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Localization of Period 1 mRNA in the ruminant oocyte and investigations of its role in ovarian function☆

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Abstract

The clock gene Period 1 (Per1) may be a prolificacy gene, because it localized to the mouse oocyte and Per1-null drosophila shed fewer eggs. Because Per1 mapped to a region of mouse chromosome 11 syntenic to bovine chromosome 19 where a quantitative trait loci (QTL) for ovulation rate existed, we hypothesized that Per1 influenced folliculogenesis and ovulation rate in ruminants. Ovarian cortex was collected at slaughter on days 5, 12, 15, 17, and 20 after estrus for real-time RT-PCR evaluation of Per1 mRNA expression in Dorset ($n = 18$), Romanov ($n = 10$), Romanov/Dorset ($n = 21$), and Composite ($n = 22$) ewes. Ovarian cortex was also collected from cows selected for increased ovulation rate ($n = 37$) or unselected controls ($n = 28$) on days 4, 5, and 6 of the estrous cycle for in situ hybridization and real-time RT-PCR. To examine the role of Per1 in early follicular development, ovarian cortex from neonatal calves ($n = 5$) was cultured for 10 days and Per1 mRNA levels were measured on day 0 and on day 10 of culture. The primers generated a 483 bp amplicon with 100% sequence homology to bovine RIGUI-like protein (Per1). In silico mapping of this sequence placed Per1 on bovine chromosome 19; however, it was 20 cM from the QTL. Per1 mRNA expression was unaffected by prolificacy, day of the cycle, or pregnancy status in ewes or cows. The riboprobe hybridized to oocytes of bovine preantral and antral follicles. In bovine ovarian cortical cultures on day 0, the tissue contained mostly primordial follicles (5.6 ± 0.6 follicles/section); however, after 10 days in culture, the number of primordial

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follicles per section decreased (0.5 follicles/section) and the number of primary follicles increased as follicles activated (day 0 = 0.5 ± 0.6 versus day 10 = 10.4 ± 0.6 primary follicles/section; $P < 0.001$). Per1 mRNA did not change over time in culture. We conclude that Per1 mRNA is expressed by ruminant oocytes in preantral and antral follicles; however, its physiological role in mammalian ovarian function remains to be elucidated. Published by Elsevier B.V.

Keywords: Oocyte; Fertility; Gene expression

1. Introduction

The mammalian oocyte regulates folliculogenesis, ovulation, fertilization, and early embryogenesis by producing factors that have key functions in these developmental processes (Fortune et al., 2000; Sirard, 2001; Hamatani et al., 2004; Echternkamp, 2005; Sirard et al., 2006). Among the known factors produced by the oocyte are growth differentiation factor-9 (GDF-9) and bone morphogenetic protein-15 (BMP-15), which control both proliferation and gene expression in granulosa cells (Hsueh et al., 2000). Other oocyte-specific factors such as maternal antigen that embryos require (Mater) and zygote arrest 1 (Zar1) are required for early embryonic development through the maternal embryonic transition (Tong et al., 2000; Wu et al., 2003). Furthermore, a recent study identified 134 oocyte-specific genes with changes in expression level associated with maternal embryonic transition in the mouse (Hamatani et al., 2004), suggesting a greater need for research to investigate the influence of oocyte quality and oocyte-specific genes on folliculogenesis and early embryonic development.

Period 1 (Per1), a clock gene involved in circadean rhythms, was localized to the oocyte in the mouse ovary but a function was not determined (Johnson et al., 2002). Because Hamatani et al. (2004) did not identify Per1 in their global study of genes differentially expressed during early embryogenesis; Per1 is more likely to be involved in folliculogenesis and oogenesis than to be involved in early embryonic development. In drosophila, Per1 was localized within the follicular cells of the ovary; however, the pattern of expression was not influenced by circadean rhythms (Beaver et al., 2003). The removal of Per1 activity resulted in a 50% decrease in the number of oocytes shed and decreased the number of offspring produced, suggesting that Per1 played a direct role in oogenesis in drosophila. Per1 mapped to a region of mouse chromosome 11 that is syntenic to bovine chromosome 19, where there is a putative quantitative trait loci (QTL) for ovulation rate (Kirkpatrick et al., 2000). To our knowledge, no one has examined expression of Per1 in the ruminant ovary or its role in prolificacy. Therefore, the objectives of the current study were to determine if Per1 mRNA was present in the ruminant ovary, and to determine if the level of expression of Per1 was associated with prolificacy in ruminants. The hypotheses to be tested were that Per1 mRNA was expressed in the ruminant oocyte and that increased Per1 mRNA expression was associated with increased follicular development and prolificacy in ruminants.

