BIOLOGY OF REPRODUCTION **60,** 381–386 (1999)

Molecular Cloning of the Ovine Growth/Differentiation Factor-9 Gene and Expression of Growth/Differentiation Factor-9 in Ovine and Bovine Ovaries[1]

K.J. Bodensteiner, C.M. Clay, C.L. Moeller, and H.R. Sawyer[2]

Animal Reproduction and Biotechnology Laboratory, Department of Physiology, Colorado State University, Fort Collins, Colorado 80523

ABSTRACT

Recently a novel member of the transforming growth factor β (TGFβ) superfamily termed growth/differentiation factor-9 (GDF-9) was shown to be expressed in ovaries of mice and humans, and to be essential for normal follicular development beyond the primary (type 2) follicle stage in mice. In the present study, the gene for ovine GDF-9 was isolated and characterized, and expression of GDF-9 mRNA in ovaries of domestic ruminants was examined. The predicted amino acid sequence of ovine GDF-9 is 77% and 66% homologous to human and mouse GDF-9, respectively. Specific hybridization using homologous ^{35}S-antisense probes was restricted to oocytes. In contrast to similar studies in mice in which GDF-9 was first detected beginning at the primary (type 2) follicle stage, in ovine and bovine ovaries GDF-9 mRNA was expressed beginning at the primordial (type 1) follicle stage. The observed timing and pattern of GDF-9 expression in oocytes of domestic ruminants is consistent with a role for GDF-9 in the initiation and maintenance of folliculogenesis in these species, and supports the general concept that early stages of follicular growth and development are regulated by intraovarian factors.

INTRODUCTION

Ovarian folliculogenesis is a complex process dependent upon the intricate interplay of numerous hormones and growth factors that regulate the growth and differentiation of oocytes, and granulosa and theca cells. After antrum formation, follicular growth and ovulation are acutely dependent on gonadotropin support. Although there is considerable information on the role of gonadotropins in the final stages of follicular development (for review see [1–3]), the signal(s) and mechanism(s) responsible for the initiation of follicular growth are unknown. In fact, elucidation of the factor or factors responsible for the initiation of folliculogenesis remains one of the major unsolved problems of ovarian physiology [4]. Initiation of follicular growth involves the transformation of primordial follicles from a quiescent, growth-arrested state to a growth-committed state. This process is characterized by differentiation and proliferation of granulosa cells and by enlargement of the oocyte [4, 5]. Once the transition into the growth phase has begun, follicles are ''committed,'' and follicular growth proceeds until the follicle is ovulated or undergoes atresia [6]. Since follicular development up to and including early antrum formation is not affected in hypophysectomized ewes [7, 8], it appears that the transition from a growth-arrested to a growth-committed state is not directly regulated by gonadotropins. Further, even though bioactive FSH is present in fetal sheep plasma beginning at Day 55 of gestation, follicular growth does not begin until around Day 90 of gestation [9]. In addition, FSH receptor mRNA is not expressed by granulosa cells until the 1–2-layer (primary) follicle stage; i.e., after follicles have begun to grow [10]. Therefore, it appears that there may be an intragonadal signal that stimulates and sustains early follicular development independent of gonadotropins (for review see [11]).

Among the key regulators of growth and differentiation in a number of tissues are members of the transforming growth factor β (TGFβ) superfamily. Members of this superfamily bind to serine/threonine kinase receptors and influence a number of differentiation processes including hematopoiesis, adipogenesis, myogenesis, and epithelial cell differentiation. In addition, several of the TGFβ family members, including inhibin, activin, and Müllerian inhibitory substance are known to play important roles in reproduction. Recently, a novel member of the TGFβ superfamily termed growth/differentiation factor-9 (GDF-9) was shown to be essential for normal follicular development beyond the primary follicle stage in mice [12]. This novel growth factor has been shown to be expressed in human and mouse ovaries, and appears to be localized exclusively to oocytes at all stages of follicular growth except primordial follicles in neonatal and adult mice [13]. Taken together, these observations suggest that GDF-9 is essential for normal folliculogenesis and may be important in the regulation of early follicular and oocyte growth. Because of the potential regulatory role of GDF-9 in folliculogenesis, the purpose of the present study was to clone the ovine gene for GDF-9 and to characterize the temporal and spatial expression of GDF-9 within the ruminant ovary.

MATERIALS AND METHODS

PCR and Analysis of Amplified Products

Genomic DNA fragments of ovine and bovine GDF-9 were isolated by polymerase chain reaction (PCR) using primers directed against homologous sequences of human and mouse GDF-9 exon 2 (5′-TAGTCAGCTGAAGTGGGACA-3′ and 5′-ACGACAGGTGCACTTTGTAG-3′). PCR was carried out using 100 ng genomic DNA and a temperature gradient of 46–60°C on a RoboCycler (Stratagene, La Jolla, CA; 2.25 mM $MgCl_2$; 40 cycles). PCR resulted in the amplification of a product of the expected size (277 base pairs [bp]) for both species. The identity of the products was confirmed by Southern analysis (described below). Since results indicated that GDF-9 was present, the remaining PCR products resulting from the optimal annealing temperatures (ovine = 58°C; bovine = 60°C) were separated by electrophoresis in a 1.2% agarose gel and isolated with the Qiaex II Agarose Gel Extraction Kit (Qiagen Inc., Valencia, CA). Gel-isolated DNA was reamplified with 20

Accepted September 18, 1998.
Received July 17, 1998.
[1]Supported by the Colorado State University Agricultural Experiment Station. K.J.B. was supported by the Colorado Institute for Research in Biotechnology, J.H. Venable Memorial Scholarship, and the Colorado State University Graduate Fellowship Award.

