Expression and regulation of secreted phosphoprotein 1 in the bovine corpus luteum and effects on T lymphocyte chemotaxis

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Abstract

Secreted phosphoprotein 1 (SPP1) in the bovine corpus luteum (CL) regulates cell function during the transitional periods of luteinization and luteal regression. The objectives were to i) characterize SPP1 expression in the CL throughout the estrous cycle, ii) determine factors that regulate SPP1 expression in luteal cells, and iii) examine the role of SPP1 in lymphocyte chemotaxis, proliferation, and function. *SPP1* mRNA was greater in fully functional (d10) CL and late cycle (d18) CL compared with developing (d4) CL. Additionally, *SPP1* mRNA increased within 1 h and remained elevated 4 and 8 h following induction of luteolysis with prostaglandin (PG)_{F_{2α}}. Expression of the SPP1 receptor, β_3 integrin, was not different throughout the estrous cycle but decreased following induction of luteolysis. Expression of *CD44* increased during the estrous cycle but did not change during luteal regression. In cultured luteal cells, *SPP1* mRNA was upregulated by PGF_{2α} and/or tumor necrosis factor α . Western blots revealed the presence of both full-length SPP1 and multiple cleavage products in cultured luteal cells and luteal tissue. Depletion of endogenous SPP1 did not hinder luteal cell-induced lymphocyte proliferation or lymphocyte phenotype but did inhibit lymphocyte migration toward luteal cells. Based on these data, it is concluded that SPP1 is initially activated to establish and maintain cellular interactions between steroidogenic and nonsteroidogenic cells during the development of the CL. Upon induction of luteolysis, SPP1 serves as a signaling molecule to recruit or activate immune cells to facilitate luteal regression and tissue degradation.

Reproduction (2013) **146** 527–537

Introduction

The corpus luteum (CL) is a unique endocrine organ with a transient lifespan that undergoes extensive tissue remodeling during luteinization and luteal regression. Luteinization is a dynamic process involving simultaneous hyperplasia, hypertrophy, and differentiation of steroidogenic cells as well as proliferation and migration of nonsteroidogenic cells, within the first 5–7 days following ovulation (Zheng *et al.* 1994). The CL of ruminants has a finite lifespan and will remain present on the ovary only until a specific signal, prostaglandin (PG)_{F_{2α}}, initiates luteal regression at the end of the estrous cycle or pregnancy. Luteolysis involves loss of progesterone (P₄) production and cellular integrity that ultimately leads to destruction of the CL (Pate 1994, Niswender *et al.* 2000). Immune cells and cytokines are thought to be involved in luteal formation and regression in a variety of species (Brännström & Norman 1993,

Pate & Keyes 2001). Understanding the mechanisms that regulate these critical periods and the role of immune cells in the CL will lead to new and innovative ways to control the estrous cycle and potentially enhance reproductive efficiency.

Secreted phosphoprotein 1 (SPP1), also known as osteopontin and early T lymphocyte activation 1, is a secreted, extracellular matrix protein that is abundant in many different cells of mesodermal and endodermal origin (Franzen & Heinegard 1985, Patarca *et al.* 1989, O'Regan & Berman 2000, Sodek *et al.* 2000, Denhardt *et al.* 2001). Binding of SPP1 to its receptors, CD44 or several integrins, initiates cell-specific signaling that activates a variety of cellular functions such as tissue remodeling, activation of extracellular matrix proteins to induce cell migration or attachment, regulation of cell-mediated immunity, and alteration of cytokine synthesis (reviewed by Sodek *et al.* (2000)). Several reports have

shown that SPP1 increases tumor cell angiogenesis in mice (Takahashi *et al.* 2002, Hirama *et al.* 2003) and enhances vascularization in ectopic bone (Asou *et al.* 2001). Xie *et al.* (2001) demonstrated that an increase in SPP1 is associated with proliferation and regeneration of renal tubular epithelial cells following renal toxic or ischemic injury. Silencing of SPP1 markedly decreased the cell motility and invasiveness of SK-Hep-1 tumor cells (Cheng *et al.* 2007) and metastasis of murine colon adenocarcinoma (Wai *et al.* 2005).

SPP1 has been shown to be highly expressed by activated macrophages, dendritic cells, and lymphocytes early in an immune response (Patarca *et al.* 1989, O'Regan & Berman 2000). Secretion of SPP1 by nonimmune cells and activated T cells enhanced recruitment and activation of macrophages and prolonged the immune response (O'Regan *et al.* 1999). Ashkar *et al.* (2000) demonstrated that SPP1 increased IL12 and suppressed IL10 production by macrophages, acting through integrin $\alpha_v\beta_3$ and CD44 receptors respectively. Further studies have demonstrated that SPP1 stimulates interferon γ and CD40 ligand expression on antigen-presenting cells, suggesting that SPP1 has a role in the proinflammatory immune response (O'Regan & Berman 2000, Denhardt *et al.* 2001).

In female reproductive tissues, this multifunctional protein has been identified in the ovary (Craig & Denhardt 1991, Brown *et al.* 1992, Brunswig-Spickenheier & Mukhopadhyay 2003), endometrium (Brown *et al.* 1992, Johnson *et al.* 2000, 2001, 2003), oviduct (Brown *et al.* 1992, Gabler *et al.* 2003), and ovarian cancers (Kim *et al.* 2002). P₄ and unknown conceptus-derived factors upregulate SPP1 in the glandular epithelium, which promotes trophoblast cell migration and attachment to luminal epithelium and may be essential for conceptus development (Dunlap *et al.* 2008, Erikson *et al.* 2009, Kim *et al.* 2010). In the bovine ovary, SPP1 mRNA was present in granulosa, theca, and luteal cells (Brunswig-Spickenheier & Mukhopadhyay 2003); however, the precise role of SPP1 and its relationship with immune cells within the CL remains unknown. Therefore, the basis of this research was to investigate a potential role of SPP1 in the bovine CL and its ability to direct lymphocyte responses. It was hypothesized that SPP1 produced by luteal cells modifies lymphocyte responses within the developing, fully functional, and regressing CL.

Materials and methods

Reagents

Powdered Hams F-12 culture medium, gentamicin, insulin–transferrin–selenium (ITS) premix, fetal bovine serum, SuperScript II Reverse Transcriptase, and TRIzol reagent were purchased from Invitrogen Corporation. Recombinant RNasin and dNTPs were purchased from Promega. Oligonucleotide primers were obtained from Operon-Eurofins (Huntsville, AL, USA).

DyNAmo HS SYBR Green qPCR kits were purchased from MJ Research (Waltham, MA, USA). QIAquick gel extraction kit was purchased from Qiagen Sciences. Type I collagenase was purchased from Worthington Biochemical Corporation (Lakewood, NJ, USA). Avidin/biotin blocking kit and biotinylated goat anti-rabbit IgG secondary antibody were purchased from Vector Laboratories (Burlingame, CA, USA). Bovine luteinizing hormone (LH; AFP II 743B) was provided by the National Hormone and Peptide Program and AF Parlow (Torrence, CA, USA). Milk-derived bovine SPP1 was purchased from R&D Systems (Minneapolis, MN, USA). Agarose was acquired from Amresco (Solon, OH, USA). Tissue culture flasks were from Corning (Corning, NY, USA). Anti-SPP1 polyclonal antibody (cat. no. ab14175) was purchased from Abcam (Cambridge, MA, USA), while peroxidase-labeled anti-rabbit secondary antibody and the ECL PLUS Western Blotting Analysis System were purchased from GE Healthcare (Piscataway, NJ, USA). Lentiviral particles directed toward the bovine SPP1 sequence (accession no. NM_174187) and scrambled sequence control were purchased from Santa Cruz Biotechnology, Inc. All antibodies for flow cytometry were purchased from VMRD, Inc. (Pullman, WA, USA) or AbD Serotec (Raleigh, NC, USA). Unless otherwise specified, all other chemicals, reagents, and supplies were purchased from Sigma Chemical Co. or VWR Scientific Products (West Chester, PA, USA).