2. Materials and methods

2.1. Ewes and cows

All procedures were approved by the USMARC Animal Care and Use Committee. Ewes were exposed to a fertile ram during the breeding season and reproductive tracts were collected at slaughter from Dorset ($n = 18$), Romanov ($n = 10$), Romanov/Dorset ($n = 21$) and Composite

($n = 22$, 1/2 Columbia, 1/4 Suffolk, 1/4 Hampshire) ewes on days 5, 12, 15, 17, and 20 after estrus. The reproductive tracts were flushed with 20 ml sterile PBS to determine pregnancy status and the total number of embryos recovered was recorded for each ewe. The total number of CL on the ovaries was counted as a measure of ovulation rate and the ovarian cortex was dissected from the medulla, frozen in liquid nitrogen, and stored at −80 °C until RNA extraction.

Bovine ovaries were collected at slaughter from cows selected for increased ovulation rate (Twinner, $n = 37$) and from unselected contemporaries (Non-Twinner, $n = 28$) on days 4, 5, and 6 of the estrous cycle. Ovarian cortex was frozen immediately in liquid nitrogen and stored at −80 °C until RNA extraction. Additional pieces of bovine ovarian cortex were fixed in 4% paraformaldehyde, embedded in paraffin, and serially sectioned at 6 μm for in situ hybridization.

2.1.1. Localization of Per1 mRNA in the bovine ovary by in situ hybridization

The cDNA generated from the Per1 primers used for real-time RT-PCR and sequencing (see below) was subcloned into the PGEM-T-Easy plasmid, transformed into competent cells, grown overnight at 37 °C, and isolated using the Qiagen mini-prep kit according to the manufacturer's directions. The isolated plasmid was linearized with *Nco I* (antisense) or *Spe I* (sense) for 1 h at 37 °C. Radioactive antisense and sense riboprobes were generated with SP6 and T7 RNA polymerase, respectively, in an in vitro transcription reaction that included S^{35}-CTP.

In situ hybridization was performed according to the methods of Spencer et al. (1995). A total of four sections (two for antisense probe and two for sense probe) were used from Twinner ($n = 8$) and Non-Twinner ($n = 7$) ovaries. Briefly, sections were hydrated in graded ethanols, washed in 0.5× SSC for 5 min and treated for 10 min with 20 μg/ml proteinase K. Following another wash with 0.5× SSC for 10 min, 100 μl of hybridization buffer was placed on each slide and the slides were pre-hybridized for 2 h at 42 °C in a humidified chamber. Slides were incubated in 0.5× SSC for 5 min, and 100 μl of fresh hybridization buffer containing antisense or sense probe at 5000 dpm/μl was added to the slides. The slides were then incubated overnight at 55 °C in a humidified chamber.

The next day, the slides were washed twice in SBE buffer for 10 min and excess probe was digested with 15 μg/ml of RNAse A for 30 min. The slides were washed twice with SBE for 10 min then with 0.1× SSC/10 mM BME/1 mM EDTA for 2 h at 55 °C. The slides were washed twice with 0.5× SSC for 10 min, dehydrated through graded ethanols and air dried for 30 min. The slides were dipped in emulsion and allowed to dry. They were then placed at 4 °C in the dark for 4 weeks, and then developed.

2.2. RNA extraction and real-time RT-PCR

Total cellular RNA was extracted from 50 mg of ovine or bovine ovarian cortex using Trizol reagent (Life Technologies Inc.). The RNA pellets were dissolved in nuclease-free water and concentrations were measured by spectrophotometry at 260 nm. Prior to quantitative real-time RT-PCR, 1 μg of total RNA per sample was incubated with 1 U Amplification grade DNAse I for 15 min at 25 °C to remove any remaining genomic contamination. Following DNAse I treatment, concentrations were measured again by spectrophotometry at 260 nm.