[2]Correspondence: H.R. Sawyer, Animal Reproduction and Biotechnology Laboratory, Foothills Campus, Colorado State University, Fort Collins, CO 80523. FAX: 970 491 3557; e-mail: hsawyer@cvmbs.colostate.edu

Downloaded from https://academic.oup.com/biolreprod/article/60/2/381/2741103 by guest on 20 August 2022

cycles of PCR at the appropriate temperatures (58 or 60°C), and fragments were subcloned into PCR 2.1 (TA Cloning Kit; Invitrogen, Carlsbad, CA). Plasmid DNA was isolated (PRIME Kit; Intermountain Scientific Corporation, Kaysville, UT) and sequenced (Macromolecular Resources, Fort Collins, CO). Nucleotide and amino acid sequences were compared with published sequences for mouse [14, 15] and human [13] GDF-9 using the GeneWorks algorithm (IntelliGenetics Inc., Mountain View, CA).

Clone Isolation and Sequencing

To isolate the gene for ovine GDF-9, an ovine genomic library constructed in λEMBL-3 (Clontech, Palo Alto, CA) was screened by phage plaque hybridization using the 277-bp fragment generated by PCR amplification of ovine genomic DNA as the radioactive probe [16]. Plaques that tested positive after the primary screen were isolated, and eluted phages were subjected to sequential screening until plaque-purified. Library screening resulted in the isolation of three potential ovine GDF-9 genomic clones, all of which were similar based on restriction mapping. One of the clones was arbitrarily selected, and a 5.3-kilobase (kb) *Bam*HI fragment that hybridized to both exon 1- and exon 2-specific probes was subcloned into the pBS/KS vector (Stratagene). Plasmid DNA was characterized by restriction mapping and Southern hybridizations to exon 1 and exon 2 specific probes. A 2.1-kb *Bam*HI/*Pst*I fragment containing part of exon 1 and a 1.5-kb *Pst*I fragment containing part of exon 2 were isolated and subcloned. To determine the sequences of the remaining portions of exons 1 and 2 and the intron, primers were designed to known segments of exon 1 and 2 (5′-CTTCTAGGGGAGAAGCTCAG-3′; and 5′-TCAGCAGCTTCTTCTCCCAC-3′) and used to sequence the original 5.3-kb insert. Nucleotide and amino acid sequences were compared with published sequences for mouse [14, 15] and human [13] GDF-9 with the GeneWorks algorithm. The sequence of the 5.3-kb *Bam*HI fragment was submitted to GenBank as ovine GDF-9 (accession number: AF078545; submitted July 15, 1998). Exon/intron boundaries were predicted on the basis of homology to human and mouse sequences and the BCM Gene Finder algorithm (Baylor College of Medicine, Houston, TX).

Southern Analyses

To ensure that no deletions or rearrangements had been introduced during cloning, restriction mapping and Southern analyses comparing the 5.3-kb insert to ovine genomic DNA were conducted. Plasmid DNA (1 μg) and ovine genomic DNA (10 μg) were digested with *Bam*HI, *Pst*I, *Nco*I, *Sac*I, and *Bgl*I restriction endonucleases alone or in combination. After electrophoresis in a 1.0% agarose gel, DNA was transferred to a nylon membrane (Hybond; Amersham Life Science, Buckinghamshire, England) via capillary action. Membranes were hybridized to α-[^{32}P]dCTP-labeled exon 1 or exon 2 specific probes at 42°C overnight. After hybridization, nonspecific binding was removed by sequential washes in SSC (highest stringency was 0.5-strength SSC [single-strength SSC = 0.15 M sodium chloride, 0.015 M sodium citrate]; 0.1% SDS at 42°C). Membranes were placed on film for 3 days. Approximate locations of restriction enzyme sites located outside of the subcloned *Bam*HI fragment were determined by restriction mapping and Southern analyses of the complete λ genomic clone.

Classification of Follicles

Criteria used to classify ovine [17] and bovine [4] follicles into specific developmental stages included the number and appearance of granulosa cells, layers of granulosa cells, and follicle/oocyte diameters. Although there are minor differences between the ovine and bovine classification systems with respect to follicle and oocyte diameter and the number of layers of granulosa cells, five well-defined stages from primordial to early antral stages are recognized. Primordial follicles are classified as type 1 follicles and are characterized by a single layer of squamous granulosa cells. Type 1A follicles are also characterized by a single layer of granulosa cells, but one or more of the granulosa cells surrounding the oocyte is cuboidal. Type 1A follicles are thought to represent a transition stage from a quiescent, growth-arrested stage into an active growth-committed stage. The granulosa layers of follicles classified as types 2–5 consist of cuboidal granulosa cells. Type 2 follicles are commonly referred to as primary follicles and have < 2 layers of cuboidal granulosa cells. Characteristics of subsequent stages using this classification scheme are as follows: type 3, small preantral follicles with 2 to < 4 layers of granulosa cells; type 4, large preantral follicles with 4–6 layers of granulosa cells; and type 5, small antral follicles with > 5 layers of granulosa cells and an antral cavity. Because of the thickness (15–20 μm) of the sections used in the present study and the small diameter of type 1 and type 1A follicles, it was not possible to hybridize the same follicles to antisense probe in one section and sense probe in the adjacent section. However, serial sections were available for type 2–5 follicles, and hybridization of ^{35}S-antisense and ^{35}S-sense GDF-9 riboprobes in these follicles were compared using adjacent sections.

