Chapter 6: Immunohistochemical localization of BMP-4 protein in the reproductive axis of sheep

6. 1. Introduction

BMP-4 is a signaling molecule that acts as a morphogen in a concentration dependent manner to influence cell fate during embryonic development. BMP-4 was first isolated for its capacity to induce ectopic cartilage and bone formation but subsequently, was implicated in various biological processes including cell proliferation and differentiation, migration, organization and apoptosis (Canalis *et al.*, 2003, Degnin *et al.*, 2004, Shimasaki *et al.*, 2004, Sopory *et al.*, 2006, Urist, 1965).

The mRNA encoding BMP-2 (Erickson & Shimasaki, 2003), BMP-3 (Erickson & Shimasaki, 2003), BMP-3b (Erickson & Shimasaki, 2003), BMP-4 (Shimasaki *et al.*, 1999, Shimizu *et al.*, 2004a), BMP-5 (Pierre *et al.*, 2005a, Shimizu *et al.*, 2004a), BMP-6 (Otsuka *et al.*, 2001a, Shimizu *et al.*, 2004a), BMP-7 (Shimasaki *et al.*, 1999) and BMP-15 (Otsuka *et al.*, 2000) has been identified in ovaries of various mammalian species. In rat ovary, the expression of BMP-4 and BMP-7 mRNA was detected in theca layer of follicles, while mRNA encoding for BMPR-IA, BMPR-IB and BMPR-II was detected in oocytes and granulosa cells (Shimasaki *et al.*, 1999). BMP-6 is mainly expressed in oocyte of the mouse (Elvin *et al.*, 2000) and cattle (Glister *et al.*, 2004) ovary but in oocyte, granulosa and theca cell layer of sheep ovary (Campbell *et al.*, 2006). In chicken ovary, BMP-4, BMP-6 and BMP-7 are expressed in granulosa and theca cells (Onagbesan *et al.*, 2003), suggesting that species differences exist in expression and functions of BMP.

In sheep, both mRNA and protein of BMPR-IA, BMPR-IB and BMPR-II have been detected in the oocytes, granulosa cell layers of primary to late antral follicle, corpus luteum and ovarian surface epithelium indicating that these cells can respond to the BMP signaling (Souza *et al.*, 2002, Wilson *et al.*, 2001). In sheep granulosa cell culture, BMP-4 and BMP-7 have been shown to increase estradiol and decrease progesterone production in both presence and absence of FSH, while in rats, similar effects are seen only in FSH induced condition (Fabre *et al.*, 2003, Glister *et al.*, 2004).

In mice, gene knockout studies have shown that BMP-4 is absolutely required for primordial germ cells (PGCs) formation as BMP-4 homozygous null embryos contain no PGCs (Lawson *et al.*, 1999). In murine ovary, BMP-4 has been shown to increase the number of developing primary follicles and subsequently, decreasing the number of primordial follicles in both *in vitro* (Nilsson & Skinner, 2003) and *in vivo* (chapter 3) conditions. In whole ovary culture system, addition of anti-BMP-4 antibodies decreased the size of ovaries associated with loss of oocytes, primordial follicles and ovarian tissue apoptosis (Nilsson & Skinner, 2003).

BMPs and their receptors are also expressed in the oviduct (Monroe *et al.*, 2000) and uterus (Erickson *et al.*, 2004, Ozkaynak *et al.*, 1997). Recently, various BMP signaling components were detected in pregnant (Ying & Zhao, 2000) and non-pregnant uterus (Erickson *et al.*, 2004). In pregnant mouse uterus, BMP-2 mRNA was detected in decidualizing stromal cells and BMP-4 mRNA was localized in vascular endothelial cells, while BMP-7 mRNA was detected in decidualizing stromal cells (Ying & Zhao, 2000). Interestingly, exogenous administration of estradiol led to down regulation of BMP-7 transcript in both oviduct (Monroe *et al.*, 2000) and uterus (Ozkaynak *et al.*, 1997) *in vivo*. In males, gene knockout studies have shown that BMP-4, BMP-7, BMP-8A and

BMP-8B are required for spermatogenesis, PGC formation, testis cord formation and epididymal development (Hu *et al.*, 2004, Zhao & Hogan, 1996, Zhao *et al.*, 1998, Zhao *et al.*, 1996).

There is considerable information available regarding the role of BMPs in pituitary differentiation and functions (Faure *et al.*, 2005, Scully & Rosenfeld, 2002). BMP-2, BMP-4, BMP-6, BMP-7 and BMP-15 are expressed in murine pituitary gland (Otsuka & Shimasaki, 2002b, Paez-Pereda *et al.*, 2003). BMP-4 gene knockout mice embryos fail to develop an ectodermal pouch placode, which is required for the development of Rathke's pouch (Takuma *et al.*, 1998). The development of anterior pituitary from oral ectoderm depends on several factors secreted by neural epithelium of ventral diencephalon such as BMP-4, Wnt5a, FGF10 and FGF 8 (Rosenfeld *et al.*, 2000).

The above studies have shown that BMP system plays a vital role in male and female fertility. Most of the studies on the role of BMPs in reproduction are in rodent species and recent studies have shown different roles of BMPs in monoovulatory and polyovulatory species (Hashimoto *et al.*, 2005). With the above points in mind, the aim of present study was to characterize the expression of BMP-4 in the fetal and adult sheep reproductive axis.

6. 2. Material and methods

6.2. 1. Tissue and section preparation

A flock of merino ewes were run with intact rams harnessed with marking crayons to determine the conception date. A group of 3 ewes were killed at 100 days of gestation and uteri were immediately collected on ice. The fetuses were sexed, weighted and their vertebral column length was measured. Ovary, oviduct, uterus, pituitary and testis were collected and fixed in Bouin's solution. The gestational ages were calculated from the dates of last marking and killing, and were confirmed using algorithms previously described (Foulds *et al.*, 1998). Fetal tissue samples were collected at 100 days of gestation as transition of primordial to primary follicles first starts in the fetal sheep ovary at this stage of development (McNatty *et al.*, 1995).