Real-time RT-PCR was performed using 134.1 ± 19.0 ng of DNAse I-treated total cellular RNA and primers designed from the published ovine Per1 EST sequence (GeneBank accession number AF044911). Primers were designed using primer 3 (Rozen and Skaletsky, 2000; code available at http://www.genome.wi.mit.edu/genome_software/other/primer3.html). The forward primer sequence was 5′-GGGCAAGGACTCAGAAAGAA-3′ and the reverse primer sequence

was 5′-AGGCTCCATTGCTGGTAGAA-3′. Relative levels of Per1 mRNA were quantified using SYBR Green and a MJ Chromo4 real-time RT-PCR thermocycler. A single-step reaction was performed under the following cycle conditions: 42 °C for 30 min for reverse transcription, 95 °C for 10 min to inactivate the reverse transcriptase, followed by 45 cycles of: 30 s at 95 °C for melting, 30 s at 58 °C for annealing, and 30 s at 72 °C for extension. Threshold cycle (CT) was determined for Per1 and for GAPDH (primer sequences published in Klipper et al., 2004) and comparisons were made as a fold difference for each gene against a pool of ovarian cortical mRNA using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), where $\Delta CT = CT$ of the sample $-CT$ of the pool and the $\Delta\Delta CT$ was determined by subtracting the ΔCT for the samples from the ΔCT of the pool.

2.3. Bovine cortical cultures

Bovine cortical cultures were performed according to previously reported methods (Wandji et al., 1996; Cushman et al., 2002). Briefly, bovine ovaries ($n = 5$ pairs) were collected from neonatal calves (44.8 ± 1.2 days of age) using a ventral mid-line laparotomy, posterior to the umbilicus. Neonatal ovaries were used because it was easier to dissect the cortex from the medulla in young ovaries and the younger ovaries had larger numbers of follicles than adult ovaries. Surgical preparation followed a 12-h fasting period and included mild sedation utilizing xylazine HCl (0.060 mg/kg), routine area preparation, and local anesthesia with infiltration of lidocaine HCl (2.0%). Each ovary was carefully exteriorized and a transfixed ligature was placed around the ovarian ligament including the vasculature and oviduct prior to excision. The incision was closed with simple interrupted sutures utilizing an absorbable suture material in the linea alba and non-absorbed suture in the skin.

Following removal of the ovaries, the ovarian cortex was dissected from the medulla and cut into 0.5 mm^3 pieces. Twenty-four cortical pieces/calf were processed immediately for morphometric analysis or gene expression analysis and 24 cortical pieces/calf were placed in serum-free culture for 10 days. Pieces were cultured in 24-well Costar (Corning Inc., Corning, NY) plates (2 pieces/well) on uncoated culture plate inserts (Millicell-CM, 0.4 μm pore size; Millipore Corporation, Bedford, MA) with 300 μl of Waymouth's medium MB 752/1 (Invitrogen Corporation) at 38.5 °C with 5% CO_2 in air. Waymouth's medium was supplemented with antibiotics (50 μg/ml streptomycin sulfate and 75 μg/ml penicillin, Sigma, St. Louis, MO), ITS+ (6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 ng/ml selenious acid, 1.25 mg/ml BSA, and 5.35 μg/ml linoleic acid; Collaborative Biomedical Products, Becton Dickinson Labware, Bedford, MA), and 25 mg/l pyruvic acid sodium salt (Sigma).

On day 0 or after termination of cultures on day 10, four cortical pieces were fixed for 1 h (2.5% glutaraldehyde, 2.5% formaldehyde in 0.075 M cacodylate buffer, pH 7.3), post-fixed for 45 min in 2% osmium tetroxide, and embedded in LR White plastic (EMS, Fort Washington, PA). Blocks were sectioned at 2 μm with a glass knife. Every other set of 10 consecutive sections was mounted on gelatin-coated slides at 60 °C, and stained with toluidine blue. Only the largest cross-section in each set of 10 consecutive sections was examined and only follicles with the germinal vesicle present in that section were counted to avoid counting the same follicle twice.

Follicles were classified as primordial or primary as described previously (Wandji et al., 1996). Follicles with an oocyte surrounded by a single layer of flattened and/or small cuboidal somatic cells were classified as primordial follicles, while in primary follicles the oocyte is surrounded by a single layer of large cuboidal granulosa cells.

For gene expression studies, an additional 20 pieces of ovarian cortex collected at day 0 and 20 pieces of ovarian cortex collected after 10 days of culture were placed into Trizol reagent and total cellular RNA was extracted as described previously in this text.

2.4. Mapping Per1 in cattle in silico

PCR amplification was performed using 25 ng of genomic bovine DNA in a total 12 μl volume containing 1.5 mM $MgCl_2$, 0.25 mM each dNTP, 0.5 U Ampli*Taq* Gold with 1× $MgCl_2$-free supplied buffer (Perkin-Elmer, Branchburg, NJ), and 0.2 μM of the same forward and reverse primers used for real-time RT-PCR. Thermal cycling conditions included an initial denaturing step at 95 °C for 5 min, followed by 40 cycles of: 95 °C for 30 s (denaturing), 58 °C for 45 s (annealing), and 72 °C for 1 min (extension), with a final extension period at 72 °C for 5 min.