Animals and tissue collection

Dairy cows exhibiting normal estrous cycles were used in accordance with protocols approved by the Institutional Laboratory Animal Care and Use Committee at The Ohio State University and The Pennsylvania State University. CLs were removed via transvaginal incision at day 4 or 5 (developing), days 10–12 (fully functional), and day 18 (late cycle) of the estrous cycle. Additional CLs were collected 0.5, 1, 4, 8, and 12 h after a luteolytic injection of PGF_{2 α} (25 mg) was administered at days 10–12 of the estrous cycle. Each CL was immediately quartered. Then one quarter was frozen in liquid N₂ and another quarter was placed in Optimal Cutting Temperature fixative (O.C.T.; Sakura Finetek USA, Inc., Torrance, CA, USA) prior to freezing in liquid N₂. Frozen samples were stored at –80 °C until further processing. The remaining two quarters were placed into ice-cold Hams F-12 media during transport to the laboratory to be dissociated for culture experiments.

Isolation of T lymphocytes

Jugular venous blood (500 ml) was collected to isolate peripheral blood mononuclear cells (PBMC), and peripheral T lymphocytes as described previously by Ndiaye *et al.* (2008). In brief, PBMC were obtained by centrifugation over Ficoll-Paque and T lymphocytes were separated immunomagnetically using the AutoMACS Cell Separator (Miltenyi Biotec, Auburn, CA, USA). T-cell purity was assessed by fluorescent labeling of the T-cell receptor (TCR, anti-CD3 VMRD, Inc.) and this separation procedure yielded ~95% pure T lymphocytes. The number of viable T cells was determined using the Guava ViaCount Flex Reagent in the Guava EasyCyte Plus (Millipore, Billerica, MA, USA).

Dissociation of corpora lutea

CLs were collected and dissociation of luteal tissue was performed according to Pate (1993). Luteal tissue was minced and placed in 24 mM HEPES-buffered Ham's F-12 culture medium (Gibco, Invitrogen Corporation) containing 0.5% BSA (Sigma–Aldrich, Inc.), 20 µg/ml gentamicin (Gentamicin Reagent Solution; Invitrogen Corporation), and 2000 U/g tissue collagenase type I (Worthington Biochemical Corporation). Following dissociation, luteal cells were resuspended in Ham's F-12 culture medium and cell viability was determined via the Guava ViaCount Flex Reagent in the Guava EasyCyte System (Millipore).

Cell culture

The luteal cells (3.5×10^5 cells/ml) were treated with LH (10 ng/ml), P_4 (0.5 µM), aminoglutethimide (AG; 50 µg/ml), $PGF_{2\alpha}$ (1, 10, or 100 ng/ml), and tumor necrosis factor (TNF; 5 or 50 ng/ml) alone or in combination. These treatment concentrations have been shown to affect bovine luteal cell function *in vitro* (Pate & Condon 1989, Benyo & Pate 1992, Townson & Pate 1996). After culture, media were removed and luteal cells were harvested to quantify SPP1 mRNA by quantitative PCR (qPCR) or to evaluate SPP1 protein expression via western blot. All cultures were carried out for 72 h at 37 °C in serum-free media supplemented with L-glutamine (200 mM) and ITS (5 µg/ml insulin, 5 µg/ml transferrin, and 5 ng/ml selenium) and 20 µg/ml gentamicin. All experiments were repeated a total of four times using CL from different animals.

SPP1 knockdown and validation

Luteal cells and lymphocytes were incubated in serum-free media overnight at 37 °C prior to transfection. All transfections were conducted according to the manufacturer's recommendations and transfection efficiency was monitored using lentiviral particles containing green fluorescent protein. Using this procedure, a transfection efficiency of $\geq 72\%$ was achieved in the luteal cell cultures and a transfection efficiency of $\geq 90\%$ was achieved in the lymphocyte cultures. Attempts to further increase the transfection efficiency of the primary luteal cell cultures resulted in increased luteal cell apoptosis. To ensure efficient knockdown of SPP1, both cell types were transfected with increasing concentrations of viral infectious units (IFU) directed toward the bovine SPP1 sequence (IFU; 1 = 12 500; 2 = 25 000; and 3 = 37 500 IFU/µl). Immunoblots for SPP1 revealed that the active isoform (45 kDa) of SPP1 in both luteal cells and lymphocytes decreased as viral particle concentration increased with optimal knockdown achieved using 25 000 IFU/µl. This concentration was used for the remaining experiments. In all knockdown experiments, luteal cells and T cells were transfected separately with either lentiviral particles directed toward SPP1 mRNA or scrambled sequence control in AIM V media containing 0.05% polybrene for 24 h at 37 °C. Following lentiviral treatment, cells were washed with serum-containing medium and incubated for 24 h. After this knockdown period, luteal cells and

lymphocytes were combined and treated with and without increasing concentrations of SPP1 (0, 50, 100, or 500 ng/ml) to determine the effects of SPP1 knockdown on lymphocyte function.

Total RNA extraction and qPCR

Total RNA was extracted from luteal tissues at various stages of the estrous cycle using TRIzol reagent. Total RNA was extracted and cDNA synthesis was performed as described previously by Ndiaye *et al.* (2008). Forward and reverse primers directed toward *Bos taurus* SPP1, hyaluronic acid receptor (CD44), $\beta 3$ integrin (ITGB3), *IL10*, and *IL12A* are described in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA fragment was amplified as a constitutively expressed gene, and mRNA from bovine uterus was used as a positive control in this study. Following the RT reaction, qPCR was performed on the MJ Research Opticon 2 (Bio-Rad Laboratories) using the DyNamo HS SYBR Green qPCR Kit under the following conditions: 95 °C for 5 min, 35 cycles of 94 °C for 30 s, 56–60 °C for 45 s, and 72 °C for 60 s, followed by an extra elongation of 5 min at 72 °C. Annealing temperature was optimized for each primer set. The qPCR amplification products were electrophoretically separated on 1.5% agarose gels and visualized with ethidium bromide under u.v. light to ensure a specific band corresponding to the size of the expected cDNA fragment. Specific bands were extracted and purified using the QIAquick Gel Extraction Kit (Qiagen Sciences) to be used for sequence confirmation. A control sample that was not reverse transcribed was used to confirm that the product obtained was not amplified from genomic DNA. For each gene of interest, a standard curve was prepared from purified cDNA PCR product and used to calculate the relative steady-state concentrations of respective mRNAs in triplicate wells for each sample. These data are presented as mRNA concentrations in arbitrary units.

Protein extraction and immunoblotting

Proteins were extracted from either luteal tissues or cultured luteal cells using the Cellytic MT Cell Lysis Reagent (Sigma–Aldrich Biotechnology) in the presence of the protease inhibitor cocktail (Sigma–Aldrich Biotechnology) following the manufacturer's protocol. Quantification of proteins was performed according to Bradford protein analysis (Bradford 1976). Protein samples were subjected to electrophoresis on a 12% SDS–polyacrylamide gel and separated proteins were blotted onto PVDF membranes (Hybond-P, Amersham Pharmacia Biotech). After 12 h of incubation in blocking solution (PBS, pH 7.4, 0.1% Tween 20, 5% heat-inactivated normal goat serum, 3% BSA) at 4 °C, membranes were washed once for 20 min in TBS–Tween 20 (20 mM Tris, pH 7.4, 150 mM NaCl, and 0.05% Tween 20). Membranes were then incubated with anti-SPP1 polyclonal antibody at a final concentration of 0.5 µg/ml in blocking solution at room temperature for 2 h. The membrane was washed three times, 20 min each, in TBS–Tween 20 and then incubated with the HRP-labeled anti-rabbit secondary antibody at a dilution of 1:20 000. The antigen–antibody complex was visualized using

Table 1 Primers directed toward *Bos taurus* mRNA targets and validated for use with quantitative PCR analysis.

