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Anthony K. McNeel

USDA-ARS, anthony.mcneel@ars.usda.gov

Robert A. Cushman

USDA-ARS, Bob.Cushman@ars.usda.gov

Jeffrey L. Vallet

USDA-ARS

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The Plasminogen Activator System in the Ovine Placentome During Late Gestation and Stage-Two of Parturition

ANTHONY K. McNEEL,* ROBERT A. CUSHMAN, AND JEFFREY L. VALLET

United States Department of Agriculture (USDA), Agricultural Research Service, U.S. Meat Animal Research Center, Reproduction Research Unit, Clay Center, Nebraska

SUMMARY

The process of placental separation is not completely understood. In domestic animals, especially cattle, it is important that expulsion of the fetal membranes takes place in a timely manner in order to achieve maximal reproductive efficiency. The activity of the matrix-metalloprotease (MMP) family of proteases is known to be reduced in placentomes from cases of retained placenta. Members of the MMP family are known to be activated by the plasminogen activator (PA) family of proteases. We hypothesized that the expression and activity of the PA family increase in the cotyledon and/or caruncle as parturition approaches, with maximal expression and activity at parturition. To test this hypothesis, we performed reverse-transcriptase quantitative PCR and plasminogen-casein zymography to detect the presence and activity of PA family members in the placentome leading up to and during parturition in spontaneous and dexamethasone-induced parturient ewes. The results from our experiments indicated that serine proteases inhibitor E1 (*SERPINE1*) mRNA abundance in the cotyledon was different between treatment groups ($P = 0.0002$). In the caruncle, gene expression for plasminogen activator urokinase-type (*PLAU*) was different ($P = 0.0154$), and there was a strong trend for differences in *SERPINE1* expression ($P = 0.0565$). These results demonstrate that expression of the PA system in the placentome changes from late pregnancy to parturition, and the presence or activity of these enzymes may occur after fetal expulsion.



*Corresponding author:
USDA
Agricultural Research Service
U.S. Meat Animal Research Center
Reproduction Research Unit
P.O. Box 166
Clay Center, NE 68933-0166.
E-mail: anthony.mcneel@ars.usda.gov

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Abbreviations: MMP, matrix metalloprotease; PA, plasminogen activator; PLAT, plasminogen activator tissue-type; PLAU[R], plasminogen activator urokinase-type [receptor]; SERPIN, serine protease inhibitor

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INTRODUCTION

Retained placenta is a reproductive disorder of significant concern to dairy producers as it has recently been estimated to reduce milk production by 753 kg over the course of a 305-day lactation period (Dubuc et al., 2011). Aside from lost milk production, retained placenta increases the risk for developing endometritis; together, these reproductive disorders are associated with reduced pregnancy rates (15% and 16%, respectively; Fourichon et al., 2000). Both are associated with an increased risk of metritis (Dubuc et al., 2010), and extend days-to-conception by 7 days (Fourichon et al., 2000). Retained placenta is also of concern for beef producers as induction of parturition (e.g. with dexamethasone) or Cesarean sections are known to increase the risk of the same disorder (Eiler and Fecteau, 2007).

Placental development in ruminants is mediated by trophoblast adhesion mainly within the placentome. Adhesion of trophoblasts to endometrial cells occurs through the action of integrins and their interaction with extracellular matrix proteins, most notably osteopontin (Johnson et al., 2003). During parturition, integrin-mediated adhesion must be disrupted in order to facilitate proper expulsion of the fetal membranes, which is an important step for the proper involution of the uterus following pregnancy. Studies that have investigated the molecular mechanism of placentome separation are limited, and have primarily focused on the collagenolytic family of proteases. Gross et al. (1985) were the first to report reduced collagenolytic activity in cases of retained placenta, but subsequent studies have produced conflicting results in terms of whether or not collagenolytic activity is reduced (Maj and Kankofer, 1997; Walter and Boos, 2001; Dilly et al., 2011).

Collagenases, also known as matrix-metalloproteases (MMPs), are a family of 26 Zn²⁺-dependent endoproteases. Four inhibitors (tissue inhibitor of matrix-metalloproteases: TIMPs) of these proteases are known to play roles in modulating the activities of the MMP. Each MMP possesses a spectrum of specificities for different extracellular matrix proteins with some functional overlap, the most common being collagen (Overall, 2002). MMPs are synthesized and secreted as inactive zymogens (pro-MMPs). Activation involves removal of the N-terminus to expose the catalytic Zn²⁺, referred to as the cysteine switch (Chakraborti et al., 2003). Once pro-MMPs are secreted, it is thought that proteases within the plasminogen activator (PA) family are responsible for MMP activation (Ny et al., 2002; Curry and Osteen, 2003). The PA family consists of the enzymes plasmin/plasminogen (PLG), plasminogen activator tissue-type (PLAT), plasminogen activator urokinase-type (PLAU), and its receptor (PLAUR). Inhibitors of the PA family include α_2 -antiplasmin, plasminogen activator inhibitor-1 (SERPINE1), plasminogen activator inhibitor-2 (SERPINB2), and protein C inhibitor (SERPINA5). These inhibitors covalently bind to their ligands, blocking activity of the enzyme, and eventually leading to their degradation (Olson et al., 2001).

