

The effects of nutrient restriction on expression of endogenous retroviruses mRNA during the establishment of pregnancy in beef heifers

Kyle J. McLean¹, Carl R. Dahlen¹, Pawel P. Borowicz¹, Larry P. Reynolds¹ and Joel S. Caton¹

The objectives of this project were to elucidate the effects of maternal nutrient restriction on endogenous retroviral genes in placental formation, placental function and establishment of pregnancy. Syncytin-Rum1 and BERV-K1 were expressed differentially during early gestation and may be important to the establishment of pregnancy.

Summary

We hypothesize that endogenous retrovirus envelope genes will be expressed differentially during early pregnancy (days 16 to 50) and will be influenced by plane of maternal nutrition. Commercial Angus crossbred heifers (n = 49; about 16 months of age; body weight [BW] = 713 ± 62 pounds) were maintained on a total mixed ration (TMR) and supplemented with dried distillers grains with solubles. All heifers were subjected to 5-day CO-Synch + CIDR estrus synchronization protocol and artificial insemination (AI) to a single Angus sire (day of breeding = day 0). On the day of breeding, heifers were assigned randomly to dietary treatments. One-half were assigned to control diet (CON) targeted to gain .45 kg/day and the remaining half were assigned to a restricted diet (RES) and received 60 percent of control diets. Heifers were subjected to ovariohysterectomy on days 16, 34 and 50 of gestation. Utero-placental tissues were obtained from the uterine horn ipsilateral (P) and contralateral (NP) to the corpora lutea (CL) and sepa-

rated into maternal caruncle (CAR); maternal endometrium, inter-caruncle (ICAR); and fetal membrane (FM). After being collected, all tissues were snap-frozen in liquid nitrogen-cooled isopentane and stored at minus 80 C. Expression of *syncytin-Rum1* was greater ($P = 0.01$) on day 16, with a 14.14 ± 2.06 -fold increase, compared with a 5.11 ± 2.06 -fold increase on day 34 and a 7.75 ± 2.06 -fold increase of expression on day 50 in P-ICAR. The effect of plane of nutrition on *BERV-K1* was dependent on the stage of gestation ($P = 0.03$) in NP-CAR. Heifers on the CON diet had expression that decreased from days 16 to 34 and reached max levels on day 50, with a 116.3 ± 22.5 increase, whereas RES heifer expression was not different throughout early gestation. We found no interactions between stage of gestation and nutritional treatment for *syncytin-Rum1* ($P > 0.22$). In conclusion, maternal nutrient restriction only influenced *BERV-K1*, but *syncytin-Rum1* and *BERV-K1* were expressed differentially in maternal and fetal tissues at critical time points during the first 50 days of gestation in beef heifers.

Introduction

Placental development is closely related to fetal growth and is sensitive to maternal nutrient supply from the earliest stages of pregnancy (Reynolds and Redmer, 2001) by facilitating the transfer of nutrient, gas and waste (Ramsey, 1982). Inadequate maternal nutrient supply leads to poor placental development, resulting in compromised fetal growth (Caton and Hess, 2010). The syncytiotrophoblast and syncytium will function as the feto-maternal interface to exchange nutrients, produce hormones and protect the conceptus from the maternal immune responses.

Syncytium formation is initiated by endogenous retroviral elements (ERV) that have been incorporated into the host genome. A significant portion of the genome is made up of ERV.

The Bovidae genome contains 24 ERV families, depending on the species (Garcia-Etxebarria and Jugo, 2013). The envelope proteins of *syncytin-Rum1* and *BERV-K1* are expressed differentially during early gestation and have been implicated as nutrient sensors (Sharif et al., 2013). Thus, expression may be influenced by the maternal plane of nutrition during early gestation.

Therefore, we hypothesize that endogenous retrovirus envelope genes will be expressed differentially during early pregnancy (days 16 to 50) and will be influenced by plane of maternal nutrition.

¹Department of Animal Sciences, NDSU

Experimental Procedures

All animal procedures were conducted with approval from the Institutional Animal Care and Use Committee at North Dakota State University (A16049). Commercial Angus crossbred heifers ($n = 49$; about 16 months of age; BW = 713 \pm 62 pounds) were transported 142 miles from the Central Grasslands Research Extension Center (Streeter, N.D.) to the Animal Nutrition and Physiology Center (North Dakota State University, Fargo, N.D.).

The heifers were housed in pens, with six heifers per pen, and individually fed daily in an electronic head gate facility (American Calan; Northwood, N.H.) at 8 a.m. Heifers were maintained on a TMR (48.4 percent dry matter [DM], 5.3 percent crude protein [CP], 29.4 percent neutral detergent fiber [NDF], 6.8 percent ash), supplemented with dried distillers grains with solubles (87.5 percent DM, 31.3 percent CP, 53.4 percent NDF, 8.2 percent ash), and granted ad libitum access to water.