Target	GenBank accession no.	Primer sequences	Amplicon size
CD44	NM_174013	FWD: 5'-TATAACCTGCCGATATGCAGG-3' REV: 5'-CAGCACAGATGGAATTGGG-3'	221
GAPDH	NM_00103403	FWD: 5'-AAGATTGTCAGCAATGCC-3' REV: 5'-ACAGACACGTTGGGAG-3'	198
IL10	NM_174088	FWD: 5'-TGTTGACCCAGTCTCTGCTG-3' REV: 5'-CCCTCTCTGGAGCTCACTG-3'	246
IL12A	U14416	FWD: 5'-GAGGCCTGTTTACCACTGGA-3' REV: 5'-CCTGCATCAGCTCAGCAATA-3'	254
ITGB3	XM_616376	FWD: 5'-TGAGGAGGTGAAGAAGCAGA-3' REV: 5'-GGTGGTGGAGGCAGAATAAT-3'	247
SPP1	NM_174187	FWD: 5'-CGATGATGATAACAGCCAGG-3' REV: 5'-ACTCGTCTTCTAGGTGCGT-3'	328

the ECL system following the manufacturer's protocol. Membranes were exposed for various times to Kodak Biomax light films and the films were developed in a SRX-101A Konica film processor (Konica Corporation). In all blots, proteins extracted from bovine uterus were used as a positive control. β -actin (ACTB) protein used as an internal control was recognized in all samples by the monoclonal anti-ACTB antibody.

Immunohistochemistry

Antibody to bovine SPP1 (rabbit polyclonal anti-SPP1) was used to localize SPP1 protein in paraffin sections of luteal tissue collected at previously identified times in the estrous cycle ($n=4$ CL per functional state). The tissues were processed, embedded in paraffin, and sectioned at a thickness of 6 μ m. Slides were prepared for staining as described previously by Bove *et al.* (2000). The tissue sections were incubated for 1 h at room temperature with rabbit polyclonal anti-SPP1 (5 μ g/ml) antiserum in PBS–1% BSA. After washing, the sections were incubated with a 1:200 dilution of biotinylated goat anti-rabbit IgG secondary antibody for 30 min at room temperature. The presence of SPP1 was detected using an avidin–biotin–peroxidase kit, and diaminobenzidine (Vector Laboratories) was used as the substrate for the enzyme reaction. To confirm positive staining of SPP1, negative controls consisted of omission of the primary antibody or replacement of the primary antibody with nonimmune rabbit IgG. Tissue sections were examined using an Olympus BX51 microscope equipped with an Olympus reflected fluorescence system. Images were collected using an Olympus MagnaFire digital camera.

T lymphocyte proliferation

Lymphocytes were loaded with 2.5 μ M carboxyfluorescein succinimidyl ester according to the manufacturer's protocol and as described by Sathiyaseelan & Baldwin (2000). Lymphocytes were placed either in culture with increasing concentrations of bovine SPP1 or in coculture with luteal cells containing increasing concentrations of bovine SPP1 (Sigma–Aldrich) and T-cell proliferation was analyzed after 24 h using Guava EasyCyte Plus (Millipore). To determine whether SPP1, produced by the steroidogenic cells, stimulates T-cell proliferation, luteal cells and T cells were transfected separately with either lentiviral particles directed toward

SPP1 mRNA or scrambled sequence control and then proliferation was analyzed after 24 h using Guava EasyCyte Plus (Millipore).

Lymphocyte chemotaxis assay

Lymphocyte migration was measured using QCM chemotaxis 96-well plates fitted with 3 μ m membrane inserts (Millipore) according to the manufacturer's instructions. Lymphocytes were placed in the upper chamber of a QCM apparatus and luteal cells and/or increasing concentrations of SPP1 (Sigma–Aldrich) were placed in the lower chamber of the QCM apparatus. To determine the role of SPP1 in luteal cell-induced T-cell chemotaxis, luteal cells and T cells were transfected separately with either lentiviral particles directed toward SPP1 mRNA or scrambled sequence control prior to coculture in the QCM apparatus. Increasing concentrations of monocyte chemoattractant protein 1 (CCL2) were used as a positive control for lymphocyte migration (Carr *et al.* 1994). After 24 h of incubation at 37 °C, T cells that migrated toward the chemoattractant were recovered from the lower chamber and unmigrated T cells from the inserts according to the manufacturer's instructions. Migrated T cells were stained with a green fluorescent dye (CyQuant GR dye, Millipore) and then transferred to a 96-well flat-bottomed ELISA microplate (Costar) and fluorescence was read at 485/535 nm using Wallac 1420 fluorescent plate reader (Perkin Elmer, Waltham, MA, USA). Data are reported in fluorescent units representing T cells that migrated into the lower chamber toward chemoattractant.

Flow cytometry

Lymphocytes were prepared for dual-color flow cytometric analysis by indirect immunofluorescence following luteal cell and T-cell coculture. Lymphocytes were diluted to 5.0×10^5 cells/ml and antibody incubations and washes were performed at 4 °C as described previously (Davis & Pate 2007). T cells were incubated with mouse anti-bovine $\gamma\delta$ (CACT61A), mouse anti-bovine CD8 α (CACT80C or BAQ111A), and mouse anti-bovine CD8 β (BAT82A) for 30 min, washed, and incubated for 30 min with the appropriate secondary antibodies. Rabbit anti-mouse IgM conjugated to FITC (102002) was used to detect $\gamma\delta$ or CD8 α T cells; rabbit anti-mouse IgG1 conjugated to RPE (STAR81PE) was used to detect CD8 α

or CD8 β T cells. Prior to analysis, lymphocytes were resuspended in ice-cold PBS + 2 mM EDTA to an optimal running concentration of ≤ 500 cells/ μ l and analyzed by flow cytometry. Unlabeled T lymphocytes, T lymphocytes labeled only with secondary antibodies, and T lymphocytes labeled with isotype-matched antibody were used to determine autofluorescence and nonspecific binding. A total of 10 000 T lymphocytes were analyzed for each sample.

Statistical analysis

All statistical analyses were performed using the mixed model of SAS (Statistical Analysis System Institute, Cary, NC, USA). Gene expression data were log-transformed and analyzed using covariate analysis, with *GAPDH* as the covariate. Two-way ANOVA followed by the Student–Newman–Keuls test was performed to determine differences among treatment groups for lymphocyte proliferation, chemotaxis, and functional phenotypes. All experiments were repeated with CL from three to five separate animals and data are presented as least-square means $\pm$ S.E.M. Differences were considered significant at $P < 0.05$.

Results

Expression of *SPP1* was significantly less in day 4 CL compared with CL collected on days 5, 10, and 18 of the estrous cycle, but there were no differences in *SPP1* mRNA among CL from days 5, 10, and 18 ($P > 0.05$; Fig. 1A). The concentration of *SPP1* mRNA significantly increased in the CL within 1 h following a luteolytic dose of PGF $_{2\alpha}$ and was further elevated by 4 and 8 h after PGF $_{2\alpha}$ ($P < 0.05$; Fig. 1B). Specific bands of the expected molecular size corresponding to the full-length bovine SPP1 protein (70 kDa) and the cleaved, active isoform

(45 kDa) were detected in CL throughout the estrous cycle and after PGF $_{2\alpha}$ (Fig. 1C and D). An additional 25 kDa cleavage product of SPP1 was observed in developing (day 5) and late cycle (day 18) CL, but this form of SPP1 was completely absent in the fully functional (day 10) CL (Fig. 1C). The day 18 sample shown in Fig. 1D had a low concentration of SPP1 overall, making the 25 kDa band undetectable, which was not representative of the other day 18 samples shown in Fig. 1C. Immunohistochemical localization revealed the presence of SPP1 in small and large steroidogenic cells as well as scattered immune cells in early (day 5, Fig. 2A), fully functional (day 10), and late cycle (day 18) CL (not shown). In addition, SPP1 was identified in large luteal cells and infiltrating immune cells at 2, 4, and 8 h after PGF $_{2\alpha}$ (Fig. 2B).

