Chapter 3: *In vivo* evidence of role of BMP-4 in the mouse ovary

3. 1. Introduction

The primary function of the ovary is to release a fertilizable egg each estrous cycle and to prepare the accessory organs for pregnancy by secreting endocrine, autocrine and paracrine factors (Gougeon, 1996, McGee & Hsueh, 2000). Folliculogenesis is a continuous process of follicle development, in which a few resting primordial follicles are converted to primary follicles, which grow in orderly progression until finally becoming ovulatory or atrectic follicles (Peters *et al.*, 1975). While much is known about the final stages of this process relatively little is known about the initiation of follicular development in a primordial follicle. The development of a primary follicle from a primordial follicle has been considered independent of gonadotrophins because these follicles have been shown to develop in FSH β subunit (Kumar *et al.*, 1997) and FSH receptor (Dierich *et al.*, 1998) knockout mice. In contrast, some studies have indicated that the development of a primary follicle is gonadotrophin dependent as chronically elevated level of LH has been shown to increase the depletion of the primordial follicle reserve in LH β subunit transgenic mice (Flaws *et al.*, 1997). In addition, increased concentrations of FSH in unilateral ovariectomized rat is associated with increased loss of primordial follicles (Meredith *et al.*, 1992). On the basis of the above studies it could be hypothesized that local regulatory factors are produced by ovary or the follicles and then these factors are responsible for development of primary follicle and gonadotrophins may modulate the activity of these factors to affect this process.

BMPs are members of the TGF-β superfamily (Shimasaki *et al.*, 2004). Recently, several BMPs have been implicated for their role in follicular development (Lee *et al.*, 2001, Vitt *et al.*, 2000). *In situ* hybridization and immunolocalization studies have shown that BMP receptors (ALK-2, BMPR-IA, BMPR-IB, ActR-II and ActR-IIB) are present in rat primordial follicles (Drummond *et al.*, 2002, Erickson & Shimasaki, 2003). Whereas rat primordial follicles do not express BMP-2, BMP-4, BMP-3, BMP-3b and BMP-6 (Erickson & Shimasaki, 2003) suggesting that these proteins are secreted by other cell types within the ovary and may promote primordial follicle development. The expression of BMP-4 has been localized to the basement membrane and stromal cells surrounding follicles in neonatal rat ovaries (Nilsson & Skinner, 2003). In addition, BMP-4 mRNA has been shown to be expressed in theca, ovarian surface epithelium and sex cords of adult rat ovaries (Erickson & Shimasaki, 2003). BMP-4 has been shown to stimulate production of estradiol and inhibit production of progesterone in the presence of FSH in rat granulosa cell culture (Shimasaki *et al.*, 1999). BMP-4 was also found to increase both basal and FSH induced production of estradiol, inhibin-A, activin-A and follistatin and inhibited both basal and IGF stimulated progesterone production in bovine granulosa cell culture (Glister *et al.*, 2004). The addition of BMP-4 was found to decrease the production of androgens from HOTT by modulating CYP 17 and 3β HSD ratio in these cells, which ultimately leads to an increase in production of progesterone with concomitant decrease in production of androstenedione (Dooley *et al.*, 2000). Apart from these paracrine effects of BMP-4 in granulosa and theca cell culture, BMP-4 null mouse embryos are devoid of primordial germ cells (Lawson *et al.*, 1999). Recently, BMP-4 has been shown to promotes transition of primordial follicle to primary follicle in whole neonatal rat ovary culture (Nilsson & Skinner, 2003).

The biological activities of BMPs are regulated at multiple levels by various extracellular and intracellular factors (Miyazono, 2000). The data from BMP-4 null mice and *in vitro* studies strongly suggest a role for BMP-4 in folliculogenesis. The aim of our study was to elucidate the biological role of BMP-4 in transition of primordial follicle to primary follicle and to observe the effect of gonadotrophin on the biological actions of BMP-4 *in vivo*.

3. 2. Materials and Methods

3.2. 1. Reagents and hormones

An antiserum against a synthetic peptide equivalent to amino acids 88-102 from mouse BMP-4 (Invitrogen Australia, Mount Waverly Vic 3149 Australia) was raised in sheep. Equine chorionic gonadotrophin (eCG, Bioniche Animal Health, Armidale NSW 2350 Australia) was used to stimulate follicle development.

3.2. 2. Animals

Female Swiss mice 21 days old (Physiology animal house, University of New England, NSW, Australia) were used in this experiment.

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 was treated with eCG (1 IU) with and without anti BMP-4 (50ug) and 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 three times and the ovaries were collected and weighed before being immersion fixed in Bouin's fluid for histological quantification of follicles.

3.2. 3. Histological analysis and follicle counting

The fixed ovaries of each treated group were dehydrated and paraffin embedded. They were serially sectioned by microtome at a thickness of 5 µm, which was previously used by Lee *et al* (Lee *et al.*, 2004) for counting of follicles in mouse ovaries. The sections were mounted on glass slides and stained with Harris's hematoxylin and eosin (fully described in appendix). Every fifth section was selected and all the follicles were

counted and classified according to Vitt *et al* (Vitt *et al.*, 2000) except the follicles larger than primary follicles were classified as developing follicles. Only follicles with visible nucleolus in oocyte were counted to avoid duplication in counting of follicles. The follicle with small oocyte and single layer of squamous cells were classified as primordial follicles. The follicles with single layer of cuboidal cells or a mixture of cuboidal and squamous cells around oocyte were counted as primary follicles.

3. 3. Results

3.3. 1. Effect of anti BMP-4 on ovarian weight

The combined ovary weight from pubertal mice treated with anti-BMP4 with or with out eCG is shown in figure 3.1. The ovarian weight of mice treated with anti BMP-4 (5.25 ± 0.572 mg) was almost half of the ovarian weight of mice belonging to the control group (10.95 ± 0.986 mg). The ovarian weight of mice treated with eCG with and without antiBMP-4 were 18.16 ± 1.21 mg and 20.95 ± 0.628 mg respectively. The ovarian weights of the three treatment groups were all significantly ($P<0.0001$) different from the control and each other. The body weight (18 ± 1 g) of the mice was not different between groups after 7 days of treatment.

3.3. 2. Effect of anti BMP-4 treatment on follicular development

The percent numbers of primordial, primary and developing follicles in each ovary from the different treatment groups are shown in figure 3.2. The number of primary follicles was significantly ($P<0.0001$) reduced in ovaries from anti BMP-4 treated mice compared with the control group. Furthermore, anti BMP-4 treated ovaries had greater numbers of primordial follicles than the ovaries of control group. The ovaries from mice treated with eCG had significantly lower numbers of primordial follicles than the control with a corresponding increase in primary follicles. Interestingly, the number of primordial follicles was higher than the control in those mice treated with a combination of eCG and anti BMP-4 and had a decreased number of primary follicles. No effect on developing follicles was seen by any of the treatments as shown in figure 3.2.

The effect of anti BMP-4 and eCG treatment on follicle development is clearly demonstrated in ovarian sections of each treated group as shown in figure 3.3. The ovarian section of anti BMP-4 treated group has more primordial and very few primary follicles. While control group ovary sections has multiple primary and primordial follicles but number of primordial follicle is less than antiBMP-4 treated group. In addition, ovarian section of eCG treated group shows more primary follicles but fewer primordial follicles compared to eCG with anti BMP-4 treated group.

