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The role of bone morphogenetic proteins 2, 4, 6 and 7 during ovarian follicular development in sheep: contrast to rat

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Abstract

The intraovarian roles of BMP family members such as BMP2, 4, 6 and 7 are not well understood, particularly in species with low ovulation rates such as sheep. Therefore, the objectives of these experiments were to determine the expression patterns of mRNAs encoding BMP2, 4, 6 and 7 during ovarian follicular development in sheep, and to determine the effects of these growth factors on ovine granulosa cell functions *in vitro*. For comparative purposes, the effects of these BMPs were also determined in rat granulosa cells since these factors have been most widely studied in this poly-ovulatory species. As assessed by *in situ* hybridization, non-atretic ovine follicles expressed mRNA for BMP6 but not 2, 4 or 7. Furthermore, expression of BMP6 was limited to the oocyte of primordial as well as primary, pre-antral and antral follicles. Reverse transcription-PCR of granulosa cell mRNA detected low levels of all the BMPs in some pools of cells. BMP2, 4, 6 and 7 each inhibited progesterone production from ovine granulosa cells without affecting cellular proliferation/survival. Similarly, these BMPs inhibited progesterone production from rat granulosa cells. However, they also stimulated cellular proliferation/survival of the rat granulosa cells highlighting a species-specific difference for these growth factors. In conclusion, in sheep, BMP2, 4, 6 and 7 inhibit granulosa cell differentiation without affecting proliferation. However, as BMP2, 4 and 7 were not detectable by *in situ* hybridization in any cells of non-atretic ovarian follicles, it seems unlikely that these proteins would have an important intraovarian role in regulating follicular development in sheep. In contrast, localization of BMP6 mRNA in the oocyte suggests that this BMP family member may have a paracrine and/or autocrine role in regulating follicular growth in sheep, as has been shown for two other oocyte derived from members of the transforming growth factor superfamily, BMP15 and growth differentiation factor 9.

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Introduction

The transforming growth factor β (TGF-β) superfamily, which includes growth differentiation factors (GDF), bone morphogenetic proteins (BMP), TGF-β, and activin/inhibin subfamilies as well as proteins, such as anti-mullerian hormone, is comprised of over 35 proteins with common structural motifs (Chang *et al.* 2002). It is known that several members of this superfamily are important regulators of ovarian follicular growth and development in many species (Matzuk 2000, Kaivo-Oja *et al.* 2003, Knight & Glister 2003, McNatty *et al.* 2003, Shimasaki *et al.* 2004). However, the precise roles that members of this family play, appear to differ between species. For example, BMP15 (also known as GDF9b) has been shown to be essential for normal follicular growth and fertility in sheep and humans (Galloway *et al.* 2000, Juengel *et al.* 2002, Di Pasquale *et al.* 2004, Hanrahan *et al.* 2004), whereas mice lacking BMP15 are fertile (Yan *et al.* 2001). In addition, while BMP15 and GDF9 have each been shown to be essential for regulating ovulation rate in sheep (Davis *et al.* 1991, Galloway *et al.* 2000, Hanrahan *et al.* 2004, Juengel *et al.* 2004), no such role has been clearly demonstrated for either factor in mice (Dong *et al.* 1996, Yan *et al.* 2001). Species differences also appear to exist in the potential roles of TGF-β1–3 as well as in their ovarian cellular origins (Juengel & McNatty 2005).

The BMP subfamily members, BMP2, 4, 6 and 7 have been shown to be expressed by follicular cells in several species such as mice, rats, chickens and cows (Shimasaki *et al.* 1999, Erickson & Shimasaki 2003, Onagbesan *et al.* 2003, Glister *et al.* 2004) and furthermore, to regulate follicular cell function *in vitro* (Shimasaki *et al.* 1999, Dooley *et al.* 2000, Lee *et al.* 2001, Mulsant *et al.* 2001, Otsuka *et al.* 2001, Souza *et al.* 2002, Fabre *et al.* 2003,

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Onagbesan *et al.* 2003, Nilsson & Skinner 2003, Glister *et al.* 2004, 2005, Lee *et al.* 2004, Pierre *et al.* 2004). While it is known that follicular cells in sheep express receptors capable of responding to these BMPs (Wilson *et al.* 2001, Souza *et al.* 2002) and that BMP2 and 4 can regulate granulosa cell function (Mulsant *et al.* 2001, Souza *et al.* 2002, Fabre *et al.* 2003, Pierre *et al.* 2004) the ovarian cell-types that express BMP2, 4, 6 and 7, as well as the effects of BMP6 and BMP7 on granulosa cell function are presently unknown in sheep. Given the importance of BMP15 and GDF9 in regulation of follicular growth and ovulation rate as well as the known effect of mutations in the BMPRIB gene on ovarian activity in sheep (Shimasaki *et al.* 2004, McNatty *et al.* 2005*a*), the characterization of the ovarian cell-types that produce other BMP family members as well as the effects of these proteins on granulosa cell function is central to further elucidation of their roles in regulating ovarian activity. The objectives of this study were to determine the patterns of expression of BMP2, 4, 6 and 7 in the ovine ovary and to determine the effects of these ligands on ovine granulosa cell proliferation and progesterone production. In addition, we determined the role of these BMPs in rat granulosa cells under identical culture conditions to be able to compare more directly the effects of BMPs on granulosa cells of sheep and the more extensively studied species, the rat.

Materials and methods

Collection of tissue samples

All experiments were performed in accordance with the 1999 Animal Welfare Act Regulations of New Zealand. All animals had access to pasture and water, and were allowed to feed *ad libitum*. Lambs were kept with their mothers until just prior to tissue collection. Romney ewes and lambs were killed by administration of a barbiturate overdose (pentobarbitone; 200 mg/kg) or by captive bolt.

