Endogenous retroviral gene elements (*syncytin-Rum1* and *BERV-K1*), *interferon-τ*, and *pregnancy associated glycoprotein-1* are differentially expressed in maternal and fetal tissues during the first 50 days of gestation in beef heifers[1]

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ABSTRACT: We hypothesized that the endogenous retroviruses [ERV: *syncytin-Rum1* and (*BERV-K1*)], and pregnancy hormones [*interferon*-τ (*IFN*-τ), and *pregnancy associated glycoprotein-1* (*PAG-1*)] would be differentially expressed whereas progesterone and insulin concentrations in maternal blood would remain steady during early gestation. To test this hypothesis Angus crossbred heifers ($n = 46$; ~15 mo of age; BW = 363 ± 35 kg) were fed native grass hay, supplemented with cracked corn to gain 0.3 kg/d, and given ad libitum access to water. All heifers were subjected to a 5-d CO-Synch + CIDR estrous synchronization protocol and AI (breeding = d 0). Ovariohysterectomies were performed on d 16, 22, 28, 34, 40, and 50 of gestation and at d 16 of the estrous cycle for non-pregnant (NP) controls. Utero-placental tissues [maternal caruncle (CAR); maternal intercaruncular endometrium (ICAR); and fetal membranes, (FM, chorion on d 16, chorioallantois on d 22 to 50)] were collected from the uterine horn ipsilateral to the corpus luteum (CL). Tissues were flash frozen and stored at –80°C. Expression of mRNA was evaluated using qPCR. In CAR, *syncytin-Rum1* expression was greater ($P < 0.01$) on d 50 (81.5-fold) compared with NP controls or any other day of early pregnancy. In contrast, *syncytin-Rum1* expression in I-CAR only tended ($P = 0.09$) to change across days of early pregnancy and did not differ ($P = 0.27$) in FM tissues. In CAR, the expression of *BERV-K1* was not different ($P > 0.79$) at d 16 and 22, was intermediate at d 28, 34, and 40, and was greatest on d 50 (108-fold increase compared with NP). Expression of *BERV-K1* in FM was increased ($P < 0.01$) on d 28, 34, and 50 compared with NP controls, but at d 40 did not differ from NP controls. The mRNA expression of *IFN*-τ in FM at d 22 was greater ($P < 0.01$) than all other days of gestation. In CAR, expression of *PAG-1* increased ($P < 0.001$) dramatically on d 40 (20,000-fold) and d 50 (86,000-fold) compared with NP heifers ($P < 0.01$). In ICAR, expression of *PAG-1* was greater ($P < 0.05$) on d 28 and 40 (fold increases of 113 and 102, respectively, compared with NP). Insulin concentrations were not different ($P = 0.53$) but progesterone was greater ($P < 0.01$) on d 16, 22, 28, 34, and 40 compared with d 50 of gestation. These data confirm differential ERV, *IFN*-τ, and *PAG-1* gene expression during critical time points of early gestation in utero-placental tissues.

Key words: bovine, early pregnancy, endogenous retroviruses, hormones, maternal recognition

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INTRODUCTION

Placental formation during early gestation is vital to the establishment and maintenance of pregnancy. The developing conceptus requires a fully functional placenta for exchange of nutrients, respiratory gases, and metabolic wastes throughout pregnancy (Meschia, 1983; Bassil et al., 1995; Reynolds and Redmer, 1995). In ruminants, trophoblast stem cells fuse to form the

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syncytial plaques, which are multinucleated cells that can contain up to 25 nuclei in sheep (Wooding, 1984) and 8 nuclei in cattle (Wooding and Wathes, 1980). In addition to nutrient and gas exchange, the syncytiotrophoblast produces hormones, including progesterone for maintenance of gestation (Bazer et al., 1991), *interferon-*τ (*IFN-*τ) for pregnancy recognition (Spencer et al., 2007), and *pregnancy associated glycoprotein-1* (*PAG-1*). The syncytiotrophoblast will also interact with the maternal immune system during early gestation and the establishment of pregnancy (reviewed in Moffett and Loke, 2004, 2006).

The *Bovidae* genome contains 24 endogenous retroviral gene elements (ERV) depending on the species (Garcia-Etxebarria and Jugo, 2013). Five ERV are expressed in bovine trophoblast cells: *syncytin-Rum1* (Cornelis et al., 2013), *BERVE-A*, *BERVE-B, BERV-K1,* and *BERV-K2* (Koshi et al., 2012) The envelope proteins of syncytin-Rum1, BERVE-A and BERV-K1 may be involved with cell-to-cell fusion that occurs in bovine trophoblast during early gestation (Cornelis et al., 2013; Nakaya et al., 2013). In addition, Sharif et al. (2013) argued that ERV function as nutrient sensors during the development of the placenta, and thus may interact with insulin, which is also indicative of animal nutrient status. Thus, we hypothesized that the mRNA of endogenous retroviruses (*syncytin-Rum1* and *BERV-K1*), *IFN-* τ, and *PAG-1* would be differentially expressed, whereas serum progesterone and insulin concentrations would remain steady during early gestation.