Plasminogen is proteolytically converted to plasmin by other proteases, most notably PLAT and PLAU (Ny et al., 2002). Both activators are also secreted as single polypeptide chains and possess little (PLAT) to no (PLAU) activity in their single chain form (Mayer, 1990). When exposed to fibrin (PLAT) or bound to their receptor (PLAU), these enzymes gain limited activity that is sufficient to initiate a reciprocal activation cascade with plasminogen/plasmin (Lijnen et al., 1990; Higazi et al., 1995). PA-dependent activation of MMPs can result in the activation of pro-PA and other MMPs, further propagating the proteolytic cascade (Orgel et al., 1998; Ny et al., 2002; Xu et al., 2010). Under normal physiological conditions, the ratio between the PA enzymes and their inhibitors favors the inhibitors. The known role of PA in activation of MMPs, combined with reports that placentomes from retained fetal membranes possess reduced MMP activity, suggests that reduced PA activity may play a role in the pathogenesis of retained placenta in cattle. To elucidate the involvement of the PA system in placental separation and retained placenta, we compared PA expression and activity in placentomal tissues from late gestation, in spontaneously lambing ewes, and in ewes induced to lamb with dexamethasone. Lastly, as tissue collection technique is more invasive than a standard Cesarean section, we documented the impact of Cesarean section/placentome collection on subsequent reproductive performance.

RESULTS

Reproductive Performance of Ewes Following Cesarean Section

Neither the number of lambs nor the sex of the lambs in the litter were different between any of our treatments ($P = 0.44$ and $P = 0.77$, respectively). None of the 13 ewes from which placentomes were collected at parturition died from the procedure, and no ewe experienced retained placenta. Of these, 12 ewes were mated via live cover, and 11 were subsequently found to be pregnant (91.7%).

Reverse Transcriptase Quantitative (Real-Time) PCR

Messenger RNA abundance in the cotyledon for *PLAU*, *PLAUR*, and *SERPINB2* was not different for any treatment group ($P > 0.05$, Fig. 1A,B,D). There was a treatment effect on mRNA abundance for *SERPINE1* ($P = 0.0002$) in cotyledonary tissue, however. Abundance of *SERPINE1* mRNA in cotyledonary tissues was greatest in those ewes treated with 10 mg of dexamethasone compared to all other treatment groups (Fig. 1C).

In the caruncle, there were no treatment effects on mRNA abundance of *PLAUR* or *SERPINB2* ($P > 0.05$; Fig. 2B,D), but there was a treatment effect on mRNA abundance of *PLAU* ($P = 0.015$) and a strong tendency for *SERPINE1* ($P = 0.0565$). Lastly, neither *PLAT* nor *PLG* expression could be detected using multiple sets of primers at different locations along the transcript.