Cloning of BMP2, 4, 6 and 7 and in situ hybridization

Except where indicated, laboratory reagents were obtained from BDH Chemicals New Zealand Ltd (Palmerston North, New Zealand), Invitrogen (Auckland, New Zealand) or Roche Diagnostics N.Z. Ltd (Auckland, New Zealand). Complimentary cDNAs encoding a portion of ovine BMP2, 4, 6 and 7 were generated using standard reverse transcription(RT)-PCR techniques. Primers and conditions for PCR are listed in Table 1. Sequences of resulting plasmids were confirmed prior to use for *in situ* hybridization. *In situ* hybridization was performed as previously described (Tisdall *et al.* 1999) with minor modifications. Briefly, 4–6 μm tissue sections were incubated overnight at 50–55 °C with 45 000 c.p.m./μl (approximately 48 000 d.p.m./μl) of ^{33}P-labelled antisense RNA. Non-specific hybridization of RNA was removed by RNase A digestion followed by stringent washes (2 × SSC, 50% formamide, 65 °C and 0.2 × SSC at 37 °C). Following washing, sections were dehydrated, air dried and coated with autoradiographic emulsion (LM-1 emulsion; Amersham Pharmacia Biotech New Zealand). Emulsion-coated slides were exposed at 4 °C for 3–4 weeks, developed for 3.5 min in D19 developer (Eastman Kodak, Rochester, NY, USA). Development was stopped using a 1 min incubation in 1% acetic acid and slides were fixed with a 10 min incubation in Ilfofix II (Ilford Limited, Cheshire, England). Sections were stained with hematoxylin and then viewed and photographed using both light and dark field illumination on an Olympus BX-50 microscope (Olympus New Zealand Ltd). At least 8 animals were examined for expression of each of the BMP genes. These included both lambs and adult ewes and no differences were noted in the pattern of gene expression related to the age of the animal. In addition, as the expression of BMP4 and 7 was not commonly observed in the ovarian sections, a positive control tissue of fetal kidney ($n = 2$) was included with each *in situ* hydridization. This tissue had been collected from female ovine fetuses collected on day 40 of gestation, which were part of another approved study. Follicles at each defined stage of development were observed in at least 3 animals for all genes examined. Classification of follicles at each developmental stage was based on the system outlined (Lundy *et al.* 1999). Briefly, type 1/1a follicles consist of an oocyte surrounded by a single layer of flattened or mixed flattened and cuboidal cells. Type 2 follicles contain $1 < 2$ layers of cuboidal granulosa cells, whereas type 3 follicles contain $2 < 4$ layers of cuboidal granulosa cells. Type 4 follicles have > 4 layers of granulosa cells and a well defined theca, but have not yet formed an antrum. Type 5 follicles have multiple layers of granulosa cells, a well defined theca and a defined antrum. All follicles with signs

Table 1 Primers used for PCR, resulting product size, annealing temperature used in PCR reaction and the reference number of the resulting sequence for the ovine bone morphogenetic proteins (BMP) 2, 4, 6 and 7.

Gene	Forward Primer (5′–3′)	Reverse Primer (5′–3′)	Product size (bp)	Annealing temperature (°C)	Genbank number (Resulting sequence)
BMP2	gagaaaccatggaagcaaaacacaaacagcgg (includes Nco I site)	gtgagatctacacccacaaccctcgacaac (includes BglII site)	353	58	DQ192012
BMP4	catcacacgactactggac	cctctactacgatctcctg	627	50	DQ192013
BMP6	cagcgcctcagactactacaac	caaccacacgctcgtacg	342	58	DQ192014
BMP7	ctccagggcaagcacaac	gttgatgctctgtccgtcc	561	50	DQ192015

of degeneration (i.e. pyknotic granulosa cells, lack of a distinct basement membrane or degenerate oocytes) were considered atretic. Non-specific hybridization was monitored by hybridizing the sense RNA for each receptor to tissue collected from at least one animal per age group. Hybridization was considered specific when the intensity of silver grains, as measured by visual assessment, over a cellular type was greater than that observed in the area of the slide not containing tissue. For all genes, hybridization of the sense RNA over the tissue section was similar or lower in intensity to that observed on the areas of the slide not containing tissue of both the sense and antisense hybridized slides and thus was considered non-specific.

Determination of expression of BMP mRNAs in freshly isolated granulosa cells

Granulosa cells were isolated from follicles 1–2 mm in diameter from ovine ovaries collected from the local abattoir. Follicles lacking vascularity or with debris in their follicular fluid were considered atretic and discarded. Granulosa cells were only used from follicles in which the oocyte was localized and removed. Three pools of granulosa cells were generated. All cells from each follicle and all follicles from each ovary were placed in a single pool of cells. RNA was collected using TRIzol according to the manufacturer's instructions. First strand cDNA was produced from DNase treated total cellular RNA using the SuperScript preamplification system for first strand cDNA synthesis. Effeciency of cDNA synthesis was analyzed using primers specific for follistatin, which is highly expressed in ovine ovarian tissue and granulosa cells (Tisdall *et al.* 1994). These primers (CTGGAAATTGCTGGCTCC and AGTCCTGGTCTTCATCTTC) are located in two separate exons of the follistatin gene. Expression of the different BMPs were determined by PCR using the following ovine primers (BMP2 CAGAACTTCAGGTCTTCGG and GCACTGAGCTCTGTTGGG; BMP4 TAACCGAATGCTGATGGTCG and CCTTGTCATACTCATCCAGG; BMP6 AGCGAGCTGAAGACGGCC and ATGTTCCTATACTTCTTCAGG; BMP7 CCTATCCCTACAAGGCCG and CTGTCGAGCAGGAACAGG). All PCRs were carried out using HotMaster Taq (Eppendorf) with the following conditions: initial denaturing cycle of 2 min at 94 °C followed by 40 cycles of denaturing at 94 °C for 20 s, annealing at 58 °C for 15 s and extension at 72 °C for 50 s and a final extension at 72 °C for 10 min. cDNA generated from a 4 week old lamb ovary, which contains many healthy preantral and antral follicles, was run as a positive control whereas replacement of cDNA with water was used as a negative control. Expression of BMP mRNAs was assessed by visualization of DNA bands of the correct size following gel electrophoresis. Identity of product (from positive control sample) was confirmed by sequencing.

Granulosa cell culture

Collection of granulosa cells

Granulosa cells were isolated from 1–2 mm follicles from ovine ovaries collected from the local abattoir and from Sprague–Dawley rats (23–26 day old; University of Otago, Dunedin, New Zealand). Granulosa cells were also collected from sheep and rats approximately 96 h after subcutaneous administration of implants containing diethylstilbestrol (DES). Rats received a single implant of approximately 1.8 cm with an internal diameter of 3.35 mm (Chen *et al.* 2001). Ewes received 2 implants based on previous results with implants containing oestradiol (McNatty *et al.* 1989). Oocytes and follicular debris were removed from the cells using a micro-glass pipette. Remaining cells were collected by centrifugation at 300 ***g*** for 5 min at room temperature, washed once in 5 ml Leibovitz media, twice in 5 ml McCoys media (Sigma, Auckland, New Zealand) and resuspended using a syringe and needle. Cell viability was determined using trypan blue exclusion, 100 000 viable cells per well (250 μl total volume) were added in McCoys media containing 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM GlutaMAX-1 and 0.1% BSA. Media for cells used for progesterone and DNA determination also contained 5 ng/ml selenium (Sigma), 10 ng/ml insulin (Sigma), 5 μg/ml apotransferrin (Sigma), 30 ng/ml androstenedione (Sigma), 3 ng/ml ovine FSH (purified in our laboratory; 1.4 X USDA-oFSH-19-SIAFP RP2), and 1 ng/ml IGF-1 (Long-R3, GroPep, Adelaide, SA, Australia).