A portion of the sample was separated on a 2% agarose gel to verify quality. Another portion of the PCR reaction (3 μl) was incubated with 0.1 U of Exonuclease I (USB, Cleveland, OH) at 37 °C for 1 h, followed by heat inactivation at 65 °C for 20 min. The product was purified using EtOH precipitation. Purified PCR products were sequenced in both directions using the same forward and reverse primers with ABI Big Dye terminator chemistry and analyzed on an ABI 3730 sequencer.

Sequence information was analyzed from chromatograms stored in the USMARC database; bases were called with Phred and assembled in contigs with Phrap (Ewing et al., 1998; Ewing and Green, 1998). The results were visually assessed using Consed (Gordon et al., 1998). Resulting sequence was aligned to the bovine genome (Btau_1.0) using NCBI megaBLAST. Genomic sequences with significant hits (*E*-value 0.0) were then aligned against the BAC end-sequences. Chromosome assignment and approximate position were obtained from the bovine BAC map (International Bovine Bac Map Consortium; http://www.bcgsc.ca/lab/mapping/bovine) integrated with bovine genetic and physical maps (Snelling, personal communication).

2.5. Statistical analysis

Ovulation rate in ewes and cows was analyzed using the MIXED procedure of SAS with breed of ewe or line of cow as the independent variable. Relative levels of Per1 mRNA in the ovine ovarian cortex were analyzed using the MIXED procedure of SAS with a model that included the main effects of breed, day after estrous, and pregnancy status with the relative level of GAPDH mRNA as a co-variate, because the efficiency of the reactions differed for GAPDH and Per1. Relative levels of Per1 mRNA in the bovine ovarian cortex were analyzed using the MIXED procedure of SAS with a model that included line with the relative level of GAPDH mRNA as a co-variate. Preliminary analysis showed that the threshold cycle for GAPDH did not differ for any of the independent variables examined. This was in agreement with Klipper et al. (2004), and suggested that the GAPDH was an effective house-keeping gene to use for standardizing relative Per1 mRNA levels.

For the morphometric analysis of cortical cultures, the total number of primordial and primary follicles per section was calculated for each culture for each day, and if Hartley's test indicated heterogeneity of variance, data were transformed to logarithms (base 10) prior to analysis. Differences among means were tested by the MIXED procedure of SAS with day as the independent variable, and numbers or primordial or primary follicles per section as the dependent variables. Relative levels of Per1 mRNA in the bovine ovarian cortical cultures were analyzed using the MIXED procedure of SAS with a model that included day of culture with the relative level of GAPDH mRNA as a co-variate. If the model was significant ($P < 0.05$), a separation of means

was performed using the Student–Newman–Keuls procedure. All results are reported as the mean and the standard error of the mean.

3. Results

3.1. Expression of Per1 mRNA in the ruminant ovary

A total of 5–15 oocytes from primordial and primary follicles were examined in each histological section, while the number of oocytes from secondary (1–3 per section) and antral follicles (1 per section) were fewer. In situ hybridization using S^{35}-labelled riboprobes showed specific hybridization of the antisense probe in the oocytes of secondary and antral bovine follicles (Fig. 1). No signal for Per1 mRNA was identified in the oocytes of primordial or primary follicles. Ovulation rate was different among breeds of ewes ($P < 0.001$; Fig. 2A) with Romanov ewes having the greatest ovulation rate and the Dorset and Composite ewes having the lowest ovulation rate. However, real-time RT-PCR showed no differences in Per1 mRNA expression in the ovarian cortex between breeds of ewe (Fig. 2B). In cows selected for multiple births, the ovulation rate tended to be greater than in unselected controls ($P = 0.10$; Fig. 3A), and similar to the results in the ewe, there was no difference in Per1 mRNA expression between the two lines of cows (Fig. 3B).

The distribution of pregnant and open ewes across the 5 days examined is presented in Table 1. The overall conception rate was 69%. Per1 mRNA expression did not change with day after estrus in ovine ovarian cortex collected during the breeding season (Fig. 4). Relative levels of ovarian Per1 mRNA expression were not influenced by pregnancy status in the early pregnancy period ($\leq$day 20 after estrus; relative level = 14.1 ± 3.6 versus 13.3 ± 2.3 in non-pregnant and pregnant ewes, respectively).

