Dynamic expression of bone morphogenetic protein 4 in reproductive organs of female mice

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Abstract

Various members of the bone morphogenetic protein (BMP) family have been shown to regulate mammalian follicular development by affecting granulosa cell proliferation and steroidogenesis. *In situ* hybridization studies have shown expression of BMPR1A, BMPR1B, and BMPR2 in the granulosa cells and oocyte of most of the follicles in the ovary, suggesting that these cells have the capacity to respond to BMP signaling. Although much is known about BMP4 signaling, its expression pattern in the female reproductive tract (FRT) is still unclear. The objective of the current study was to characterize the expression of BMP4 and its downstream target proteins (pSMAD1/5/8) in the FRT. In the ovary, BMP4 protein was detected in all the stages of follicular development. Staining for pSMAD1/5/8 was observed in granulosa cells and oocytes of all the stages of follicular development including primordial follicles, suggesting that these follicles are responsive to autocrine/paracrine BMP signaling. In the uterus, BMP4 and pSMAD1/5/8 staining was observed in all three compartments and strongest expression was observed during the estrus phase. BMP4- and pSMAD1/5/8-specific staining was also observed in oviductal epithelium. Different forms (apparent MW: 50, 35, and 15 kDa) of BMP4 were detected in mouse ovary by western blot analysis. In conclusion, these results have defined BMP4 and pSMAD1/5/8 protein expression in the mouse FRT and highlighted the importance of BMP4 in folliculogenesis. *Reproduction* (2011) **142** 573–579

Introduction

The bone morphogenetic proteins (BMPs) are the largest family of growth factors in the transforming growth factor β (TGFB) superfamily. They are involved in multiple cellular functions such as proliferation, differentiation, migration, organization, and death (Shimasaki *et al.* 2004, Kayamori *et al.* 2009). The biological actions of BMPs are mediated through a hetero-oligomeric complex of the BMP type 1 (BMPR1A and BMPR1B) and type 2 (BMPR2) receptors. This complex allows phosphorylation of type 1 receptor by type 2 receptor, which subsequently leads to transphosphorylation of intracytoplasmic signaling molecules SMAD1, 5, and 8 (Massagué 1998, Miyazono 2000). Several BMP family members including BMP2, BMP3, BMP4, BMP5, BMP6, BMP7, and BMP15 have been identified in the mammalian ovary (Takao *et al.* 1996, Dube *et al.* 1998, Shimasaki *et al.* 1999, Erickson & Shimasaki 2003, Pierre *et al.* 2005, Abir *et al.* 2008). In rat ovary, *Bmp2* mRNA is expressed in granulosa cells of atretic and graafian follicles (Erickson & Shimasaki 2003). In bovine ovary, BMP2 protein has been detected in the theca and oocyte of antral follicles (Fatehi *et al.* 2005).

Bmp4 mRNA has been detected in the theca, corpus luteum, ovarian surface epithelium, and sex cords of the rat ovary (Erickson & Shimasaki 2003). In contrast, *BMP4* mRNA is reported to be expressed in both granulosa and thecal cells of bovine, chicken, and mouse ovary (Onagbesan *et al.* 2003, Fatehi *et al.* 2005, von Schalburg *et al.* 2006). Recently, BMP4 protein was detected in oocytes and granulosa cells of human ovary (Abir *et al.* 2008), while growth differentiation factor 9 (GDF9) and BMP15 expression appear to be limited to the oocyte of the follicles throughout follicular development (Bodensteiner *et al.* 1999, Erickson & Shimasaki 2003, Juengel *et al.* 2004) except in primates, where GDF9 is also expressed in the cumulus and mural granulosa cells (Duffy 2003). These studies indicate significant differences in the expression pattern of BMPs across different species and sometimes within the same species. These differences might be partially attributed to the differences in sensitivity of the various techniques/reagents (BMP antibodies) used by different laboratories.

The mRNA encoding various BMP ligands including *Bmp2*, *Bmp4*, *Bmp6*, and *Bmp7* have also been detected in rat uterus (Erickson *et al.* 2004). The expression of BMP receptor 1A (*Bmpr1a*), *Bmpr1b*, and *Bmpr2* mRNA

has been detected in uterine epithelia. *Bmpr1a* and *Bmpr2* mRNA have also been detected in periluminal stroma and smooth muscle (Erickson *et al.* 2004).