Determination of ^{3}H-thymidine incorporation

Cells (100 000 viable cells per well) were incubated in media described above with the addition of 6-^{3}H-thymidine (Perkin Elmer, Boston, MA, 20 Ci/mmol, 1.0 μCi per well) with or without human (h) BMP2, hBMP4, hBMP6 or hBMP7 (3 μg/ml; R&D Systems Inc, MN USA) for 48 h at 37 °C in a 5% CO_2 incubator. This standardized dose of BMP added to the cultures was chosen after analysis of dose-response assays with both sheep and rat granulosa cells (see Fig. 1 for representative data from sheep). At the termination of culture, cells were harvested with a cell harvester onto a thick filter mat. Incorporation of ^{3}H-thymidine was determined using a Wallac Trilux MicroBeta 1450 liquid scintillation counter (Biolab, Auckland, New Zealand). Average values for incorporation of ^{3}H-thymidine were determined for each treatment, outlier replicates (outside of 30% of the mean) were identified and discarded.

Determination of progesterone and DNA content

Cells were cultured at 37 °C in a 5% CO_2 incubator in media specified above with or without 300 ng/ml of hBMP2, hBMP4, hBMP6, or hBMP7 for 6 days. The BMP dose of 300 ng/ml was also chosen for these studies following analysis of preliminary dose-response studies

(see Fig. 1 for representative data from sheep). Every 48 h, 200 μl of media was removed from each well and replaced with 200 μl of warmed media that had been prepared at the start of the culture and stored at 4 °C. Media samples from the first (rat only) and last 48 h of treatment were collected on the appropriate day of treatment and frozen at −20 °C for later determination of progesterone concentrations by RIA. Unattached cells were removed by 2 washes with McCoys media at 37 °C. Attached cells were lysed by incubating cells at 37 °C in 100 μl distilled water for 1 − 2 h followed by freezing at −70 °C.

Measurement of progesterone

Concentrations of progesterone in media were determined by RIA as described (Lun *et al.* 1998). The sensitivity of the assay (90% maximum binding) was 16 pg/ml and the intra- and interassay co-efficients of variation (CV), averaged for a standard pool sample at approximately 20, 50 and 80% binding, was 11 and 16%, respectively. No samples were below the sensitivity of the assay. Average values for progesterone concentrations were determined for each treatment within each independent granulosa cell bioassay, outlier replicates (outside of 20% of the mean) were identified and discarded.

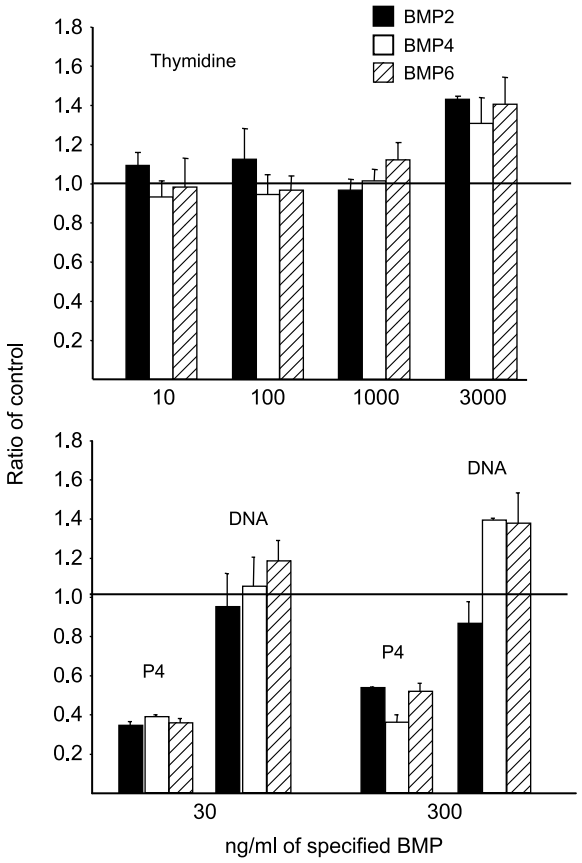

Figure 1 Effects of varying doses of bone morphogenetic protein (BMP) 2, 4 and 6 on thymidine uptake (top panel) and progesterone and DNA concentrations (bottom panel). Granulosa cells were collected from sheep not treated with DES. Values (mean+S.E.M. of replicate wells of a single pool of granulosa cells) are expressed as a ratio of the control value, which is indicated with the horizontal line at 1.0.

Measurement of DNA

The amount of DNA present in each well was determined by comparing binding of Hoechst 33258 dye (Sigma, final concentration of 10 μg/ml in well) in samples to calf thymus DNA standard measured with a Wallac 1420 plate reader at 350 nm for excitation and 460 nm for emission. Sensitivity of the assay (+ two S.D. of control buffer value) was 20 ng per well and the intra- and interassay CV, based on variability of the 100, 250, 1000 and 2500 ng standard curve points were 6 and 6%, respectively. No samples were below the sensitivity of the assay.

Statistical analysis

Amount of progesterone produced per μg DNA was calculated individually for each well. Replicates that were not within 30% of the mean were discarded. For each variable, effects of specified treatment were determined by comparison to the untreated control sample using the 2-tailed paired *t*-test function in Microsoft Excel 2003. As the BMPs were not always run in the same assay, no statistical comparisons were made between the effects of the various BMPs. For ease of presentation, all values have been converted to a ratio of the appropriate controls which were assigned a value of 1.00.

Results

Expression of BMP2, 4, 6 and 7 as assessed by in situ hybridization

BMP2

Expression of mRNA encoding BMP2 was not observed in the granulosa cells, thecal cells, cumulus cells or oocytes of non-atretic follicles of any size class (Fig. 2). Atretic type 5 follicles expressed BMP2 mRNA in the degenerating granulosa cells and this was most pronounced in follicles in the late stages of atresia (Fig. 2).

BMP4

BMP4 mRNA was not evident in granulosa cell, theca interna, cumulus cells or oocytes of non-atretic follicles of any size examined (Fig. 3). Expression of BMP4 mRNA was often observed in the surface epithelium and around blood vessels. In addition, expression was observed in a concentric circle around some antral follicles. This expression did not appear to be in the theca interna or externa of these follicles although the boundary between the theca externa and stromal cells of the ovary can be difficult to discern. Often, these follicles were atretic. The positive controls (i.e. sections of kidney tissue collected on day 40 of fetal life) expressed mRNA encoding BMP4 (Fig. 3).

