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Growth Differentiation Factor 9 and Bone Morphogenetic Protein 15 Are Essential for Ovarian Follicular Development in Sheep[1]

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ABSTRACT

The aim of this study was to test the hypothesis that both growth differential factor 9 (GDF9) and bone morphogenetic protein (BMP15; also known as GDF9B) are essential for normal ovarian follicular development in mammals with a low ovulation rate phenotype. Sheep (9–10 per group) were immunized with keyhole limpet hemocyanin (KLH; control), a GDF9-specific peptide conjugated to KLH (GDF9 peptide), a BMP15-specific peptide conjugated to KLH (BMP15 peptide), or the mature region of oBMP15 conjugated to KLH (oBMP15 mature protein) for a period of 7 mo and the effects of these treatments on various ovarian parameters such as ovarian follicular development, ovulation rate, and plasma progesterone concentrations evaluated. Also in the present study, we examined, by immunohistochemistry, the cellular localizations of GDF9 and BMP15 proteins in the ovaries of lambs. Both GDF9 and BMP15 proteins were localized specifically within ovarian follicles to the oocyte, thereby establishing for the sheep that the oocyte is the only intraovarian source of these growth factors. Immunization with either GDF9 peptide or BMP15 peptide caused anovulation in 7 of 10 and 9 of 10 ewes, respectively, when assessed at ovarian collection. Most ewes (7 of 10) immunized with oBMP15 mature protein had a least one observable estrus during the experimental period, and ovulation rate at this estrus was higher in these ewes compared with those immunized with KLH alone. In both the KLH-GDF9 peptide- and KLH-BMP15 peptide-treated ewes, histological examination of the ovaries at recovery (i.e., ~7 mo after the primary immunization) showed that most animals had few, if any, normal follicles beyond the primary (i.e., type 2) stage of development. In addition, abnormalities such as enlarged oocytes surrounded by a single layer of flattened and/or cuboidal granulosa cells or oocyte-free nodules of granulosa cells were often observed, especially in the anovulatory ewes. Passive immunization of ewes, each given 100 ml of a pool of plasma from the GDF9 peptide- or BMP15 peptide-immunized ewes at 4 days before induction of luteal regression also disrupted ovarian function. The ewes given the plasma against the GDF9 peptide formed 1–2 corpora lutea but 3 of 5 animals did not display normal luteal phase patterns of progesterone concentrations. The effect of plasma against the BMP15 peptide was more dramatic, with 4 of 5 animals failing to ovulate and 3 of 5 ewes lacking surface-visible antral follicles at laparoscopy. By contrast, administration of plasma against KLH did not affect ovulation rate or luteal function in any animal. In conclusion, these findings support the hypothesis that, in mammals with a low ovulation rate phenotype, both oocyte-derived GDF9 and BMP15 proteins are essential for normal follicular development, including both the early and later stages of growth.

follicular development, granulosa cells, growth factors, oocyte development, ovulation

INTRODUCTION

Ovarian follicular growth is a process involving a complex exchange of hormonal signals between the pituitary gland and the ovary and by a localized exchange of growth factors and hormones within ovarian follicles including the oocyte and its adjacent somatic cells. In recent years, evidence has been provided to show that the oocyte plays an important role in regulating its adjacent somatic cells during follicular growth as well as the ovulation rate [1–3]. If the oocyte has such a major effect on ovulation rate, then it follows that the relative importance and indeed actions of certain growth factors may vary between species with a high-ovulation-rate phenotype (e.g., rats, mice, dogs, and pigs) and those with a low ovulation rate phenotype (e.g., humans, cattle, and sheep). Whether this hypothesis has any validity remains to be tested.

The oocyte produces two closely related growth factors that are known to be essential for normal follicular development, namely growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15; also known as GDF9B) [4–7]. These proteins are members of the transforming growth factor β (TGFβ) superfamily. The TGFβ superfamily is comprised of over 30 proteins, including TGFβ, activin/inhibin, growth differentiation factors, bone morphogenetic proteins, anti-Müllerian hormone, and others, that share common structural motifs. TGFβ superfamily members are pivotal in controlling cellular growth and differentiation during fetal and adult life [8, 9].

Mice lacking the GDF9 gene (GDF9KO) are infertile and follicular growth is arrested at the primary stage of development (an oocyte surrounded by 1 to <2 complete layers of cuboidal granulosa cells) [6]. Consistent with this finding, GDF9 has been shown under in vitro conditions to

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stimulate growth of preantral rat follicles [10], including the proliferation of rat granulosa cells [11, 12]. Moreover, GDF9 is involved in inducing the cumulus cell phenotype [2, 12]. The evidence for the latter is that GDF9 stimulates progesterone production (by increasing steroidogenic acute regulatory gene [StAR] mRNA), cyclooxygenase 2 mRNA synthesis, hyaluronan synthase 2, and cumulus expansion but inhibits urokinase plasminogen activator mRNA expression and FSH-stimulated LH receptor mRNA expression.

In contrast with the mouse GDF9KO, mice lacking a functional BMP15 gene are fertile [13]. Although litter size was reduced, this appeared to be due to a failure of oocytes to be released from follicles and reduced fertilization rate and not to an alteration in the number of preovulatory follicles. No discernible effects were noted in mice with one copy of a functional BMP15 gene. Under in vitro conditions, BMP15 stimulates mitosis of rat granulosa cells in a manner similar to that described for GDF9 [14]. However, in contrast with GDF9, BMP15 suppressed progesterone production from rat granulosa cells (through suppression of StAR mRNA) and also suppressed the expression of FSH-R mRNA [15].

In contrast with results with mice, sheep with inactivating mutations of the BMP15 gene are infertile, with follicular development being arrested at the primary stage of growth [7]. However, sheep with a single copy of an inactivating BMP15 mutation are fertile with an increased ovulation rate. In contrast with BMP15, the role of GDF9 in regulating follicular development and ovulation rate in sheep is not known.

The aim of this study was to test the hypothesis that GDF9 and BMP15 are essential for normal ovarian follicular development in mammals with a low ovulation rate phenotype. To test the validity of this hypothesis, sexually mature sheep were immunized against either GDF9 or BMP15 and the effects of these treatments on various ovarian parameters such as ovarian follicular development, ovulation rate, and plasma progesterone concentration were evaluated. In addition, the cellular localizations of GDF9 and BMP15 proteins in the sheep ovary were examined by immunohistochemistry.

MATERIALS AND METHODS

All experiments were performed with the approval of the Animals Ethics Committee at Wallaceville Animal Research Centre in accordance with the 1987 Animal Protection (Codes of Ethical Conduct) Regulations of New Zealand. The animals used in the immunization studies (n = 55) were 5- to 6-yr-old parous Romney ewes. For the immunohistochemical studies, ovaries were recovered from 4-wk-old Romney lambs (n = 5 animals). Except where indicated, laboratory chemicals 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).

Expression of the Mature Region of Ovine BMP15 Protein in Escherichia coli

Total cellular RNA was isolated from ovine ovarian tissue using Trizol according to manufacturer's instructions. First strand cDNA was produced using 5 μg of ovarian total cellular RNA using the SuperScript preamplification system for first strand cDNA synthesis. The cDNA encoding the mature region of ovine BMP15 was generated using the Qiagen PCR system (Biolab Scientific Limited, Christchurch, New Zealand) and forward and primers corresponding to bases 870–887 and 1227–1244 based on published sequence data (GenBank accession number AF236079; [7]). Following denaturation at 94°C for 2 min, ovine mature region BMP15 cDNA was amplified using 40 cycles of denaturing for 0.5 min at 94°C, 0.5 min of annealing at 55°C, and extension at 72°C for 1.0 min. The sequence of the 370-base pair product was verified [16] and the cDNA subcloned into pET23b. The resulting plasmid was transformed into BL21DE3 cells for production of protein using isopropylthio-β-galactoside induction as described previously [17].

Immunohistochemistry

The GDF9 monoclonal antibody (C37) was generated as follows. A synthetic peptide corresponding to a region near the C-terminal of human GDF-9, sequence VPAKYSPLSVLTIEPDGSIAYKEYEDMIATKC, was synthesized and the peptide was coupled to a purified protein derivative of tuberculin [18]. Outbred Tyler original (T/O; Southend on Sea, Essex, U.K.) mice were immunized and boosted over a 4-mo period. The animals were killed and the spleens removed. Splenocytes were fused to Sp2/0 myeloma cells following a standard procedure [19]. Hybridoma supernatants were screened by ELISA using the peptide coated onto Nunc immunoplates following standard protocols [20]. Positive clones were expanded and recloned by limiting dilution.

The subsequent clones were further screened against recombinant GDF-9 and the best used for immunostaining and Western blotting. Upon selection, each clone was expanded and isotyped, and all were found to be IgG1. Each was purified on a protein A column using a high-salt protocol [21] before being reassessed and clone 37 selected as giving the strongest specific signal. This was then confirmed by blocking studies.

The BMP15 polyclonal antibody (M10-9B) was generated by immunizing mice with the mature region of recombinant ovine (o) BMP15 mature protein expressed in *Escherichia coli*. Mice were injected (i.p.) with 200 μg of an oBMP15 mature protein in Freund complete adjuvant (0.22 ml). Three additional injections of 100 μg oBMP15 mature protein were given (i.p.) at two weekly intervals in a Span/Tween/Marcol solution (STM; 0.22 ml, 9/0.5/0.5 v/v/v) [22]. One week after the final injection, the mice were anesthetized (i.p.) with 0.2 ml of 12 mg/ml Nembutal before exsanguination.