MATERIALS AND METHODS

All animal procedures were conducted with approval from the Institutional Animal Care and Use Committee at North Dakota State University (A14053). Commercial Angus crossbred heifers (n = 46; ~ 15 mo of age; BW = 362.3 ± 34.7 kg) were transported 229 km from Central Grasslands Research Extension Center (Streeter, ND) to the Animal Nutrition and Physiology Center (North Dakota State University, Fargo). Heifers were housed in pens with 6 heifers per pen and fed daily at 0800 h. Heifers were maintained on an ad libitum native grass hay diet, granted ad libitum access to water, and supplemented with cracked corn to maintain a positive energy balance. All heifers were subject to 5-d CO-Synch + CIDR (Zoetis Inc., Parsippany, NJ) estrus synchronization protocol and AI to a single Angus sire (day of breeding = d 0; Bridges et al., 2008). Heifers were ovariohysterectomized on d 16, 22, 28, 34, 40, or 50 (n = 9, 6, 6, 7, 6, and 5 respectively) of gestation and at d 16 of the estrus cycle for non-bred, non-pregnant controls (NP; n = 7). During surgery, the left and right uterine arteries, the left and right spiral arteries, and the cervix were ligated, and then the uterus was removed. Uterine contents were held in place with a 24-cm Crafoord Coarctation Clamp (Integra-Miltex; Plainsboro, NJ), placed just cranial to the cervical ligatures, during and after removal from the body cavity. Following surgery, heifers were kept in individual pens during recovery and stitches were removed 14 d after surgery (McLean et al., 2016a). Heifers were randomly selected for ovariohysterectomy on d 16 based on the inability to confirm viability of pregnancy via ultrasound. Heifers without any evidence of a conceptus in the uterus on d 16 were deemed not pregnant and removed from the study. Pregnancy was confirmed via transrectal ultrasonography on d 22 and again on the d of surgery (d > 28).

Tissue Collecting and Processing

Immediately on removal from the body cavity, tissues were trimmed of excess broad ligament, fat, and non-reproductive tissues. Utero-placental tissues [maternal caruncle (CAR); maternal intercaruncular endometrium, (ICAR), fetal membranes (FM; chorioallantois, d 22 and later)] were obtained from the uterine horn containing the conceptus, as previously described (Grazul-Bilska et al., 2010). There were no FM collected until d 22 due to insufficient development of tissues for adequate collection, extraction, and analysis on d 16. After collection, all tissues were snap frozen in liquid nitrogen cooled isopentane (Sigma-Aldrich; St. Louis, MO) and stored at –80°C.

Blood samples were taken via jugular venipuncture on d 16, 22, 28, 34, 40, and 50 of gestation until the heifer underwent ovariohysterectomy. Non-bred, non-pregnant control heifers were sampled on d 16 of the estrous cycle. Blood samples were collected in 10 mL vacutainer tubes (Becton Dickinson Healthcare; Franklin Lakes, NJ), allowed to clot, and stored at 4°C until processing. Samples were centrifuged for 30 min at 3,000 rpm and 4°C after which serum was removed and stored at –20°C. Concentrations of progesterone and insulin in serum were determined using an Immulite 1,000 (Siemens AG; Munich, Germany). Sensitivity of the assays was 0.2 ng/ml and 2 μIU/ml for progesterone and insulin, respectively. Intra-assay CV for progesterone and insulin were 4.08% and 19.25%, respectively.

Real-time Reverse Transcriptase Quantitative PCR

The RNA was extracted and purified via an RNeasy Mini Kit (Qiagen Inc., Valencia, CA). The concentration of RNA extracted was determined using Take3 module of a Synergy H1 Microplate Reader (BioTek Instruments Inc., Winooski, VT). A total of 1 μg of

RNA was used for cDNA synthesis via a QuantiTect Reverse Transcription Kit (Qiagen Inc.). Primer sequences (Table 1) were obtained from previous literature for *syncytin-Rum1* (Cornelis et al., 2013), *BERV-K1* (Nakaya et al., 2013), *IFN-* τ (Hickman et al., 2013), and *PAG-1* (Patel et al., 2004). Primer validation for optimum cDNA concentration and primer efficiency for each tissue type was completed before qPCR analysis. Gene expression was analyzed using a 7500 Fast Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific Inc., Grand Island, NY) with SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA). Gene expression for maternal tissues was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) with β-*actin* as the reference gene and the average of NP expression as the control (set to 1) within each tissue. Fetal membrane gene expression was calculated using the same methods with the exception that the average of d 22 FM expression as the control (set to 1) within each gene. Gene expression analysis of *syncytin-Rum1*, *BERV-K1*, *IFN*-τ, and *PAG-1* across day was performed separately from analysis of *syncytin-Rum1*, *BERV-K1*, *IFN*-τ, and *PAG-1* expression across tissues within a given day of gestation to compare expression between tissues. Across tissue gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) with β-*actin* as the reference gene and the average of ICAR expression as the control (set to 1) on each day of gestation.

Statistical Analysis

Statistical analyses were conducted via the GLM procedure of SAS version 9.4 (SAS Inst. Inc., Cary, NY), with individual heifer as the experimental unit. During pregnancy, relative pattern of mRNA expression for *syncytin-Rum1*, *BERV-K1*, *IFN*-τ, and *PAG-1* in CAR, ICAR, and FM was determined via the REG procedure of SAS. Regression analyses were conducted to determine if coefficients for linear, quadratic, cubic, exponential models were significantly different than zero. When multiple models were found significant, best fit was determined from the regression analyses by which had the smallest *P* value and the greatest r^2. Concentrations of progesterone and insulin were analyzed as repeated measures using the MIXED procedure of SAS with day as the variable and cow as the subject for a repeated measure. Progesterone was analyzed with a Compound Symmetry covariance structure. Insulin was analyzed with a Toeplitz covariance structure. Means were separated using the LSMEANS statement of SAS with differences determined at a *P*-values ≤ 0.05.

