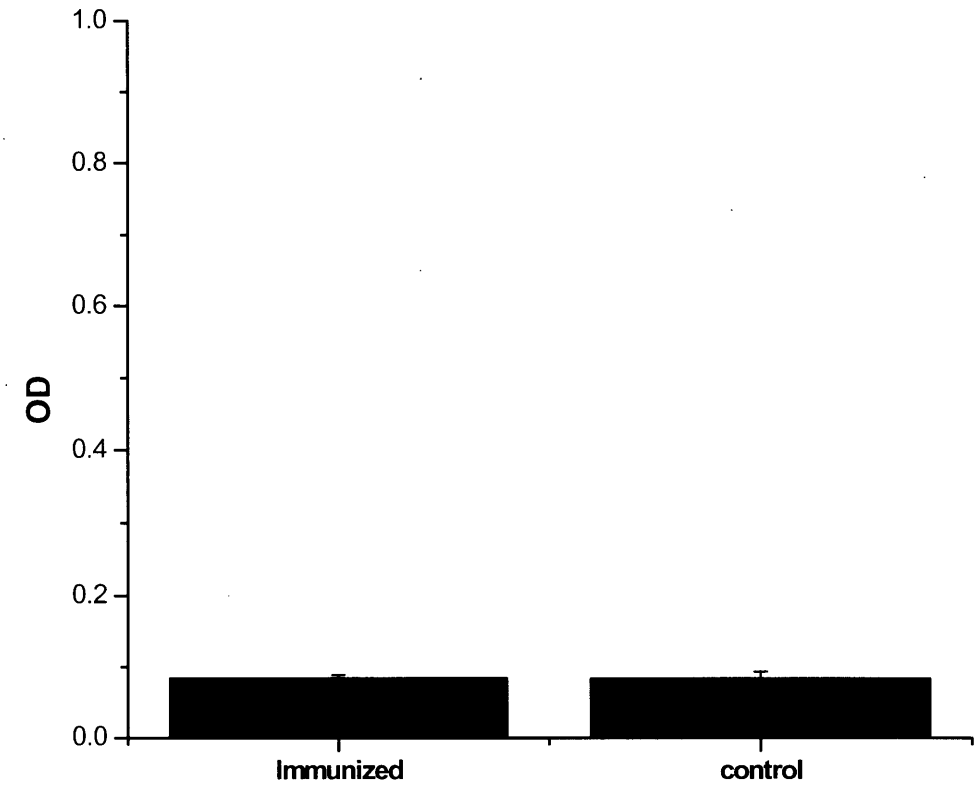

Figure 7. 3. Plasma from different treatment groups was tested for cross reactivity with BMP-2 peptide. No cross reactivity was detected to BMP-2 peptide.