To determine the immunolocalization of BMP15 protein, 12 individual follicles from paraformaldehye-fixed ovaries of four lambs (4 wk of age) were examined, whereas for GDF9 protein, 64 individual follicles from the paraformaldehye-fixed ovaries of three lambs (4 wk of age) were examined; 2 of the lambs were the same animals as those examined for BMP15 protein and the third was a different animal. Localization of GDF9 and BMP15 proteins was as previously described [17, 23] with the exception that the three washes between incubation steps were replaced with two washes with the Tris-HCl wash buffer described with the addition of 0.3% (v/v) Triton X-100 followed by two washes in Tris-HCl wash buffer. Antibodies C37 (anti-GDF-9) and M10-9B (anti-BMP15) were used at a dilution of 5 μg IgG/ml and 1:5000, respectively. To determine specificity of individual antibodies, the antibodies were incubated overnight with 10 μg/ml of the immunizing peptide/protein or 100 μg of the mature region of oGDF9 produced in *E. coli* (kindly donated by Dr. H. Sawyer, Colorado State University, Fort Collins, CO) or oBMP15 (described above). Following incubation, antibody-antigen preparations were centrifuged and the supernatant applied to the tissue sections.

Generation of Antigens for Immunization of Sheep

The oBMP15 mature protein was found primarily in inclusion bodies so the protein solubilization and refolding method of Kohno et al. [24] was employed to obtain protein suitable for conjugation. Analysis of the purified protein by silver staining indicated that the resulting product was approximately 90% pure. Equal quantities of the oBMP15 mature protein and keyhole limpet hemocyanin (KLH; Sigma, Auckland, New Zealand) were conjugated using gluteraldehyde as described previously [25]. Analysis by SDS-PAGE revealed that approximately 90% of the oBMP15 mature protein was conjugated to KLH.

Peptides KKPLVPASVNLSEYFC (GDF9) and SEVPGPSREHDGPESC (BMP15) were synthesized and conjugated to KLH through the C-terminal cysteine residue by Macromolecular Resources (Colorado State University, Fort Collins, CO).

Active Immunization of Ewes Against GDF9 and BMP15

Ewes were injected (i.m.) with 0.4 mg KLH (control; n = 10), 0.4 mg KLH-GDF9 peptide conjugate (GDF9 peptide; n = 10), 0.4 mg KLH-oBMP15 peptide conjugate (BMP15 peptide; n = 10), or 0.8 mg KLH-oBMP15 mature protein conjugate (oBMP15 mature protein; n = 10) in 1 ml of Freund complete adjuvant for the initial immunization, which was given 4–5 mo before the onset of the breeding season. Thereafter, ewes were immunized once monthly with 0.2 mg KLH, GDF9 peptide, BMP15

peptide, or 0.4 mg oBMP15 mature protein in 1 ml of saline mixed with 1.25 ml of STM for 6 mo. One ewe in the BMP15 peptide-immunized group was replaced after the first injection and thus only received a total of six immunization injections. In addition, one ewe in the KLH group died 3 mo into the project and one ewe in the oBMP15 mature protein group died in the sixth month of the study. Data from the control ewe that died was excluded from analysis; however, when available, data from the oBMP15 mature protein-immunized ewe that died was included in analysis. After the fifth injection, vasectomized rams with marking harnesses were run with the ewes to monitor estrous cycles. The length of the estrous cycle was calculated as the days between first observed markings of successive cycles by the vasectomized ram. In addition, blood samples were collected via the jugular vein three times a week for determination of plasma progesterone concentrations. Ovulation rates of the ewes that displayed estrous behavior were determined by laparoscopy once all of the control ewes had been observed in estrus and for each successive estrous cycle. In addition, ovulation rate of all ewes was determined by laparoscopy 3–4 wk prior to ovarian collection. Thus, the control ewes underwent laparoscopy two to three times and the treated groups most commonly once, although some were subjected to laparoscopy two to three times also. Approximately 2 wk following the final injection, ewes were killed using a captive bolt and exsanguinated. The blood collected from all ewes was to be used in subsequent passive immunization studies. Both ovaries were recovered and the number of corpora lutea (CL) present was recorded, and one ovary from each ewe was fixed in Bouin fluid for morphological examination and analysis of follicular populations.

Ovarian Studies

All the Bouin-fixed ovaries were serially sectioned at 5 μm and stained with hemotoxylin and eosin. From the ewes actively immunized with KLH, GDF9 peptide and BMP15 peptide, or mature protein, ovarian volumes were measured and the numbers of ovarian follicles at each stage of follicular development were counted. In addition, individual follicles were classified as nonatretic, atretic, or abnormal and the diameters of both the oocyte and the follicle determined. The stages of ovarian follicular development were classified using the optical plane through the largest cross-section using a modification of the follicular-type criteria described by Lundy et al. [26], namely, type 1 (primordial) follicles, with one layer of flattened granulosa cells; type 1a (transitory) follicles, with one layer of a mixture of flattened and cuboidal granulosa cells; type 2 (primary) follicles, with 1 to <2 complete layers of cuboidal granulosa cells; type 3 (small preantral) follicles, with 2 to <4 layers of granulosa cells; type 4 (large preantral) follicles, with ≥4 layers of granulosa cells but no antral cavity; type 5 (small antral) follicles, with a fully formed antrum but ≤1000 μm in diameter; and type 5+ (large antral) follicles, with a fully formed antrum and >1000 μm in diameter. When classifying abnormal follicles, the criteria for types 1, 1a, 5, and 5+ were the same as those described above for nonatretic or atretic follicles. The major problem arose with respect to types 2, 3, and 4 follicles, where the configurations of granulosa cells were often irregular around the oocyte. In these instances, the classification given was that for the greatest number of layers of granulosa cells.

Ovarian volume (μm^3) was measured by the Cavalieiri principle using the formula $V = \Sigma ah$, where a = the cross-sectional area of every 10th section (μm^2) and h = the distance between sections (μm) [27]. The number of follicles was counted using stereological principles [27, 28]. For the type 1, 1a, and 2 follicles, an unbiased counting frame of area $a_{(f)}$ was placed over projected images from every 10th or 12th ovarian section (each section thickness being 5 μm) and then the number of follicles exclusive to one section of each pair (Q) was described. Only follicles in which the oocyte cytoplasm was present in the section were counted. The total follicular count (N) for each follicular type was derived using the formula $N = [\Sigma Q/\Sigma (a_{(f)}h_{(p)})]V$, where $h_{(p)}$ = the distance between each pair and V = the ovarian volume. For type 3, 4, 5, and 5+ follicles, all serial sections were examined and every follicle was counted. For all follicles counted, a note was made as to whether the follicle was nonatretic, atretic, or abnormal. A follicle was considered atretic if any one of the following criteria in the largest cross-section were observed: >5 pyknotic granulosa cells, >10 pyknotic thecal cells, loss of integrity of the basement membrane, or a nonspherical oocyte. Abnormal follicles were recorded as those having an unusually large oocyte for a follicular type, an unusual or irregular configuration of granulosa cells, an unusual or irregular arrangement of granulosa or cumulus cells around the oocyte, a highly irregular but continuous basement membrane, or an unusual or irregular configuration of theca interna. These abnormal follicles, in most instances, could not be described as atretic by any of the criteria described above.

For measurements of follicular or oocyte diameter, nonatretic or abnormal follicles were selected; measurements were not made of atretic follicles. Type 1, 1a, and 2 follicular or oocyte diameters were measured on six sections per animal using the systematic random sampling method described by Smith et al. [27]. The oocyte and follicular diameters of the type 3, 4, 5, and 5+ follicles were made for every nonatretic or abnormal follicle as they appeared on any section. In all cases, the diameters of the oocyte and follicle were obtained by averaging the two diameters at right angles to each other in the largest cross-section of the follicle and/or in the section in which the oocyte was at its largest. All measurements were performed using an Olympus BH-2 microscope (Olympus New Zealand Limited, Upper Hutt, New Zealand) linked to a video camera, color monitor, and computer with National Institute of Health's (Bethesda, MD) image analysis software (version 1.6).

Passive Immunization of Ewes Against KLH, KLH-GDF9 Peptide, and KLH-BMP15 Peptide

Pools of antiplasma from KLH (n = 9, all ewes) KLH-GDF9 peptide treated (GDF9 peptide; n = 7, all anovulatory ewes), and KLH-BMP15 peptide- (BMP15 peptide; n = 9, all anovulatory ewes) treated ewes were generated by combining the plasmas obtained during the final 3 mo of the experiment from some of the actively immunized ewes within each treatment group. The estrous cycles of ewes were synchronized by using a prostaglandin $F_{2\alpha}$ derivative (Estrumate; 125 μg). Estrus was detected with the aid of a vasectomized ram wearing a marking harness. On Day 4 or 5 of the estrous cycle (estrus = day 0), ewes were subjected to laparoscopy to determine ovulation rate and fitted with an indwelling jugular cannula. The following day, ewes (n = 4–5 per group) were administered 100 ml of antiplasma to KLH, GDF9 peptide, or BMP15 peptide through the indwelling jugular cannula. Ewes were given another injection of Estrumate at 96 h after administration of the antiplasma to induce a follicular phase, and ovulation rate was determined by laparoscopy at 10 days after the injection of Estrumate and every 15–18 days thereafter until the end of the breeding season (as assessed by lack of estrous activity in nonexperimental sheep). Blood samples were collected from the ewes at 5 min, 1 h, and 96 h after injection of the antiplasma and thereafter three times a week from the second injection of Estrumate for determination of antibody titers and concentrations of progesterone in plasma.