All heifers were subjected to 5-day CO-Synch + CIDR estrus synchronization protocol and AI to a single Angus sire (day of breeding = day 0; Bridges et al., 2008). On the day of breeding, heifers were assigned randomly to dietary treatments.

One half were assigned to a control diet (CON) targeted to gain .45 kg/day and the remaining half were assigned to a restricted diet (RES) and received 60 percent of control diets.

Heifers were subjected to ovariectomy on days 16, 34 and 50, as previously described, McLean et al., (2016a). Thus, experimental design for the pregnancy analysis was a 2 \times 3 factorial design. Nonbred, nonpregnant control heifers (NP; $n = 6$) were ovariectomized on day 16 of the luteal cycle

following the synchronization cycle. The NP heifers and heifers that were ovariectomized on days 16, 34 and 50 and fed the CON diet were used in a CRD to address comparisons of pregnancy status and establishment.

Pregnancy was confirmed via trans-rectal ultrasonography on day 28 and again on the day of surgery (day beyond 28). During surgery, the left and right uterine arteries, 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-centimeter (cm) Crafoord Coarctation Clamp (Integra-Miltex; Plainsboro, N.J.) 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 days after surgery (McLean et al., 2016a), then the animals were returned to the control diets.

Immediately upon removal from the body cavity, tissues were trimmed of excess broad ligament, fat and nonreproductive tissues. Gravid uterus, individual and total ovarian, and CL weights were taken, as well as CL measurements for CL area before fixation, freezing and storage.

Utero-placental tissues were obtained from the uterine horn ipsilateral (containing the embryo) to the CL (pregnant uterine horn), maternal caruncle (P-CAR) and maternal endometrium, inter-caruncle, (P-ICAR).

Tissues were obtained in the uterine horn contralateral (opposite the embryo) to the CL (nonpregnant horn), maternal caruncle (NP-CAR) and maternal endometrium, inter-caruncle, (NP-ICAR). Fetal membranes (FM) were collected on days 16, 34 and 50. After being collected, all tissues were snap-frozen in liquid nitrogen-cooled isopentane and

stored at minus 80 C.

Gene expression was analyzed for threshold cycle using a 7500 Fast Real-Time PCR System (Applied Biosystems, Grand Island, N.Y.) with SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, Calif.). Gene expression for maternal tissues was calculated using the $\Delta\Delta CT$ method, 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 $\Delta\Delta CT$ method, with β -actin as the reference gene and of the ΔCT for uterine endometrium of each individual gene as the control (set to 1) within each tissue.

Statistical analyses for gene expression of *syncytin-Rum1* and *BERV-K1* were conducted as a 2 \times 3 factorial with individual heifer as the experimental unit via the GLM procedure of SAS version 9.4 (SAS Inst. Inc., Cary, N.Y.) to test for a stage of gestation \times nutritional plane interaction or the main effects of nutritional plane and stage of gestation. Means were separated using the LSMEANS statement of SAS with differences determined at a P -values \leq 0.05.

Results and Discussion

Factors that influence fetal and placental growth and development include maternal plane of nutrition, number of fetuses, maternal parity and age, maternal and fetal genotype, and maternal stress (Reynolds et al., 2010). The long-term effects of restricted nutrient intake during early gestation may be associated with impaired placental development or poor contact during the establishment of the fetomaternal interface, resulting in intrauterine growth retardation (Zhang et al., 2015).

In P-CAR, we found no interactions between the stage of gestation and nutritional treatment for *syncy-*

tin-Rum1 or *BERV-K1* ($P > 0.49$), so the main effects of day of gestation and nutritional treatment will be presented. Expression of *syncytin-Rum1* tended ($P = 0.10$) to be greater at day 50 (17.7 ± 3.8), compared with days 16 and 34 (6.9 and 7.9 ± 3.9 , respectively; data not shown).

In P-ICAR, we found no interactions between plane of nutrition and day of gestation or any effects of nutritional treatment for either gene expression ($P > 0.12$). Expression of *syncytin-Rum1* was greater ($P = 0.01$) on day 16, compared with day 34 and day 50 (Figure 1A). Expression of *syncytin-Rum1* increased ($P = 0.03$) in FM at day 50, compared with days 16 and 34 (Figure 1B).

In NP-CAR, neither stage nor nutritional treatment influenced *syncytin-Rum1* ($P > 0.11$). We found a plane of nutrition \times stage of gestation ($P = 0.03$) on *BERV-K1* expression. Heifers on the CON diet had expression that decreased from days 16 to 34 and increased on day 50; whereas, RES heifer expression was not different throughout early gestation (Figure 2A).