RESULTS

Endogenous Retroviruses

Expression of *syncytin-Rum1* in CAR was greater ($P < 0.01$; Fig. 1A) by 81.5-fold on d 50 compared with NP controls and all other days of gestation. In ICAR, s*yncytin-Rum1* expression tended ($P = 0.09$; Fig. 1B) to increase until d 28 and then decrease as pregnancy progressed from d 28 to 50. The expression of *syncytin-Rum1* in FM during the first 50 d of gestation did not change over time ($P = 0.27$; Fig. 1C). The expression of *BERV-K1* was not different ($P > 0.79$) at d 16 and 22 compared with NP control heifers. The mRNA levels of *BERV-K1* in CAR were intermediate at d 28, 34, and 40 and greater ($P < 0.01$) on d 40 and 50 compared with NP, d 16, and d 22 heifers; whereas d 28 and 34 were intermediate. In addition, d 50 was greater ($P < 0.01$) compared with d 28 and 34 (Fig. 2A). In ICAR, *BERV-K1* was less ($P = 0.003$) on d 16 and 22 compared with d 40 and 28 and greatest ($P = 0.003$) on d 28 with a 12.9-fold increase but then returned to NP levels (Fig. 2B) during the first 50 d of gestation. In FM, the expression of *BERV-K1* increased ($P = 0.001$) from d 22 to d 34 with a 27.4-fold increase compared with d

Table 1. Primer sequences of *syncytin-Rum1, bovine endogenous retrovirus-K1 (BERV-K1), interferon–*τ (*IFN-τ*), and *pregnancy associated glycoprotein-1* (*PAG-1*)[1]

Gene of interest	Primer direction	Product size (bp)	Sequence[2]	GenBank accession number
Syncytin-Rum1	Forward	2464	TGGTATGACTATCTTGCTGGCTTC	NM_001305454
	Reverse		TGGGCTGTGAGTAGTTCTAAT	
BERV-K1	Forward	2142	GGAAATCACGATGTCCT	NM_001245951
	Reverse		GGAGAGGAGGCGCTTACCTG	
IFN-τ	Forward	1313	CAGGACAGAAAGACTTTGG	NM_001015511
	Reverse		GTGCTCTGTGTAGAAGAGGTTG	
PAG-1	Forward	1295	TCCAGCCTGTTCTACACACGTT	NM_174411
	Reverse		AGGTGATCCTGAAGGTCTTATTGG	

[1]Primer sequences were obtained from Cornelis et al., 2013 (syncytin-Rum1), Nakaya et al., 2013 (BERV-K1), Hickman et al., 2013 (IFN-τ), and Patel et al., 2004 (PAG-1).

[2]All sequences are represented from 5' to 3'.

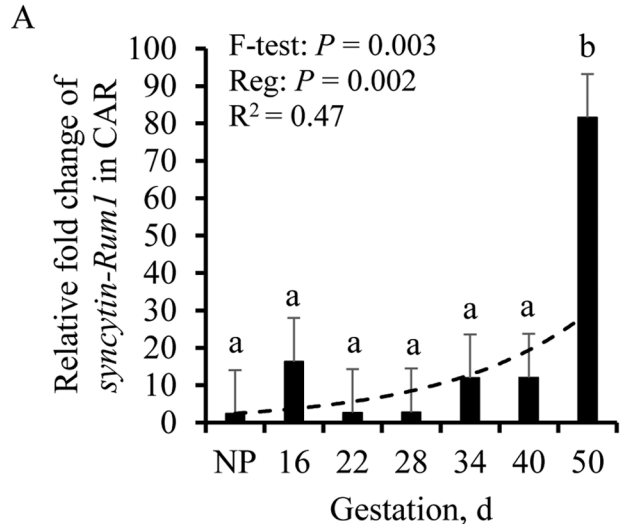

Figure 1. Expression of *syncytin-Rum1* in reproductive tissues during the establishment of pregnancy in beef heifers: A) *syncytin-Rum1* in maternal caruncles (CAR), B) *syncytin-Rum1* in uterine endometrium (ICAR), and C) *syncytin-Rum1* in fetal membranes (FM). Data presented as a $2^{-\Delta\Delta CT}$-fold change normalized to β-*Actin* and the average of non-pregnant (NP; maternal tissues) or d 22 FM (fetal tissues). Expression pattern line (— —) via regression ($P < 0.05$); regression analysis does not include NP heifers. Means without a common superscript differ ($P < 0.05$).

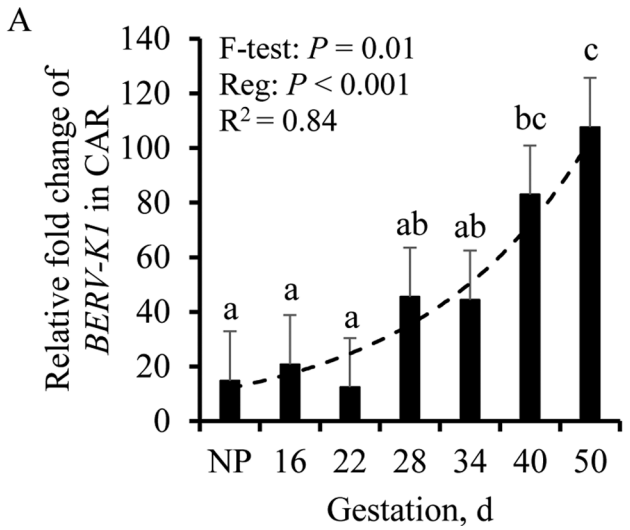

Figure 2. Expression of *bovine endogenous retrovirus-K1* (*BERV-K1)* in reproductive tissues during the establishment of pregnancy in beef heifers: A) *BERV-K1* in maternal caruncles (CAR), B) *BERV-K1* in uterine endometrium (ICAR), and C) *BERV-K1* in fetal membranes (FM). Data presented as a $2^{-\Delta\Delta CT}$-fold change normalized to β-*Actin* and the average of non-pregnant (NP; maternal tissues) or d 22 FM (fetal tissues). Expression pattern line (— —) via regression ($P < 0.05$); regression analysis does not include NP heifers. Means without a common superscript differ ($P < 0.05$).