Determination of Antibody Titers and Cross-Reactivity

Individual plasma samples from the actively immunized sheep collected at the time of the laparoscopy 3–4 wk prior to ovarian collection and when the ovaries were collected, together with the KLH and GDF9 peptide and BMP15 peptide pooled antiplasmas used in the passive immunization experiment were tested for reactivity to both oGDF9 and oBMP15 mature proteins. To do this, wells of microtiter plates were coated at 4°C overnight with increasing concentrations (0–10 μg) of oGDF9 mature protein or oBMP15 mature protein (described above) in coating buffer (50 mM carbonate-bicarbonate buffer, pH 9.6). Nonspecific binding was blocked by incubating the wells with 0.25 ml coating buffer containing 0.5% gelatin for 30 min at room temperature. Wells were then washed three times (3 min) in wash buffer (PBS containing 0.05% Tween 20). Plasma samples from the actively immunized ewes were appropriately diluted (1:10 000–1:20 000) in GAB (PBS containing 0.05% Tween and 0.1% gelatin), and 0.2 ml of these dilutions were then incubated in the wells of the microtiter plates coated with increasing concentrations of oGDF9 or oBMP15 mature proteins for 1.5 h at 37°C. Following this incubation, the appropriately diluted plasma samples were transferred to microtiter plates previously coated, as described above, with 100 ng/well of oGDF9 or oBMP15 mature proteins and incubated at 37°C for 1.5 h. Wells were washed three times in wash buffer and incubated with 0.1 ml of HRP-conjugated rabbit anti-sheep IgG (1:10 000 dilution in GAB; Scientific Supplies, Auckland, New Zealand) for 1 h at 37°C. Wells were then washed three times and incubated with 0.1 ml of 50 mM citrate buffer containing 0.4 mg/ml of o-phenylenediamine and 0.04% H_2O_2 for 30 min in the dark. Reactions were stopped by the addition of 50 μl of 2.5 M H_2SO_4, and absorbance at 490 nm was determined on a Bio-Tek EL311 microplate autoreader (Bio-Tek Instruments Inc., Winooski, VT).

The concentration of oGDF9 or oBMP15 mature protein in the first plate that caused a 50% decrease in specific binding of the antiplasma when reacted with 100 ng oGDF9 or oBMP15 mature protein, respectively, on the second plate was calculated for all ewes where specific displacement was observed. To determine cross-reactivity, the concentration of oGDF9 or oBMP15 mature protein on the first plate that caused a corresponding decrease in specific binding as that shown above when reacted

with oBMP15 or oGDF9 mature protein on the second plate was calculated for the ewes immunized against BMP15 (peptide and mature protein) or GDF9 peptide, respectively. If this point was not reached with the concentrations of oGDF9 or oBMP15 mature protein used for coating the first plate, the highest concentration of protein tested was used to calculate the cross-reactivity.

Titers in plasma collected from passively immunized ewes collected 5 min, 96 h (time of prostaglandin injection), approximately 144 h, and approximately 452 h after injection of 100 ml of pooled antiplasma against KLH, GDF9 peptide, and BMP15 peptide were determined by ELISA. Briefly, plasma samples were diluted 1:1000 in GAB and 0.2 ml of these dilutions were then incubated in the wells of the microtiter plates previously coated, as described above, with 100 ng/well of oGDF9 or oBMP15 mature protein and incubated at 37°C for 1.5 h. Determination of bound antibody was as described above. Plasma samples from ewes given KLH antiplasma were reacted with wells containing both oGDF9 and oBMP15 mature proteins, whereas plasma samples from ewes receiving GDF9 peptide or BMP15 peptide antiplasma were reacted with wells containing oGDF9 or oBMP15 mature protein, respectively.

Determination of Progesterone Concentrations

Concentrations of progesterone in plasma were determined by RIA as described previously [29]. The inter- and intraassay coefficients of variation were <10% and assay sensitivity was 0.1 ng/ml. All samples below the sensitivity of the assay were assigned a value of 0.1 ng/ml for statistical analysis.

Statistical Analysis

For the actively immunized ewes, ovulation rate for individual ewes was calculated as the mean of the number of CL observed at all observations for that ewe when at least one CL was present (i.e., observations of no CL were excluded from the calculation). The Kruskal-Wallis test was used to compare ovulation rates between the oBMP15 mature protein- and the KLH-treated groups. No other groups were included in this comparison because none had sufficient numbers of ewes ovulating. The chi-squared test was used to compare the proportion of ewes observed in estrus by the time all the control ewes had been observed in estrus. In addition, the chi-squared test was used to compare the proportion of ewes with CL on their ovaries 3–4 wk before and at ovarian collection.

When examining the effects of active immunization treatments on ovarian volumes, numbers of follicles, or oocyte or follicular diameters, the data were analyzed within each follicle type after normalizing the data by log transformation. For each parameter, a one-way ANOVA was performed, taking out within-animal effects where appropriate, with differences among treatment groups determined by least significant difference.

For the passively immunized ewes, differences in the number of ewes with CL at each laparoscopy were determined using the Fisher exact test. The areas under the curves were calculated with Genstat (NAG Ltd, Oxford, U.K.) using the area function for progesterone values from 2 to 19 days following injection of Estrumate that was given 4 days after administration of plasma. Resulting values were analyzed with one-way ANOVA and differences between the control and treated ewes determined with the Fisher pairwise comparisons.

RESULTS

Immunohistochemistry

Evidence that GDF9 and BMP15 proteins are localized specifically within the ovary to oocytes is shown in Figure 1. The specificity of the oocyte staining was determined by replacement of the primary antibody with an irrelevant in-house monoclonal antibody to ovine IgE (YD3) or polyclonal antibody raised in mice to bovine α-lactalbumin (n = 32 follicles for GDF9 and 10 for BMP15). No specific staining was observed with either control antibody (data not shown). Immunostaining was abolished when GDF9 and BMP15 antisera were preincubated with GDF9 immunizing peptide (10 μg/ml; n = 30 follicles) and oBMP15 mature protein (10 μg/ml; n = 12 follicles), respectively. Conversely, preincubating GDF9 antisera with oBMP15 mature protein (100 μg/ml; n = 56 follicles) or BMP15 antisera with oGDF9 mature protein (100 μg/ml; n = 6 follicles) failed to abolish specific staining. Thus, each antibody was able to identify its primary antigen without significant cross-reaction from its closely related growth factor.

Antibody Titers

In the active immunization experiments, antibody titers and cross-reactions were evaluated at two different time points, namely at laparoscopy (i.e., 3–4 wk before ovarian collection) and at ovarian collection. All animals immunized against KLH showed no specific cross-reaction with either oGDF9 or oBMP15 (data not shown). Ewes immunized with the GDF9 peptide had antibodies against oGDF9 and showed no cross-reactivity (<1%) to oBMP15 (Table 1). Ewes immunized with BMP15 peptide had antibodies specific for oBMP15 and showed no cross-reactivity (<1%) with oGDF9 (Table 1). Ewes immunized with oBMP15 mature protein had antibodies against oBMP15 and, with the exception of a single ewe with 5%-cross reactivity, had <1% cross-reactivity with oGDF9 (Table 1).

In the passive immunization experiments, the pools of antiplasma generated from the KLH-immunized ewes did not show any specific binding to either oGDF9 or oBMP15 (Fig. 2). Preabsorption with oGDF9, but not with oBMP15, decreased the binding of the pooled GDF9 plasma to GDF9. Likewise, oBMP15, but not oGDF9, was able to inhibit binding of plasma from BMP15-immunized ewes to oBMP15 (Fig. 2). Antibodies for GDF9 or BMP15 were detectable within 5 min of administration and remained detectable for approximately 144 h in the plasma of ewes administered 100 ml of GDF9 or BMP15 antiplasma, respectively (Fig. 3). Binding of neither oGDF9 nor oBMP15 was observed in the ewes that received the KLH antiplasma (Fig. 3).

Active Immunizations

Ovarian function. Ewes immunized with KLH maintained regular estrous cycles throughout the study, with an average cycle length of 17 ± 2 days (Fig. 4). On average, each ewe was observed in estrus three times and analysis of the progesterone data indicated that one estrus was not observed in each of two control ewes. On average, the control ewes had an ovulation rate of 1.6 (range 1–2; 3–4 observations per ewe).

In contrast with the KLH-immunized ewes, cyclic estrous behavior in those immunized against GDF9 peptide, BMP15 peptide, or oBMP15 mature protein was rare (Table 2) and normal patterns of progesterone concentration were uncommon (Fig. 4). No ewe immunized with GDF9 peptide displayed cyclic estrous behavior and an erratic pattern of progesterone concentration was common, with high levels noted for only one or two samples (Fig. 4). Two ewes had luteal-like structures (one or two) on the ovarian surface 3–4 wk before ovarian collection and three had luteal tissue at ovarian recovery; however, a normal luteal phase pattern of progesterone was not observed. In addition, a single luteal structure was recorded for two other ewes over the course of the experiment following marking by a vasectomized ram. However, in only one of the ewes was a normal luteal phase observed (Fig. 4). In addition, many of the ewes did not have any visible antral follicles at laparoscopy or at ovarian collection.