In Situ Hybridization

Genomic fragments (277 bp) representing coding sequences in exon 2 of the ovine and bovine GDF-9 genes were isolated from PCR 2.1 (Invitrogen) by digestion with *Xba*I and *Bam*HI, and ligated into the *Xba*I and *Bam*HI sites in pBS/KS (Stratagene). Plasmid DNA was digested either with *Bam*HI (T7; antisense) or *Xba*I (T3; sense) restriction endonucleases for the production of riboprobes. Distribution and relative abundance of GDF-9 mRNA was examined using in situ hybridization as previously described [18]. Frozen sections of ovine and bovine ovaries embedded in OCT were cut at a thickness of 15 or 20 μm, fixed for 5 min in 4% paraformaldehyde, washed twice in double-strength SSC for 5 min, rinsed in distilled water, dipped in triethanolamine (0.1 M, pH 8.0), incubated in 0.25% acetic anhydride in 0.1 M triethanolamine 10 min, dipped in double-strength SSC, dehydrated through graded alcohols (50–100%), and air-dried. After linearization of plasmid, antisense and sense [^{35}S]UTP-labeled riboprobes were generated using T7 and T3 promoters. Sixty microliters (2×10^7 cpm/ml) of RNA probe in hybridization buffer (for 40 ml: 25 ml deionized formamide, 3.75 ml 4 M NaCl, 0.5 ml 1 M Tris pH 8.0, 1 ml 50-strength Denhardt's solution, 10 ml 50% dextran sulfate, 0.75 ml double-distilled H_2O, and 200 mM dithiothreitol) was added to each section and covered with a glass coverslip. Sections were incubated overnight at 45°C in a humidified chamber. After hybridization, coverslips were removed by soaking in double-strength SSC. Sections were then washed in double-strength SSC for 10 min, incubated in a ribonuclease (RNase)-A solution (Sigma Chemical, St. Louis, MO; 600 μl

Downloaded from https://academic.oup.com/biolreprod/article/60/2/381/2741103 by guest on 20 August 2022

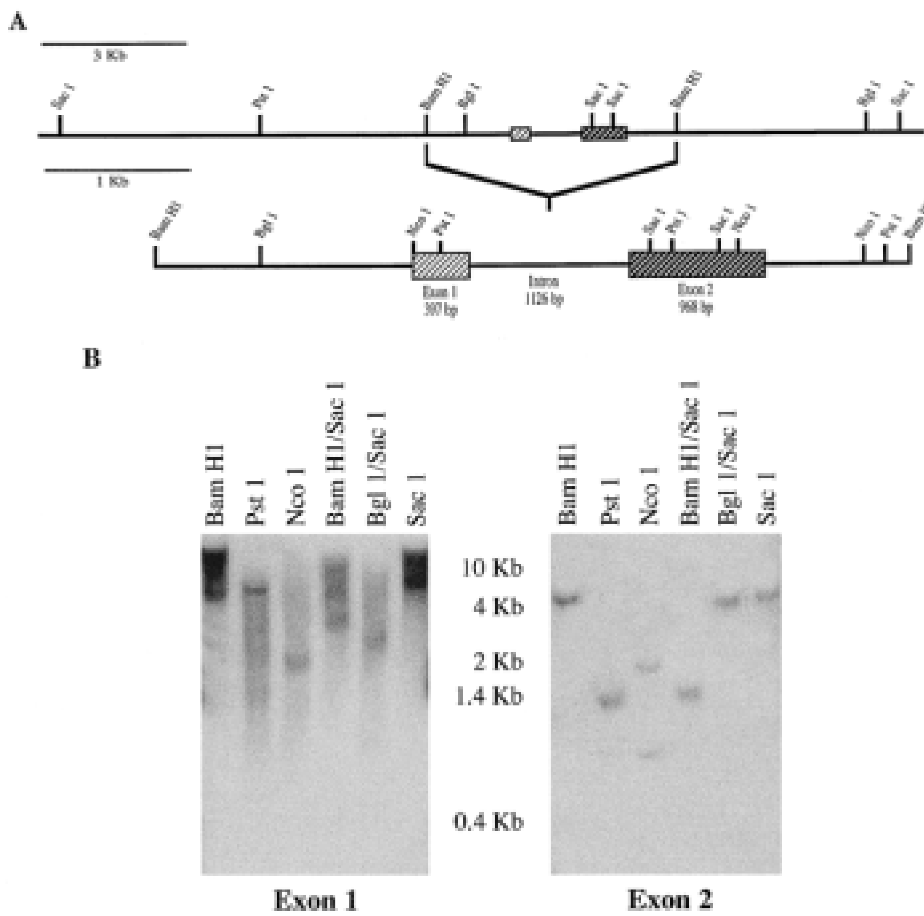

FIG. 1. Analyses of the ovine genomic clone. **A**) Map of the ovine λ genomic clone illustrating known restriction enzyme sites. A detailed map of the 5.3-kb *Bam*HI fragment is also presented. Location and sizes of exon 1 and 2 and the intron are illustrated. **B**) Detection of ovine GDF-9 in ovine genomic DNA by Southern analyses. The migration pattern of the molecular weight markers is shown in the center of the Southern blots (GenBank accession number: AF078545).

