Effect of bone morphogenetic protein 2 (BMP2) on oestradiol and inhibin A production by sheep granulosa cells, and localization of BMP receptors in the ovary by immunohistochemistry

C. J. H. Souza¹, B. K. Campbell², A. S. McNeilly³ and D. T. Baird¹

¹Department of Reproductive and Developmental Sciences, and ³MRC Human Reproductive Sciences Unit, Centre for Reproductive Biology, University of Edinburgh, 37 Chalmers Street, Edinburgh EH3 9ET, UK; and ²School of Human Development, Queen's Medical Centre, Nottingham NG7 2UH, UK

The bone morphogenetic proteins (BMPs) have been implicated in the paracrine regulation of ovarian follicular development. In this study, we investigated the expression of the BMP receptors (BMPRs) in sheep ovaries by immunohistochemistry and the effect of BMP2, a natural ligand for these receptors, on granulosa cells cultured *in vitro*. Ovaries from cyclic ewes were fixed, embedded in paraffin wax and cut into sections. The sections were rehydrated, submitted to microwave antigen retrieval and treated with polyclonal antibodies against BMPR1A, BMPR1B and BMPR2. Strong immunostaining for all three receptors was observed in the granulosa cell layer of follicles from the primary to late antral stages of development. Staining was also present in the oocyte, corpus luteum, ovarian surface epithelium and, to a lesser

extent, the theca layer of antral follicles. For functional studies, granulosa cells were obtained from immature follicles 1–3 mm in diameter. The cells were cultured for 6 days in serum-free medium containing 1 ng oFSH-20 ml⁻¹ in the presence of 0, 3, 10 or 30 ng ml⁻¹ human recombinant BMP2. The medium was replaced every 2 days and oestradiol and inhibin A concentrations were measured in the spent medium. In the absence of BMP2, oestradiol and inhibin A production increased as the granulosa cells differentiated *in vitro*. The addition of the highest dose of BMP2 enhanced oestradiol production ($P < 0.05$) without affecting the proliferation of the cells. It is concluded that BMP receptors are present in sheep ovaries and that BMPs may have a role in the differentiation of granulosa cells by enhancing the action of FSH.

Introduction

The bone morphogenetic proteins (BMPs) are members of the transforming growth factor β (TGF- β) superfamily of peptides, which have recently been implicated in the paracrine regulation of ovarian follicular development. These peptides signal through a hetero-oligomeric complex of the BMP receptor 2 (BMPR2) and BMPR type 1A or 1B. The complex allows the type 2 receptor to phosphorylate the type 1 receptor, which leads to phosphorylation, heterodimerization and nuclear migration of the Smad proteins, and subsequent alteration in the transcription of target genes (for a review, see Massague, 1998; Miyazono *et al.*, 2001).

Several BMP genes are expressed in the mammalian ovary. In rodents, BMP4 and BMP7 are expressed in the theca layer of rat follicles (Shimasaki *et al.*, 1999), and BMP6 is expressed in mouse oocytes (Lyons *et al.*, 1989). Normal cyclic rats express mRNA of the BMP receptor types 1A, 1B and 2 in the granulosa cells and oocytes of most follicles in the ovary (Shimasaki *et al.*, 1999). Furthermore, a

functional role for this local system in the ovary is suggested by the observation that rat granulosa cells cultured *in vitro* show increased oestradiol production and a reduction in progesterone secretion when treated with BMP4 or BMP7 (Shimasaki *et al.*, 1999), and that null mutations of the BMP receptor type 1B are infertile and hypo-oestrogenic (Yi *et al.*, 2001).

The importance of this putative regulatory system in species in which the ovulatory quota is controlled rigorously has been highlighted by recent findings showing that naturally occurring mutations in the BMP pathways can result in marked perturbations in ovarian function in sheep. The Inverdale phenotype is a result of a mutation in the BMP15 (GDF 9b) gene and causes increased ovulation rate and multiple births in heterozygotes, but primary ovarian failure in homozygotes because of an impairment of follicular development beyond the primary stage (Galloway *et al.*, 2000). In contrast, the Booroola phenotype is a result of a point mutation in the kinase domain of the BMP receptor type 1B (Alk 6) and results in deregulation of the follicle selection mechanisms, with 'precocious' development of a large number of small antral follicles, leading to greatly increased ovulation rate and multiple births (Mulsant *et al.*, 2001; Souza *et al.*, 2001; Wilson *et al.*, 2001).