During the breeding season, tissues (ovary, oviduct, uterus and pituitary) were collected from normal cyclic adult merino ewes. In males, testes were excised from young rams (aged day 1, 7, 27, 38, 56, 126 days) under local anesthesia by infiltration of scrotal wall and spermatic cord with anesthetics (Lignocaine hydrochloride, 20mg/ml; Troy laboratories, Smithfield, Australia) or from adult rams under light general anesthesia (Thiopentone sodium, Rhone Meriex Aust. Pty. Ltd., Pinkenba, Australia) with local anesthesia. Epididymides were also collected in adult animals. All the tissues were further processed and sectioned as described in chapter 2.

6.2. 2. Immunohistochemistry

Method described in chapter 2

6.2. 3. Western blotting

Ovine follicular fluid was collected by laparoscopy from the normal cyclic ewes. Follicular fluid samples were diluted (1:30) in non-reducing and reducing sample buffer and further processed as described in chapter 2.

6. 3. Results

In fetal ovary, expression of BMP-4 was observed in oocytes, pre-granulosa cells, and the basal membrane surrounding follicles and in stroma (Fig. 6.1A). BMP-4 expression was observed in primordial and primary follicle in fetal ovary, which is consistent with previous finding showing that BMP-4, BMPR-IA, BMPR-II, smad-1, smad-4 and smad-5 mRNA is present as early as 32 day post coitum in developing ovine ovary (Fig. 6.1A) (Fabre *et al.*, 2006) . In adult sheep ovary, BMP-4 expression was observed in preantral, antral follicle, corpus luteum, ovarian surface epithelium (OSE) and blood vessels (Fig. 6.3C, 6.4D and 6.6F). In preantral and antral follicles, high intensity staining was observed for BMP-4 in oocytes and granulosa cells with much fainter staining in theca cells (Fig. 6.3C and 6.4D). There was a significant increase in expression of BMP-4 with increase in follicular size. In contrast to fetal ovaries, no expression of BMP-4 was observed in primordial and primary follicle (Fig. 6.2B and 6.5E).

In both fetal and adult sheep oviduct, BMP-4 protein expression was exclusively limited to the luminal epithelium and blood vessels (Fig. 6.7G). In fetal uterus, BMP-4 expression was observed in endometrial surface epithelium, circular muscles and blood vessels (Fig. 6.8H) and in endometrial surface epithelium, epithelium of endometrial glands and blood vessels of adult sheep uterus (Fig. 6.9I).

In adult sheep pituitary, BMP-4 expression was predominantly observed in basophilic cells (Fig. 6.10J and 6.11K). In contrast, no expression of BMP-4 was detected in fetal pituitary (Fig. not shown).

In males, no expression of BMP-4 was detected at any stage of testicular development (Fig. 6.13M) while strong expression for BMP-4 was detected in basal cells of the epididymis (Fig. 6.12L).

A range of different isoforms of BMP-4 was detected with western blotting of ovine follicular fluid (Fig. 6.15). Bands corresponding to 100 and 42 kDa were detected in follicular fluid samples analyzed under non reducing condition (Fig. 6.15 membrane 1 and lane A). Bands migrating at 50.1, 38.5, 33.8, 23.9 and 15.1 kDa (Fig. 6.15, membrane 1 and lane B) were detected in follicular fluid sample analyzed under reducing condition. 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.6.15 membrane2).

Figure 6. 1(A). Expression of BMP-4 protein in fetal ovary (Oocyte: Oc; Pregranulosa cells: Pg; Basal membrane: Bm; Stroma: S; Primordial: Pri and Primary follicles: Pra) (400X).

Figure 6. 2(B). No expression of BMP-4 in primordial and primary follicles of adult ovary (Primary follicles: Pra) (400X).

Figure 6. 3(C). Expression of BMP-4 in preantral follicle (Pan) of adult ovary (Granulosa cells: G; Theca cells: T; Blood vessels: Bv; Oocyte: O)(400X).