Downloaded from https://academic.oup.com/biolreprod/article/60/2/381/2741103 by guest on 20 August 2022

10 mg/ml RNase-A into 300 ml double-strength SSC) at 37°C for 30 min, washed in double-strength SSC for 10 min, washed in stringent wash solution (0.1-strength SSC, 0.09% β-mercaptoethanol, 1 mM EDTA) at 55°C for 2 h, and rinsed twice in 0.5-strength SSC for 10 min. Sections were dehydrated through graded ethanol-sodium acetate solutions (50–100% ethanols; 0.3 M NH_4OAc) and air-dried. Slides were then coated with emulsion (type NTB2; Eastman Kodak, Rochester, NY) and stored for 32 days at 4°C. The emulsion was developed, and tissue sections were stained with hematoxylin and eosin and examined using both brightfield and darkfield optics. The experiment was performed twice using sections from ovaries obtained from adult nonpregnant ewes (n = 2) and cows (n =4). Ovaries used in each experiment were from different animals and were processed on separate occasions.

RESULTS

Molecular Cloning of the Ovine GDF-9 Gene

Amplification of ovine and bovine genomic DNA with primers directed against homologous sequences of human and mouse GDF-9 exon 2 resulted in the isolation of a 277-bp fragment for both species. Nucleotide and amino acid sequences of the isolated fragments demonstrated 96% homology between species. In addition, sequences of the 277-bp fragments were approximately 91% and 83% homologous to human and mouse GDF-9, respectively. The genomic fragments corresponded to amino acids 362–454 of the predicted human sequence [13] and 349–441 of the predicted mouse sequence [14, 15].

The entire coding sequence of the ovine GDF-9 gene was isolated in a 5.3-kb *Bam*HI fragment from a single λ clone (Fig. 1A). Like the human and mouse genes, ovine GDF-9 spans approximately 2.5 kb and contains 2 exons and 1 intron. Exon 1 spans 397 bp and encodes for amino acids 1–134 while exon 2 spans 968 bp and encodes for amino acids 135–456. The single intron spans 1126 bp. The hybridization pattern for ovine genomic DNA (Fig. 1B) was consistent with that predicted by sequence analysis of the recombinant λ clone. Prominent hybridizing DNA fragments were observed at 5.3 kb with *Bam*HI, 5.5 kb with *Pst*I, 2.9 kb with *Nco*I, 3.5 kb with *Bam*HI/*Sac*I, 2.7 kb with *Bgl*I/*Sac*I, and 11.2 kb with *Sac*I for exon 1. Prominent hybridizing fragments were observed at 5.3 kb with *Bam*HI, 1.5 kb with *Pst*I, 2.9 kb and 0.9 kb with *Nco*I, 1.3 kb with *Bam*HI/*Sac*I, 5.3 kb with *Bgl*I/*Sac*I, and 6.0 kb with *Sac*I for exon 2. Predicted amino acid sequences are 77% and 66% homologous to human and mouse GDF-9, respectively (Fig. 2). In addition, there are four potential *N*-glycosylation sites and six cysteine residues that appear to be conserved across species (Fig. 2).

Expression of GDF-9 mRNA in Ovaries of Domestic Ruminants

In situ hybridization was used to determine the distribution and relative abundance of mRNA for ovine and bovine GDF-9. The distribution of silver grains over the sections of ovine and bovine ovaries incubated in the presence of the ^{35}S-radiolabeled antisense probe appeared to be oocyte-specific (Fig. 3). Specific hybridization was not evident in any other cell type, including granulosa and theca cells. Oocytes in follicles from the primordial follicle stage