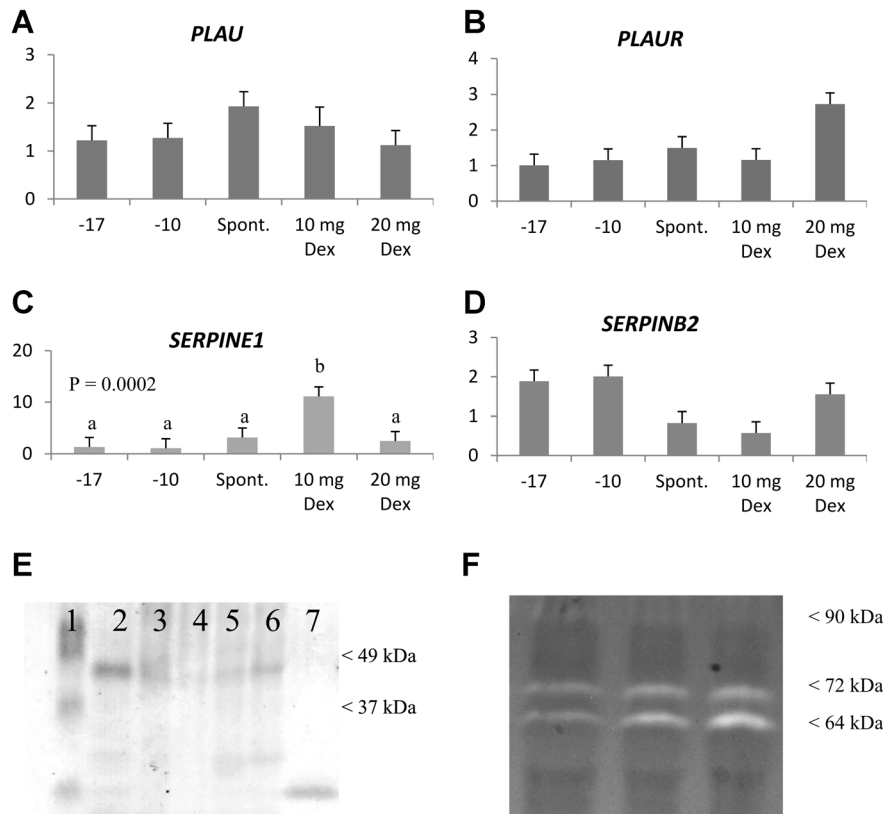


Figure 1. A–E: Least-squares means of the relative mRNA abundance for genes of interest in cotyledonary tissue from ewes collected 17 days before predicted parturition (–17d; $n = 5$); 10 days before predicted parturition (–10d; $n = 5$); at spontaneous parturition (Spont.; $n = 5$); and from ewes induced to lamb with 10 mg (10 mg Dex; $n = 3$) and 20 mg (20 mg Dex; $n = 5$) dexamethasone. Groups with different superscripts are significantly different ($P < 0.05$). F: Representative immunoblot of 80 μ g of protein from homogenates of ovine cotyledons with anti-PLAU-specific goat IgG (Lane 1: pre-stained ladder; Lanes 2–6: cotyledonary tissue extracts; Lanes 7: human PLAU). Band at ~ 48 kDa is presumed to be ovine IgG, as exclusion of a primary antibody also resulted in staining at this molecular weight. G: Representative gelatin zymograph for gelatinase activity in 80 μ g of protein from cotyledon homogenates.

Immunoblotting and Casein-Plasminogen Zymography

Plasminogen casein zymography failed to detect PA activity in cotyledons and caruncles at all time points (data not shown), even though purified human PLAU activity was detectable at 24 hr of incubation to levels as low as 25 mIU. Using immunoblotting, only the positive control (50 IU purified human urokinase-type PA) was detected. Gelatin zymography (as described by Maj and Kankofer, 1997) of a selected number of cotyledonary and caruncular tissue samples indicated that the samples do possess gelatinolytic capacity, indicating that the samples were not compromised during tissue collection or extraction (data not shown).

DISCUSSION

While we found no differences in expression for *PLAU*, *PLAUR*, and *SERPINB2* in the cotyledon at any time point

or treatment examined, differences in mRNA abundance of *SERPINE1* were observed. Serine protease inhibitor E1 (also called plasminogen activator inhibitor-1, or PAI-1) along with *SERPINB2* (PAI-2), are the main inhibitors of PAs (Blasi et al., 1987; Conese and Blasi, 1995). Elevated expression of *SERPINE1* during parturition is to be expected, as it is known to be regulated by dexamethasone in vivo (Medcalf et al., 1988) and circulating concentrations of glucocorticoids are elevated during parturition. Aside from its ability to bind to and ablate PA activity, *SERPINE1* is also capable of regulating cell adhesion to the extracellular matrix by obscuring the RGD integrin recognition tripeptide found within vitronectin (Deng et al., 2001). Localized secretion of *SERPINE1* to the fetal–maternal interface would disrupt adhesion of the cotyledon to the caruncle, which would mediate placental separation/expulsion. While vitronectin does not appear to be distributed along the fetal–maternal interface (Burghardt et al., 2009), osteopontin is present, though it is unknown if a similar