Only one ewe in the BMP15 peptide group displayed cyclic estrous behavior (data not shown, but see Table 2). This ewe had three estrous cycles, with an average ovulation rate of 6.3. The remaining ewes did not have any vis-

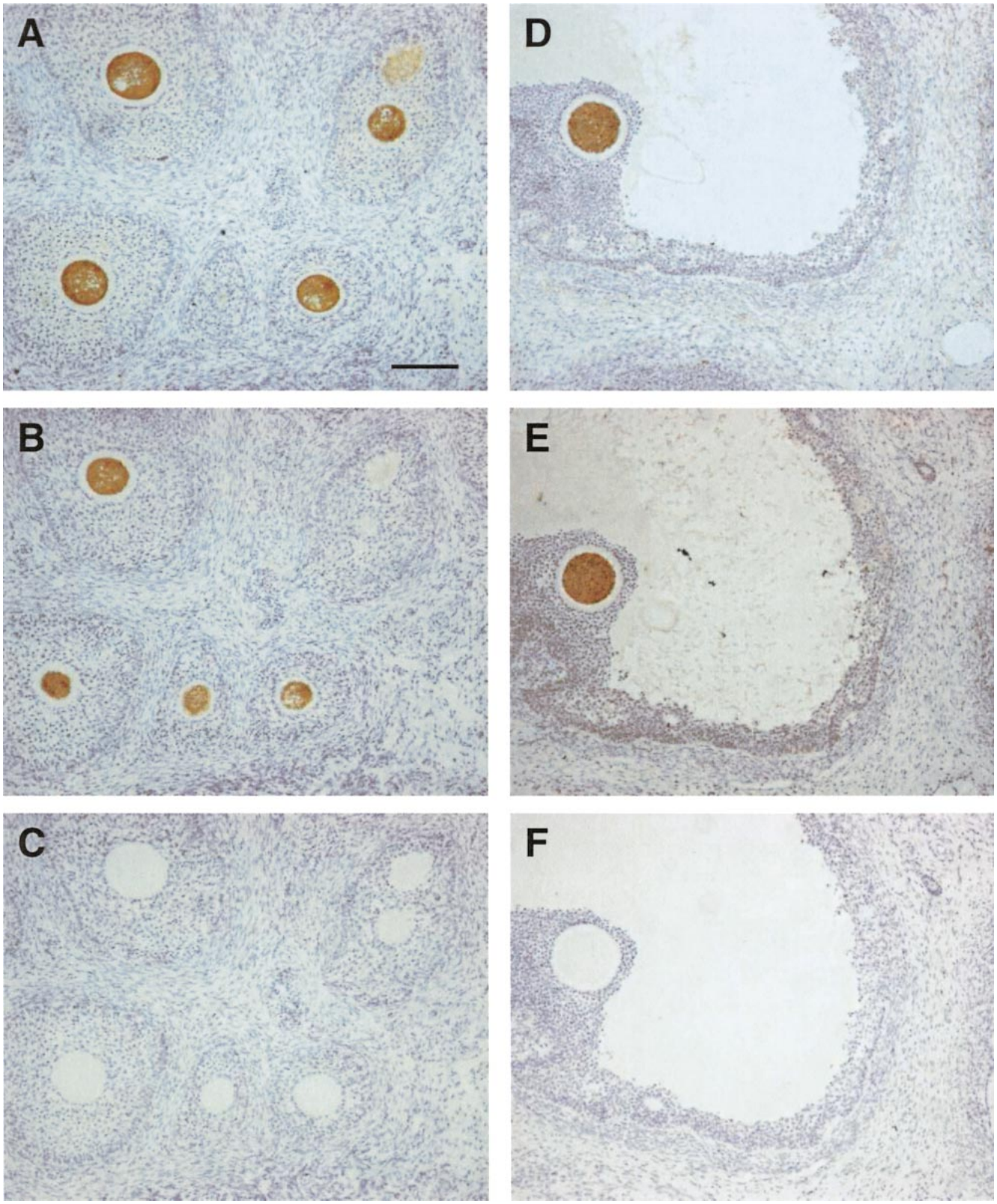

FIG. 1. Immunolocalization of GDF9 and BMP15 proteins in ovine ovaries. **A**) Localization of GDF9 protein in oocytes of preantral and antral follicles. Preincubation of the antibody with 100 μg/ml of oBMP15 mature protein did not abolish staining (**B**). Immunostaining was abolished when the antibody was preabsorbed with the 10 μg/ml of the antigen used for immunization (**C**). **D**) Localization of BMP15 protein within an oocyte of an antral follicle. Preincubation with 100 μg/ml of oGDF9 mature protein had no effect on staining (**E**). Preabsorption with 10 μg/ml of oBMP15 mature protein (**F**) abolished staining. Bar = 108 μm.

TABLE 1. Average amount of oGDF9 mature protein (ng) that reduced binding of plasma from the KLH-GDF9 peptide-immunized ewes to 100 ng of oGDF9 mature protein by 50% (50% displacement) and average amount of oBMP15 mature protein (ng) that reduced binding of plasma from the KLH-BMP15 peptide- or KLH-oBMP15 mature protein-immunized ewes to 100 ng of oBMP15 mature protein by 50% (50% displacement). Also shown is the average cross-reactivity (% CR) of plasma from KLH-GDF9 peptide-immunized ewes to oBMP15 mature protein and of plasma from KLH-BMP15 peptide- and KLH-oBMP15 mature protein-immunized ewes to oGDF9 mature protein.

Immunized group	50% Displacement (ng)	% CR
KLH-GDF9 peptide	342.5	<0.34
KLH-BMP15 peptide	34.0	<0.34
KLH-oBMP15 mature protein	28.7	<0.56

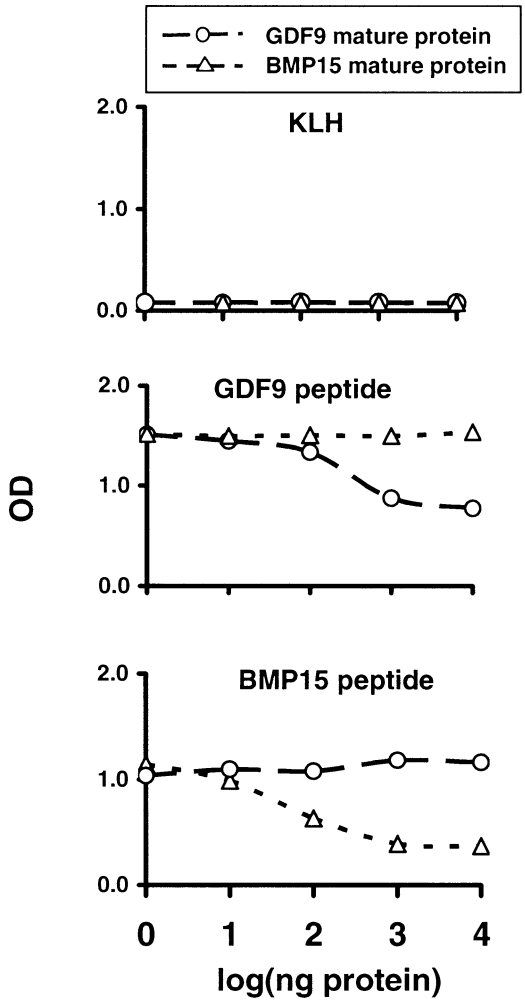

FIG. 2. The top panel shows binding of antiplasma pooled from the KLH-immunized ewes to 100 ng of oGDF9 or oBMP15 mature protein (see legend) after they had been preabsorbed with increasing amounts of oGDF9 or oBMP15 mature protein, respectively (see legend). The middle panel shows binding of antiplasma pooled from the KLH-GDF9 peptide (GDF9 peptide)-immunized ewes to 100 ng of oGDF9 mature protein after the antiplasma had been preabsorbed with increasing amounts of oGDF9 or oBMP15 mature proteins (see legend). The bottom panel shows binding of antiplasma pooled from the KLH-BMP15 peptide (BMP15 peptide)-immunized ewes to 100 ng of oBMP15 mature protein after the antiplasma had been preabsorbed with increasing amounts of oGDF9 or oBMP15 mature proteins (see legend). The amounts of GDF9 or BMP15 mature protein used in the absorptions studies are indicted on the x-axis with the value at zero representing preabsorption with no hormone.

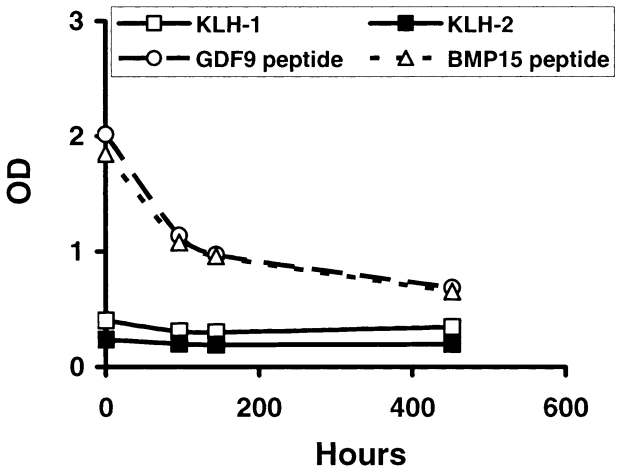

FIG. 3. Average optical density readings of plasma with respect to the time (hours) after treatment of ewes given KLH, KLH-GDF9 peptide (GDF9 peptide), or KLH-BMP15 peptide (BMP15 peptide) antiplasma. The binding of plasma from the KLH antiplasma-treated ewes was tested against the oGDF9 (KLH-1) and oBMP15 (KLH-2) mature proteins, whereas the plasma from the KLH-GDF9 peptide and KLH-BMP15 peptide antiplasma-treated ewes was tested against oGDF9 or oBMP15 mature proteins, respectively. The first time point is at 5 min following administration of the antiplasmas.

ible luteal structures on their ovaries at either inspection and, as was observed in the GDF9-immunized ewes, often no visible follicular structures were apparent either. However, occasionally, a luteal phase pattern of progesterone concentrations or an erratic pattern of progesterone was noted (Fig. 4).