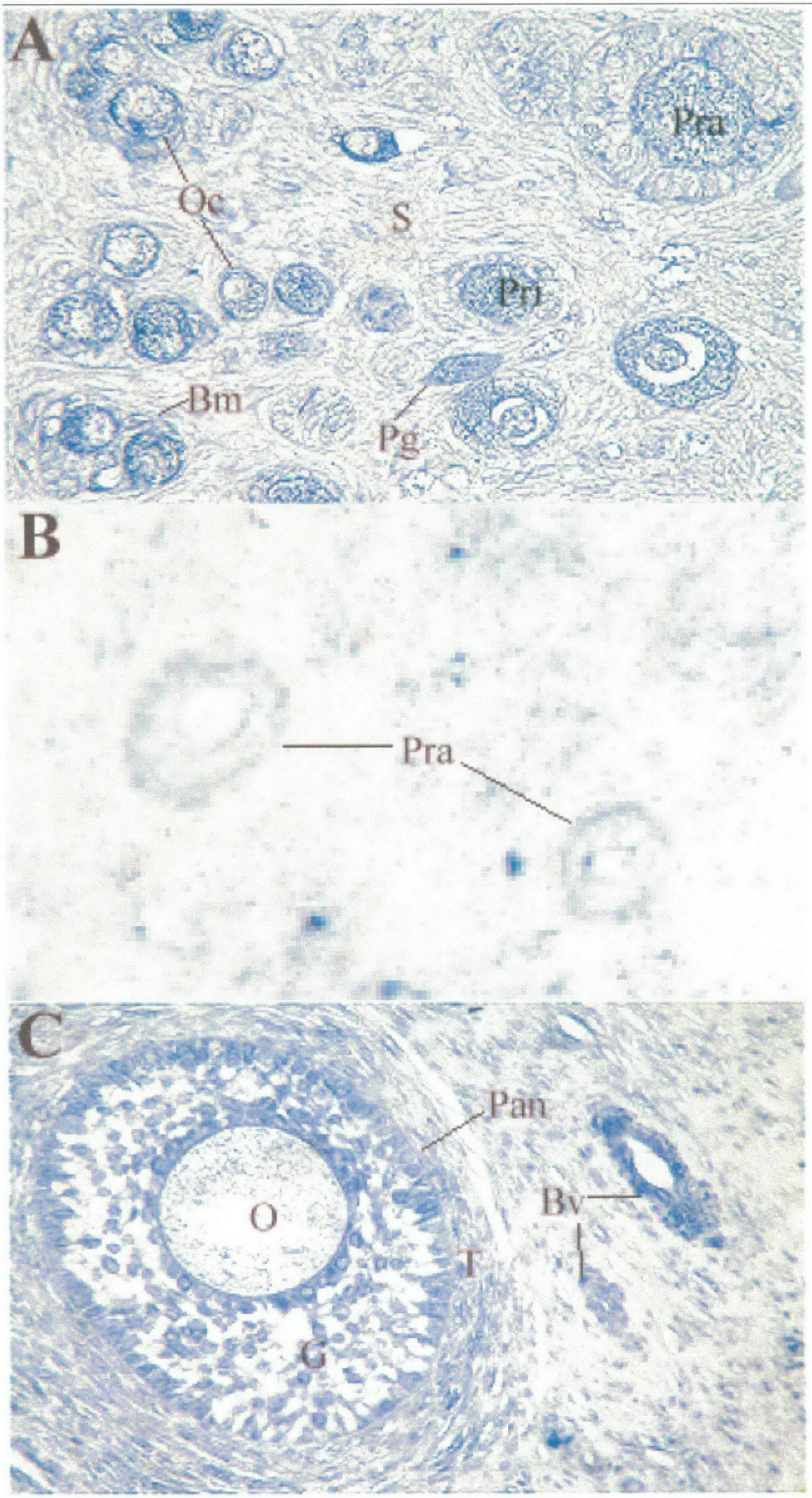

A
Pra
Oc
S
Pri
Bm
Pg
B
Pra
C
Pan
O
Bv
T
G

Figure 6. 4(D). Expression of BMP-4 in large antral follicle of adult ovary (400X)

Figure 6. 5(E). Localization of BMP-4 in ovarian surface epithelium (OSE) of adult ovary (Ovarian surface epithelium: OSE; Primordial: Pri)(400X)

Figure 6. 6(F). Expression of BMP-4 in corpus luteum in adult ovary (400X)