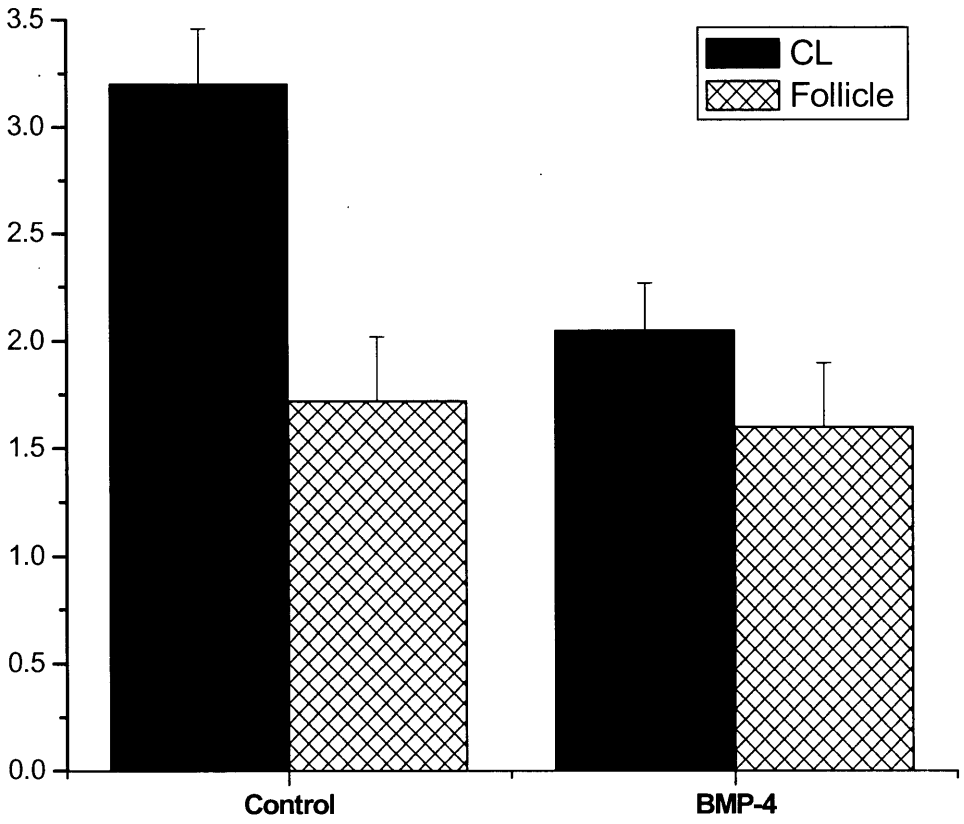

Figure 7. 4 Mean of number of CL and follicles observed at laparoscopic examination of ewes immunized against BMP-4 and controls. Number of corpus lutea was significantly lower in ewes immunized against BMP-4 than in controls ($P < 0.05$). No significant difference was observed between numbers of follicles in different treatment groups ($P > 0.05$).