22 heifers, while d 28 was intermediate. Expression of *BERV-K1* in FM decreased from d 34 to 40 and increased again at d 50. The d 50 increase in mRNA expression of *BERV-K1* in FM represents a 32.3-fold increase compared with d 22 FM ($P = 0.001$; Fig. 2C).

Pregnancy Hormones

The maternal recognition signal in ruminants, *IFN*-τ, was not detected in maternal tissues, CAR and ICAR; thus, analysis of *IFN*-τ expression as pregnancy progressed was only conducted in FM and no across tissue comparisons were made. The mRNA expressions of *IFN*-τ at d 22, which was used as baseline for all FM tissues, was greater ($P < 0.01$) than all other days of gestation (Fig. 3). Expression levels of *PAG-1* increased dramatically with d 40 and 50 being 20,000 and 86,000-fold, respectively, greater than NP heifers in CAR (Fig. 4A). Due to the magnitude of relative fold change on d 40 and 50 in CAR for *PAG-1* they were removed and the same analysis was conducted to determine if differences existed early in gestation (d 16, 22, 28, and 34) compared with NP heifers. Relative expression of *PAG-1* was increased ($P < 0.001$) on d 22 and 34 (3,876- and 5,368-fold, respectively) but was not different ($P > 0.10$) on d 16 and 28 compared with NP heifers. In ICAR, expression of *PAG-1* was greater ($P < 0.05$) on d 28 and 40 compared with NP with fold increases of 113 and 102, respectively, and d 22, 34, and 50 were intermediate (Fig. 4B). Expression of *PAG-1* in FM tissue was similar ($P = 0.33$) across all days evaluated

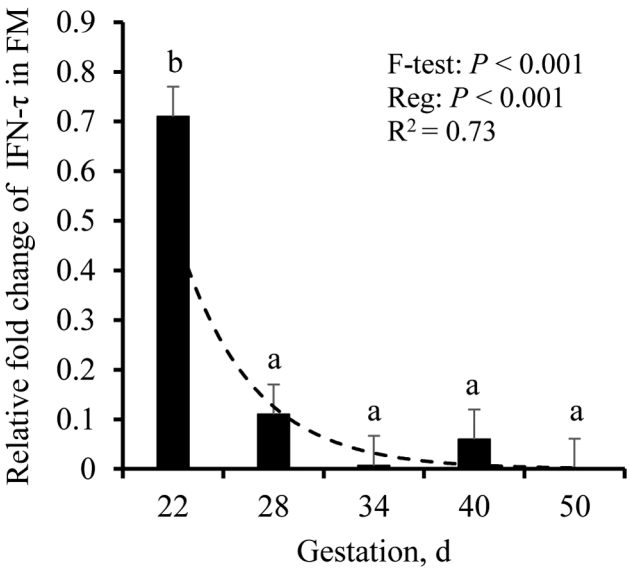

Figure 3. Expression of (IFN-τ) in fetal membranes (FM) during the establishment of pregnancy in beef heifers. Data presented as a $2^{-\Delta\Delta CT}$-fold change normalized to β-*Actin* and the average of d 22 FM (fetal tissues). Expression pattern line (– –) via regression ($P < 0.05$); regression analysis does not include NP heifers. Means without a common superscript differ ($P < 0.05$).