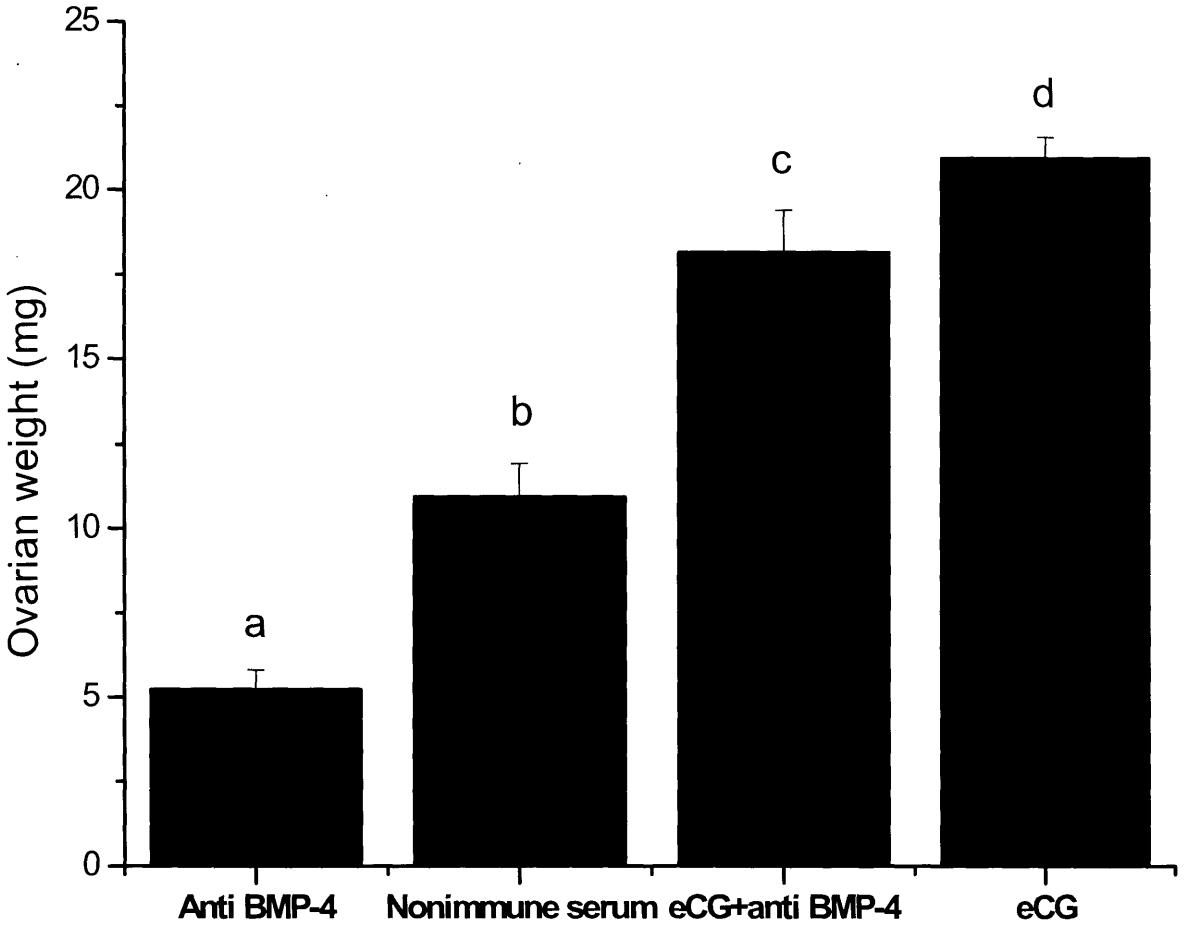


Figure 3. 1. The *in vivo* effects of anti BMP-4 treatment on ovarian weight in the presence or absence of eCG. The data are shown as mean ± SEM for individual ovaries of five mice of each treatment group repeated 3 times. Different lettered subscripts represent significant differences in ovarian weight of each treatment group ($P < 0.0001$).

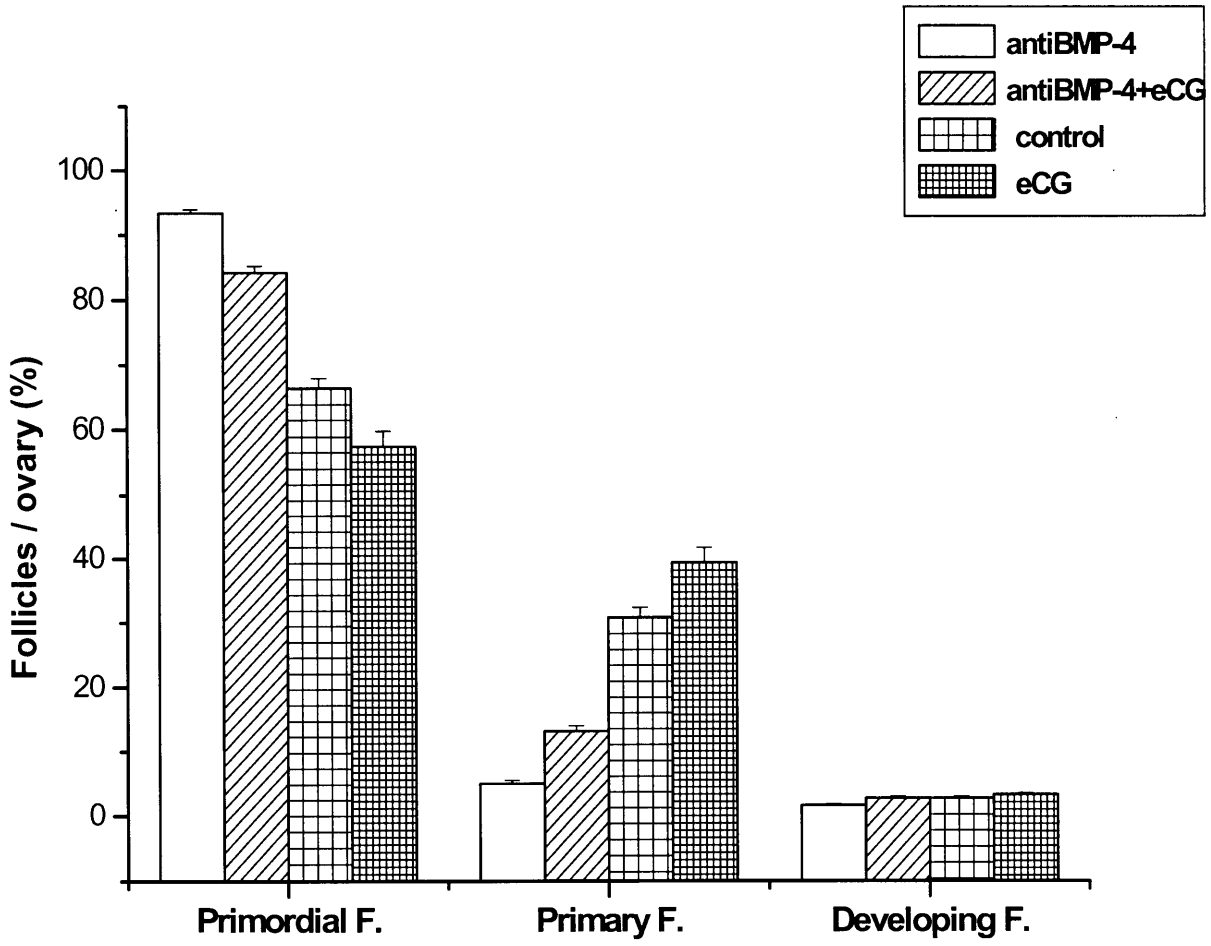


Figure 3. 2. The effects of anti BMP-4 treatment on follicular development in the presence or absence of a low dose of eCG. The data are displayed as percent of total follicle number per ovary in each treatment group. The data are shown as mean ± SEM for five mice in each treatment group repeated 3 times ($P < 0.0001$).