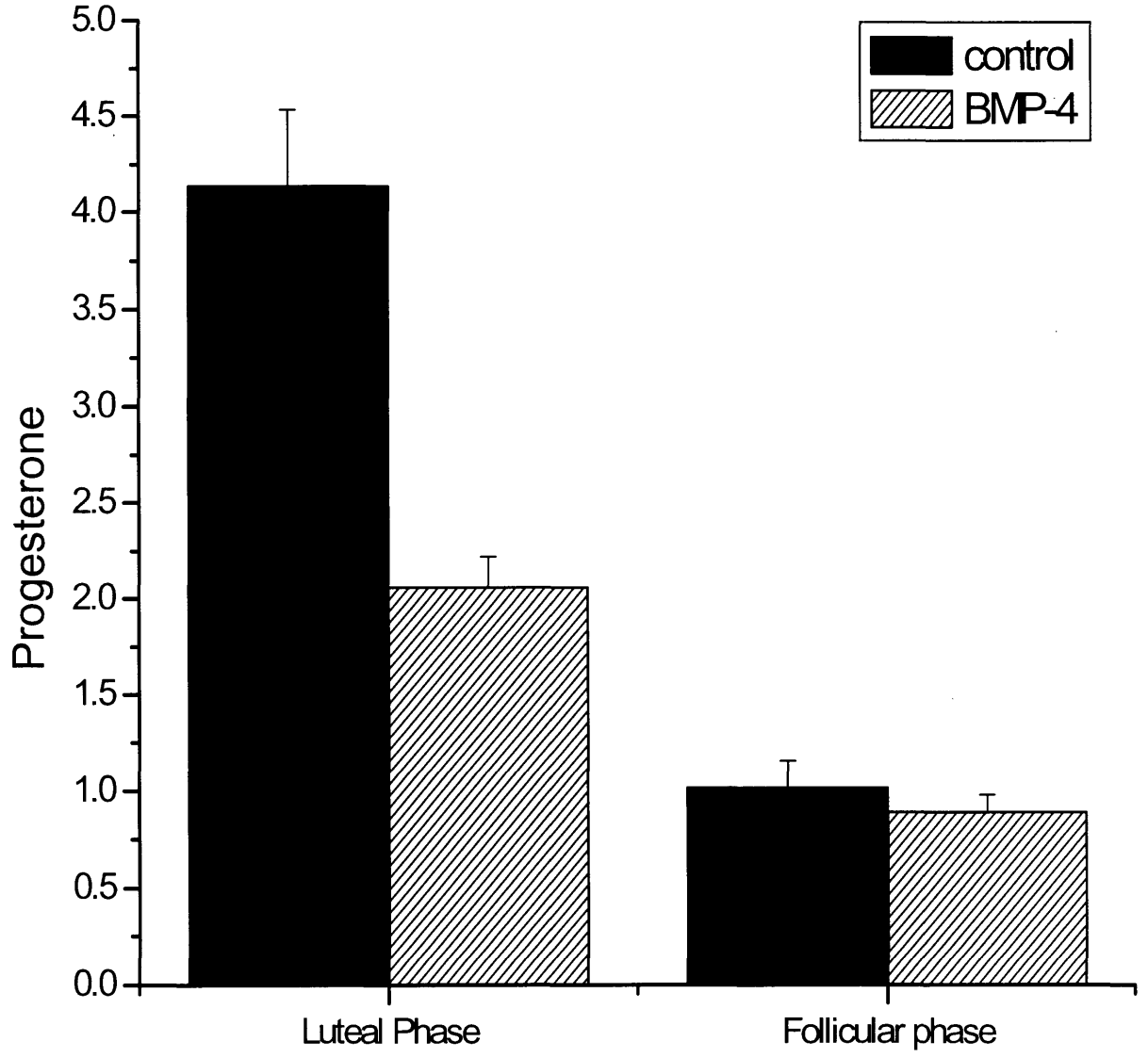

Figure 7. 5 Mean concentration of progesterone in plasma of ewes immunized against BMP-4 and control. Progesterone concentration was lower in luteal phase of the estrous cycle in immunized ewes than in controls ($P < 0.05$), but no difference in progesterone concentration was observed in follicular phase of estrous cycle ($P > 0.05$).

7. 4. Discussion

Our studies have shown that active immunization against BMP-4 decreases ovulation rate in ewes. The decrease in ovulation rate in BMP-4 immunized animals is accompanied by a decrease in progesterone secretion in plasma in luteal phase of the estrous cycle. The decrease in ovulation rate following immunization was due to specific neutralization of BMP-4, because no cross reaction was observed when tested against its most closely related family member, BMP-2 (Fig. 7.2 and 7.3.).

In the last few years, several major genes have been identified to affect ovulation rate in sheep (Fabre *et al.*, 2006). Inverdale and Hanna breed of sheep were shown to have mutation in the cDNA coding for the BMP-15 (Fabre *et al.*, 2006, Galloway *et al.*, 2000). Similarly, mutations in coding sequence of BMP-15 and GDF-9 gene have been identified and correlated with presence of sterility (homozygous) and enhanced fertility (heterozygous) in Belclare and Cambridge sheep (Hanrahan *et al.*, 2004). In addition, mutation in BMPR-IB has been shown to influence ovulation rate of Booroola merino sheep (Mulsant *et al.*, 2001, Souza *et al.*, 2001, Wilson *et al.*, 2001). Recently, active immunizations of ewes against GDF-9 peptide, BMP-15 peptide, and BMP-15 mature protein have been shown to cause arrested normal follicular development at transitory/ primary stage of follicular development indicating the role of these proteins in normal follicular development in sheep (Juengel *et al.*, 2002). Some ewes immunized against the mature region of BMP-15 had estrous cycles with increased ovulation rates compared to the control group. The phenotype observed in these immunized ewes is similar to Inverdale and Hanna sheep (Juengel *et al.*, 2004a, Juengel *et al.*, 2002).

Recently, a mutation in the BMP-15 gene has been shown cause infertility in humans, demonstrating the critical role of BMP-15 in fertility of woman (Di Pasquale *et*

al., 2004). Similar to mutation in sheep, this mutation occurs in the proregion of the BMP-15 protein, rather than in the functional mature region, indicating that the human BMP-15 mutation might affect the posttranslational processing of the human BMP-15 proprotein (Liao *et al.*, 2003, Yoshino *et al.*, 2006). However, in contrast to monoovulatory ewes and humans, BMP-15 gene deleted mice showed no defects in folliculogenesis, completing all stages of follicular development and having multiple corpora luteum except their litter size is smaller compared with the wild-type mice (Yan *et al.*, 2001). Similar phenotypic differences were observed in Booroola merino sheep (naturally occurring point mutation in BMPR-IB) (Mulsant *et al.*, 2001, Souza *et al.*, 2001, Wilson *et al.*, 2001), BMPR-IB knockout mice (Yi *et al.*, 2001) and in humans with naturally occurring mutation(s) in BMPR-IB (Demirhan *et al.*, 2005, Lehmann *et al.*, 2003). BMPR-IB null mice were infertile due to defective cumulus expansion, but follicular development was normal (Yi *et al.*, 2001). A human female with defective BMPR-IB was presented with absence of ovaries (ultrasonographically), hypoplastic uterus and with hypogonadotropic hypogonadism (Demirhan *et al.*, 2005). These discrepancies in the function of these genes might be because of species differences and/or different type of gene alteration (natural mutations / gene knockout).