At least one estrus was observed in most of the ewes immunized with the BMP15 mature protein, and this was often followed by a normal luteal phase pattern of progesterone concentrations (Fig. 4). The average ovulation rate of the BMP15 ewes that ovulated was 4.4 (range 1–10) (Fig. 5) and was higher ($P < 0.001$) than that observed in the control ewes (Fig. 5). However, regular cyclic activity was not common and only five of the ewes immunized against the oBMP15 mature protein had shown estrous activity by the time all the control ewes had been in estrus (Table 2). As seen in many of the animals immunized against BMP15 peptide, the ovaries of some animals immunized against the oBMP15 mature protein were devoid of any identifiable antral follicles.

Ovarian histology. Ovaries collected from ewes immunized against KLH appeared normal in all aspects (Fig. 6). Immunization against either GDF9 peptide or BMP15 peptide decreased mean ovarian volume compared with that for the KLH ewes, whereas the mean ovarian volume for ewes immunized against oBMP15 mature protein was intermediate (Table 3; see also Fig. 6). The mean total numbers of follicular types 1/1a (separate or pooled), 2, or 3 were not different among the treatment groups. However, the mean total numbers of type 4, 5, and 5+ follicles were lower in the GDF9 peptide- and BMP15 peptide-immunized animals compared with that for the KLH (i.e., control) animals (see also Fig. 6 for type 5 and 5+ follicles). The mean total numbers of follicles for the oBMP15 mature protein groups were similar to the KLH-treated animals for the type 4 and 5 group but lower for the type 5+ group (Table 3). The lower number for the type 5+ group is likely due to combining follicle numbers from anovulatory as well as ovulatory ewes. No differences in the percentage

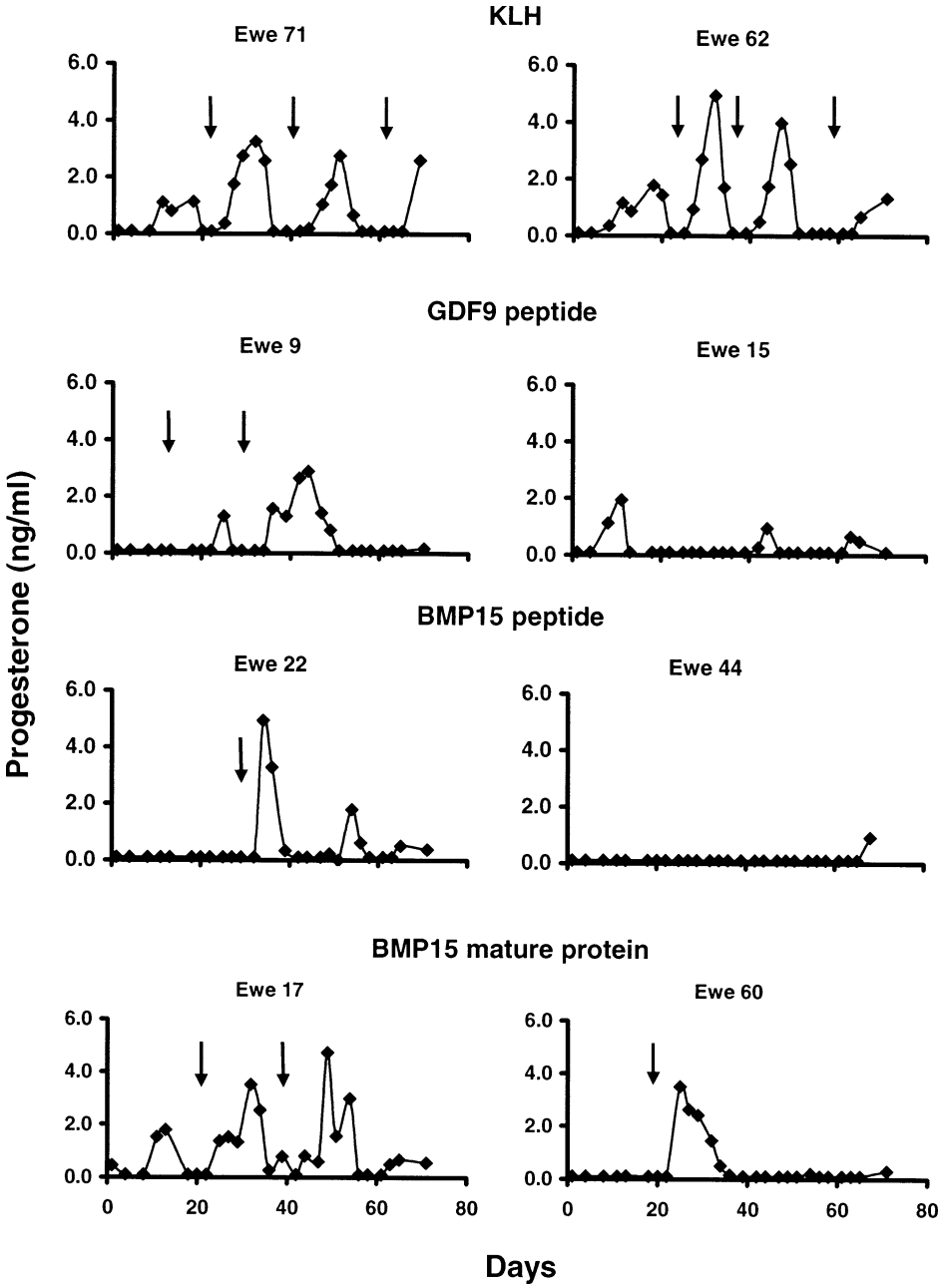

FIG. 4. Examples of the pattern of progesterone concentrations in plasma of actively immunized ewes. Antigen used for immunization and the ewe identification numbers are shown at the top of each graph. The GDF9 and BMP15 peptide and BMP15 mature protein antigens were each conjugated to KLH. Markings by vasectomized rams are indicated with arrows. Day 0 corresponds to the beginning of thrice weekly sampling period.

of atretic follicles were noted between the treatment groups. Most follicles that had developed to the type 2 stage or beyond in the ewes immunized with GDF9 peptide, BMP15 peptide, or oBMP15 mature protein were abnormal in appearance (see Figs. 7 and 8). In GDF9 peptide-, BMP15 peptide-, and oBMP15 mature protein-treatment groups, some of the oocytes from the type 1a stage and more commonly from the type 2 to 4 stage appeared to have abnormally large diameters relative to those in the KLH-treated animals (Fig. 7). Also from the type 2 stage onward, the organization of the granulosa cells and/or the cumulus cells around the oocyte was abnormal (Figs. 7 and 8). In addition, oocyte-free nodules of granulosa cells containing large amounts of zona pellucida-like material (not shown) or inclusions of zona-like material among the granulosa cells were observed (not shown). The effects of the treatments on mean oocyte diameters at each stage of follicular development from type 1 to type 5+ are shown in Table 4. No effects of treatment were noted on type 1 follicles. However, at the type 1a stage, the BMP15 peptide animals had a significantly larger mean oocyte diameter than that for the other treatment groups (Table 4). For the type 2 to type 4 stages of growth, the KLH animals had a mean oo-

TABLE 2. Proportions of ewes immunized against KLH, KLH-GDF9 peptide, KLH-BMP15 peptide, or KLH-oBMP15 mature protein that had been in estrus before the first laparoscopy (1st), with visible luteal structures at laparoscopy 3–4 wk before collection (2nd) and at ovarian collection (3rd).

Immunized group	1st	2nd	3rd
KLH	9/9	9/9	9/9
KLH-GDF9 peptide	2/10*	2/10*	3/10*
KLH-BMP15 peptide	1/10*	1/10*	1/10*
KLH-BMP15 mature protein	5/10*	4/9*	4/9*

* Signifies a value that is different from the control (KLH) value ($P < 0.05$).

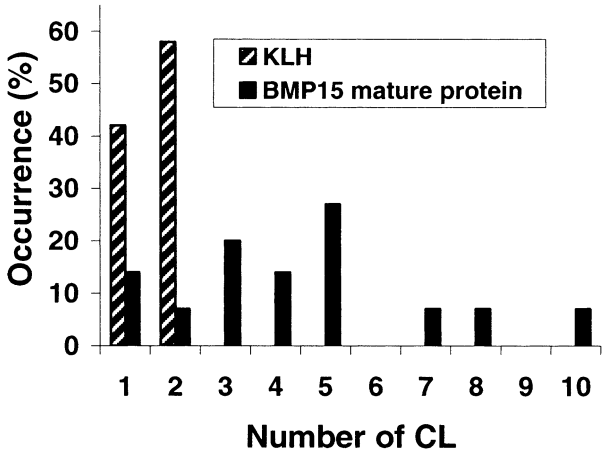

FIG. 5. Percent occurrence of ewes with a given ovulation rate after immunization against KLH or KLH-oBMP15 mature protein (BMP15 mature protein). Data were collected at laparoscopy and/or at ovarian recovery and refer only to those ewes that ovulated (n = 9 ewes and 31 observations for the KLH group and 7 ewes and 15 observations for the KLH-oBMP15 mature protein group). The average ovulation rate in the oBMP mature protein-immunized ewes was higher ($P < 0.001$) than that in the KLH group.

cyte diameter significantly smaller than for all other treatments. At the type 5 and 5+ stages, no differences in oocyte diameter were noted between the KLH and oBMP15 mature protein groups: too few type 5 or type 5+ follicles were available from the GDF9 peptide and BMP15 peptide animals for comparisons.