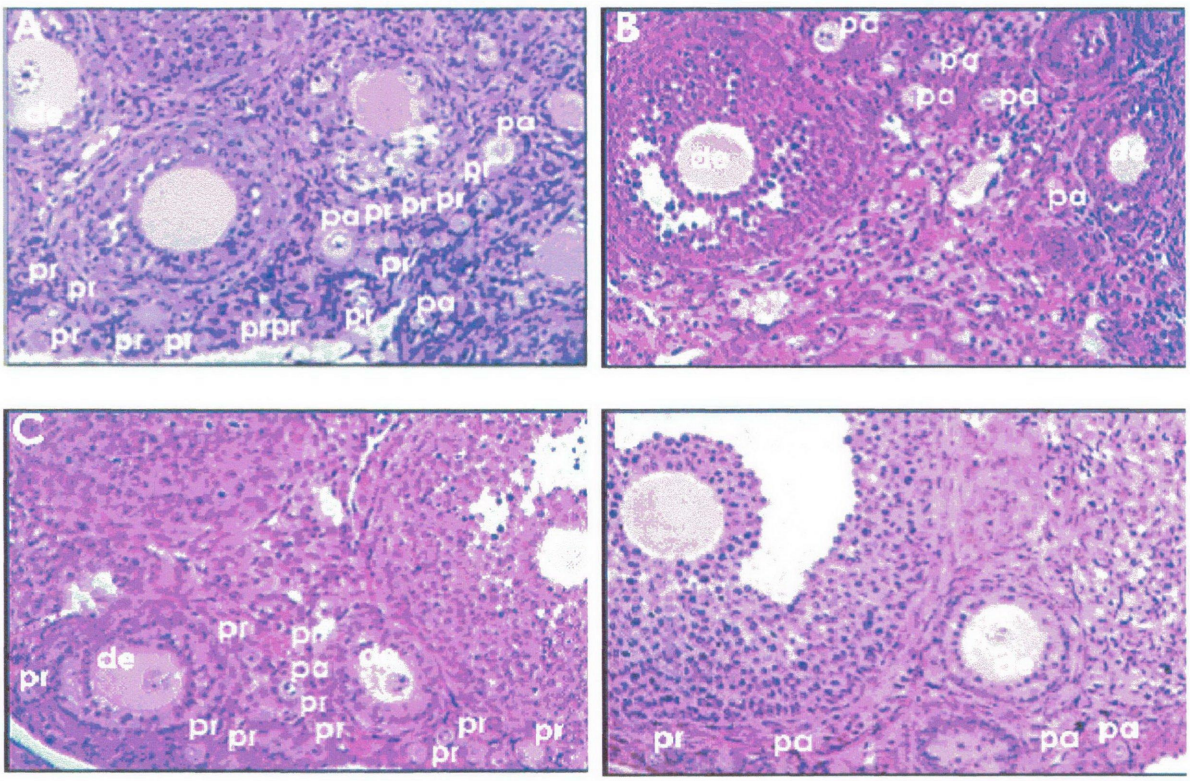


Figure 3.3. Representative ovarian sections from pubertal mouse ovaries after 7 days of treatment with anti BMP-4, eCG with or without anti BMP-4 or non immune serum. Each panel displays a partial view of ovarian section of each treatment group at 200X magnification. (A) Ovarian section from anti BMP-4 treated group (B) Ovarian section from control group (C) ovarian section from anti BMP-4 with eCG treated group (D) Ovarian section from eCG treated group. Primordial follicle (pr), Primary follicle (pa), Developing follicle (de)

3. 4. Discussion

The results of our present study strongly suggest that BMP-4 is an important permissive factor in the transition of primordial to primary follicles and that this effect is independent of gonadotrophins. A similar conclusion was drawn by Nilsson *et al* based on their *in vitro* studies showing that anti BMP-4 treatment decreases the size of the ovary compared to the control group, while addition of BMP-4 to culture media promotes transition of primordial follicle to primary follicles in neonatal rat ovaries (Nilsson & Skinner, 2003).

Several growth factors have been shown to be potentially involved in the regulation of initial follicular development. Kit ligand (KL) (Nilsson & Skinner, 2004), basic fibroblast growth factor (bFGF) (Nilsson & Skinner, 2004), leukemia inhibitory factor (LIF) (Nilsson *et al.*, 2002), insulin (Kezele *et al.*, 2002) and keratinocyte growth factor (KGF) (Kezele *et al.*, 2005) have all been shown to promotes primordial to primary follicle transition in a rat ovary culture system. BMP-4, BMP-7 and GDF-9 have also been shown to influence initial follicular development in various culture conditions (Lee *et al.*, 2004, Nilsson & Skinner, 2002, 2003). Recently, Lee *et al* have reported that *in vivo* injection of BMP-7 into the ovarian bursa of the rat ovary decreases number of primordial follicles with a subsequent increase in number of primary, pre-antral and antral follicles suggesting that BMP-7 can also act as facilitating agent in different stages of follicular development (Lee *et al.*, 2001). Similarly, Vitt *et al* have shown that the treatment of immature mice with GDF-9 increases the weight of ovaries with concomitant increase in progression of primordial and primary follicle to small preantral follicle (Vitt *et al.*, 2000). In contrast to these above studies, our results have for the first time demonstrated that BMP-4 is involved in the transition of

primordial to primary follicle without having any apparent effect on other stages of follicular development; however this needs to be confirmed with further study.

In this current study anti BMP-4 significantly reduced the number of primary follicles present in the ovary after stimulation with eCG supported by the fact that there was an increased number of primordial follicles. This suggests that at least a major part of the effect is not related to an effect on gonadotrophins since if BMP-4 suppresses FSH production in the mouse pituitary as has been shown in sheep pituitary cultures (Faure *et al.*, 2005), it would be expected that circulating FSH concentrations would rise in response to treatment with anti BMP-4. However, there appears to be considerable differences in the action of BMPs between different species. Recently, Faure *et al* reported that BMP-4 and BMP-6 suppresses FSH production and the expression of FSH β mRNA in sheep pituitary cell cultures (Faure *et al.*, 2005). In contrast, Huang *et al* reported stimulatory effect of BMP-6 and BMP-7 in mouse pituitary culture (Huang *et al.*, 2001a). While Otsuka and Shimasaki have demonstrated that BMP-6 and BMP-7 have no affect on FSH production in rat pituitary cell culture (Otsuka & Shimasaki, 2002b). These studies constitutively indicate that extreme caution should be applied when comparing actions of BMPs in different species of animals and further studies are required to specify physiological role of these proteins in different species of animals.

While results from this and previous studies clearly demonstrate an effect of BMP-4 on follicular development, these experiments do not pin point the site of action. The bulk of the evidence suggests that BMP-4 acts in a paracrine manner but it is possible that BMP-4 has an endocrine role in the regulation of reproductive function and future studies examining this aspect will be important in elucidating our knowledge of BMP function.