There was a slight but significant increase in the expression of the mRNA for the SPP1 receptor, hyaluronic acid receptor (*CD44*), which paralleled the increase in SPP1 from day 4 to day 5 CL, and remained elevated through day 18 ($P < 0.05$; Fig. 3A). However, *CD44* mRNA did not change during luteal regression ($P > 0.05$; Fig. 3B). β 3 integrin (*ITGB3*), which has been shown to colocalize with SPP1 in other reproductive tissues, was expressed in the bovine CL but remained unchanged throughout the estrous cycle ($P > 0.05$; Fig. 3A). However, the induction of luteolysis with PGF $_{2\alpha}$ resulted in a decrease in *ITGB3* mRNA at 4 and 8 h compared with control (0 h; $P < 0.05$; Fig. 3B). Expression of interleukin 10 (*IL10*) and *IL12A* mRNA in luteal tissue was significantly greater on days 5 and 10 compared with day 4 ($P < 0.05$; Fig. 3C), paralleling the changes in SPP1 and its receptors. Both *IL10* and *IL12A* declined between days 10 and 18 ($P < 0.05$; Fig. 3C).

Figure 1 Steady-state concentrations of mRNA encoding *SPP1* in CL during the estrous cycle (A; $n=4$) and after PGF $_{2\alpha}$ injection (B; $n=4$). Bars represent log-transformed least-squared mean values $\pm$ S.E.M. Different letters indicate significant differences ($P < 0.05$). Representative western blots of bovine luteal tissue using antibodies against SPP1 during different functional stages of the estrous cycle (developing (day 5), fully functional (day 10), and late cycle (day 18)) and at specific times after PGF $_{2\alpha}$ injection; C and D). This experiment was repeated four times and proteins isolated from bovine uterus (UT) served as a positive control.

Figure 2 Immunohistochemical localization of SPP1 in developing CL (A; day 5) and 8 h after PGF_{2α} (B). Solid arrows and diffuse brown staining indicate the presence of SPP1 in large and small steroidogenic cells. More intense staining was observed in scattered small cells that are likely immune cells (dashed arrows). No staining was observed in the IgG-negative control (C). Images presented are representative of four CL per time point examined taken at 400× magnification.

IL10, but not *IL12A*, mRNA was reduced at 1 and 8 h after PGF_{2α} ($P<0.05$; Fig. 3D).

Luteal cells were cultured with factors known to regulate luteal function to evaluate their effect on SPP1 production. The addition of LH, P₄, and/or AG (to inhibit P₄ production) had no effect on *SPP1* mRNA in cultured luteal cells ($P>0.05$; Fig. 4A). However, the addition of PGF_{2α} and/or TNF significantly increased *SPP1* mRNA ($P<0.05$; Fig. 4B and C). Western blot analysis revealed specific bands of the 45 and 70 kDa forms of SPP1 in all cultured luteal cells treated with LH, P₄, PGF_{2α}, and TNF, alone or in combination with LH (Fig. 4D).

To determine whether SPP1 is involved in luteal cell–T-cell communication, luteal cells and T lymphocytes were cultured for 24 h with increasing concentrations (12 500, 25 000, or 37 500 IFU/μl) of viral IFU containing either *SPP1* siRNA or scrambled sequence controls. Immunoblots for SPP1 revealed that the active isoform (45 kDa) of SPP1 in both luteal cells and lymphocytes decreased as viral particle concentration increased (Fig. 5A and B). Ablation and replacement studies were conducted to determine the effects of SPP1 on luteal cell-induced T-cell proliferation, chemotaxis, and cellular function. Luteal cells stimulated lymphocyte proliferation *in vitro*, regardless of treatment ($P<0.05$; Fig. 5C). Neither a reduction of endogenous nor addition of exogenous SPP1 altered luteal cell-stimulated lymphocyte proliferation ($P>0.05$; Fig. 5C). In lymphocyte-only cultures, knockdown of *SPP1* did not alter lymphocyte migration compared with control lymphocytes ($P>0.05$; Fig. 5D). However, lymphocyte migration was stimulated by luteal cells ($P<0.05$; Fig. 5D) and lymphocyte chemotaxis toward luteal cells was decreased when SPP1 was reduced ($P<0.05$; Fig. 5D). However, the addition of exogenous SPP1 did not increase T-cell migration (Fig. 5D). Moreover, luteal cells were capable of inducing even greater lymphocyte migration than CCL2.

Treatment with *SPP1* siRNA or scrambled sequence control siRNA in the lentiviral vector increased the proportion of $\gamma\delta^+$ and $\gamma\delta^+\text{CD8}^+$ lymphocytes ($P<0.05$; Fig. 6A and B). However, this effect was diminished in the presence of luteal cells ($P<0.05$;

Fig. 6B). In addition, the number of $\text{CD8}\alpha\alpha^+$ T cells increased when T cells, cultured alone or with luteal cells, were treated with either the *SPP1* siRNA or the scrambled sequence control siRNA ($P<0.05$; Fig. 7A). The shift in $\text{CD8}\alpha\alpha^+$ T cells resulted in a small reduction in the number of $\text{CD8}\alpha\beta^+$ T cells when cultured alone, but not when T cells were cultured with luteal cells ($P<0.05$; Fig. 7A).

Discussion

Following ovulation, the theca and granulosa cells of the ovulated follicle undergo extensive tissue remodeling and differentiation to form the CL. Silvester & Luck (1999) proposed that luteinization may be best understood as a wound repair-like process. SPP1 has an important role in cell-to-cell and cell-to-extracellular matrix signaling (Giachelli & Steitz 2000) and can be expected to be highly expressed in tissues undergoing extensive structural development such as during formation of the CL. SPP1 and thrombin colocalize in highly vascularized areas, including sites of tissue damage, such as in wounds, tumors, and at sites of inflammation (Dvorak 1986). Cleavage of the full-length SPP1 protein by thrombin and/or other proteases exposes the glycine–arginine–glycine–aspartic acid–serine (GRGDS) region of the protein, which is critical for proper binding to its receptors and mediating cell-to-cell and cell-to-extracellular matrix interactions (Senger *et al.* 1994, Denhardt & Noda 1998). Gabler *et al.* (2003) identified three SPP1 products of different molecular mass (55, 48, and 25 kDa) in bovine oviductal fluid. In addition, Johnson *et al.* (1999) reported a similar pattern of three SPP1 products (70, 45, and 25 kDa) in ovine endometrial

Figure 3 Steady-state concentrations of mRNA encoding *CD44* (A and B; open bars), integrin $\beta 3$ (*ITGB3*; A and B; solid bars), *IL10* (C and D; open bars), and *IL12A* (C and D; solid bars) in CL during the estrous cycle (A and C; $n=4$) or luteal regression (B and D; $n=4$). Bars represent log-transformed least-squared mean values. Different letters indicate significant differences ($P<0.05$).