A point mutation in BMPR1B has been shown to increase ovulation in Booroola sheep (Mulsant *et al.* 2001, Souza *et al.* 2001, Wilson *et al.* 2001). Gene knockout and naturally occurring mutations in BMPR1B have adverse effects on female fertility in mice and humans (Yi *et al.* 2001, Demirhan *et al.* 2005). These findings underscore the importance of BMPs in female fertility. The cellular localization of BMPs in female reproductive organs is still unclear because of the contrasting results reported by different laboratories (Erickson & Shimasaki 2003, Shimasaki *et al.* 2004, Fatehi *et al.* 2005, Juengel *et al.* 2006, Abir *et al.* 2008). In this study, we have developed a specific antibody against BMP4 and analyzed the expression of BMP4 and pSMAD1/5/8 proteins in the reproductive organs of female mice.

Results

Specificity of BMP4 antibody

Most of the commercially available antibodies detect both BMP2 and BMP4 proteins. The anti-BMP4 antibody used in this study has been shown to be specific against BMP4 in previous studies (Tanwar & McFarlane 2005, Tanwar *et al.* 2007). In this study, we tested the cross-reactivity of the anti-BMP4 antibody against BMP2 by using indirect ELISA. The anti-BMP4 antibody showed specific binding to BMP4 peptide and had no cross-reactivity with BMP2 peptide (Fig. 1).

Figure 1 Binding of anti-BMP4 antibody to BMP4 peptide in comparison with increasing amount of BMP2 and BMP4 peptides. The amounts of BMP2 and BMP4 peptides used to compete with BMP4 peptide coated on plate are indicated on the X-axis. The experiment was repeated three times.

BMP4 protein expression pattern in ovary, uterus, and oviduct

Immunohistochemical analyses of adult mouse ovary showed strong BMP4 protein expression in all stages of follicular development across the estrous cycle (Fig. 2A–D). In primary and primordial follicles, strong staining for BMP4 was observed in both oocyte and granulosa cells (Fig. 2E–H). Intense staining for BMP4 protein was also observed in granulosa cells of antral and preantral follicles (Fig. 2E–H). Interestingly, very high levels of BMP4 protein expression were observed in the ovarian surface epithelium (Fig. 2E–H). Strong staining was also observed in the corpus luteum and stromal cells around follicles (Fig. 2A–H).

In mouse oviduct, BMP4 protein was mainly detected in epithelium (Fig. 2I–L). Within the adult mouse uterus, BMP4 was detected in all three compartments of uterus and the strongest staining was observed in the uterine luminal epithelium during estrus phase (Fig. 2M–P). No staining was observed in uterine tissue exposed to sheep IgG instead of primary antibody (Fig. 3).

Detection of pSMAD1/5/8 proteins in female reproductive tract

The activation of the BMP signaling pathway can be detected by analyzing the expression of pSMAD1/5/8 proteins (Massagué 1998, Miyazono 2000). We detected the expression of pSMAD1/5/8 in mouse ovaries, oviducts, and uteri ($n=3$ /each) across the estrous cycle by an antibody against pSMAD1/5/8. These pSMAD1/5/8 proteins were also detected in oocyte and granulosa cells at all the stages of follicular development including primordial follicles (Fig. 4A–H). However, no difference in expression of pSMAD1/5/8 was detected in ovaries ($n=3$) collected at different stages of estrous cycle (Fig. 4A–H). Similar to BMP4, pSMAD1/5/8-specific staining was also observed in the stromal, theca, and ovarian surface epithelial cells of the ovary (Fig. 4A–H), suggesting that BMP signaling is also active in these cells. We also analyzed the expression of pSMAD1/5/8 in oviduct and uterus (Fig. 4D and E) and found that staining was mainly observed in the epithelium of oviduct (Fig. 4I–L). In the uterus, pSMAD1/5/8 staining was observed in all three compartments and the strongest staining was observed during the estrus phase (Fig. 4M–P). No staining was observed in the controls (Fig. 3).

BMP4 protein isoforms in mouse ovary

The presence of BMP4 in the ovary was confirmed by western blot analyses. Under non-reducing conditions, a band migrating at 50 kDa was detected (Fig. 5 lane A) while bands migrating at 15 and 35 kDa were observed under reducing conditions (Fig. 5 lane B). The membrane

Figure 2 Immunolocalization of BMP4 protein in the mouse ovary, oviduct, and uterus. (A–H) Expression of BMP4 protein in ovary. (I–L) Expression of BMP4 in oviduct (M–P) BMP4 protein localization in uterus. Tissues from three different adult animals were analyzed to study BMP4 expression pattern. Bars: 50 μ m.

treated with non-specific purified sheep immunoglobulin instead of anti-BMP4 antibody showed no bands and was used as a negative control (Fig. 5 lane C and D). The observed band sizes reported here are similar with those reported in previous studies (Cui *et al.* 2001).