Activin, inhibin and follistatin are important regulators of female fertility (de Kretser *et al.*, 2002). In mouse limb bud cell line (MLB13MYC clone 17), BMP-2 treatment increased follistatin and activin βA mRNA (Kearns & Demay, 2000). In addition, BMP-2 stimulates inhibin-B production in human granulosa luteal cells (Jaatinen *et al.*, 2002) and inhibin-A secretion by ovine granulosa cells (Souza *et al.*, 2002). In folliculostellate cells (FS/D 1h), BMP-4 and IL-1β have shown dose dependent effects on follistatin secretion (Bilezikjian *et al.*, 2006). Treatment of bovine granulosa cells with BMP-4, BMP-6 and BMP-7 has been shown to increase inhibin-A, activin-A,

and follistatin production (Glister *et al.*, 2004). Moreover, Follistatin can bind to BMP-4, BMP-6 and BMP-7 and inhibits their effects on bovine granulosa cells (Glister *et al.*, 2004). Follistatin can also prevent the inhibitory actions of BMP-15 on FSH receptor expression (Otsuka *et al.*, 2001b). Furthermore, inhibin-A has been shown to block the effects of BMP-2, BMP-7, BMP-9 and GDF-5 in hepatocyte HepG2 and mouse sertoli TM4 cell line (Wiater & Vale, 2003). Collectively, these studies indicate that the interplay of these proteins might influence the functional output of these factors and further studies are required to elucidate their role in modulation of ovarian functions.

In summary, active immunization against BMP-4 in ewes lowered ovulation rate and decreased progesterone secretion (luteal phase) in plasma indicating that BMP-4 plays a significant role in regulation of ovarian functions in sheep.

Chapter 8: General Discussion

The follicle is a basic functional unit of the ovary and consists of an oocyte surrounded by granulosa and theca cells (Hirshfield, 1991). Initiation of follicular growth occurs within the first week of life indicating that follicle recruitment is not limited to sexual maturity (Peters *et al.*, 1975). In addition, follicular development also occurs in FSH-β and FSH-R knockout, hypogonadal, gonadotrophin depleted, hemiovariectomized, and pregnant mice (Cattanach *et al.*, 1977, Dierich *et al.*, 1998, Kumar *et al.*, 1997, Peters *et al.*, 1975, Peters *et al.*, 1973, Radovick *et al.*, 1991). These studies have shown that the initiation of follicular development also occurs in full or partial absence of gonadotrophin, and is most likely controlled by intra-ovarian factors. Recently, BMP-4 and BMP-7 have been shown to promote transition of primordial to primary follicle (Lee *et al.*, 2001, Nilsson & Skinner, 2003). It has been shown in this thesis that anti-BMP-4 treated mice ovaries had a significantly higher proportion of primordial follicle and fewer developing primary follicle than control ovaries (Chapter 3). These findings are important for the treatment of human infertility/ subfertlity (genetic or pathological disorders). Current ovarian stimulation treatment for infertility or premature ovarian failure (POF) mainly uses gonadotrophins (Diedrich & Felberbaum, 1998, Zafeiriou *et al.*, 2000). The ovaries of most of the women with POF only contain primordial follicles (Olivar, 1996) and gonadotrophins only stimulate the growth of antral follicle so it is very difficult to achieve the required ovulation rate with current protocols in these cases. Results from chapter-3 with previous studies (Nilsson & Skinner, 2003) have indicated that BMP-4 can be used as an alternative treatment to stimulate the transition of primordial to primary follicle. More importantly, these factors may be used in human reproduction (i) to increase the longevity of female fertility by increasing the initial primordial follicle population; (ii) to delay menopause by delaying the development of

primordial follicles; (iii) to regulate the onset and timing of menopause by manipulating primordial follicle pool size and (iv) to increase fertility in subfertile women by stimulating primordial follicular development (Skinner, 2005).