FIG. 2. Comparison of the predicted amino acid sequences for ovine, human, and murine GDF-9. Numbers indicate amino acid positions relative to the amino termini. Amino acids in boldface type are different between species. Dashes indicate a gap in sequence between species. Boxed sequences indicate the four potential *N*-glycosylation sites and six cysteine residues that are conserved across species.

```
Ovine   MALPNKFFLW FCCFAWLCFP ISLDSLPSRG EAQIVARTAL ESEAETWSLL   50
Human   MARPNKFLLW FCCFAWLCFP ISLGSQASGG EAQIAASAEL ESGAMPWSLL   50
Murine  MALPSNFLLG VCCFAWLCFL SSLSSQASTE ESQSGASENV ESEADPWSLL   50

Ovine   NHLGGRHRPG LLSPLLEVLY DGHGEPPSCS QDDRALRYMK KLYKAYATKE   100
Human   QHIDERDRAG LLPALFKVLS VGRGGSPRLQ PDSRALHYMK KLYKTYATKE   100
Murine  LPVDGTDRSG LLPPLFKVLS DRRGETPKLQ PDSRALYYMK KLYKTYATKE   100

Ovine   GTPKSNRRHL YNTVRLFTPC AQHKQAPGDL AARTFPSVIL LFNLNRVTVV   150
Human   GIPKSNRSHL YNTVRLFTPC TRHKQAPGDQ VTGILPSVEL LFNLDRITTV   150
Murine  GVPKPSRSHL YNTVRLFSPC AQQEQAPSNQ VTGPLPMVDL LFNLDRVTAM   150

Ovine   EHLFKSVLLY TFNNSISFPF PVKCICNLVI KEPEFSSKTL PRAPYSFTYN   200
Human   EHLLKSVLLY NINNSVSFSS AVKCVCNLMI KEPKSSSRTL GRAPYSFTFN   200
Murine  EHLLKSVLLY TLNNSASSSS TVTCMCDLVV KEAMSSGRAP PRAPYSFTL-   199

Ovine   SQFEFRKKYK WMEIDVTAPL EPLVASHKRN IHMSVNFTCA EDQLQHPSAR   250
Human   SQFEFGKKHK WIQIDVTSLL QPLVASNKRS IHMSINFTCM KDQLEHPSAQ   250
Murine  ------KKHR WIEIDVTSLL QPLVTSSERS IHLSVNFTCT KDQVP----E   239

Ovine   DSLFNMTLLV APSLLLYLND TSAQAFHRWH SLHPKRKPSQ GPDQKRGLSA   300
Human   NGLFNMTLV- SPSLILYLND TSAQAYHSWY SLHYKRRPSQ GPDQERSLSA   299
Murine  DGVFSMPLSV PPSLILYLND TSTQAYHSWQ SLQSTWRPLQ HPGQA-GVAA   288

Ovine   YPVGEEAAEG VRSSRHRQRA KE-SASSELK KPLVPASVNL SEYFKQFLFP   349
Human   YPVGEEAAED GRSSHHRHRR GQETVSSELK KPLGPASFNL SEYFRQFLLP   349
Murine  RPVKEEATEV ERSP--RRRR GQKAIRSEAK GPLLTASFNL SEYFKQFLFP   336

Ovine   QNECELHDFR LSFSQLKWDN WIVAPHKYNP RYCKGDCPRA VGHRYGSPVH   399
Human   QNECELHDFR LSFSQLKWDN WIVAPHRYNP RYCKGDCPRA VGHRYGSPVH   399
Murine  QNECELHDFR LSFSQLKWDN WIVAPHRYNP RYCKGDCPRA VRHRYGSPVH   386

Ovine   TMVQNIIHEK LDSSVPRPSC VPAKYSPLSV LSIEPDGSIA YKEYEDMIAT   449
Human   TMVQNIIYEK LDSSVPRPSC VPAKYSPLSV LTIEPDGSIA YKEYEDMIAT   449
Murine  TMVQNIIYEK LDPSVPRPSC VPGKYSPLSV LTIEPDGSIA YKEYEDMIAT   436

Ovine   KCTCR      454
Human   KCTCR      454
Murine  RCTCR      441
```

(type 1) onward were labeled (Fig. 3, A–C and G and H). Specific labeling was not observed in sections incubated with ^{35}S-radiolabeled sense probe (Fig. 3, D–F and I).

DISCUSSION

Although considerable information exists on the role of gonadotropins in the final stages of follicular development, the signal(s) and mechanism(s) involved in the initiation and maintenance of gonadotropin independent folliculogenesis are unknown. Perhaps one of the more interesting developments in this area of ovarian physiology is the isolation of GDF-9, a member of the TGFβ superfamily that is essential for early follicular growth in mice [12]. Unfortunately, there is little information on the presence or function of GDF-9 in domestic ruminants. Herein, we report the isolation of the first ruminant GDF-9 genomic clone and characterization of GDF-9 gene expression in the ruminant ovary.

Consistent with the reported sequences for the human [13] and mouse [14, 15] GDF-9 genes, the ovine gene for GDF-9 consists of two exons separated by a single intron. Exon 1 spans 397 bp and exon 2 spans 968 bp. Exon sizes correlate well with those reported for the human [13] and mouse [14, 15]. Amino acid sequence for the ovine GDF-9 gene is approximately 77% homologous to human and 66% homologous to mouse with increased homology in the mature portion of the gene. In addition, there are four potential *N*-glycosylation sites and six cysteine residues that appear to be conserved across species. Isolation of the ovine GDF-9 gene and knowledge of its structure will allow for further studies into the regulation of GDF-9 gene expression within the ruminant ovary.

To begin to address this issue, we used in situ hybridization to examine the distribution and relative abundance of GDF-9 mRNA in ovine and bovine ovarian tissue. Specific hybridization using the antisense probe was localized exclusively to the oocytes of both species. The oocyte-specific expression of GDF-9 in the ovaries of sheep and cattle was similar to that reported for mice [13, 19]. However, antisense hybridization was present in ovine and bovine follicles at all stages of development, including type 1 (primordial) follicles. This is in contrast to results for mice in which initiation of GDF-9 expression was reported to coincide with the formation of type 2 (primary) follicles [13, 19]. The discrepancy with respect to the onset of GDF-9 expression may be due to differences in follicular classification criteria, to the sensitivity of the in situ hybridization system used, or to species differences.