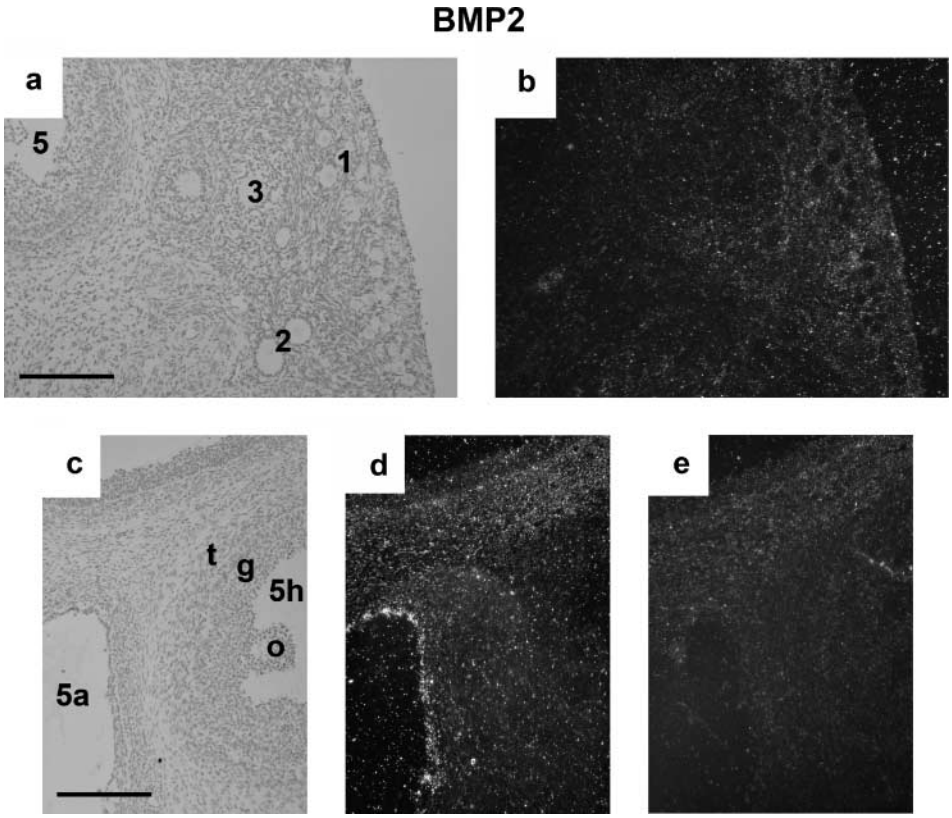

Figure 2 Corresponding bright field (a, c) and dark field (b, d, e) views of ovaries collected from 4 week old lambs following hybridization to BMP2 antisense (a–d) or sense (e) RNA. (a, b) No specific signal is observed in the healthy type 1, 2, 3 or 5 follicles. (c, d) Signal can be observed in the atretic type 5 follicle (5a) but is not observed in the healthy type 5 follicles (5 h). (e) No specific signal was observed when the sense RNA was hybridized to the follicles shown in panels c and d. Scale bar equals approximately 200 μm for all panels. o, oocyte; g, granulosa; t, theca.

BMP6

Oocytes of all sizes of follicles that were examined expressed mRNA encoding BMP6 (Fig. 4). BMP6 mRNA was not evident in either the granulosa or thecal cells of follicles of any size (Fig. 4).

BMP7

Expression of BMP7 mRNA was not observed in the granulosa cells, thecal cells, cumulus cells or oocytes of healthy follicles of any sized examined (Fig. 5). When rete tubules were present on the slide ($n = 3$), positive expression of BMP7 mRNA was noted. The positive controls (i.e. sections of kidney tissue collected on day 40 of fetal life) expressed mRNA encoding BMP7 (Fig. 5).

Expression of BMPs in isolated granulosa cells as assessed by RT-PCR

Expression of BMP2, 4, 6 or 7 was observed in at least some pools of granulosa cells (Fig. 6). However, in all cases, the strength of the signal was weak compared with the positive control sample.

Effects of BMPs on granulosa cell cultures

Sheep

Without DES

Overall, the effects of BMP2, 4, 6 and 7 were similar. None of these BMPs affected cellular proliferation (Fig. 7) as assessed either over the first 48 h of culture by thymidine incorporation or during the longer term (6 days) through direct measurement of DNA. In contrast, to the lack of effect observed on cellular proliferation, concentrations of progesterone in media were decreased when compared with control cultures following treatment of granulosa cells with either BMP2, 4, 6 or 7. When the effects of the BMPs were assessed on a per cell basis, concentrations of progesterone/ng DNA were decreased in granulosa cells treated with BMP4, 6 or 7 when compared with control cultures (Fig. 8).

With DES

Similar to the effects observed on granulosa cells that had not been exposed to DES, none of the BMPs affected proliferation of DES treated ovine granulosa cells (Fig. 7).