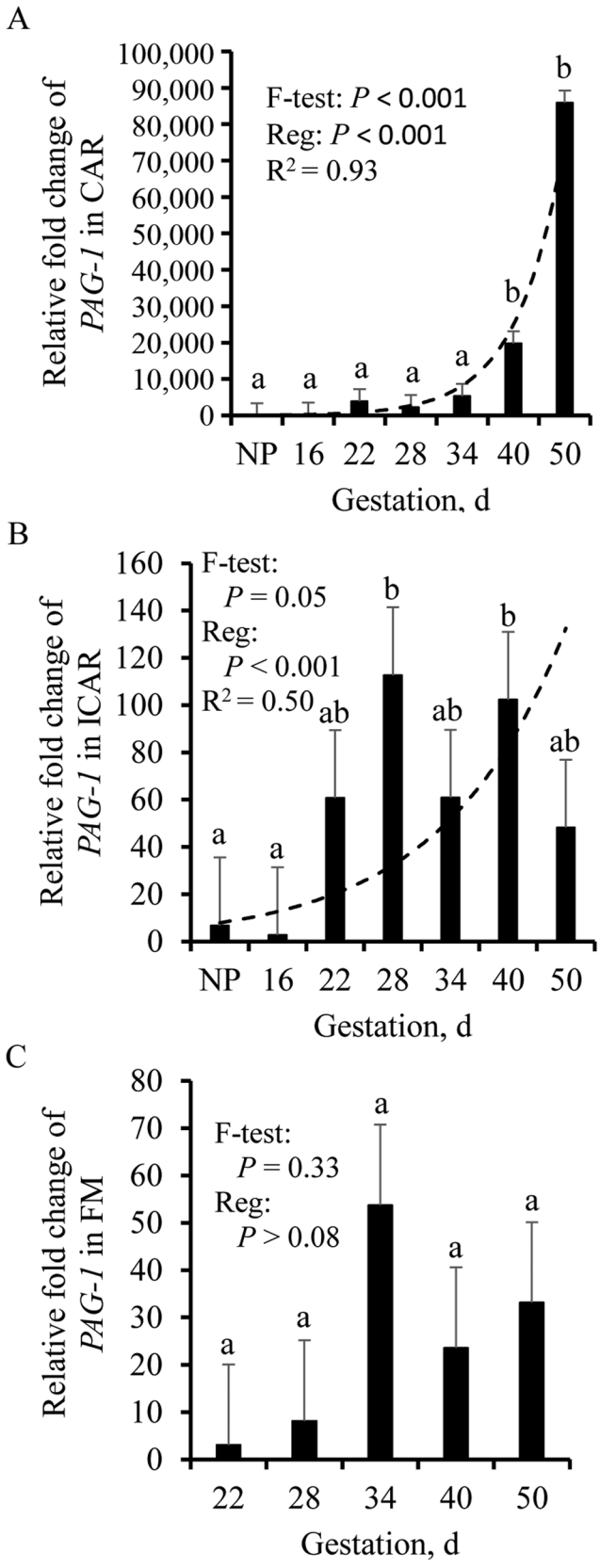

Figure 4. Expression of *pregnancy associated glycoprotein-1* (*PAG-1*) in reproductive tissues during the establishment of pregnancy in beef heifers: A) *PAG-1* in maternal caruncles (CAR), B) *PAG-1* in uterine endometrium (ICAR), and C) *PAG-1* in fetal membranes (FM). Data presented as a $2^{-\Delta\Delta CT}$-fold change normalized to β-*Actin* and the average of non-pregnant (NP; maternal tissues) or d 22 FM (fetal tissues). Expression pattern line (– –) via regression ($P < 0.05$); regression analysis does not include NP heifers. Means without a common superscript differ ($P < 0.05$).

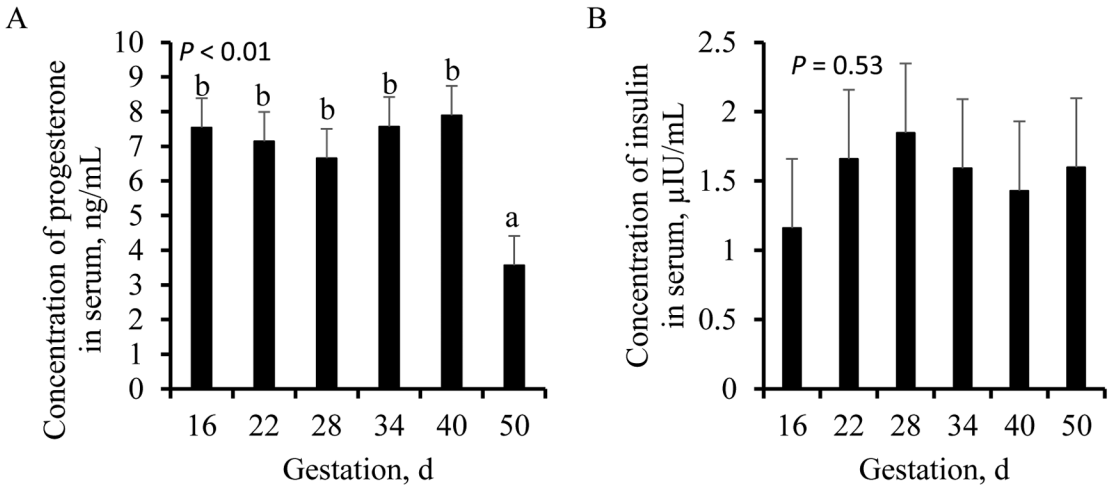

Figure 5. Concentrations of hormones in serum of beef heifers during early gestation. A) Concentrations of circulating progesterone in serum of beef heifers B) Concentrations of circulating insulin in serum of beef heifers. Hormones are reported as a pooled mean from heifers (n = 38, 30, 25, 18, 11, and 5 for d 16, 22, 28, 34, 40, and 50; respectively) on each d. Means without a common superscript differ ($P < 0.05$).

(Fig. 4C). Concentrations of progesterone (Fig. 5A) on d 50 (1.8 ± 1.8 and 3.6 ± 1.0 ng/mL; respectively) was decreased ($P < 0.01$) compared with all other days (7.4 ± 0.6 ng/mL). Concentrations of insulin were similar ($P = 0.53$) among all days of gestation evaluated (Fig. 5B).

Temporal Patterns of Expression

Regression analysis for expression of *syncytin-Rum1*, *BERV-K1*, *IFN*-τ, and *PAG-1* across days of gestation was used to determine temporal changes in expression pattern during the first 50 d of pregnancy (Table 2). In CAR, *syncytin-Rum1* ($P = 0.002$), *BERV-K1* ($P < 0.001$), and *PAG-1* ($P < 0.001$) all had exponential expression patterns during the first 50 d of gestation. Expression patterns for *BERV-K1* ($P = 0.009$), and *PAG-1* ($P < 0.001$) in ICAR were also exponential; however, *syncytin-Rum1* expression in ICAR had no ($P > 0.19$) pattern of expression. In FM, a linear pattern ($P = 0.03$) was observed in *syncytin-Rum1* expression but a cubic pattern was found for *BERV-K1* ($P = 0.01$). The pattern of expression for *IFN*-τ in FM was exponential ($P < 0.001$) but there was only a tendency for an exponential pattern of expression for *PAG-1* ($P > 0.08$; Table 2).