Oocyte-specific expression of GDF-9 mRNA in the ovaries of a number of species indicates that this growth factor may be important in the regulation of folliculogenesis and oocyte growth. In fact, targeted deletion of GDF-9 exon 2 creates a block to normal folliculogenesis in transgenic mice at the primary follicle stage, which, in turn, leads to

Downloaded from https://academic.oup.com/biolreprod/article/60/2/381/2741103 by guest on 20 August 2022

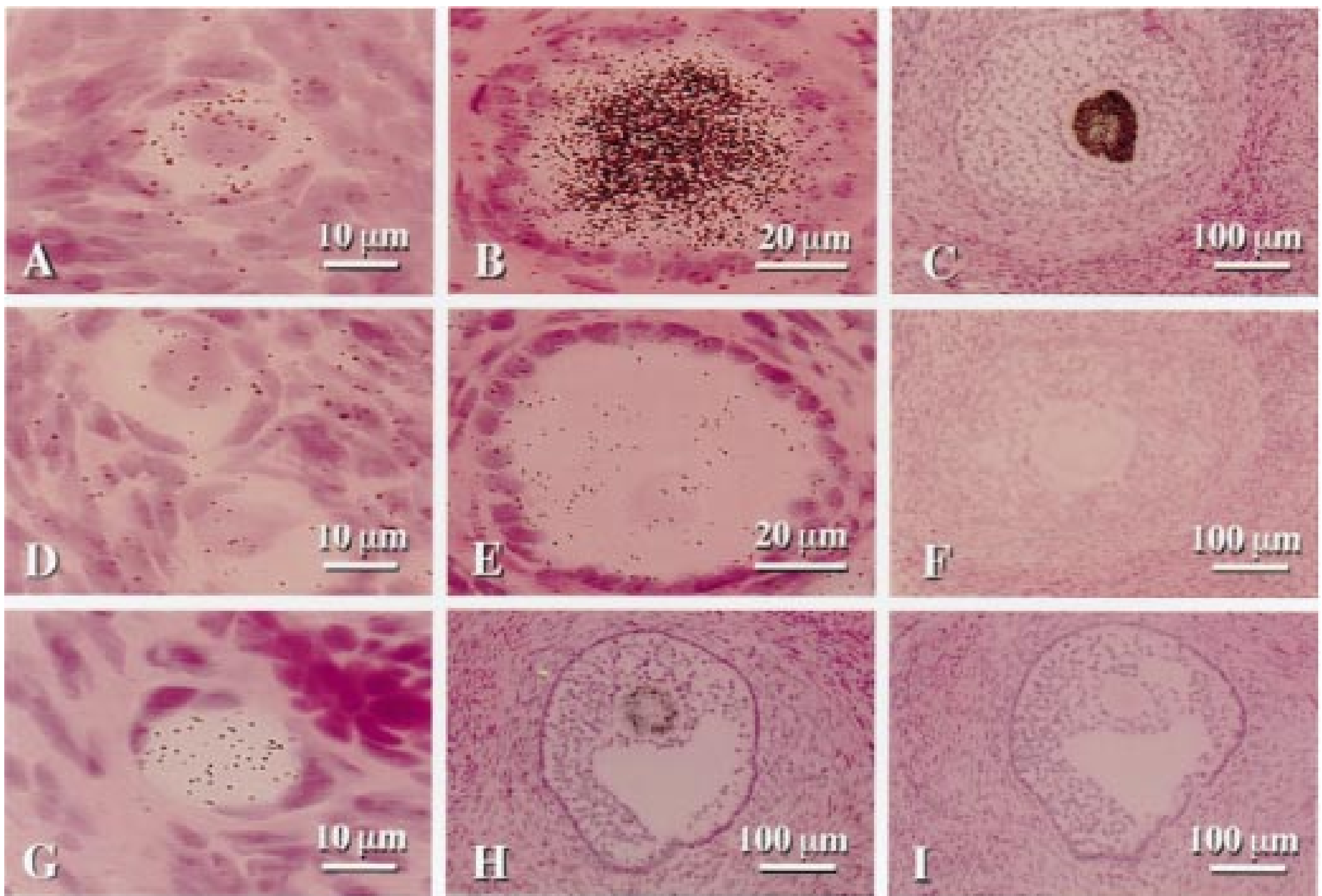

FIG. 3. In situ localization of mRNA for GDF-9 in oocytes present in ovine and bovine follicles. **A–C**) Ovine follicles from the same section hybridized to ovine GDF-9 ^{35}S-antisense probe: **A**) ovine type 1 follicle; **B**) ovine type 2 follicle; **C**) ovine type 4 follicle. **D–F**) Adjacent serial section incubated in the presence of ovine GDF-9 ^{35}S-sense probe: **D**) ovine type 1 follicles; **E**) ovine type 2 follicle; **F**) ovine type 4 follicle. **G**) Bovine type 1 follicle hybridized to bovine GDF-9 ^{35}S-antisense probe. **H**) Bovine type 5 follicle, antisense probe. **I**) Adjacent serial section of the bovine type 5 follicle incubated in the presence of bovine GDF-9 ^{35}S-sense probe.

complete infertility [12]. Although oocytes from the follicles of these mice reached normal size, they rarely contained cortical granules and did not achieve meiotic competence. In addition, granulosa cells surrounding these follicles appeared abnormal and theca layers were absent [12]. Thus, it appears that GDF-9 is essential for the maintenance of normal follicular growth and oocyte development soon after the transition into a growth-committed state. Since GDF-9 mRNA expression is restricted to oocytes, it appears that early folliculogenesis and oocyte growth are not only regulated by factors from the somatic cells of the ovary, but by factors from the oocyte itself. However, the biological function(s) and site(s) of action of GDF-9 remain unknown.