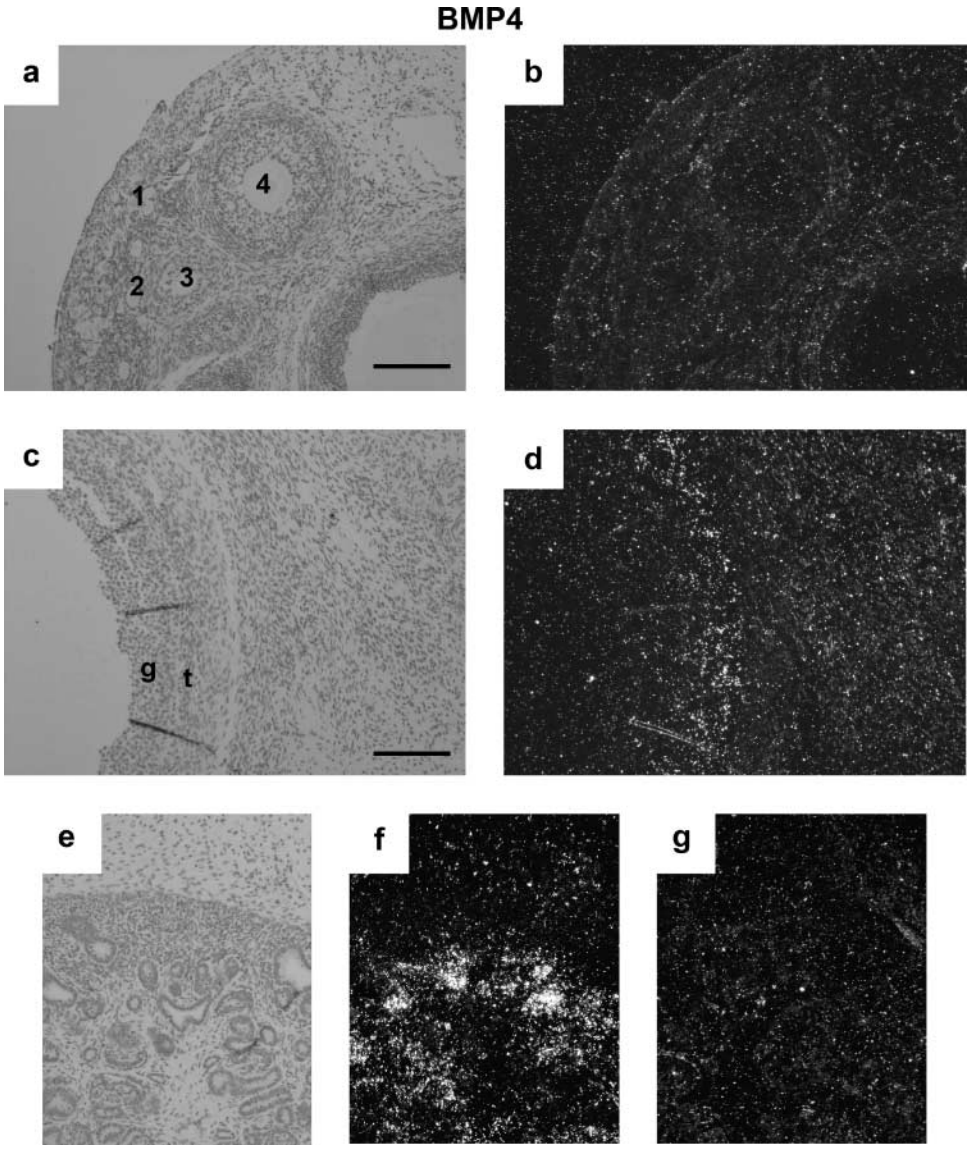

Figure 3 Corresponding bright field (a, c, e) and dark field (b, d, f, g) views of ovaries collected from 4 week old lambs (a, b), adult ewes (c, d) or kidney collected on day 40 of gestation (e–g) following hybridization to BMP4 antisense (a–f) or sense (g) RNA. (a, b) No specific signal is observed in the healthy type 1, 2, 3 or 4 follicles. (c, d) A healthy type 5 follicle with signal observed in stroma cells just outside the theca layer of the follicle (g, granulosa; t, theca). (e, f) Positive expression is also observed in the tubules of the developing kidney. (g) No specific signal was observed when the sense RNA was hybridized to the kidney shown in panels e and f. Scale bar equals approximately 200 μm for all panels.

However, the concentrations of progesterone in media were decreased by all BMPs and a similar trend ($P < 0.10$) was observed when progesterone was expressed on a per cell basis (Fig. 8).

Rat

Without DES

Treatment of rat granulosa cells with either BMP4, 6 or 7, but not BMP2, resulted in an increased thymidine uptake over the first 48 h of culture (Fig. 9). The amount of DNA at the end of culture was not significantly higher than observed for control cultures, although the absolute values were increased between 40–60% (Fig. 9). All BMPs reduced progesterone concentrations after 2 days in culture and progesterone produced per cell at 6 days in culture (Fig. 10).

With DES

Treatment of DES-exposed rat granulosa cells with BMP4 resulted in an increased thymidine uptake over the first 48 h of culture and BMP4, 6 and 7 all increased DNA concentrations following 6 days of culture (Fig. 9). BMP2 had

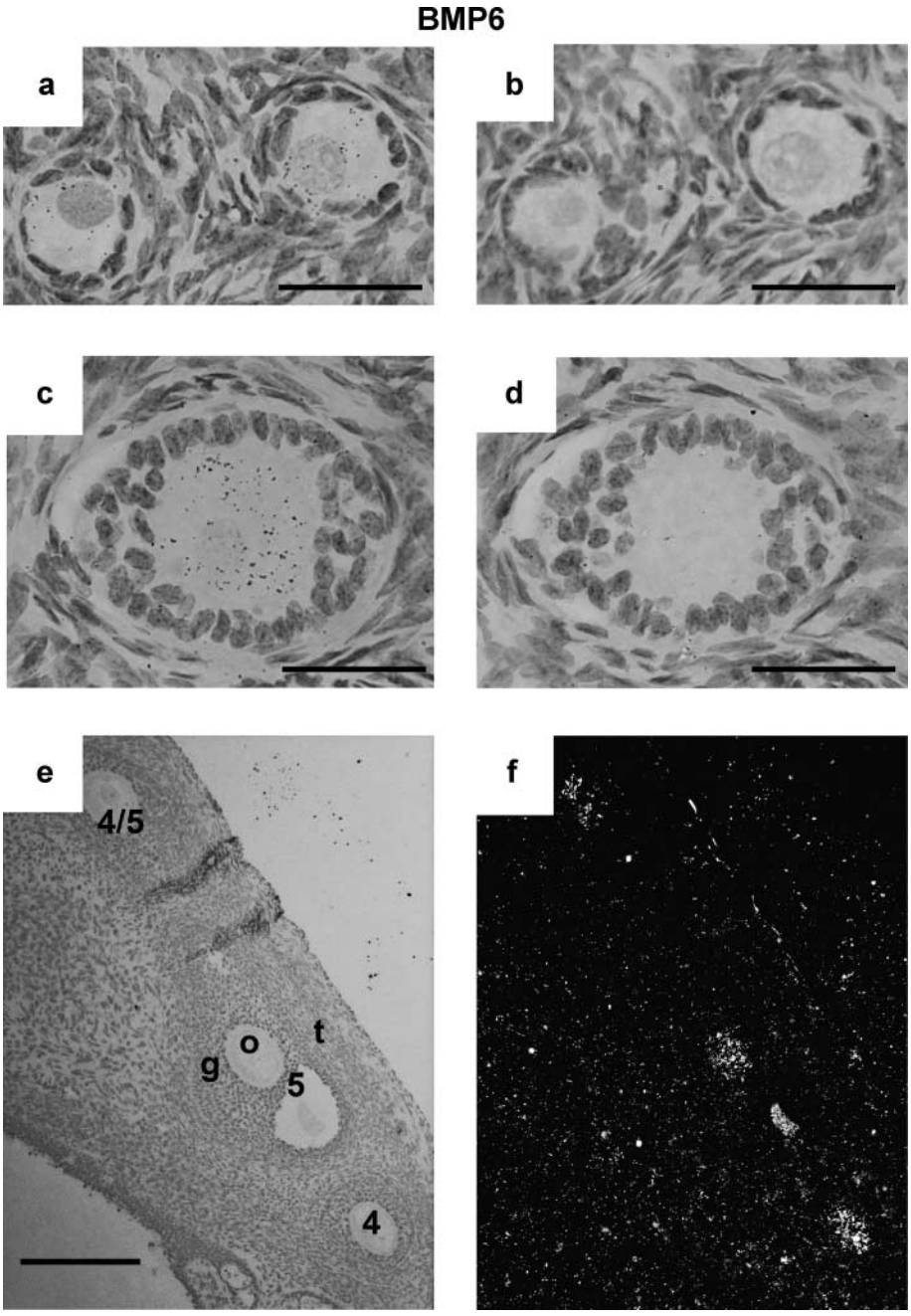

Figure 4 Corresponding bright field (a–e) and dark field (f) views of ovaries collected from an adult ewe (a–d) or 4 week-old lamb (e–f) following hybridization to BMP6 antisense (a, c, e, f) or sense (b, d) RNA. (a, c) Silver grains can be observed specifically in the oocyte of the type 1/1a (a) and type 2 (c) follicles. (b, d) No silver grains are observed over the type 1/1a or type 2 follicles in the negative control sense hybridized slides. (e, f) Healthy type 4 and 5 follicles with signal observed in oocytes (o) of the follicles but not in granulosa (g) or theca (t) of the follicles. Scale bar equals approximately 50 μm for panels a–d and 200 μm for panels e and f.

no effect on thymidine uptake or DNA concentrations. Concentrations of progesterone in media were decreased in all BMP treated cultures when compared with control cultures following 2 days of culture but only BMP6 showed a decreased concentration of progesterone compared with controls at 6 days of culture (Fig. 10). However, all BMPs tested decreased progesterone synthesis at 6 days of culture when the concentrations of progesterone were expressed relative to DNA concentration (Fig. 10).