In mammals, PGCs are the precursors of oocytes and pre-spermatogonia. In mice, gene knockout studies have revealed that BMP-4 and BMP-8B from extraembryonic ectoderm, and BMP-2 from visceral endoderm are required for development of PGCs (Lawson *et al.*, 1999, Ying & Zhao, 2001, Ying *et al.*, 2001). During embryonic development, these cells proliferate and remain in clusters and do not consistently attach with somatic cells (Pepling & Spradling, 2001). In later life (during fetal development in sheep, cow and human, and postnatally in the rodents), these cells disassociate themselves from the clusters and then assemble with precursor of squamous granulosa cells to form primordial follicles (McNatty *et al.*, 1995, McNatty *et al.*, 2000, Pepling & Spradling, 2001, Skinner, 2005). At present, factors controlling the formation of primordial follicle assembly are unknown. In chapter-6, we did immunolocalization of BMP-4 in sheep fetal ovaries at 100 day of gestation, a stage at which primordial follicle first appear in the sheep ovary (McNatty *et al.*, 1995) and found expression of BMP-4 protein in naked oocyte and precursor pregranulosa cells. These studies indicated that BMP-4 is involved in primordial follicle formation in sheep. Presently, no culture system allows efficient and complete *in vitro* culture of early stages of folliculogenesis in large domestic animals and humans (Cortvrindt & Smitz, 2001). Recently, Farini *et al.* used media with cocktails of soluble growth factors such as KL, LIF, BMP-4, stromal cell derived factor-1 (SDF-1), bFGF and compounds (L-cysteine, forskolin, retinoic acid) to grow mouse PGCs in the absence of somatic cells (Farini *et al.*, 2005). Most of these growth factors used in their germ cell culture media have already been shown to promote primordial follicle development (Nilsson & Skinner, 2003, 2004, Nilsson *et al.*, 2002, Tanwar & McFarlane,

2005) (Chapter 3). Collectively, these studies have suggested that similar cocktails of growth factor can be used to grow the initial stages of folliculogenesis in *in vitro* condition, which could provide mature oocytes for assisted reproductive treatments.

In situ hybridization and/or immunolocalization studies have shown expression of BMP ligands (BMP-2, BMP-3, BMP-3b, BMP-4, BMP-5, BMP-6, BMP-7, BMP-15 and GDF-9 and BMP receptors and downstream signaling molecules (ALK-2, BMPR-IA, BMPR-IB, ActR-II, ActR-IIB, β-glycan, smad-5, and smad-8) in ovaries of various mammalian species (Aaltonen *et al.*, 1999, Brankin *et al.*, 2005, Cameron *et al.*, 1994, Drummond *et al.*, 2002, Erickson & Shimasaki, 2003, Juengel *et al.*, 2002, McGrath *et al.*, 1995, Shimasaki *et al.*, 1999, Shimizu *et al.*, 2004a, Souza *et al.*, 2002, Wilson *et al.*, 2001, Xu *et al.*, 2002). In chapter 4 and 6 we have detected expression of BMP-4 protein in mouse and sheep ovaries. Interestingly, BMP-4 protein expression in the sheep ovaries starts in the preantral follicle and remains in preovulatory follicle and corpus luteum (Chapter 6). While, in mouse ovaries, BMP-4 protein expression was detected in all stages of follicular development except primordial follicle (Chapter 4). These studies have indicated that species difference exist in expression of BMP-4 protein in mice and sheep. Similar differences in expression of BMPs and their receptors have also been observed in mice, rat, human and sheep ovaries (Juengel *et al.*, 2006, Pangas *et al.*, 2002, Souza *et al.*, 2002, Wilson *et al.*, 2001, Yi *et al.*, 2001). In addition, for the first time we have detected expression of BMP-4 protein in oviduct and uterus of mice and sheep (Chapter 4 and 6). Recent studies by Hashimoto *et al.* have shown that processing of BMP-15 protein in mice and human is different in an *in vitro* system of transfected cells and, on the basis of their studies, the authors predicted that this difference in processing of BMP-15 protein could correlate with ovulation quota of mice and humans (Hashimoto *et al.*, 2005). In present studies (Chapter 4 and 6), in western analysis of mouse

ovaries and sheep follicular fluid, we observed some differences in the molecular weight of isoforms of BMP-4 between mouse ovaries and sheep follicular fluid. Unfortunately, further analysis of these isoforms was beyond the scope of present study and further studies are required to elucidate their role in different reproductive processes.