The pattern of GDF-9 expression and results from GDF-9 gene ablation studies in mice suggest that GDF-9 may play an autocrine role in the regulation of oocyte development and maturation and/or a paracrine role in the regulation of granulosa cell differentiation and proliferation. An autocrine and/or paracrine role for GDF-9 within the ovary is further supported by the presence of a putative signal sequence for secretion [13]. GDF-9 has also been shown to be expressed after ovulation and up to 1.5 days after fertilization [13]. This continued expression implies an additional role of GDF-9 in fertilization and early embryonic development. GDF-9 was also recently shown to be expressed in a variety of nongonadal tissues, which suggests possible roles for GDF-9 outside of the ovary [19]. However, expression of GDF-9 within the ovary is cell-specific and is necessary for normal follicular and oocyte development. Therefore, although GDF-9 may have physiological roles in other tissues, it is critical to understand the role of GDF-9 in cellular differentiation and/or proliferation within the ovary. Further research into the site(s) of action of GDF-9 within the mammalian ovary is needed to determine the precise role of GDF-9 in the initiation and regulation of folliculogenesis.

The potential role of a number of growth factors and hormones and their receptors on the initiation of folliculogenesis has been examined (for review see [17]). In contrast to GDF-9, the majority of these factors are expressed after follicles have entered the growth phase, i.e., after the transition from type 1 (primordial) to type 2 (primary) follicles. As discussed above, the oocyte specificity and timing of GDF-9 expression indicate that this growth factor may be important in the initiation of folliculogenesis. To our knowledge, the tyrosine kinase receptor c-*kit* and its ligand, stem cell factor, are the only other factors expressed by cells in the ovary during this critical stage of development that have been shown to play a role in folliculogenesis. Expression of c-*kit* was detected in mouse oocytes starting at the diplotene stage [20] and in fetal sheep oocytes from the

Downloaded from https://academic.oup.com/biolreprod/article/60/2/381/2741103 by guest on 20 August 2022

primordial follicle stage onward [21]. Mutations in the c-*kit* receptor lead to varying degrees of infertility in mice and have been shown to affect gametogenesis (for review see [22]). In addition, blockage of c-*kit* function via the administration of an anti-c-*kit* antibody, ACK2, prevented the onset of follicular growth [23]. Further, the ligand for the c-*kit* receptor, stem cell factor, appears to be necessary for mouse primordial germ cell survival in culture [24] and is expressed in granulosa cells at all stages of follicular development, from primordial through antral follicles in sheep [25]. However, the presence of stem cell factor alone did not induce proliferation of mouse primordial germ cells in culture; and it was suggested that perhaps stem cell factor was involved in regulating a survival signal rather than a proliferation signal [24, 26]. Tisdall et al. [25] have proposed that stem cell factor may act as an inhibitor of apoptosis in granulosa cells and oocytes during follicular formation and early growth in sheep [25]. It is possible, therefore, that c-*kit* and stem cell factor act in combination with another factor to stimulate granulosa cell proliferation, oocyte growth, and follicular survival. For example, once the interaction between c-*kit* and its ligand had been achieved and primary follicles had formed, administration of ACK2 did not prevent further development [23]. In addition, c-*kit* is expressed only by oocytes, but administration of ACK2 prevents granulosa cell proliferation [23]. Since the stage of follicular growth disrupted by ACK2 administration is similar to that disrupted by the GDF-9 mutation in mice, GDF-9 may be a signal for granulosa cell proliferation derived from oocytes that is activated via stem cell factor and c-*kit* interaction [23]. The timing of GDF-9 expression observed in the present study indicates that it may play a role in oocyte growth and granulosa cell proliferation and differentiation. Thus, GDF-9, c-*kit*, and stem cell factor may act together to promote follicular growth and survival.

In summary, the members of the TGFβ superfamily influence a wide variety of growth and differentiation processes in a number of tissues and species. The newest member of this superfamily, GDF-9, appears to be no exception. In the present study, we report that the expression of GDF-9 is oocyte-specific in domestic ruminants. The oocyte specificity of GDF-9 mRNA expression is consistent with the concept that the oocyte itself may play a role in the regulation of follicular development and/or oocyte maturation.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Se-Jin Lee at John's Hopkins School of Medicine for generously providing the cDNAs for human and mouse GDF-9. Technical assistance provided by Ms. Bernadette Meberg and Dr. Scott Nelson is gratefully acknowledged.