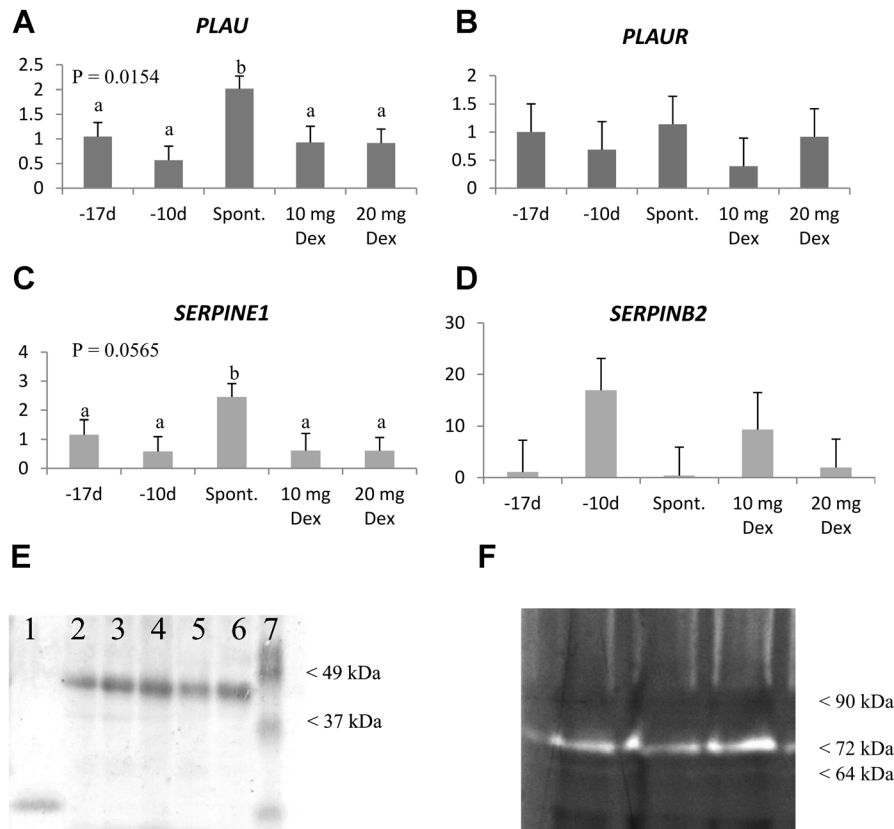


Figure 2. A–E: Least-squares means of the relative mRNA abundance for genes of interest in caruncular tissue from ewes collected 17 days before predicted parturition (–17d; $n = 4$); 10 days before predicted parturition (–10d; $n = 4$); at spontaneous parturition (Spont.; $n = 5$); and from ewes induced to lamb with 10 mg (10 mg Dex; $n = 3$) and 20 mg (20 mg dex; $n = 5$) dexamethasone. Groups with different superscripts are significantly different ($P < 0.05$). **F:** Representative immunoblot of 80 μ g of protein from homogenates of ovine cotyledons stained with anti-PLAU-specific goat IgG (Lane 1: human PLAU; Lanes 2–6: caruncular tissue extracts; Lane 7: pre-stained ladder). No specific binding for PLAU was observed in either tissue. Band at ~48 kDa is presumed to be ovine IgG, as exclusion of a primary antibody also resulted in staining at this molecular weight. **G:** Representative gelatin zymograph for gelatinase activity in 80 μ g of protein from caruncle homogenates.

interaction takes place between osteopontin and SERPINE1. Further work investigating the distribution of SERPINE1 in the placentome and its ability to bind to osteopontin is needed to clarify its role in placental separation in ruminants.

Differences in *PLAU* mRNA abundance in the caruncle may reflect the anticipation of placental separation, increased leukocyte prevalence within the placentome, and/or extracellular matrix remodeling as part of uterine involution following parturition. While *PLAU* mRNA expression was detected using reverse transcriptase quantitative PCR, *PLAU* was not detected by immunoblotting or plasminogen-casein zymography. It is unlikely that our samples contain meaningful *PLAU* activity as we were able to routinely detect the positive control down to 25 mIU (9.25 ng) of human *PLAU*, and overloading of samples in the zymograph (up to 360 μ g of protein) also failed to detect *PLAU* activity at any time point. The antibody used for immuno-

blotting has been previously demonstrated to recognize active sheep *PLAU* (Colgin and Murdoch, 1997). Thus, this discrepancy between the presence of *PLAU* mRNA and *PLAU* protein suggests that there are additional factors controlling translation of the transcript into protein. MicroRNAs are known to repress both translation and increase mRNA decay (Huntzinger and Izaurralde, 2001). Additionally, the number of ribosomes associated with a transcript has been shown to influence the abundance of a protein (Van Der Kelen et al., 2009). Additional experiments utilizing serial collections of placentomes over the course of stage-three of parturition are needed to elucidate what mechanisms are responsible for impaired translation of *PLAU*. To that end, we have recently developed an inexpensive instrument and methodology to make these collections transvaginally in cattle (McNeel et al., 2013).

We were unable to detect PA and plasmin activity at any time point or treatment examined. Our protein data support

observations by other investigators, who noted a lack of differences in fibrinolysis or caseinolysis in whole tissue minces (Gross et al., 1985). While these authors failed to detect differences in fibrinolysis (e.g., plasmin activity in whole placentomes), this is not the only avenue by which the PA system can activate MMPs as PLAUI is known to directly cleave MMP-9 (Zhao et al., 2008). The presence of MMP activity in the samples indicates that proteolytic activity of the samples had not been compromised during tissue collection or protein extraction.

Our observations of the PA system suggest that if transcription of the entire PA system changes between late gestation and parturition, as observed by other investigators (Strey et al., 2012), these changes depend on the fetus traversing the birth canal. An endocrine signal(s) has been recently proposed for such an event (Kamada et al., 2012), which appear to be secreted after fetal expulsion; this lag may account for the variation in time between expulsion of the calf and expulsion of the placenta. Differences between our data and that from other investigators (Strey et al., 2012) may be due to differences in timing of tissue collection, in cDNA preparation (oligo-dT vs. oligo-dT and random decamers), in the mechanisms responsible for placental separation between cattle and sheep, or in method of assessment (e.g., inherent differences between microarray and reverse transcriptase quantitative PCR).