When pieces of neonatal calf ovarian cortex were placed in culture for 10 days, there was a significant decrease in the number of primordial follicles per section and an increase in the number of primary follicles per section ($P < 0.001$; Fig. 5A); however, there was no change in Per1 mRNA expression in the ovarian cortex after the primordial follicles activated (Fig. 5B).

3.2. Sequencing and mapping of the Per1 amplicon generated from bovine genomic DNA

The primers generated a 483 bp amplicon from bovine genomic DNA, which was sequenced (Fig. 6), and compared against the NCBI database. The sequence showed 100% homology with

Table 1
Distribution of ewes among days and pregnancy status

Days after estrus	Pregnancy status	Number of ewes
5	Open	1
5	Pregnant	7
12	Open	4
12	Pregnant	7
15	Open	15
15	Pregnant	13
17	Open	2
17	Pregnant	11
20	Open	0
20	Pregnant	11

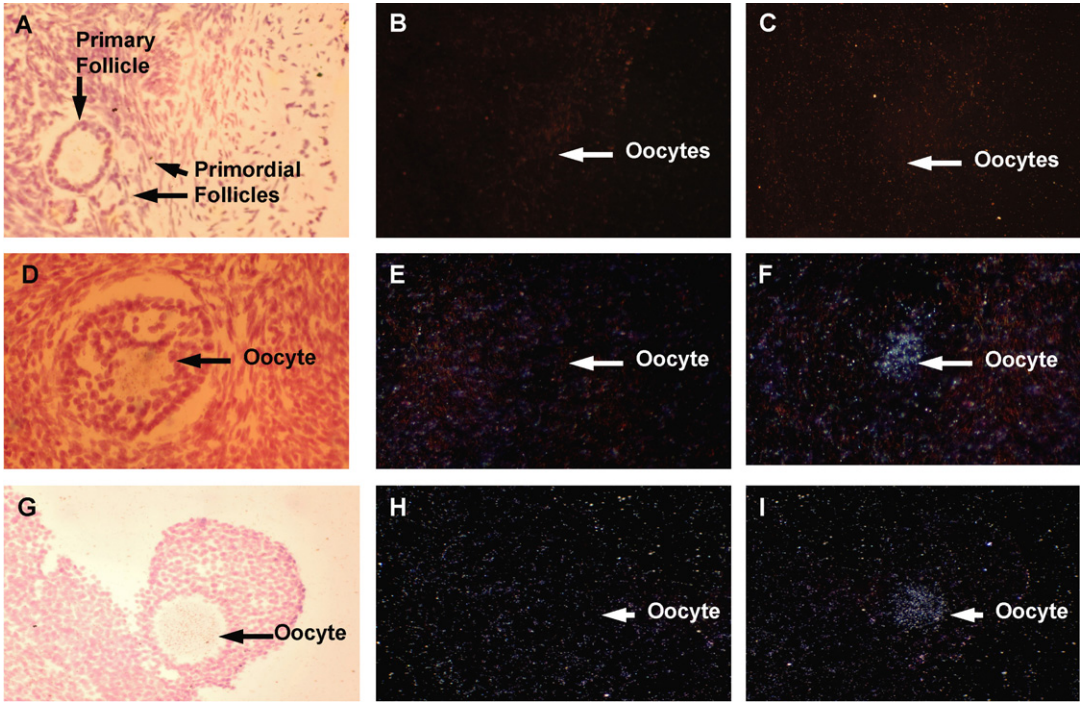

Fig. 1. Localization of Per1 mRNA by in situ hybridization in the bovine ovary. (A) Bright field image of a primary follicle (40 μm in diameter) and two primordial follicles (20 μm in diameter). (B) Dark field image of the same follicles with sense probe. (C) Dark field image of the same follicles with antisense probe. There is no specific signal for Per1 mRNA within the oocytes of these early preantral follicles. (D) Bright field image of a secondary follicle, 60 μm in diameter with multiple layers of granulosa cells surrounding the oocyte. (E) Dark field image of the same secondary follicle with sense probe. (F) Dark field image of the same secondary follicle with antisense probe showing specific signal for Per1 mRNA localized in the oocyte. (G) Bright field image of the cumulus oophorus of a small antral follicle 3 mm in diameter. (H) Dark field image of the same cumulus oophorus with sense probe. (I) Dark field image of the same cumulus oophorus with antisense probe showing Per1 mRNA localized in the oocyte.