Chapter 4: Immunolocalization of BMP- 4 in reproductive organs of mice

4. 1. Introduction

The BMPs are the largest family of growth factors in the TGF- β superfamily. They are involved in multiple cellular functions such as proliferation, differentiation, migration, organization and death (Shimasaki *et al.*, 2004). Recently, several BMP family members including BMP-2 (Erickson & Shimasaki, 2003), BMP-3 (Takao *et al.*, 1996), BMP-4 (Shimasaki *et al.*, 1999), BMP-5 (Pierre *et al.*, 2005a), BMP-6 (Erickson & Shimasaki, 2003), BMP-7 (Shimasaki *et al.*, 1999) and BMP-15 (Dube *et al.*, 1998) have been identified in the mammalian ovary. BMP-2 mRNA is expressed in granulosa cells of atrectic and graffian follicles in rat ovary (Erickson & Shimasaki, 2003) while BMP-2 protein is detected in theca and oocyte of bovine ovary (Fatehi *et al.*, 2005). BMP-4 mRNA is detected in theca, corpus luteum, ovarian surface epithelium of rat ovary (Erickson & Shimasaki, 2003). In contrast, BMP-4 mRNA is expressed in both granulosa and thecal cells of bovine, chicken and mouse ovary (Fatehi *et al.*, 2005, Onagbesan *et al.*, 2003, von Schalburg *et al.*, 2006). Similarly, GDF-9 and BMP-15 expression is detected in the oocyte of the follicles throughout follicular development (Bodensteiner *et al.*, 1999, Erickson & Shimasaki, 2003, Juengel *et al.*, 2004b) except in primates, where GDF-9 expression was reported in the cumulus and mural granulosa cells (Duffy, 2003). The mRNA encoding various BMP ligands including BMP-2, BMP-4, BMP-6 and BMP-7 have also been detected in rat uterus (Erickson *et al.*, 2004). In addition, BMPR-IA, BMPR-IB and BMPR-II mRNA are detected in uterine epithelial

cells, and BMPR-IA and BMPR-II mRNA are detected in periluminal stroma and smooth muscle (Erickson *et al.*, 2004).

The biological actions of BMPs are mediated through a hetero-oligomeric complex of the BMP receptor type I (BMPR-IA and BMPR-IB) and type II (BMPR-II) receptors. This complex allows phosphorylation of type I receptor by type II receptor, which subsequently leads to transphosphorylation of intracytoplasmic signaling molecules smad 1, 5, and 8 (Massague, 1998, 2000, Miyazono, 2000). Recently, the expression of BMP receptor type I A, I B and II were detected in granulosa cells and oocytes of primordial follicles in the rat and sheep ovary (Shimasaki *et al.*, 1999, Souza *et al.*, 2002). A point mutation in BMPRIB has been shown to increase ovulation in Booroola sheep (Mulsant *et al.*, 2001, Souza *et al.*, 2001, Wilson *et al.*, 2001). However, gene knockout and naturally occurring mutation in BMPRIB have adverse effects on female fertility in mouse (Yi *et al.*, 2001) and humans (Demirhan *et al.*, 2005).

The mature BMP-4 ligand is a functional dimer. It contains seven cystine residues, six of which are involved in intramolecular disulphide linkage, and the seventh of which is responsible for dimer formation (McDonald & Hendrickson, 1993, Vitt *et al.*, 2001). BMP-4 is secreted as a large inactive precursor protein (408 aa). The precursor protein consists of a signal sequence (19 aa), a proregion (273 aa), and a mature segment (116 aa) (Wozney *et al.*, 1988). Both the precursor and mature form of BMP-4 contains two potential N-linked glycosylation sites (Cui *et al.*, 2001). The precursor form of BMP-4 is proteolytically cleaved by members of the proprotein convertase family of serine proteases following a multibasic motif (-R-X-K-R-, termed the S1 site) to generate active carboxy terminal ligand (Table 4.1)(Aono *et al.*, 1995, Degnin *et al.*, 2004, Sopory *et al.*, 2006). BMP-4 also undergoes a second cleavage at a minimal furin consensus motif (-R-X-X-R-, the S2 site) located upstream of the S1 site

within the prodomain (Table 4.1) (Constam & Robertson, 1999, Cui *et al.*, 1998, Cui *et al.*, 2001). Cleavage of pro-BMP-4 occurs sequentially (S1 and then S2), and the initial cleavage at the S1 site releases the mature ligand , while the subsequent cleavage at the S2 site regulates the activity and signaling range of mature BMP-4 (Cui *et al.*, 2001, Sopory *et al.*, 2006). In *Xenopus* embryos, BMP-4 synthesized from exogenous precursor in which the S2 is non-cleavable is less active and signals over shorter range and accumulates at lower levels than does BMP-4 cleaved from native precursors. Whereas proBMP-4 in which the upstream site is mutated to an optimal furin motif, is rapidly and simultaneously cleaved at both sites and this generate a ligand that is more active and signals over greater range than does BMP-4 cleaved from native precursor (Cui *et al.*, 2001, Sopory *et al.*, 2006). Recent biochemical analysis of proBMP-4 in the *Xenopus* oocytes have suggested that the BMP-4 precursor is first cleaved at the S1 site in the trans golgi network and at this stage the prodomain remains non-covalently associated with the mature ligand (Degnin *et al.*, 2004, Sopory *et al.*, 2006). This complex then traffics to the post trans golgi network compartment, where the more acidic environment unmasks and facilitates cleaving at the second site. The S2 cleaves breaks the non-covalent association between mature BMP-4 and the prodomain, and the free ligand is stable and able to signal over long range. If the cleavage at the S2 site doesn't occur, the prodomain/ligand complex is targeted to lysosome degradation and because of this mature BMP-4 non-covalently associated with the prodomain signals to nearby cells (short range activity). These studies indicate that cleavage at S2 site determine how much BMP-4 is available for signaling (Degnin *et al.*, 2004, Sopory *et al.*, 2006).

Recently, analysis of mice carrying a point mutation that prevents S2 processing has shown that cleavage of S2 site is essential for normal development and this site

might be selectively cleaved in a tissue specific fashion (Goldman *et al.*, 2006, Sopory *et al.*, 2006). Specifically, these mice showed severe loss of BMP-4 activity in some tissues, such as testes and germ cells, whereas other tissues that are sensitive to BMP-4 dosage, such as the limb, dorsal vertebrae and the kidney, developed normally (Goldman *et al.*, 2006, Sopory *et al.*, 2006). In a haploinsufficient background (C57BL/6J), inability to cleave the S2 site leads to embryonic and postnatal lethality due to defects in multiple organ systems including allantois, placental vasculature, ventral body wall, eye and heart (Goldman *et al.*, 2006, Sopory *et al.*, 2006). These findings supports that differential use of S2 cleavage site provides a mechanism for tissue specific regulation of BMP-4 activity (Goldman *et al.*, 2006, Sopory *et al.*, 2006).

Recent studies have observed significant differences in the activity of prodomain of TGF-β and BMPs (Gray & Mason, 1990, Gregory *et al.*, 2005, Sopory *et al.*, 2006). In TGF-β family, prodomians have been shown to affect the activity of mature ligand by stabilizing it, maintaining in inactive and latent state, and by targeting it to extracellular matrix (ECM). Following TGF- β cleavage, the prodomain non-covalently associated with the mature ligand in a small latent complex that cannot interact with its receptor (Todorovic *et al.*, 2005). This complex is targeted to the ECM via an interaction between the prodomain and the members of the latent TGF-β binding protein (LTBP) family of matrix proteins. Similar to TGF-β, the prodomain of BMP-7 remains non-covalently attached with the mature domain following cleavage but unlike TGF-β, this complex is capable of binding to and activating its receptors (Jones *et al.*, 1994). The prodomain of BMP-4 is unique in its ability to bind to, and target the mature domain for lysosomal degradation after initial cleavage at S1 site. However, it releases mature BMP-4 following a subsequent cleavage at S2 site. Like BMP-7, mature BMP-4 is signaling competent when complexed with its prodomain, but its signals over a shorter

distance due to targeted lysosomal degradation (Goldman *et al.*, 2006, Sopory *et al.*, 2006).