Email: carlos@obg93.obg.ed.ac.uk

Therefore, the BMP system appears to be a major intraovarian regulatory system central to the follicle selection mechanisms in species such as large domestic ruminants and humans. However, little is known about the functional basis of the ovarian BMP system in these species, although BMP3 is expressed in granulosa-luteal cells in humans (Jaatinen *et al.*, 1996) and mRNA for the BMPR1B and BMPR2 genes is expressed in the granulosa cells and oocytes of most follicles in sheep ovaries (Wilson *et al.*, 2001). In the present study, we investigated the protein expression of the BMP receptor types 2, 1A and 1B in sheep ovaries, and the effect of BMP2, a natural ligand for these receptors (Liu *et al.*, 1995), on FSH-induced differentiation of cultured sheep granulosa cells.

Materials and Methods

Immunohistochemistry

The ovaries were obtained from sheep that were killed for other reasons. The presence of a corpus luteum in one of the ovaries in non-pregnant animals was used as an indication that the animals were cyclic. Ovaries from cyclic ewes were fixed overnight in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS; 0.01 mol l⁻¹, pH 7.6; Sigma, Poole) and transferred to 70% (v/v) ethanol until processing. The fixed tissue was embedded in paraffin wax after dehydration.

Sections (5 µm thickness) were dewaxed and rehydrated in decreasing concentrations of alcohol (90, 70 and 30%, respectively, and distilled water), followed by two washes of 5 min each in PBS. Antigen retrieval was performed by microwaving the sections in citrate buffer (0.01 mol l⁻¹, pH 6.0) at full power (800 W) for 10 min, and the sections were kept in the buffer for 20 min until cool. After a wash in PBS, the sections were incubated in 3% hydrogen peroxide in water for 15 min. After a wash in PBS, a combined avidin-biotin block was performed according to the manufacturer's instructions (Vector Laboratories, Peterborough). In brief, avidin was added to the blocking buffer, which consisted of 20% normal swine serum (NSS; Diagnostics Scotland, Edinburgh) and 5% (w/v) bovine serum albumin (BSA; Sigma) in PBS, the slides were incubated for 30 min and then washed in PBS. Biotin was added to the blocking buffer and the slides were treated for 15 min and washed in PBS. Rabbit polyclonal antibodies directed against synthetic peptides of the BMPR2 (amino acid 185–202; Rosenzweig *et al.*, 1995), BMPR1A (amino acid 181–202; ten Dijke *et al.*, 1994) and BMPR1B (amino acid 151–168; ten Dijke *et al.*, 1994), which had previously been shown to be specific to the target receptors, were used in blocking buffer diluted to 1:400, 1:1000 and 1:400 for BMPR2, BMPR1A and BMPR1B, respectively. Negative controls were performed by replacing the primary antibody with normal rabbit serum (DAKO, Cambridge) at matching protein concentrations. Slides with serial sections were incubated with one of these antibodies in a humidified chamber at 4°C overnight. Slides

were washed twice for 5 min in 0.1% (v/v) Tween 20 (Sigma) in PBS (PBST), and incubated with swine anti-rabbit biotinylated antibody (DAKO) diluted 1:500 in blocking buffer for 1 h at room temperature. The sections were washed twice in PBST for 5 min, incubated in ABC-HRP (Vector Laboratories) for 1 h at room temperature and washed again in PBST. Bound antibodies were visualized by incubation in 3-3'-diaminobenzidine (brown stain; Vector Laboratories); the sections were counterstained with Harris' haematoxylin (BDH, Poole), dehydrated through increasing concentrations of alcohol and mounted with coverslips.

Sections from rat ovaries were used as positive controls, as the pattern of mRNA expression has been established previously (Shimasaki *et al.*, 1999) and these antisera have been validated for immunohistochemistry in rats (Obata *et al.*, 1999).

The slides were examined using an Olympus Provis microscope (Olympus Optical Co., London) and the images were captured with a Kodak DCS420 camera (Eastman Kodak) and assembled in Photoshop 5.0 software (Adobe).