With respect to mean follicular diameter, no differences were noted between the treatment groups for the type 1 follicles. There was a tendency for a greater variability in diameters in the GDF9 peptide, BMP15 peptide, and/or oBMP15 mature protein animals between the type 1a to type 3 stages of growth; this effect of treatment was observed to be significant at the type 1a stage for the GDF9 peptide and BMP15 peptide groups, which had larger follicles (both $P < 0.01$) compared with the KLH animals, and also at the type 3 stage for the GDF9 ($P < 0.05$) and oBMP15 mature protein ($P < 0.01$) groups, which similarly had larger follicles compared with the KLH animals (data not shown). No other treatment effects were noted at each follicular type.

Some animals immunized against GDF9 peptide, BMP15 peptide, or oBMP15 mature protein ovulated with normal CL. However, many animals had ovaries with luteal bodies being invisible from the ovarian surface (Fig. 6). On occasion, some animals had both surface-visible and surface-invisible structures (Fig. 6).

Passively Immunized Ewes

There were no differences in ovulation rates among the groups before administration of the antiplasma (Table 5). Administration of antiplasma against GDF9 peptide 4 days before induction of the follicular phase did not affect ovulation rate. However, at laparoscopy, the CL of two of the animals treated with GDF9 peptide antiplasma appeared smaller than normal. In addition, the overall mean concentration of progesterone during the subsequent luteal phase was less ($P < 0.05$) than that observed in the control animals (Fig. 9). This was the result of the progesterone concentrations being normal in two of the animals (see animal 151, Fig. 10), but in the other three animals, the postovulation rise in progesterone was delayed even though luteolysis occurred at the normal time (see animal 161, Fig. 10). In four of the five ewes treated with the BMP15 peptide antiplasma, no CL were observed at laparoscopy some 10 days following induction of the follicular phase. Furthermore, in three of these four animals, no surface-visible antral follicles were observed either. While the ewe with CL present at the time of laparoscopy had a normal luteal phase pattern of progesterone concentration following prostaglandin synchronization (data not shown), no such luteal phase pattern was observed in the other four ewes (see ewes 158 and 168, Fig. 10). Overall, the mean concentration of progesterone in the ewes treated with BMP15 peptide antiplasma during the synchronized luteal phase was lower (*P*

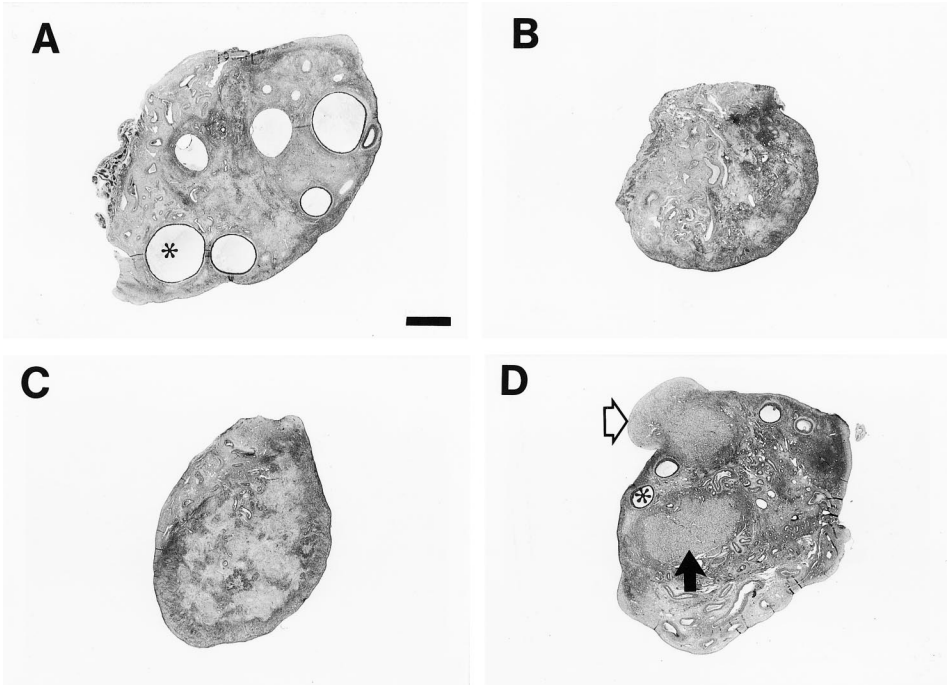

FIG. 6. Examples of normal and abnormal ovaries in ewes treated with KLH alone (**A**) or with KLH-GDF9 peptide (**B**), KLH-BMP15 peptide (**C**), or KLH-oBMP15 mature protein (**D**). **A**) Numerous or large antral follicles (asterisk); **B**, **C**) ovaries devoid of large antral follicles and no small antral follicles visible in these sections; **D**) antral follicles (asterisk) together with a normal surface-visible CL (open arrow) and a luteal body submerged within the ovary (solid arrow) and invisible from the ovarian surface. Bar = 2 mm.

TABLE 3. Effect of immunizing ewes against KLH, KLH-GDF9 peptide, KLH-BMP15 peptide, or KLH-oBMP mature protein on ovarian volume and total numbers of follicles at specific stages of development (i.e., follicular type). Values are geometric means (and 95% confidence limits).*

Treatment	Volume (μm^3)	Follicular type					
		1/1a	2	3	4	5	5+
KLH	446[a] (357, 557)	15 503[a] (11 232, 21 397)	117[a] (13 962)	20[a] (16, 24)	29[a] (25, 33)	17[a] (13, 23)	10[a] (8, 12)
KLH-GDF9 peptide	279[b] (254, 307)	12 045[a] (8 230, 17 635)	685[a] (469, 1000)	15[a] (10, 23)	11[c] (5, 24)	2[c] (0, 5)	2[c] (0, 6)
KLH-BMP15 peptide	278[b] (202, 380)	10 508[a] (6979, 15 822)	205[a] (46, 903)	18[a] (11, 29)	10[c] (4, 21)	1[c] (0, 4)	0.3[c] (0, 1)
KLH-oBMP15 mature protein	347[a,b] (276, 439)	13 331[a] (10 563, 16 823)	258[a] (29, 2248)	23[a] (16, 32)	19[b,c] (11, 32)	10[a] (3, 30)	1[c] (0, 3)

* Values in columns with different alphabetical superscripts are significantly different from one another: a versus b, $P < 0.05$; a versus c, $P < 0.01$.