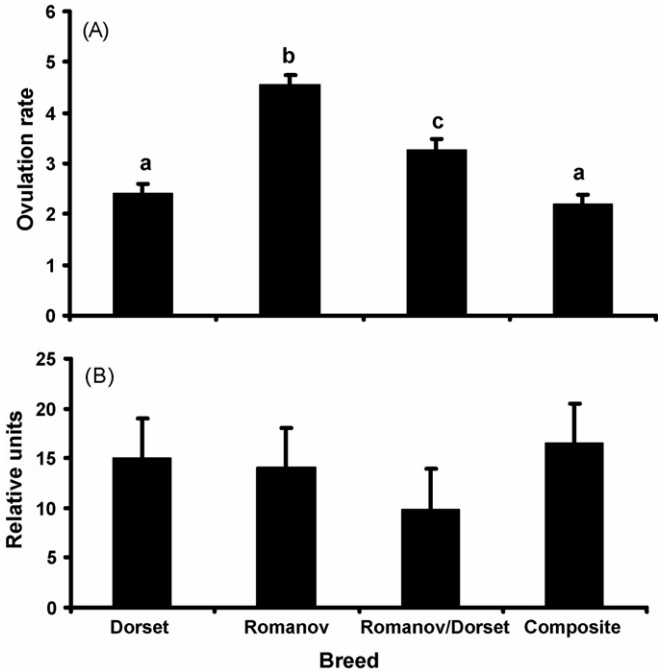

Fig. 2. (A) Ovulation rate and (B) relative levels of Per1 mRNA in ovaries of different breeds of ewes (Dorset $n = 18$, Romanov $n = 10$, Romanov/Dorset $n = 21$, Composite $n = 22$). There was a significant effect of breed on ovulation rate ($P < 0.001$); however, there was no effect of breed on Per1 expression in the ovarian cortex. Bars with different superscipts are different.

170 bp region of exon 5 and 33 bp of exon 6 of the mammalian ortholog to the drosophila Per1 gene, RIGUI-like protein (Per1; accession# XM_594471) with an intron of 313 bp spanning between the two exons. In silico mapping of Per1 in the bovine placed the gene in scaffold NW_165741.1, ∼15 kb in length (NCBI Bos Taurus build 1.0), which aligned with CHORI-240_3L2 end-sequence. This BAC clone is in close proximity to clones containing Fragile X related protein 2 (FXR2) on the bovine BAC map (International Bovine Bac Map Consortium, http://www.bcgsc.ca/lab/mapping/bovine).

4. Discussion

To our knowledge, this was the first study to demonstrate expression of Per1 mRNA in the ruminant ovary and to localize Per1 mRNA expression to the bovine oocyte. Localization of Per1 to the bovine oocyte agreed with previous work, which localized Per1 to the oocyte in the mouse ovary but did not propose a function for ovarian Per1 (Johnson et al., 2002). The hypothesis in the present study was that Per1 was associated with enhanced follicular development and increased ovulation rate; however, the results did not support this hypothesis, because neither the prolific Romanov ewes nor cows selected for multiple ovulations had increased Per1 mRNA expression in their ovaries.

The primers used generated a 169 bp cDNA from bovine ovarian mRNA and a 483 bp amplicon from bovine genomic DNA. Sequencing of the 483 bp amplicon allowed us to map Per1 in silico to bovine chromosome 19 close to FXR2. However, based on its current mapping position, Per1

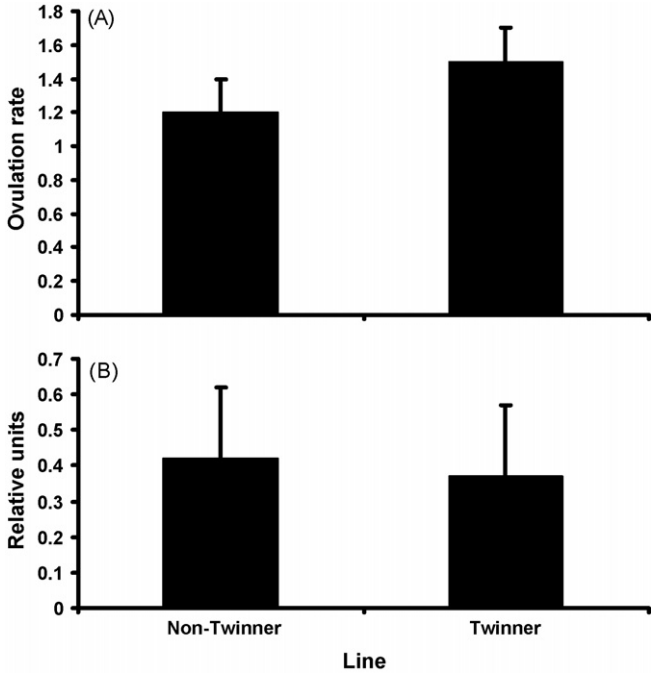

Fig. 3. (A) Ovulation rate and (B) relative levels of Per1 mRNA in ovaries of unselected controls (Non-Twinners, $n = 28$) and cows selected for increased ovulation rate (Twinners, $n = 38$). Ovulation rate tended to be greater in Twinner than Non-Twinner cows ($P = 0.10$); however, there was no effect of line on Per1 expression in the ovarian cortex.