Discussion

In females, BMPs and their receptors have been shown to regulate granulosa cell function (Shimasaki *et al.* 1999, Gilchrist *et al.* 2006, Kayamori *et al.* 2009). Various BMP signaling antagonists and agonists such as dragon, gremlin, and follistatin are expressed in the mammalian ovary and are likely to play an important role in the regulation of ovarian function (Pangas *et al.* 2004, Xia *et al.* 2004, 2005, Kimura *et al.* 2010), suggesting that modulation of BMP signaling by various factors is required for normal ovarian function. The significance of BMP signaling in the ovary is further highlighted by the increased ovulation rates in Booroola sheep with a point mutation in *BMPR1B* gene (Mulsant *et al.* 2001, Souza *et al.* 2001, Wilson *et al.* 2001). Previous *in situ* hybridization and PCR-based studies have suggested that BMP4 is mainly synthesized in theca cells, corpus

luteum, and other cells including stromal cells and acts in a paracrine manner to regulate granulosa cell function (Shimasaki *et al.* 1999, Erickson & Shimasaki 2003, von Schalburg *et al.* 2006, Abir *et al.* 2008). Our previous report demonstrated that BMP4 promotes the transition of the primordial to primary follicle during follicular development (Tanwar *et al.* 2007). Interestingly, in this study, we have detected BMP4 protein in all stages of follicular development including primordial follicles. In addition, pSMAD1/5/8 proteins were also detected in the oocyte and granulosa cells of primordial follicles, suggesting that BMP4 might act in paracrine/autocrine manner to affect the transition of primordial to primary follicles.

Figure 3 Negative antibody controls for BMP4 (A) and pSMAD1/5/8 (B) staining. Bars: 50 μ m.

Figure 4 pSMAD1/5/8 protein expression in female reproductive tract. The pSMAD1/5/8 proteins were detected in ovary (A–H), oviduct (I–L), and uterus (M–P). Tissues from three different adult animals were analyzed to study pSMAD1/5/8 expression. Arrow: ovarian surface epithelium; arrowheads: primordial and primary follicles. L, lumen; Es, endometrial stroma; M, myometrium. Bars: 50 μ m.

Various BMP signaling components are expressed in various cells in the mammalian uterus (Kim *et al.* 2003, Erickson *et al.* 2004). The expression of BMP ligands including BMP2, BMP4, BMP7, and BMP8a has been detected in the murine uterus during decidualization (Ying & Zhao 2000). In *in situ* hybridization studies, BMP2 expression was detected in the periluminal stroma. In comparison, BMP7 was detected in periluminal stroma and glandular epithelial cells (Erickson *et al.* 2004). Moreover, *Bmp4* and *Bmp6* mRNA expression was detected in myometrium and vascular endothelial cells (Ying & Zhao 2000, Erickson *et al.* 2004). The mRNA for *Bmpr1a*, *Bmpr1b*, and *Bmpr2* was also localized in luminal and glandular epithelium (Erickson *et al.* 2004). Yi *et al.* (2001) have reported that deletion of BMPR1B in mice leads to endometrial thinning and a reduced number of endometrial glands, which further underlines the significance of BMP signaling in uterine physiology (Yi *et al.* 2001). We observed BMP4 and pSMAD1/5/8 protein expression in endometrial glandular and luminal epithelia, stroma, and myometrium. Taken together, these results suggest that BMP4 potentially plays an important role in the maintenance of the uterine endometrium.

The interrelationship between estradiol, progesterone, and various growth factor pathways plays an important

role in the normal function of reproductive tissues. Exogenous treatment with estradiol has been shown to decrease expression of uterine *Bmp7* mRNA *in vivo* (Ozkaynak *et al.* 1997). Similarly, estradiol treatment has been shown to repress *BMP7* mRNA levels in chicken oviduct (Monroe *et al.* 2000). In addition, BMP7 is shown to regulate apoptosis in primary oviductal cells (Monroe *et al.* 2000). Interestingly, we observed BMP4 and pSMAD1/5/8 protein expression in the epithelium of murine oviduct. It will be of interest for future studies to find a functional link between estradiol, progesterone, and BMP4 in oviduct and uterus physiology because both BMP4 and BMP7 have been shown to regulate the production of estradiol and progesterone in ovarian cell cultures (Shimasaki *et al.* 1999, Campbell *et al.* 2006).