Figure 4 Steady-state concentrations of mRNA encoding *SPP1* in bovine luteal cells following treatment with LH (50 ng/ml), AG (50 ng/ml), and P_4 (0.5 μ M; $n=4$). Effect of $PGF_{2\alpha}$ and TNF separately (B) and in combination (C) on the steady-state concentration of *SPP1* mRNA ($n=4$). Bars represent log-transformed least squared mean values and different letters indicate significant differences ($P<0.05$). Representative western blot for SPP1 in cultured bovine luteal cells (D). Treatments consisted of LH, P_4 , AG, P_4 + AG, $PGF_{2\alpha}$, $PGF_{2\alpha}$ + LH, TNF, and TNF + LH or no treatment (control, lane 1).

extracts. The SPP1 protein contains a thrombin cleavage site region (Senger *et al.* 1994, Denhardt & Noda 1998) and when cleaved results in a 23–25 kDa cleavage product (Zhang *et al.* 1990). Western blot analyses demonstrated that the full-length (70 kDa) and the cleaved, activated (45 kDa) isoform of bovine SPP1 are present in CL throughout the estrous cycle. However, the presence of a 25 kDa cleavage product of SPP1 was also observed in developing and late cycle CL, which suggests a potential role of SPP1 signaling during periods of luteal tissue remodeling.

Craig & Denhardt (1991) first demonstrated that sex steroids, primarily P_4 , upregulated *Spp1* mRNA in the mouse endometrium. Moreover, *SPP1* expression was

greatest 6 h after treatment with P_4 but expression was diminished by 24 h after treatment (Craig & Denhardt 1991). In addition, P_4 stimulated *SPP1* expression in the developing ovine uterine glandular epithelium during the peri-implantation period (Johnson *et al.* 2000). P_4 stimulates hypertrophy and hyperplasia of the glandular epithelium and development of the uterine glands to produce numerous growth factors, in addition to SPP1, to provide hematotrophic nutrition to the conceptus (Gray *et al.* 2002). Johnson *et al.* (2000) suggested that P_4 upregulation of SPP1 expression within the uterus may be mediated through additional paracrine factors, such as placental lactogen. Dunlap *et al.* (2008) concluded that placental factors, including placental

Figure 5 Luteal cells and T lymphocytes were cultured for 24 h with increasing concentrations of viral IFU containing either *SPP1* siRNA or scrambled sequence controls (lane 1 = 12 500; lane 2 = 25 000; and lane 3 = 37 500 IFU/ μ l). Representative western blot analyses using antibodies against SPP1 for protein expression in bovine luteal cells (A) and lymphocytes (B) display effective knockdown of endogenous *SPP1*. Luteal cells (LC) and lymphocytes (TC) were treated with lentiviral particles, either directed against *SPP1* mRNA (black bars) or scrambled sequence control (gray bars) or no lentiviral particles (control; white bars) prior to culture. Following *SPP1* knockdown, lymphocytes were then placed in culture for 24 h with increasing concentrations of SPP1 (0, 50, 100, or 500 ng/ml) or in coculture with LC and increasing concentrations of SPP1 (0, 50, 100, or 500 ng/ml) to evaluate the effect of SPP1 on T-cell proliferation (C) or chemotaxis (D). Bars represent least-squared mean values ($n=4$) and different letters indicate significant differences ($P<0.05$).

Figure 6 T cells (TC) were treated with lentiviral particles, either directed against *SPP1* mRNA (black bars) or scrambled sequence control (gray bars) or no lentiviral particles (control; white bars) prior to culture. The proportions of total gamma delta ($\gamma\delta^+ CD8^{+/-}$; A) and $\gamma\delta^+ CD8^+$ (B) TC either cultured alone (TC only) or in the presence of luteal cells (TC+LC) are depicted. Bars represent least-squared mean values and different letters indicate significant differences ($P < 0.05$, $n = 4$). Representative scatter diagrams of control and SPP1 lentiviral-treated lymphocytes dual-labeled for $\gamma\delta$ and $CD8^+$ (C).

lactogen, increase glandular epithelium hyperplasia, which lead to increased *SPP1* gene expression in addition to P_4 alone. In the current study, the increase in *SPP1* expression following P_4 treatment was not observed in luteal cells. This response could be attributed to either the length of P_4 exposure or the absence in our cultures of additional paracrine factors that would have been present within the uterus.

In cattle, the CL is resistant to the luteolytic effects of $PGF_{2\alpha}$ before day 5 of the estrous cycle (Rowson *et al.* 1972), and therefore the CL must acquire the capacity to regress in response to $PGF_{2\alpha}$ (luteolytic capacity; Tsai & Wiltbank (1998)). Several factors including endothelin 1 (EDN1, Wright *et al.* 2001), CCL2 (Townson *et al.* 2002), and 15-hydroxyprostaglandin dehydrogenase (Silva *et al.* 2000) have been proposed mediators for acquisition of luteolysis. Increases in these factors were observed in fully functional (day 10) CL when compared with early (day 4) CL, thus suggesting their role in acquisition of luteolytic capacity. In the current study, there was a significant increase in *SPP1* mRNA during this transition period between days 4 and 5 of the estrous cycle, which is the time during which CL begin to acquire luteolytic capacity (Tsai & Wiltbank 1998). It is suggested that the increase in *SPP1* in day 5 CL facilitates luteal sensitivity to $PGF_{2\alpha}$ and luteolytic capacity.

In addition to the changes in *SPP1* expression early in the estrous cycle, there was an immediate increase in *SPP1* mRNA within 1 h of a luteolytic injection of $PGF_{2\alpha}$. Furthermore, *SPP1* expression continued to increase 4 and 8 h after $PGF_{2\alpha}$. *In vitro* experiments demonstrated that two luteolytic factors, $PGF_{2\alpha}$ and TNF, increased *SPP1* expression in cultured luteal cells. The upregulation of *SPP1* during luteolysis may serve as a signaling molecule to recruit immune cells into the CL to facilitate luteal regression and tissue degradation.

Two types of receptors have been shown to mediate *SPP1* actions. First, *SPP1* can bind to integrins via interaction with the GRGDS region of the protein (Denhardt & Noda 1998). Johnson *et al.* (2001) demonstrated that $\alpha_v\beta_3$, $\alpha_v\beta_1$, $\alpha_v\beta_5$, $\alpha_4\beta_1$, and $\alpha_5\beta_1$ integrin heterodimers serve as *SPP1* receptors in the

ovine uterus to initiate outside-in signaling and mediate attachment of the conceptus. Alternatively, *SPP1* can also bind to CD44, the hyaluronic acid receptor, to activate immune cell responses (Weber *et al.* 1996).

Figure 7 (A) T cells (TC) were treated with lentiviral particles, either directed against *SPP1* mRNA (black bars) or scrambled sequence control (gray bars) or no lentiviral particles (control; white bars) prior to culture. The proportions of $CD8\alpha^+$ and $CD8\alpha\beta^+$ TC either cultured alone (TC only) or in the presence of luteal cells (TC+LC) are depicted. Bars represent least-squared mean values and different letters indicate significant differences ($P < 0.05$, $n = 4$). Representative scatter diagrams of control and SPP1 lentiviral-treated lymphocytes dual-labeled for $CD8\alpha$ and $CD8\beta$ (B).