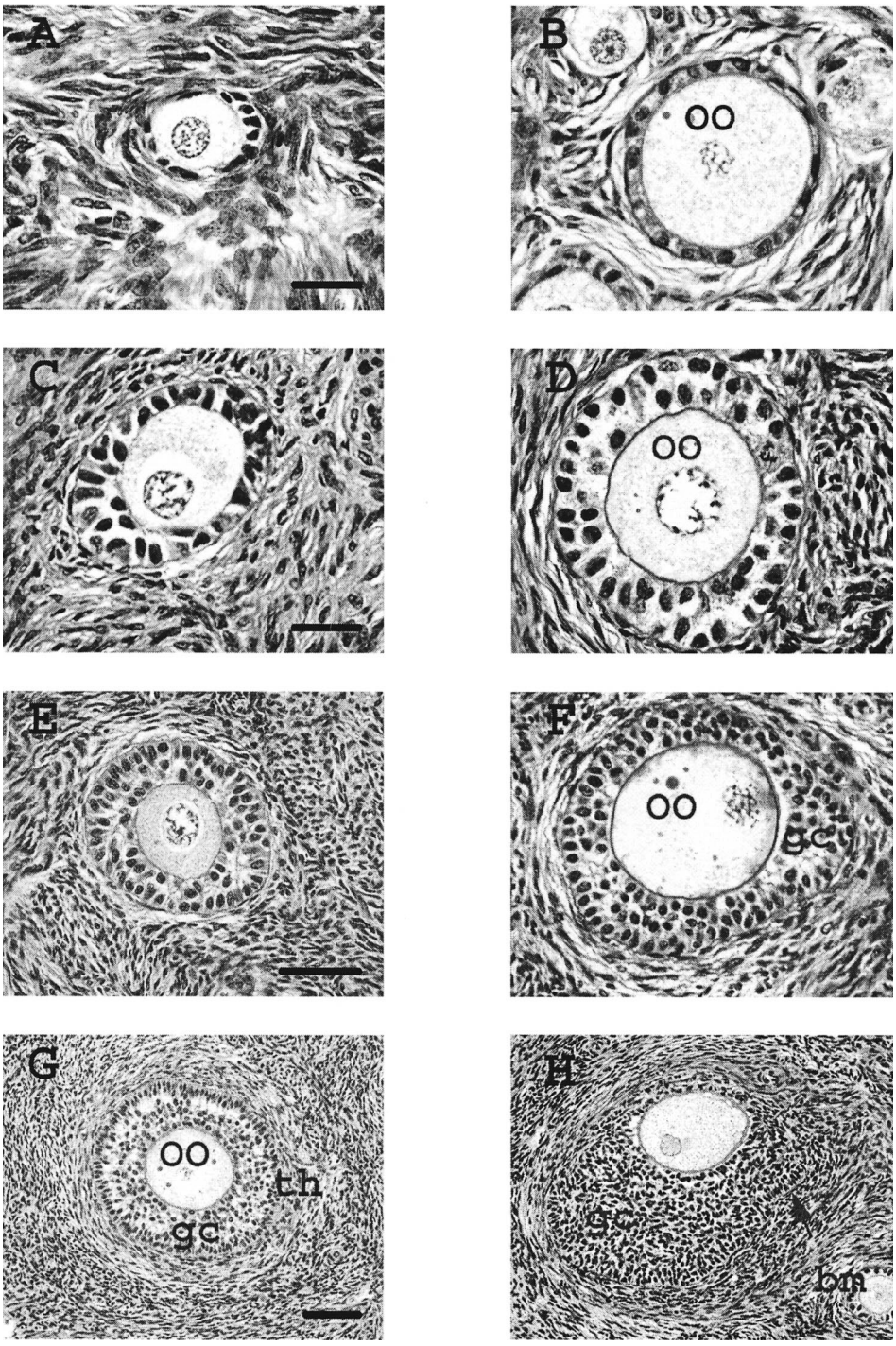

FIG. 7. Examples of normal and abnormal preantral ovarian follicles in ewes treated with KLH alone (**A**, **C**, **E**, **G**) or with KLH-BMP15 mature protein (**D**, **H**), KLH-GDF9 peptide (**B**), or KLH-BMP15 peptide (**F**). **A**) Normal type 1a follicle; **B**) abnormal type 1a follicle with enlarged oocyte (oo); **C**) normal type 2 follicle; **D**) abnormal type 2 follicle with enlarged oocyte (oo); **E**) normal type 3 follicle; **F**) abnormal type 3 follicle with enlarged oocyte (oo) and irregular granulosa configuration (gc); **G**) normal type 4 follicle with well-defined oocyte (oo), granulosa cell (gc), and thecal cell (th) layers; and **H**) abnormal type 4 follicle with highly irregular arrangement of granulosa cells (gc) around the oocyte and highly irregular basement membrane (bm) and configuration of theca interna. **A**–**D**) Bars = 25 μm; **E**–**H**) bars = 50 μm.

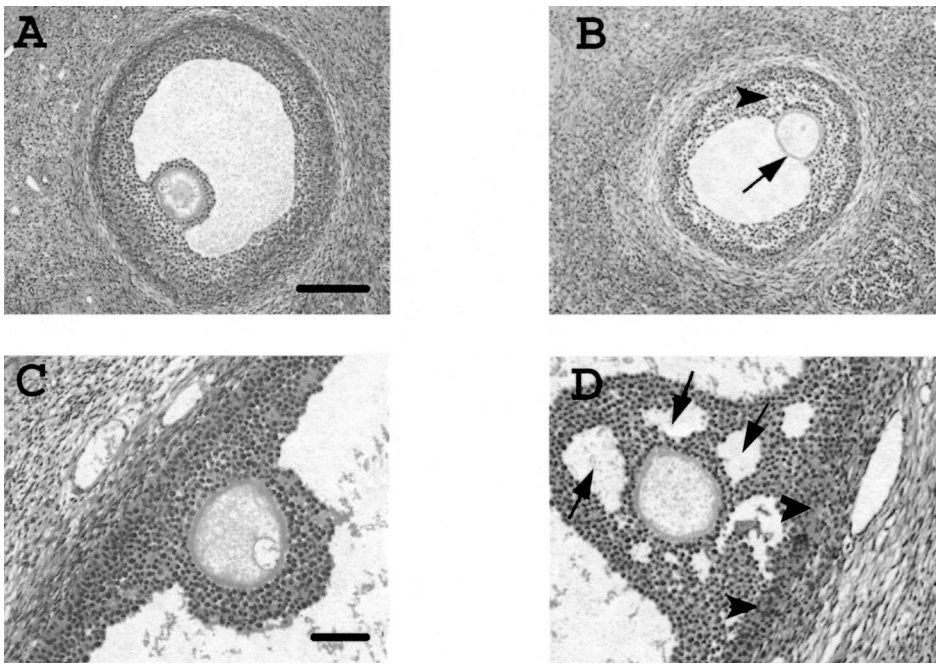

FIG. 8. Examples of normal and abnormal ovarian antral follicles in ewes treated with KLH alone (**A**, **C**) or with KLH-BMP15 peptide (**B**, **D**). **A**) Normal type 5 follicle; **B**) abnormal type 5 follicle with oocyte (arrowed) not completely surrounded by cumulus cells (arrow) and irregular spaces within the granulosa cell layers (arrow head); **C**) normal type 5+ follicle; and **D**) abnormal type 5+ follicle with large holes (arrow) in the cumulus cell arrangement around the oocyte and disorganized granulosa cell layer adjacent to the basement membrane (arrow head). **A**, **B**) Bar = 100 μm; **C**, **D**) bar = 50 μm.

< 0.01) than that observed in the control ewes (Fig. 9). Most of the BMP15 peptide-treated ewes established normal cyclical ovarian activity after a variable length of time. However, some irregularities such as shortened luteal phases were still observed. Moreover, two of the ewes had four CL in subsequent cycles, which was abnormally high, as the control ewes never ovulated more than two follicles per cycle.

DISCUSSION

Using highly specific antisera to either GDF9 or BMP15, we have established the presence of both proteins exclusively in oocytes, thus confirming the evidence from gene expression studies that the oocyte is the only intraovarian source of these growth factors [7, 30].

The major findings from these studies are that both GDF9 and BMP15 have effects on follicular growth and ovulation rate in sheep. The immunization of sheep against GDF9 peptide, BMP15 peptide, or oBMP15 mature protein arrested normal follicular development at the type 1a (transitory) or type 2 (primary) stage. These effects were specific for GDF9 or BMP15, as plasma from sheep immunized with the GDF9 peptide specifically recognized GDF9 and not BMP15 and vice versa. Even those animals immunized with the mature region of oBMP15, which shares considerable homology with GDF9, did not, in most instances, produce antibodies that recognized GDF9. These findings show that both GDF9 and BMP15 are essential for follicular development in sheep, in contrast with what is observed in mice [13].

Our results do not support the proposal of Yan et al. [13] that either GDF9 or BMP15, but not both, were essential for normal follicular development and that whichever protein was essential was dependent on the species, e.g., GDF9 in mice and BMP15 in sheep. Instead our results are consistent with the alternative suggestions raised by Galloway et al. [7] and Yan et al. [13] that both GDF9 and BMP15 are essential in sheep as homodimers or that it is the heterodimer of GDF9 and BMP15 that is the active protein in sheep. Although mice and human GDF9 and BMP15 have been speculated to form heterodimers in vitro [13], the forms of the GDF9 and BMP15 proteins in vivo are presently unknown for all species, including the sheep.

Neutralization of either GDF9 or BMP15 resulted in abnormal ovarian morphology, with many structures reminiscent of those observed in the GDF9KO mouse [6] or sheep lacking an active BMP15 gene [7, 31]. This abnormal morphology included the presence of enlarged oocytes sur-

TABLE 4. Effect of immunizing ewes against KLH, KLH-GDF9 peptide, KLH-BMP15 peptide, or KLH-oBMP15 mature protein on oocyte diameter (μm) with respect to follicular type. Values are geometric means (and 95% confidence limits).*

	Follicular type						
Treatment	1	1a	2	3	4	5	5+
KLH	24 (23, 25)[a] [57]	27 (26, 28)[a] [24]	37 (34, 40)[a] [41]	61 (59, 63)[a] [84]	85 (83, 87)[a] [155]	105 (102, 107)[a] [64]	107 (103, 111)[a] [22]
KLH-GDF9 peptide	24 (24, 25)[a] [83]	29 (27, 30)[a] [67]	49 (45, 52)[b] [111]	85 (78, 92)[c] [41]	99 (94, 104)[c] [36]	106 (100, 113)[a] [25]	127[†] [1]
KLH-BMP15 peptide	25 (25, 26)[a] [75]	31 (29, 33)[c] [63]	48 (46, 50)[b] [135]	80 (70, 92)[c] [31]	98 (91, 106)[c] [23]	115 (108, 123)[a] [20]	[0]
KLH-oBMP15 mature protein	25 (24, 25)[a] [73]	27 (26, 28)[a] [40]	48 (45, 51)[b] [103]	86 (84, 89)[c] [85]	98 (95, 102)[c] [94]	110 (108, 113)[a] [116]	112 (98, 128)[a] [6]

* Values in brackets refer to number of follicles measured. Values for each column with different alphabetical superscripts are different from one another: a versus b, $P < 0.05$; a versus c, $P < 0.01$.

[†] Excluded from analysis.

TABLE 5. Average ovulation rate (range) of ewes before and after treatment with KLH, KLH-GDF9 peptide, or KLH-BMP15 peptide antiplasma as measured by laparoscopy during the midluteal phase of the estrous cycle (four to five animals per treatment group).

	Days relative to administration of plasma (Day 0)				
Treatment	−1	13	30	45	63
KLH	1.3 (1–2)	1.5 (1–2)	1.3 (1–2)	2.0 (2)	1.0 (0–2)
KLH-GDF9 peptide	1.2 (1–2)	1.4 (1–2)	1.8 (1–3)	1.2 (1–2)	0.8 (0–1)
KLH-BMP15 peptide	1.0 (1)	0.2* (0–2)	1.6 (0–4)	2.3† (1–4)	1.4 (1–2)

* Signifies a value that is different from the control (KLH) value ($P < 0.05$).
† Data from four ewes only.