While cattle and sheep share identical placental micro-anatomy, these species differ in their placentomal gross morphology. In the ewe, the caruncle encapsulates the cotyledon whereas the cotyledon encapsulates the caruncle in the bovine. Furthermore, it is known that the morphological features of adjacent placentomes from sheep and cattle differ during the last trimester (Rici et al., 2011). In sheep, single placentomes are distinct during the last third of gestation, while in cattle it appears that there is some degree of fusion between multiple adjacent placentomes. This fusion appears to be exclusively of fetal origin, as adjacent caruncles are distinctly separate (Rici et al., 2011). The impact that these differences in placentomal morphology might have on the mechanisms of placentomal separation is unknown.

In conclusion, our data demonstrate that differences in expression of PA system components during late gestation and stage-two of parturition in the ovine placentome are of fetal and maternal origin, but neither PLAUI protein nor activity can be detected using Western blotting or plasminogen-casein zymography. Based on changes in gene expression within the PA system between late gestation and parturition, the molecular mechanisms regulating separation of the placentome in the ewe appear to follow a different time course than cattle.

MATERIALS AND METHODS

Animals

All procedures were approved by the U.S. Meat Animal Research Center (USMARC) Institutional Animal Care and

Use Committee, and were in accordance with Federation for Animal Science Society (FASS) guidelines for the use of agricultural animals in research. Placentomes were collected from 23 pregnant ewes at the following five different time points: at 17 days prior to parturition (–17 days; $n = 5$); at 10 days prior to parturition (–10 days; $n = 5$); at spontaneous parturition (0 days; $n = 5$); at induced parturition using 10 mg dexamethasone (10Dex; $n = 3$); and at induced parturition using 20 mg dexamethasone (20Dex; $n = 5$). Ewes induced to lamb were treated with dexamethasone 48 hr prior to their projected lambing date, which was calculated using recorded grease harness marking records and average gestational length of the flock from previous lambing seasons.

Ewes at 17 and 10 days prior to parturition were sacrificed at the USMARC abattoir after stunning with a captive bolt followed by exsanguination. The reproductive tract was removed from the ewe, and an incision was made along the anti-mesometrial side of the uterus to expose the fetus and associated fetal membranes. A single, intact representative placentome was excised, excess fetal membranes and endometrium were trimmed, the placentome was separated into cotyledonary and caruncular components, placed into 2.0 ml cryovials, and snap frozen in liquid nitrogen within 15 min of stunning. Samples were stored at -80°C until RNA and protein extraction.

Ewes spontaneously lambing and those induced to lamb with dexamethasone were observed for signs of parturition (e.g. protrusion of the chorioallantoic membrane from the vulva). Within 30 min of the rupture of the fetal membranes, a line block using 30 ml of 2% lidocaine injected subcutaneously was performed, an incision for a Cesarean section was made, any remaining lambs were removed, and the endometrium was exposed. A single representative placentome was then selected, clamped at the base of the caruncle, and excised using a scalpel. Harvested tissues were handled and stored as described in the previous section. Following tissue collection, normal Cesarean section procedures for closing the uterus, muscle layer, and skin were performed. Animals were provided with flunixin meglumine IV (50 mg/45 kg), penicillin G IM (300 kIU/ml at 1.05 MIU/45 kg of BW), and 40 IU oxytocin immediately following the procedure. For the 48 hr following surgery, ewes were treated daily with penicillin G IM (150 k IU/ml at 450 k IU/45 kg of BW), followed by 1.5 ml of penicillin G and benzathine IM (150 k IU/ml each at 1.5 ml/45 kg). All ewes expelled their fetal membranes within 24 hr of the procedure. Ewes and lambs were then returned to the flock to continue the production cycle. Six months later, ewes were pasture-bred and checked for pregnancy via ultrasonography 60 days following removal of the rams.

RNA Extraction and Quantification

Cotyledonary and caruncular tissues were homogenized, and RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Purified RNA was suspended in autoclaved distilled water and quantified on a Nanodrop 1000 (Thermoscientific,

TABLE 1. Primers Used to Determine Relative Quantification of mRNA Expression Using RT-qPCR

Gene	Forward	Reverse	Amplicon size	T _m	Ref. accession #
<i>PLAU</i>	5'-ACCATCTGCCTGCCCCCACT	5'-GGGTCAGCCGCACACAGCAT	206 bp	60°C	254692795
<i>PLAUR</i>	5'-AGCGCCACAGGTGTTCCCTC	5'-CCCTTGCGGGGCTGGGCGTC	248 bp	58°C	254692821
<i>SERPINE1</i>	5'-GGTCCGCGTTTCATGCCCA	5'-TACCAGGACCAGGCGCGTCA	184 bp	59°C	291621639
<i>SERPINB2</i>	5'-GGGCGCCCGGGAAACTG	5'-GGACGCGTTGATGGCGTTGC	220 bp	59°C	290795367
<i>GAPDH</i>	5'-CCACCAACTGCTTGGCCCCC	5'-GGGATGACCTTGCCACGGC	208 bp	60°C	296785214

Wilmington, DE). Only samples with a 280/260 absorbance greater than 1.7 were used for reverse transcription.