REFERENCES

1. Richards JS. Maturation of ovarian follicles: actions and interactions of pituitary and ovarian hormones on follicular cell differentiation. Physiol Rev 1980; 60:55.
2. Fortune JE. Ovarian follicular growth and development in mammals. Biol Reprod 1994; 50:225–232.
3. Roche JF. Control and regulation of folliculogenesis—a symposium in perspective. Rev Reprod 1996; 1:19–27.
4. Braw-Tal R, Yossefi S. Studies in vivo and in vitro on the initiation of follicle growth in the bovine ovary. J Reprod Fertil 1997; 109:165–171.
5. Hirshfield AN. Development of follicles in the mammalian ovary. Int Rev Cytol 1991; 124:43–101.
6. Tsafriri A, Reich R, Abisogun AO. The ovarian egg and ovulation. In: Lamming GE (ed.), Marshall's Physiology of Reproduction, vol.3, 4th ed. London: Chapman and Hall; 1994: 3–58.
7. Dufour J, Cahill LP, Mauléon P. Short- and long-term effects of hypophysectomy and unilateral ovariectomy on ovarian follicular populations in sheep. J Reprod Fertil 1979; 57:301–309.
8. Driancourt MA, Fry RC, Clarke IJ, Cahill LP. Follicular growth and regression during the 8 days after hypophysectomy in sheep. J Reprod Fertil 1987; 79:635–641.
9. Smith P, O W-S, Hudson NL, Shaw L, Heath DA, Condell L, Phillips DJ, McNatty KP. Effects of the Booroola gene (Fec^B) on body weight, ovarian development and hormone concentrations during fetal life. J Reprod Fertil 1993; 98:41–54.
10. Tisdall DJ, Wantanabe K, Hudson NL, Smith P, McNatty KP. FSH receptor gene expression during ovarian follicle development in sheep. J Mol Endocrinol 1995; 15:273–281.
11. Findlay JK. Peripheral and local regulators of folliculogenesis. Reprod Fertil Dev 1994; 6:127–139.
12. Dong J, Albertini DF, Nishimori K, Kumar TR, Lu N, Matzuk MM. Growth differentiation factor-9 is required during early ovarian folliculogenesis. Nature 1996; 383:531–535.
13. McGrath SA, Esquela AF, Lee S-J. Oocyte-specific expression of growth/differentiation factor-9. Mol Endocrinol 1995; 9:131–136.
14. McPherron AC, Lee S-J. GDF-3 and GDF-9: two new members of the transforming growth factor-β superfamily containing a novel pattern of cysteines. J Biol Chem 1993; 268:3444–3449.
15. Incerti B, Dong J, Borsani G, Matzuk MM. Structure of the mouse growth/differentiation factor 9 gene. Biochem Biophys Acta 1994; 1222:125–128.
16. Campion CE, Turzillo AM, Clay CM. The gene encoding the ovine gonadotropin-releasing hormone (GnRH) receptor: cloning and initial characterization. Gene 1996; 170:277–280.
17. McNatty KP, Heath DA, Lundy T, Fidler AE, Quirke L, O'Connell A, Smith P, Groome N, Tisdall DJ. Control of early ovarian follicular development. J Reprod Fertil Suppl 1998; (in press).
18. Eckery DC, Moeller CL, Nett TM, Sawyer HR. Localization and quantification of binding sites for follicle-stimulating hormone, luteinizing hormone, growth hormone, and insulin-like growth factor 1 in sheep ovarian follicles. Biol Reprod 1997; 57:507–513.
19. Fitzpatrick SL, Sindoni DM, Shughrue PJ, Lane MV, Merchenthaler IJ, Frail DE. Expression of growth differentiation factor-9 messenger ribonucleic acid in ovarian and nonovarian rodent and human tissues. Endocrinology 1998; 139:2571–2578.
20. Manova K, Huang EJ, Angeles M, DeLeon V, Sanchez S, Pronovost SM, Besmer P, Bachvarova RF. The expression of the c-*kit* ligand in gonads of mice supports a role for the c-*kit* receptor in oocyte growth and in proliferation of spermatogonia. Dev Biol 1993; 157:85–99.
21. Clark DE, Tisdall DJ, Fidler AE, McNatty KP. Localization of mRNA encoding c-*kit* during the initiation of folliculogenesis in ovine fetal ovaries. J Reprod Fertil 1996; 106:329–335.
22. Besmer P, Manova K, Duttlinger R, Huang EJ, Packer A, Gyssler C, Bachvarova RF. The kit-ligand (steel factor) and its receptor c-*kit*/W: pleiotropic roles in gametogenesis and melanogenesis. Dev Suppl 1993; 125–137.
23. Yoshida H, Takakura N, Kataoka H, Kunisada T, Okamura H, Nishikawa S-I. Stepwise requirement of c-*kit* tyrosine kinase in mouse ovarian follicle development. Dev Biol 1997; 184:122–137.
24. Godin I, Deed R, Cooke J, Zsebo K, Dexter M, Wylie CC. Effects of the steel gene product on mouse primordial germ cells in culture. Nature 1991; 352:807–809.
25. Tisdall DJ, Quirke LD, Smith P, McNatty KP. Expression of the ovine stem cell factor gene during folliculogenesis in late fetal and adult ovaries. J Mol Endocrinol 1997; 18:127–135.
26. Dolci S, Williams DE, Ernst MK, Resnick JL, Brannan CI, Lock LF, Lyman SD, Boswell HS, Donovan PJ. Requirement for mast cell growth factor for primordial germ cell survival in culture. Nature 1991; 352:809–811.

Downloaded from https://academic.oup.com/biolreprod/article/60/2/381/2741103 by guest on 20 August 2022