Discussion

As judged by *in situ* hybridization, BMP6, but not BMP2, 4 or 7 mRNA was expressed in non-atretic growing

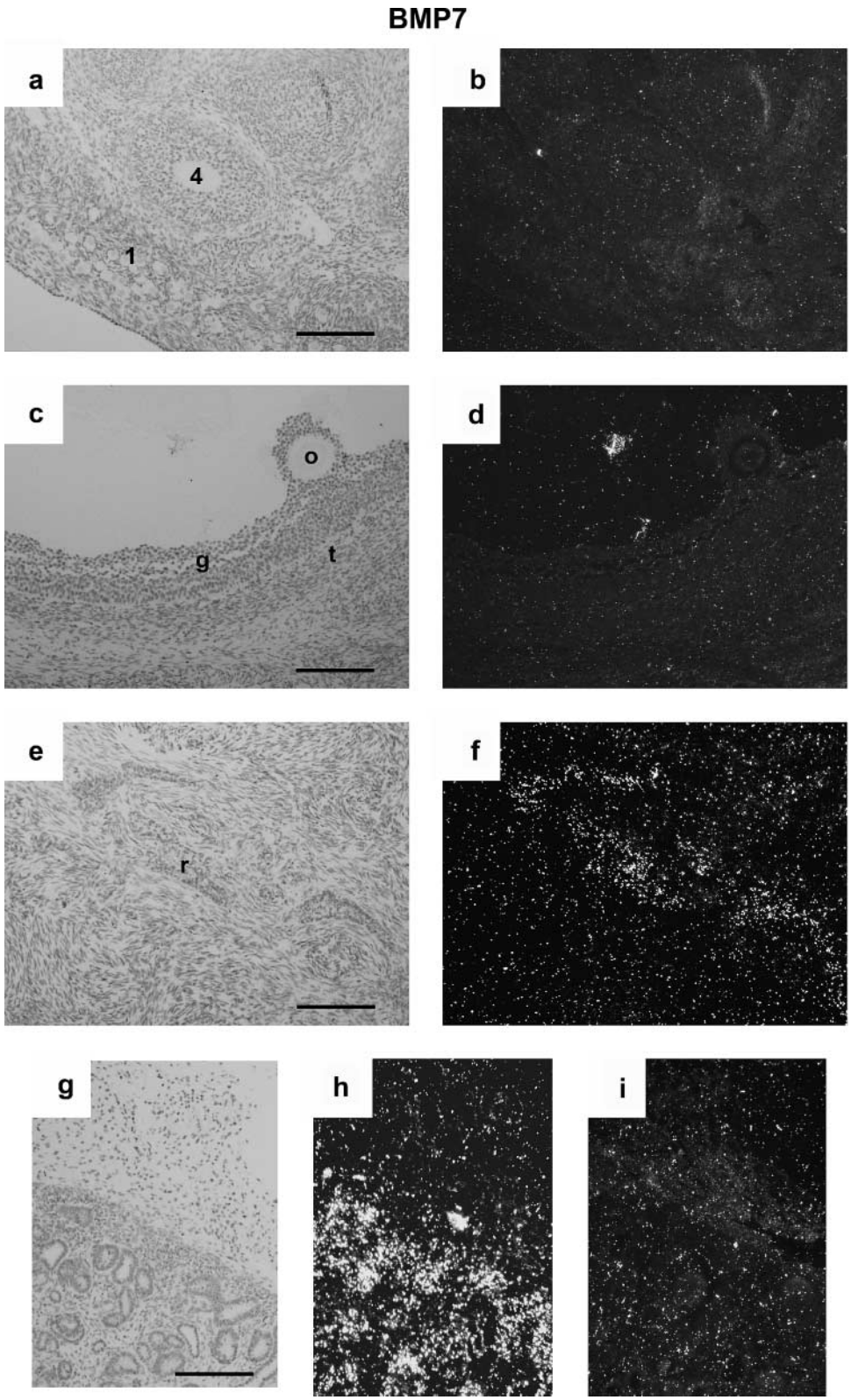

Figure 5 Corresponding bright field (a, c, e, g) and dark field (b, d, f, h, i) views of ovaries collected from 4 week old lambs (a, b), adult ewes (c–f) or kidney collected on day 40 of gestation (g–i) following hybridization to BMP7 antisense (a–h) or sense (i) RNA. (a, b) No specific signal is observed in the healthy type 1 or 4 follicles. (c, d) A healthy type 5 follicle with no signal observed in the oocyte (o), granulosa (g) or theca (t). (e, f) Positive expression is observed in the rete (r). (g, h) Positive expression is also observed in the tubules and collecting ducts of the developing kidney. (i) No specific signal was observed when the sense RNA was hybridized to the kidney shown in panels g and h. Scale bar equals approximately 200 μm for all panels.

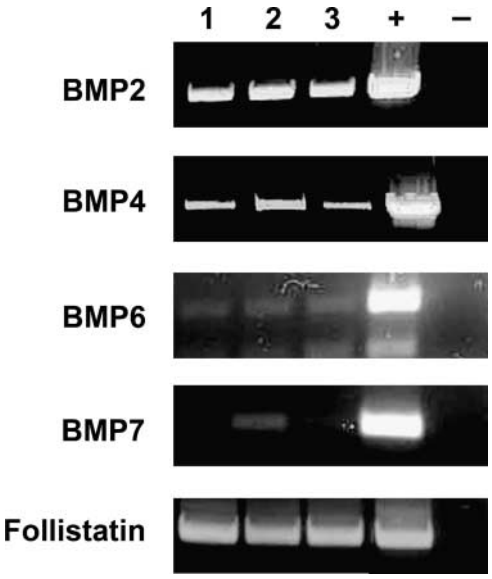

Figure 6 Determination of expression mRNA encoding BMP2, -4, 6 and 7 as assessed by RT-PCR in granulosa cells isolated from non-DES-treated sheep. Lanes 1-3, 3 separate pools of granulosa cells, + positive control sample, − negative control water blank. Identity of BMP product is indicated on the left. Expression of follistatin is indicated at the bottom as a positive control for cDNA synthesis.

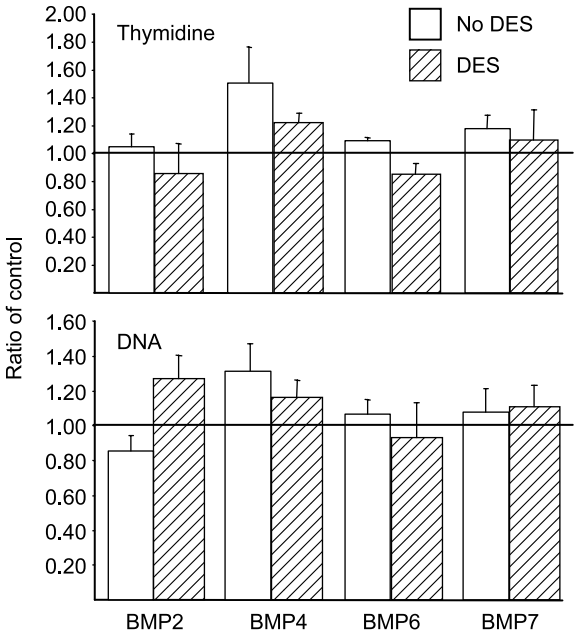

Figure 7 Effects of BMP 2, 4, 6 and 7 on thymidine uptake (top panel) and DNA concentrations (bottom panel). Granulosa cells were collected from sheep not treated with DES (open bar) or sheep treated with DES (striped bar). Values (mean+S.E.M., $n=3-4$ independent pools of granulosa cells) are expressed as a ratio of the control value, which is indicated with the horizontal line at 1.0. None of the treatments had an effect on either thymidine uptake or DNA concentrations ($P > 0.05$).