Sharif et al. (2013) reported that, in the developing placenta, ERV likely function as nutrient sensors that may be turned on during periods of hypomethylation, which occurs very early in gestation. *BERV-K1* has been reported to have greater fusogenic capabilities than *BERVE-A* or *syncytinRum-1* (Nakaya et al., 2013). This may explain why *BERVE-K1* was increased in the normal placental cytotrophoblast cell line (NPC) as the placental development spreads into the contralateral uterine horn, which may indicate a greater role in cell-to-cell fusion and the formation of syncytial plaques.

Thus, the function of *BERVE-K1* may be similar to *syncytin-A*, which altered trophoblast stem cell (TSC) fusion, causing inefficient placental transport, decreased vascularity

and growth retardation, ultimately terminating gestation between days 11.5 and 13.5 of gestation (Dupres-soir et al., 2009). The stage of gestation and plane of nutrition did not influence *syncytin-Rum1* or *BERV-K1*

($P > 0.14$). The mRNA expression levels of *BERV-K1* in FM increased ($P < 0.01$) at day 34, compared with day 16, and then decreased to day 50; however, day 50 was greater ($P < 0.01$) than day 16 (Figure 2B).

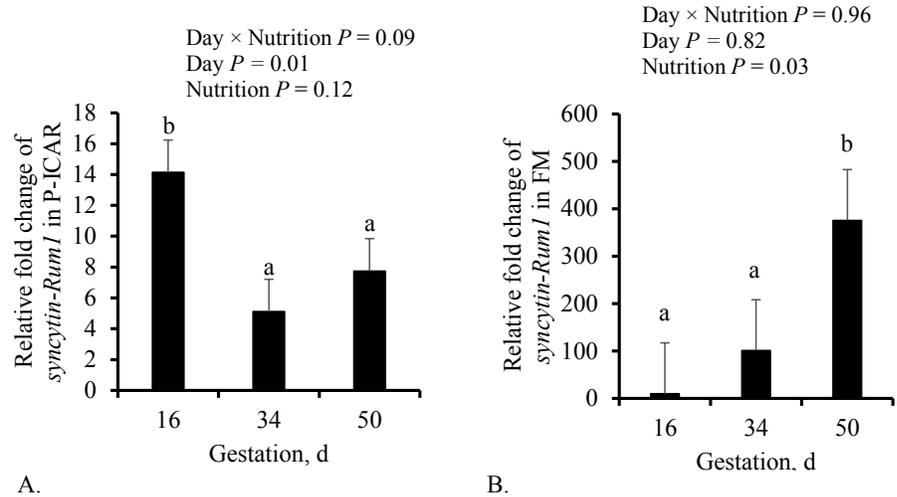


Figure 1. Expression of *syncytin-Rum1* in pregnant uterine horn endometrium (P-ICAR) and fetal membranes during the establishment of pregnancy in beef heifers: A) *syncytin-Rum1* in P-ICAR and B) Expression of *BERV-K1* in FM. Data presented as a $2^{\Delta\Delta CT}$ fold change normalized to β -Actin and the average of NP. ^{a,b}Means without a common superscript differ ($P < 0.05$)

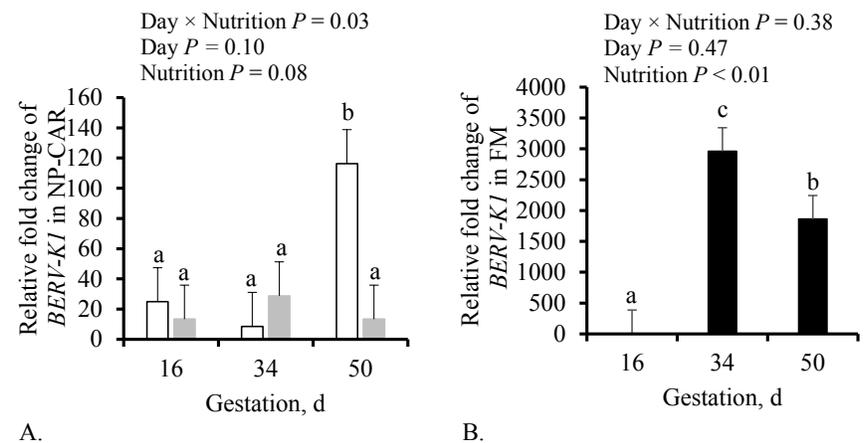


Figure 2. Expression of *BERV-K1* in caruncles of the contralateral uterine horn to the conceptus (NP-CAR) and fetal membranes (FM) during the establishment of pregnancy in beef heifers: A) Expression of *BERV-K1* in NP-CAR where white bars outlined in black represent control heifers and gray bars represent restricted heifers and B) Expression of *BERV-K1* in FM. Data presented as a $2^{\Delta\Delta CT}$ fold change normalized to β -Actin and the average of NP. ^{a,b,c}Means without a common superscript differ ($P < 0.05$)

As in rodents and humans, our data has determined that cattle have at least two ERV, *syncytin-Rum1* and *BERV-K1*, that are expressed differentially in reproductive tissues during the establishment of pregnancy. However, in contrast to previous findings (Cornelis et al., 2013), these data indicated that maternal tissues do express mRNA for ERV and at times have increased mRNA expression. This is in agreement with previous data from our laboratory (McLean et al., 2016b).