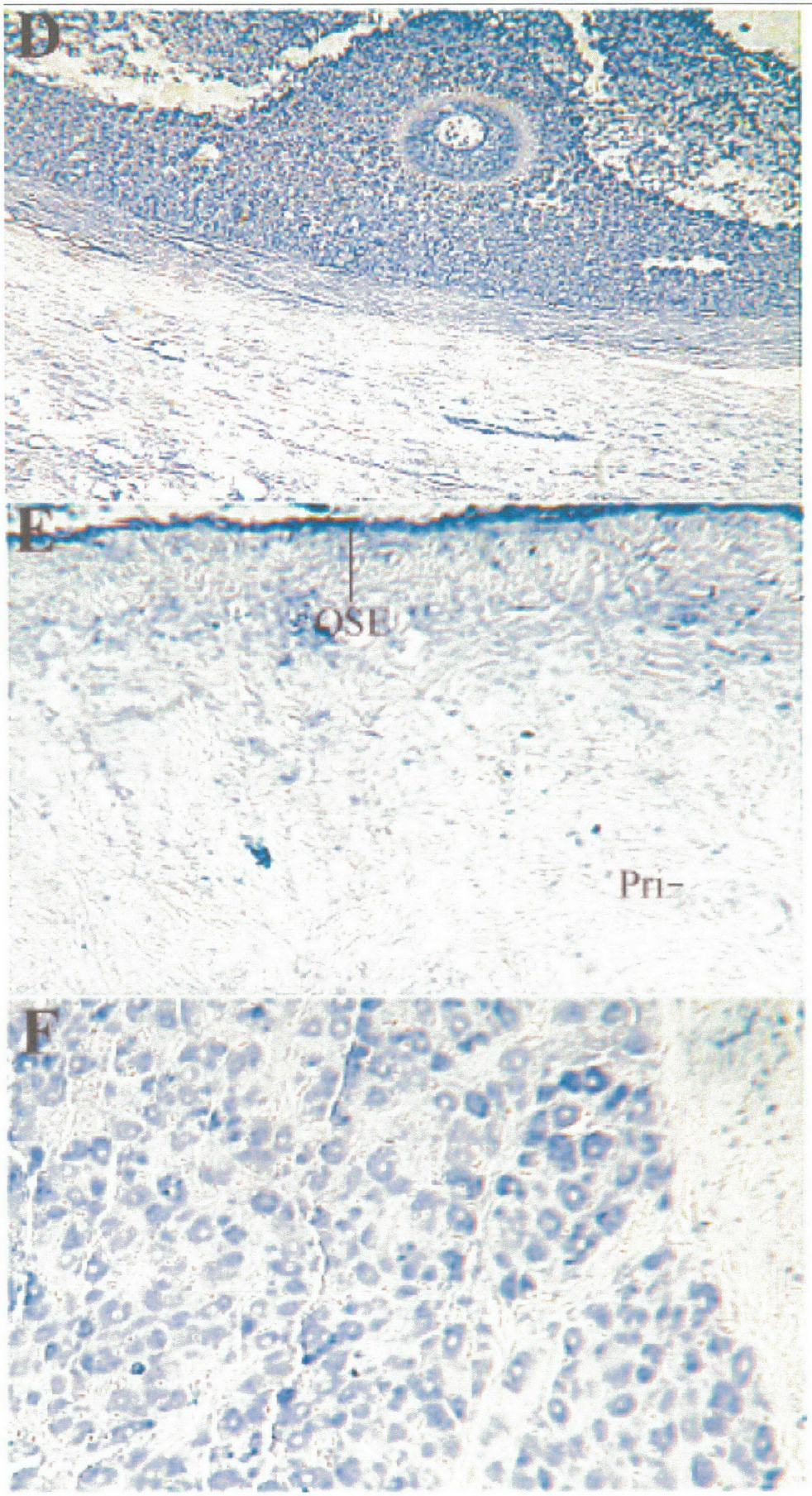

D
E
OSE
Pri-
F

Figure 6. 7(G). BMP-4 protein localization in fetal oviduct (BMP-4 protein expression in adult oviduct is similar to fetal oviduct. Fig. not shown) (Epithelium: Epi; Blood vessels: Bv) (400X)

Figure 6. 8(H). Expression of BMP-4 in fetal uterus (Blood vessels: Bv; Surface epithelium of endometrium: Sep) (200X)

Figure 6. 9(I). BMP-4 expression in adult sheep uterus (Surface epithelium of endometrium: Sep; Endometrial gland: Eg) (200X)

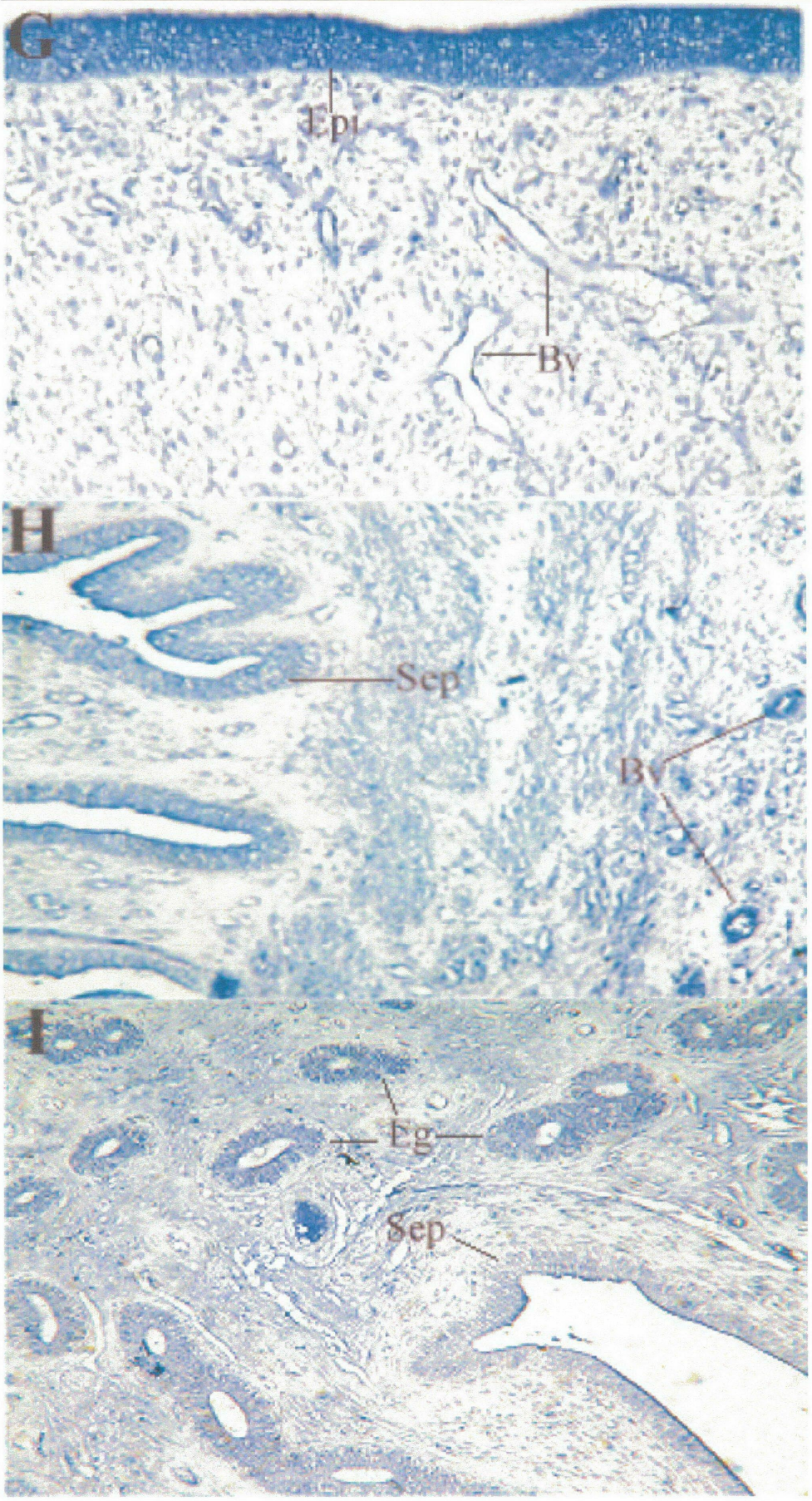

G
Epi
Bv
H
Sep
Bv
I
Eg
Sep

Figure 6. 10(J). Hematoxylin and Eosin stain section of adult sheep pituitary, arrowhead indicate basophilic cells while arrow indicate acidophilic cells (next section was used in immunostaining; 1000X)

Figure 6. 11(K). BMP-4 expression in pituitary gland, arrowhead showing staining in basophilic cells while arrow indicate acidophilic cells (1000X)

Figure 6. 12(L). BMP-4 expression in epididymis (Basal cell: Ba)(400X)