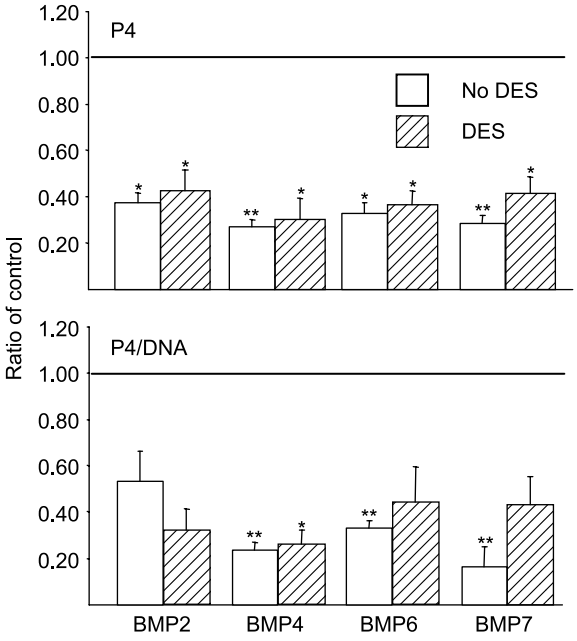

Figure 8 Effects of BMP 2, 4, 6 and 7 on progesterone concentrations in media (top panel) and progesterone concentrations corrected for DNA concentrations (bottom panel). Granulosa cells were collected from sheep not treated with DES (open bar) or sheep treated with DES (striped bar). Values (mean+S.E.M., $n=3-4$ independent pools of granulosa cells) are expressed as a ratio of the control value, which is indicated with the horizontal line at 1.0. $^{*}P < 0.05$, $^{**}P < 0.01$ when compared with control values.

follicles. Furthermore, BMP6 mRNA was evident only in the oocyte and was expressed in primordial as well as primary, pre-antral and antral follicles. Using RT-PCR, message for all BMPs were detected in at least some pools of granulosa cells. The detection of these mRNAs by RT-PCR but not by *in situ* hybridization may be related to the different sensitivities of the methods or to changes induced in the granulosa cells by the isolation procedure. It is also important to note that while follicles used for isolation of granulosa cells were assessed to be non-atretic, it is likely that follicles in the early stages of atresia were not identified. This is particularly relevant for expression of BMP2 as this gene was shown to be expressed in granulosa cells of atretic follicles by *in situ* hybridization.

The expression patterns for BMP2, 4 and 7 were more restricted in sheep follicles when compared with other species that have been examined to date. For instance, while expression of BMP2 mRNA was not observed in granulosa cells, theca cells, cumulus cells or oocytes of healthy follicles and was restricted to atretic follicles in the sheep ovary *in situ*, both the granulosa and thecal cells of non-atretic rat follicles as well as atretic follices expressed BMP2 as detected by *in situ* hybridization (Erickson & Shimasaki 2003). In chickens, expression of BMP2 mRNA was detected in both the theca and granulosa of non-atretic antral follicles using RT-PCR (Onagbesan *et al.* 2003) while

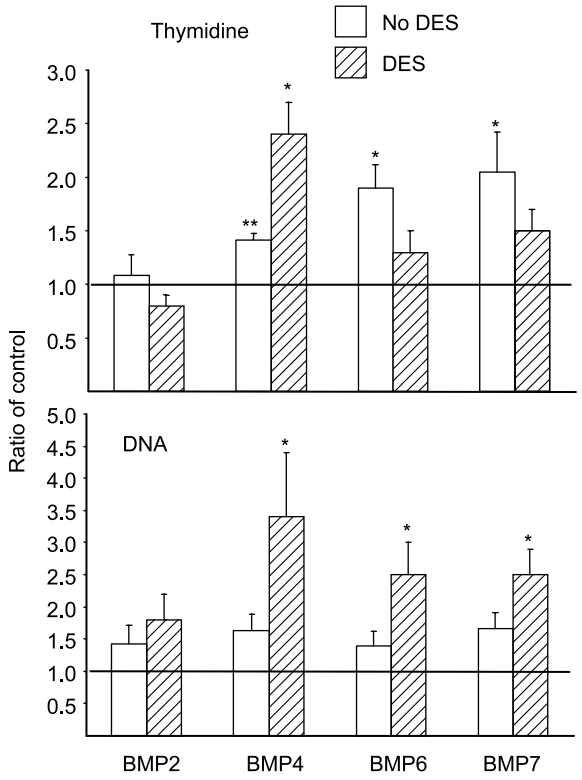

Figure 9 Effects of BMP2, 4, 6 and 7 on thymidine uptake (top panel) and DNA concentrations (bottom panel). Granulosa cells were collected from rats not treated with DES (open bar) or rats treated with DES (striped bar). Values (mean+S.E.M., $n>3$ independent pools of granulosa cells per treatment) are expressed as a ratio of the control value, which is indicated with the horizontal line at 1.0. $*P < 0.05$, $**P < 0.01$ when compared with control values.