In this study, there was a decrease in *ITGB3* mRNA, but not *CD44* mRNA, in luteal tissue following a luteolytic dose of $\text{PGF}_{2\alpha}$. The loss of *ITGB3* following $\text{PGF}_{2\alpha}$ could be associated with the degradation of the extracellular matrix and altered cellular environment that occurs during luteal regression as reported by Smith *et al.* (1999). In the CL, expression of *CD44* mRNA paralleled *SPP1* mRNA throughout the estrous cycle whereas *ITGB3* mRNA remained unchanged. The cell surface glycoprotein, CD44, is expressed in many cell types, including leukocytes, erythrocytes, fibroblasts, endothelial and epithelial cells, and a variety of tumor cells (Lesley *et al.* 1993) and is responsible for many cellular responses including cell-to-cell and cell-to-ECM interactions and stimulation of immune cells (reviewed by Cichy & Puré (2003)). Activation of macrophage surface CD44 via antibody stimulation resulted in increased phagocytosis of apoptotic neutrophils *in vitro* (Vivers *et al.* 2004). Within the pig ovary, Miyake *et al.* (2006) examined the role of CD44 in ovarian macrophages and concluded that CD44 may be responsible for removing apoptotic granulosa cells in atretic follicles. Binding of SPP1 to its receptors influences cytokine production (O'Regan *et al.* 1999, O'Regan & Berman 2000). Ashkar *et al.* (2000) demonstrated that SPP1 increased IL12 and suppressed IL10 production by macrophages, acting through $\alpha_v\beta_3$ and CD44 receptors respectively. Thus, the effect of SPP1 on the IL10:IL12 ratio may depend on the relative abundance of these two receptors. In the current study, both *IL10* and *IL12A* mRNA increase in the developing CL reaching maximal expression in the fully functional CL. The expression of the mRNA for the anti-inflammatory cytokine, IL10, was greater than that for the proinflammatory cytokine, IL12. If protein concentrations (not quantified in this study) are reflective of differences in *IL10/IL12* mRNA concentrations, it would suggest that the developing CL establishes a microenvironment suitable to ensure luteal homeostasis. During luteolysis, reduced *ITGB3* and *IL10* would allow for a proinflammatory environment, leading to destruction of the tissue and leukocyte chemotaxis.

Functional interactions exist between T cells and luteal cells *in vitro* including luteal cell induction of T-cell proliferation and inhibition of steroidogenesis by immune cell-derived proinflammatory cytokines (reviewed by Pate & Keyes (2001)). Patarca *et al.* (1989) demonstrated that SPP1 stimulated lymphocyte activation at the initiation of an immune response. Thus, the increase in luteal SPP1 in response to $\text{PGF}_{2\alpha}$ both *in vitro* and *in vivo* could serve as a signaling molecule to modulate lymphocyte function in the regressing CL, but based on the current results, any such response is not likely to involve T-cell proliferation.

The addition of exogenous SPP1 had no effect on lymphocyte chemotaxis, possibly because it dispersed throughout the media, failing to create a desirable

concentration gradient or because the SPP1 was not cleaved into bioactive fragments (Senger *et al.* 1994). Regardless, knockdown of *SPP1* reduced luteal cell-induced lymphocyte migration and removing SPP1 from the culture system significantly reduced T-cell migration, providing support for the hypothesis that SPP1 serves as a lymphocyte chemoattractant within the CL during periods of luteal tissue remodeling. A similar role for SPP1 was reported by Cheng *et al.* (2007).

Lentiviral vectors can provide an effective means for gene transfer into human lymphocytes without T-cell activation, thus preserving the phenotype and functional characteristics of the transduced T cells (Cavalieri *et al.* 2003). However, lentiviral particles containing either *SPP1* or scrambled sequence siRNA increased the proportion of total $\gamma\delta^+$, $\gamma\delta^+\text{CD8}^+$, and $\text{CD8}\alpha\alpha^+$ lymphocytes and decreased the proportion of $\text{CD8}\alpha\beta^+$ lymphocytes in this study. The vast majority of peripheral blood lymphocytes of humans and mice express the $\alpha\beta$ TCR, whereas lymphocytes expressing the $\gamma\delta$ TCR are prominent in peripheral blood of ruminants (Mackay & Hein 1989). Unlike the $\alpha\beta$ T cells, $\gamma\delta$ T cells recognize, but are not restricted to, class I and II MHC molecules and have been shown to respond to a wide range of molecules (reviewed by Chien *et al.* (1996)). Gamma delta T cells are capable of killing virus-infected cells *in vitro* (Bukowski *et al.* 1994) and are activated in response to viral infections *in vivo* (De Paoli *et al.* 1990, Wallace *et al.* 1995), which likely explains the results obtained in this study in response to lentiviral particles.

During times of tissue remodeling, such as during luteal development and luteolysis, SPP1 facilitates recruitment and/or activation of immune cells and SPP1 may influence the responsiveness of the CL to the luteolytic effects of $\text{PGF}_{2\alpha}$. The increase in *SPP1* mRNA between day 4 and 5 of the estrous cycle may be essential for proper luteal development and sensitivity to the luteolytic effects of $\text{PGF}_{2\alpha}$. Furthermore, inhibition of endogenous SPP1 reduced luteal cell-induced lymphocyte chemotaxis, and there was an increase in luteal SPP1 in response to $\text{PGF}_{2\alpha}$ both *in vitro* and *in vivo*, providing evidence that SPP1 plays an active role as a signaling molecule to recruit lymphocytes into the regressing CL. Based on these data, it is concluded that this diverse protein is initially upregulated, allowing for acquisition of luteolytic capacity in the developing CL. Additionally, SPP1 increases during luteolysis, to serve as a signaling molecule to recruit immune cells into the CL to facilitate luteal regression and tissue degradation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by National Research Initiative Competitive grant number 2008-35203-04617 from the USDA National Institute of Food and Agriculture to J L Pate.

Acknowledgements

The authors wish to thank Jodi Winkler, Justin Fear, Sadhat Walusimbi, and Edyta Brzezicka for their assistance with sample and PCR data collection and Dr Gary Killian for his generous donation of bovine SPP1 antibody.

