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)F2α. Expression of the SPP1 receptor, β3 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 PGF2α 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)F2α, 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 αβ 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 trophoderm 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 (Lake-wood, 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 (cat. no. ab14175) 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 PGF2α (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 1 (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 × 10⁶ cells/ml) were treated with LH (10 ng/ml), P₄ (0.5 μM), aminoglutethimide (AG; 50 μg/ml), PGF₂α (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 & Condgon 1989, Benyo & Pate 1992, Townsend & 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 ≥72% was achieved in the luteal cell cultures and a transfection efficiency of ≥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), β3 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 Celllytic 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 Pharma 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...
separately with either lentiviral particles directed toward SPP1, proliferation, luteal cells and T cells were transfected with increasing concentrations of bovine SPP1 (Sigma–Aldrich) and T-cell proliferation was analyzed after 24 h using Guava EasyCyte Plus (Millipore).

**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 mm. 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 μg/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 μM 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 μm 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 un migrated 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 5.0x10^5 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 γδ (CACT61A), mouse anti-bovine CD8α (CACT80C or BAQ111A), and mouse anti-bovine CD8β (BATB2A) for 30 min, washed, and incubated for 30 min with the appropriate secondary antibodies. Rabbit anti-mouse IgG conjugated to FITC (102002) was used to detect γδ or CD8α T cells; rabbit anti-mouse IgG1 conjugated to RPE (STAR81PE) was used to detect CD8β.
or CD8β T cells. Prior to analysis, lymphocytes were resuspended in ice-cold PBS +2 mM EDTA to an optimal running concentration of ≤500 cells/μl 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 ± 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 PGF2α and was further elevated by 4 and 8 h after PGF2α (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 PGF2α (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 PGF2α (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). β3 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 PGF2α 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).
IL10, but not IL12A, mRNA was reduced at 1 and 8 h after PGF$_{2\alpha}$ (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, P$_4$, and/or AG (to inhibit P$_4$ production) had no effect on SPP1 mRNA in cultured luteal cells (P>0.05; Fig. 4A). However, the addition of PGF$_{2\alpha}$ 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, P$_4$, PGF$_{2\alpha}$, 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 transcript 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

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 β3 (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).
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 P₄, upregulated Spp1 mRNA in the mouse endometrium. Moreover, SPP1 expression was greatest 6 h after treatment with P₄ but expression was diminished by 24 h after treatment (Craig & Denhardt 1991). In addition, P₄ stimulated SPP1 expression in the developing ovine uterine glandular epithelium during the peri-implantation period (Johnson et al. 2000). P₄ 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. 1991). In addition, P₄ stimulated SPP1 expression in cultured bovine luteal cells (Dunlap et al. 2008) concluded that placental factors, including placental...
lactogen, increase glandular epithelium hyperplasia, which lead to increased SPP1 gene expression in addition to P₄ alone. In the current study, the increase in SPP1 expression following P₄ treatment was not observed in luteal cells. This response could be attributed to either the length of P₄ 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 PGF₂α before day 5 of the estrous cycle (Rowson et al. 1972), and therefore the CL must acquire the capacity to regress in response to PGF₂α (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 PGF₂α 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 PGF₂α. Furthermore, SPP1 expression continued to increase 4 and 8 h after PGF₂α. In vitro experiments demonstrated that two luteolytic factors, PGF₂α 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 GRGD5 region of the protein (Denhardt & Noda 1998). Johnson et al. (2001) demonstrated that α₁β₃, α₂β₁, α₃β₁, α₄β₁, and α₅β₁ 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 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 (γδ⁺, CD8⁺⁻; A) and γδ⁺ 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 γδ and CD8⁺⁺ (C).

**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 CD8α⁺⁺ and CD8β⁺⁺ 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 CD8α and CD8β (B).
In this study, there was a decrease in ITGB3 mRNA, but not CD44 mRNA, in luteal tissue following a luteolytic dose of PGF$_{2\alpha}$. The loss of ITGB3 following 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 CD4+ T cells, but based on the current results, any such response is not to modulate lymphocyte function in the regressing CL, as a signaling molecule to recruit immune cells into 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 (Cavaliere et al. 2003). However, lentiviral particles containing either SPP1 or scrambled sequence siRNA increased the proportion of total γδ T, γδ+CD8+ T, and CD8αα+ lymphocytes and decreased the proportion of CD8ββ+ lymphocytes in this study. The vast majority of peripheral blood lymphocytes of humans and mice express the αβ TCR, whereas lymphocytes expressing the γδ TCR are prominent in peripheral blood of ruminants (Mackay & Hein 1989). Unlike the αβ T cells, γδ 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 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 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 PGF$_{2\alpha}$ 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.

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Reproduction (2013) 146 527–537

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