These studies underscored the importance of BMPs in female fertility but the cellular localization of BMPs in female reproductive organs is still unclear. In present study we have analyzed the expression of BMP-4 protein in female reproductive organs of mice and detected different isoforms of BMP-4 in mouse ovary by western blot analysis.

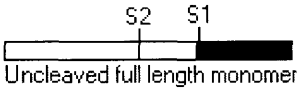
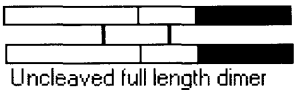
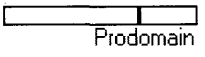
Reported Isoforms of BMP-4	MW	Reference
	NG: 50 kDa G: 58 kDa	(Cui *et al.*, 2001) Calculated
	NG:100 kDa G: 108-110 kDa	(Constam & Robertson, 1999) Calculated
	NG: 30 kDa G: 40-42 kDa	(Constam & Robertson, 1999, Hammonds *et al.*, 1991)
	NG: 15 kDa G: 23-27 kDa	(Cui *et al.*, 2001, Wordinger *et al.*, 2002)
	NG: 32-35 kDa	(Cui *et al.*, 2001)

Table 4. 1. The schematic representation of different BMP-4 isoforms (S1: first cleavage site, S2: second cleavage site, NG: non glycosylated, G: glycosylated). (The molecular weights of some isoforms are calculated on the basis of previously reported studies and only represent full glycosylation forms of BMP-4).

4. 2. Material and Methods

4.2. 1. Tissues and sections preparation

All the tissues (ovary, oviduct and uterus) used in this study were collected from female Swiss mice (Discipline of Physiology, University of New England, NSW, Australia). All the tissues were further processed and sectioned as described in chapter 2.

4.2. 2. ELISA

To test the cross reactivity of anti BMP-4 antibody against BMP-2, we used indirect ELISA technique as described in chapter 2.

4.2. 3. Immunohistochemistry

Method is described in chapter 2

4.2. 4. Western blotting

Mouse ovaries were homogenized in four times (V/W) of a homogenization buffer (benzamidine 5mM, EDTA 5 mM and sodium azide 0.02%). After centrifugation for 10 min at 10000 rpm, the supernatant was collected and diluted in reducing and nonreducing buffer. AntiBMP-4 antibody used in this experiment can detect all BMP-4 isoforms with BMP-4 mature domain. Samples were further processed as described in chapter 2.

4. 3. Results

Before immunohistochemical localization studies, cross-reactivity of anti BMP-4 antibody was determined against BMP-2 peptide. Anti BMP-4 antibodies showed no cross-reactivity with BMP-2 peptide (as described in chapter 2).

Immunohistochemical analyses of adult mouse ovary showed strong immunostaining for BMP-4 in all stages of follicular development except primordial follicles (Fig. 4.1A). Staining was observed in granulosa cells membrane of primary follicles while both oocyte and granulosa cells membrane were stained in preantral follicles. In tertiary follicles (Fig. 4.2B and 4.3C), strong immunostaining was observed in oocytes and granulosa cells membrane while lower intensity staining was also present in theca cells, especially in theca interna cells. The intensity of staining increased with the development of follicle and very strong staining was observed in tertiary follicles. The intensity of staining was very low in atrectic follicles as compared to tertiary follicles (Fig. 4.4D). Interestingly, the ovarian surface epithelium overlying atrectic follicles was stained while staining was absent in the surface epithelium of tertiary follicles (Fig. 4.4D). Strong staining was also observed in the corpus luteum (Fig. 4.5E) and stromal cells around follicles (Fig. 4.2B).

Within the adult mouse uterus, BMP-4 was detected in blood vessels, endometrial gland and surface epithelium of endometrium (Fig. 4.7G). In mouse oviduct, BMP-4 protein was mainly detected in epithelium and blood vessels (Fig 4.6F).

The presence of BMP-4 in ovary was confirmed by western blotting, where a band migrating at 50 kDa was detected in nonreducing condition (Fig. 4.9 lane A) while bands migrating at 15 and 35 kDa were observed in reducing condition (Fig. 4.9 lane B). The membrane treated with non specific purified sheep immunoglobulin instead of anti

BMP-4 antibody showed no bands and was considered as negative control (Fig. 4.9 lane C and D). The observed band sizes are consistent with previous studies (Cui *et al.*, 2001).

Figure 4. 1(A). Expression of BMP-4 protein in primary (Pra), preantral (Pan) and antral (An) but not in primordial (Pri) follicle of mouse ovary (200 X)

Figure 4. 2(B). Expression of BMP-4 of tertiary follicle (Granulosa cells: G; Theca cells: T; Surface epithelium: SE; Stromal tissue: S; Oocyte:O) (200 X)

Figure 4. 3(C). Expression of BMP-4 in granulosa and theca cells of tertiary follicle (Granulosa cells: G; Theca cells: T) (1000X)