References

- Ashkar S, Weber GF, Panoutsakopoulou V, Sanchirico ME, Jansson M, Zawaideh S, Rittling SR, Denhardt DT, Glimcher MJ & Cantor H 2000 SPP1 (osteopontin): an early component of type-1 (cell-mediated) immunity. *Science* **287** 860–864. (doi:10.1126/science.287.5454.860)
- Asou Y, Rittling SR, Yoshitake H, Tsuji K, Shinomiya K, Nifuji A, Denhardt DT & Noda M 2001 Osteopontin facilitates angiogenesis, accumulation of osteoclasts, and resorption in ectopic bone. *Endocrinology* **142** 1325–1332. (doi:10.1210/en.142.3.1325)
- Benyo DF & Pate JL 1992 Tumor necrosis factor- α alters bovine luteal cell synthetic capacity and viability. *Endocrinology* **130** 854–860. (doi:10.1210/en.130.2.854)
- Bove SE, Petroff MG, Nishibori M & Pate JL 2000 Macrophage migration inhibitory factor in the bovine corpus luteum: characterization of steady-state messenger ribonucleic acid and immunohistochemical localization. *Biology of Reproduction* **62** 857–885. (doi:10.1095/biolreprod62.4.879)
- Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72** 248–254. (doi:10.1016/0003-2697(76)90527-3)
- Brännström M & Norman RJ 1993 Involvement of leukocytes and cytokines in the ovulatory process and corpus luteum function. *Human Reproduction* **8** 1762–1775.
- Brown LF, Berse B, Van de Water L, Papadopoulos-Sergiou A, Perruzzi CA, Manseau EJ, Dvorak HF & Senger DR 1992 Expression and distribution of osteopontin in human tissues: widespread association with luminal epithelial surfaces. *Molecular Biology of the Cell* **3** 1169–1180. (doi:10.1091/mbc.3.10.1169)
- Brunswig-Spickenheier B & Mukhopadhyay AK 2003 Expression of osteopontin (OPN) mRNA in bovine ovarian follicles and corpora lutea. *Reproduction in Domestic Animals* **38** 175–181. (doi:10.1046/j.1439-0531.2003.00413.x)
- Bukowski JF, Morita CT & Brenner MB 1994 Recognition and destruction of virus-infected cells by human gamma delta CTL. *Journal of Immunology* **153** 5133–5140.
- Carr MW, Roth SJ, Luther E, Rose SS & Springer TA 1994 Monocyte chemoattractant protein 1 acts as a T-lymphocyte chemoattractant. *PNAS* **91** 3652–3656. (doi:10.1073/pnas.91.9.3652)
- Cavaliere S, Cazzaniga S, Geuna M, Magnani Z, Bordignon C, Naldini L & Bonini C 2003 Human T lymphocytes transduced by lentiviral vectors in the absence of TCR activation maintain an intact immune competence. *Blood* **102** 497–505. (doi:10.1182/blood-2003-01-0297)
- Cheng J, Huo DH, Kuang DM, Yang J, Zheng L & Zhuang SM 2007 Human macrophages promote the motility and invasiveness of osteopontin-knockdown tumor cells. *Cancer Research* **67** 5141–5147. (doi:10.1158/0008-5472.CAN-06-4763)
- Chien YH, Jores R & Crowley MP 1996 Recognition by gamma/delta T cells. *Annual Reviews in Immunology* **14** 511–532. (doi:10.1146/annurev.immunol.14.1.511)
- Cichy J & Puré E 2003 The liberation of CD44. *Journal of Cell Biology* **161** 839–843. Review. (doi:10.1083/jcb.200302098)
- Craig AM & Denhardt DT 1991 The murine gene encoding secreted phosphoprotein 1 (osteopontin): promoter structure, activity, and induction *in vivo* by estrogen and progesterone. *Gene* **100** 163–171. (doi:10.1016/0378-1119(91)90362-F)
- Davis TL & Pate JL 2007 Bovine luteal cells stimulate proliferation of major histocompatibility nonrestricted gamma delta T cells. *Biology of Reproduction* **77** 914–922. (doi:10.1095/biolreprod.106.059824)
- Denhardt DT & Noda M 1998 Osteopontin expression and function: role in bone remodeling. *Journal of Cellular Biochemistry. Supplement* **30–31** 92–102. (doi:10.1002/(SICI)1097-4644(1998)72:30/31<92::AID-JCB13>3.0.CO;2-A)
- Denhardt DT, Giachelli CM & Rittling SR 2001 Role of osteopontin in cellular signaling and toxicant injury. *Annual Review of Pharmacology and Toxicology* **41** 723–749. (doi:10.1146/annurev.pharmtox.41.1.723)
- De Paoli P, Gennari D, Martelli P, Cavarzerani V, Comoretto R & Santini G 1990 $\gamma\delta$ T cell receptor-bearing lymphocytes during Epstein–Barr virus infection. *Journal of Infectious Diseases* **161** 1013–1016. (doi:10.1093/infdis/161.5.1013)
- Dunlap KA, Erikson DW, Burghardt RC, White FJ, Reed KM, Farmer JL, Spencer TE, Magness RR, Bazer FW, Bayless KJ *et al.* 2008 Progesterone and placentation increase secreted phosphoprotein one (SPP1 or osteopontin) in uterine glands and stroma for histotrophic and hematotrophic support of ovine pregnancy. *Biology of Reproduction* **79** 983–990. (doi:10.1095/biolreprod.108.071068)
- Dvorak HF 1986 Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *New England Journal of Medicine* **315** 1650–1659. (doi:10.1056/NEJM198612253152606)
- Erikson DW, Burghardt RC, Bayless KJ & Johnson GA 2009 Secreted phosphoprotein 1 (SPP1, osteopontin) binds to integrin α v β 6 on porcine trophectoderm cells and integrin α v β 3 on uterine luminal epithelial cells, and promotes trophectoderm cell adhesion and migration. *Biology of Reproduction* **81** 814–825. (doi:10.1095/biolreprod.109.078600)
- Franzen A & Heinegard D 1985 Isolation and characterization of two sialoproteins present only in bone calcified matrix. *Biochemical Journal* **232** 715–724.
- Gabler C, Chapman DA & Killian GJ 2003 Expression and presence of osteopontin and integrins in the bovine oviduct during the estrous cycle. *Reproduction* **126** 721–729. (doi:10.1530/rep.0.1260721)
- Giachelli CM & Steitz S 2000 Osteopontin: a versatile regulator of inflammation and biomineralization. *Matrix Biology* **19** 615–622. (doi:10.1016/S0945-053X(00)00108-6)
- Gray CA, Burghardt RC, Johnson GA, Bazer FW & Spencer TE 2002 Evidence that absence of endometrial gland secretions in uterine gland knockout ewes compromises conceptus survival and elongation. *Reproduction* **124** 289–300. (doi:10.1530/rep.0.1240289)
- Hirama M, Takahashi F, Takahashi K, Akutagawa S, Shimizu K, Soma S, Shimanuki Y, Nishio K & Fukuchi Y 2003 Osteopontin overproduced by tumor cells acts as a potent angiogenic factor contributing to tumor growth. *Cancer Letters* **198** 107–117. (doi:10.1016/S0304-3835(03)00286-6)
- Johnson GA, Spencer TE, Burghardt RC & Bazer FW 1999 Ovine osteopontin: I. Cloning and expression of messenger ribonucleic acid in the uterus during the periimplantation. *Biology of Reproduction* **6** 884–891.
- Johnson GA, Spencer TE, Burghardt RC, Taylor KM, Gray CA & Bazer FW 2000 Progesterone modulation of osteopontin gene expression in the ovine uterus. *Biology of Reproduction* **62** 1315–1321. (doi:10.1095/biolreprod62.5.1315)
- Johnson GA, Bazer FW, Jaeger LA, Ka H, Garlow JE, Pfarrer C, Spencer TE & Burghardt RC 2001 Muc-1, integrin, and osteopontin expression during the implantation cascade in sheep. *Biology of Reproduction* **65** 820–828. (doi:10.1095/biolreprod65.3.820)
- Johnson GA, Burghardt RC, Bazer FW & Spencer TE 2003 Osteopontin: roles in implantation and placentation. *Biology of Reproduction* **69** 1458–1471. (doi:10.1095/biolreprod.103.020651)
- Kim JH, Skates SJ, Uede T, Wong KK, Schorge JO, Feltmate CM, Berkowitz RS, Cramer DW & Mok SC 2002 Osteopontin as a potential diagnostic biomarker for ovarian cancer. *Journal of the American Medical Association* **287** 1671–1679. (doi:10.1001/jama.287.13.1671)
- Kim J, Erikson DW, Burghardt RC, Spencer TE, Wu G, Bayless KJ, Johnson GA & Bazer FW 2010 Secreted phosphoprotein 1 binds integrins