Reverse Transcription and Primer Design

One microgram of RNA was reverse transcribed using the Superscript III kit (Invitrogen), according to manufacturer's instructions, and cDNA was diluted to 100 µl with autoclaved distilled water. Primers for quantitative PCR (Table 1) were designed using PRIMER-BLAST (Rozen and Skaletsky, 2000) with ovine sequences when available (*PLAU*, *PLAUR*, *SERPINE1*, *SERPINB2*, *GAPDH*). Otherwise, alignments of the mouse, rat, human, and bovine sequences were used to search for regions of high homology, with the preference given to the bovine sequence (*PLG*, *PLAT*, *SERPINA5*). Intron-spanning primer pairs were chosen based on the annotation of the gene of interest in the bovine genome (*Btau* 4.6.1).

Relative mRNA Expression Using Quantitative PCR

Relative gene expression was conducted using Platinum Taq SYBR Green with UDG (Invitrogen), according to manufacturer's instructions. Briefly, 1.0 µl of cDNA was combined with 19 µl of a master mix containing 200 nM of each primer, 10 µl Platinum Taq master mix (400 µM dATP, dCTP, dGTP, 800 µM TTP, 6 mM MgCl₂, Hot-Start Platinum Taq) and autoclaved distilled water. Polymerase chain reactions were subjected to 40 cycles of the following: 95°C for 10 sec; 30 sec at an annealing temperature indicated in Table 1; 10 sec at 72°C. Reactions were conducted in a 96-well plate on a Roche Light Cycler 480 (Roche Applied Science, Indianapolis, IN). Negative controls for each primer pair consistently failed to generate signal. Primer pairs generated a single peak during melting curve analysis, and visualization of the PCR products by 2% gel electrophoresis produced a single band at the predicted size. Gene expression was calculated using the 2^{-ΔΔC_t} method (Livak and Schmittgen, 2001) with *GAPDH* serving as the reference gene and day -17 serving as the reference group.

Protein Extraction and Quantification

Protein was extracted from cotyledonary and caruncular tissues as described previously (Dow et al., 2002). Briefly, one-quarter of a cotyledon or caruncle was weighed and homogenized in the appropriate amount of extraction buffer

(1 ml/500 mg) with a tissue homogenizer, then centrifuged at 9,000g for 30 min at 4°C. The supernatant was removed and stored at -20°C for later assay. Protein samples were subjected to a maximum of three freeze-thaw cycles in order to reduce variation in enzyme activity. Protein concentrations were measured using the Pierce BCA kit (Thermoscientific) in a microplate, and read at 550 nm on a Elx808^{IU} ultra microplate reader (Biotek instruments, Inc., Winooski, VT).

Casein-Plasminogen Zymography

Casein-plasminogen zymography was performed as described previously (Dow et al., 2002). Briefly, 80 µg of protein from caruncle or cotyledon and a standard curve of high molecular weight human PLAU (American Diagnostica, Stamford, CT) were loaded onto castellated 4% polyacrylamide gels (0.375 M Tris, 0.1% SDS, pH 8.6) cast on top of a 10% polyacrylamide gels (0.375 M Tris, 0.1% SDS, pH 8.6) containing 0.025% hammerstein-casein (Sigma-Aldrich, St. Louis, MO) and 25 mU/ml bovine plasminogen (Sigma-Aldrich). Samples were electrophoresed for 1 hr at 140 V in a Mini-Protean Tetra-cell (Bio-Rad, Hercules, CA). Gels were removed from the apparatus, the stacking gel was removed, and the running gel was washed in fresh 2.5% Triton X-100 for 45 min to remove SDS. Gels were then washed once in incubation buffer (50 mM Tris, 100 mM NaCl, pH 7.6), placed in fresh incubation buffer, and incubated for 17 hr at 37°C. Gels were then washed once in water, placed in staining solution (40% methanol, 40% distilled H₂O, 10% acetic acid, 0.025% Coomassie Brilliant Blue) for 45 min, and destained in staining solution without Coomassie. MMP-mediated caseinolysis is unlikely with this assay as both the electrophoresis and incubation buffers lack the metal ions (Zn²⁺, Ca²⁺) necessary for MMP activity.