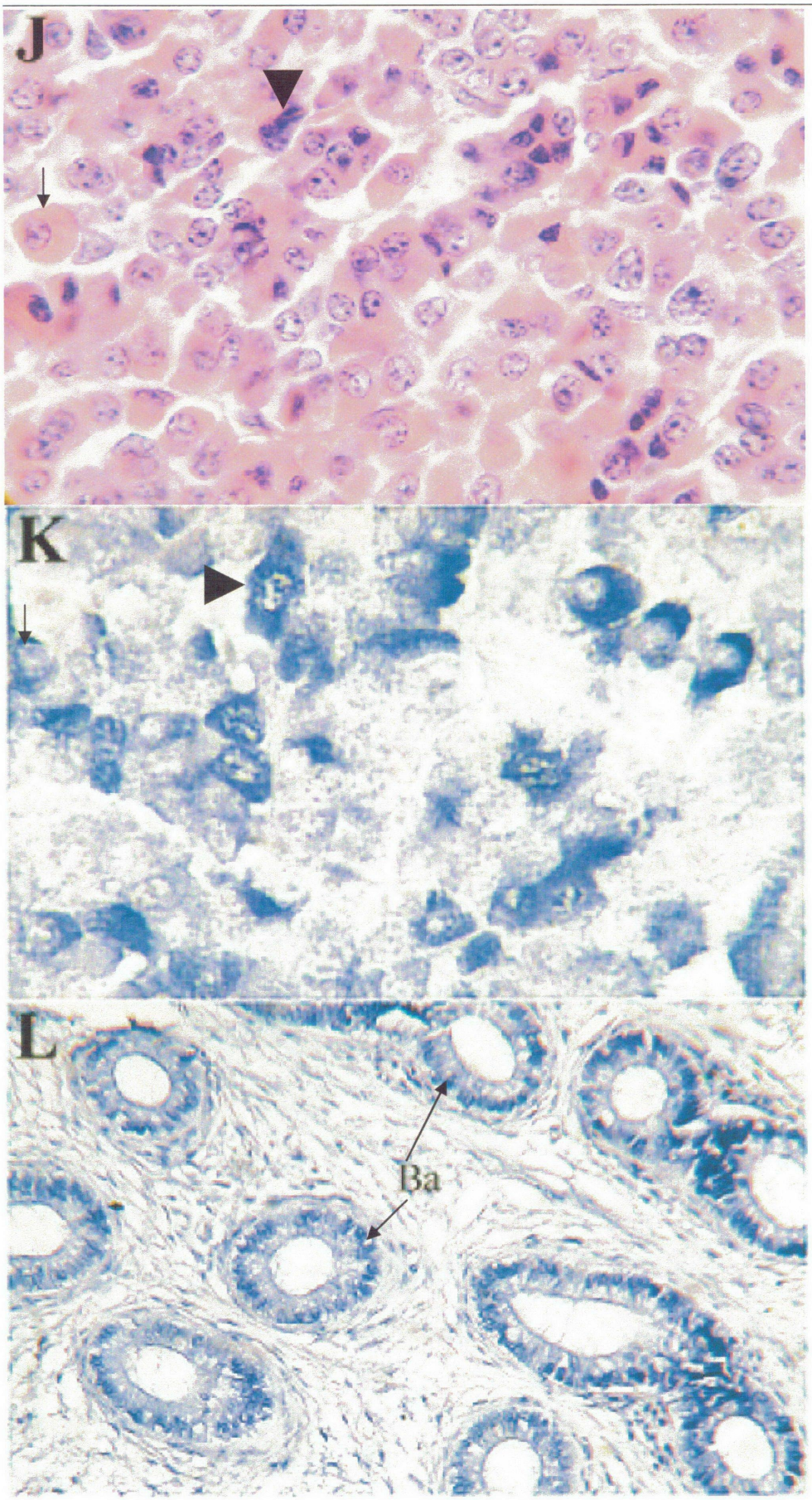
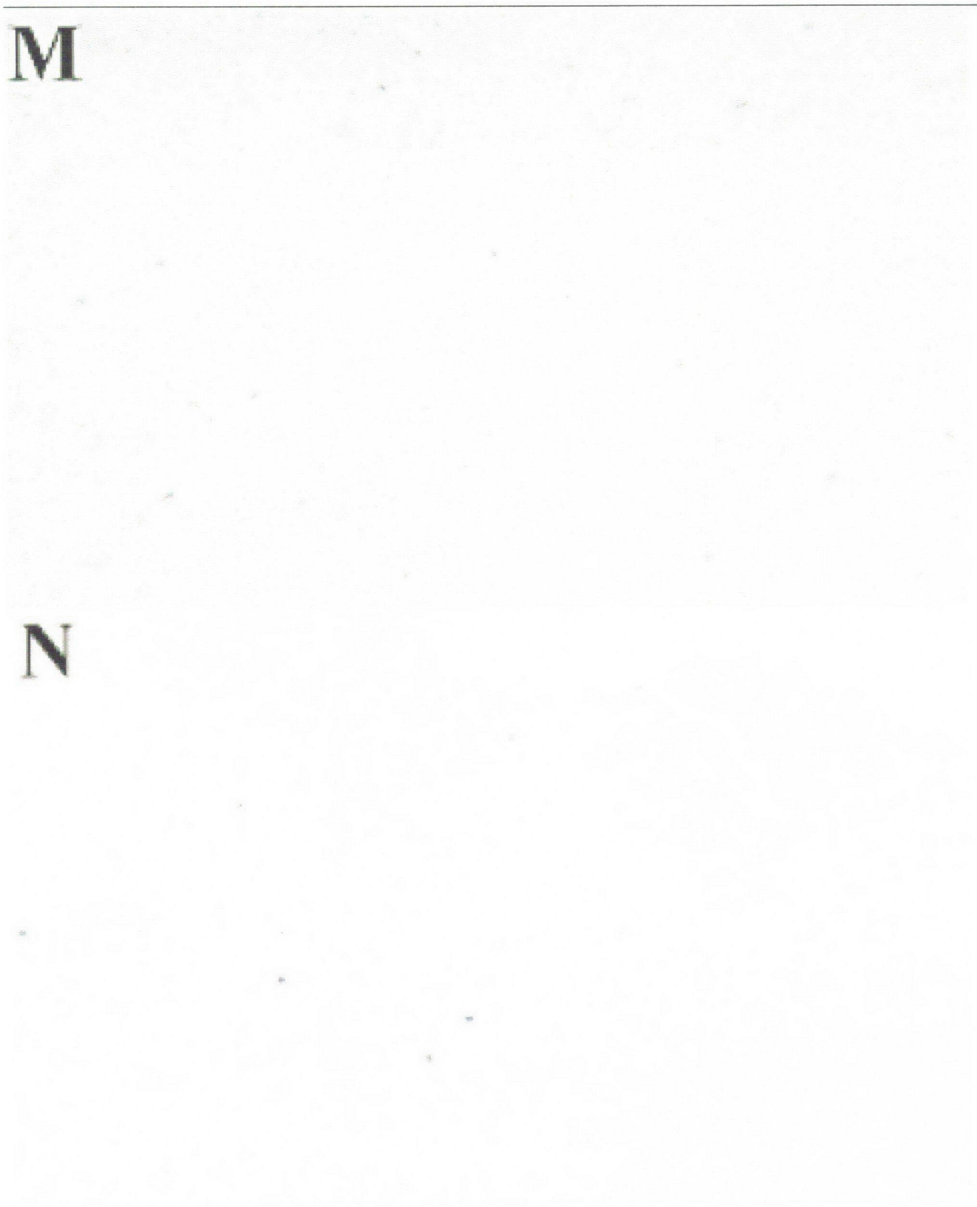