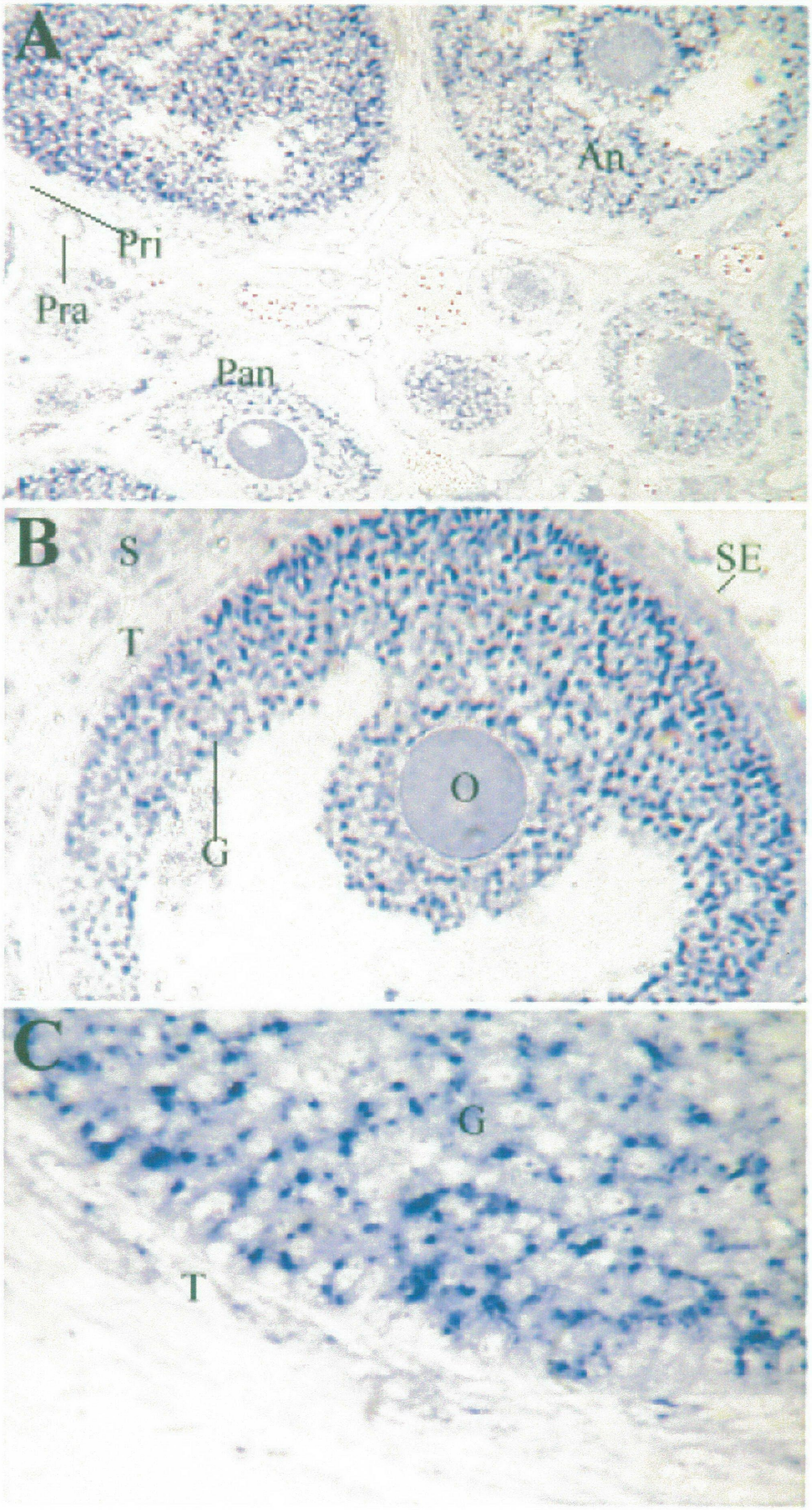
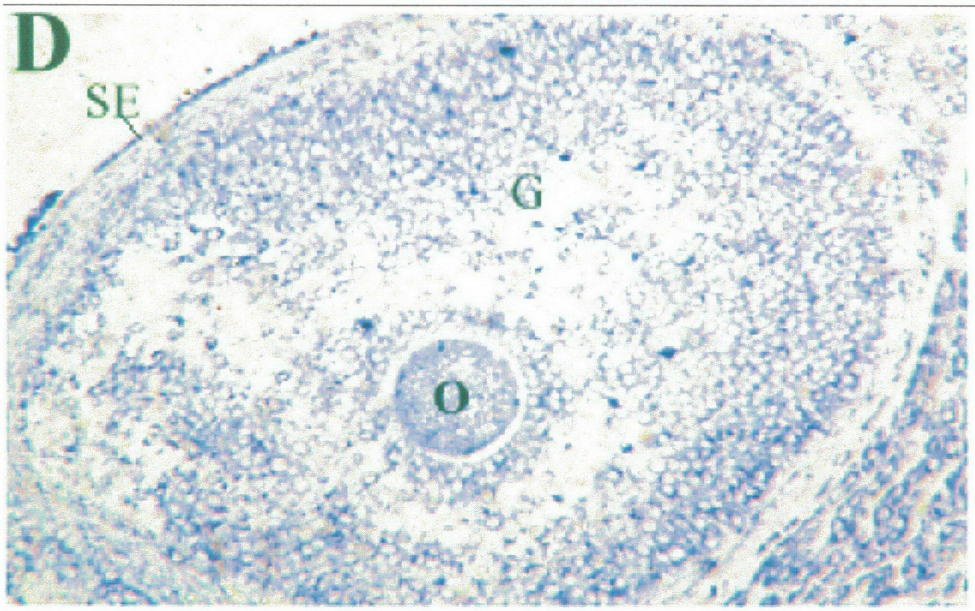
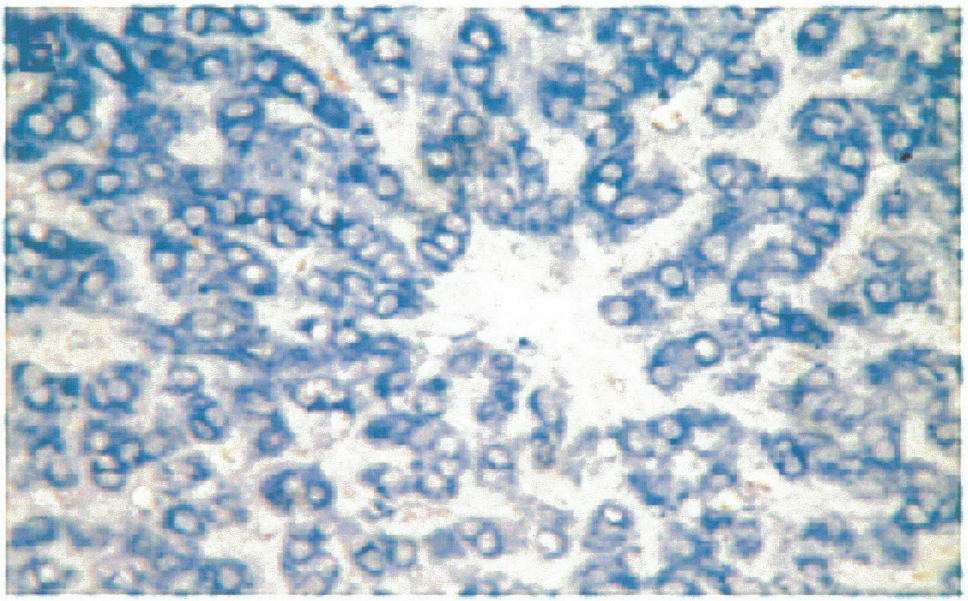

A
An
Pri
Pra
Pan
B
S
SE
T
O
G
C
G
T

Figure 4. 4(D). Expression of BMP-4 in atrectic follicle (Granulosa cells: G; Theca cells: T; Surface epithelium: SE; Oocyte: O) (200X)

Figure 4. 5(E). Localization of BMP-4 in corpus luteum (400X)

Figure 4. 6(F). Expression of BMP-4 in oviduct (Epithelium: Ep; Connective tissue: Ct; Blood vessels: Bv) (200X)