Immunoblotting

Eighty micrograms of cotyledonary or caruncular protein and 10 IU of active, high molecular weight PLAU (American Diagnostica) were subjected to SDS-PAGE as described previously (Buhi et al., 1989), with slight modifications. Gels were blotted onto nylon-supported nitrocellulose, then blocked in 50 mM Tris, 1% Triton X-100, and 1 M NaCl, pH 8.0. Membranes were incubated overnight with polyclonal goat anti-PLAU (American Diagnostica) with known cross-reactivity for ovine PLAU (Colgin and Murdoch, 1997) (1:1,000 dilution in blocking buffer). The

next day, membranes were removed from the primary antibody, washed five times, and incubated in secondary rabbit anti-goat IgG conjugated to horseradish peroxidase (Sigma-Aldrich) (1:10,000 dilution) for 1 hr, and washed five times in blocking buffer and once in 0.9% NaCl. Membranes were then stained with NovaRed (Vector Labs, Burlingame, CA) according to kit instructions, using a tenfold dilution of detection substrate. Membranes were scanned using a Canon MX880.

Statistical Analysis

Relative gene expression was analyzed using the MIXED procedure of SAS version 9.2 (SAS Institute, Inc., Cary, NC), with treatment as the main effect. Differences were considered significant when $P \leq 0.05$.

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REFERENCES

- Blasi F, Vassalli J-D, Danø K. 1987. Urokinase-type plasminogen activator: Proenzyme, receptor, and inhibitors. *J Cell Biol* 104:801–804.
- Buhi WC, Vallet JL, Bazer FW. 1989. De novo synthesis and release of polypeptides from cyclic and early pregnant porcine oviductal tissue in explant culture. *J Exp Zool* 252:79–88.
- Burghardt RC, Burghardt JR, Taylor JD II, Reeder AT, Nguen BT, Spencer TE, Bayless KJ, Johnson GA. 2009. Enhanced focal adhesion assembly reflects increased mechanosensation and mechanotransduction at maternal-conceptus interface and uterine wall during ovine pregnancy. *Reproduction* 137:567–582.
- Chakraborti S, Mandal M, Das S, Mandal A, Chakraborti T. 2003. Regulation of matrix metalloproteinases: An overview. *Mol Cell Biochem* 253:269–285.
- Colgin DC, Murdoch WJ. 1997. Evidence for a role of the ovarian surface epithelium in the ovulatory mechanism of the sheep: Secretion of urokinase-type plasminogen activator. *Anim Reprod Sci* 47:197–204.
- Conese M, Blasi F. 1995. Urokinase/urokinase receptor system: Internalization/degradation of urokinase-serpin complexes: Mechanism and regulation. *Biol Chem Hoppe Seyler* 376: 143–155.
- Curry TE, Jr., Osteen KG. 2003. The matrix metalloproteinase system: Changes, regulation, and impact throughout the ovarian and uterine reproductive cycle. *Endocr Rev* 24: 428–465.
- Deng G, Curriden SA, Hu G, Czekay R-P, Loskutoff DJ. 2001. Plasminogen activator inhibitor-1 regulates cell adhesion by binding to the somatomedin B domain of vitronectin. *J Cell Physiol* 189:23–33.
- Dilly M, Hambruch N, Shenavai S, Schuler G, Froehlich R, Haeger J-D, Ozalp GR, Pfarrer C. 2011. Expression of matrix metalloproteinase (MMP)-2, MMP-14 and tissue inhibitor of matrix metalloproteinase (TIMP)-2 during bovine placentation and at term with or without placental retention. *Theriogenology* 75:1104–1114.
- Dow MPD, Bakke LJ, Cassar CA, Peters MW, Pursley JR, Smith GW. 2002. Gonadotropin surge-induced up-regulation of the plasminogen activators (tissue plasminogen activator and urokinase plasminogen activator) and the urokinase plasminogen activator receptor within bovine periovulatory follicular and luteal tissue. *Biol Reprod* 66:1413–1421.
- Dubuc J, Duffield TF, Leslie KE, Walton JS, LeBlanc SJ. 2010. Risk factors for postpartum uterine diseases in dairy cows. *J Dairy Sci* 93:5764–5771.
- Dubuc J, Duffield TF, Leslie KE, Walton JS, LeBlanc SJ. 2011. Effects of postpartum uterine diseases on milk production and culling in dairy cows. *J Dairy Sci* 94:1339–1346.
- Eiler H, Fecteau KA. 2007. Retained placenta. In: Youngquist RS, Threlfall WR, editors. *Current Therapy in large animal theriogenology*, 2nd edition. St. Louis, MO: Saunders Elsevier. pp 345–354.
- Fourichon C, Seegers H, Malher X. 2000. Effect of disease on reproduction in the dairy cow: A meta-analysis. *Theriogenology* 53:1729–1759.
- Gross TS, Williams WF, Manspeaker JE, Russek E. 1985. In vitro proteolytic activity of the late pregnant and peripartum bovine placenta. *J Anim Sci* 61:391 (Abstract # 448).
- Higazi AA-R, Cohen RL, Henkin J, Kniss D, Schwartz BS, Cines DB. 1995. Enhancement of the enzymatic activity of single-chain urokinase plasminogen activator by soluble urokinase receptor. *J Biol Chem* 270:17375–17380.
- Huntzinger E, Izaurralde E. 2001. Gene silencing by microRNAs: Contributions of translational repression and mRNA decay. *Nat Rev Genet* 12:99–110.
- Johnson GA, Burghardt RC, Bazer FW, Spencer TE. 2003. Osteopontin: Roles in implantation and placentation. *Biol Reprod* 69:1458–1471.
- Kamada H, Matsui Y, Sakurai Y, Tanigawa T, Itoh M, Kawamoto S, Kai K, Sasaki T, Takahashi K, Hayashi M, Takayama Y, Nakamura M, Kadokawa H, Ueda Y, Sutoh M, Murai M. 2012. Twelve oxo-eicosatetraenoic acid induces fetal membrane release after delivery in cows. *Placenta* 33:106–113.
- Lijnen HR, Van Hoef B, De Cock F, Collen D. 1990. Effect of fibrin-like stimulators on the activation of plasminogen by tissue-type plasminogen activator (t-PA)---Studies with active site mutagenized plasminogen and plasmin resistant t-PA. *Thromb Haemost* 64:61–68.

- Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) Method. *Methods* 25:402–408.
- Maj JG, Kankofer M. 1997. Activity of 72-kDa and 92-kDa matrix metalloproteinases in placental tissues of cows with and without retained fetal membranes. *Placenta* 18:683–687.
- Mayer M. 1990. Biochemical and biological aspects of the plasminogen activation system. *Clin Biochem* 23:197–211.
- McNeel AK, Ondrak JD, Cushman RA. 2013. Technical report: Serial collections of placentomes during parturition in cattle and subsequent reproductive performance. *J Anim Sci* (in press). doi: 10.2527/jas.2012-5876
- Medcalf RL, Van den Berg E, Schleuning W-D. 1988. Glucocorticoid-modulated gene expression of tissue- and urinary-type plasminogen activator and plasminogen activator inhibitor 1 and 2. *J Cell Biol* 106:971–978.
- Ny T, Wahlberg P, Brändström IJM. 2002. Matrix remodeling in the ovary: Regulation and functional role of the plasminogen activator and matrix metalloproteinase systems. *Mol Cell Endocrinol* 187:29–38.
- Olson ST, Swanson R, Day D, Verhamme I, Kvassman J, Shore JD. 2001. Resolution of Michaelis complex, acylation, and conformational change steps in the reactions of the serpin, plasminogen activator inhibitor-1, with tissue plasminogen activator and trypsin. *Biochemistry* 40:11742–11756.
- Orgel D, Schröder W, Hecker-Kia A, Weithmann KU, Kolkenbrock H, Ulbrich N. 1998. The cleavage of pro-urokinase type plasminogen activator by stromelysin-1. *Clin Chem Lab Med* 36:697–702.
- Overall CM. 2002. Molecular determinants of metalloproteinase substrate specificity. Matrix metalloproteinase substrate binding domains, modules, and exosites. *Mol Biotechnol* 22:51–86.
- Rici REG, Facciotti PR, Maria DA, Fernandes VM, Ambrosio CE, Miglino MA. 2011. Evaluation of the contribution of the placental fusion during gestation in cattle. *Anim Reprod Sci* 126:143–150.
- Rozen S, Skaletsky H. 2000. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* 132:365–386.
- Streyl D, Kenngott R, Herbach N, Wanke R, Blum H, Sinowatz F, Wolf E, Zerbe H, Bauersachs S. 2012. Gene expression profiling of bovine periparturient placentomes: Detection of molecular pathways potentially involved in the release of foetal membranes. *Reproduction* 143:85–105.
- Van Der Kelen K, Beyaert R, Inzé D, De Veylder L. 2009. Translational control of eukaryotic gene expression. *Crit Rev Biochem Mol Biol* 44:143–168.
- Walter I, Boos A. 2001. Matrix metalloproteinases (MMP-2 and MMP-9) and tissue inhibitor-2 of matrix metalloproteinases (TIMP-2) in the placenta and interplacental uterine wall in normal cows and in cattle with retention of fetal membranes. *Placenta* 22:473–483.
- Xu D, McKee CM, Cao Y, Ding Y, Kessler BM, Muschel RJ. 2010. Matrix metalloproteinase-9 regulates tumor cell invasion through cleavage of protease nexin-1. *Cancer Res* 70:6988–6998.
- Zhao Y, Lyons CE, Jr., Xiao A, Templeton DJ, Sang QA, Brew K, Hussaini IM. 2008. Urokinase directly activates matrix metalloproteinases-9: A potential role in glioblastoma invasion. *Biochem Biophys Res Commun* 369:1215–1220.