Cell culture

Granulosa cells from follicles 1–3 mm in diameter were cultured as described by Campbell *et al.* (1996). Unless specified otherwise, all reagents were purchased from Sigma (Poole). In brief, ovaries were obtained from an abattoir and were transported to the laboratory at 37°C in tissue culture medium 199 (TCM199) supplemented with Hepes (20 nmol l⁻¹), penicillin (100 000 iu l⁻¹), streptomycin (0.1 µg l⁻¹) and amphotericin (1 mg l⁻¹). The follicles were dissected from the stromal tissue and placed in DPBS (Dulbecco's PBS without calcium and magnesium), hemisected and the follicle shells were flushed repeatedly with a 1 ml syringe. The thecal cells were allowed to settle, the granulosa cell-rich supernatant was removed and the flushing procedure was repeated. The granulosa cells were centrifuged at 800 g for 5 min, and the cell pellet was resuspended in culture medium (McCoy's 5A supplemented with Hepes (20 nmol l⁻¹), penicillin (100 000 iu l⁻¹), streptomycin (0.1 µg l⁻¹), L-glutamine (3 mmol l⁻¹), BSA (0.1%, w/v), transferrin (2.5 mg l⁻¹), selenium (4 µg l⁻¹), androstenedione (10 µmol l⁻¹), bovine insulin (10 µg l⁻¹), long R3 insulin-like growth factor (LR3-IGF, 10 µg l⁻¹) and ovine FSH (1 µg l⁻¹, NIDDK-oFSH-20, *in vivo* biological potency 175 × NIH-oFSH-S1; A. Parlow, NIDDK, Torrance, CA).

The cells were plated at a density of 7.5 × 10⁴ viable cells per well in a 96-well plate, in 250 µl of culture medium containing 0, 3, 10 or 30 µg human recombinant BMP2 l⁻¹ (W. Sebald, Würzburg University). The plates were incubated in a humidified atmosphere with 5% CO₂ in air at 37°C for 6 days. The medium was changed at 48 h intervals and, to minimize disturbance of the cells, 175 µl medium was removed and replenished each time and the spent media was stored frozen until assayed. At the

completion of the culture period, the number of viable cells was estimated using neutral red uptake (Campbell *et al.*, 1996); the results are expressed as hormone production per 1000 cells per 48 h.

Assay

The concentration of oestradiol was determined in unextracted culture medium using a double antibody radioimmunoassay (Campbell *et al.*, 1996) and inhibin A was measured by two-site ELISA (Souza *et al.*, 1997). The sensitivities of the oestradiol and inhibin A assays were 50 pmol l⁻¹ and 30 ng l⁻¹ of 32 kDa immunopurified bovine inhibin A, respectively. The intra- and interassay coefficients of variation were < 15%.

Statistical analysis

Four independent experiments from different ovaries were performed with at least four replicates of each treatment. The effect of the treatments was determined by repeated measurement ANOVA and the comparisons between the treatment means were made by Tukey's test using SYSTAT software (SYSTAT Inc., Evanston, IL).

Results

Expression of BMPR protein

Approximately 100 follicles were examined in sections from the ovaries of six ewes. Strong immunostaining for BMPR2, BMPR1A and BMPR1B was observed in the granulosa layer of follicles from the primary to preovulatory stages (Fig. 1). Staining was also observed in most oocytes of these follicles. In the antral follicles, the theca layer also stained, but at a lower intensity than in the granulosa. There was very strong staining of the ovarian surface epithelium and in the corpus luteum for all three receptor types, but staining was not observed in the stroma. The same pattern of staining was observed in rat ovaries (data not shown).

Cell culture

Addition of BMP2 at any concentration had no effect on granulosa cell proliferation (data not shown). Oestradiol production in the cultured cells increased with time in culture ($P < 0.05$), as the cells differentiated *in vitro* (Fig. 2). Addition of BMP2 at the highest dose enhanced oestradiol production by the cells ($P < 0.05$). This increase was evident from day 2 of culture and remained significantly increased throughout the culture period.

There were significant differences in the basal rate of oestradiol production between experiments ($P < 0.05$). In experiments in which basal production was low ('slow rate of differentiation'), addition of BMP showed a more marked effect (Fig. 3). In this set of cultures, there was an increase in the production of inhibin A, as well as oestradiol, indicating that the granulosa phenotype is preserved in the culture system.