Similar to other TGFB superfamily members, BMPs are produced as precursor proteins. The precursor protein consists of a hydrophobic secreted leader sequence followed by a pro-region and a mature region protein. The mature region of protein is located at the carboxy terminus of the precursor molecule and contains seven cysteine amino acids involved in intra- and inter-molecular disulfide bonds between two monomers to form a dimeric precursor protein (Chang *et al.* 2002, Canalis *et al.* 2003, Zhao 2003). Similar to other

Figure 5 Western blot analysis of mouse ovary for detection of BMP4. The samples were subjected to 12.5% SDS-PAGE under non-reducing (lane A) and reducing condition (lane B). The membranes were treated with equal concentration of anti-BMP4 antibody (lanes A and B) and non-specific purified sheep Ig (lanes C and D). The approximate molecular weight of bands detected is shown.

members of the TGFB family, BMP4 is also synthesized *in vivo* as a pre-pro-protein (Constam & Robertson 1999), and the sequential cleavage of pre-pro-protein at two distinct sites (S_1 and S_2) by furin and furin-like proteases is required for the activation of BMP4 precursor (Cui *et al.* 2001, Degnin *et al.* 2004). Both the precursor and the mature protein of BMP4 contain two potential N-linked glycosylation sites (Cui *et al.* 2001). The precursor forms of BMP2 and BMP6 have been detected in porcine follicular fluid (Brankin *et al.* 2005). Similarly, the precursors of GDF9 and BMP15 and mature GDF9 have been detected in monkey follicular fluid (Duffy 2003). Based on the predicted molecular weights of BMP4 isoforms and the studies discussed above, it is likely that in mouse ovaries, we have detected the BMP4 precursor (50 kDa) protein under non-reducing conditions. Under reducing conditions, it is likely we have detected a non-glycosylated mature BMP4 monomer (15 kDa) and the mature BMP4 with an attached fragment of the pro-domain (35 kDa).

In conclusion, we have characterized the expression of BMP4 and pSMAD1/5/8 proteins in ovary, oviduct, and uterus of mice. Additionally, we have detected different isoforms of BMP4 protein in the mouse ovary by western blot analysis. These findings, along with our previous study (Tanwar *et al.* 2007), support the idea that BMP4 is an important regulator of the female fertility.

Materials and Methods

Tissue and section preparation

All the tissues (ovary, oviduct, and uterus) used in this study were collected from female Swiss mice (University of New England, NSW, Australia). The estrous cycle of the animals was

determined by vaginal smear cytology. The tissues were immersion fixed in Bouin's fluid or 4% paraformaldehyde for 10–12 h and then transferred to 70% alcohol until processing. The fixed tissues were dehydrated in a graded ethanol series, cleared in xylene, and embedded in paraffin wax. Embedded tissue samples were sectioned at 5 μ m thickness and were mounted on slides previously coated with 3-aminopropyl triethoxy-silane (Sigma Chemical Co.).

Animals used in this study were housed under standard animal housing conditions and all experimentation was approved by the Animal Ethics Committee, University of New England, Armidale, NSW, Australia.

Determination of cross-reactivity of anti-BMP4 antibody with BMP2

The specificity of the anti-BMP4 antibody (JMS1301) used in this study has been demonstrated in our previous studies (Tanwar & McFarlane 2005, Tanwar *et al.* 2007). In this study, we determined the cross-reactivity of anti-BMP4 antibody with its closely related family member BMP2 by using indirect ELISA technique as described below. Briefly, flat-bottomed microtiter plates (Greiner Labortechnik, Kremsmünster, Austria) were coated with 30 ng/well recombinant BMP4 in binding buffer (0.05 M bicarbonate, 0.02% sodium azide, pH 9.6). Excess binding sites were blocked with 200 μ l 5% skim milk in PBS for 1 h at 37 °C. The plates were washed five times with 0.9% saline and 0.05% Triton-X 100, using a Titertek Microplate washer (Labsystems, Helsinki, Finland). Serial double dilutions (1000 ng/ml $\rightarrow$ 0.48 ng/ml) of BMP2 and BMP4 peptide were prepared in phosphate buffer, pH 8.0, containing 0.5 M sodium chloride, 0.5% Tween-20, and 0.1% BSA (ELISA buffer) and were added to the ELISA plate in a volume of 100 μ l. Biotinylated anti-BMP4 antibody (2 μ g/50 μ l) was added to the ELISA plate and incubated overnight at 37 °C. The following day, the plates were washed and streptavidin conjugated to alkaline phosphatase at a dilution of 1:10 000 and was incubated for 1 h at 37 °C. The plates were developed with *p*-nitrophenylphosphate disodium salt hexahydrate and read at 405 nm using a Titertek Multiskan Plus microplate reader (Labsystems).