In sheep, large variation in litter size has been observed among different breeds and within breeds. Recently, Booroola sheep have been shown to have a mutation in the kinase domain of BMPR-IB and this mutation has been correlated with their higher ovulation rate than Merino sheep (Mulsant *et al.*, 2001, Souza *et al.*, 2001, Wilson *et al.*, 2001). Previous studies have shown that in Booroola ewes follicles mature and ovulate at significantly smaller size than in merino ewes (McNatty *et al.*, 1986). In addition, Booroola ewes have a higher concentration of FSH than merino ewes in the estrous cycle (McNatty *et al.*, 1991, Xia *et al.*, 2003). Recent *in vitro* studies have shown that granulosa cells from Booroola ewes (carrier) were less responsive than those from noncarrier Booroola ewes to the inhibitory effects of BMP-4 and GDF-9 on steroidogenesis (Fabre *et al.*, 2003, Mulsant *et al.*, 2001). In rat and bovine granulosa cell cultures, BMP-4 has been shown to inhibit progesterone and stimulate estradiol secretion (Glister *et al.*, 2004, Shimasaki *et al.*, 1999). In chapter-7, active immunization against BMP-4 of heterozygous Booroola ewes resulted in a lower ovulation rate than that of control ewes. In addition, plasma progesterone concentration in luteal phase in immunized ewes was lower than in control ewes. This is first study to show that BMP-4 has an important role in regulation of follicular development in sheep.

In males, mRNA of BMP-4, BMP-8a, BMP-8b, and GDF-9 has been detected in murine testis (Fitzpatrick *et al.*, 1998, Hu *et al.*, 2004, Zhao *et al.*, 1998, Zhao *et al.*, 1996). Moreover, expression of BMPR-IA, ActR-II was shown in pachytene spermatocytes and round spermatids, and no expression of BMPR-IB has been detected in murine testis

(Cameron *et al.*, 1994, Zhao *et al.*, 2001). *In situ* hybridization studies have also revealed the presence of BMP ligands (BMP-7 and BMP-8a), BMP receptors (BMPR-IA, BMPR-IB, and BMPR-II), and smad-1 mRNA in epithelial cells of mouse epididymis (Chen *et al.*, 1999, Hu *et al.*, 2004, Zhao & Hogan, 1996, Zhao *et al.*, 1998, Zhao *et al.*, 1996, Zhao *et al.*, 2001). Knockout mice of BMP-4, BMP-7, BMP-8a and BMP-8b had defects in spermatogenesis and in epididymal development, indicating the role of these BMPs in regulation of male reproductive functions. In chapter-5 we localized expression of BMP-4 protein in testis, epididymis, vas deferens, and prostate. In contrast, we did not find any expression of BMP-4 in ram testis; although, strong immunostaining for BMP-4 was observed in epithelial cells of ram epididymis (Chapter 6). Hu *et al.* have shown that, unlike BMP-7 and BMP-8a, no granuloma formation occurs with degeneration of epididymal epithelium of BMP-4 heterozygous knockout mice (Hu *et al.*, 2004). These findings suggest that targeted disruption of BMP-4 signals in epididymis of adult males can be used as a birth control measure and to develop new male contraceptive treatments. Further studies are required to elucidate the role of BMPs in human reproduction as most of the knowledge regarding BMPs in reproduction is from murine or ruminant models, and studies have shown species specific differences in the function of these proteins.

The development of the prostate and other male accessory internal structures is androgen dependent (Pointis *et al.*, 1980, Resko, 1970, Siiteri & Wilson, 1974). Ablation or surgical removal of testis during fetal development inhibits development of male accessory sex glands (Cunha *et al.*, 1987). Similarly, treatment with estrogens and anti-androgens also inhibits development of male accessory sex glands including prostate (Cunha *et al.*, 1987). BMP-4 has been regarded as a negative regulator of branching in the early prostate (Miyazaki *et al.*, 2000). Enlargement of prostate glands occurs in BMP-4 heterozygous mutant mice

(Almahbobi *et al.*, 2005). In chapter-5 strong expression of BMP-4 protein in prostate gland was shown. Additionally, passive immunization against BMP-4 decreased and increased testosterone secretion in prepubertal and adult testis, respectively. Collectively, these studies have indicated that BMP-4 plays an important role in regulation of male reproductive functions.

In conclusion, the present studies have shown that BMP-4 is an important regulator of male and female fertility in both mice and sheep.