While differing expression levels are intriguing, the limited knowledge of function and pathways in which ERV are influencing the formation of the placenta and fetus and the establishment of pregnancy hinder the elucidation of the importance of ERV during early gestation.

In conclusion, 50 days of 40 percent nutrient restriction may not be severe or long enough of a restriction to influence ERV expression in utero-placental tissues. Our previous data and this work confirmed differential expression of *syncytin-Rum1* and *BERV-K1* in maternal and fetal tissues during the first 50 days of gestation. These differences in expression are at critical time points during the establishment of pregnancy, specifically, maternal recognition (day 16), completion of fetal adhesion (day 34) and rapid placental development (day 50). While exact functions during early gestation remain to be elucidated, ERV may complete vital steps for the successful establishment of pregnancy in beef heifers.

Literature Cited

- Caton, J.S., and B.W. Hess. 2010. Maternal plane of nutrition: Impacts on fetal outcomes and postnatal offspring responses. in: Proc 4th Grazing Livestock Nutrition Conference. West Sect. Am. Soc. Anim. Sci. 39:104-122.
- Cornelis, G., O. Heidmann, S.A. Degrelle, C. Vernochet, C. Lavialle, C. Letzelter, S. Bernard-Stoecklin, A. Hassanin, B. Mulot, M. Guillomot, I. Hue, T. Heidmann and A. Dupressoir. 2013. Captured retroviral envelope syncytin gene associated with the unique placental structure of higher ruminants. Proc. Natl. Acad. Sci. USA 110:E828-E837.
- Dupressoir, A., C. Vernochet, O. Bawa, F. Harper, G. Pierron, P. Opolon and T. Heidmann. 2009. Syncytin-A knockout mice demonstrate the critical role in placentation of a fusogenic, endogenous retrovirus-derived, envelope gene. Proc. Natl. Acad. Sci. USA 106:12127-12132.
- Garcia-Etxebarria, K., and B.M. Jugo. 2013. Evolutionary history of bovine endogenous retroviruses in the Bovidae family. BMC Evol. Bio. 13:256-267.
- McLean, K.J., C.R. Dahlen, P.P. Borowicz, L.R. Reynolds, B.W. Neville, S.D. Walden and J.S. Caton. 2016a. Technical note: A new surgical technique for ovariohysterectomy during early pregnancy in beef cattle. J. Anim. Sci (Submitted, JAS # E-2016-0761).
- McLean, K.J., M.R. Crouse, M.S. Crosswhite, N. Negrin Pereira, C.R. Dahlen, P.P. Borowicz, L.R. Reynolds, A.K. Ward, B.W. Neville and J.S. Caton. 2016b. Rapid Communication: Expression of an endogenous retroviral element, *syncytin-Rum1*, during early gestation in beef heifers. J. Anim. Sci. (Submitted, JAS # E-2016-0793).
- Nakaya, Y., K. Koshi, S. Nakagawa, K. Hashizume and T. Miyazawa. 2013. Fematrin-1 is involved in fetomaternal cell-to-cell fusion in Bovine placenta and has contributed to diversity of ruminant placentation. J. Virol. 87:10563-10572.
- Ramsey, E.M. 1982. The placenta, human and animal. Praeger, New York.
- Reynolds, L. P. and D. A. Redmer. 2001. Angiogenesis in the placenta. Biol. Reprod. 64:1033-1040.
- Reynolds, L- P., P.-P. Borowicz, J.S. Caton, K.A. Vonnahme, J.S. Luther, D.S. Buchanan, S.A. Hafez, A.T. Grazul-Bilska and D.A. Redmer. 2010. Uteroplacental vascular development and placental function: An update. Invited review. Internat. J. Develop. Biol. Special Issue "Placental Developmental Biology," J.S. Hunt and K.L. Thornburg (eds.) 54:355-366.
- Sharif, J., Y Shinkai and H. Koseki. 2013. Is there a role for endogenous retroviruses to mediate long-term adaptive phenotypic response upon environmental inputs? Phil. Trans. R. Soc. B. 368:20110340-20110353.
- Zhang, S., T.R.H. Regnault, P.L. Barker, K.J. Botting, I.C. McMillen, C.M. McMillan, C.T. Roberts and J.L. Morrison. 2015. Placental adaptation in growth restriction. Nutrients. 7:360-389.