D
SE
G
O


E

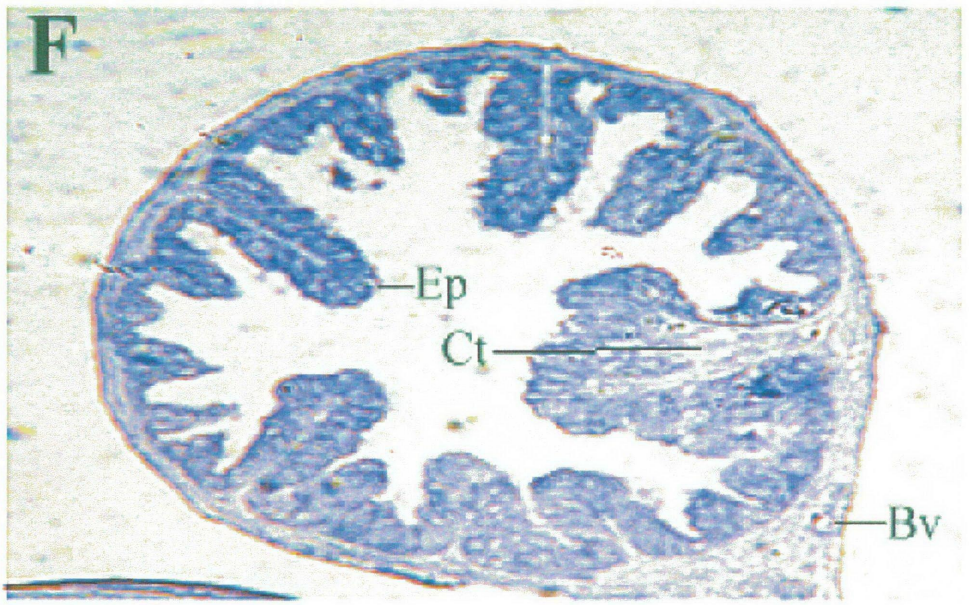
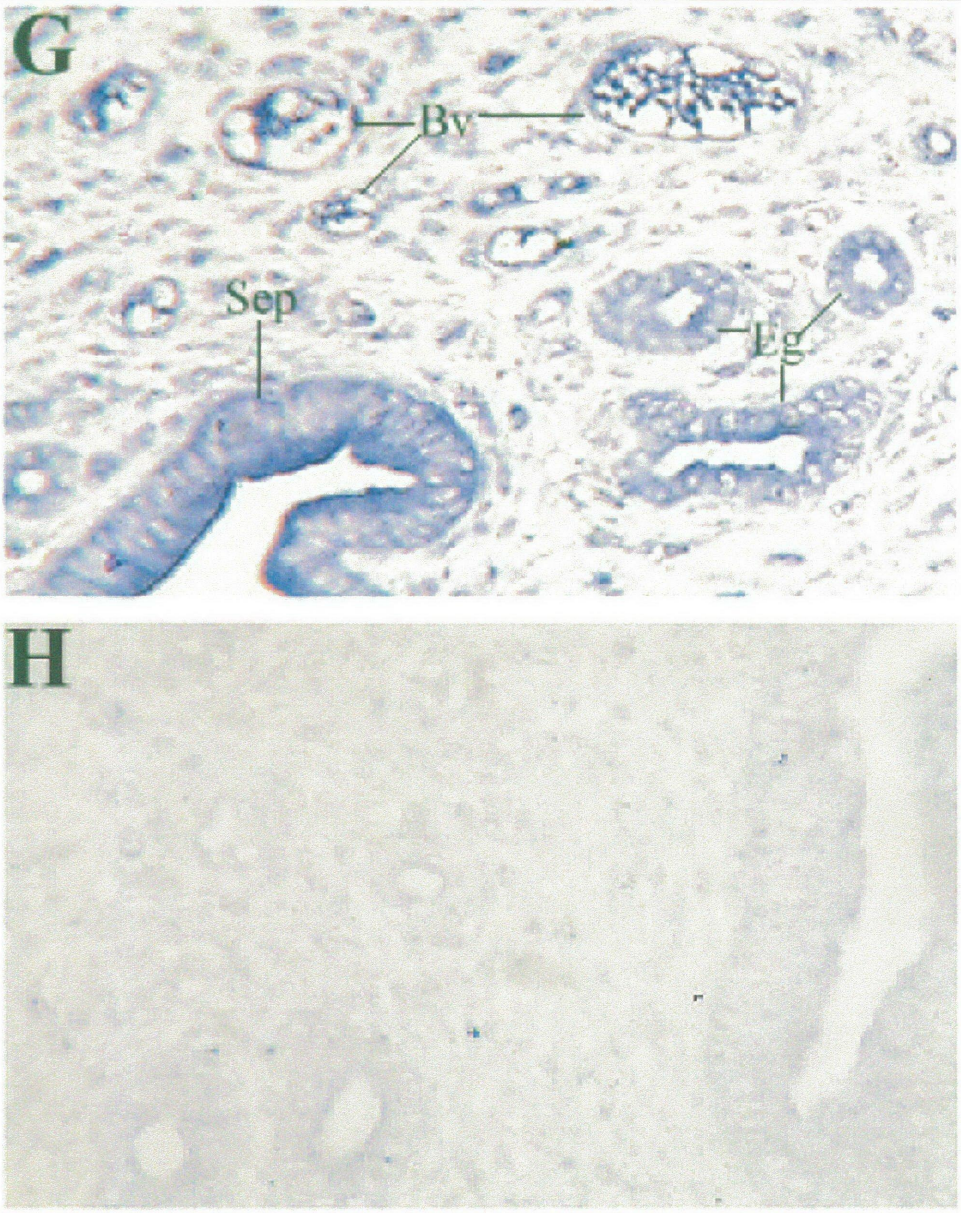

F
Ep
Ct
Bv

Figure 4. 7(G). BMP-4 protein localization in uterus (Blood vessels: Bv; Surface epithelium of endometrium: Sep; Endometrial gland: Eg)(200X)

Figure 4. 8(H). Negative control (200X)


G
Bv
Sep
Eg
H

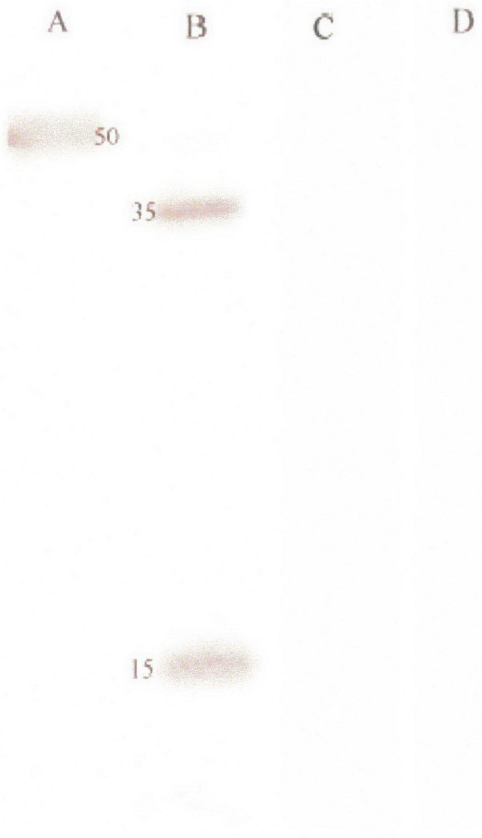


Figure 4.9. Western blot analysis of mouse ovary for detection of BMP-4. The samples were subjected to 12.5% SDS-PAGE under non-reducing (lane A) and reducing conditions (lane B). The membranes were treated with equal concentration of anti BMP-4 antibody (lane A and B) and non specific purified sheep Ig (lane C and D). The approximate molecular weight of bands detected is shown.

4. 4. Discussion

In females, both BMPs and their receptors have been shown to regulate granulosa cell functions (Shimasaki *et al.*, 2004). Interestingly, we found immunostaining of BMP-4 in granulosa cells (intense staining in granulosa cell membrane and faint staining in their cytoplasm) and oocyte, supporting a paracrine role of BMP-4 protein after its production from theca cells, the corpus luteum and other cells within the ovary (Erickson & Shimasaki, 2003). Interestingly, PCR studies in mouse ovary have shown the presence of BMP-4 mRNA in granulosa cells (von Schalburg *et al.*, 2006). However, the results of our present study indicate that granulosa cells contribution to BMP-4 protein secretion is limited. In addition, PCR is a far more sensitive technique than *in situ* hybridization. The significance of BMP signaling in ovarian functions is further highlighted by the altered ovulation rates in Booroola sheep with a point mutation in BMPR-IB gene (Mulsant *et al.*, 2001, Souza *et al.*, 2001, Wilson *et al.*, 2001) and in the Inverdale sheep with a point mutation in the BMP-15 gene (Galloway *et al.*, 2000). BMP-4 and BMP-7 have been shown to promote transition of primordial to primary follicle during follicular development (Lee *et al.*, 2001, Nilsson & Skinner, 2003, Tanwar & McFarlane, 2005). Our results have demonstrated that in the ovary, BMP-4 protein is expressed in all the stages of follicular development except primordial follicle and the expression of BMP-4 protein increases with follicular development. We observed very high intensity staining in granulosa cells, oocyte and low intensity staining in theca and stromal cells of tertiary follicle. These findings are consistent with *in situ* hybridization studies in rat ovary, in which BMP-4 expression first appeared in primary follicles and tertiary follicles were shown to have higher BMP-4 expression than any other stage of follicular development (Erickson & Shimasaki, 2003). This, together with the role of BMP-4, BMP-6, BMP-7 and BMP-15 in