- to initiate multiple cell signaling pathways, including FRAP1/mTOR, to support attachment and force-generated migration of trophectoderm cells. *Matrix Biology* **29** 369–382. (doi:10.1016/j.matbio.2010.04.001)
- Lesley J, Hyman R & Kincade PW** 1993 CD44 and its interaction with extracellular matrix. *Advances in Immunology* **54** 271–335.
- Mackay CR & Hein WR** 1989 A large proportion of bovine T cells express the gamma delta T cell receptor and show a distinct tissue distribution and surface phenotype. *International Immunology* **1** 540–545. (doi:10.1093/intimm/1.5.540)
- Miyake Y, Matsumoto H, Yokoo M, Miyazawa K, Kimura N, Tunjung WA, Shimizu T, Sasada H, Aso H, Yamaguchi T et al.** 2006 Expression and glycosylation with polylectosamine of CD44 antigen on macrophages during follicular atresia in pig ovaries. *Biology of Reproduction* **74** 501–510. (doi:10.1095/biolreprod.105.045641)
- Ndiaye K, Poole DH & Pate JL** 2008 Expression and regulation of functional oxytocin receptors in bovine T lymphocytes. *Biology of Reproduction* **78** 786–793. (doi:10.1095/biolreprod.107.065938)
- Niswender G, Juengel J, Silva P, Rollyson M & McIntush E** 2000 Mechanisms controlling the function and life span of the corpus luteum. *Physiological Reviews* **80** 1–29.
- O'Regan A & Berman JS** 2000 Osteopontin: a key cytokine in cell-mediated and granulomatous inflammation. *International Journal of Experimental Pathology* **81** 373–390. (doi:10.1046/j.1365-2613.2000.00163.x)
- O'Regan AW, Chupp GL, Lowry JA, Goetschkes M, Mulligan N & Berman JS** 1999 Osteopontin is associated with T cells in sarcoid granulomas and has T cell adhesive and cytokine-like properties *in vitro*. *Journal of Immunology* **162** 1024–1031.
- Patarca R, Freeman GJ, Singh RP, Wei FY, Durfee T, Blattner F, Regnier DC, Kozak CA, Mock BA & Morse HC** 1989 Structural and functional studies of the early T lymphocyte activation 1 (Eta-1) gene. Definition of a novel T cell-dependent response associated with genetic resistance to bacterial infection. *Journal of Experimental Medicine* **170** 145–161. (doi:10.1084/jem.170.1.145)
- Pate JL** 1993 Isolation and culture of fully differentiated bovine luteal cells. *Methods in Toxicology* **3B** 360–370.
- Pate JL** 1994 Cellular components involved in luteolysis. *Journal of Animal Science* **72** 1884–1890.
- Pate JL & Condon WA** 1989 Regulation of steroidogenesis and cholesterol synthesis by prostaglandin $F_{2\alpha}$ and lipoproteins in bovine luteal cells. *Journal of Reproduction and Fertility* **87** 439–446. (doi:10.1530/jrf.0.0870439)
- Pate JL & Keyes LP** 2001 Immune cells in the corpus luteum: friends or foes? *Reproduction* **122** 665–676. (doi:10.1530/rep.0.1220665)
- Rowson LE, Tervit R & Brand A** 1972 The use of prostaglandins for synchronization of oestrus in cattle. *Journal of Reproduction and Fertility* **29** 145. (doi:10.1530/jrf.0.0290145)
- Sathiyaseelan T & Baldwin CL** 2000 Evaluation of cell replication by bovine T cells in polyclonally activated cultures using carboxy-fluorescein succinimidyl ester (CFSE) loading and flow cytometric analysis. *Research in Veterinary Science* **69** 275–281. (doi:10.1053/rvsc.2000.0429)
- Senger DR, Perruzzi CA, Papadopoulos-Sergiou A & Van de Water L** 1994 Adhesive properties of osteopontin: regulation by a naturally occurring thrombin-cleavage in close proximity to the GRGDS cell-binding domain. *Molecular Biology of the Cell* **5** 565–574. (doi:10.1091/mbc.5.5.565)
- Silva PJ, Juengel JL, Rollyson MK & Niswender GD** 2000 Prostaglandin metabolism in the ovine corpus luteum: catabolism of prostaglandin $F(2\alpha)$ ($PGF(2\alpha)$) coincides with resistance of the corpus luteum to $PGF(2\alpha)$. *Biology of Reproduction* **63** 1229–1236. (doi:10.1095/biolreprod63.5.1229)
- Silvester LM & Luck MR** 1999 Distribution of extracellular matrix components in the developing ruminant corpus luteum: a wound repair hypothesis for luteinization. *Journal of Reproduction and Fertility* **116** 187–198. (doi:10.1530/jrf.0.1160187)
- Smith ME, McIntush EW, Ricke WA, Kojima FN & Smith GW** 1999 Regulation of ovarian extracellular matrix remodelling by metalloproteinases and their tissue inhibitors: effects on follicular development, ovulation and luteal function. *Journal of Reproduction and Fertility. Supplement* **54** 367–381.
- Sodek J, Ganss B & McKee MD** 2000 Osteopontin. *Critical Reviews in Oral Biology and Medicine* **11** 279–303. (doi:10.1177/10454411000110030101)
- Takahashi F, Akutagawa S, Fukumoto H, Tsukiyama S, Ohe Y, Takahashi K, Fukuchi Y, Saijo N & Nishio K** 2002 Osteopontin induces angiogenesis of murine neuroblastoma cells in mice. *International Journal of Cancer* **98** 707–712. (doi:10.1002/ijc.10261)
- Townson DH & Pate JL** 1996 Mechanism of action of $TNF-\alpha$ -stimulated prostaglandin production in cultured bovine luteal cells. *Prostaglandins* **52** 361–373.
- Townson DH, O'Connor CL & Pru JK** 2002 Expression of monocyte chemoattractant protein-1 and distribution of immune cell populations in the bovine corpus luteum throughout the estrous cycle. *Biology of Reproduction* **66** 361–366. (doi:10.1095/biolreprod66.2.361)
- Tsai SJ & Wiltbank MC** 1998 Prostaglandin $F_{2\alpha}$ regulates distinct physiological changes in early and mid-cycle bovine corpora lutea. *Biology of Reproduction* **58** 346–352. (doi:10.1095/biolreprod58.2.346)
- Vivers S, Heasman SJ, Hart SP & Dransfield I** 2004 Divalent cation-dependent and -independent augmentation of macrophage phagocytosis of apoptotic neutrophils by CD44 antibody. *Clinical and Experimental Immunology* **138** 447–452. (doi:10.1111/j.1365-2249.2004.02638.x)
- Wai PY, Mi Z, Guo H, Sarraf-Yazdi S, Gao C, Wei J, Marroquin CE, Clary B & Kuo PC** 2005 Osteopontin silencing by small interfering RNA suppresses *in vitro* and *in vivo* CT26 murine colon adenocarcinoma metastasis. *Carcinogenesis* **26** 741–751. (doi:10.1093/carcin/bgi027)
- Wallace M, Malkovsky M & Carding SR** 1995 Gamma/delta T lymphocytes in viral infections. *Journal of Leukocyte Biology* **58** 277–283.
- Weber GF, Ashkar S & Cantor H** 1996 Interaction between CD44 and osteopontin as a potential basis for metastasis formation. *Proceedings of the Association of American Physicians* **109** 1–9.
- Wright MF, Sayre B, Keith Inskeep EK & Flores JA** 2001 Prostaglandin $F(2\alpha)$ regulation of the bovine corpus luteum endothelin system during the early and midluteal phase. *Biology of Reproduction* **65** 1710–1717. (doi:10.1095/biolreprod65.6.1710)
- Xie Y, Nishi S, Iguchi S, Imai N, Sakatsume M, Saito A, Ikegame M, Iino N, Shimada H, Ueno M et al.** 2001 Expression of osteopontin in gentamicin-induced acute tubular necrosis and its recovery process. *Kidney International* **59** 959–974. (doi:10.1046/j.1523-1755.2001.059003959.x)
- Zhang Q, Domenicucci C, Goldberg HA, Wrana JL & Sodek J** 1990 Characterization of fetal porcine bone sialoproteins, secreted phosphoprotein I (SPPI, osteopontin), bone sialoprotein, and a 23-kDa glycoprotein. Demonstration that the 23-kDa glycoprotein is derived from the carboxyl terminus of SPPI. *Journal of Biological Chemistry* **265** 7583–7589.
- Zheng J, Fricke P, Reynolds L & Redmer D** 1994 Evaluation of growth, cell proliferation, and cell death in bovine corpora lutea throughout the estrous cycle. *Biology of Reproduction* **51** 623–632. (doi:10.1095/biolreprod51.4.623)

Received 3 May 2013

First decision 3 June 2013

Revised manuscript received 16 August 2013

Accepted 9 September 2013