Table 2. Relative expression patterns for *syncytin-Rum1*, *bovine endogenous retrovirus-K1* (*BERV-K1)*, *interferon*-τ (*IFN*-τ), and *pregnancy associated glycoprotein-1* (*PAG-1*) during early pregnancy in beef heifers

Gene[1]	Equation for the best fit regression model[2,3]	*P*-value	R^2
Syncytin-Rum1			
CAR (Fig. 1A)	$y = 1.6298e^{0.4125x}$	0.002	0.47
FM (Fig. 1C)	$y = 0.504x + 0.61$	0.03	0.92
BERV-K1			
CAR (Fig. 2A)	$y = 8.4859e^{0.3567x}$	< 0.001	0.84
ICAR (Fig. 2B)	$y = 0.9091e^{0.2619x}$	0.009	0.23
FM (Fig. 2C)	$y = 3.8167x^3 - 35.62x^2 + 102.79x - 68.984$	0.01	0.89
IFN-τ			
FM (Fig. 3)	$y = 3.8167e^{-1.374x}$	< 0.001	0.73
PAG-1			
CAR (Fig. 4A)	$y = 32.007e^{1.1099x}$	< 0.001	0.93
ICAR (Fig. 4B)	$y = 4.967e^{0.4691x}$	< 0.001	0.50

[1]Tissue expression in maternal caruncles (CAR), uterine endometrium (ICAR), and fetal membranes (FM).

[2]Regression analysis does not include non-pregnant heifers.

[3]Equation variables are y = gene and x = day.

Tissue Comparisons

For comparison of gene expression across tissues on a given day the expression of *syncytin-Rum1*, *BERV-K1*, *IFN*-τ, and *PAG-1* was normalized to their average expression in ICAR tissues. In maternal tissues, CAR and ICAR had similar mRNA expression of *syncytin-Rum1* from d 16 to 40 and in NP tissues ($P > 0.32$; Table 3). However, at d 50 *syncytin-Rum1* mRNA expression in CAR was greater than ICAR ($P < 0.05$) and increased ($P < 0.0001$) by 190.3-fold over NP baseline (Table 3). Expression of *syncytin-Rum1* mRNA was greater in FM compared with ICAR ($P < 0.002$) and CAR ($P < 0.004$) from on d 22, 28, and 34 of gestation. At d 40 *syncytin-Rum1* in CAR and FM tissues were similar ($P = 0.34$) compared with ICAR. However, at d 50 *syncytin-Rum1* expression in CAR was greater ($P = 0.01$) compared with ICAR and FM tissues (Table 3).

There were no differences between tissues ($P > 0.36$; Table 4) in *BERV-K1* on d 16 and 22 in pregnant heifers and NP expression. On d 28 of gestation expression of BERV-K1 in FM was greater ($P < 0.01$) than that of maternal tissues CAR and ICAR. Expression of *BERV-K1*

Table 3. Relative fold change of *syncytin-Rum1* expression in maternal caruncles (CAR), uterine endometrium (ICAR), and fetal membranes (FM) during the first 50 d of pregnancy in beef heifers[1]

	Tissue type				
Gestation, d	CAR	ICAR	FM	SEM	*P*-value
NP[2]	5.1	1.5	–	2.8	0.67
16	2.4	2.2	–	0.5	0.77
22	1.2[a]	0.6[a]	4.8[b]	0.8	< 0.01
28	1.9[a]	1.5[a]	11.0[b]	1.4	< 0.01
34	14.1[a]	6.8[a]	128.5[b]	11.7	< 0.01
40	13.9	1.5	23.6	7.5	0.10
50	190.3[b]	1.1[a]	71.7[a]	28.6	< 0.01

[a,b]Means within rows without a common superscript differ ($P < 0.05$).

[1]Data normalized to β-*Actin* and the average for normalized ICAR for $2^{-\Delta\Delta CT}$ values.

[2]NP: non-pregnant controls ovariohysterectomized at d 16 of luteal cycle.

Table 4. Relative fold change of *bovine endogenous retrovirus-K1* (*BERV-K1)* expression in maternal caruncles (CAR), uterine endometrium (ICAR), and fetal membranes (FM) during the first 50 d of pregnancy in beef heifers[1]

	Tissue type				
Gestation, d	CAR	ICAR	FM	SEM	*P*-value
NP[2]	0.47	2.33	–	1.03	0.48
16	3.06	1.32	–	0.60	0.06
22	1.90	1.44	8.37	1.10	0.36
28	0.98[a]	1.33[a]	6.73[b]	3.87	< 0.01
34	10.06[a]	1.44[a]	45.01[b]	6.27	< 0.01
40	5.63[a]	2.29[a]	16.04[b]	4.27	0.04
50	21.04[a]	1.26[a]	62.55[b]	11.12	< 0.01

[a,b]Means within rows without a common superscript differ ($P < 0.05$).

[1]Data normalized to β-*Actin* and the average for normalized ICAR for $2^{-\Delta\Delta CT}$ values.

[2]NP: non-pregnant controls ovariohysterectomized at d 16 of luteal cycle.

in fetal membranes remained elevated ($P < 0.04$) on d 34, 40, and 50 of gestation (45.01, 16.04, and 62.55, respectively; Table 4) compared with maternal tissues. When comparing *PAG-1* expression across tissues, there were no differences in maternal tissues in NP or d 16 of gestation or across CAR, ICAR, or FM on d 28, 34, or 40 of gestation. On d 22 and 50, however, *PAG-1* expression was increased in CAR with a fold increase of 10.27 and 143.98, respectively (Table 5) compared to NP.

Table 5. Relative fold change of *pregnancy associated glycoprotein-1* expression in maternal caruncles (CAR), uterine endometrium (ICAR), and fetal membranes (FM) during the first 50 d of pregnancy in beef heifers[1]

	Tissue type				
Gestation, d	CAR	ICAR	FM	SEM	*P*-value
NP[2]	0.01	6.84	–	4.44	0.55
16	29.67	9.93	–	8.50	0.12
22	10.27[b]	3.16[a]	1.69[a]	2.41	0.05
28	1.47	1.45	1.11	0.46	0.83
34	4.98	1.10	10.30	3.01	0.13
40	11.10	2.58	2.73	2.45	0.05
50	143.98[b]	1.23[a]	8.91[a]	13.84	< 0.01

[a,b]Means within rows without a common superscript differ ($P < 0.05$).