J
K
L
Ba

Figure 6. 13(M). Lack of expression of BMP-4 in testis (200X)

Figure 6. 14(N). Negative control (200X).


M
N

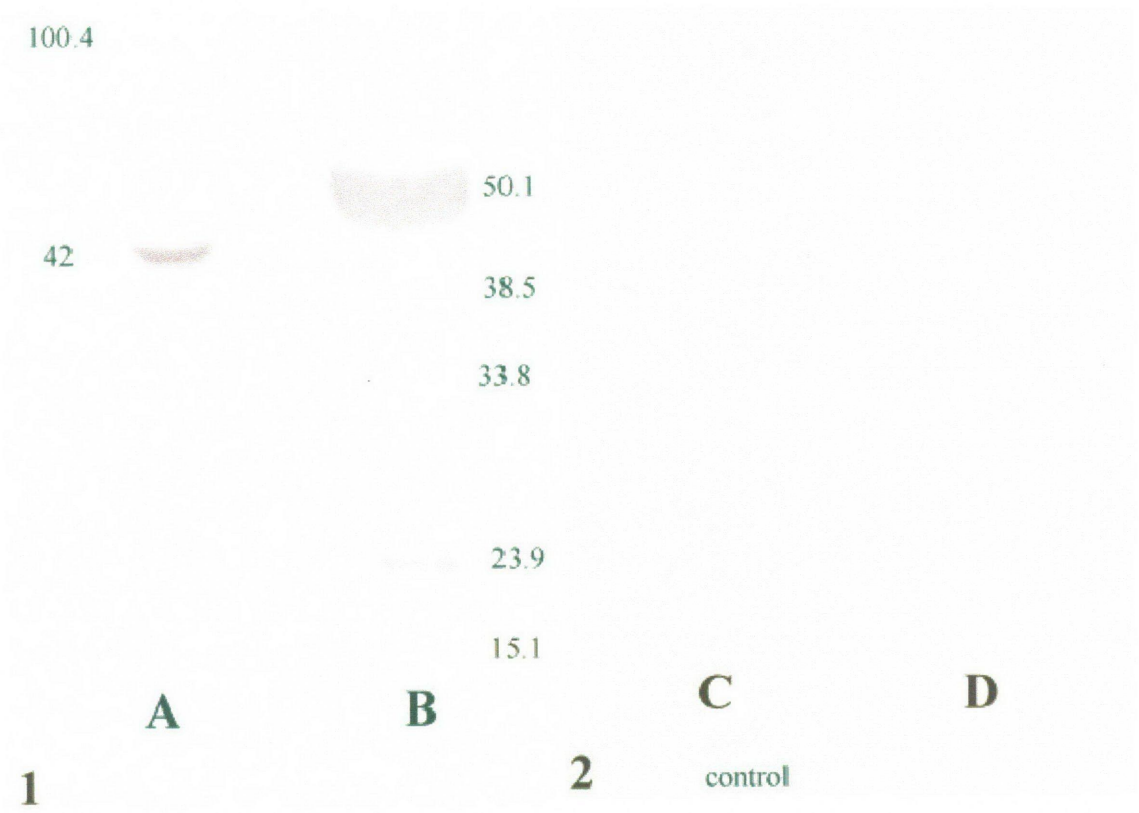


Figure 6.15. Western blot analysis of ovine follicular fluid for detection of BMP-4. The samples were subjected to 12.5% SDS-PAGE under non-reducing (lane A and C) and reducing condition (lane B and D). The membranes were treated with equal concentration of anti BMP-4 antibody (membrane 1) and non specific purified sheep Ig (membrane 2). The approximate molecular weight of bands detected is shown.