Discussion

BMP receptors are expressed in the oocyte, granulosa and thecal layer in sheep follicles from the primary to the large antral stage, and in the ovarian surface epithelium. The response of the granulosa cells to BMP2 demonstrates that these receptors are functional in sheep ovaries. The pattern of protein distribution of the BMP receptors in sheep ovaries is similar to the RNA distribution in rat ovaries (Shimasaki *et al.*, 1999) and to the RNA expression of BMPR2 and BMPR1B in sheep ovaries (Wilson *et al.*, 2001).

The granulosa cells increased oestradiol and inhibin A production during the culture period, indicating that these cells were differentiating *in vitro*. BMP2 enhanced differentiation of granulosa cells, as indicated by the fact that oestradiol production was increased to a greater extent in the experiment in which basal oestradiol production was low. These effects also point to the actions of the BMP receptors in ovarian paracrine regulation in sheep, as mice deficient in BMPR1B are infertile and have impaired oestradiol production (Yi *et al.*, 2001).

In rat granulosa cells from mature follicles cultured *in vitro*, BMP4 and BMP7 enhanced FSH action by stimulating oestradiol and decreasing progesterone production (Shimasaki *et al.*, 1999). This action of BMP4 of reducing progesterone production is also observed in luteinizing sheep granulosa cells *in vitro* (Mulsant *et al.*, 2001). However, in ruminants, oestradiol and inhibin A are better markers of granulosa cell differentiation which, in many culture systems, luteinize spontaneously, resulting in decreased or absent oestradiol, but increased progesterone production (Campbell *et al.*, 1996; Campbell and Baird, 2001). In addition, the BMPR1B mutation present in the Booroola phenotype reduced the effect of BMP4 on progesterone production *in vitro* (Mulsant *et al.*, 2001), indicating that the intracellular signal generated by BMP4 through the mutated receptor may be reduced, decreasing the effect of the ligand. In contrast, our result showing that BMP2 increased oestradiol and inhibin A production, and enhanced the differentiation of the immature granulosa cells *in vitro*, supports the hypothesis that the Booroola mutation in the receptor BMPR1B would enhance the signalling downstream from the receptor. This would result in the characteristic phenotype of Booroola sheep, in which there is advanced differentiation of the granulosa cells, leading to a higher number of follicles that ovulate 'precociously' (Souza *et al.*, 1997). The effects of BMPs on cellular function are ligand- and tissue-specific. Human theca-derived tumour cells show reduced androstenedione production *in vitro* when treated with BMP4, and when cyclic AMP agonists are associated with BMP4, the effect of the BMP4 in cultured cells is increased (Dooley *et al.*, 2000). In the same model, BMP4 also increased progesterone production, indicating a possible autocrine effect in theca cells. BMP15 (GDF9b) stimulates the proliferation of rat granulosa cells *in vitro*, an effect which is

Fig. 1. Immunohistochemistry of sheep ovaries showing protein expression of bone morphogenetic protein receptor (BMPR) type (a,e) 1A, (b,f) 1B and (c) 2, and (d) negative control, incubation with normal rabbit serum. Arrowheads: primary follicles; pa: preantral follicles; sa: small antral follicles; la: large antral follicles; se: surface epithelium. Scale bars represent (a–d) 100 μ m and (e,f) 50 μ m.

Fig. 2. Mean $\pm$ SE oestradiol concentration per well from sheep granulosa cells treated *in vitro* with 0 (■), 3 (□), 10 (▨) or 30 (▩) $\mu\text{g l}^{-1}$ human recombinant bone morphogenetic protein (BMP) 2 ($n = 4$ experiments). Granulosa cells from antral follicles 1–3 mm in diameter were cultured for 6 days in serum-free conditions in McCoy's 5A medium supplemented with Hepes (20 nmol l^{-1}), penicillin (100 000 iu l^{-1}), streptomycin (0.1 $\mu\text{g l}^{-1}$), L-glutamine (3 mmol l^{-1}), BSA (0.1%, w/v), transferrin (2.5 mg l^{-1}), selenium (4 $\mu\text{g l}^{-1}$), androstenedione (10 $\mu\text{mol l}^{-1}$), bovine insulin (10 $\mu\text{g l}^{-1}$), long R3 insulin-like growth factor (LR3-IGF, 10 $\mu\text{g l}^{-1}$) and ovine FSH (1 $\mu\text{g l}^{-1}$, NIDDK-oFSH-20). ^{ab}Means on the same day of culture with different letters are significantly different ($P < 0.05$).