controlling level of mitosis and FSH receptor signaling in granulosa cells (Otsuka & Shimasaki, 2002a, Otsuka *et al.*, 2001a, Shimasaki *et al.*, 1999), supports the view that BMP-4 might play an important role in selection of the dominant follicle. This view is further supported by the findings that follicle with an average diameter of 15 mm and higher estradiol concentration (higher estradiol to progesterone ratio) have higher expression level of ActRII, ALK-2, ALK-5 and BMPR-II mRNA than follicle with an average diameter of 7 mm and lower estradiol concentration (Jayawardana *et al.*, 2006, Shimizu *et al.*, 2006). In our studies we also observed BMP-4 expression in corpus luteum and in some stromal cells surrounding follicles. These finding are in agreement with mRNA studies in rodent ovary (Erickson & Shimasaki, 2003, von Schalburg *et al.*, 2006).

The presence of atrectic follicles is a prominent feature of adult cyclic mammalian ovaries (Hsueh *et al.*, 1994). Most of the follicles present in the ovary at birth become atrectic with less than 1% achieving ovulation (Hsueh *et al.*, 1994). In histological studies, the appearance of pkynotic granulosa cells in follicles is the most common criterion for identification of atrectic follicles (Hirshfield, 1988). Our results suggest a possible role of BMP-4 in follicle atresia. We observed low intensity staining in atrectic follicles compared to healthy follicle. In contrast, strong BMP-4 immunostaining was detected in ovarian surface epithelium overlying atrectic follicles, and in corpus luteum, while no BMP-4 expression was detected in other areas of ovarian surface epithelium. This suggests a role for BMP-4 in regeneration of ovarian surface epithelium. These finding are consistent with the role of these proteins in inhibition of apoptosis and stimulation of DNA synthesis in various cell systems (Lee *et al.*, 2001, Luo *et al.*, 1995, Oxburgh *et al.*, 2005). In addition, expression of BMP-4 mRNA has been detected in cells lines derived from ovarian surface epithelium and ovarian cancer

cells (Shepherd & Nachtigal, 2003). Furthermore, ovarian cancer cell lines have been shown to produce mature BMP-4 protein in *in vitro* conditions (Shepherd & Nachtigal, 2003). BMP-4 treatment of primary ovarian cells (CaOV3, SkOV3, and HeyC2) and normal ovarian surface epithelium (IOSE80 and IOSE144) culture leads to induction of ID1 and ID2 gene expression (Shepherd & Nachtigal, 2003). These ID proteins are involved in normal growth regulation and deregulation of these proteins leads to abnormal cell growth. Recently, Moll *et al.* have shown that chordin, a BMP antagonist, is under expressed in epithelial ovary cancer and epithelial cancer cell lines as compared with normal tissues and ovarian surface epithelium, respectively (Moll *et al.*, 2006). Interestingly, the expression of chordin and expression of BMP-4 are similar in normal ovarian surface epithelium of rat ovaries (Moll *et al.*, 2006). These findings are important considering that the majority (90%) of human ovarian cancer arises from the ovarian surface epithelium (Auersperg *et al.*, 2001).

Recent investigations have shown that BMP signaling components are expressed in a variety of cells in mammalian uterus (Erickson *et al.*, 2004, Kim *et al.*, 2003). Expression of various BMP ligands including BMP-2, BMP-4, BMP-7 and BMP-8a was detected in murine uterus during decidualization (Ying & Zhao, 2000). In *in situ* hybridization studies, BMP-2 expression was detected in periluminal stroma while expression for BMP-7 were detected in periluminal stroma and glandular epithelial cells (Erickson *et al.*, 2004). Moreover, BMP-4 and BMP-6 expression were detected in myometrium and in vascular endothelial cells (Erickson *et al.*, 2004, Ying & Zhao, 2000). The mRNA for BMPR-IA, BMPR-IB and BMPR-II were also localized in luminal and glandular epithelium (Erickson *et al.*, 2004). Recently, Yi *et al.* have reported that deletion of BMPR-IB gene in mice leads to endometrial thinning and a reduced number of endometrial glands, which further underlines the significance of

BMP signaling in uterine activity (Yi *et al.*, 2001). We observed BMP-4 protein expression in blood vessels, endometrial gland and surface epithelium of endometrium. Our results have demonstrated for the first time the expression of BMP-4 protein in the uterus.

The interrelationship between estradiol, progesterone and various growth factor pathways plays an important role in normal functioning of reproductive tissues. Exogenous treatment with estradiol has been shown to decrease expression of uterine BMP-7 mRNA *in vivo* (Ozkaynak *et al.*, 1997). Similarly, estradiol treatment has been shown to repress BMP-7 mRNA levels in chicken oviduct (Monroe *et al.*, 2000). In addition, BMP-7 is shown to regulate apoptosis in primary oviductal cells (Monroe *et al.*, 2000). Interestingly, we observed BMP-4 expression in epithelium and blood vessels of murine oviduct. It will be of interest for future studies to find the possible link between estradiol, progesterone and BMP-4 in oviduct and uterus as both BMP-4 and BMP-7 have been shown to regulate production of estradiol and progesterone in ovary cell cultures (Shimasaki *et al.*, 1999).

In recent years, several studies have reported various isoforms of BMPs in different reproductive tissues but there is limited or conflicting information available in regard to their physiological functions (Brankin *et al.*, 2005, Duffy, 2003, Silva *et al.*, 2003). The precursor forms of BMP-2 (45 kDa) and BMP-6 (55 kDa) have been detected in porcine follicular fluid (Brankin *et al.*, 2005). In addition, smaller isoforms (approximately 10 kDa) of BMP-2 were also observed in the porcine granulosa and theca cells. Similarly, precursors of GDF-9 (54 MW) and BMP-15 (single band of 45 MW) and mature GDF-9 (22 MW) have been detected in monkey's follicular fluid (Duffy, 2003). Interestingly, no mature form of BMP-15 was observed in monkey's follicular fluid (follicle size > 4mm). Our results have demonstrated for the first time

that the BMP-4 protein exists in ovary. We detected a 50 kDa band in non reducing conditions. In reducing condition, we have detected non glycosylated mature BMP-4 monomer (15 kDa) and glycosylated mature BMP-4 monomer with an attached fragment of pro-domain (35 kDa). Our finding are consistent with a previous study (Cui *et al.*, 2001), who have shown that *in vitro* incubation of 50 kDa proBMP-4 with recombinant furin yields a 15 kDa mature BMP-14 peptide and a 35 kDa pro-domain (Cui *et al.*, 2001). However, a 32 kDa band was also observed upon longer incubation with recombinant furin (Cui *et al.*, 2001).

In conclusion, we have shown the expression of BMP-4 in ovary, oviduct and uterus of mice by using immunohistochemistry and have detected different isoforms of BMP-4 in mouse ovary by western analysis, supporting the view that BMP-4 is an important regulator of female fertility.