is about 20 cM from the putative QTL for ovulation rate reported by Kirkpatrick et al. (2000). Additional supporting evidence is provided by the human map with Per1 and FXR2 within 0.5 Mb of each other. FXR2 has been mapped to 40 cM on BTA19 on the USMARC bovine linkage map (Snelling et al., 2005). Taken together, the expression data and chromosomal location of the Per1 gene would lead us to conclude that Per1 is not associated with increased ovulation rate in ruminants. However, mRNA levels do not always predict protein levels and there could be sequence variation which could result in functional differences at the protein level. Future studies will consider this as we investigate the physiological role of Per1 in the ruminant oocyte.

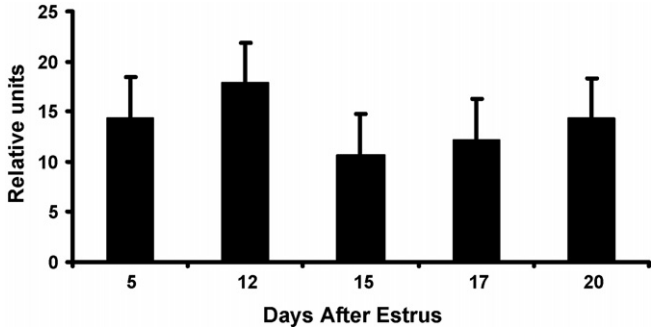

Fig. 4. Relative levels of Per1 mRNA expression in the ovarian cortex of sheep on days 5 ($n = 8$), 12 ($n = 11$), 15 ($n = 28$), 17 ($n = 13$), and 20 ($n = 11$) after estrus. There was no difference in Per1 mRNA expression due to day after estrus.

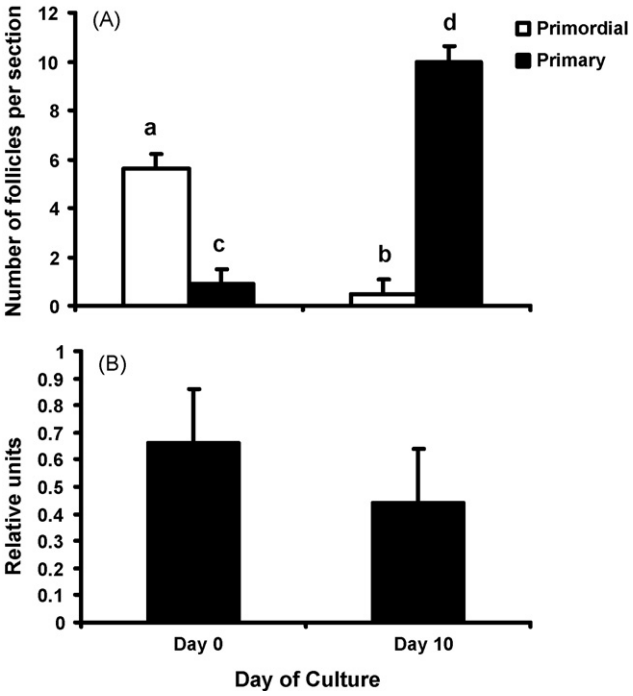

Fig. 5. (A) Numbers of primordial and primary follicles in neonatal bovine ovarian cortex ($n = 5$ calves) on day 0 and after 10 days in serum-free culture. The number of primordial follicles decreased after 10 days in culture and the number of primary follicles increased after 10 days in culture ($P < 0.001$). The number of sections evaluated per culture ranged from 11 to 26. (B) Relative levels of Per1 mRNA in bovine ovarian cortex on day 0 and after 10 days in serum-free culture. Within a follicle stage, bars with different superscripts are different.