6. 4. Discussion

Different breeds or sometime within same breed, of sheep have different ovulation rate and litter size. The ovulation rate of breeds *viz.* Romanov and Finnish-landrace, Booroola merino, Javanese, Olkuska, Belclare, Cambridge, Inverdale and Hanna, Woodlands and Lacaune is regulated by naturally occurring mutations in different genes belonging to BMP family (Souza *et al.*, 2004). In Inverdale, Hanna, Cambridge and Belclare mutations in the BMP-15 and GDF-9 gene lead to higher ovulation rate in heterozygous and sterility in homozygous ewes (Galloway *et al.*, 2000, Hanrahan *et al.*, 2004). Similarly, mutation in highly conserved intracellular kinase signaling domain of BMPR-1B (Q249R) has been shown to be responsible for higher ovulation rate in Booroola (FecB), Garole and Javanese sheep (Mulsant *et al.*, 2001, Souza *et al.*, 2001, Wilson *et al.*, 2001). In contrast, BMPR-1B gene knockout (Yi *et al.*, 2001) and naturally occurring mutations in BMR-1B (Demirhan *et al.*, 2005) lead to infertility in mice and humans, respectively. In *Xenopus laevis* mesoderm induction assay, BMP-4 has been shown as a physiological ligand of BMPR-1B (Aaltonen *et al.*, 1999). *In vitro*, ovarian granulosa cells from FecB ewes have shown a decrease in inhibitory effect of BMP-4 on progesterone production compared with granulosa cells from normal ewes, suggesting that FecB mutation alters the activity of BMP-4 ligand and further underscores the importance of BMP-4 in ovarian functions (Mulsant *et al.*, 2001).

In the present study, we observed BMP-4 protein expression in oocytes, granulosa and theca cells of preantral and antral follicle. BMP-4 protein expression detected in our studies is consistent with role of BMP-4 in bovine granulosa (Mulsant *et al.*, 2001) and theca cell steroidogenesis (Glister *et al.*, 2005). Results observed in our study are different from reported expression of BMP-4 in cattle ovary (Glister *et al.*, 2004). In their studies,

granulosa and theca cells were first isolated and then cultured for six days before immunolocalization (Glister *et al.*, 2004). Recently, Campbell *et al.* have shown that protein expression differ in whole ovary sections and in isolated cultured cells (Campbell *et al.*, 2006). In our study, BMP-4 expression was significantly increased with increase in follicular size indicating its role in selection of dominant follicle. On the basis of our study, we hypothesized that production of BMP-4 increases with increase in size of follicle and helps in selection of dominant follicle by increasing estradiol and decreasing progesterone production. This hypothesis is further supported by findings that the dominant follicle in cattle has higher estradiol: progesterone ratio (Fortune, 1994) and in monoovular sheep, one follicle per wave has higher estradiol: progesterone ratio than other follicles of same wave (Evans, 2003). Furthermore, treatment of bovine granulosa cells (follicle diameter 4-7 mm) with estradiol has been shown to increase expression of ActR-II, ALK-2, ALK-5 and BMPR-II mRNA (Jayawardana *et al.*, 2006, Shimizu *et al.*, 2006).

The transition of primordial to primary follicle is an important physiological process for female fertility and 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. Recently, BMP-4 (Nilsson & Skinner, 2003), BMP-7 (Lee *et al.*, 2001) and GDF-9 (Vitt *et al.*, 2000) have been shown to promote transition of primordial to primary follicle. In sheep, development of primordial and primary follicles occurs during fetal development (100 days) (Sawyer *et al.*, 2002). In our study, BMP-4 protein expression was detected in pre-granulosa, naked oocyte, primordial and primary follicles suggesting its involvement in development of primordial and primary follicles. In contrast, there was no expression of BMP-4 in

primordial and primary follicles in adult ovary. The most likely explanation of these differences in expression of BMP-4 in fetal and adult ovary is suggested by intense expression of BMP-4 in pre-granulosa cells and naked oocyte, indicating that BMP-4 might be involved in attracting pre-granulosa cells to naked oocyte for follicle formation in the fetal ovary.

The mRNAs encoding BMPR-IA, BMPR-IB and BMPR-II are expressed in various cell types in the uterus including luminal and glandular epithelial cells (Erickson *et al.*, 2004). BMP-2 mRNA is expressed in periluminal stroma and BMP-7 mRNA is expressed in periluminal stroma and glandular epithelium while BMP-4, BMP-6 are expressed in endothelial cells of large blood vessels of myometrium (Erickson *et al.*, 2004). Interestingly, expression of BMP-7 mRNA decreases during pregnancy (Ozkaynak *et al.*, 1997). The importance of the BMP system in uterus is further underscored by the low number of uterine glands and thinning of endometrial lining in BMPR-1B knockout mice (Yi *et al.*, 2001). In our study, BMP-4 expression was observed in endometrial epithelium, epithelium of endometrial glands and blood vessels of uterus and in luminal epithelium and blood vessels of oviduct. Detection of BMP-4 in oviduct and uterus is quite important considering that these are extremely estrogen sensitive reproductive tissues and BMP-4 has been shown to affect estradiol production (Glister *et al.*, 2004, Shimasaki *et al.*, 1999). It will be of interest in future studies to characterize the interaction of BMP-4 and estradiol in the uterus.

The expression of various BMP receptors including BMPR-1A, BMPR-1B and BMPR-II has been detected in sheep pituitary (Faure *et al.*, 2005). *In vitro*, BMP-6 and BMP-7 have been shown to increase FSH production in mouse pituitary cell culture and gonadotrope cell line (Huang *et al.*, 2001a). Similarly, Otsuka and Shimasaki have shown

an increase in FSH production with addition of BMP-15 but not with BMP-6 and BMP-7 in rat pituitary cell culture (Otsuka & Shimasaki, 2002b). In contrast, BMP-4 and BMP-6 have been shown to decrease FSH production and expression of FSH β mRNA in sheep pituitary cell cultures (Faure *et al.*, 2005). The differences in these results are further complicated by presence and absence of BMPR-1B in sheep (Faure *et al.*, 2005) and mouse (Yi *et al.*, 2001) pituitary, respectively. In previous studies, it has been shown that FSH and progesterone concentrations are higher in plasma of Booroola merino ewes than in normal merino ewes (Xia *et al.*, 2003). Interestingly, the production of FSH and progesterone is inhibited by BMP-4 in sheep pituitary (Faure *et al.*, 2005) and granulosa cell cultures (Glister *et al.*, 2004), respectively. In the present study, BMP-4 protein expression was localized predominantly in basophilic cells suggesting that BMP-4 affects production of gonadotrophins. Our findings are consistent with previous findings reporting the presence of BMP receptors in gonadotropes (Faure *et al.*, 2005) and BMP-4 mRNA in sheep pituitary (Souza *et al.*, 2003).