Immunohistochemistry

Serial sections of tissues from three different animals were deparaffinized with xylene and rehydrated with graded series of ethanol (absolute, 80 and 50%, respectively, and distilled water), followed by two washes for 5 min each in PBS containing 0.05% Tween-20 (PBS-T). Tissue sections were incubated for 10 min in 3% (v/v) hydrogen peroxide in methanol to block the endogenous peroxidase activity. Antigen retrieval was performed by microwaving the sections in 0.01 M citrate buffer (pH 6) in a domestic microwave oven (750 W) at full power for 15 min and the sections were kept in buffer until cool (20–30 min). The sections were washed for 5 min in PBS-T and blocked at room temperature for 1 h by using 5% normal rabbit/donkey serum in PBS. Tissues sections were incubated in humidified chamber for overnight incubation at 4 °C with anti-BMP4 and pSMAD1/5/8 antibody (Cell Signaling Technology, Danvers, MA, USA) diluted in PBS containing 5%

donkey/rabbit serum and 0.1% BSA. BMP4 antibody was raised against a synthetic BMP4 peptide (aa 88–102, Invitrogen Australia) in sheep and had previously been shown to be specific against BMP4 (Tanwar & McFarlane 2005, Tanwar *et al.* 2007). Sections were subsequently washed with PBS-T and incubated at 37 °C for 1 h with 1:800 dilution of biotinylated rabbit anti-sheep (BMP4 ab) and donkey anti-rabbit (pSMAD1/5/8 ab) IgG in 0.05 M PBS containing 0.3% BSA. The sections were incubated at 37 °C for 1 h with diluted alkaline phosphatase-conjugated streptavidin at 1:800 in PBS. Bound antibodies were visualized by incubation in DAB (Dark brown color, Vector Labs, Burlingame, CA, USA). The sections were washed in distilled water, dehydrated through increasing concentration of alcohol, and mounted with coverslips using DPX (BDH Laboratory Supplies, Poole, England). For negative control, primary antibodies were replaced with sheep or rabbit IgG at similar protein concentration.

Western blot analysis

Mouse ovaries ($n=3$) were homogenized four times (V/V) with a homogenization buffer (benzamidine 5 mM, EDTA 5 mM and sodium azide 0.02%). After centrifugation for 10 min at 6000 *g*, the supernatant was collected. The supernatant was diluted in non-reducing (0.125 M Tris–HCl, 0.07% SDS, 0.6% glycerol, 1.5% Nonidet P-40 detergent, and bromophenol blue) and reducing sample buffer (0.125 M Tris–HCl, 0.07% SDS, 0.6% glycerol, 10% 2-mercaptoethanol, and bromophenol blue) and was heated at 95 °C for 10 min and loaded on to a SDS polyacrylamide gel (12.5% resolving and 4.06% stacking gel) using a slab electrophoresis unit (Model SE 250-Mighty Small II; Hoefer, Holliston, MA, USA). The proteins were transferred from gel to polyvinylidene fluoride membrane (Micron Separations, Inc., Westborough, MA, USA) by using the transfer electrophoresis unit (Model TE 22-Mighty Small, Hoefer) in a transfer buffer (25 mM Tris base, 200 mM glycine, and 15% methanol). After protein transfer, the membrane was slightly stained with Ponceau S (0.2% Ponceau, 3% trichloroacetic acid, and 3% sulfosalicylic acid) to see the successful transfer of protein. The membrane was blocked for 1 h with 5% skimmed milk powder in PBS-T. The membrane was incubated overnight with anti-BMP4 antibody (0.5 µg/ml) diluted in high-salt ELISA buffer. The following day, the membrane was incubated for 1 h with biotinylated rabbit anti-sheep immunoglobulin (1:10 000) diluted in high-salt ELISA buffer. The membrane was incubated with streptavidin alkaline phosphatase conjugate (1:10 000) for 1 h. The appearance of specific bands on the membrane was detected by using BCIP/NBT one-step reagent (Chemicon International, Temecula, CA, USA). The specificity of bands was determined by using pre-immune sheep immunoglobulin in place of primary antibody.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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