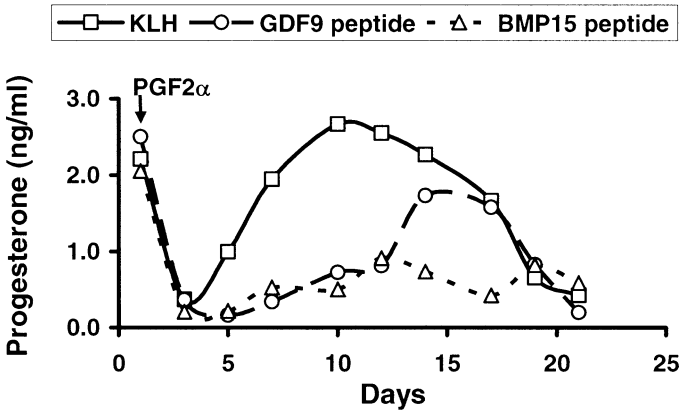

FIG. 9. Average concentrations of progesterone in plasma following synchronization of luteal regression. Ewes were administered 100 ml of KLH, KLH-GDF9 peptide (GDF9 peptide), or KLH-BMP15 peptide (BMP15 peptide) antiplasma i.v. 4 days before synchronization with Estrumate (i.e., $PGF_{2\alpha}$, arrowed).

rounded by a single layer of flattened and/or cuboidal granulosa cells and oocyte-free nodules containing remnants of zona pellucida. While follicles beyond the primary stage of development are not observed in sheep or mice lacking BMP15 or GDF9, respectively, larger abnormal follicles were observed in ovaries from the GDF9 peptide-, BMP15 peptide-, or oBMP15 mature protein-immunized sheep. These large follicles shared many of the abnormalities observed in the smaller follicles, including an oocyte abnormally large for the number of layers of granulosa cell, remnants of zona pellucida-like material with the membrana granulosa, and disorganization of the membrana granulosa or cumulus cells. The degree of abnormality observed in the follicles ranged from very subtle to the obvious, and those animals with more subtle abnormalities tended to be the ones with intraovarian luteal bodies or the normal-looking CL at the time of ovarian collection. These subtle abnormalities were often observed in the organization of the cumulus cells. It will be of interest to determine whether fecundity is compromised in animals that continue to ovulate after being immunized against GDF9 or BMP15.

Some ewes immunized against oBMP15 mature protein had estrous cycle(s) with increased ovulation rates com-

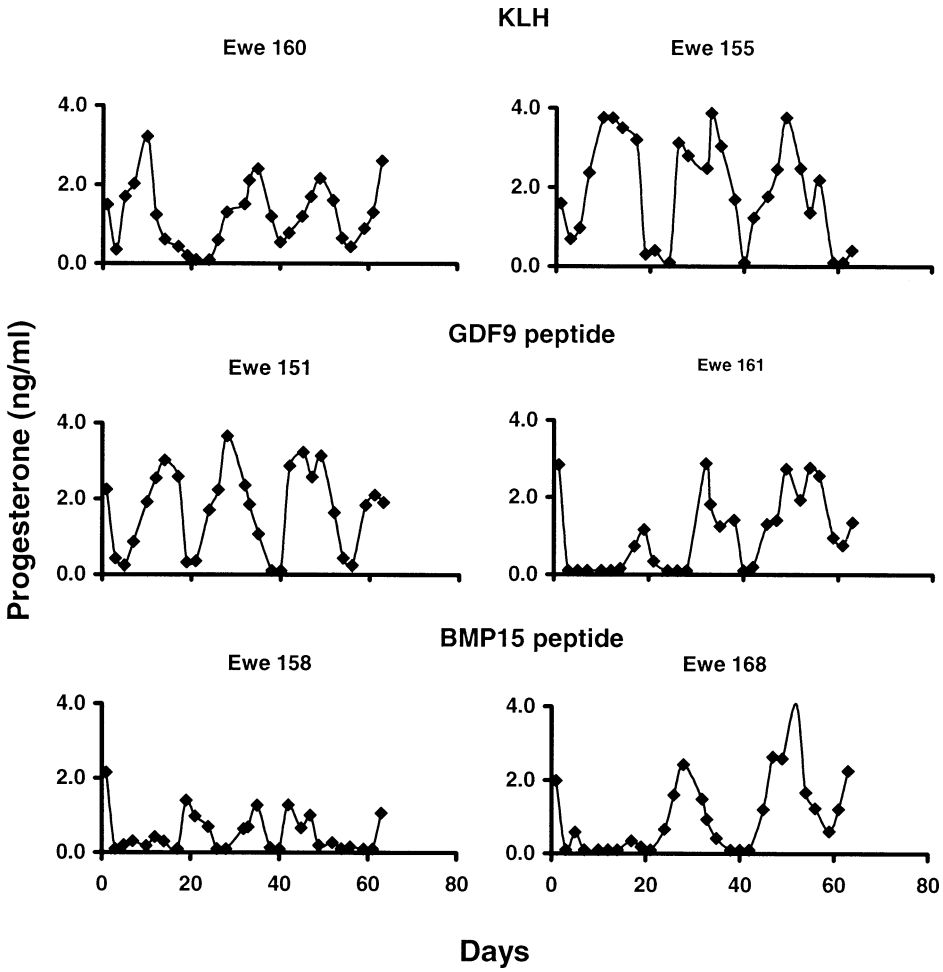

FIG. 10. Examples of the pattern of concentration of progesterone in plasma in passively immunized ewes. Antigens that the antiplasma samples were raised against are listed at the top of each graph. Both the GDF9 and BMP15 peptide antigens were conjugated to KLH. Day 0 = day of Estrumate administration. Normal cyclic progesterone concentrations were evident in the KLH antiplasma-treated ewes. In the ewes treated with GDF9 peptide antiplasma, two animals had normal cyclic progesterone concentrations (e.g., ewe 151) whereas the other three animals had a delayed and muted rise in progesterone followed by a normal cyclic pattern (e.g., ewe 161). In the ewes treated with BMP15 peptide antiplasma, two animals had delayed as well as severely muted progesterone concentrations over the treatment period (e.g., ewe 158), two animals had a severely delayed onset to cyclic progesterone concentrations (e.g., ewe 168), and another animal had normal cyclic progesterone concentrations.

pared with those immunized against KLH (i.e., control group). This is reminiscent of the increased ovulation rate observed in Inverdale and Hanna sheep that are heterozygous for a mutation in BMP15 [32, 33]. The increased ovulation rate observed in the heterozygous Inverdale or Hanna sheep has been hypothesized to be either a dosage effect of BMP15 protein (i.e., half of the amount of active BMP15) [7] or due to a dominant negative effect of the BMP15 propeptide interfering with the formation of GDF9 homodimers, BMP15 homodimers, and/or GDF9/BMP15 heterodimers [13]. Because antibodies directed against the mature region of the protein are able to mimic the increased ovulation rate observed in heterozygous Hanna and Inverdale sheep, it seems likely that the increased ovulation rate observed in these ewes is the result of a decrease in the amount of active BMP15 protein available, as proposed by Galloway et al. [7], and not to any dominant negative actions of the BMP15 proregion, as suggested by Yan et al. [13]. Phenotypically, the effect was more dramatic in the immunized sheep, with an increase in ovulation rate of approximately three more eggs per cycle versus an increase of only one egg in the ewes with a single copy of the Inverdale or Hanna mutation [32, 33]. This is probably due to neutralization of a different proportion of the active protein in the immunized sheep. Interestingly, those ewes immunized with the mature region of the protein seemed more likely to continue to cycle and have increased ovulation rates when compared with those immunized with the peptide. The reason for this is not known but may be due to the relative abilities of the antiplasmas to neutralize the protein. Possibly, antiplasma directed against the N-terminal region of the mature protein prevents binding of BMP15 to its receptor, whereas antibodies against other regions of the protein are less effective.

Insufficient evidence was obtained from this study to support a role for GDF9 in increasing ovulation rate in either the actively immunized or passively immunized ewes. Because multiple luteal structures were observed in the ovary of one ewe immunized with the GDF9 peptide, a possible role for GDF9 in regulating ovulation rate in sheep cannot be discounted but will require further study.

In addition to the effects of long-term immunization on follicular development, neutralization of GDF9 or BMP15 for a relatively short period of time also affected ovarian function. It seems that both GDF9 and BMP15 are important in regulating the later stages of follicular development as well as being essential during early follicular growth. While passive immunization against GDF9 did not affect ovulation rate, luteal function was abnormal. In vitro GDF9 has been shown to stimulate progesterone production and has been proposed to be the oocyte-derived factor responsible for maintaining cumulus cell function [1, 2, 12]. The results of the present study suggest that GDF9 has a more widespread function in regulating differentiation of mural granulosa cells, including the luteinization process as well. Most of the ewes treated with anti-BMP15 peptide plasma did not ovulate for an extended period of time; in the most severe cases, no antral follicles were observed at laparoscopy some 10 days following induction of luteolysis. Thus, it would seem that the later stages of follicular development are dependent on a continued paracrine role for BMP15. It is important to note that both GDF9 and BMP15 mRNA have been detected in the pituitary glands of some species [7, 34, 35], albeit at much lower levels than is observed in the ovary. In addition, several members of the TGFβ superfamily have been shown to regulate FSH secretion [36, 37]. Thus, the effects of neutralization of GDF9 or BMP15 on maturation of antral follicles could be direct and/or indirect through regulation of pituitary function.

In conclusion, immunoneutralization of either GDF9 or BMP15 severely interferes with normal follicular development in sheep. Furthermore, immunization against GDF9 or BMP15 affects ovulation rate and/or normal luteal function. Collectively, these findings support the notion that the oocyte-derived growth factors GDF9 and BMP15 have different biological roles in mammals with low or high ovulation rate phenotypes. They also support the hypothesis that, in mammals with a low ovulation rate phenotype, both GDF9 and BMP15 proteins are essential for normal ovarian follicular development.

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