BMP-4 gene knockout studies in mice have shown that BMP-4 heterozygous males have compromised fertility due to degeneration of germ cells, reduced sperm count and sperm motility (Hu *et al.*, 2004), indicating its role in maintenance of spermatogenesis. BMP-4, BMP-7 and BMP-8A are expressed in epididymis and their gene knockouts have shown degeneration of the epididymal epithelium suggesting a role for BMPs in maintenance of epididymal functions (Hu *et al.*, 2004, Zhao, 2003, Zhao *et al.*, 1998, Zhao *et al.*, 1996). In our studies, no expression of BMP-4 was detected at any stage of testicular development but very strong expression of BMP-4 was observed in basal cells of epididymis. The absence of BMP-4 expression in the testis was unexpected as studies in mice have indicated its role in maintenance of spermatogenesis. To our

knowledge at present there are no reports of presence of BMP-4 protein in ram testis. Furthermore, recent study has shown that the expression and functions of BMP-4 are different in rat and sheep (Juengel *et al.*, 2006). In epididymis, expression of BMP-4 protein is consistent with previous studies (Hu *et al.*, 2004).

Israel *et al.* have shown that different isoforms of BMP-2, including a propeptide: 40-45 kDa, a mature homodimer: 18-22 kDa and a precursor protein: 60 kDa are secreted by Chinese hamster ovary cells (Israel *et al.*, 1992). Similarly, Klosch *et al.* have shown monomer, dimer and polymer forms of recombinant rat BMP-4 using western blot analysis (Klosch *et al.*, 2005). Our results have demonstrated for the first time that the different isoforms of BMP-4 exist in follicular fluid. We detected BMP-4 precursor (dimer) (100.4 kDa) and glycosylated mature BMP-4 protein (dimer) (42kDa) in nonreducing condition. In reducing condition, we have detected precursor BMP-4 monomer (50.1 kDa), glycosylated mature BMP-4 monomer (23.9 kDa) and non glycosylated mature BMP-4 monomer (15.1 kDa). We have also detected two other isoforms of BMP-4 (33.5 and 38.5 kDa) in reducing condition. We believe that these are the non-glycosylated (33.5 kDa) and glycosylated mature (38.5 kDa) BMP-4 monomer with an attached fragment of prodomain formed during processing as our antibody recognizes only mature BMP-4 protein. Similar isoforms has been formed during cleavage of BMP-4 pre-pro-protein in *in-vitro* conditions (Cui *et al.*, 2001). To our knowledge there are no other reports about presence of BMP-4 in follicular fluid but the presence of other BMP family members (BMP-2, BMP-6, BMP-15 and GDF-9) in porcine and monkey follicular fluid is well documented (described in chapter 4) (Brankin *et al.*, 2005, Duffy, 2003).

The molecular weight of some of the bands observed in sheep follicular fluid is different from the molecular weight of bands observed in mouse ovary (details in

chapter 4). We believe that the observed differences are because of the following reasons: (a) Differences in posttranslational processing of BMP-4 in monoovular (sheep) and polyovular (mouse) species as recent study has shown that human BMP-15 mature protein is readily produced but there are defects in production of mouse BMP-15 mature protein in an *in vitro* system of transfected cells (Hashimoto *et al.*, 2005). Furthermore, the generation of chimeric constructs consisting of different combinations of mouse and human BMP-15 proregions, cleavage sites, and mature regions indicates that the defects in production of mouse BMP-15 mature protein depend on presence of mouse BMP-15 proregion as mouse BMP-15 proregion also caused significant reduction in the production of human BMP-15 mature protein (Hashimoto *et al.*, 2005) (b) Differences in level and pattern of expression of BMP-4 in the mouse and sheep as shown in *in situ* hybridization studies of rat (Juengel *et al.*, 2006, Shimasaki *et al.*, 1999) and sheep (Juengel *et al.*, 2006) ovaries. Similar differences are also observed in immunolocalization studies (Chapter 4, 5, and 6) (c) Differences in functions of these proteins in monoovular and polyovular species as BMPR-IB knockout mice are subfertile (Yi *et al.*, 2001) whereas Booroola sheep have increased fertility due to mutation in BMPR-IB (Mulsant *et al.*, 2001, Souza *et al.*, 2001, Wilson *et al.*, 2001). Similarly, BMP-15 gene deleted mice are subfertile and have normal follicular development (Yan *et al.*, 2001) whereas Inverdale sheep are infertile (homozygous) or hyperprolific (heterozygous) due to mutation in BMP-15 gene (Galloway *et al.*, 2000) (d) Intracellular interaction between different BMPs affects the processing and secretion of these proteins as studies by Liao *et al.* have shown that co-expression of BMP-15 and GDF-9 leads to formation of BMP-15/GDF-9 heterodimers (Liao *et al.*, 2003). Interestingly, co-expression of BMP-15 and GDF-9 proproteins significantly impaired processing of both proproteins compared with that of singly expressed proproteins (Liao

et al., 2003). Similarly, the presence of heterodimers of BMP-4 and BMP-7 has been shown in *vitro* conditions (Aono *et al.*, 1995). Further studies are required to characterize the role of these isoforms of BMP-4 in reproductive processes and further studies are also required to study the differences in functions of these proteins in monoovular and polyovular species.

In conclusion we have demonstrated for first time the expression of BMP-4 protein in the sheep reproductive axis and have shown the presence of different isoforms of BMP-4 in ovine follicular fluid.