FSH-independent. In contrast, BMP15 alone has no effect on steroidogenesis but resulted in a marked reduction in FSH-induced progesterone production, without any effect on FSH-stimulated oestradiol production (Otsuka *et al.*, 2000). Treatment with BMP15 alone has no effect on the basal expression of mRNAs encoding steroidogenic acute regulatory protein, P450 side chain cleavage enzyme, P450 aromatase, 3 β -hydroxysteroid dehydrogenase, LH receptor and inhibin/activin subunits. However, BMP15 markedly inhibited the FSH-induced increases in these mRNAs, but did not affect the levels of these transcripts after induction by forskolin. Furthermore, BMP15 severely reduced the expression of FSH receptor mRNA in granulosa cells and inhibited the biological response of FSH, but not that of forskolin (Otsuka *et al.*, 2001). Mice deficient in BMP15 are fertile but have reduced litter size (Yan *et al.*, 2001), while the sheep orthologue appears to be essential for follicle development, as ewes homozygous for the Inverdale and Hanna mutations, resulting in a loss of functional BMP15, are completely sterile and fail to produce follicles beyond the primary stage (Galloway *et al.*, 2000). This finding

indicates that there may be species differences in the role of BMP in folliculogenesis.

BMP3, which is expressed in human granulosa cells (Jaatinen *et al.*, 1996), is an antagonist of BMP action in other tissues such as bone and kidney (Daluiski *et al.*, 2001), providing an opportunity at the granulosa cell for modulation of BMPs coming from the theca layer and from the oocyte. The modulation of BMP3 signalling must be downstream from the BMP receptors, as it appears to activate signalling through the activin pathway via activin receptor type 2 and activin receptor 1b (Alk4; Daluiski *et al.*, 2001). We propose that, in sheep, the BMPs induce differentiation of the granulosa cells of antral follicles, and that the origin of the ligands is probably the neighbouring theca layer (BMP4 and BMP7). BMPs originating from the oocyte (BMP6 and BMP15) may also exert an action, but this is probably more important in the preantral stages of follicle development, if the same pattern of gradient diffusion and modulation by antagonist expression observed in other tissue systems is also operating in the ovary (Christian, 2000; Miyazono, 2000).

Fig. 3. Profile of (a) oestradiol and (b) inhibin A concentration *in vitro* in an experiment with granulosa cells from sheep follicles 1–3 mm in diameter that showed a slow rate of differentiation. Granulosa cells from antral follicles 1–3 mm in diameter were cultured for 6 days in serum-free conditions in McCoy's 5A medium supplemented with Hepes (20 nmol l⁻¹), penicillin (100 000 iu l⁻¹), streptomycin (0.1 μg l⁻¹), L-glutamine (3 mmol l⁻¹), BSA (0.1%, w/v), transferrin (2.5 mg l⁻¹), selenium (4 μg l⁻¹), androstenedione (10 μmol l⁻¹), bovine insulin (10 μg l⁻¹), long R3 insulin-like growth factor (LR3-IGF, 10 μg l⁻¹) and ovine FSH (1 μg l⁻¹, NIDDK-oFSH-20) and treated with human recombinant bone morphogenetic protein 2 (BMP2) at concentrations of 0 (○), 3 (●), 10 (□) and 30 (■) μg l⁻¹. There was a significant increase in the concentration of both hormones following the addition of BMP2 at concentrations of 3, 10 or 30 μg l⁻¹ after 4 and 6 days in culture. ^{a-c}Means on the same day of culture with different letters are significantly different (*P* < 0.05).

This work was supported by JRF Ltd Research Fellowship 2000-2/3 and MRC program grant G8929853. The authors would like to thank Carl-Henrik Heldin from the Ludwig Institute for Cancer Research, Uppsala, Sweden for the gift of the BMP receptor antibodies and Walter Sebald from Würzburg University, Germany for kindly providing the human recombinant BMP2. The authors are grateful to Brigid Orr and Caroline Mutch for technical assistance, and to Joan Docherty, Norah Anderson and Marjorie Thomson for care of the animals.

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Received 7 September 2001.

First decision 23 October 2001.

Accepted 11 December 2001.