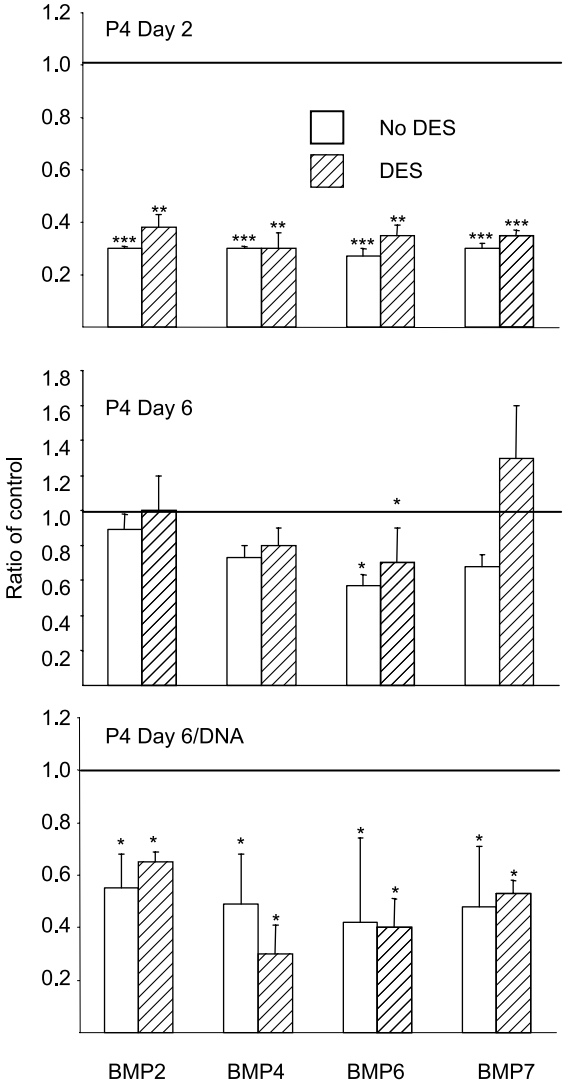

Figure 10 Effects of bone morphogenetic protein (BMP) 2, 4, 6 and 7 on concentrations of progesterone (P4) after 2 days of culture (top panel), P4 after 6 days of culture (middle panel) and P4 after 6 days of culture corrected for DNA concentration (bottom panel). Granulosa cells were collected from rats not treated with DES (open bar) or rats treated with DES (striped bar). Values (mean+S.E.M., $n>3$ independent pools of granulosa cells per treatment) are expressed as a ratio of the control value, which is indicated with the horizontal line at 1.0. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ when compared with control values.

in cattle BMP2 protein was predominately observed in thecal cells of antral follicles with some oocytes also staining positive using immunocytochemistry (Fatehi *et al.* 2005). In the present study, BMP4 and 7 mRNA was not observed in granulosa cells, theca cells, cumulus cells or oocytes of non-atretic ovine follicles *in situ*. In contrast, both BMP4 and 7 mRNA was detected in rat thecal cells using *in situ* hybridization (Erickson & Shimasaki 2003) and in both granulosa and thecal cells in chickens as detected by RT-PCR (Onagbesan *et al.* 2003). Moreover, both BMP4 and 7 immunoreactive proteins were detected in bovine thecal cells by immunocytochemistry (Glister *et al.* 2004, Fatehi *et al.* 2005). In the present study, BMP6 mRNA was observed only in oocytes of sheep follicles and was not observed in granulosa cells, theca cells or cumulus cells as determined by *in situ* hybridization, whereas in cows (Glister *et al.* 2004) and rats (Erickson & Shimasaki 2003) BMP6 was detected in both the oocytes and granulosa cells.

In some instances, these findings may represent true species differences. However, there is also a possibility that the differences are due to different methodologies. One key difference between the present study and that of Glister *et al.* (2004) is that the bovine granulosa and thecal cells had been cultured for 6 days before examination compared with *in situ* examination of ovine granulosa and theca cells at tissue recovery. Given the low level of expression detected by RT-PCR for these genes in isolated granulosa cells, it is possible that the culture conditions may have induced expression of the BMPs in bovine granulosa and theca cells.

In ovine granulosa cells, neither BMP2, 4, 6 nor 7 stimulate granulosa cell proliferation/survival. In contrast, while some differences were noted among the rat assays according to animal treatment and end measurement overall,

BMP4, 6 and 7 all stimulated granulosa cell proliferation/survival in rat granulosa cells and the effects of BMP2 on DNA content at the end of culture approached significance for the DES treated rat cells ($P < 0.06$). Previously, BMP4, 6 and 7 have been shown to increase cell numbers in cultures of bovine granulosa cells (Glister *et al.* 2004) and BMP 7 stimulated thymidine uptake and numbers of cultured granulosa cells in rats (Lee *et al.* 2001) and chickens (Onagbesan *et al.* 2003) whereas BMP4 or 6 had no effect on cell numbers in the chicken (Onagbesan *et al.* 2003) or thymidine incorporation of rat granulosa cells (Otsuka *et al.* 2001), respectively. The reasons for the differences between these findings, including the differences between the DES-treated and non-treated rats in the present study, may relate to differences in methodologies. Multiple factors, including the stage of maturation of the granulosa cells, differences in media composition such as addition of factors such as FSH and IGF-1, or differences in length of the culture period may influence the responsiveness to BMPs. In addition, determination of cell numbers/DNA content will take into account both cell mitosis and cell death, whereas measuring thymidine uptake only measures the intent for cell division. However, it is important to note that in the present study, rat and ovine granulosa cells were cultured under identical conditions suggesting that true differences between species in their responses to BMPs exist. Interestingly, the effects of BMP15 and GDF9, other TGFβ superfamily members, have also been shown to differ subtly between even the closely related species sheep and cow when tested under identical conditions in the same laboratory suggesting the roles of the BMPs may vary even between closely related species (McNatty *et al.* 2005*b*).

BMP2, 4, 6 and 7 all suppressed progesterone production. This is in agreement with the suppressive effects of the BMPs in a number of mammalian species with respect to progesterone production by granulosa cells (Shimasaki *et al.* 1999, Otsuka *et al.* 2001, Fabre *et al.* 2003, Glister *et al.* 2004, Pierre *et al.* 2004). In chickens, BMP4 and 7 stimulate progesterone production by granulosa cells but this difference may be related to the differing roles of progesterone in regulation of gonadotrophins between mammalian and avian species (Onagbesan *et al.* 2003).

Sheep with a mutation in activin receptor-like kinase 6 (Alk6, also known as BMPRIB) have increased ovulation rates (Mulsant *et al.* 2001, Souza *et al.* 2001, Wilson *et al.* 2001) and the Alk6 receptor is found in both oocytes and granulosa cells (Wilson *et al.* 2001, Souza *et al.* 2002). The mechanisms by which this mutation results in increased ovulation rate, including the preferred ligand for this receptor in sheep is not known. Based on the expression patterns of BMP2, 4 and 7, these proteins do not appear to be strong candidates as the preferred physiological ligands for Alk6 in sheep granulosa cells. However, BMP6, which has been shown to be able to signal through Alk6 (Juengel & McNatty 2005), might be one of the preferred ligands regulating follicular maturation and ovulation rate through this receptor (Shimasaki *et al.* 2004). However, it is important to note that BMP15 also has been shown to act through Alk6 (Moore *et al.* 2003). Futhermore, the BMP15 and Alk6 mutations in sheep have been shown to have synergistic effects on ovulation rate (Davis *et al.* 1999). Since mice lacking an active BMP6 gene show no apparent fertility phenotype (Solloway *et al.* 1998), the role that BMP6 plays in regulating ovarian follicular development and ovulation rate in sheep needs further clarification.

In conclusion, several members of the BMP subfamily of the TGFβ superfamily, namely BMP2, 4, 6 and 7 were shown to have similar actions in regulating ovine granulosa cells. Specifically, these proteins suppressed concentrations of progesterone secreted into the media without affecting cell number. This is consistent with a luteinization inhibition factor. However, based on their localization patterns in developing ovarian follicles, BMP6 would be the most likely candidate to play a physiological role in the local regulation of follicular growth in sheep, as mRNA encoding BMP2, 4 and 7 could not be detected by *in situ* hybridization in non-atretic follicles.

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