[1]Data normalized to β-*Actin* and the average for normalized ICAR for $2^{-\Delta\Delta CT}$ values.

[2]NP: non-pregnant controls ovariohysterectomized at d 16 of luteal cycle.

DISCUSSION

Endogenous retroviral gene elements contribute to the formation of the multinucleation within placental formation in a wide variety of mammals with many different placental morphologies (Blond et al., 2000; Mi et al., 2000; Dupressoir et al., 2005, 2009; Heidmann et al., 2009; Dupressoir et al., 2011; Cornelis et al., 2012, 2013, 2014, 2015). To date, mammalian placentas found to form syncytium exhibit 2 morphologies, syncytiotrophblast and syncytial plaques. The syncytiotrophoblast is a multinucleated tissue layer of the placenta whereas syncytial plaques are multinucleated cells that form at the feto-maternal interface. Formation of syncytial plaques, which consist of both fetal and maternal cells, is unique to ruminants among eutherian mammals. The conjoining of cells of fetal and maternal origin makes separation of maternal and fetal transcriptome extremely difficult and is a limitation of this data but expression of ERV in maternal tissues, CAR and ICAR as reported here, is understandable in the uterus. The classical functions for ERV of immunosuppressive and cell to cell fusion (Dupressoir et al., 2011; Cornelis et al., 2013) and previously established ERV expression in fetal tissues (Blond et al., 2000; Mi et al., 2000; Dupressoir et al., 2011; Cornelis et al., 2013) and now, from this study, ERV expression in the maternal endometrium are intriguing to potential roles in the establishment of pregnancy such as maternal recognition, uterine immunotolerance, and overall placental development.

The measurement of basal mRNA expression during the first 50 d of gestation is entirely novel for the *ERV*, *BERV-K1*. McLean et al. (2016b) reported across tissue and day of gestation expression during early gestation but did not establish general mRNA expression patterns. These data are necessary to begin understanding the roles of ERV in pregnancy success. As stated earlier, we hypothesized that the ERV (*syncytin-Rum1* and *BERV-K1*), *IFN*-τ, and *PAG-1* would be differentially expressed while progesterone and insulin concentrations would remain steady during early gestation. In keeping with our hypothesis, we found *BERV-K1* began to increase near d 28; whereas, *syncytin-Rum1* expression

was only different at d 50 of gestation in CAR but both exhibited exponential patterns of expression from d 16 to 50 of gestation. This coincides with the time period when Winters et al. (1942) reported the greatest amount of multinucleated cells and syncytial plaque formations. The early increase of *BERV-K1* over *syncytin-Rum1* may be due to the increased cell to cell fusion capabilities of *BERV-K1*, which agrees with data from Nakaya et al. (2013). Cornelis et al. (2013), Nakaya et al. (2013) and our data presented here indicate that ruminants have at least 2 ERV, *syncytin-Rum1* and *BERV-K1*. This finding is similar to ERV in the rodent placenta, *syncytin-A* and *-B* (Dupressoir et al., 2011) and the human placenta *syncytin-1* and *-2* (Fisher et al., 1989). While *syncytin-A* and *-B* and *syncytin-1* and *-2* are homologous genes it is currently unknown if *syncytin-Rum1* and *BERV-K1* are also homologous to the mouse and human genes. Knockout mice for *syncytin-A* exhibit abnormal embryogenesis, ultimately terminating gestation between d 11.5 and 13.5 of gestation (Dupressoir et al., 2009). While termination of rodent gestation occurs later in pregnancy, comparatively, than the timeframe in this study; these data may be taken to imply that these *BERV-K1* and *syncytin-Rum1* are important to placentation, placentome formation, and successful pregnancy in beef cattle. However, more work remains to be completed to determine roles for *BERV-K1* and *syncytin-Rum1* during gestation.

The increased mRNA expression occurred in ICAR earlier (d 28) during pregnancy than in CAR for *BERV-K1*. The *BERV-K1* increase in ICAR occurred at the end of the adhesion phase of implantation, which further supports previous data that demonstrated *BERV-K1* has increased expression during early gestation and fusogenic functions (Koshi et al., 2012; Nakaya et al., 2013). Thus, the role of *BERV-K1* in placental formation is likely in cotyledon formation and subsequent syncytial plaque development. However, the presence of *syncytin-Rum1* and *BERV-K1* in maternal tissues is not in agreement with previous data for syncytin genes in cattle (Cornelis et al., 2013) or *BERV-K1* expression in trophoblast cells (Koshi et al., 2012). The increase earlier in gestation of *BERV-K1* expression compared with *syncytin-Rum1* may indicate a greater role for *BERV-K1* for cell to cell fusion not only between trophoblast cells of the fetus but also in syncytial plaque formation and the combination of maternal and fetal cells. These data may also support previous data (Imakawa et al., 2015) that suggested *BERV-K1* is replacing *syncytin-Rum1*as the main catalyst in placental cell to cell fusion.

Although means were not different from d 22 to 50 of gestation the linear increase in mRNA expression of *syncytin-Rum1* in FM could be aiding in cell to cell fusion occurring during placental development, which is rapidly occurring during this time (Winters et al., 1942). Expression pattern of *BERV-K1* was cubic in nature with peaks at d 34 and 50. These data agree with characteristic functions of *BERV-K1* for cell to cell fusion (Nakaya et al., 2013) during binucleate cell formation within placental trophoblast cells and syncytial plaques between fetal and maternal cells. As well as known events during early gestation such as maternal recognition, embryonic adhesion with the uterine endometrium, and placentation. Placental development is necessary for the transfer of nutrients responsible for the rapid fetal growth that must occur during late gestation.