Disruption of clock genes in the mouse has led to several reproductive phenotypes, including reduced fecundity, prolonged estrous cycles, and increased time in estrus (Kennaway, 2005); however, due to the wide-spread expression of these genes in the suprachiasmatic nucleus and in peripheral tissues throughout the body (Lincoln et al., 2003), it is difficult to interpret the results from these mutant mice. Production of transgenic farm animals is not easily accomplished at this

5′GGGCAAGGACTCAGAAAGAACTCATGACGGCACTGCGAGAGCTCAAGCTTCGGCTGCCCCCGG
AGCGCAGGGGCAAGGGCCGCTCCGGGACTCTGGCCACACTGCAGTACGCGCTGGCCTGTGTCAAG
CAGGTGCAAGGTGAGTGGTGCTCCTGGGAGGATAATGGTCAGGTTCTGGGTGACCGTGGGGAGCT
GTCTCCTCGGTAAATGCGCTGCTGCCCGCACCCCATAGAGATTCCCTTCTAGCTTAATCATTAAA
GTATCTGCTTGCAATGCAGGACACCTGGGTTCCATCCCTGGGTTGGGAGGATCCCCTGGAGTAGG
GAATGGCAACCCACTCCAGTATTCTTGCCTGGGAAGTCCCATGGACAGAGGAGCCTGGTGGGCTG
CAGTCCATGGGGTTGCAAAGAGTCGGACATGACTGAGTGACTAAACCACTACCCACCCCTGCAGC
CAACCAGGAGTTCTACCAGCAATGGAGCCT3′

Fig. 6. Sequence of the 483 bp amplicon generated from bovine genomic DNA. Forward and reverse primers are highlighted in red and exonic sequence is highlighted in cyan.

point. Therefore, we took a more conventional approach by examining ovarian expression of the gene on various days of the estrous cycle in the ewe. Ovarian expression of Per1 mRNA did not change with day of the estrous cycle. Therefore, if Per1 mRNA expression levels play a role in timing of events in the ovary, it does not appear to be associated with timing of the ovarian cycle. Recently, an abstract reported an increase in expression of the clock gene Bmal1 in granulosa cells approximately 8 h following the LH surge in the mouse (Klementiev and Tischkau, 2005). Therefore, the clock genes could play a role in the ovulatory cascade of the Graafian follicle(s) in the mammalian ovary; however, in the present series of experiments, ovarian samples were not collected at the correct time of the estrous cycle to investigate this possibility.

A heifer is born with approximately 140,000 oocytes in her ovaries, and the majority of these are in the dormant pool of primordial follicles, termed the ovarian reserve (Erickson, 1966). Entry into the growing pool, the so-called primordial to primary follicle transition (also known as activation of the primordial follicles), is a poorly understood process involving both inhibitory and stimulatory factors (Fortune et al., 2000). The best characterized of these factors is anti-Müllerian hormone which was demonstrated to be an inhibitor of the primordial to primary follicle transition in the mouse and, most likely, the cow (Durlinger et al., 2002; Gigli et al., 2005). Because a number of oocyte-specific factors control growth and proliferation of the granulosa cells (Hsueh et al., 2000) and because the primordial to primary follicle transition is difficult to study in vivo in ruminants where activation begins prior to birth (Fortune et al., 1999), we decided to use the bovine ovarian cortical culture model to examine Per1 expression around the time of primordial follicle activation. In this culture system, the ovarian cortical pieces placed in the culture on day 0 contain almost exclusively primordial follicles; however, those primordial follicles activate and become primary follicles within a very short time after being placed in culture (Wandji et al., 1996). From these experiments, we concluded that oocyte Per1 is not involved in controlling the timing of primordial follicle activation in the ovary, because Per1 mRNA expression did not change when primordial follicles activated. It could be that any change in Per1 expression associated with activation of primordial follicles is transient, and, therefore, we were unable to detect it with these two time points; however, that seems unlikely based on the in situ hybridization results. When Per1 mRNA was localized by in situ hybridization in the bovine ovary, no clear signal above background was detectable in the oocytes of primordial and primary follicles; however, oocytes of secondary and antral follicles clearly expressed Per1 mRNA. This would suggest that, whatever the role of Per1 in ovarian function, it does not occur until after formation of multiple layers of granulosa cells around the oocyte during the secondary stage of follicular development.

5. Conclusion

Per1 mRNA was localized to the ruminant oocyte in secondary and antral bovine follicles. There was no relationship between transcription levels and prolificacy in sheep or cattle and the gene did not map to a known QTL for ovulation rate in cattle. Further research will be needed to elucidate the role of Per1 in mammalian ovarian function, but from the present results, it does not appear to be involved in the primordial to primary follicle transition, regulation of the ovarian cycle, or prolificacy in ruminants.

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