The secretion of IFN-τ from the trophoblast is widely accepted as the ruminant signal for pregnancy recognition and inhibition of luteolysis (Thatcher et al., 1989; Bazer et al., 1991; Bazer, 1992; Mann et al., 1999; Spencer and Bazer, 2004; Spencer et al., 2007). The secretion of IFN-τ must occur before the initiation of luteolysis on d 18 of the estrous cycle. After this point concentrations of IFN-τ decreased dramatically back to basal levels which is in agreement with data from the current research where *IFN*-τ mRNA expression at d 22 was greater ($P < 0.01$) compared with all other days of gestation and exhibited a negative exponential pattern of expression in FM. Interferon-τ stimulates the production of many other proteins such as: ubiquitin-like interferon stimulated gene 15, myxovirus resistance 1, and 2'-,5'-oligoadenylate synthetase 1, which may be necessary for the establishment of pregnancy (Glaucia Teixeira et al., 1997; Perry et al., 1999; Binelli et al., 2001; Bazer et al., 2015). Another such protein, PSP-B, is produced in detectable quantities as early as d 15 of gestation (Butler et al., 1982; Sasser et al., 1986); however, concentrations vary greatly until after d 30 (Sasser et al.,1986; Humblot et al., 1988a; Sasser et al., 1991; Vasques et al., 1995). The limited secretion of PSP-B early in gestation agrees with our data in which we observed mRNA expression of *PAG-1* during the first 34 d of gestation.

The exponential increase in expression of *PAG-1* may indicate a greater prevalence in placental development as gestation progresses and may be stimulated by IFN-τ and ERV. However, secondary functions may be to aide IFN-τ and ERV in fetal protection during implantation via immune suppression (Wooding et al., 2005). In addition, expression pattern for *PAG-1* in CAR was exponential with a mean fold change of 18,000 compared with NP. Pregnancy associated glycoproteins from binucleated cells seem to interact extensively with maternal connective tissue which develops during placental villi formation (Wooding et al., 2005). It has been speculated that PAG may possibly be involved in proteolytic activation of growth factors and other molecules specific to pregnancy, protection of fetal tissues from maternal immune response, transport of hormones between fetal and maternal tissues, and cell to cell fusion (Wooding et al., 2005). Our data presented

here confirms expression of *PAG-1* during pregnancy, which would support Wooding et al. (2005) suggested roles of *PAG-1* in cell to cell fusion during placentation. Combined these data may also indicate an interaction with ERV to promote the cell to cell fusion needed for syncytial plaques formation and placental development to support fetal growth throughout gestation.

Progesterone must also be present for IFN-τ to suppress the release of $PGF_{2\alpha}$ stimulated by oxytocin (Meyer et al., 1995) to maintain pregnancy (Mann and Lamming, 2001; Green et al., 2005; Mann et al., 2006; Bazer et al., 2015). Our data clearly demonstrates elevated circulating progesterone concentrations (> 5 ng/mL) in pregnant heifers on all days except for d 50. Pregnant cattle will not only maintain a functional corpus luteum (CL) but also have greater progesterone concentrations compared with non-pregnant cattle (Henricks et al., 1971; Humblot et al., 1988b; Humblot, 2001). The drop in progesterone, regardless of treatment, on d 50 is intriguing; while the placenta does become the major source of progesterone in sheep and horses this does not occur in cattle (reviewed in Hoffmann and Schuler, 2002). Our data could be interpreted to mean that by d 50 the CL has begun to share progesterone secretion with the placenta but progesterone synthesis within the placenta remains to be completely elucidated (reviewed in Hoffmann and Schuler, 2002).

The time points assessed in this study, specifically d 16, 34, and 50 of gestation are influential to the expression of *syncytin-Rum1*, *BERV-K1*, *IFN*-τ, and *PAG-1* and may be important for the establishment of pregnancy. On d 16 the embryo must support the maintenance of a functional CL and as such this has been termed the period of maternal recognition. Day 34 is the approximate end of adhesion when the embryo has successfully completed implantation. Finally, d 50 is when embryogenesis is nearing completion and during rapid placentation when formation of bi- and multinucleated cells is at its peak. The differences in mRNA expression among tissues also provides insight into the functions of *syncytin-Rum1*, *BERV-K1*, *IFN*-τ, and *PAG-1* most of which remain to be completely understood.

In conclusion, the mRNA expression of *syncytin-Rum1*, *BERV-K1*, *IFN*-τ, and *PAG-1* was differentially present in utero-placental tissues during the first 50 d of gestation. We established 3 times, d 16, 34, and 50, during early gestation which had differences in gene expression and should be a focus of research in the future. Expression of *IFN*-τ was increased during the time of maternal recognition (~d 16). Level of *BERV-K1* was increased in ICAR on d 28, which coincides with fetal adhesion and the completion of implantation (~d 30; Winters et al., 1942; Guillomot, 1995). Gene expression *syncytin-Rum1*, *BERV-K1* and *PAG-1* in CAR was increased on d 50 supporting roles in cell to cell fusion and placental development. This research also established basal expression patterns for *syncytin-Rum1*, *BERV-K1*, *IFN*-τ, and *PAG-1* which can be used in future research to determine the influence of treatments on pregnancy. While these data provide evidence for differential expression the functions and interactions between *syncytin-Rum1*, *BERV-K1*, *IFN*- τ, and *PAG-1* remain to be elucidated and should be the focus in future studies to determine the importance in fetal and placental development and the establishment of pregnancy.

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